Oligomeric HIV-1 Integrase Structures Reveal Functional Plasticity for Intasome Assembly and RNA Binding

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Abstract

Integrase (IN) performs dual essential roles during HIV-1 replication. During ingress, IN functions within an oligomeric “intasome” assembly to catalyze viral DNA integration into host chromatin. During late stages of infection, tetrameric IN binds viral RNA and orchestrates the condensation of ribonucleoprotein complexes into the capsid core. The molecular architectures of HIV-1 IN assemblies that mediate these distinct events remain unknown. Furthermore, the tetramer is an important antiviral target for allosteric IN inhibitors. Here, we determined cryo-EM structures of wildtype HIV-1 IN tetramers and intasome hexadecamers. Our structures unveil a remarkable plasticity that leverages IN C-terminal domains and abutting linkers to assemble functionally distinct oligomeric forms. Alteration of a newly recognized conserved interface revealed that both IN functions track with tetramerization *in vitro* and during HIV-1 infection. Collectively, our findings reveal how IN plasticity orchestrates its diverse molecular functions, suggest a working model for IN-viral RNA binding, and provide atomic blueprints for allosteric IN inhibitor development.

Introduction:

Human immunodeficiency virus 1 (HIV-1) currently infects ~40 million people worldwide, and the number of infected individuals continues to rise. Left alone, HIV-1 will eventually lead to acquired immunodeficiency syndrome through a series of progressive events that weaken the immune system and make individuals susceptible to opportunistic infections. Although there are now numerous therapies for people living with HIV that block different stages of the viral replication cycle, HIV-1 infection afflicts individuals for life, and there remains no cure[1].

After cell entry, HIV-1 RNA is reverse transcribed into double-stranded linear DNA containing a copy of the viral long terminal repeat (LTR) at each end. A hallmark of HIV-1 infection is the covalent insertion of this viral DNA (vDNA) into host chromatin, the terminal step in viral ingress that is necessary to establish a persistent infection[2,3]. This key step, termed integration, distinguishes HIV-1 and related retroviruses from other animal viruses and poses a central challenge to therapeutic intervention. Concerted integration of both LTR ends is catalyzed by IN[4] and requires the formation of a nucleoprotein complex containing multimers of IN bound to the vDNA ends, termed "intasomes"[5]. Intasomes can be assembled *in vitro* from purified IN and vDNA oligonucleotides and are catalytically competent to perform two-ended integration[6,7]. Structural biology efforts elucidated how a conserved HIV-1 intasome core consisting of two IN dimers scaffolds and intertwines the vDNA ends[8]. However, that same study revealed a putative larger intasome architecture, consisting of up to sixteen protomers of IN, which could not be fully resolved or modeled into the experimental density[8]. Based on structures of related lentiviral intasomes from Maedi Visna virus (MVV)[9,10], which are more stable and yield homogeneous assemblies, it was suggested that lentiviral intasomes might be generally hexadecameric, i.e. arise from sixteen copies of viral IN[11]. However, all HIV-1 intasome structures resolved to date[8,12–14] lack key IN flanking subunits that assemble on the intasome periphery, and atomic models constitute only ~40% of the total protein mass. These limitations can be attributed to the dynamic nature of HIV-1 IN[15,16] (also observed with simian immunodeficiency virus IN[17]), characteristics that confer significant challenges to structural biology attempts. Thus, whether the hexadecameric intasome architecture is conserved in HIV-1 remains unclear.

Viral genetics initially revealed pleiotropic outcomes of HIV-1 IN mutagenesis. Some substitutions, notably those that affect active site residues (i.e., Asp64, Asp116, and Glu152), could specifically block integration in infected cells, consistent with selective abrogation of intasome function[18–21]. These were subsequently termed class I IN mutations[22]. In contrast, the majority of IN substitutions impair additional processes, including reverse transcription as well as particle assembly and release from transfected cells (reviewed in reference [22]). These were termed class II IN mutations[22]. The presence of multiple classes of mutations affecting distinct stages of viral replication highlighted the complexity of IN and suggested multifaceted functionalities during HIV-1 infection[23].

Recent work has shown that the compendium of class II substitutions can be explained by a second essential function of IN that exerts itself during the late stage of viral replication. HIV-1 IN binds directly to viral RNA (vRNA) in virions, and impairing the IN-vRNA interaction yields eccentric, non-infectious virions characterized by mis-localization of ribonucleoprotein complexes (RNPs) outside of the protective capsid core[24]. Biochemical assays suggested that IN tetramers, but not dimers or monomers, effectively bind cognate vRNA segments, as amino acid substitutions that impaired IN tetramerization also inhibited IN-vRNA binding *in vitro* and in virions[25]. Collectively, these results proposed a novel biological role for IN-vRNA binding during virion morphogenesis[24,25]. However, the structural organization of the IN tetramer, which mediates vRNA binding, remains unknown.

Furthermore, IN tetramers are the antiviral target for investigational allosteric IN inhibitors[26], which are currently in Phase 2 clinical trials[27]. ALLINIs potently impair proper virion maturation by inducing aberrant or hyper multimerization of IN, which inhibits vRNA binding[24,26,28,29]. Consequently, the virions produced in the presence of ALLINIs have RNPs mis-localized outside of the mature capsid and are non-infectious. While previous structural studies have elucidated the mode of action of ALLINIs in the context of full-length dimeric IN mutant Y15A/F185H, which is substantially resistant to ALLINIs[30], the authentic antiviral target of these inhibitors is the full-length wildtype (WT) IN tetramer[26]. Therefore, elucidating the structure of IN tetramers is of significant pharmacological importance.

HIV-1 IN consists of three functional domains including the N-terminal domain (NTD), catalytic core domain (CCD), and C-terminal domain (CTD)[31]. The NTD harbors a zinc-finger

binding motif that stabilizes its fold and helps coordinate the assembly of the multimeric intasome complex[32]. The CCD harbors the conserved DDE motif containing the Asp64-Asp116-Glu152 catalytic triad[33] and is involved in the cleavage and joining of vDNA strands for integration. The CTD plays an essential role in DNA binding, IN multimerization, and the proper positioning of IN on the vDNA ends to promote the assembly of the higher-order intasome[6,8,34]. Notably, the CTD is also primarily responsible for vRNA binding[24,25]. All three domains must be present and largely WT for IN to properly execute its two distinct functions. However, despite nearly three decades of structural biology work that contributed to our growing understanding of IN function (reviewed in [2]), structures of architecturally complete HIV-1 IN assemblies have remained critically absent.

Here, we used cryo-EM to determine structures of two oligomeric assemblies formed by WT full-length HIV-1 IN. First, using the scaffolding domain from an IN host factor to stabilize and solubilize tetrameric IN, we present the structure of the IN tetramer, which is characterized by interlocked placement of all four CTDs that maintain the architectural integrity of the tetrameric assembly. Next, using a biochemical strategy to isolate stabilized cross-linked intasomes and focused classification approaches during image analysis of cryo-EM data, we deciphered the architectural assembly of the hexadecameric WT HIV-1 intasome, which revealed how coordinated structural rearrangements that arise within the IN tetramer facilitate assembly into the complete intasome. Inspection of the contacts mediated by the inter-domain interactions revealed two conserved residues, E35 and K240, which form a salt bridge that contributes to both the structural integrity of IN tetramers and the interactions observed within the hexadecameric intasome. Our reverse-charge guided mutagenesis studies targeting these interactions have revealed that both catalytic and non-catalytic functions of IN *in vitro* and in infected cells closely correlated with the ability of the mutant proteins to form tetramers. Finally, using molecular modeling, we suggest how vRNA may bind the IN tetramer. These data highlight how the plastic molecular architecture of HIV-1 IN can mediate its diverse functions and provide a molecular blueprint for the design of novel inhibitors that can block IN function at distinct stages of the viral replication cycle.

