

1 **Characterization of Vif domains that mediate Feline Immunodeficiency Virus**
2 **antagonism of APOBEC3-H and APOBEC3-CH restriction.**

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37

38 **Abstract**

39 Feline immunodeficiency virus (FIV) Vif mediates degradation of two anti-lentiviral feline
40 APOBEC3 (fA3) proteins, fA3Z3 and fA3Z2bZ3. HIV-1 Vif targets the restriction factor
41 human APOBEC3G (A3G, hA3Z2g-Z1c) for proteasome degradation to mediate viral
42 evasion. Despite this similarity, FIV and HIV-1 Vif share limited homology. Vif binds
43 hA3Z2g-Z1c through its N-terminal region, while its C-terminal region binds to an E3-
44 ligase complex containing Cullin5 and Elongin B/C. Further, HIV-1 Vif contains critical
45 domains in its C-terminus, including an adjacent BC box, the only shared domain
46 between FIV and HIV-1 Vif, and a non-classical zinc finger (HCCH) domain. Felid
47 lentivirus Vif, however, contains a highly conserved KCCC motif. While both Vifs have
48 evolved to counteract select A3 antiretroviral proteins, the FIV Vif domains necessary to
49 target fA3s for degradation are incompletely understood. To identify these domains, we
50 used the well-characterized HIV-1 Vif domains to show that distinct mutations within the
51 BC box of FIV Vif prevent fA3Z3 and fA3Z2bZ3 degradation and reduce virion
52 infectivity. We also found that mutating any single residue in the KCCC motif blocked
53 fA3 targeting and impaired FIV infectivity and replication. These mutations also failed to
54 disrupt the FIV Vif and Cullin5 interaction. Further, we showed that, in contrast to the
55 HCCH domain in HIV-1 Vif, the KCCC domain of FIV Vif does not bind zinc. However,
56 unlike HIV-1 Vif, FIV Vif (C36 isolate) reduces intracellular levels of co-expressed
57 Cullin5 proteins, a novel finding. Our results reveal important C-terminal residues in FIV
58 Vif and show that the BC box and KCCC regions are critical for fA3 degradation,
59 infectivity, and spreading replication.

60

61 Introduction

62 Lentiviruses cause chronic degenerative diseases in primate, ungulate, and
63 feline species. The current AIDS pandemics in *Homo sapiens* and *Felis catus* are
64 caused by human immunodeficiency virus type-1 (HIV-1) and feline immunodeficiency
65 virus (FIV), respectively. All primate and non-primate lentiviruses encode a Vif protein,
66 except the equine virus (1), that is essential for viral replication. HIV-1 Vif counters the
67 antiviral activity of human APOBEC3G (A3G, hA3Z2g-Z1c) (2-11). In the absence of Vif,
68 hA3Z2g-Z1c incorporates into virion particles of the virus-producing cell, where it inhibits
69 viral replication by cytidine deamination of the viral minus-strand cDNA. This
70 mechanism produces a plus-strand G-to-A hypermutation and interference during
71 reverse transcription (4, 12-15).

72 A3 proteins, such as hA3Z2g-Z1c, are targeted to the proteasome for
73 degradation (2, 16-22) via a Vif-dependent mechanism. The N-terminus of HIV-1 Vif, the
74 ¹⁴DRMR¹⁷ and ⁴⁰YHHY⁴⁴ motifs (23-26), mediates the interaction with hA3Z2g-Z1c.
75 However, the C-terminal region of Vif coordinates recruitment of a cellular E3 ligase
76 complex consisting of Cullin5 (Cul5), Elongin C, Elongin B, an E2 ligase, and a Ring box
77 (Rbx) protein (2, 5, 6, 18, 27). Recruitment of the E3 ligase occurs via several
78 evolutionarily conserved domains in Vif, including a non-classical zinc-finger domain
79 that binds to Cul5, SOCS (suppressors-of-cytokine-signaling-like domain) box, BC box,
80 that interacts with Elongin C (2, 5-9, 15, 28-30), and a PPLP domain that interacts with
81 Elongin B (1, 6, 31). While there are seven different human Cullin proteins (32, 33), HIV-
82 1 Vif selectively interacts with Cul5 to stabilize the E3 ligase complex (2).

83 The half-life of both HIV-1 and simian immunodeficiency virus (SIV) Vif is
84 stabilized and extended by core-binding factor- β (CBF- β) (34), which promotes the
85 interaction between HIV-1/SIV Vif and Cul5 needed to form the E3-ligase complex (27,
86 35-39). The interface that mediates CBF- β binding to and stabilization of Vif were solved
87 using the Vif/CBF- β /Cul5/Rbx2/EloBC and, separately, the Vif/CBF- β /N-terminal
88 hA3Z2g-Z1c/EloBC complexes with X-ray crystallography and NMR spectroscopy,
89 respectively (37, 40). CBF- β forms multiple interactions with the HIV-1 Vif N- and C-
90 terminus, particularly with the BC box and HCCH domains (40-42). Mutating HCCH
91 residues prevents the HIV-1 Vif/CBF- β /hCul5 interaction and significantly suppresses
92 hA3Z2g-Z1c antiviral activity (41).

93 Non-primate mammals also encode antiviral APOBEC3 proteins (11, 43-50). Two
94 *Felis catus* A3 proteins, fA3Z3 (fA3H) and fA3Z2bZ3 (fA3CH), restrict Δ vif FIV (11, 43-
95 46), a FIV vif-deletion mutant. FIV Vif promotes HIV-1 replication by mediating
96 degradation of fA3Z3 or fA3Z2bZ3 when expressed in *cis* (45, 46) or *trans* (45) in
97 Crandall-Rees Feline Kidney (CrFK) cells. FIV Vif also recruits an E3-ligase complex
98 containing Cul5, EloC, and EloB (51). Although FIV Vif lacks the HCCH motif, a region
99 adjacent to the BC box contains a strongly conserved KCCC motif (Fig. 1A) (45) that
100 (45, 51) may serve as an atypical putative zinc-binding domain. Other lentiviral proteins
101 contain an atypical zinc finger, such as the HIV-1 nucleocapsid (NC), that is important
102 for transferring DNA intermediate strands during reverse transcription (52-54).
103 Additionally, FIV Vif interacts with human and feline Cul5 (fCul5) (51); however, the
104 residues governing the interaction between Vif and Cul5 are different between HIV-1
105 and FIV Vif. Indeed, replacing the two most C-terminal cysteine residues in the KCCC

106 motif of FIV Vif with serine (KCSS) no longer rescues Δ Vif HIV-1 infectivity in the
107 presence of fA3 proteins, despite maintaining the interaction between FIV Vif and Cul5
108 (51).

109 The zinc chelator PTEN (N, N, N', N'-tetrakis-(2-pyridylmethyl)-ethylenediamine)
110 further distinguishes FIV from HIV-1. PTEN disrupts the interaction between HIV-1 Vif
111 and hCul5, but not between FIV Vif and fCul5, suggesting that FIV Vif might not be a
112 bona fide zinc-binding protein (9, 29, 51). However, PTEN can chelate iron and copper
113 (albeit it has a greater affinity for copper) and alter their cellular levels (55-57). While
114 earlier data suggested that FIV Vif does not bind zinc, biochemical data using purified
115 proteins were needed to verify this notion. Because HIV-1 NC has a non-canonical zinc
116 motif, we posited that the KCCC domain in FIV Vif might be the major zinc-binding site
117 and may mediate a functionally important Vif-Cul5 interaction. To test this hypothesis,
118 we interrogated the biomolecular interactions between FIV Vif and Cul5 using
119 isothermal titration calorimetry with and without zinc. Our studies were designed to
120 decipher whether FIV Vif is a zinc-binding protein and whether additional determinants
121 in the BC box and KCCC domain are essential for this interaction.

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125

126 **Materials and Methods**

127 **Cells**

128 293T and CrFK cells were obtained from the American Type Culture Collection (ATCC).

129 Cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) with

130 10% fetal bovine serum, 1% penicillin/streptomycin, and L-glutamine.

131

132 **Plasmids**

133 The expression plasmid containing codon-optimized wild-type (WT) FIV Vif C36 tagged

134 with a hemagglutinin (HA) epitope (FIV C36 Vif-HA) was generated as previously

135 described (45). Primer pairs (Table 1) were used for site-directed mutagenesis within

136 FIV C36 HA-tagged Vif using sense and antisense primers to generate the following FIV

137 Vif mutants: TLQ/AAA, TLQ/TAQ, A205L, A205S, A205T, K157R, C161S, and C187S.

138 To generate the FIV Vif KCCC/RSSS mutant, a multisite-directed mutagenesis kit

139 (Invitrogen) was used with two sense primers outlined in the primer table. Overlap-

140 extension PCR deleted the KCCC domain using FIV C36 Vif-HA as a template with the

141 outer sense (OS) and inner antisense (IAS) primers to generate the first PCR product.

142 The second PCR reaction used the inner sense (IS) and outer antisense (OAS) primers.

143 Combined PCR products were amplified with OS and OAS primers to generate Δ KCCC

144 FIV C36 Vif-HA. PCR products were ligated into p1012 using EcoRI and KpnI restriction

145 sites. The expression plasmid containing VR1012 human Cul5 Myc was a generous gift

146 from Dr. Xiao Fang Yu at John Hopkins University School of Public Health (2).

147 Construction of expression plasmids containing fA3Z3 and fA3Z2bZ3 FLAG was
 148 previously described (45). The VR1012 feline Cul5 Myc was a generous gift of Dr. Yu
 149 (51). All expression plasmids were verified by Sanger DNA sequencing.

