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CBFβ stabilizes HIV Vif to counteract APOBEC3 at the expense of RUNX1 target gene expression

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SUMMARY

The HIV-1 accessory protein Vif hijacks a cellular cullin-RING ubiquitin ligase, CRL5, to promote degradation of the APOBEC3 family of restriction factors. Recently, the cellular transcription cofactor CBFβ was shown to form a complex with CRL5-Vif and be essential for APOBEC3 degradation and viral infectivity. We now demonstrate that CBFβ is required for assembling a well-ordered CRL5-Vif complex by inhibiting Vif oligomerization and activating CRL5-Vif by direct interaction. The CRL5-Vif-CBFβ holoenzyme forms a well-defined heterohexamer, indicating that Vif simultaneously hijacks CRL5 and CBFβ. Heterodimers of CBFβ and RUNX transcription factors contribute towards the regulation of genes, including those with immune system functions. We show that binding of Vif to CBFβ is mutually exclusive of RUNX heterodimerization and impacts expression of genes whose regulatory domains are associated with RUNX1. Our results provide a mechanism by which a pathogen with limiting coding capacity uses one factor to hijack multiple host pathways.

INTRODUCTION

Viruses interact with host factors to promote replication and achieve persistent infection. Systematic studies have revealed the global landscape of human protein interactions with HIV proteins, indicating several hundred new interactions that could impact essential pathways such as transcription, splicing, mRNA export, translation, budding and modulation of innate immunity (Bushman et al., 2009; Jäger et al., 2012a). HIV-1 and related

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lentiviruses encode just over a dozen proteins, which creates the conundrum of how a pathogen with limiting protein coding capacity efficiently leverages such a large network of physical contacts (Frankel and Young, 1998). Structural disorder in isolated HIV proteins may derive from their dependence on host partners to nucleate well-folded, functional complexes whereas flexibility may allow exchange between host complexes during different steps in the viral lifecycle or regulatory process (Daugherty et al., 2008; He et al., 2010; Tahirov et al., 2010; Xue et al., 2011).

Alternatively, a viral protein could affect multiple pathways by recruiting separate host factors to the same complex. Evidence is provided by recent studies on the lentiviral accessory protein Vif that plays a critical role in suppressing the APOBEC3 (A3) cytidine deaminase family of retroviral restriction factors. In the absence of Vif, A3 enzymes dominantly block viral replication by massive editing of viral cDNA generated during reverse transcription (reviewed by (Albin and Harris,2010)). Vif promotes the degradation of A3 family members by hijacking an ubiquitin ligase complex termed CRL5, which consists of CUL5, RBX2 and the adaptors ELOB and ELOC. The CRL5-Vif complex catalyzes formation of polyubiquitin chains on A3 substrates, targeting them for degradation by the 26S proteasome (Yu, 2003). This activity was recently shown to require the transcription cofactor CBFβ, which associates with Vif to form a CRL5-Vif- CBFβ complex (Jäger et al., 2012b; Zhang et al., 2012). CBFβ normally associates with one of three RUNX transcription factors to form obligate heterodimers that contribute towards genetic regulation of processes that include hematopoiesis, T-cell development, osteogenesis and neurogenesis (Collins et al., 2009; Shimizu et al., 2007; Stifani and Ma, 2009).

Several observations have established that Vif hijacks CBFβ to the CRL5 complex and the CRL5-Vif-CBFβ complex is required for Vif mediated A3 degradation and viral infectivity. First, CBFβ co-immunoprecipitated with CRL5 only in the presence of Vif (Jäger et al., 2012b; Zhang et al., 2012). Second, tandem affinity purification experiments revealed that APOBEC3G (A3G) substrate and CBFβ simultaneously bound CRL5-Vif (Jäger et al., 2012b). Third, mixing of co-expressed Vif, CBFβ and ELOBC with CUL5/RBX2 allowed reconstitution of the CRL5-Vif-CBFβ complex *in vitro*. This complex was capable of polyubiquitinating A3G, which recapitulated the substrate and ubiquitin chain linkage specificity observed in cells (Jäger et al., 2012b). Finally, knockdown of CBFβ reduced the stability of Vif and impaired its ability to promote polyubiquitination and degradation of A3G, reducing viral infectivity in the presence of A3G (Jäger et al., 2012b; Zhang et al., 2012).

The initial characterizations between Vif and CBFβ lead to two unresolved issues. First, although CBFβ is required for Vif-mediated A3G degradation, the mechanistic basis of this requirement is not known. We evaluated whether CBFβ promoted CRL5-Vif activity *in vitro* and investigated the molecular mechanism of this effect. Direct binding of CBFβ to CRL5-Vif enhances catalytic activity, blocks Vif oligomerization and promotes formation of a well-ordered Vif E3 ligase whose overall architecture is revealed by SAXS. Second, the CBFβ-RUNX heterodimers function to regulate classes of genes, some of which have roles in immune system function. Some observations suggest that Vif may be capable of interacting with RUNX and CBFβ proteins simultaneously (Zhang et al., 2012). On the contrary, we find that incorporation of CBFβ into CRL5 precludes its association with the RUNX family of transcription factors. We show that through this sequestration mechanism, Vif can perturb transcription of genes with RUNX1-associated regulatory domains. We suggest that primate lentiviral Vif has evolved to make use of two cellular pathways, down regulating the innate immunity provided by the APOBEC3 family of restriction factors and perturbing expression of RUNX target genes, an act that is presumably beneficial for the

virus. This dual-hijacking mechanism may be a common strategy for pathogens with a limited protein coding capacity to affect multiple cellular pathways.

RESULTS

CBFβ activates CRL5-Vif in vitro by inhibiting Vif oligomerization

To evaluate the requirement of CBFβ for the function of Vif, we compared the activity of CRL5-Vif-CBFβ with CRL5-Vif. Whereas both E3s had measureable autoubiquitination activity, only CRL5-Vif-CBFβ was able to promote ubiquitin chain synthesis on A3G substrate. Moreover, the average molecular weight of polyubiquitinated Vif was reduced in the absence of CBFβ (Figures 1A and 1B), indicating a decrease in ubiquitin ligase activity. These findings indicate CBFβ activates CRL5-Vif *in vitro*.

The inability of CRL5-Vif to ubiquitinate its substrate could be due to a defect in the assembly of Vif with CUL5/RBX2 or in the structural organization of Vif. To address the former possibility, we analyzed Vif-ELOBC and CBFβ-Vif-ELOBC by size-exclusion chromatography and assayed the ability of these Vif complexes to bind CUL5/RBX2. Analytical size exclusion chromatography indicated Vif-ELOBC was heterogeneous and oligomeric whereas CBFβ-Vif-ELOBC was relatively monodisperse and eluted at a molecular mass consistent with single-copy stoichiometry of each subunit (Figure 1C). Pull-down experiments indicated both Vif complexes can bind GST tagged-CUL5/RBX2, though CRL5-Vif was oligomeric in the absence of CBFβ (Figures 1D **and S1A**). Limited proteolysis revealed that Vif was protected from degradation when in complex with both ELOBC and CBFβ whereas in the absence of CBFβ, Vif was susceptible to proteolysis (Figures 1E and 1F). Notably, the ELOBC heterodimer was refractory to proteolysis in both complexes, consistent with it forming a structured module with the BC box helix of Vif (Stanley et al., 2008). Since Vif-ELOBC was more sensitive to proteolysis by chymotrypsin than CBFβ-Vif-ELOBC (Figure 1E), we conclude that CBFβ may shield hydrophobic residues of Vif to prevent oligomerization, possibly by stabilizing its fold (**Figure S1B-E**).

CBFβ stabilizes the Vif substrate receptor by binding a conserved hydrophobic region

Previous studies indicated the complex containing Vif, ELOBC and CBFβ functions as a substrate receptor because its binding specificity recapitulated that of the CRL5-Vif- CBFβ E3 ligase *in vitro* and in cells (Jäger et al., 2012b). We found residues 1–143 are necessary and sufficient for Vif to co-purify with CBFβ when co-expressed with ELOBC (**Figure S2**). This is in agreement with a recent report indicating the interaction between CBFβ and Vif is direct, using surface distinct from the ELOC binding sites (Zhou et al., 2012).