Results:

Interlocked CTDs maintain the architectural integrity of the IN tetramer

Prior studies suggested that the formation of a core IN tetramer is relevant for the proper execution of two distinct functions mediated by IN, integration[9] and RNA binding[24,25,35]. We set out to determine the structure of the WT IN tetramer in apo form without nucleic acids bound. Previous work showed that the IN-binding domain (IBD) of the IN host factor lens epithelium-derived growth factor (LEDGF)/transcriptional co-activator p75 can solubilize and stabilize tetrameric IN[16]. We thus included IBD in our bacterial co-expression system, which was expected to tightly bind IN and stabilize a multimeric form to facilitate structural studies. In the absence of the IBD, IN migrated in a gel filtration column predominantly as dimers and tetramers in the presence and absence of CHAPS detergent, respectively. As anticipated[16], IBD co-expression predominantly yielded IN tetramers (**Supplementary Fig. 1**). The IN-IBD complex was purified using nickel affinity chromatography followed by size-exclusion chromatography (SEC), yielding a peak corresponding to the appropriate molecular weight of the tetramer and containing both IN and IBD polypeptides (**Supplementary Figure 2**). We advanced this complex for structural biology studies. The IN-IBD particles were characterized by severe preferred orientation on cryo-EM grids. To address this limitation, we tilted the stage during data collection[36–38]. We collected 10,076 movies of the IN-IBD complex and incorporated iterative 2D and 3D classification in cryoSPARC[39] and cryoDRGN[40], producing a final map resolved to ~3.0 Å and an atomic model derived from this map (**Supplementary Figure 3** and **Supplementary Table 1**).

The structure of IN-IBD is defined by two-fold rotational symmetry and contains four IN protomers with one IBD monomer bound to each of the four corners of the tetrameric assembly (**Figure 1a-b**). All three of the functionally relevant IN domains, the NTD, CCD, and CTD, are fully resolved within the density. The NTDs and CCDs are connected by ~15-residue flexibly dynamic linkers[23]. Accordingly, these linkers were not modeled, although density at low threshold was apparent, which informed their connectivity to the respective CCDs. In contrast, all four linkers connecting the CCDs to the CTDs are alpha-helical and fully defined within the cryo-EM density. As expected, the conserved CCD-CCD interface[41] scaffolds each of the two IN dimers[6,8]. The tetrameric organization is held together by the organization of the CTDs, which extend outward

and interlock with one another through the CCD-CTD linkers. The role of the CTDs in maintaining the integrity of the tetramer is well appreciated when the structures are colored by individual IN domains (**Figure 1c-d**). Displayed as such, it becomes apparent that all four CTDs are arranged at the "top" of the assembly, whereas the CCD:CCD dimers reside underneath. Each of the two NTDs are bound to the CCD:CCD dimer interface, as previously observed for two-domain assemblies lacking the CTD[16,42]. The primary binding mode for each IBD resembles prior crystal structures with partial CCD and NTD-CCD constructs (**Supplementary Figure 4a**)[43,44]. The binding mode is asymmetric, with density for IBD_1/IBD_3 being more pronounced than the density for IBD_2/IBD_4 on the opposing molecule (**Supplementary Figure 4b**). We attribute this asymmetry, at least in part, to the fact that $IBD_{1,3}$ interface with both $NTD_{1,3}$ and $CTD_{3,1}$, respectively, whereas $IBD_{2,4}$ engage $NTD_{2,4}$ only weakly. Notably, the additional $IBD_{1,3}$–$CTD_{3,1}$ interactions explain why IBD helps stabilize and solubilize the tetrameric form of IN[15,16].

Collectively, our IN-IBD structure reveals how the CTDs bridge two independent IN dimers through alpha-helical linkers. The numerous inter-domain and inter-protomer interfaces explains why all three domains are required for functional IN oligomerization.

Conformational plasticity within inter-domain linkers leads to context-dependent interfaces made by the CTDs

The CTDs exhibit a highly flexible positional configuration in relation to the dimeric CCD scaffold. Superposition of each IN dimer within the tetrameric IN-IBD assembly to previously determined crystal structures of two-domain HIV-1 IN CCD-CTD constructs (PDBs 1EX4[45] and 5HOT[30]) or from lentiviruses MVV (PDB 5T3A[10]) or SIV (PDB 1C6V[46]) revealed rearrangements in both position and configuration of the CTDs, mediated by the helical linkers (**Figure 2a**). The most dramatic demonstration of this structural plasticity is observed when comparing the configurations of the CTDs from the HIV-1 IN tetramer with six independent modeled IN protomers from the MVV intasome, which are structurally homologous (PDB 7U32[10]; two of these eight protomers did not contain atomic models of the CTD) (**Figure 2b**). These comparisons imply that the conformational plasticity of the CCD-CTD linker plays a key role in choreographing diverse assemblies, including the formation of higher-order IN structures and the intasome.

As a consequence of the structural plasticity, the CTDs can engage in diverse interactions (**Figure 2c**). There are two prominent interactions between CTD_1/NTD_3 (and analogously

CTD_3/NTD_1) from two separate protomers, each residing on opposite IN dimers (**Figure 2c**, top left). These are key for maintaining the architectural integrity of the complex. This interface is also conserved in the HIV-1 intasome, although it was not previously appreciated (**Supplementary Fig. 5**). The IN tetramer is also stabilized by interactions between the CTD of one protomer and the CCD-CTD linker region of another protomer (**Figure 2c**, top right). Lastly, the CTDs engage one another at the twofold axis through hydrophobic contacts (**Figure 2c**, bottom left). This engagement is anti-parallel, which differs from the original dimeric CTD solution NMR structure (PDB 1IHV)[47], but resembles recently described crystallographic packing interactions within IN assemblies containing bound ALLINI (PDB 8CTA)[48] (**Figure 2c**, bottom right). Notably, the fact that the anti-parallel CTD:CTD interface is now observed within the IN tetramer indicates that this interaction mode is biologically relevant, which could not be concluded from packing assemblies observed crystallographically[48]. Such diverse contacts contributed by CTDs help stabilize and maintain the tetrameric conformation and add to our understanding of the promiscuity of the CTD, which additionally engages with other protein domains and/or nucleic acids in the intasome[8].

In contrast to the promiscuous CTD interactome, the positions of the NTDs are more conserved. The NTDs are typically bound to the two-fold interface of each CCD dimer, a conserved location among IN structures, including intasome assemblies (**Supplementary Fig. 6**). The NTDs do rearrange upon intasome assembly, which is required for intertwining the vDNA, though the resulting interfaces are maintained[6–8,10,49–52]. The CCDs are universally conserved and engage through the canonical dimerization domain[41].

These data highlight the propensity of the CTD to form distinct context-dependent interactions, which explains why this domain is key to higher-order IN assembly and mediates its functional plasticity.

The IN tetramer forms the core organizational unit of the hexadecameric HIV-1 intasome

Prior lentiviral intasome structures have revealed a diversity of oligomeric IN species ranging from tetramer to hexadecamer[8–10,12,13,17], and recent results suggested that functional IN tetramerization is essential for lentiviral intasome assembly[9]. However, owing to the propensity of HIV-1 IN to aggregate *in vitro*, and to challenges with biochemical intasome assembly, structures of HIV-1 intasomes containing fully resolved flanking subunits have remained elusive. This

limitation has precluded an understanding of how HIV-1 IN assembles into the complete intasome, and also whether sixteen IN subunits indeed comprise the functional unit.

To address this limitation, we set out to determine the complete structure of the HIV-1 intasome and resolve the missing flanking regions. Unlike prior efforts that employed the chimeric Sso7d-IN fusion protein (with Sso7d appended to the IN NTD), which cannot form a hexadecameric intasome due to steric restraints[8], here we used WT HIV-1 IN. Intasomes were assembled using a similar strategy to those previously described[8,12,13], but we additionally used the drug Dolutegravir to prevent spurious strand transfer[53] and crosslinked the sample to stabilize discrete multimeric forms and isolate assemblies only within the expected ~600 kDa range (**Supplementary Figure 7**). The eluted mixture was vitrified on grids and subjected to cryo-EM analysis. We collected a dataset consisting of 775 movies and used standard protocols for image refinement and classification, yielding a map resolved to 3.5 Å within the intasome core. However, despite the crosslinking and extensive biochemical purification, density for the flanking regions remained unresolved (**Supplementary Figure 8**). We thus sought to recover the densities computationally. We subjected the dataset to multiple rounds of global classification, followed by density subtraction and focused classification (**Supplementary Figure 9**). Using an iterative approach, we could resolve each half of the intasome separately and use rigid body docking to fit all constituent domains. Importantly, independent analysis of both halves of the intasome yielded nearly identical domain docking, indicating, as expected, that the architectural integrity and mode of IN oligomerization is conserved across each half of the two-fold symmetric complex (**Supplementary Figure 10**). We could then derive a composite map and model of the hexadecameric HIV-1 intasome, assembled from two independently refined halves (**Supplementary Figure 11**).