150 **Table 1. Primer Sequences to Generate FIV Vif Expression Plasmids.**

| Name | Primer Sequences for FIV Vif Mutants |
|---------------|--|
| TLQ/AAA | 5' TCTCCACCCGGCGCCGCGCCGCCCCGCTGGCCATG 3' CATGGCCAGGCGGGCGGCGGCGCCGGGTGGAGA |
| TLQ/TAQ | 5' CCACCCGGCACTGCCAACGCCTGGCCATGCTGGCCTGTGGCC 3' GGCCACAGGCCAGCATGGCCAGGCGTTGGGCAGTGCCGGGTGG |
| A205L | 5' CCACCCGGCACTCTGCAACGCCTGCTGATGCTGGCCTGTGGCCGC 3' GCGGCCACAGGCCAGCATCAGCAGGCGTTGCAGAGTGCCGGGTGG |
| A205S | 5' CCACCCGGCACTCTGCAACGCCTGTCCATGCTGGCCTGTGGCCGC 3' GCGGCCACAGGCCAGCATGGACAGGCGTTGCAGAGTGCCGGGTGG |
| A205T | 5' CCACCCGGCACTCTGCAACGCCTGACCATGCTGGCCTGTGGCCGC 3' GCGGCCACAGGCCAGCATGGTCAGGCGTTGCAGAGTGCCGGGTGG |
| K157R | 5' GGCATGGTTGGTATCGCAATCCGCGCCTTCTCCTGCGGGGAGCGC 3' GCGCTCCCCGCAGGAGAAGGCGCGGATTGCGATACCAACCATGCC |
| C161S | 5' ATCGCAATCAAAGCCTTCTCCTCCGGGGAGCGCAAATTGAAGCA 3' TGCTTCAATTTTGCCTCCCCGGAGGAGAAGGCTTTGATTGCGAT |
| C184S | 5' GAAATTGATCCGAAAAAATGGTCCGGCGACTGCTGGAACCTGATG 3' CATCAGGTTCCAGCAGTCGCCGACCATTTTTTCGGATCAAT TTC |
| C187S | 5' CCGAAAAAATGGTGCGGCGACTCCTGGAACCTGATGTGCCTGCGC 3' GCGCAGGCACATCAGGTTCCAGGAGTCGCCGCACCATTTTTTCGG |
| KCCC/RSSS | 5' GGTGGTATCGCAATCCGCGCCTTCTCCTCCGGGGAGCGCAAATTG 5' GATCCGAAAAAATGGTCCGGCGACTCCTGGAACCTGATGTGC |
| Δ KCCC | <i>Overlap Extension Primers to Generate FIV Vif ΔKCCC Mutant</i> |
| OS | 5' ATATATGAATTCCACCACACTGGACTAGTGGATCCATGGG |
| IAS | 3'GCAGGCACATCAGGTTCCAGATTGCGATACCAACCATGCCCGGCCCCC AG |
| IS | 5' GCATGGTTGGTATCGCAATCTGGAACCTGATGTGCCTGCGCAATTCTCC |
| OAS | 3' ATATATGGTACCCATAGAGCCCACCGCATCCCCAGCATGC |

151
 152
 153 **Construction of Vif-Mutant Infectious Molecular Clones**
 154
 155 **Generation of 34TF10 C184S_187S Vif.** The human cytomegalovirus driven infectious
 156 molecular clone 34TF10 (CT5) (58) containing a functional Orf2 was subcloned into
 157 pcDNA3.1(-) using EcoRI and KpnI (pcDNA3.1-CT5). pcDNA3.1-CT5 has a partial

158 fragment of Gag and full length Pol, Vif, and Orf2 genes (hereafter referred to as
159 pcDNA3.1-CT5Vif) (58). To introduce the C184_187S mutation into pcDNA3.1(-)
160 CT5Vif, the OS and IAS primers were used to generate the first PCR amplicon. The
161 second PCR product was synthesized with OAS and IS primers. Combined PCR
162 products were amplified with the OS and OAS primers, and the overlap product was
163 inserted into pCT5 with AcclII and KpnI.

164

165 **Creation of KCCC/RSSS.** To construct KCCC/RSSS, the C184_187S amplicon
166 described above was ligated into pcDNA3.1(-) empty vector using EcoRI and KpnI. To
167 generate KCCC/RSSS Vif, the pcDNA3.1-C184S_187S Vif plasmid and OS and IAS
168 primers were used to amplify the first PCR product. The pcDNA3.1-C184_187S plasmid
169 and OAS primer and the IS primers generated the second PCR amplicon. Combined
170 PCR products were amplified using OS and OAS primers to generate the CT5 Vif
171 KCCC/RSSS. The KCCC/RSSS PCR product was ligated into CT5 with AcclII and KpnI.

172

173 **Creation of 34TF10 TLQ/AAA Vif.** To generate the TLQ/AAA Vif mutant, the pcDNA
174 3.1-CT5 Vif plasmid and the OS and IAS primers were used to amplify the first PCR
175 product. The pcDNA3.1-CT5 Vif template and OAS and IS primers were used to amplify
176 the second PCR product. Combined PCR products were amplified with OS and OAS
177 primers to generate the TLQ/AAA amplicon. The TLQ/AAA PCR product was ligated
178 into CT5 with AcclII and KpnI.

179 **Creation of 34TF10 Δ KCCC_TLQ/AAA Vif.** To generate the Δ KCCC_TLQ/AAA Vif
180 mutant, the CT5 TLQ/AAA amplicon was ligated into pcDNA3.1(-) using EcoRI and

181 KpnI. The CT5 TLQ/AAA CT5 Vif template and OS and IAS primers were used to
 182 amplify the first PCR product. The second PCR product was amplified using the CT5
 183 TLQ/AAA CT5Vif template DNA with OAS and IS primers. Combined PCR products
 184 were amplified using OS and OAS primers to generate the full-length Δ KCCC_TLQ/AAA
 185 Vif amplicon. The primers used to generate mutants with overlap-extension PCR are
 186 listed in Table 2. All mutants were verified by Sanger DNA sequencing.

187

188 **Table 2. Overlap-Extension Primers to Generate Molecular Clones of Vif Mutant**

189 **CT5.**

| Name | Mutant CT5 Primers Sequences |
|---------------------------------------|---|
| C184/187S | |
| OS | ATAT TCCGGATGAAAGAGAAAAGTATAAAAAAGAC |
| IAS | ACACATTAATTCCATGAATCTCCTGACCATTTTTTTGGATC |
| IS | GATCCAAAAAATGG TCA GGAGAT TCA TGGAATTTAATGTGT |
| OAS | ATAT GGTACC CTAATGGGTTTACTCCTGGATTTAG |
| KCCC/RSSS | |
| OS | ATAT TCCGGATGAAAGAGAAAAGTATAAAAAAGAC |
| IAS | CTTTCTTTCGCCTGAACTAAAAGCGCGTATTGCTATACC |
| IS | GGTATAGCAATA CGC GCTTTTAGT TCA GGCGAAAGAAAG |
| OAS | ATAT GGTACCCTAAATGGGTTTACTCCTGGATTTAG |
| TLQ/AAA | |
| OS | ATAT TCCGGATGAAAGAGAAAAGTATAAAAAAGAC |
| IAS | CATAGCGAGTCTAGCAGCAGCCTTTGGAGGTG |
| IS | CACCTCCAAGGCTGCTGCT AGACTCGCTATG |
| OAS | ATAT GGTACCCTAAATGGGTTTACTCCTGGATTTAG |
| ΔKCCC_TLQ/AA | |
| OS | ATAT TCCGGATGAAAGAGAAAAGTATAAAAAAGAC |
| IAS | GTTTCTAAGACACATTAATTCCATATTGCTATACCTACCATCC CTGGTCCCAT |
| IS | GGATGGTAGGTATAGCAATATGGAATTTAATGTGTCTTAGAACTCACC TCCAAAG |
| OAS | ATATGGTACC CTAATGGGTTTACTCCTGGATTTAG |

190

191

192

193 **Cloning, Expression and Purification of Recombinant FIV C36 Vif**

194 Expression constructs containing FIV C36 Vif (residues 156–200 and 156–213) were
195 generated by insertion into the EcoRI-Sbf I site of pMal c-5X (NEB). The resulting
196 mannose-binding protein (MBP)-Vif fusion proteins were expressed and purified as
197 previously described (59). Purified fusion proteins were concentrated in 20 mM HEPES
198 pH 7.4, 150 mM NaCl, and 200 μ M Tris (2-carboxyethyl) phosphine and used
199 immediately for zinc-binding studies.

200

201 **ITC Analysis of Zinc Binding to Recombinant FIV C36 Vif**

202 Isothermal titration calorimetry (ITC) experiments were performed at 25°C using a
203 MicroCal iTC200 microcalorimeter (GE Healthcare) with a stirring rate of 1000 rpm.
204 Complete details of the ITC assay have been described (59). Briefly, purified FIV Vif
205 proteins were loaded into the ITC cell. A standardized stock solution of $Zn(NO_3)_2$ was
206 loaded in the ITC syringe and injected step-wise into the cell. After the first injection of
207 0.4 μ l $Zn(NO_3)_2$, 19 additional injections of 2 μ L were made into the cell at 2 minute
208 intervals to allow the system to return to equilibrium. Data were analyzed using
209 MicroCal Origin software.