We next determined which residues within 1–143 of Vif are required for CBFβ binding. Since at least three different A3 enzymes and CUL5 are implicated in binding separate epitopes within residues 1–143 of Vif, it is unclear how CBFβ can co-occupy this region (Figure 2A) (Chen et al., 2009; Zhen et al., 2010) and reviewed in (Smith et al., 2009). Clues to where CBFβ binds are provided by several segregation of function mutants that preclude Vif mediated degradation of A3G but permit degradation of A3F, and *vice-versa* (Smith et al., 2009). In contrast, mutation of residues in a conserved hydrophobic region spanned by residues 58–72 impair the ability of Vif to promote degradation of A3G, A3F and A3H (the FGH box) (He et al., 2008; Zhen et al., 2010) (Figure 2A and 2B), suggesting a general loss of function. Since CBFβ protects hydrophobic residues of Vif from proteolysis (Figure 1E) and is required for Vif-mediated degradation of all APOBEC3 family members (Hultquist et al., 2012), we hypothesized that the FGH box could be required for CBFβ binding and not A3 substrates *per se*. To test this possibility, we made alanine or serine substitutions in the A3 boxes and assayed the ability of CBFβ to copurify with Vif-ELOBC coexpressed in *E.*

coli (Figures 2A and 2C). Substitution of Leu64 or Ile66 with serine abolished the ability of CBFβ to co-purify with Vif-ELOBC but did not disrupt the Vif-ELOBC interaction. These mutations also reduced the amount of CBFβ that co-immunoprecipitated with Vif expressed in 293T cells (**Figure S2D**). In contrast, lesions in the G box (the Arg41/His42 to alanine double mutant) have no effect on copurification of CBFβ-Vif-ELOBC. Vif complexes expressed without ELOBC or containing lesions in the F1 box (the Asp14/Arg15 to alanine double mutant) do not efficiently bind the affinity matrix, possibly due to reduced solubility and aggregation. Substitutions in Thr68 and Tyr69 had an intermediate effect on the ability of CBFβ to copurify with Vif-ELOBC. We conclude that CBFβ may promote ubiquitination of A3 substrates by interacting with hydrophobic residues in the FGH box of Vif, possibly to configure the substrate receptor for specific interactions with A3 proteins.

The Vif substrate receptor is an elongated, rod shaped particle

Vif is a small protein of only 192 amino acids. To understand how it nucleates interactions with CRL5, CBFβ and substrates, we sought to determine the molecular envelope of the Vif substrate receptor (CBFβ-Vif-ELOBC). First, we determined its stoichiometry using size-exclusion chromatography followed by multi-angle laser light scattering (SEC-MALLS). We found that the calculated molecular weight of the complex was about 68 kDa, indicating the substrate receptor was monomeric in solution with each subunit at single copy stoichiometry (Figure 2D and Table S2). We subjected the substrate receptor to small angle x-ray scattering in solution and found it to be monodisperse and folded under conditions of the experiment and therefore suitable for envelope analysis (**Figures S2D-F and** Table S2). As a control, we also analyzed a prototypical substrate receptor for the CRL5 complex, SOCS2/ELOBC, for there is a known crystal structure (Bullock et al., 2006). The pair-wise distance distribution function computed from the raw SAXS intensity data indicated that the Vif substrate receptor was an elongated particle in solution whereas SOCS2/ELOBC was more compact (Figure 2E). The molecular envelope for SOCS2/ELOBC was in good agreement with its crystal structure (Figure 2F). The extended structure of the Vif substrate receptor may allow it to accommodate A3 substrates using different epitopes (Figures 2G, **S2G and S2H**).

CRL5-Vif-CBFβ is a monomeric particle with three lobes

Previous reports indicated Vif to be oligomeric in solution but our findings suggested it formed a discrete, well-ordered monomeric complex when bound by CBFβ and ELOBC (Auclair et al., 2007; Marcsisin and Engen, 2010; Paul et al., 2006; Techtmann et al., 2012) (Figure 1). To determine if assembly with CUL5/RBX2 caused a major change in oligomerization or conformation of the Vif substrate receptor, we performed SEC-MALLS and SAXS analysis on the CRL5-Vif-CBFβ complex. SEC-MALLS and Coomassiestained SDS-PAGE gels indicated that the Vif holoenzyme was a monomer consisting of a single copy of each protein (Figure 3A and Table S2) (Jäger et al., 2012b). Consistent with this interpretation, isothermal titration calorimetry indicated the substrate receptor bound CUL5/RBX2 with 1:1 stoichiometry and high binding affinity (Figures 3B and 3C). Taken together, these findings indicated the hexameric assembly was well-folded, monodisperse and suitable for SAXS analysis (**Figures S3A-C and** Table S2). Accordingly, distance distribution functions and molecular envelopes were computed (Figures 3D-G). CUL5/RBX2 and a related cellular complex, CRL5-SOCS2, were used as controls for the envelope quality and to guide mapping of the Vif substrate receptor onto CUL5/RBX2. The envelope model revealed that the hetero-hexameric complex of CRL5-Vif-CBFβ had a tri-lobed architecture. The additional rod-like density in CRL5-Vif- CBFβ, which remained after superposition with CUL5/RBX2, was consistent with the envelope of CBFβ-Vif-ELOBC (Figures 2G, 3E and 3G). Comparison with CRL5- SOCS2 suggested that CBFβ was located on the tip of the substrate receptor pointing away from CUL5/RBX2. This observation was

in line with the observation that CBFβ did not co-IP with CRL5 in the absence of Vif (Figures 3F and 3G) (Jäger et al., 2012b; Zhang et al., 2012). Altogether, these results indicated Vif directly bound CBFβ and recruited it into a complex with CRL5 whose envelope structure, including the large distance between substrate receptor and RING domain, was qualitatively similar to cellular Cullin-RING ligases (Zheng et al., 2002). This observation is consistent with the requirement of post-translational modification of CRL5 by NEDD8 for activation of Vif (Duda et al., 2008; Saha and Deshaies, 2008; Stanley et al., in Press; Yu, 2003).

Binding between Vif and RUNX proteins to CBFβ is mutually exclusive

Since CBFβ is hijacked by Vif to CRL5, we next asked if assembly of the Vif substrate receptor containing CBFβ was mutually exclusive with RUNX binding. Accordingly, we incubated the Runt domain of RUNX 1 (RUNT1) with a preassembled CBFβ-Vif-ELOBC complex and subjected the mixture to analytical size-exclusion chromatography. Increasing concentrations of RUNT1 up to 16-fold molar excess failed to bind CBFβ-Vif ELOBC (Figure 4A). However, under similar conditions RUNT1 formed a stable complex with CBFβ at one molar equivalent when incubated in the absence of Vif/ELOBC (Figure 4B). Similar results were obtained with the Runt domains of RUNX2 and RUNX3 (**Figures S4A-D**). These observations suggest binding of CBFβ to Vif and the Runt domains of the RUNX family of transcription factors were mutually exclusive. Since a previous report indicated overexpressed RUNX1 could co-IP with Vif (Zhang et al., 2012), we examined this possibility in HEK293T cells but the interpretation was confounded by nonspecific binding of RUNX1 and Vif to the affinity resins used for our experiments (**Figures S4E and S4F**). To further address this issue, we assayed the ability of endogenous RUNX proteins co-IP with Vif or CBFβ stably expressed in Jurkat T cells. Epitope tagged CBFβ did co-IP with RUNX proteins, whereas epitope tagged Vif did not (Figure 4C). These observations are consistent with extensive affinity-purification and MS analysis of Vif interaction partners in Jurkat T-cells that revealed endogenous CBFβ did co-purify with Vif whereas RUNX proteins did not (Jäger et al., 2012b). Together, these results are consistent with the notion that Vif binds CBFβ mutually exclusive of endogenous RUNX transcription factors in cells.

Previous truncation deletion analysis of CBFβ were interpreted to suggest Vif bound CBFβ at an epitope outside of the RUNX binding site; however, expression of all the CBFβ truncation mutants were severely reduced in this study, suggesting the protein may not have folded properly when elements of regular secondary structure were deleted (Zhang et al., 2012). Since we found that the association of Vif and Runt domains with CBFβ was mutually exclusive, we reasoned that residues on CBFβ lining the interface with RUNT1 might contact Vif. To test this possibility, we generated single and double alanine substitutions of residues R3, G61, Q67, and N104 on CBFβ (Figure 4D) and determined if these mutants co-purified with Vif-ELOBC expressed in *E. coli*. Previous functional, biochemical and structural studies implicated these residues in RUNT domain binding. For example, single alanine substitutions in these residues reduced RUNT binding by 7, 30, 10 and 40-fold respectively, while retaining the native conformation as judged by HSQC NMR (Huang et al., 1999; Tahirov et al., 2001; Tang, 2000). We found substitution of N104 with alanine completely blocked the ability of Vif to co-purify with CBFβ whereas mutation of R3, Q61 and Q67 to alanine did not disrupt the interaction (Figure 4E). The 69–90 loop of CBFβ was previously found to be necessary for CBFβ binding to Vif (Zhang et al., 2012). We found deletion of this region affected the oligomeric state of CBFβ, likely by altering its structure (**Figure S4G**). We conclude that the Vif binding surface on CBFβ at least partially overlapped with the Runt domain of the RUNX family of transcription factors, though we cannot exclude the possibility that additional surfaces are involved.