Four IN tetramers comprise the hexadecameric HIV-1 intasome (**Figure 3a**). The two-fold symmetry of the intasome makes tetramers I/II and III/IV identical. Each of the two unique tetramers have two CCD:CCD dimers that are juxtaposed about a central set of interconnected CTDs, with the CTD-CCD linkers interlocking the arrangement into an architecture that is similar to that observed for the tetrameric IN-IBD structure. Notably, the conformational flexibility of CTD-CCD linkers facilitates the incorporation of each tetramer into the intasome hexadecamer. The major structural rearrangements involve the “compression” of each tetramer about the interlocked CTDs, bringing the two dimers into closer proximity when compared to the apo tetramer (**Figure 3b-c**). Accompanying changes include small repositioning of the CTDs residing on the inner CCD

protomers, which in turn interface with other regions of the intasome, as well as a well-known and conserved rearrangement of the NTDs around the vDNAs[2,3] (depicted by arrows). As expected, the HIV-1 intasome has an overall similar architectural arrangement and protomer organization as the MVV intasome, albeit with weaker interactions maintaining tetramer-tetramers contacts through the CTD bridge (**Supplementary Figure 12**). Guided by prominent experimental densities for alpha helical linker assignments, we could also fully assign individual domains for the HIV-1 intasome, thus establishing the complete lentiviral intasome architecture. Collectively, these results highlight complex structural rearrangements, spanning individual IN domains and quaternary assemblies, that must arise to ensure proper intasome formation.

Functional implications of the tetrameric assembly in vitro and in cells

IN tetramerization is thought to play an important role both in the early and the late stages of HIV-1 replication[9,24,54]. To critically test this model, we searched for likely stabilizing interactions within the apo IN tetramer and identified an intermolecular salt bridge between E35 of the NTD from one IN protomer and K240 of the CTD from a separate IN protomer (**Figure 2c** and **4a**). This interaction is conserved in the HIV-1 intasome, although its functional relevance was previously untested (**Supplementary Fig. 5**). We posited that the interaction between E35 and K240 may in fact be relevant to the biochemical and functional properties of IN, and we sought to interrogate the relevance of this interaction.

We designed reverse-charge amino acid substitutions E35K, K240E and the charge-swapped double mutation E35K/K240E, expressed the mutant IN proteins in *E. coli* cells, and purified them using nickel affinity and heparin chromatography (**Supplementary Figure 13**). The purified proteins were analyzed by SEC to assess their multimeric states. Individual IN changes E35K and K240E led to modestly and dramatically reduced levels of the tetramer, respectively. Strikingly, the E35K change in the context of the charge-swapped double mutant substantially restored K240E IN tetramerization (**Figure 4b**). We next examined the effect of these changes on the ability of IN to catalyze two-end vDNA integration *in vitro*. We found that the catalytic activities of mutant INs tracked closely with their ability to form IN tetramers. The concerted integration products were reduced for E35K IN and markedly impaired for the K240E IN. However, the charge-swap double mutant E35K/K240E was substantially more active than its K240E counterpart (**Figure 4c**). Additionally, we examined the effect of these mutations on the ability of IN to bind and bridge vRNA[24,25]. We used the AlphaScreen-based assay[24] to probe IN-RNA

binding and bridging as a proxy for IN's role in facilitating the localization of RNPs into capsid lattices. Again, the ability of IN to bind and bridge RNA closely correlated with the tetramerization patterns of the mutant proteins. The K240E IN was substantially compromised for bridging RNA molecules, whereas E35K and E35K/K240E IN exhibited partly reduced RNA bridging activity (**Figure 4d**). Collectively, these experiments indicate that the tetrameric organization of IN, maintained in part through interactions between E35K and K240E, are relevant for performing the two distinct functional roles of IN *in vitro*.

We next analyzed the consequences of E35K, K240E, and E35K/K240E IN changes in the context of HIV-1 replication. Mutations were introduced into the IN-coding portion of a single-round luciferase reporter virus. Previously analyzed class I and class II IN mutant viruses D64N/D116N and H12N, respectively, were included for comparison[21,22]. Viruses were assessed for particle release from transfected HEK293T by p24 ELISA, and p24-normalized levels of WT and mutant viruses were used in subsequent infection assays. While H12N displayed a ~3-fold defect in particle release, the other viruses released from cells comparably to the WT (**Figure 5a**). As previously reported, the K240E and E35K changes instilled severe (~10,000-fold) and comparatively mild (~2-fold) HIV-1 infection defects, respectively (**Figure 5b**)[8]. Although the E35K/K240E double mutant was also significantly defective (~350-fold), we note that the addition of the E35K change boosted K240E infectivity and hence the completion of the early events of the viral replication cycle by ~28-fold.

To assess effects of the IN changes on HIV-1 structure, we first quantified IN protein content of virion extracts by immunoblotting. As described previously[21,55], H12N IN was unstable in virions. While the ~4-fold reduction observed in K240E virions was also statistically significant (**Figure 5c**), the IN content of the remaining viruses was generally similar to WT. Thus, the addition of the E35K change increased the steady-state level of K240E virus IN by about 2-fold. The RNP complex presents as electron-density by transmission electron microscopy (TEM), and ALLINI treatment and class II IN mutations yield a predominance of eccentric virion particles with the RNP situated outside of comparatively lucent capsid structures[24,25,29,54]. As expected, the vast majority of WT particles harbored centrally located electron density congruent with conical cores (**Figure 5d**, left; "Mature"); we noted comparatively minor populations of immature, empty, and eccentric particles. Whereas class I D64N/D116N and E35K particles appeared broadly similar to the WT, H12N revealed the sharp increase in eccentric particles typical of class II mutant virions. As K240E virion morphologies largely mimicked H12N, we conclude that K240E is a replication-defective class II IN mutant virus. The addition of the E35K change significantly improved the

proportion of mature K240E mutant particles. We conclude the charge swapped mutation partially corrects K240E virion structure by increasing the steady state level of IN protein and proportion of mature virion particles.

Discussion

HIV-1 IN structural dynamics help choreograph complex functions

HIV-1 IN is a highly dynamic protein[15,16]. Purified IN protein stoichiometry generally ranges from monomer to tetramer, with the tetramer being favored at higher protein concentrations or at low salt. However, the native form of HIV-1 IN in virions is likely tetrameric[26]. Here we solve the structure of the apo IN tetramer stabilized by the LEDGF/p75 IBD and elucidate snapshots for how the tetramer rearranges to assemble the intasome hexadecamer. Whereas the conformational changes necessary for intasome formation within and between individual IN domains, mediated by flexible NTD-CCD and CCD-CTD linkers, have been well-described and are generally conserved[6–8,10,49–52], changes to the tetrameric organization of lentiviral IN have remained unknown. Our structural biology data implies that the two IN dimers must contract while tethered through the CTD scaffold. An analogy would be the wing movement of a butterfly, with the tetrameric CTD arrangement representing the body and the two NTD:CCD dimers the wings. IN dynamics about the central interlocked CTDs, which are expected to be more pronounced in the absence of the IBD, may also be relevant to engaging/disengaging the viral RNA and for recognizing diverse RNA lengths. The data also informs a better understanding for why intasomes from HIV-1 (and likely by extension SIV), are more pleiotropic than those from MVV. Both hexadecameric intasome assemblies maintain a "CTD bridge" between tetramers I/III and II/IV, respectively; however, the inter-tetramer interaction interface made by the CTDs is weaker in HIV-1 in comparison to MVV (**Supplementary Figure 12**). Thus, it is not surprising that the flanking HIV-1 INs frequently dissociate, in turn making the whole complex compositionally pleiotropic. Collectively, these data add to our understanding of IN structural dynamics and suggest mechanisms by which these properties may help choreograph its complex functions during HIV-1 replication.