210

211 **Western Blotting**

212 Following washing and trypsinization, cells lysates were obtained by suspending cell
213 pellets in a volume of 10 μ l per 100,000 cells with protease inhibitor (Complete-Mini,
214 Roche) and an equivalent volume of 6X Laemmli buffer per sample. Lysates were
215 boiled and clarified. Proteins were resolved on a sodium dodecyl sulfate–polyacrylamide

216 electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene fluoride (PVDF)
217 membrane (Thermo Scientific). Membranes were blocked in 10% milk and probed with
218 primary antibodies. Membranes were incubated with appropriate secondary HRP-
219 conjugated antibodies (dilutions 1: 5000–1:10000). Chemiluminescent substrates were
220 used for visualization (Thermo Scientific 34080 or 34095).

221

222 **Western Antibodies**

223 Rat anti-HA (Roche, high-affinity clone 3F10), rabbit anti-HA (Santa Cruz), mouse anti-
224 HA (Sigma H3663), polyclonal rabbit anti-Myc (Santa Cruz), rabbit anti-tubulin (Santa
225 Cruz Product 9104), and mouse anti-Flag (Sigma F3165) antibodies were used at a
226 dilution between 1:500 and 1:1000.

227

228 **Proteasome Inhibition**

229 Plasmid DNAs were transfected into 293T cells using calcium phosphate co-
230 precipitation and medium was changed 12–16 hours later. Cells were treated with
231 dimethyl sulfoxide (DMSO) or 10 μ M MG132 (Sigma) 48 hours post-transfection, and
232 cell lysates were harvested 22–24 hours later. Proteins were separated via SDS-PAGE
233 as previously described.

234

235 **Co-Immunoprecipitation**

236 Co-transfections of 293T cells were performed as above. Transfection media was
237 replaced with DMEM 12–16 hours later. Then, 48–52 hours post-transfection, cells were

238 washed with ice-cold PBS and lysed with ice-cold lysis buffer [150 mM NaCl, 50 mM
239 TRIS HCl (pH 7.5), 1% Triton X, complete mini-protease inhibitor (Roche, Germany),
240 and 200 mM phosphatase inhibitor (Sigma: Sodium Pervanadate)]. Sheep anti-mouse
241 Immunoglobulin (Ig) magnetic dynabeads (sheep anti-mouse IgG, Invitrogen cat
242 no.110.31; Oslo, Norway) were blocked with 0.01% bovine serum albumin (BSA) and 2
243 mM EDTA. Cell lysates were stored at -20°C. To eliminate non-specific binding, beads
244 were incubated with cell lysates at 4°C for 1 hour. Beads from previous adsorption were
245 discarded, and lysate were incubated with 1.0 µg mouse anti-HA antibody (Sigma
246 H3663) to immunoprecipitate HA-tagged proteins. Pre-cleared lysates were incubated
247 with 30 µl of pre-blocked dynabeads for 1 hour. Beads were washed three times with
248 ice-cold co-immunoprecipitation lysis buffer. Immune complexes were eluted from
249 beads with 30 µl of 2X β-mercaptoethanol Laemmli sample buffer and boiled at 95°C for
250 9 minutes. Proteins were separated by SDS-PAGE. Immunoblots were probed with a
251 polyclonal rabbit antibody directed against the Myc epitope to assess the interaction
252 between feline Cul5 Myc and FIV Vif HA-tagged (WT or mutant) proteins.

253

254 **Immunofluorescence**

255 The 293T cells were seeded onto Nunc LabTEK II chamber slides (Thermofisher,
256 Catalog Number 154461). Adhered 293T cells were transfected with Fugene (Roche).
257 Then, 48 hours post-transfection, cells were fixed with 4% formaldehyde, washed with
258 PBS, and perforated with ice-cold methanol. Cells were stained with primary antibodies
259 against HA- (high-affinity rat anti-HA antibody; Roche) or Myc- (rabbit anti-Myc; Santa
260 Cruz) tagged proteins and probed with secondary antibodies goat anti-rabbit Alexa

261 Flour (AF) 488 or goat anti-rat AF 594 (Invitrogen) to analyze cellular distribution of
262 proteins. Cells were stained with prolonged gold DAPI (Invitrogen) and mounted with a
263 glass coverslip. All images were taken with a 510 laser-scanning confocal microscope
264 (LSM 510).

265

266 **Single-Cycle FIV-Replication Assays**

267 The 293T producer cells were co-transfected using the calcium phosphate method with
268 the following plasmids: transfer (FIV human cytomegalovirus (CMV)–driven luciferase
269 reporter) (60), vesicular stomatitis virus glycoprotein (VSV-G), FP93 packaging (express
270 FIV: gag, pol, and rev, but lacks Vif and envelope proteins) (61), fA3s (either fA3Z3 or
271 fA3Z2bZ3), and codon-optimized FIV C36 Vif-HA (WT or mutant; or empty vector
272 parental control) plasmids. Then, 16–20 hours post-transfection, media was changed;
273 supernatants were collected with a 0.45 μ M filter 32 hours later. Freshly plated 293T
274 cells were transduced with equal amounts of virus normalized to reverse transcriptase
275 (RT). Media was changed 16–20 hours after infection. Seventy-two hours post-infection,
276 supernatants were removed, cells were washed with PBS, and then lysed with 1%
277 Tween. Quantification of luciferase activity was measured by the relative light units
278 emitted from each sample. Error bars reflect standard deviation from the mean.

279

280 **Spreading Replication**

281 The 293T producer cells were transfected with infectious molecular clones of WT or
282 mutant 34TF10 Orf2 (CT5) using polyethylenimine (PEI) transfection reagent (Salk

283 Institute). To boost first-round infectivity, vesicular stomatitis virus glycoprotein G (VSV-
284 G) was co-expressed during production. Media was changed 6–8 hours later. Viruses
285 were harvested, filtered (0.45 μ M), and pelleted on a 20% sucrose cushion 48 hours
286 post-transfection. Viral titers were determined using a focal infectivity assay (FIA).
287 Freshly plated CrFk cells were infected, and 12–16 hours later, they were washed
288 thoroughly to eliminate input virus. Every 72 hours, viral supernatant was harvested.
289 Simultaneously, feline cells were divided, cultured, and passaged with fresh media. The
290 RT activity of viral supernatants was determined as previously described (62).

291

292 **FIV Focal Infectivity Assay**

293 The focal infectivity assay (FIA) assay has been previously described (63). In brief,
294 CrFK cells were serially infected in 24-well plates. Sixteen hours post-infection, media
295 was changed, and 40–42 hours post-infection, cells were washed and fixed with
296 methanol. Cells were washed with 1X TNE (15.8 g Tris-HCl, 87 g NaCl, 7.44 g EDTA,
297 700 ml H₂O pH to 7.5.), blocked (1X TNE, 10% FBS), and probed with PPR antisera (a
298 generous gift from Peggy Barr) and goat anti-cat HRP secondary antibody (MP
299 Biomedicals 55293). Cells were stained with aminoethyl carbazol (AEC Sigma A-6926)
300 solution to visualize infected cells. Infected cells were counted with a hemocytometer to
301 empirically determine the viral titer.

302 Results

303 Vif mutations affect fA3 protein degradation

304 Given that fA3Z2bZ3 is more active than fA3Z3 in preventing □Vif FIV replication (44-
305 46), we used fA3Z2bZ3 in the majority of our experiments. We initially tested fA3Z2bZ3
306 with FIV Vif C36 harboring mutations in the BC box (TLQ/AAA, TLQ/TAQ, A205L,
307 A205S, and A205T) and KCCC motif (K162R, C166S, C189S, C192S, KCCC/RSSS,
308 and ΔKCCC) (Fig 1A and 1B). Wild-type (WT) and mutant FIV Vif proteins had similar
309 intracellular patterns (Fig 1C) and expression levels (Fig 1D). In contrast, BC-box
310 mutants TLQ/AAA, TLQ/TAQ, and A205L lost A3-degradative function (Fig 2A and B),
311 while A205S and A205T maintained their function. Moreover, all KCCC mutants failed to
312 direct fA3 degradation (Fig 2C and 2D). Treatment with the proteasome inhibitor MG132
313 partially rescued degradation of FIV Vif-mediated fA3Z2bZ3 and HIV-1 Vif-mediated
314 hA3Z2g-Z1c (Fig 2E and 2F). Notably, Vif was poorly expressed when co-transfected
315 with fA3Z2bZ3 and hA3Z2g-Z1c in DMSO-treated cells (Fig 2E). These findings suggest
316 that Vif might be degraded when complexed with A3 proteins, consistent with rapid
317 turnover of Vif and concomitant degradation when associated with A3 proteins (51, 64).

318

319 Fig 1. Vif protein alignments, mutations, cellular locations and expression levels.

320 A, Amino-acid alignment of the C-terminus of HIV, SIVagm, and FIV Vif. B, FIV Vif C36
321 mutants. C, 293T cells were transfected with HA-tagged wild-type (WT) or mutant Vif
322 with or without FLAG-tagged fA3Z2bZ3. Cells were subjected to immunofluorescence
323 with antibodies to the respective epitope tags to visualize the cellular distribution of FIV
324 Vif or fA3Z2bZ3 proteins alone (top row, left column) or together. Cellular DNA was

325 stained with DAPI. *D*, Immunoblots demonstrating equivalent expression of HA-tagged
326 FIV Vif mutants.