Vif inhibits transcription of a RUNX1 reporter gene by competition with CBFβ

Key surface residues (e.g. N104) on CBFβ mediate the interaction with the RUNX proteins. Given that Vif binds this surface of CBFβ and prevents assembly of Runt-CBFβ complexes *in vitro*, we determined how Vif affects the transcription of a reporter gene whose expression requires RUNX1 association at its promoter. We performed this assay in HEK 293T cells, and transfection of RUNX1 alone stimulated the reporter gene by 40- fold whereas control transfections lacking RUNX1 but containing CBFβ, Vif or empty vector did not activate the reporter gene (Figure 5A **and data not shown**). Co-transfection of RUNX1 and increasing amounts of Vif reduced reporter gene transcription in a dose-dependent manner by roughly four-fold while overexpression of CBFβ alleviated the inhibitory effect of Vif (Figure 5A). Critically, overexpression of N104A mutant of CBFβ did not alleviate inhibition by Vif, indicating Vif repression of transcription can be rescued by CBFβ only if it can form a functional complex with RUNX1 (Figure 5A).

To confirm that endogenous CBFβ contributed towards the RUNX1-mediated expression of the RUNX reporter gene, we employed a cell line in which CBFβ was stably knocked down (Figure 5B) (Jäger et al., 2012b). Knockdown of CBFβ reduced reporter gene expression by more than 2-fold and over-expression of CBFβ restored transcription of the reporter gene (Figure 5B). Overexpression of CBFβ in the presence of increasing amounts of transfected Vif also resulted in a dose-dependent decrease in reporter gene expression (Figure 5B). Interestingly, steady-state levels of CBFβ were reduced when the amount of Vif in transfection reactions was increased, which suggested that Vif can promote degradation of CBFβ (Figure 5B). Consistent with this observation, CBFβ was autoubiquitinated when in complex with CRL5-Vif (**Figure S5A**). The same effects were observed in a control cell line in which CBFβ was not knocked down, although expression of the reporter gene was augmented due to the presence of endogenous CBFβ (Figure 5C **and Figure S5B**). Importantly, residues 100–192 of Vif containing the BC and CUL5 boxes did not repress transcription whereas residues 1–100 or 1–143 were capable of repressing transcription, but to a lesser degree than full-length Vif (Figure 5B and 5C). These data were consistent with the notion that the N-terminal region of Vif acts by sequestering CBFβ from binding RUNX and suggest that Vif targets CBFβ for degradation by the proteasome.

Vif modulates the expression of genes regulated by RUNX1 in T-lymphocytes

Two observations suggest the Vif-CBFβ interaction may modulate gene expression in the host cell. First, CBFβ cannot bind to the Runt domain and Vif simultaneously (Figure 4). Second, RUNX1-mediated reporter gene expression was repressed by Vif and restored by overexpression of CBFβ (Figure 5). It had been previously shown that CBFβ-RUNX heterodimers regulate the expression of a variety of genes, including those involved in immune processes (Collins et al., 2009; Taniuchi et al., 2002; Wong et al., 2011). Cells infected with HIV experience changes in gene expression, and the mechanisms that promote these changes are not fully understood (Chang et al., 2011; Giri et al., 2006). The interaction between Vif and the transcription cofactor CBFβ represents a potential pathway through which the virus modulates host gene expression. We sought to determine the existence of this potential phenomenon by studying which host genes are affected by Vif and whether the regulatory domains of these genes are associated with RUNX1.

First, we first determined where RUNX1 binds chromatin in a Jurkat T cell lymphoblast line via chromatin immunoprecipitation (ChIP) using polyclonal antibodies against endogenous RUNX1, followed by high throughput sequencing of co-precipitating DNA (ChIP-seq). Analysis of consensus RUNX1 enrichment regions found in two biological replicates revealed that the top scoring motif closely resembled the consensus motif recognized by the DNA binding domain of RUNX1 (Figure 6A) (Melnikova et al., 1993). The majority of

these RUNX1 enrichment regions were associated with 4,122 genes at a median distance of 12,043 bp from transcription start sites, suggesting enrichment for promoter regions (Figure 6B). These results suggest we have defined a high confidence set of gene regulatory domains bound by RUNX1 *in vivo*.

We determined the effects of Vif on gene expression in a derivative of a T cell line that carries a stably integrated, inducible Vif gene construct (Jäger et al., 2012a). The cell line lacking a Vif expression construct was used as a reference for determining Vif-dependent gene expression changes. Based on attribute term enrichment analyses (McLean et al., 2010) that we performed on our set of genes with RUNX1-associated regulatory domains and because RUNX1 has demonstrated roles in regulation of genes tied to the immune system, we reasoned that Vif-CBFβ modulation of gene expression may only become apparent in immunologically activated cells. Therefore, we chemically activated cells by phorbol 12-myristate 13-acetate (PMA) and phytohemagluttinin (PHA) treatments for 4 and 6 hours prior assaying gene expression changes. In total, 288 differentially expressed genes that had a minimum absolute change of at least 1.5 fold after 4 hours of PMA/PHA treatment were identified, which increased to 355 after 6 hours (Figure 6C). Based on our RUNX1 genome-wide association data in nonactivated cells, we determined that 101 of 288 genes (hypergeometric $p=5.6\times10^{-6}$) at 4 hours and 118 of 355 genes ($p=1.7\times10^{-5}$) at 6 hours had RUNX1-associated regulatory domains. In sum, a total of 155 differentially expressed genes with RUNX1-associated regulatory domains were observed after 4 or 6 hours of PMA/PHA treatment (Figure 6D and Table S3). Interestingly, these include interferon gamma (IFNG), the MHC II DQ beta subunit HLA-DQB1, several proteins implicated in transcription (e.g., T-bet/TBX21, KLF2), interleukins and their receptors (e.g., IL4, IL10, IL7R), proteins associated of the TNF superfamily (e.g., TNFRSF10D, TNFSF8), metallopeptidases (ADAMTS19, MME) and STATH, which is a salivary protein. Significant changes in gene expression from 4 to 6 hours were observed in a few cases, possibly due to phenomenon such as adaptation in signaling pathways. Analysis of enriched gene ontology terms (DAVID FAT biological processes) revealed that at both 4 and 6 hours of activation, “immune response” was the top scoring ontology term for differentially expressed genes associated with RUNX1 (**Figure S6**) (Huang da et al., 2009). These findings indicated Vif can affect expression of genes associated with RUNX1 binding sites in activated T-cells.

HIV infection represses transcription of a RUNX gene in a Vif-dependent manner

Since overexpression of Vif in Jurkat T-cells perturbed expression of genes whose regulatory domains are associated with RUNX1, we determined whether activation of HIV-infected T-cells by PMA and ionomycin similarly affects RUNX-mediated gene expression in a Vif-dependent manner. We studied expression of the T-box transcription factor T-bet because ChIP-seq indicated it has a RUNX1 regulatory domain and is differentially expressed in Jurkat cells overexpressing Vif (Figures 7A,C and Table S3). We infected SupT11 T-cells with HIV-1 NL4-3 reference virus and mutant NL4-3 viruses that either cannot express Vif (HIV_{NL4-3} ΔVif) or express a Vif mutant that cannot bind CBFβ (HIV_{NL4-3} Vif I64S-L66S). We confirmed that the spreading kinetics of HIV_{NL4-3} Vif I64S-L66S infection was impacted in an A3G-dependent manner as would be expected if Vif cannot bind CBFβ (**Fig S7**).

At an multiplicity of infection (MOI) of one, induction of T-bet expression by 4 hours of PMA/ionomycin stimulation was impacted in SupT11 cells acutely infected with HIV_{NL4-3} but not in cells acutely infected with HIV_{NL4-3} ΔVif or HIV_{NL4-3} Vif I64S-L66S (Figure 7B). Parallel immunoblot analyses for T-bet in these activated cells indicated that cells infected with HIV_{NL4-3} had reduced levels of T-bet protein compared to cells mock infected or infected with HIV_{NL4-3} ΔVif or HIV_{NL4-3} Vif I64S-L66S (Figure 7D). Expression of Vif

was impaired by the I64S,L66S mutations, consistent with the notion that interaction with CBFβ stabilizes Vif (Figure 7D and (Jäger et al., 2012b). When cells were acutely infected (MOI=5), there was a more significant restoration of T-bet mRNA levels with HIV_{NL4-3} ΔVif or HIV_{NL4-3} Vif L64S-I66S (**Figure S7C**). The repression of T-bet by HIV_{NL4-3} depended on MOI in a dose dependent manner that was partially alleviated when infections were performed with HIV_{NL4-3} ΔVif (**Figure S7D**). Altogether these data indicate that activation-dependent expression of T-bet, which has a RUNX1 binding site in its regulatory domain, does not proceed normally in HIV-infected cells. This abnormal T-bet expression appears to be dependent on Vif and the interaction between Vif and the RUNX1 binding partner CBFβ.