The oligomeric forms of HIV-1 IN relevant to function

Since the discovery of IN, there have been ongoing debates about its oligomeric form required for catalytic integration. Structures of PFV intasomes contained four IN protomers assembled around vDNA ends[6]. However, the first structures of HIV-1 intasomes complicated interpretations; whereas the conserved tetrameric core was analogous to PFV, larger species, including octamers, decamers, and dodecamers, were identified in particle subsets by cryo-EM[8].

The intasome from MVV, which is biochemically more stable and hence homogeneous, was a hexadecamer, although smaller species were also observed[10]. The pleiotropic nature of lentiviral IN / intasomes is well-established[8,10,12,17]. Loss-of-function experiments using MVV IN and corresponding viruses showed reduced ability of IN mutants to assemble into tetramers and form intasomes to catalyze strand transfer, with concomitant defects in viral infectivity[9]. Analogously, numerous loss-of-function and mutagenesis studies suggested that the composition of IN required for RNA binding is tetrameric. However, all loss-of-function experiments are inherently inconclusive because mutations may have pleiotropic effects, complicating conclusions. Thus, both the nature of the multimeric lentiviral IN assembly relevant for integration and for vRNA binding have remained unclear. In this regard, our identification of a key salt-bridge interaction between residues E35 and K240, and *in vitro* experiments with E35K, K240E and E35K/K240E mutant INs demonstrating that catalytic activity and RNA binding closely correlate with tetramerization, are noteworthy. Additionally, our virology experiments with these mutants highlight the indispensable role of the IN tetramer for viral infectivity. We note that the effects of E35K and K240E mutations alone are non-equivalent, and the phenotypic effect of K240E is more severe across all experimental assays. Although the introduction of either individual E35K or K240E mutations is expected to comparably compromise the salt-bridge interaction, there are two additional sets of E35/K240 residues in the tetramer structure outside the salt-bridge interface (**Figure 4a**). The effect of K240E must therefore extend beyond the salt bridge interface to exert pleiotropic effects on both intasome formation and on IN-mediated RNA binding, which would affect HIV-1 particle morphogenesis. Collectively, we conclude that the most biologically active form of the HIV-1 intasome is hexadecameric, and the relevant form of HIV-1 IN for vRNA binding is tetrameric.

How might the IN tetramer engage vRNA?

The arrangement of the tetrameric IN assembly has important implications for a working model of its binding to RNA. The four CTDs, which are crucial for RNA binding[24,25,35], zigzag along the interface of two IN dimers (**Figure 6a**). These CTDs provide a highly positively charged surface stretching over 80 Å, which could effectively bind RNA (**Figure 6b**). The central cavity (~40 Å) comprised of inner CTD_2 and CTD_4 is suitably sized to engage both single-stranded and double stranded RNAs while CTD_1-CTD_4 and CTD_3-CTD_2 are connected through narrow channels that could only accommodate single-stranded segments. Distinct vRNA structural

features, including stem/bulges relevant to IN binding[24], would be expected to dictate engagement of these sites on IN. The structure of the IN tetramer reveals a putative vRNA binding channel and provides initial clues for experimental observations that IN tetramers, rather than dimers (where two CTDs are completely separated from one another) or monomers, can effectively bind vRNA *in vitro* and in virions[24,25]. Class II IN mutant proteins exhibit a pronounced defect in IN's ability to bind and bridge RNA molecules, in part because they compromise IN tetramerization. Taken together, our findings strongly support the idea that proper IN tetramerization is an essential prerequisite for IN-vRNA binding in virions and plays a crucial role in IN's function during virion morphogenesis. The IN tetramer structure should also help to guide efforts to optimize clinically-relevant ALLINI compounds[26].

Study limitations

Because our structures were based on IN oligomers assembled from purified, recombinant components and not complexes isolated from infected cells, we can only infer biological relevance. Critically, our mutagenesis strategy indicates that the E35K-K240E salt bridge and, by extension, the IN tetramer structure, is biologically relevant. At the same time, our ability to inform the pathway of intasome assembly from the tetramer structure is limited through comparison of two independently resolved cryo-EM structures. Similarly, although our data strongly support the conclusion that intasomes that fail to multimerize will exhibit compromised integration efficiency, we cannot unequivocally know the oligomeric form of intasomes in cells, especially under conditions of stress. Although our model represents the most straightforward and accordingly likely biologically relevant pathway, it does not consider more elaborate scenarios that, for example, might require tetramer disassembly into composite components to assemble an active HIV-1 intasome.

Materials and Methods

Protein expression and purification of WT IN and IBD complex

The pCPH6P-IN plasmid contains full-length of HIV-1 NL4-3 IN with a N-terminal 6His tag, followed by a HRV 3C protease cleavage site. The pES-IBD-3C7 plasmid contains the LEDGF/p75 IBD with a N-terminal 6His-SUMO tag, followed by a SUMO protease cleavage site. These two plasmids were co-transformed into *E. coli* Rosetta DE3 competent cells (NEB) to co-express full-length HIV-1 IN and the IBD. The cells were cultured in LB medium supplemented with 50 μM $ZnCl_2$ and appropriate amount of antibiotics kanamycin and ampicillin. When the OD of the culture reached ~1.0 at 37°C, the temperature was dropped to 16°C, and expression was induced overnight with the addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Following overnight expression, cells were harvested for protein purification by centrifugation at 6,000 *g*.

To purify the IN-IBD complex, the cells were sonicated in a buffer containing 1 M NaCl, 20 mM imidazole, 0.2 mM PMSF, 50 mM Tris-HCl, 5mM 2-Mercaptoethanol, pH 7.6, then the cell lysate was subjected to centrifugation at 39,000 *g* for 30 minutes. The supernatant was immediately loaded into a 5 mL HisTrap column (Cytiva), which was attached to a FPLC system for purification. The complex was washed at 8% and 20% buffer B containing 250 mM imidazole to remove non-specific bound proteins, followed by an imidazole gradient wash and elution from 20%-100% of buffer B. The proteins were eluted in 1 M NaCl, 250 mM imidazole, 50 mM Tris-HCl, pH 7.6. The 6His tag on IN and the 6His-SUMO tag on the IBD were cleaved in the presence of 5 mM 2-mercaptoethanol using SUMO and HRV 3C proteases, respectively. The protein solution was then subjected to another 5 mL HisTrap column purification to remove the cleaved tags. The final IN/IBD complex was purified by SEC using a Superdex-200 column in 750 mM NaCl, 50 mM Tris-HCl, pH 7.6, 5 mM DTT and 10% glycerol.

In general, we found that the IN-IBD complex does not need additional buffer components, such as CHAPS detergent, to remain soluble and tetrameric during purification. In contrast, when we purified IN alone, all the initial steps, including resuspension, lysis, and HisTrap and heparin column purifications, required CHAPS to be present.

Cryo-EM sample preparation for the IN-IBD tetramer

IN-IBD complex (0.1 mg/mL) was applied onto freshly plasma cleaned (20s, Solarus plasma cleaner) holey grids (Quantifoil UltrAufoil R 1.2/1.3 300 mesh) at 4°C in the cold room with ~90% humidity. The sample was settled onto grids for 30 sec before manually blotting for 8 sec, then plunged into the liquid ethane using a manual plunger. The vitrified grids were transferred to liquid nitrogen for storage and data collection.

Cryo-EM data collection and processing for the IN-IBD tetramer

The IN/IBD complex cryo-EM dataset was collected on a Titan Krios transmission electron microscope (Thermo Fisher Scientific) operating at 300 keV, equipped with K3 direct electron detector (Gatan) with a GIF Quantum Filter with a slit width of 20 eV. The data collection was performed using Leginon[56,57] with the stage tilt set to 30°–50°[36,38,58]. Movies were recorded at a nominal magnification of 105 kx, corresponding to a calibrated pixel size of 0.83 Å. The total dose for this dataset is 49.5 e^-/ Å^2, using dose rate of 22.7 e^-/ pix/s. The imaging parameters for this dataset are summarized in **Supplementary Table 1**.