327

328 **Vif mutations influence viral infectivity**

329 To determine whether loss of fA3 degradation correlates with increased restriction, we
330 produced a virus containing a Δvif FIV reporter both with and without fA3Z2bZ3 and the
331 various Vif proteins. Consistent with their ability to target fA3Z2bZ3 for degradation (Fig
332 2A), A205S and A205T rescued FIV infectivity by 50% in the presence of fA3Z2bZ3 (Fig
333 3A). In contrast, TLQ/AAA, TLQ/TAQ, and A205L reduced FIV infectivity by 80% in the
334 presence of fA3Z2bZ3 (Fig 3A). None of the KCCC mutants counteracted fA3Z2bZ3's
335 antiviral activity (Fig 3B). We confirmed the correlation between fA3 levels and infectivity
336 with immunoblotting (Fig 3A and 3B). These data reveal novel residues within the BC
337 box (TAQ, A205L, A205S, and A205T) and KCCC domain (K162R, C166S, C189S,
338 C192S, KCCC/RSSS, and Δ KCCC) that are required for FIV Vif activity against fA3s
339 and viral infectivity.

340

341 **Fig 2. Effects of FIV Vif mutations on fA3 protein degradation.** *A* and *B*, Specific FIV
342 Vif BC-box mutants target fA3s for destruction. 293T cells were co-transfected with 0.5
343 μ g untagged p1012, wild-type (WT) or mutant FIV Vif HA, and 1 μ g of untagged
344 pcDNA3.1(-) or FLAG-tagged fA3 (fA3Z3 or fA3Z2bZ3). Protein separation was
345 analyzed by SDS-PAGE. Immunoblot membranes were probed with antibodies
346 recognizing HA, FLAG, or tubulin proteins. *C* and *D*, FIV Vif with mutations in the BC
347 box were unable to target fA3s for degradation. 293T cells were co-transfected with or

348 without fA3s or untagged parental empty vectors with similar ratios of plasmid DNA as
349 detailed in Fig 2A and 2B. A separate set of experiments showed FIV Vif mediates
350 fA3Z2bZ3 proteasomal degradation. *E* and *F*, 293T cells were co-transfected with 0.5
351 μg of p1012 and 1 μg fA3Z2bZ3 or pcDNA3.1 (-) hA3Z2g-Z1c expression plasmids with
352 0.5 μg of WT FIV Vif or HIV-1 Vif with DMSO or MG132.

353

354 **Fig 3. Effects of FIV Vif mutants on fA3CH restriction.** *A* and *B*, Reporter viruses
355 containing FIV Δ Vif were produced in 293T cells transfected with 0.4 μg transfer vector,
356 0.2 μg pFP93 (FIV Gag-Pol and Rev) packaging vector, 0.2 μg pMD.G (VSV-G), with or
357 without 0.3 μg of fA3Z2bZ3-FLAG and/or 0.5 μg Vif-HA [wild-type (WT) or mutant]
358 plasmids, or the empty p1012 parental plasmid. The reverse transcription (RT) activity
359 of each viral supernatant was determined, and equivalent amounts of RT-normalized
360 virus were used to infect freshly plated 293T cells. Seventy-two hours after infection,
361 lysates were analyzed for luciferase activity in triplicate. Standard deviation between
362 triplicate samples was determined. FIV alone was set as 100%.

363

364 **Vif mutations alter spreading replication**

365 We then introduced four Vif mutants (KCCC/RSSS, Δ KCCC+TLQ/AAA, TLQ/AAA, and
366 C184+187S) into the full-length CT5 molecular clone to determine their effects on
367 spreading viral replication. We chose these mutants because all of the KCCC mutants
368 were defective for single-round infectivity. Stocks of mutant FIV virus were produced in
369 293T cells and tested in CrFk cells. All four mutants exhibited similar reverse
370 transcriptase (RT) activities (Fig 4A), as 293T cells lack antiviral hA3 activity, and they

371 did not negatively impact the infectious titer of the viruses (Fig 4B). Next, we infected
372 CrFK cells with WT or mutant viruses at MOIs of 0.01 and 0.1. None of the four mutant
373 viruses could replicate, while the WT virus replicated at 0.01 MOI, with large amounts of
374 RT activity at 0.1 MOI (Fig 4C and 4D).

375

376 **Fig 4. Effects of FIV Vif mutants block spreading replication of FIV.** 293T producer
377 cells were transiently transfected with VSVG-pseudotyped wild-type (WT) FIV or FIV
378 Vif-mutant molecular clones (pCT5-based). A and B, Reverse transcription (RT) activity
379 of viral supernatants and titers. C and D, Spreading replication: 4.5×10^4 CrFK cells
380 were infected at two different MOIs (0.01 and 0.1). Every 72 hours, viral supernatant
381 was collected and stored at -80°C until RT activity was determined.

382

383 **Mutant Vif proteins co-localize and interact with feline Cul5**

384 To date, the intracellular distribution of feline Cul5 (fCul5) has not been well
385 characterized. Using confocal microscopy, we found fCul5 predominately in the
386 cytoplasm, similar to Vif (Fig 5A). The KCCC mutants also display a slight intra-nuclear
387 presence, in agreement with HIV-1 Vif, which has been shown to have a minimal Vif
388 nuclear localization (65, 66). Co-immunoprecipitation studies assessed which FIV Vif
389 domains were responsible for the interaction with Cul5. While the KCCC mutants
390 interacted with fCul5 (Fig 5C, lanes 6–7), the TLQ/AAA mutant interacted poorly with
391 fCul5 (Fig 5C, lane 8), as previously reported (51). Notably, when WT or mutant FIV Vif
392 was co-expressed with fCul5 or hCul5, Cul5 expression significantly decreased (Fig 5B,
393 lanes 5–8). As a result, less FIV Vif proteins were immunoprecipitated (Fig 5C, lanes 5–

394 8), an effect not observed if hCul5 and HIV-1 Vif are co-expressed (Fig 5B, lane 4).
395 Despite multiple attempts, we were unable to detect an interaction between feline
396 Elongin B and C (fEloB and fEloC, respectively) in the presence of FIV Vif and fCul5.
397 The latter may be due to reduced Cul5, which could have limited detection of the FIV
398 Vif/E3 ligase constituents (e.g., fEloB and fEloC). Nonetheless, with these data, we
399 verified the FIV Vif/fCul5 interaction reported by Wang and colleagues (51).

400

401 **Fig 5. The FIV Vif KCCC domain does not mediate fCul5 interaction.** A, 293T cells
402 were co-transfected with 1 µg feline Cul5-Myc plasmid and 1 µg wild-type (WT) or
403 mutant FIV Vif-HA plasmid using PEI. Then, 48 hours later, cells were immunolabeled
404 and imaged using confocal microscopy. B, Cell lysates from 293T cells co-transfected
405 with 3 µg of each expression plasmid were analyzed 48 hours post-transfection C,
406 Protein-protein interactions. Lysates were immunoprecipitated with mouse anti-HA, and
407 then incubated with sheep anti-mouse beads. Immune complexes were eluted and
408 subjected to SDS-PAGE. Immunoblots were probed with a rabbit anti-Myc antibody to
409 evaluate the interaction between fCul5-Myc and WT or mutant FIV Vif-HA proteins.

410

411 **Analysis of zinc binding to FIV Vif**

412 A HCCH C-terminal motif in primate Vif proteins binds zinc and is required for Cul5
413 recognition. Feline Vifs have a similarly positioned KCCC motif. With isothermal titration
414 calorimetry (ITC), we evaluated whether the KCCC domain of FIV Vif also binds zinc
415 (59). FIV Vif proteins (Fig 6A) were expressed (Fig 6B) from the pMal-c5X vector and
416 purified. All mannose binding protein (MBP)-Vif constructs showed no evidence of

417 protein degradation (Fig 6B). Further, with size-exclusion chromatography, we
418 separated and determined the protein quality of the purified MBP-Vif mutants. Notably,
419 most of the N-terminal constructs did not bind to the amylose resin, suggesting that they
420 may have formed aggregates that were unable to bind to the matrix (Fig 6C). Only two
421 MBP-Vif mutants were monomeric (Fig 6D and 6E), as the rest formed aggregates (Fig
422 6D). Because protein aggregates can impair the biological function of a protein (67), the
423 monomeric peaks that contained the intact KCCC-domain and BC-box motifs (156–
424 213), as well as the domain that lacks the BC-box motif (156–200), were collected,
425 concentrated, and analyzed further by ITC.

426

427 **Fig 6. Expression, Purification, and Size Exclusion of FIV Vifs.** A, Schematic map
428 and numbering of purified and codon-optimized FIV_{C36} Vif. B, Expression of pre- and
429 post-induction samples analyzed by SDS-PAGE. C, Verification of size exclusion
430 chromatography SEC elutant and supernatant alongside pre- and post-induction
431 samples were analyzed by SDS-PAGE. For each construct, the first lane is the induced
432 (I) sample from Fig 6B, the second lane is the supernatant (S), the third lane is the
433 unbound (U) flow-thru from the amylose column, and the fourth lane is the protein that
434 bound (B) to and was eluted from the amylose column. D, Table of elution volumes and
435 folding status of purified Vif MBP proteins. E, Monomeric resolution of purified Vif
436 proteins (156–213 and 156–200) at 17 mL.

437

438 MBP-Vif proteins were concentrated in buffer containing excess reductant (tris-
439 carboxyethyl phosphine) to maintain Cys residues in the thiol state. These thermogram

440 data indicate that zinc failed to bind to either FIV Vif construct (Fig 7A and 7B),
441 consistent with a recent study in which the zinc chelator PTEN failed to inhibit fA3
442 degradation by FIV Vif (51). These results suggest that FIV Vif must degrade fA3
443 proteins via a mechanism distinct from that of the zinc-binding primate Vif.