DISCUSSION

Recently it was shown that the transcription cofactor CBFβ is recruited by Vif to form a CRL5-Vif-CBFβ E3 ubiquitin ligase complex required for the degradation of APOBEC3 family members and viral infectivity (Hultquist et al., 2012; Jäger et al., 2012b; Zhang et al., 2012). The mechanism by which CBFβ promotes Vif-mediated APOBEC3 degradation has not been determined. Here, we have shown that CBFβ stimulates activity of CRL5-Vif *in vitro* by promoting a well-ordered substrate receptor. Numerous reports indicate Vif is intrinsically unstructured on its own and that the C-terminal half of Vif undergoes a disorder-to-order transition when binding ELOBC and CUL5 (Auclair et al., 2007; Bergeron et al., 2010; Marcsisin and Engen, 2010; Marcsisin et al., 2011; Paul et al., 2006; Stanley et al., 2008; Techtmann et al., 2012). Our limited proteolysis data are consistent with the idea that CBFβ could promote local folding of the N-terminus of Vif by binding a separate epitope. However, we cannot disregard the possibility that CBFβ may protect Vif from proteolysis by simply shielding a hydrophobic binding surface on Vif. In either case, it is clear that CBFβ prevents oligomerization of CRL5-Vif and allows formation of an active Vif E3 holoenzyme *in vitro* and in cells.

The combined biochemical and SAXS data suggest CBFβ and ELOBC bind Vif on separate epitopes to form a stable substrate receptor, suggesting that Vif is sandwiched between ELOC and CBFβ. This model explains why multivalent interactions of host factors and Vif are required to obtain a well-ordered complex that recapitulates the specificity for A3 substrates (Jäger et al., 2012b). This modular receptor can bind CUL5/RBX2 without drastic alterations to its rod like structure. It has a large surface area, which may be crucial for forming interactions with different A3 substrates using separate epitopes. Though recent mutational studies of CBFβ indicate separate regions of the protein are important for RUNX mediated transcription and stabilization of Vif (Hultquist et al., 2012), the simplest interpretation of the available data is that Vif binds CBFβ on a surface that partially overlaps with RUNX proteins. High-resolution structural studies will be required to fully explain the interactions that promote assembly of CRL5- Vif-CBFβ and the interactions of the substrate receptor with A3 enzymes.

In uninfected cells, CBFβ forms an obligate heterodimer with the RUNX family of transcription factors (Wang et al., 1996), which raises the possibility that the hijacking of CBFβ by Vif could modulate gene expression mediated by CBFβ-RUNX. Previously it was suggested that CBFβ and RUNX proteins bind Vif at distinct sites (Zhang et al., 2012). This conclusion was reached by creating a panel of CBFβ deletion mutants and then determining whether these mutants co-precipitated with Vif. It was found that residues comprising loop 3 or helix 4 of CBFβ were required for coprecipitation of Vif and CBFβ. Based on the solved RUNX1-CBFβ structure, loop 3 and helix 4 of CBFβ do not interface with RUNX1, and deletions of residues in CBFβ that are required for RUNX1-CBFβ binding did not prevent coprecipitation of Vif and CBFβ (Zhang et al., 2012). We argue that entire deletion of

internal loops or elements of regular secondary structure could alter the fold of a protein and result in non-specific interactions, confounding the interpretation. Accordingly, we used competition assays and structure guided sitedirected mutagenesis of CBFβ residues to evaluate which surfaces on CBFβ are required for binding to Vif. We determined that binding of Vif to CBFβ is mutually exclusive of RUNX based on several observations. First, preassembled substrate receptor (CBFβ-Vif-ELOBC) is unable to bind the Runt domains of RUNX1, RUNX2 and RUNX3, and the Runt domain is necessary for heterodimerization of RUNX and CBFβ proteins. Second, CBFβ point mutants that block interaction with RUNX transcription factors and coactivation of RUNX transcription fail to co-purify with Vif. Third, Vif is able to immunoprecipitate CBFβ, but not RUNX proteins from T-cells. Fourth, Vif inhibits transcription of a RUNX1 reporter gene in a dose dependent manner, and concomitant overexpression of CBFβ alleviates the transcriptional repression. Our evidence supports a model in which there is a mutually exclusive association of RUNX and Vif proteins with CBFβ.

The inability for CBFβ to simultaneously bind Vif and RUNX suggests that the association of CBFβ with Vif may impact the pool of CBFβ-RUNX heterodimers that are involved in gene expression regulation. To test this, we employed a microarray platform to conduct differential gene expression analysis to understand how Vif modulates host gene expression. Jurkat T-cell lymphocytes expressing Vif were chemically stimulated with PMA and PHA to mimic downstream effects of the T cell receptor signaling pathway. This system is appropriate for study in the context of HIV infection because the virus infects and ultimately kills CD4+ T-cells, each of which has a unique T cell receptor from which immune response signals nucleate when a pathogen is detected. We discovered that Vif contributes towards the regulation of gene expression and determined that the Vif-CBFβ interaction is seemingly the cause for at least some observed gene expression changes because a statistically significant number of these genes have regulatory domains associated with RUNX1. Among these, T-bet expression was found to be downregulated during HIV infection in a Vif dependent manner (Table S3). T-bet is a master transcription factor that represses expression of interleukin-2 (IL-2) which is important for T-cell growth, proliferation and differentiation after antigen presentation (Szabo et al., 2000). Though we had difficulty reliably detecting IL-2 mRNA in mock infected SupT11 cells or in cells infected with mutant or Vif deficient virus we did observe a reciprocal relationship between T-bet and IL2 mRNA levels in Jurkat cells when Vif was expressed. These findings suggest Vif could impact transcription factor networks that affect cytokine production by interfering with RUNX function.

Other differentially expressed genes may be regulated by the RUNX2 or RUNX3 transcription factors that heterodimerize with CBFβ. Other host factors regulated by Vif may also be impinging on the transcriptional regulation of host genes. For example, we had previously found other transcription and chromatin regulators, including the HDAC3/NCOR complex, physically associated with Vif in our proteomic screen (Jäger et al., 2012a). Further examination of the network of genes modulated by Vif will reveal how the changes contribute to HIV replication and infectivity, and the Vif-CBFβ interaction provides mechanistic insight in how HIV modulates gene expression.

In a typical viral hijack of the ubiquitin-proteasome pathway, a viral protein binds a cellular ubiquitin ligase by acting as a molecular mimic of a cellular substrate specificity factor, relegating a divergent epitope for recognition of a host factor that normally impedes infection (Li et al., 2006; Li et al., 2009). We have shown that Vif extends this paradigm by recruiting CBFβ to CRL5 to form a new complex (CRL5-Vif-CBFβ) that promotes elimination of A3 restriction factors and interferes with RUNX mediated transcription by sequestering CBFβ. Binding of CBFβ to Vif is coupled to Vif stabilization with concomitant occlusion of the RUNX binding site. We propose a sequestration model in which the

association of Vif and CBFβ depletes the pool of CBFβ available for forming CBFβ-RUNX heterodimers (Figure 7E). In this way, Vif depends on CBFβ for ubiquitination at the expense of normal transcription of RUNX target genes. This dual hijack mechanism is an economical strategy for a pathogen with limited protein coding capacity to perturb multiple host pathways. The biochemical, structural and genomewide expression studies reported here lay the foundation for exploring the hypothesis that Vif could modulate a subset of RUNX-controlled genes during infection to affect Tcell development or the immune response to HIV.

METHODS

Pull down experiments

Bacterial (*E. coli*) or mammalian (HEK293T) lysates were obtained after respective transformation or transfection with vectors expressing Vif, CBFβ and ELOBC. CBFβ-Vif-ELOBC complexes were purified with Strep-Tactin resin. GST pull-down assays were conducted by first attaching GST-RBX2-CUL5 to GST resin and then loading Vif_{HXB2}-ELOBC or CBFβ-Vif_{HXB2}-ELOBC.

Limited proteolysis

Either chymotrypsin or trypsin was incubated with Vif substrate receptor complexes (Vif-ELOBC and CBFβ-Vif-ELOBC) at a 1:100 ratio. The reaction was quenched after 2, 5, 10, 30 and 60 minutes by adding SDS buffer. The proteolysis reactions were fractionated by SDS-PAGE and visualized by Coomassie staining.

Ubiquitination assays

NEDD8ylated CUL5/RBX2 was incubated in buffer containing UBE1, E2 enzymes UBE2R1 or UbcH5b, substrate receptors Vif-ELOBC or CBFβ-Vif- ELOBC, HA-tagged APOBEC3G, ubiquitin and ATP (Jäger et al., 2012b). Antibodies against Vif, CBFβ or HA were used to detect high MW species representative of substrate ubiquitination.

Small angle X-ray scattering (SAXS)

SAXS measurements were performed at the SIBYLS beamline (BL-12.3.1) of Advanced Light Source. Proteins in the concentration range of 5–30 mg/ml were exposed for 0.5 second and buffer scattering was subtracted. Details of the SAXS analyses and modeling can be found in the Supplemental Material.