For data processing, the dose-weighted[59] motion correction was performed in Relion 4.0-beta-2[60] using a gain reference that was generated during data collection with a B factor of 150 Å^2 on 5-by-5 patch squares. The aligned and dose-weighted micrographs were transferred to Warp 1.0.9[61] to conduct contrast transfer function (CTF) estimation and particle selection. After CTF estimation, a re-trained BoxNet model was used for particle picking with constraint settings of particle diameter of 130 Å and a minimum distance between particles of 30 Å. To avoid contaminants, the particles that scored above 0.9 were selected and extracted using a boxsize of 320 pixels to generate the particle star file. The motion-corrected micrographs in Relion and particle star file, generated in Warp, were imported into cryoSPARC V3.3.2[39] for the next steps of data processing. The particles were re-extracted in cryoSPARC using boxsize of 320 pixels for 2D classification. After one round of 2D classification, the majority of particles in 2D classes were selected except those particles without discernable particle features. The particles selected from 2D averages were subjected to multiple rounds of heterogeneous refinement using an ab-initio low-resolution map as the initial volume to enrich good particles until no further improvements were observed, as indicated by the Fourier shell correlation (FSC)[62]. After several rounds of heterogeneous refinement, particles contributing to the highest-resolution map were selected, whereas the remaining particles were discarded. The selected particles from the last round of heterogeneous refinement were subjected to a non-uniform refinement to generate a high-

resolution map. These particles were then imported back to Relion to perform Bayesian polishing using default parameters[63]. The polished particles were re-imported back to cryoSPARC to perform one round of per-particle CTF refinement and one round of non-uniform refinement. We repeated the procedure – Bayesian polishing in Relion followed by CTF refinement and non-uniform refinement in cryoSPARC iteratively, until no further improvements were observed in terms of map quality. The optics parameters, per-particle defoci, and the map resolutions converged after two rounds of polishing and CTF refinement. The map was generated from 442,964 particles at this stage.

A close inspection of the map generated from the above procedure indicated the presence of residual noise that affected its interpretation. We thus proceeded to further clean the particle stack using 3D classification in cryoDRGN. To do this, we used the 442,964 particles from above for training cryoDRGN[40]. The particle box size was initially cropped from 320 Å to 192 Å and then downsampled to 96 Å. Subsequently, the variational autoencoder neural network (256x3 architecture) implemented in cryoDRGN was trained on the downsampled particle stack. The latent space was visualized using PCA and UMAP embeddings, and 3D density maps (volumes) were generated from the latent embeddings at 35 kmeans cluster-centers using the analysis tools in the cryoDRGN suite. Subsequently, particles were filtered using the template Jupyter notebook in cryoDRGN and visual inspection of the resultant 35 volumes corresponding to the 35 kmeans clusters, filtering out the particles corresponding to worst volumes and keeping a relatively high number of particles in the stack for subsequent processing. This resulted in a filtered stack of 355,028 particles, which were then imported into cryoSPARC for a last round of non-uniform refinement, producing the final density map displayed here (**Supplementary Table 1** and **Figure 1**). The directional resolution of the map was evaluated using the 3D FSC server (3dfsc.salk.edu)[36], and the quality of the orientation distribution was evaluated using the Sampling Compensation Function (SCF)[64,65]. All images were made using UCSF Chimera[66].

Model building for the IN-IBD tetramer

The atomic model of HIV-1 IN tetramer was constructed in a stepwise manner. We first docked all individual domains into density, beginning with high-resolution crystal structures of the CCD:CCD dimer[41], the NTD[67], and the CTD[47], which were rigid body docked into the 3.0 Å cryo-EM map using UCSF Chimera. The alpha helical linkers connecting the CCD to the CTD were built automatically using Rosetta[68]. Subsequently, the model was subjected to one round of real-

space refinement in the Phenix suite using phenix.real_space_refine with restraint weights[69]. The model was manually adjusted in Coot[70], and the discrepant parts in the model were removed. The final model was generated by iterative inspection and adjustment in Coot and Phenix using phenix.real_space_refine. The statistics of the final model are shown in **Supplementary Table 1**.

Computational classification and refinement of the hexadecameric HIV-1 intasome

Based on the assumption that the two flanking regions are independently flexible, we proceeded with resolving each flanking region within the intasome core separately in cryoSPARC[39]. The map was divided into a "top" half and a "bottom" half, as shown in **Supplementary Figure 9**. To resolve the top flanking region withing core, 147,569 particles were subjected to density subtraction to first remove signal in the bottom flanking region. The subtracted particles were classified using global 3D classification into five classes. From these classes, 70,680 particles from the best three classes had density within the top flanking region. These particles were selected and subjected to a second round of 3D classification using a mask focused on the top flanking region. From focused classification, we selected 11,658 particles that contained clearly resolved density for the entirety of the flanking region, whereby linker density and density for all domains was evident. These particles were then subjected to local CTF refinement, which was followed by a round of local refinement using a mask on the entirety of the volume. This procedure yielded a final map of the top half of the HIV-1 intasome. We resolved the bottom half of the intasome using an identical method, with 12,124 particles contributing to the final map. The procedure is demonstrated in **Supplementary Figure 9**. Both maps were validated using global and local resolution estimates in cryoSPARC. The 3DFSC was generated using the 3D FSC server following standard procedures[36,71]. The surface sampling plot for the Euler angle distribution was generated using standalone scripts, as previously described[58,65]. The composite map was stitched together from two independently refined halves of the intasome after aligning each half to the intasome core and summing the two maps using maximum pixel intensities as the summation criterion. The validation for both maps is shown in **Supplementary Figure 10**, and the generation of the composite map is shown in **Supplementary Figure 11**. The visualization of all maps was performed in UCSF Chimera and ChimeraX[66,72].

Expression and purification of WT and mutant IN proteins

All of the mutations were introduced into a plasmid backbone expressing 6xHis tagged pNL4-3-derived IN by QuikChange site-directed mutagenesis kit (Agilent). The plasmids containing full-length WT and mutants were transformed into BL21 (DE3) *E. coli* cells. The cells were cultured in LB medium supplemented with 50 μM $ZnCl_2$ and appropriate amount of antibiotics kanamycin and ampicillin. When the OD of the culture reached ~1.0 at 37°C, the temperature was dropped to 16°C, and expression was induced overnight with the addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Next day, the cells were harvested by centrifugation at 4200 *g*, 15min and suspended in Ni buffer A containing 45 mM HEPES, pH 7.5, 6.75 mM CHAPS, 0.9 M NaCl, 10% glycerol, 3 mM 2-mercaptoethanol, 20 mM imidazole. The resuspended cells were sonicated and subjected to centrifugation at 39,000 *g* for 30 min. The supernatant was immediately loaded into a 5 mL HisTrap column, attached to a FPLC system at a flow rate of 1 ml/min. After sample loading, a gradient washing/elution was carried out using buffer A and B (45 mM HEPES, pH 7.5, 6.75 mM CHAPS, 0.9 M NaCl, 10% glycerol, 3 mM 2-mercaptoethanol, 500 mM imidazole). 1.5ml of each fraction was collected. The fractions containing target proteins were pooled and diluted to 300 mM salt and loaded onto a second Heparin column equilibrated with 30% Heparin buffer B containing 50 mM HEPES, pH 7.5, 7.5 mM CHAPS, 1 M NaCl, 10% glycerol, 3 mM 2-mercaptoethanol a flow rate of 3 ml/min followed by gradient wash from 30% buffer B to 100%. Fractions across the peak were run on SDS-PAGE and clean fractions containing target proteins were pooled and concentrated for downstream assays.