444 **Fig 7. Isothermal titration calorimetry data for zinc titrations into FIV C36 Vif. A**
445 and *B*, Integrated heat data for MBP-Vifs (156–200 and 156–213, respectively).

446

447

448

449 **Discussion**

450 With our data, we revealed novel residues within the FIV Vif BC box and KCCC
451 domain that are required for infectivity and fA3 degradation. We also verified that the
452 KCCC domain does not mediate the interaction with Cul5. In addition, we showed that
453 FIV Vif does not bind zinc, and we are the first to demonstrate that FIV Vif (C36) targets
454 human or feline Cul5 for degradation by an unknown mechanism. Interestingly, this
455 phenomenon does not occur with HIV-1 Vif. These results collectively highlight a
456 complex interplay between antiviral fA3 proteins, FIV Vif, and Cul5 that impacts FIV
457 infectivity and replication.

458 Cullin proteins have an elongated structure that supports their conformational
459 flexibility and facilitates binding to a broad range of substrate adaptors, including Vif. Vif
460 usurps the host's E3-ligase complex (which contains Cul5, Rbx, E2, Elongin B, and
461 Elongin C) to steer A3 proteins to the proteasome. HIV-1 Vif contains a HCCH domain
462 that coordinates zinc binding and mediates hCul5 binding (4, 8, 17, 19). However, the
463 domain of FIV Vif that interacts with the fCul E3 ligase to target the two active anti-FIV
464 fA3 proteins (fA3Z3 and fA3Z2bZ3) is not known (11). We hypothesized that residues in
465 the KCCC motif, which has a similar location and composition as the HCCH motif in
466 HIV-1 Vif, would bind to Cul5.

467 Prior to our studies, it was unknown whether introducing the BC box and KCCC
468 mutants would disrupt the cytoplasmic distribution of Vif. Toward this end, we performed
469 confocal microscopy, which showed that the KCCC and BC-box mutants were stably
470 expressed (Fig 1D) and predominantly distributed in the cytoplasm (Fig 1C) with

471 fA3Z2bZ3 (Fig 1C). The mutants co-localized with fA3Z2bZ3 in the absence of a
472 proteasome inhibitor (Fig 1C). We believe that these FIV Vif/fA3Z2bZ3 complexes
473 represent non-degraded populations that are incompletely targeted by the proteasome
474 (51). We also tested whether previously unreported residues within the FIV Vif BC box
475 would function similarly to HIV-1 Vif. Our results showed that two FIV Vif BC residues,
476 TLQ/TAQ and A205L, were unable to target fA3 proteins for degradation (Fig 2A and
477 2B) as well as the reported TLQ/AAA mutant (48, 51). Interestingly, the A205S mutant
478 targeted fA3s and partially rescued infectivity (Fig 2A, 2B and 3A). These results are
479 consistent with the analogous A149S in HIV-1 Vif, which supports HIV-1 infectivity and
480 hA3Z2g-Z1c elimination (15). In contrast, A205T restored FIV infectivity (Fig 3A), unlike
481 the analogous A149T mutation in HIV-1 Vif (15). KCCC-domain mutants did not rescue
482 Δ vif FIV infectivity in the presence of fA3Z2bZ3 (Fig 3B), which correlates with their
483 inability to mediate fA3Z2bZ3 degradation in virus-producing cells (Fig 3B). Collectively,
484 these data support that the cytoplasmic localization of Vif proteins was not perturbed by
485 mutations in the BC box and KCCC domain. Further, we demonstrated that both
486 domains are necessary for targeting fA3s for degradation and single-round infectivity.
487 We also provide data that conserving the alanine at residue 205 significantly contributes
488 to the ability of FIV Vif to target fA3s for degradation. This finding suggests that the
489 contact interface between Vif and fA3 proteins may not exactly mimic those within HIV-1
490 Vif and should be further studied.

491 An earlier report by Wang and colleagues showed that a triple alanine mutation
492 (TLQ/AAA) and a KCCC/KCSS mutation in FIV Vif were able to block \square Vif HIV-1
493 infectivity in human cells. Here, we aimed to determine the impact of FIV infectivity and

494 spreading replication in human and feline cells, respectively. When we expressed a
495 single-cycle, Δ vif reporter virus, FIV infectivity was incompletely restored, despite WT
496 Vif being expressed in *trans*. Unfortunately, we found that studying FIV Vif
497 complementation was technically challenging. In our hands, Δ vif FIV infectivity in the
498 presence of ectopically expressed Vif was only restored by 50–70%, despite multiple
499 attempts to optimize the FIV packaging-VSVG-Vif-A3 plasmid ratios. Previous studies
500 were able to complement Δ Vif FIV infectivity with expression of WT Vif in *trans*;
501 however, different FIV Vifs that were derived from distinct isolates and packaging
502 vectors were used (44, 46). Further, Wang and colleagues restored Δ vif HIV-1 infectivity
503 with WT FIV Vif_{34TF10} expressed in *trans* (51), while we used FIV_{C36} Vif. These
504 experimental differences may explain the variation in restoring FIV infectivity between
505 studies. Moreover, our FIV Vif mutants abolished spreading replication in CrFK cells,
506 with a significant delay in FIV replication kinetics compared to WT Vif. Our data support
507 previous work showing that Δ vif FIV mutants are unable to restore FIV infectivity relative
508 to WT controls in studies of spreading replication (68, 69). Further, we observed similar
509 delays in spreading replication in CrFKs compared to peripheral blood mononuclear
510 cells (PBMCs) derived from domestic cats and co-cultured with CrFK cells infected with
511 Δ vif FIV (69). CrFK cells express all fA3 proteins; hence, the delay in spreading WT
512 replication may have been due to the antiviral activity of fA3 proteins in the absence of
513 Vif (70). In support of this, all Vif mutant viruses were replication-incompetent, and the
514 block in replication was sustained over 18 and 40 days at two different MOI (Fig 4C and
515 4D). Based on these data, the KCCC domain and BC box residues contribute to the
516 essential function of Vif activity to enable FIV replication. Although, we do not formally

517 show the mechanisms here, presumably our Vif mutants may be unable to overcome
518 the restriction of fA3 proteins necessary to recruit an E3 ligase Cullin complex to steer
519 these antiviral proteins to the proteasome.

520 Previously, localization of fCul5 was unknown. We found that fCul5 localizes to
521 the cytoplasm, similar to hCul5 (Fig 5A), and that WT and mutant Vif proteins co-
522 localize with Cul5 (Fig 5A). We determined that the BC-box mutant TLQ/AAA has a
523 reduced interaction with fCul5 and examined whether the KCCC domain served as a
524 binding motif for fCul5. By co-immunoprecipitation, we found that the KCCC mutants
525 retained their ability to interact with fCul5 (Fig 5C), demonstrating that the KCCC motif is
526 not required for the Vif-Cul5 interaction. While we cannot exclude that deleting the
527 KCCC domain alters proper folding of FIV Vif, the KCCC/RSSS mutant produced similar
528 results in that the KCCC/RSSS mutant retained its interaction with Cul5.

529 We verified that FIV Vif and fCul5 interact (Fig 5B, lane 5). Interestingly, we
530 observed reduced fCul5 and hCul5 expression in the presence of WT and FIV Vif
531 mutants, but not in the presence of HIV-1 Vif (Fig 5B, lanes 4–9). We consistently
532 observed robust co-immunoprecipitation between HIV-1 Vif and hCul5 (Fig 5C). We
533 suspect that an unidentified factor that is not expressed in fibroblasts may influence the
534 stability of the FIV Vif/fCul5 complex.

535 Curiously, fCul5 and hCul5 are 99% homologous (51). However, Wang and
536 colleagues did not observe reduced fCul5 expression in the presence of codon-
537 optimized FIV_{34TF10} Vif in their interaction studies (51), as we showed with codon-
538 optimized FIV_{C36} Vif in our co-immunoprecipitation studies (Fig 5A). Because the
539 affinities of these protein-protein interactions are largely unknown, we question whether

540 these two Vif variants differ in their ability to interact with fCul5. hA3s and HIV-1 Vif
541 require different binding motifs to interact with fA3s and FIV Vif (71). Further, Troy and
542 colleagues showed that the pathogenicity of FIV depends on the Vif isolate (70), which
543 might correlate with the interaction, or lack thereof, between FIV_{C36} Vif and fCul5. Thus,
544 our results demonstrate that a single residue within the BC box, TLQ/TAQ or A205L, of
545 FIV Vif can induce fA3Z3 and fA3Z2bZ3 degradation, and that the KCCC motif does not
546 mediate the interaction between fCul5 and FIV Vif.

547 Lastly, our biochemical ITC data showed that the KCCC domain of FIV Vif does
548 not bind zinc (Fig 7B). We were unable to express a purified form of the full-length FIV
549 Vif protein (Fig 6D), because purifying Vif is technically challenging (37, 72, 73).
550 Nonetheless, our finding that FIV Vif does not bind to zinc agrees with studies of the
551 PTEN zinc chelator carried out by Wang and colleagues (51). However, this result
552 contrasts with the zinc binding we observed for HIV-1 Vif using ITC (59). Zinc binding to
553 HIV-1 Vif changes the protein conformation and promotes interaction with Cul5. In the
554 case of FIV Vif, such a conformational change is either not required or driven by other
555 processes. Curiously, non-primate lentiviral BIV Vif requires zinc and has a novel zinc-
556 binding domain, C-x1-C-x1-H-x19-C. Visna Vif, however, does not use zinc for its
557 activity, but rather relies on a novel protein-protein interaction (74, 75).