Transcription activity assay

HEK293T cells were transfected with plasmid sets comprised of the indicated plasmids and lysed 36 hours later with passive lysis buffer (Promega). Green fluorescence and firefly luminescence were recorded with an Ultra Evolution plate reader (Tecan) using Luciferase Assay Reagent II (Promega).

Chromatin immunoprecipitation and sequencing

ChIP experiments were conducted using lysate obtained from the Jurkat TRex cell line (Invitrogen); there was no prior PMA/PHA treatment. The ChIP procedure was modified after a previously described procedure (Lee et al., 2006), and full details are provided in the Supplementary Methods.

Differential gene expression analysis

Differential gene expression due to Vif was studied in a Jurkat TRex cell line (Invitrogen) carrying a stably integrated, tetracycline inducible gene construct consisting of Vif with 2 copies of Strep and 3xFLAG fused to its carboxyl terminal (Vif-SF) (Jäger et al., 2012a; Jäger et al., 2012b). Gene expression changes were controlled with reference Jurkat TRex. Cells were treated with 50 ng/mL PMA and 1 μg/mL PHA and collected 4 and 6 hours later. Total RNA was isolated and converted into cDNA for gene expression analysis using the Affymetrix GeneChip Human Gene 1.0 ST microarray platform. A Bayesian statistical approach utilizing the MMBGX software platform was used for differential gene expression analysis (Turro et al., 2010). A gene was considered differentially expressed if it met a Z score threshold defined by MMBGX and a minimum, absolute fold change of 1.5.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- Action of a viral hijacked E3 requires binding to a cellular transcription cofactor
- E3 assembly prevents binding of the transcription cofactor to regulatory partners
- A viral protein can interfere with multiple cellular pathways by these mechanisms

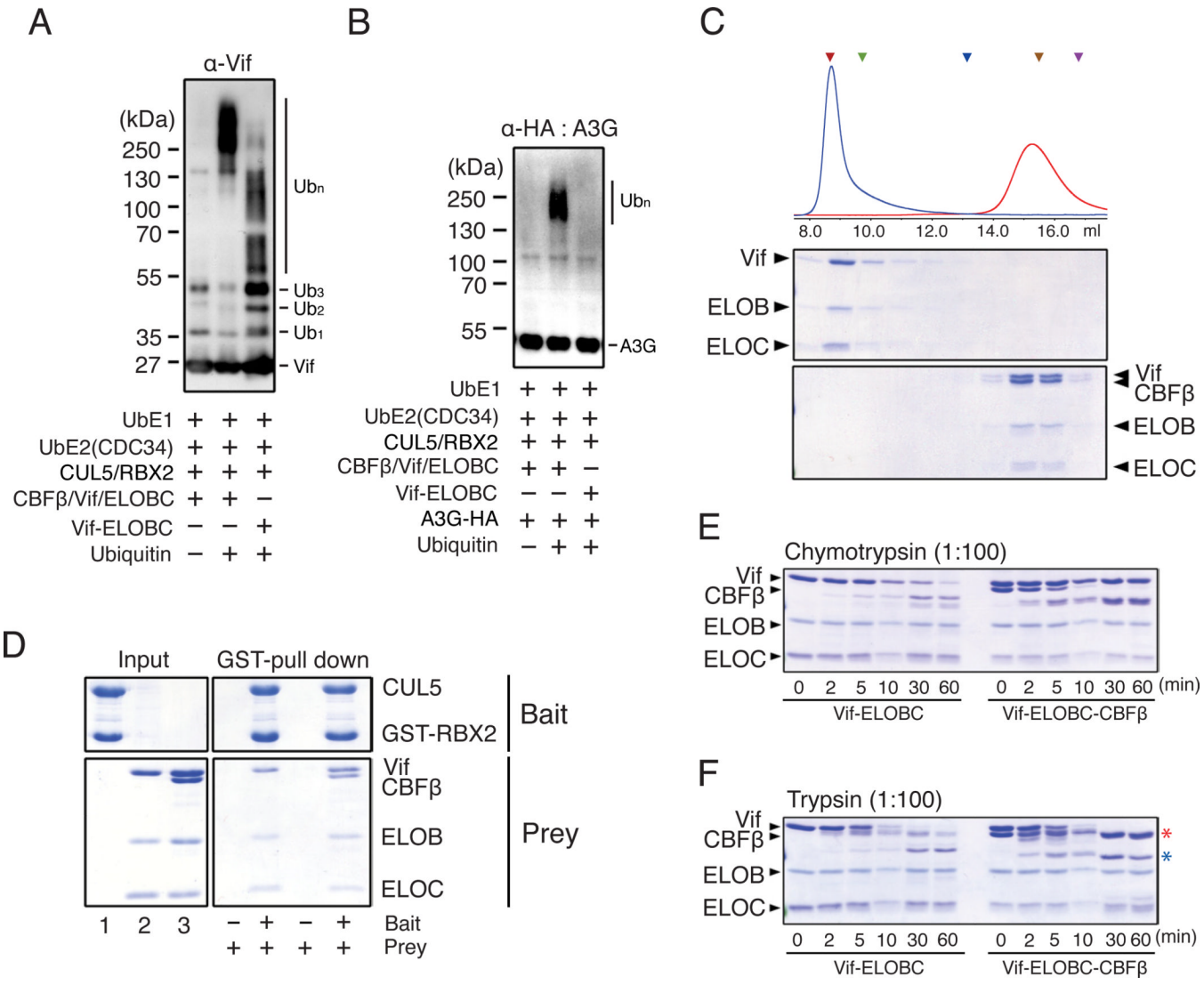

Figure 1. CBFβ controls the oligomeric state of Vif together with ELOBC by protecting a hydrophobic region

(A) When CBFβ was present, Vif was highly polyubiquitinated when mixed with CRL5. (B) CRL5-Vif-CBFβ promoted poly-ubiquitination of HA-tagged APOBEC3G (second lane) *in vitro*, while CRL5-Vif did not (third lane). (C) Shown are the results of size exclusion chromatography of purified Vif-ELOBC (blue curve) and CBFβ-Vif-ELOBC (red curve). The triangles indicate UV absorbance peaks of void (red), thyroglobulin (green; 670 kDa), γ-globulin (blue; 158 kDa), ovalbumin (brown; 44 kDa), and myoglobulin (purple; 17 kDa). Vif and ELOBC formed a heterogeneous oligomer in the absence of CBFβ, while Vif, ELOBC and CBFβ formed a smaller homogenous complex. (D) Both Vif-ELOBC and CBFβ-Vif-ELOBC were capable of associating with CUL5/RBX2-GST based on GST pull-down assays. The left panel depicts the input fractions of recombinant purified CUL5/GST-RBX2 (lane 1), Vif-ELOBC (lane 2) and CBFβ-Vif-ELOBC (lane 3). The right panel shows proteins found in the eluate after GST-pull down. (E, F) Limited proteolysis assays demonstrate that CBFβ-Vif-ELOBC was more resistant to chymotrypsin, which recognizes aromatic residues in the P1 position of substrate (E). CBFβ binding to Vif does not change the degradation pattern of Vif by trypsin (F). Red and blue asterisks denote resistant Vif and CBFβ fragments, respectively.