In vitro IN concerted integration assay

Integration assays were conducted essentially as described[14] with slight modifications. Briefly, 2.0 µM IN and 1.0 µM vDNA were preincubated on ice in 20 mM HEPES (pH 7.5), 25% glycerol, 10 mM DTT, 5 mM $MgCl_2$, 4 µM $ZnCl_2$, 50 mM 3-(Benzyldimethyl-ammonio) propanesulfonate (Sigma), 3.0 µM polyethylenimine (Sigma) and 100 mM NaCl in a 20-µl reaction volume. The vDNA (Integrated DNA Technologies) was fluorescently labeled with a 6-carboxyfluorescein (6-FAM) fluorophore attached to the 5′-end of the oligonucleotide, yielding 5'-Fam-AGCGTGGGCGGGAAAATCTCTAGCA, which was annealed with 5'-ACTGCTAGAGATTTTCCCGCCCACGCT-3' to form the precut vDNA substrate. Target plasmid DNA pGEM-9zf (300 ng) was then added and the reaction was initiated by transferring to 37°C, and incubated for 2 h. The integration reactions were stopped by addition of SDS and EDTA to 0.2% and 10 mM, respectively, together with 5 µg of proteinase K and further incubated at 37°C

for an additional 1 h. The DNA was then recovered by ethanol precipitation and subjected to electrophoresis in a 1.5% agarose gel in 1× tris-boric acid–EDTA buffer. DNA was visualized by fluorescence using a Typhoon 8600 fluorescence scanner (GE Healthcare). Densitometry was used to quantify IN activity. The density of the bands corresponding to concerted integration on fluorescence-scanned gels was quantified by ImageQuant (GE Healthcare). The integration activity of WT IN was set to 100%, and mutant activities are expressed as percentages of WT activity. Error bars were calculated from triplicate samples.

Size Exclusion chromatography (SEC)

Purified IN proteins were diluted to 1.5 mg/ml with running buffer containing 20 mM HEPES (pH 7.5), 750 mM NaCl, 10% glycerol, 5 mM CHAPS and 5 mM 2-mercaptoethanol and incubated for 1 h at 4°C followed by centrifugation at 10,000 g for 10 min at 4°C. Multimeric state of WT and mutant INs were analyzed on Superdex 200 10/300 GL column (GE Healthcare) equilibrated with running buffer at a flow rate of 0.2 ml/min.

IN-RNA interactions

To monitor IN-RNA interactions we utilized AlphaScreen-based assay[24], which monitors the ability for IN to bind and bridge between two trans-activation response element (TAR) RNAs. Briefly, equal concentrations (1 nM) of two synthetic TAR RNA oligonucleotides labeled either with biotin or digoxin (DIG) were mixed and then streptavidin donor and anti-DIG acceptor beads at 0.02 mg/mL concentration were supplied in a buffer containing 100 mM NaCl, 1 mM MgCl2, 1 mM DTT, 1 mg/mL BSA, and 25 mM Tris (pH 7.4). After 2 h incubation at 4°C, 320 nM IN was added to the reaction mixture and incubated further for 1.5 h at 4°C. AlphaScreen signals were recorded with a PerkinElmer Life Sciences Enspire multimode plate reader.

Crosslinked HIV-1 Intasome preparation

Intasomes containing pre-cleaved vDNA ends were assembled using essentially the same conditions as for the concerted integration assay descried above, except the target plasmid DNA was omitted, 50 µM dolutegravir was present in the reaction buffer, and the vDNA substrate was substituted by U5-69T, which was prepared by annealing LTR-29 (5'-AAAAAAAAGTGTGGAAAATCTCTAGCA-3') with U5-69R (5'-ACTGCTAGAGATTTTCCACACTTT

TTTTTTTTTTTTTTTTTTTTTTTT-3'). The reaction was carried at 37°C for 120 min and stopped on ice, then, NaCl (1.0 M final) was added to the reaction mixtures to solubilize the intasomes. Purification of intasomes was essentially as previously described[13]. The reaction mix was loaded onto a HisTrap column (GE Healthcare) equilibrated with 20 mM HEPES pH 8.0, 5 mM 2-mercaptoethanol, 1.0 M NaCl, 20% (w/v) glycerol. Intasomes were then eluted with a linear gradient of 0 mM to 500 mM imidazole in the same buffer. Pooled intasomes were crosslinked by 2.0 mM BS3 (Pierce). The crosslinking reaction was quenched by adding 100 mM Tris, pH 8.0. Aggregates/stacks of intasomes and free IN were then removed by gel filtration on a TSKgel UltraSW Aggregate HPLC column (Tosoh Bioscience) in 20 mM Tris pH 6.2, 0.5 mM TCEP, 1.0 M NaCl, 5.0 mM MgCl2, and 10% (w/v) glycerol. Intasomes corresponding to single species were concentrated to 0.4 mg/ml for EM studies.

Virological assays

To build IN mutant viral constructs, the larger DNA fragment of pNLX.Luc.R-.ΔAvrII[73] following digestion with PflMI and AgeI restriction enzymes (New England Biolabs) was assembled with 1.85-kb synthetic fragments (Twist Bioscience) containing single or double E35K and K240E changes using NEBuilder HiFi DNA Assembly MasterMix under conditions recommended by the manufacturer. Analogous H12N[74] and D116N/D64N[75] mutant constructs were previously described.

HEK293T cells obtained from America Type Culture Collection were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (DMEM) at 37 °C in the presence of 5% CO_2. Viruses were generated by co-transfecting 10^7 cells plated the previous day in 15 cm dishes with 30 μg of total plasmid DNA (pNLX.Luc.R-.ΔAvrII and a vesicular stomatitis virus G envelope expressor at 6:1 ratio using PolyJet™ DNA transfection reagent; SignaGen Laboratories). Two days post-transfection, virus-containing cell supernatants were clarified by centrifugation at 800 *xg* for 15 min prior to filtration by gravity flow through 0.45 μm filters. Capsid (CA) levels were assessed by p24 ELISA using a commercial kit (Advanced Bioscience Laboratories). Viruses were pelleted by ultracentrifugation using a Beckman SW32-Ti rotor at 26,000 rpm for 2 h for western blotting and TEM.

Cells (1.5 X 10^5) were infected with 37.5 ng p24 in 100 μL DMEM for 1 h at 37 °C, after which 400 μl DMEM was added and the mixtures were transferred to 24-well plates. After 6 h at 37 °C, the media was replaced with fresh DMEM. At 48 h post-infection, cells were washed twice

with phosphate-buffered saline, removed from the plate, and treated with passive lysis buffer (Promega) by freezing for 1 h at -80 °C and thawing at 37 °C for 10 min. Cell lysates were vortexed and centrifuged at 21,100 × *g* for 10 min. Luciferase activity, assessed as relative light units per μg of total protein in the cell extracts (RLU/μg), was determined as previously described[76].

For immunoblotting, equal volumes of viral lysates separated by electrophoresis on Bolt 4–12% Bis-Tris Plus gels (Life Technologies) were transferred using Trans-Blo Turbo Mini PVDF Transfer Packs (Bio-Rad). Membranes blocked for 1 h at room temperature were probed overnight at 4 °C in 5% non-fat dry milk with 1:2,000 anti-CA (Abcam ab63917) or 1:5,000 anti-IN[77]. The following day, immunoblots were probed using goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Agilent P0448), treated with enhanced chemiluminescence detection reagents (Cytiva), and imaged on ChemiDoc Imaging System (Bio-Rad). Adjusted relative band intensity values for anti-IN immunoblots were determined using ImageJ software with anti-CA bands acting as loading controls. A vertical rectangular selection was made over each lane and plotted as a histogram for both immunoblots. For anti-CA, p24 and p25 bands were combined. The area under the curve representing the protein of interest was calculated using the "wand" tracing tool. A "percentage value" was assigned to each sample by dividing the area of each individual peak by the sum of all peaks. Relative band intensity to WT was calculated for each immunoblot. Finally, adjusted relative density was determined by dividing anti-IN relative density by respective anti-CA relative density.