558 While we were preparing this manuscript, Kane and colleagues showed that
559 FIV_{34TF10} Vif interacts with both hCul2 and hCul5, albeit its binding to hCul2 requires the
560 Vif N-terminal tag (75). Recruitment of Cul5 is mediated by the N-terminus of HIV-1 Vif
561 (76), and stabilization between primate Vifs and Cul5 requires CBF- β , which targets
562 hA3Z2g-Z1c for proteasomal degradation (27, 39, 72). Interestingly, FIV Vif does not

563 require CBF- β to stabilize Vif, form the fE3-ligase complex, or mediate fA3-mediated
564 degradation (39, 75), despite the identical homology between feline and human CBF- β
565 (75). A non-primate Visna Vif has evolved to use a novel cellular co-factor, Cylophilin A,
566 to promote the Visna Vif/Cul2 E3-ligase complex needed to degrade ovine A3 proteins,
567 whereas BIV Vif does not require a co-factor to eliminate bovine A3 via the proteasome
568 (75).

569 These studies illustrate the complex selection criteria that lentiviruses have
570 undergone to allow species-specific Vif to adapt to host A3 proteins (71, 75, 77, 78). We
571 hypothesize that the domestic cat may have flexible fCul2/fCul5 usage, unlike HIV-1 Vif,
572 which predominantly interacts with hCul5. The reported study by Kane and colleagues
573 focused on the FIV Vif–host interactome in human 293T cells (75). However, further
574 studies in feline cells should determine whether Vif preferentially binds fCul2 or fCul5,
575 examine if other feline Cullins interact with FIV Vif, and identify the elusive cellular co-
576 factor that stabilizes FIV Vif. Based on our results, we believe that the lentiviral/host
577 interactome hijacks novel cellular co-factors needed to eliminate the antiviral properties
578 of A3 proteins, as is the case with FIV Vif, despite 100% homology between human and
579 feline CBF- β (75). Data from these studies may serve as a platform for future
580 experiments to provide mechanistic insight important for the development of novel
581 antiviral HIV-1 therapies.

582

583

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588

589 **References**

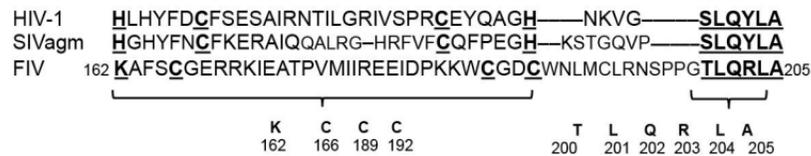
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- 809
- 810
- 811

A**B**

| | K | C | C | C | T | L | Q | R | L | A | |
|------------------|------------------|----------|----------|----------|------------------|----------|----------|-----|-----|----------|----------------|
| | 162 | 166 | 189 | 192 | 200 | 201 | 202 | 203 | 204 | 205 | |
| Mutations | | | | | Mutations | | | | | | |
| K162R | B | C | C | C | A | A | A | R | L | A | TLQ/AAA |
| | 162 | 166 | 189 | 192 | 200 | 201 | 202 | 203 | 204 | 205 | |
| C166S | K | S | C | C | T | A | Q | R | L | A | L201A |
| | 162 | 166 | 189 | 192 | 200 | 201 | 202 | 203 | 204 | 205 | |
| C189S | K | C | S | C | T | L | Q | R | L | L | A205L |
| | 162 | 166 | 189 | 192 | 200 | 201 | 202 | 203 | 204 | 205 | |
| C192S | K | C | C | S | T | L | Q | R | L | S | A205S |
| | 162 | 166 | 189 | 192 | 200 | 201 | 202 | 203 | 204 | 205 | |
| KCCC/RSSS | R | S | S | S | T | L | Q | R | L | I | A205T |
| | 162 | 166 | 189 | 192 | 200 | 201 | 202 | 203 | 204 | 205 | |
| | ΔKCCC | | | | | | | | | | |
| | Deletion 162-192 | | | | | | | | | | |

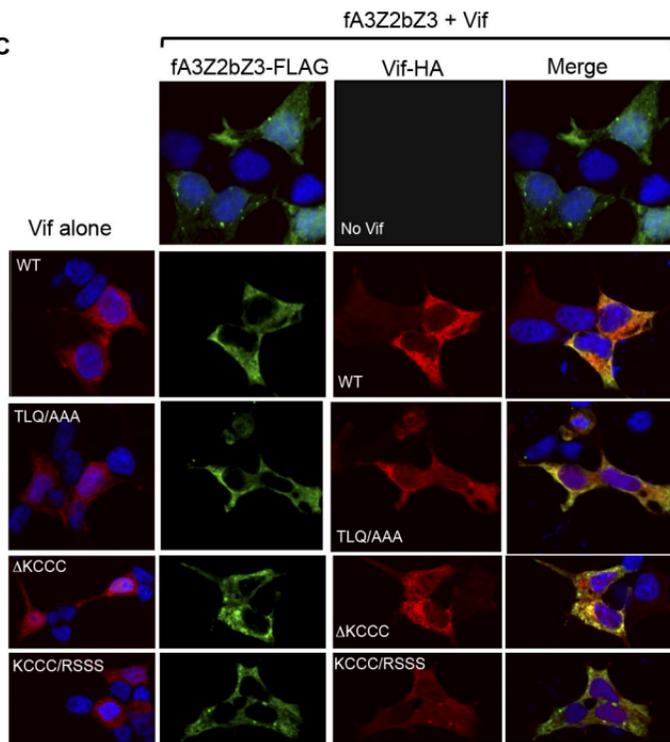
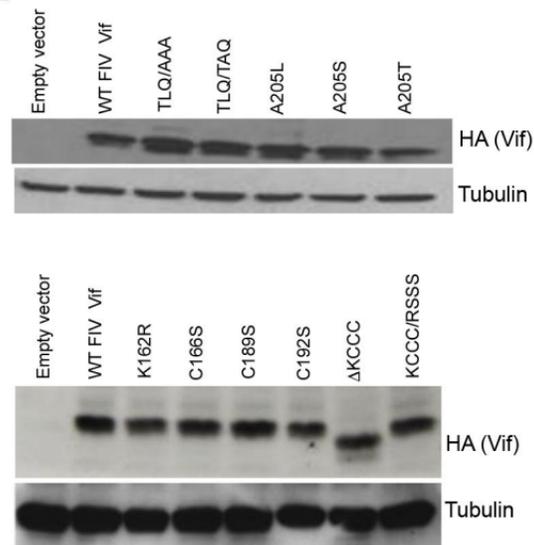
C**D**