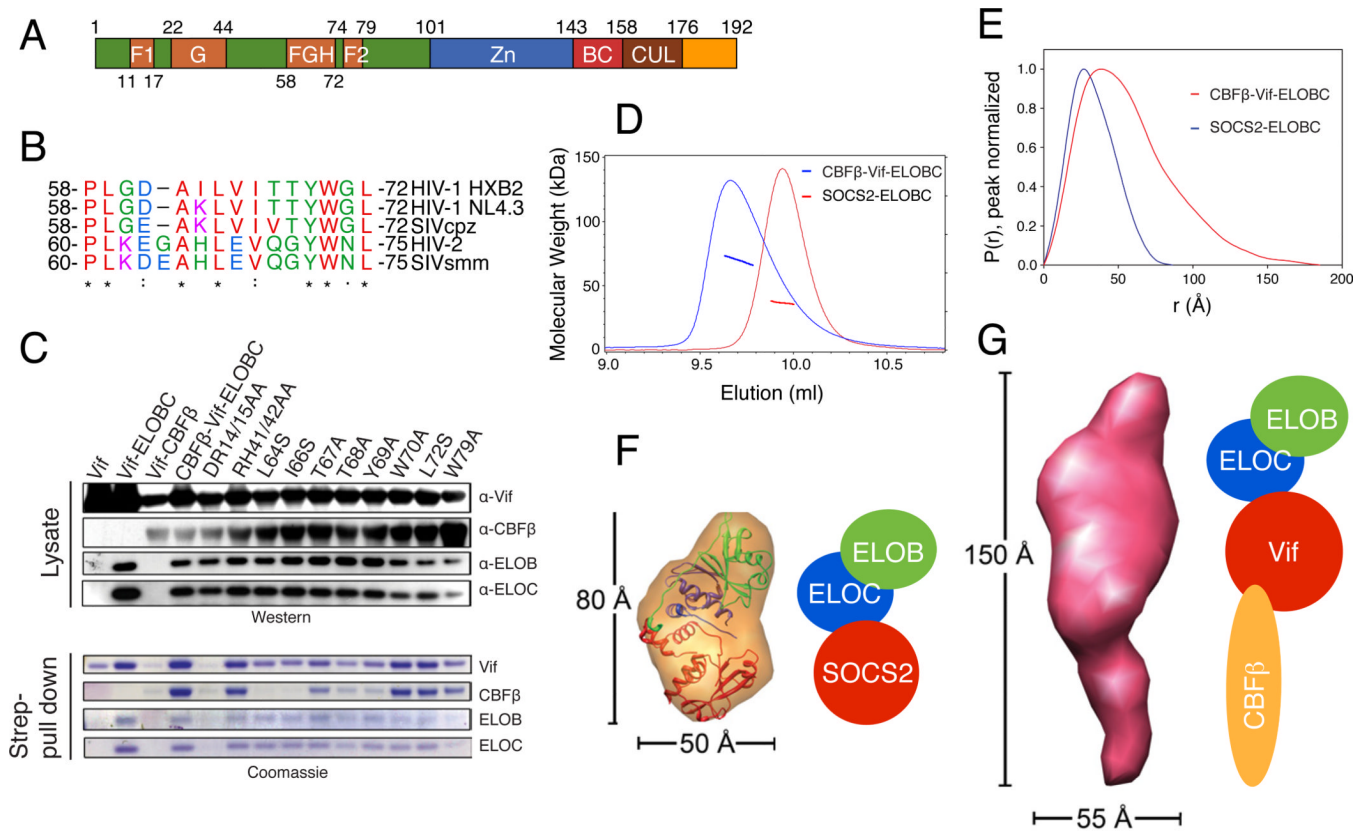

Figure 2. CBFβ and ELOC stabilize the Vif substrate adaptor through separate binding surfaces

(A) Shown is a schematic of known binding motifs in Vif. The terms Zn, BC and CUL indicate the Zn binding cluster, ELOBC binding and CUL5 binding motifs, respectively (Mehle, 2004; Mehle et al., 2006; Stanley et al., 2008). Sites implicated in the degradation of A3F or A3G are denoted as F or G, respectively, while the site implicated in the degradation of A3F, A3G and A3H is denoted as FGH ((Zhen et al., 2010) and reviewed in (Smith et al., 2009). (B) Sequence alignment of FGH motifs in HIV and SIV Vif sequences. (C) Shown are the results of affinity-purification assays in *E. coli*. Wild-type and mutant Strep-Vif, ELOBC and CBFβ were co-expressed in *E. coli*, Vif was purified using Strep-Tactin resin and co-purifying proteins were visualized by Coomassie stained SDS-PAGE gels. Lesions in the FGH box of Vif impaired the ability of CBFβ to co-purify with Vif whereas a control mutation in the G box did not effect copurification of CBFβ. (D) Size exclusion chromatography with multi-angle laser light scattering (SEC-MALLS) indicated that both CBFβ-Vif-ELOBC and SOCS2-ELOBC were monodisperse complexes with single copy stoichiometry of each subunit. (E) Pair distance distribution function (P(r)) calculated from SAXS intensity data. (F) Molecular envelopes of CBFβ-Vif-ELOBC and SOCS2-ELOBC calculated from P(r). Superimposition of the crystal structure of SOCS2-ELOBC (PDB ID; 2C9W) revealed good fit with its experimentally determined molecular envelope (depicted as a drawing on the right). (G) Cartoon schematic based on our biochemical studies (B, C) of the molecular envelope of the CBFβ-Vif-ELOBC substrate receptor and proposed configuration of subunits.

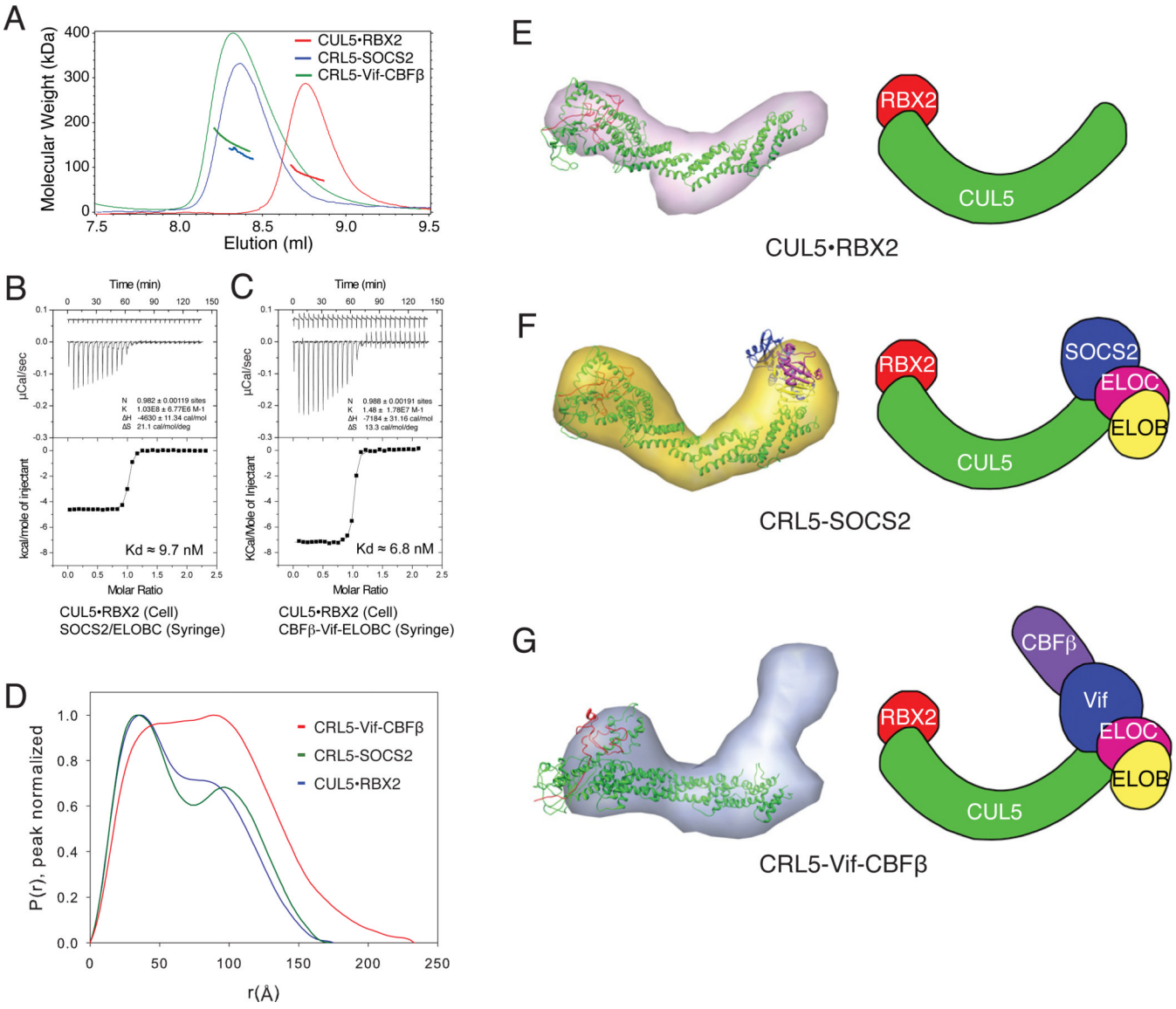

Figure 3. CRL5-Vif-CBFβ is a monodisperse particle with three lobes

(A) SEC-MALLS of CUL5/RBX2 (red), CRL5-SOCS2 (blue) and CRL5-Vif-CBFβ (green) indicated that the CRL5-Vif-CBFβ E3 ligase is a heterohexamer consisting of a single-copy of each subunit (Table S1). Isothermal titration calorimetry (ITC) data show that CUL5 bound to SOCS2-ELOBC (B) and CBFβ-Vif-ELOBC (C) with comparable affinities. (D) P(r) function of CRL complexes. Molecular envelopes of CUL5/RBX2 (E), CRL5-SOCS2 (F) and CRL5-Vif-CBFβ (G). Homology models of CUL5/RBX2 and CRL5-SOCS2 were superimposed onto their experimentally determined molecular envelopes.