For TEM, virus pellets were resuspended in 1 mL fixative solution (2.5% glutaraldehyde, 1.25% paraformaldehyde, 0.03% picric acid, 0.1 M sodium cacodylate, pH 7.4) and incubated at 4 °C overnight. The preparation and sectioning of fixed virus pellets was performed at Harvard Medical School Electron Microscopy core facility as previously described[78]. Sections (50 nm) were imaged using a JEOL 1200EX transmission electron microscope operated at 80 kV. All images were taken at 30,000X magnification. Micrographs were uploaded to ImageJ and analyzed using the Cell Counter plugin, which allows numbers to be assigned to individual virions. Categories "Mature", "Immature", "Empty", and "Eccentric" were assigned based on previously described IN mutant phenotypes[54].

Acknowledgements

Funding: We are grateful for funding from the following sources: NIH grants U01 AI136680 awarded to D.L. and M.K., R01 AI146017 awarded to D.L., U54 AI170855 awarded to D.L, M.K., and R.C., R37 AI039394 and U54 AI170791 awarded to A.N.E., the Intramural Program of the National Institute of Diabetes and Digestive Diseases, NIH to R.C. D.L. is also grateful to the Margaret T. Morris Foundation and the Hearst Foundations for funding support. The molecular graphics and analyses were performed with the USCF Chimera package (supported by NIH P41 GM103311). The cryo-EM data for this project was collected at Scripps Research with equipment supported by NIH grant S10OD032467.

Competing interests:

None.

Data and materials availability:

All data needed to evaluate the conclusions in the paper are present in the paper and/or the **Supplementary Materials**.

Figure Legends

Figure 1. Cryo-EM structure of the tetrameric HIV-1 IN-IBD complex. (**a**) Cryo-EM reconstruction. Colors refer to individual IN protomers (orange, cyan, blue, red) and IBD (brown). (**b**) Segmented cryo-EM density colored by IN domains (NTD, green; CCD, yellow; CCD-CTD linker, magenta; CTD, purple) and IBD (brown). (**c** and **d**) Atomic model colored by IN promoters and IN domains, respectively, as in **a** and **b**. Subscripts denote the four different IN chains.

Figure 2. The structure of full-length IN reveals novel conformational rearrangements and inter-domain interactions. (**a**) Comparison of the CCD-CTD dimer from the IN tetramer to previously determined two-domain IN structures from HIV-1 or other lentiviruses. All prior structures are colored in gray and correspond to HIV-1 (1EX4[45] and 5HOT[30]), MVV (5T3A)[10], or SIV (1C6V[46]). (**b**) Comparison of a single CCD-CTD protomer from the IN tetramer to individual fully modeled IN protomers from the MVV intasome[10]. The conformation of the CCD-CTD protomer from HIV-1 is distinct from all modeled MVV conformations, implying a large degree of structural plasticity. (**c**) The structure of the tetramer is displayed in the center, with key interactions interfaces shown within inset panels. The interaction mediated by the central CTD:CTD dimer (bottom left panel) resides in an anti-parallel configuration, which is distinct from the parallel configuration previously encountered in CCD-CTD oligomeric assemblies with bound inhibitors[30] or within dimeric CTD:CTD structures in solution[67], but resembles the crystal packing interactions observed in PDB 8CTA[48]. The CTD also mediates other diverse interactions, including with the NTD (upper left) and with the CCD-CTD linker (upper right) within the IN tetramer.

Figure 3. The IN tetramer undergoes compression and domain rearrangements to assemble the hexadecameric HIV-1 intasome. (**a**) Composite map of the complete HIV-1 intasome with four IN tetramers assembled with two vDNA ends. Each of four IN protomers are colored in red, orange, blue, or cyan; the vDNA is in gray. (**b**) Two distinct IN tetramers from the hexadecameric HIV-1 intasome are displayed as isosurfaces (left) and with the apo IN tetramer docked into the density (right). The two IN dimers constituting the apo tetramer must move closer

together, or compress, to fit into each of the tetrameric building blocks within the HIV-1 intasome. This is apparent from the bottom dimer, which resides outside of the EM density, whereas the top dimer nearly perfectly superimposes. The curved arrows indicate rearrangements of NTDs and CTDs. (**c**) Schematic showing all major structural changes of the HIV-1 IN tetramer to accommodate intasome assembly. Boxes around the CCDs highlight the compression of individual IN dimers, whereas the curved arrows indicate rearrangements of NTDs and CTDs.

Figure 4. The E35K change restores K240E IN functionality in the context of charge-swapped E35K/K240E *in vitro*. (**a**) Salt bridge interaction formed between E35 in NTD_1/NTD_3 and K240 in CTD_1/CTD_3, within the outer two regions of IN tetramer. (**b**) SEC analysis demonstrating the restoration of K240E tetramerization by the E35K/K240E mutant (T: tetramer, D: dimer). (**c**) Concerted integration assay. Left panel: schematic representation of the assay using vDNA ends, supercoiled plasmid DNA, and purified IN. Both half site and concerted integration products are generated as outputs. Middle panel: integration products were detected on 1.5% agarose gel using a fluorescence scanner. The bands corresponding to half site and concerted products are indicated. Right panel: bar graph showing the quantification of concerted integration within the middle panel. The integration activity of WT IN was set to 100%, and the activity of mutant IN was presented as a percentage of WT. The error bars represent SDs of independent experiments, n = 3, performed in triplicate. (**d**) Left panel: Schematic of an AlphaScreen-based RNA bridging assay[24]. Each oval corresponds to an IN multimer. “A” and “D” indicate anti-digoxin Acceptor and streptavidin coated Donor beads bound to either digoxin (DIG) or biotin, respectively. Right panel: quantification of purified IN protein interactions with HIV-1 TAR RNA. Average values are from three independent experiments, with error bars indicating SDs. Statistically significant differences in comparison to WT and between experimental groups are represented with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 5. E35K partially restores K240E IN defects during early and late stages of HIV-1 replication. (**a**) IN mutant p24 values (µg/mL) normalized to WT. (**b**) IN mutant infectivities (RLU/µg) normalized to WT. (**c**) Immunoblot analysis of IN and Gag/p24 processing intermediates. Representative anti-IN and anti-CA blots from three independent experiments are shown; numbers to the left are mass standards in kDa. Right, quantification of IN mutant band intensities relative to p24/p25. (**d**) Frequencies of WT and IN mutant virus morphologies. For each

experiment, >200 virions were counted. Categories were assigned based on the following criteria (1) Mature; vRNPs congruent with the CA lattice, (2) Immature; radially arranged electron density, (3) Empty; membrane with no contents, (4) Eccentric; vRNPs outside the CA lattice. Results are avg. ± SEM of n=3 (panels a-c) or n=2 (panel d) experiments. Statistically significant differences in comparison to WT virus are represented with asterisks (*, $P < 0.05$, ** $P < 0.01$).

Figure 6. Electrostatic environment of the IN tetramer. (**a**) Atomic model of the IN tetramer, colored by domain. The four CTDs, which are crucial for RNA binding[24–26], zigzag along the interface of two IN dimers. (**b**) The model is colored by the electrostatic charge distribution. The organization of the CTDs provide a highly positively charged surface stretching over 80 Å, which could effectively bind RNA. A ~40 Å central cavity comprised of inner CTD_2 and CTD_4 is suitably sized to engage both single- and double-stranded RNAs. The putative path of RNA binding to the IN tetramer is shown in yellow.

Supplementary Figure 1. SEC analysis of WT IN and IN-IBD complex in two buffer conditions. The IN-IBD complex or IN alone was eluted in buffers containing 750 mM NaCl in the presence or absence of CHAPS. In the absence of the IBD, the multimeric state of IN changed as a function of CHAPS, but the IN-IBD complex eluted as a tetramer irrespective of CHAPS in the buffer. T: tetramer, D: dimer. The migration positions of protein standards are indicated atop the chromatograms.

Supplementary Figure 2. Purification of the IN-IBD complex. (**a**) The IN-IBD complex was initially purified and eluted from SEC, represented by the blue trace. The peak fraction from the 2nd peak of the blue trace was re-injected into the same gel filtration column and eluted again (orange trace), yielding one peak. (**b**) SDS-PAGE analysis of the individual fractions of the initial SEC run (blue trace). The boxed fraction was selected for cryo-EM.