Fig. 1

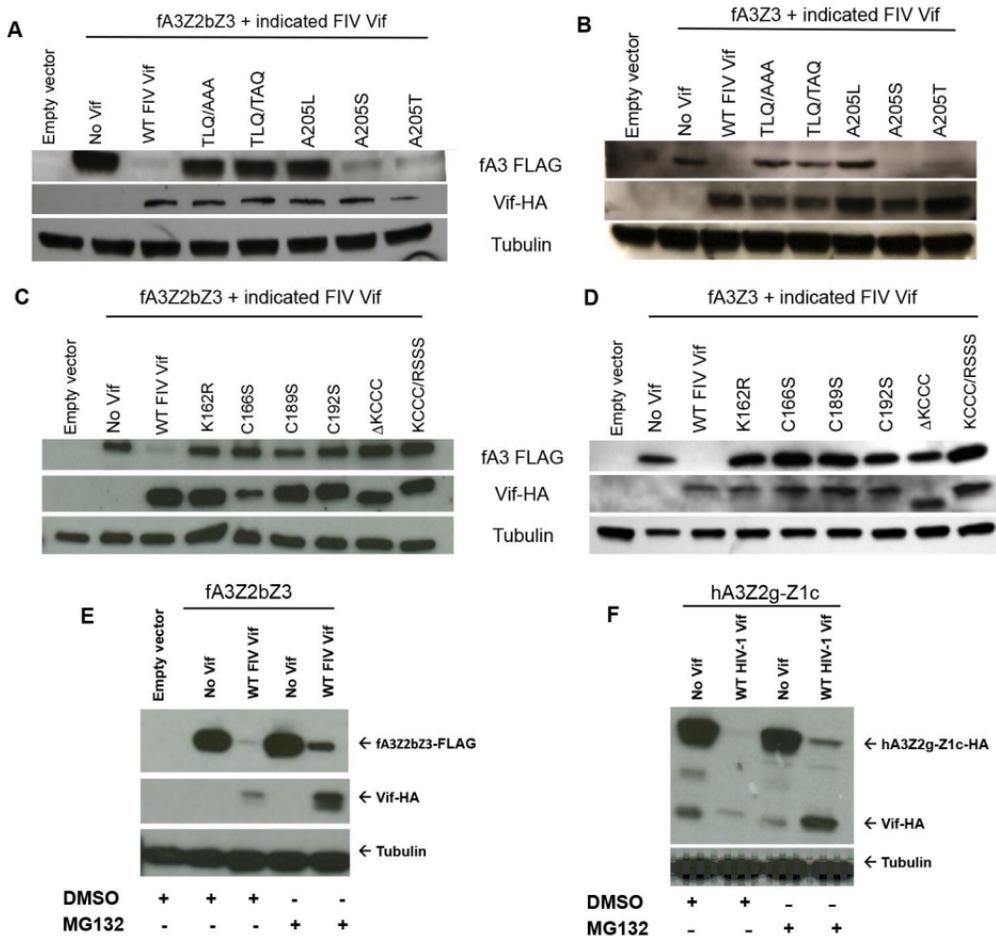


Fig. 2

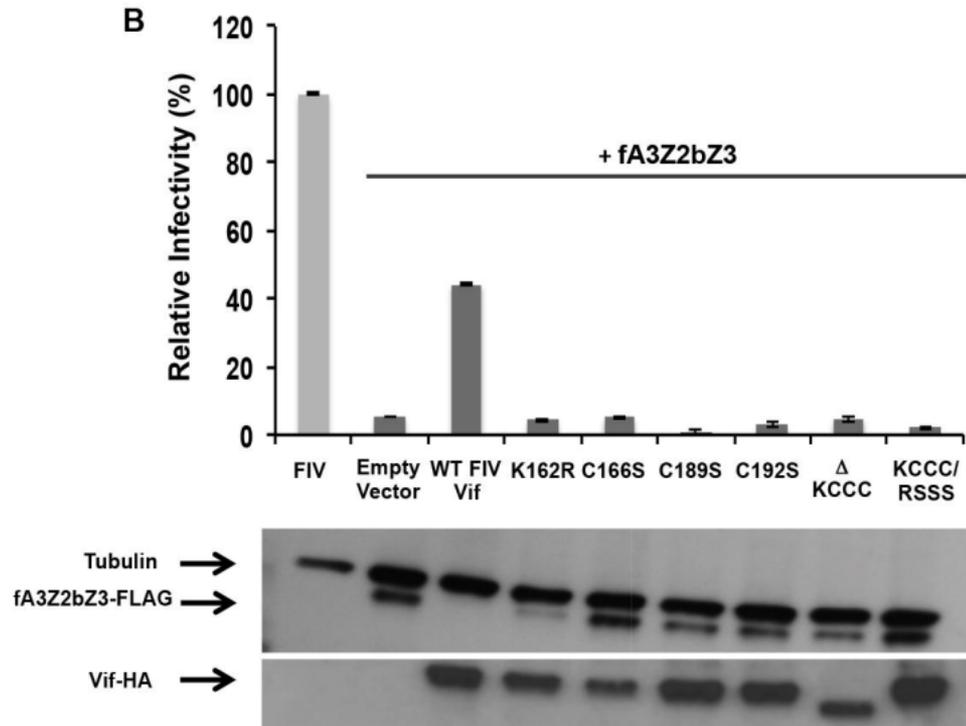
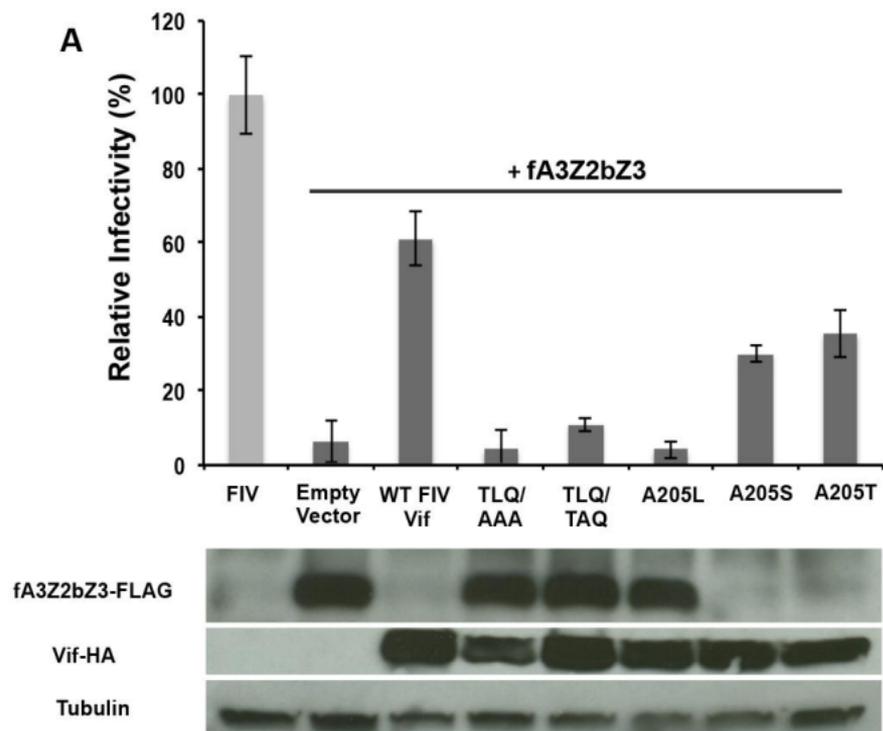
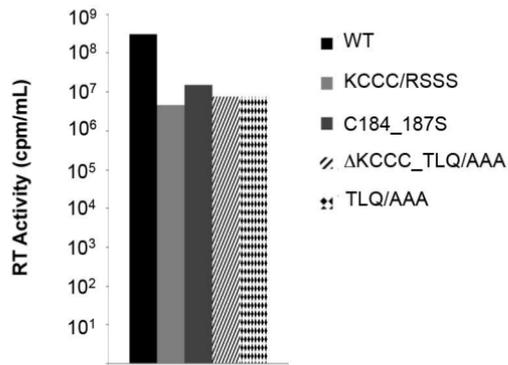
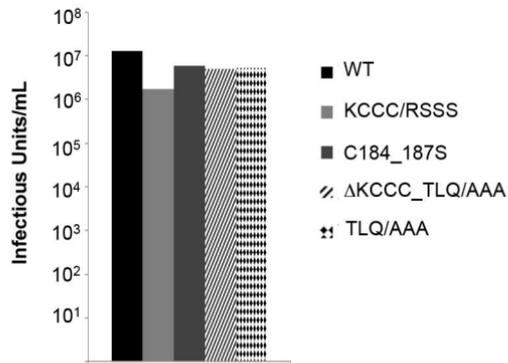
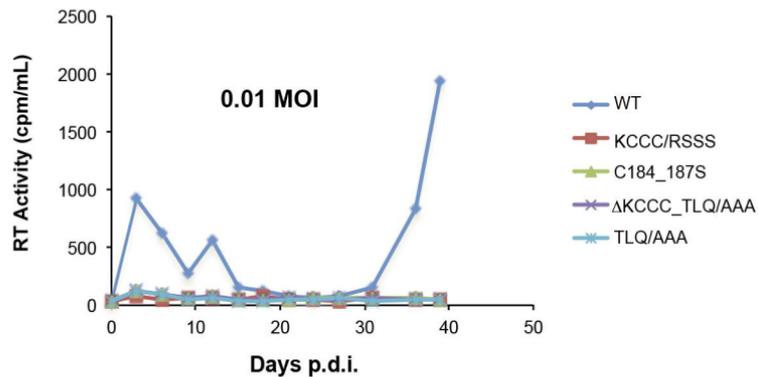
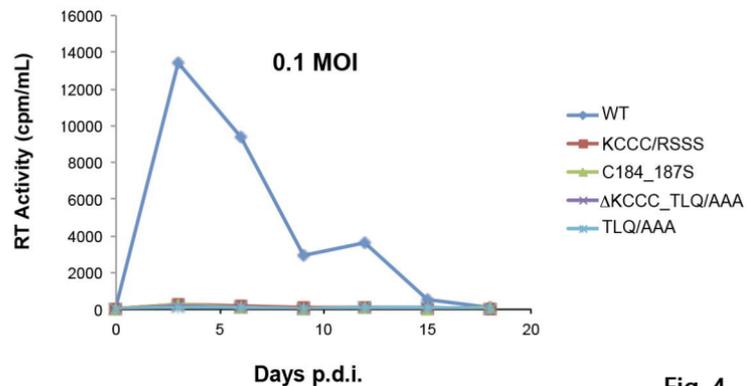