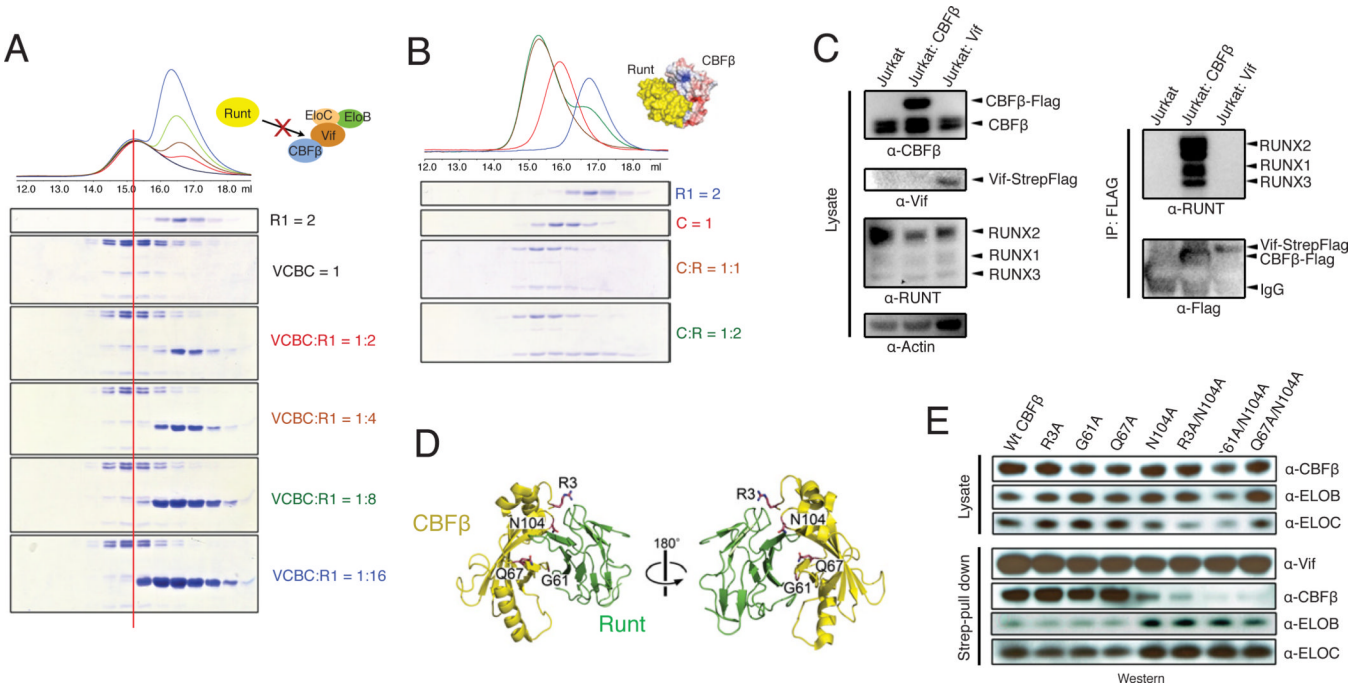

Figure 4. Binding between Vif and RUNX to CBFβ is mutually exclusive

SEC experiments assaying the ability of the Runt domain of RUNX1 to copurify with preformed CBFβ-Vif-ELOBC complex (A) or CBFβ in isolation (B). R1, C, and VCBC indicate RUNT1, CBFβ and CBFβ-Vif-ELOBC. The ratio indicates molar equivalents of proteins. RUNT1 did not bind to CBFβ when preassembled with Vif, ELOBC, even when in 16-fold excess (A) whereas RUNT1 bound CBFβ at 1:1 ratio (B). (C) RUNX proteins co-IP with CBFβ but not Vif stably expressed in Jurkat T-cells. Cell lines expressing empty vector control, CBFβ-FLAG or Vif-Strep-FLAG where lysed and subjected to immunoprecipitation using anti-FLAG resin. Proteins were detected with antibodies indicated below the immunoblots. (D) The crystal structure of CBFβ (yellow)/RUNT (green) complex (PDB ID; 1E50) was used to guide mutational analysis evaluating the ability of CBFβ to co-purify with Vif-ELOBC. (E) In isolation, the N104A mutation of CBFβ reduced Vif binding whereas other residues implicated in RUNT binding did not.

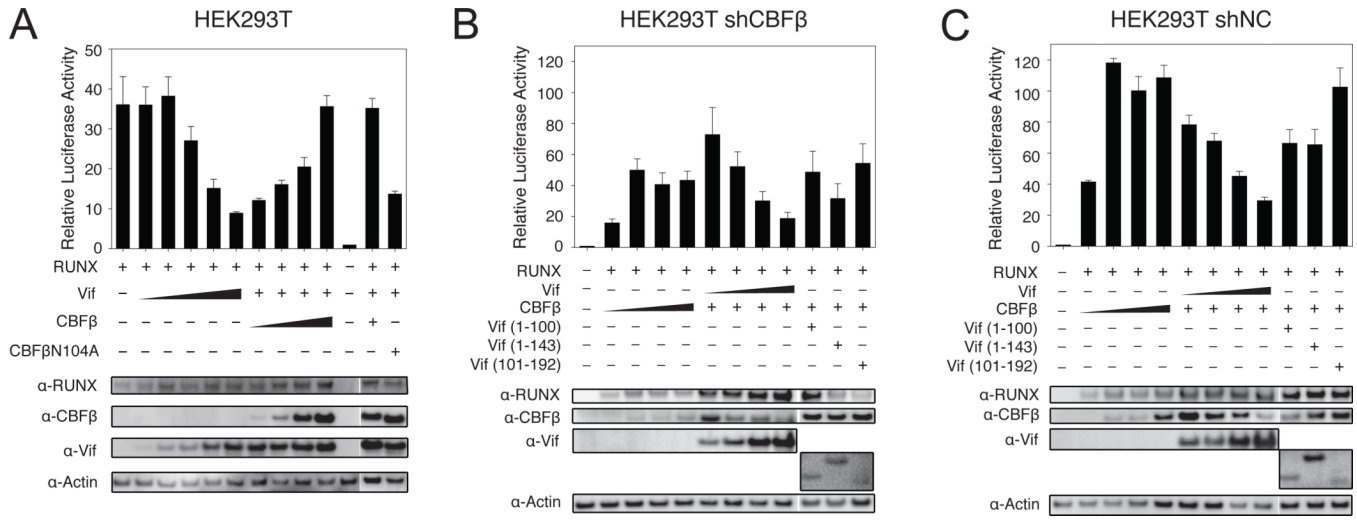

Figure 5. Vif antagonizes transcription of a RUNX reporter gene by competition with CBFβ

(A) Vif inhibited RUNX1 transcriptional activity in a dose dependent manner and can be restored by overexpression of wild-type but not N104A mutant of CBFβ. HEK293T cells were transiently co-transfected with a RUNX1 reporter plasmid (firefly luciferase gene with IL17 promoter) and plasmids encoding RUNX1, Vif and/or CBFβ. The luciferase activity was normalized by fluorescence of co-transfected eGFP. (B) Transcription of the RUNX reporter gene was attenuated in cells where CBFβ was stably knocked down by shRNA (HEK293-shCBFβ) but increased by overexpression of CBFβ. Increasing concentrations of Vif plasmid used in transfection reactions resulted in a decrease in transcription activity with a concomitant decrease in steady-state levels of CBFβ as detected by parallel immunoblot analysis. Expression of the CBFβ binding domain of Vif (residues 1–143) partially inhibited transcription of the RUNX1 reporter gene. Assays were performed as in (A). (C) Experiments performed as in (B) but in nonsilencing control cell line. Error bars of each reporter assay represent the mean ± standard deviation of independent triplicates. The expression level of proteins is indicated by parallel immunoblots.