Fig. 3

A**B****C****D****Fig. 4**

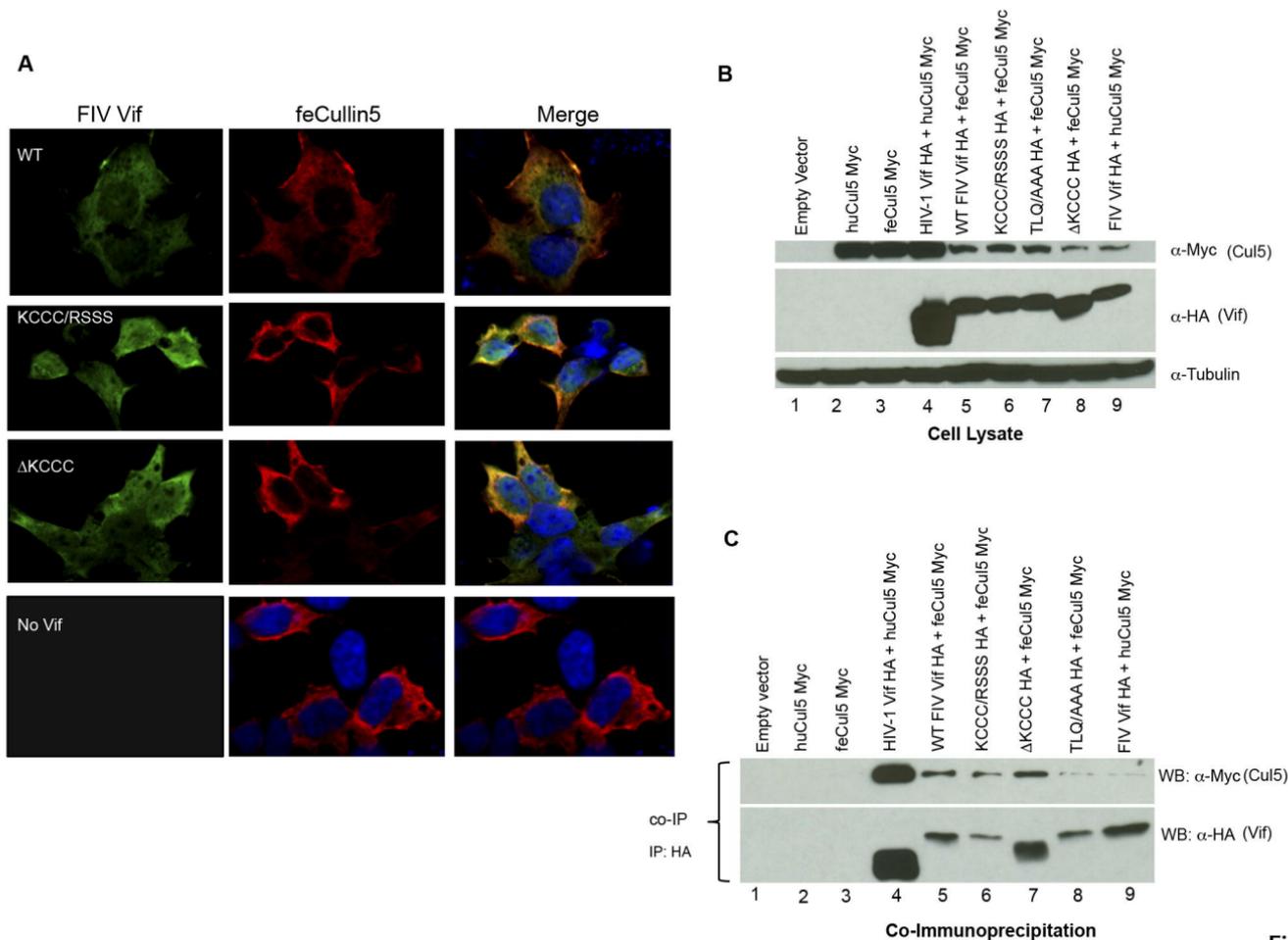


Fig. 5

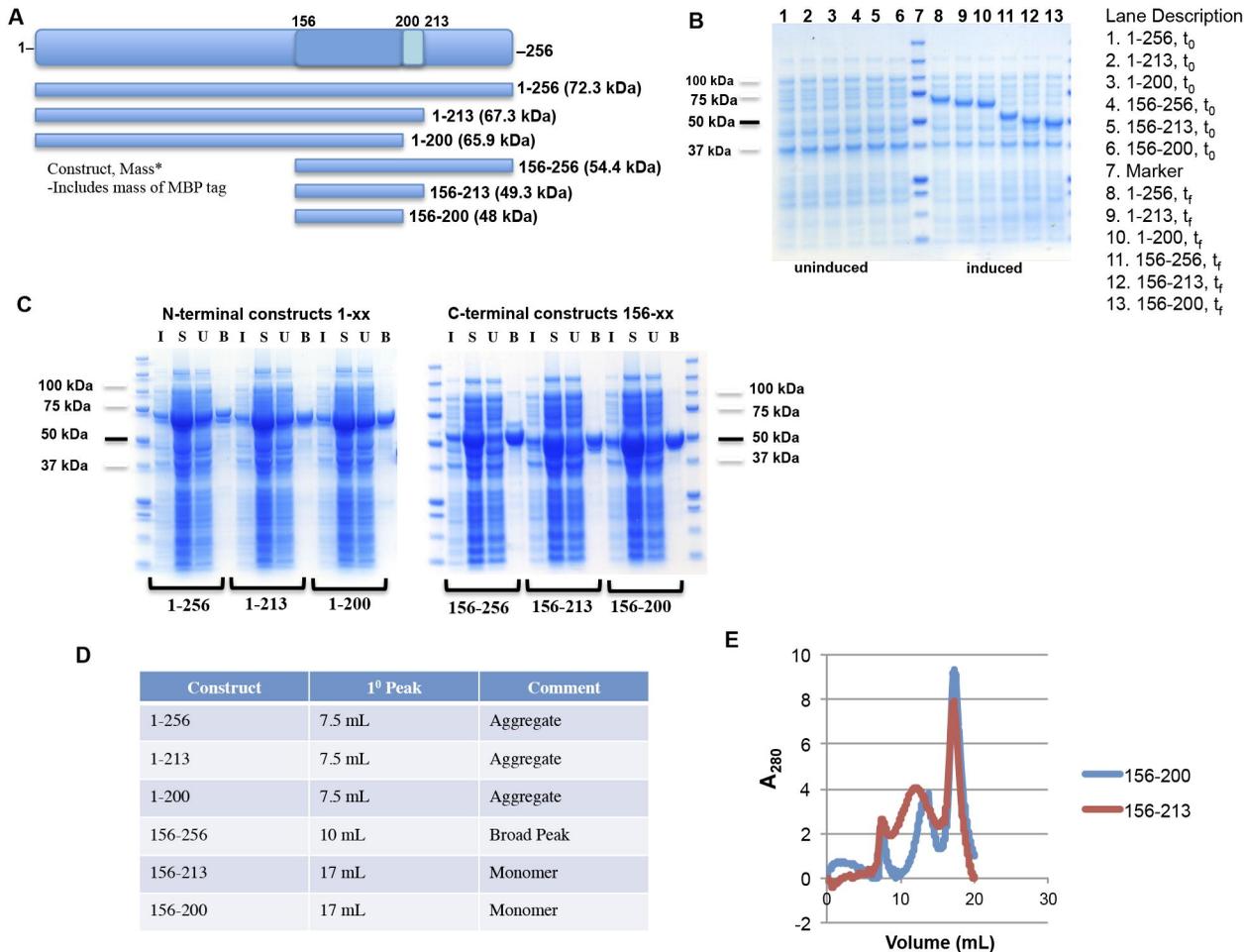


Fig. 6

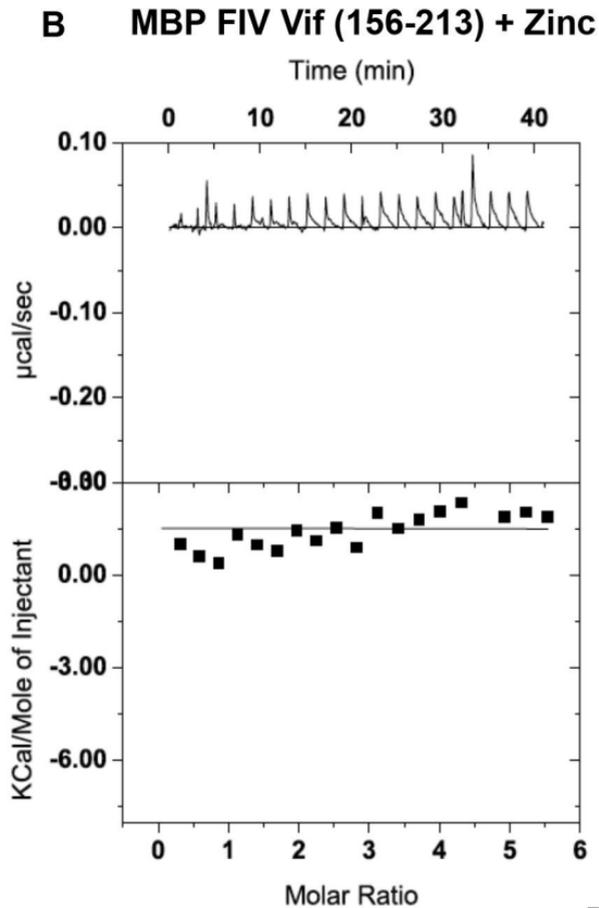
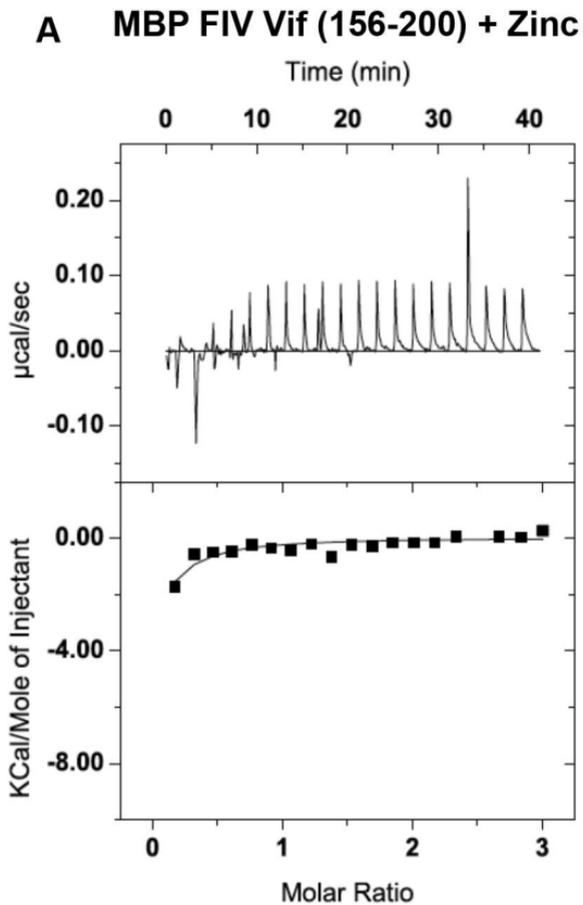


Fig. 7