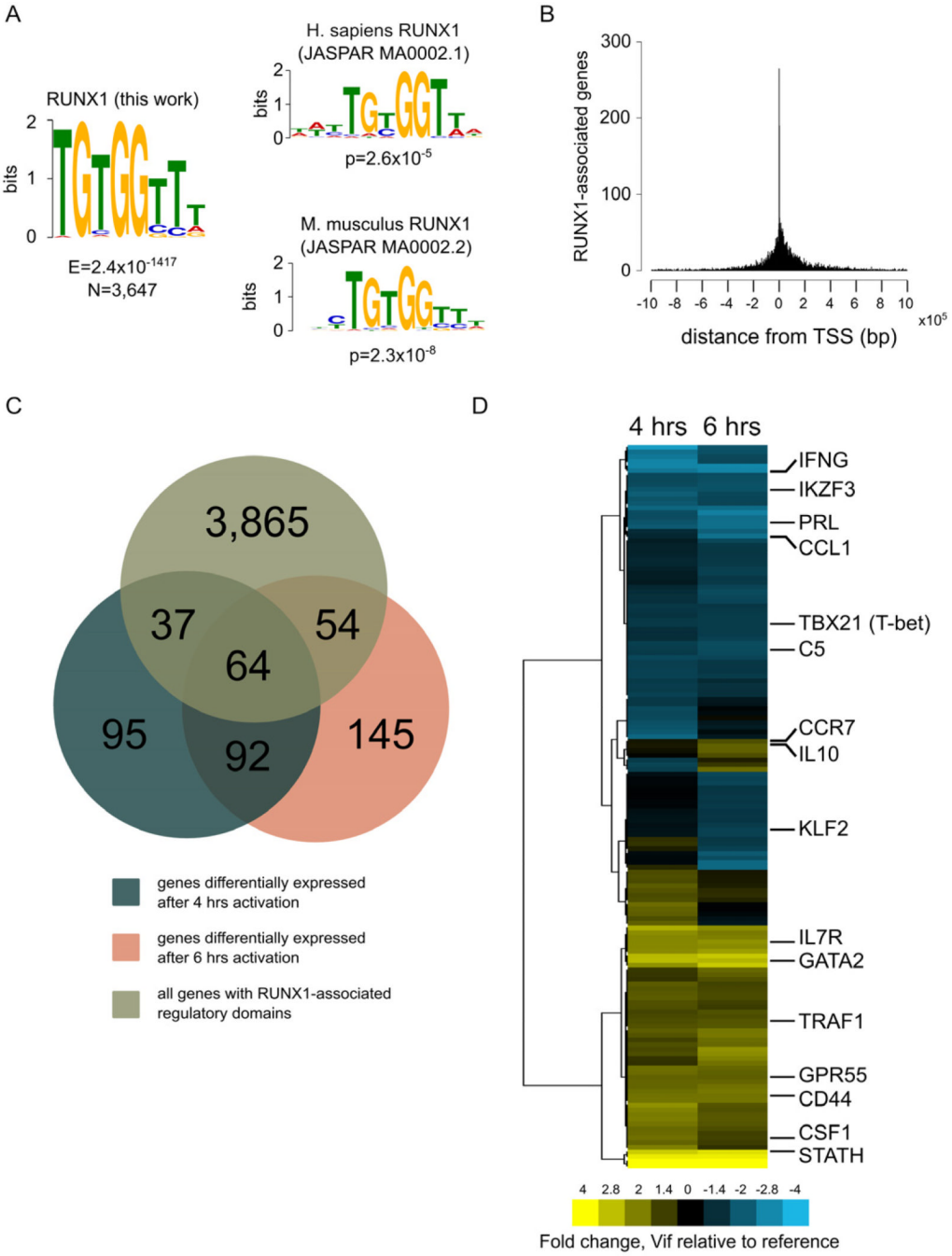

Figure 6. Vif modulates the expression of genes, including those whose regulatory domains are associated with RUNX1

(A) A set of 3,647 RUNX1-enriched genomic regions were analyzed using CUDA-MEME to identify the top scoring enriched motif depicted in the left panel. A database of previously identified motifs was queried to find similar motifs, and the right panel depicts the two most similar motifs; both are RUNX1 consensus motifs.

(B) The 3,647 RUNX1-enriched genomic regions were filtered to remove those that lacked the consensus sequence TGYGGYY to yield a final set of 3,320 RUNX1-enriched genomic sites. A total of 3,290 out of 3,320 sites formed 5,951 associations with 4,122 genes, and the distances of each RUNX1 binding site from its associated gene transcription start sites

(TSSs) were determined and plotted as a histogram (1,000 bins, N=5,951). (C) Venn diagram showing the relationships among genes with RUNX1- associated regulatory domains in nonactivated Jurkat T cells that do not express Vif and genes with Vif-dependent differential expression after 4 or 6 hours of PMA/PHA treatment. The total number of genes under analysis is 17,081. (D) Hierarchical cluster based on Euclidean distance of expression measures for 155 genes with Vif-dependent differential expression at 4 or 6 hours PMA/PHA treatment. A list of these genes is provided as Table S3, and genes of interest are indicated.

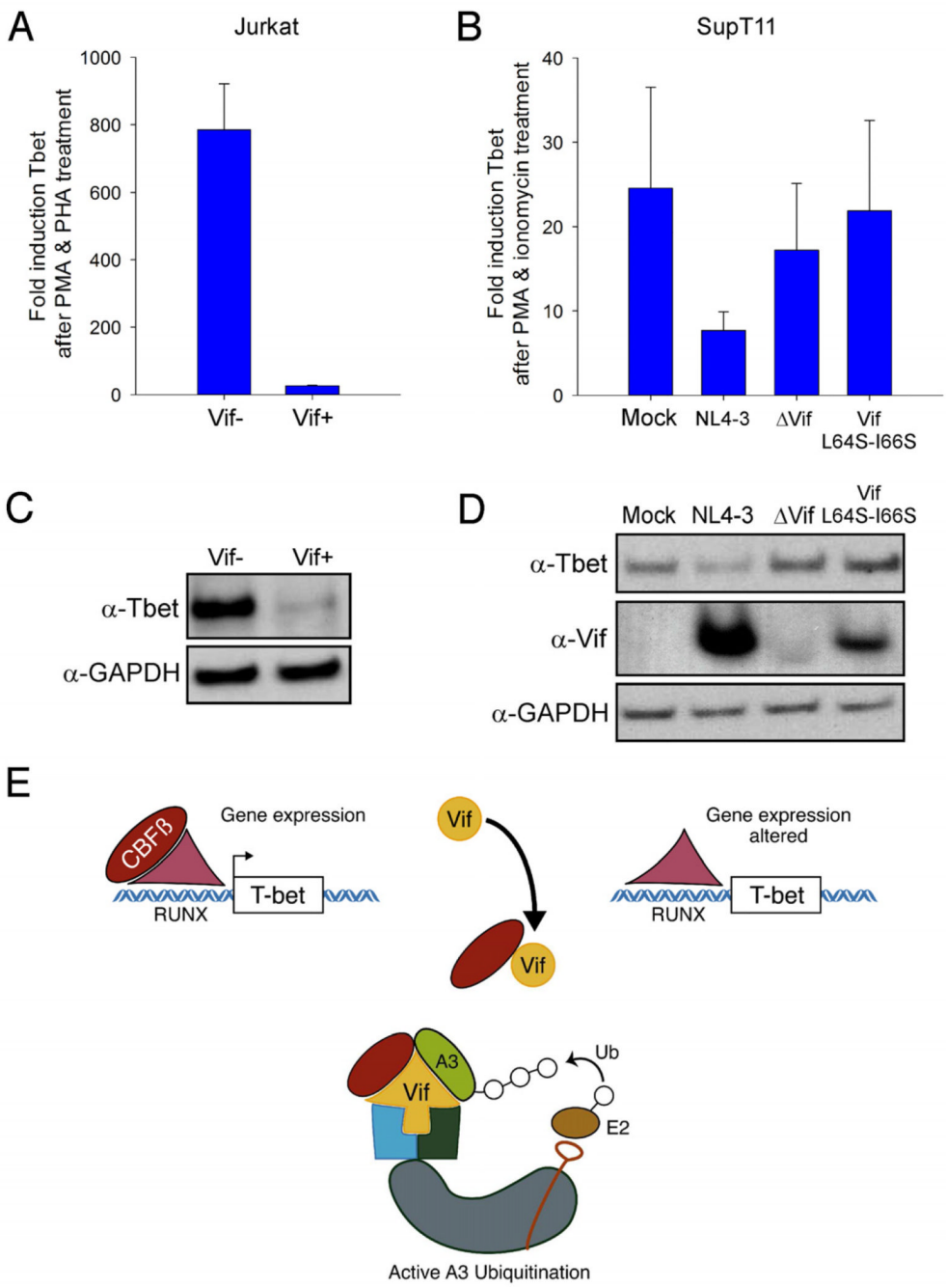

Figure 7.

(A) Vif represses induction of T-bet in Jurkats stimulated with PMA and PHA. Shown are the average (N=3) fold change increases of T-bet expression after 6 hours of stimulation relative to before stimulation. The bars represent the 95% confidence interval. Experiments were performed with Jurkats stably expressing Vif or the reference line lacking Vif. (B) Vif represses PMA and ionomycin dependent induction of T-bet in SupT11 cells infected at MOI=1.0 with the indicated HIV virus or mock treatment. Cells were infected or mock treated for 48 hours prior to stimulation. Shown are the average (N=3) fold change increases of T-bet expression after 4 hours of stimulation relative to before stimulation. The bars represent the 95% confidence interval. (C) Western blots of T-bet and GAPDH in Jurkats

stimulated with PMA and PHA for 6 hours. (D) Western blots of T-bet, GAPDH and Vif in SupT11 cells infected or mock treated prior to stimulation with PMA and ionomycin for 4 hours. Protein extracts were obtained from the same experiment represented in panel (B) by combining equal amounts of the three replicates. (E) Model figure showing how CBFβ promotes ubiquitination of A3 substrates at the expense of RUNX mediated gene expression by sequestration of Vif. Color code: CUL5, grey; RBX2, red colored ring; ELOBC, blue and green. Other factors are labeled with text.