Supplementary Figure 3. Cryo-EM validations. (**a**) Representative cryo-EM image of IN-IBD complex. (**b**) Representative of 2D class of IN-IBD complex. (**c**) Cryo-EM map colored by local resolution. (**d**) Fourier shell correlation (FSC) curves derived from two half-maps with FSC cutoffs at the 0.143 threshold indicated. (**e**) Global FSC curve shown alongside the range of directional resolution conical FSC curves (in green, +/-1 standard deviation) and overlaid on a histogram of directional resolution values taken at 0.143 threshold.

Supplementary Figure 4. Comparison of IBD organization between the IN tetramer with prior structures. (**a**) Left panel: an individual IN-IBD dimer extracted from the full-length IN-IBD tetramer. Middle panel: IN-IBD dimer from crystalized HIV-1 IN_{CCD} dimer complexed to IBD (PDB 2B4J)[43]. Right panel: one IBD bound to the IN_{CCD} dimer from the conserved intasome core of the MVV intasome (PDB 7U32)[9]. (**b**) An individual IN-IBD dimer is extracted from the full-length IN-IBD tetramer (as in panel **a**), with the two segmented cryo-EM densities for the IBDs displayed in transparency. The enclosed volume of top and bottom IBD densities are calculated respectively in Chimera, and the relative volumes are labeled next to the density map. The CCD dimers are colored in yellow and IBD are in brown. For clarity, NTD and CTD were omitted.

Supplementary Figure 5. Comparison of CTD:NTD interface between the IN tetramer and the HIV-1 intasome. (**a**) CTD-NTD interface in the IN tetramer showing interaction between E35 and K240. (**b**) CTD-NTD interface in the conserved core of the HIV-1 intasome (PDB 6PUT)[12]

showing interaction between E35 and K240. The colors are as in Figure 1c-d (NTD, green; CCD, yellow; CCD-CTD, magenta; CTD, purple). Other regions of the HIV-1 conserved intasome core are shown in gray.

Supplementary Figure 6. Comparison of NTD and CCD organization between the IN tetramer and prior structures. (**a**) NTD-CCD dimer from HIV-1 full-length IN tetramer. (**b**) NTD-CCD dimer from HIV-1 NTD-CCD two-domain construct, which crystalized in a tetrameric form (PDB 1K6Y)[42]. (**c-d**) NTD-CCD dimer from two crystallographic forms of MVV IN tetramers, PDB 3HPH and 3HPG, respectively. (**e**) NTD-CCD dimer selected from an MVV intasome solved by cryo-EM (PDB 7U32). The three other unique dimers within the intasome assembly similarly had density occupying the NTD:CCD interface. (**f**) Crystal structure of mouse mammary tumor virus (MMTV) IN NTD-CCD construct (PDB 5CZ2). In all cases, the NTD and CCD domains are colored by forest green and yellow, respectively. All structures are presented in the same orientation. For clarity, the CTD domains in panels **a** and **e** were omitted.

Supplementary Figure 7. Purification of the crosslinked WT HIV-1 intasome. Crosslinked intasomes were purified by gel filtration on a TSKgel UltraSW HPLC column in 20 mM Tris pH 6.2, 0.5 mM TCEP, 1.0 M NaCl, 5.0 mM MgCl2, and 10% (w/v) glycerol. The gel filtration profile for the purification is shown. All species eluting earlier than ~7 mL retention volume correspond to intasome stacks, which have been previously described for HIV-1[12], SIV[17], and MVV intasomes[10]. These stacks contain multiple vDNAs copies and are not physiologically relevant. Intasomes used for cryo-EM studies are indicated by the red arrow.

Supplementary Figure 8. Initial results from cryo-EM processing of the crosslinked HIV-1 intasome. (**a**) Representative micrograph of the crosslinked HIV-1 intasome (**b**) Representative 2D class averages from the cryo-EM data (**c**) Cryo-EM map generated in cryoSPARC[39] using a conventional data processing strategy and after global 3D classification. (**d**) Cross-section of the reconstructed map from 3D classification, showing heterogeneous density for the poorly resolved flanking regions, which correspond to the outer IN subunits. We were unable to recover the missing data using any combination of global classification approaches, with and without symmetry applied.

Supplementary Figure 9. Workflow to resolve the flanking regions within the hexadecameric HIV-1 Intasome. 147,569 particles were subjected to density subtraction to remove signal in the bottom flanking region. The subtracted particles were classified using global 3D classification into five classes. Among them, three classes with potential top flanking densities were selected (dashed box), and the particles within these classes were subsequently subjected to a second round of 3D classification, using a mask focused on the top flanking region. The 11,658 particles from the best class (dashed box) were selected. Finally, we conducted one round of local CTF refinement, followed by a final round of non-uniform refinement[79], using a mask encompassing the entire volume.

Supplementary Figure 10. Validation metrics for each independently refined half of the HIV-1 intasome. Evaluation of the two maps containing the top and bottom flanking regions of the hexadecameric intasome. (**a-c**) The top panel corresponds to the map derived from focused classification on the upper flank, and the bottom panel corresponds to the map derived using the same approach on the bottom flank. (**a**) Central slice through the 3DFSC, colored by resolution. (**b**) Surface sampling plot for the Euler angle distribution, with the sampling compensation factor (SCF) value indicated below. (**c**) Global FSC curve derived from the two half-maps. (**d**) Refined cryo-EM map colored by local resolution.

Supplementary Figure 11. Derivation of the composite map of the hexadecameric Intasome. (**a**) The composite map of the HIV-1 intasome hexadecamer was derived from two independently refined halves, summed using maximum pixel intensities for each of the aligned halves. (**b**) Pseudo-atomic model of the HIV-1 intasome hexadecamer, derived through rigid-body docking of individual NTD, CCD, and CTD domains into cryo-EM density. The model is colored by IN (left) protomer and (right) domain.

Supplementary Figure 12. Comparison of the intasome hexadecamers between HIV-1 and MVV. The pseudo-atomic model of the HIV-1 intasome is displayed alongside the atomic model of the MVV intasome (PDB 7U32)[9]. For each model (top), a molecular map (bottom), generated in Chimera at 8 Å resolution, is displayed below to show a surface view of the assembly. The models and maps are colored by IN domain. The inter-tetramer CTD-CTD bridge, which has a smaller interface in the HIV-1 intasome compared to the MVV intasome, is circled.

Supplementary Figure 13. Purification of WT and mutant IN. SDS PAGE showing purified WT and mutant IN proteins.

Cryo-EM data collection, refinement and validation statistics

	IN-IBD complex (EMDB-XXXX) (PDB-XXXX)	Crosslinked WT Intasome (EMDB-XXXX) (PDB-XXXX)
Data collection and processing		
Magnification	105,000x	29,000
Voltage (kV)	300	300
Electron exposure (e–/Å2)	49.5	60.0
Defocus range (μm)	-0.8 to -2.2	-1.0 to -3.0
Pixel size (Å)	0.83	1.015
Symmetry imposed	C2	C2 → C1
Number of particles (in final map).	340,383	11,658
Map resolution (Å)	3.0	5.9
FSC threshold	0.143	0.143
Map resolution range (Å)	3.0 – 4.0	3.0 – 12.0
Refinement		
Initial model used (PDB codes)	1ITG, 1IHV, 1WJA	
Model resolution (Å)	4.0	
FSC threshold	0.5	
Model resolution range (Å)	3.5 – 4.5	
Map sharpening *B* factor ($Å^2$)	-140	
Model composition		
Non-hydrogen atoms	11,176	
Protein residues	1,392	
B factors ($Å^2$)		
Protein	92	
R.m.s. deviations		
Bond lengths (Å)	0.007	
Bond angles (°)	1.007	
Validation		
MolProbity score	2.4	
Clashscore	14.9	
Poor rotamers (%)	2.3	
Ramachandran plot		
Favored (%)	93.1	
Allowed (%)	6.8	
Disallowed (%)	0.1	

Supplementary Table 1. Cryo-EM data collection, refinement, and validation statistics.

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Figure 1

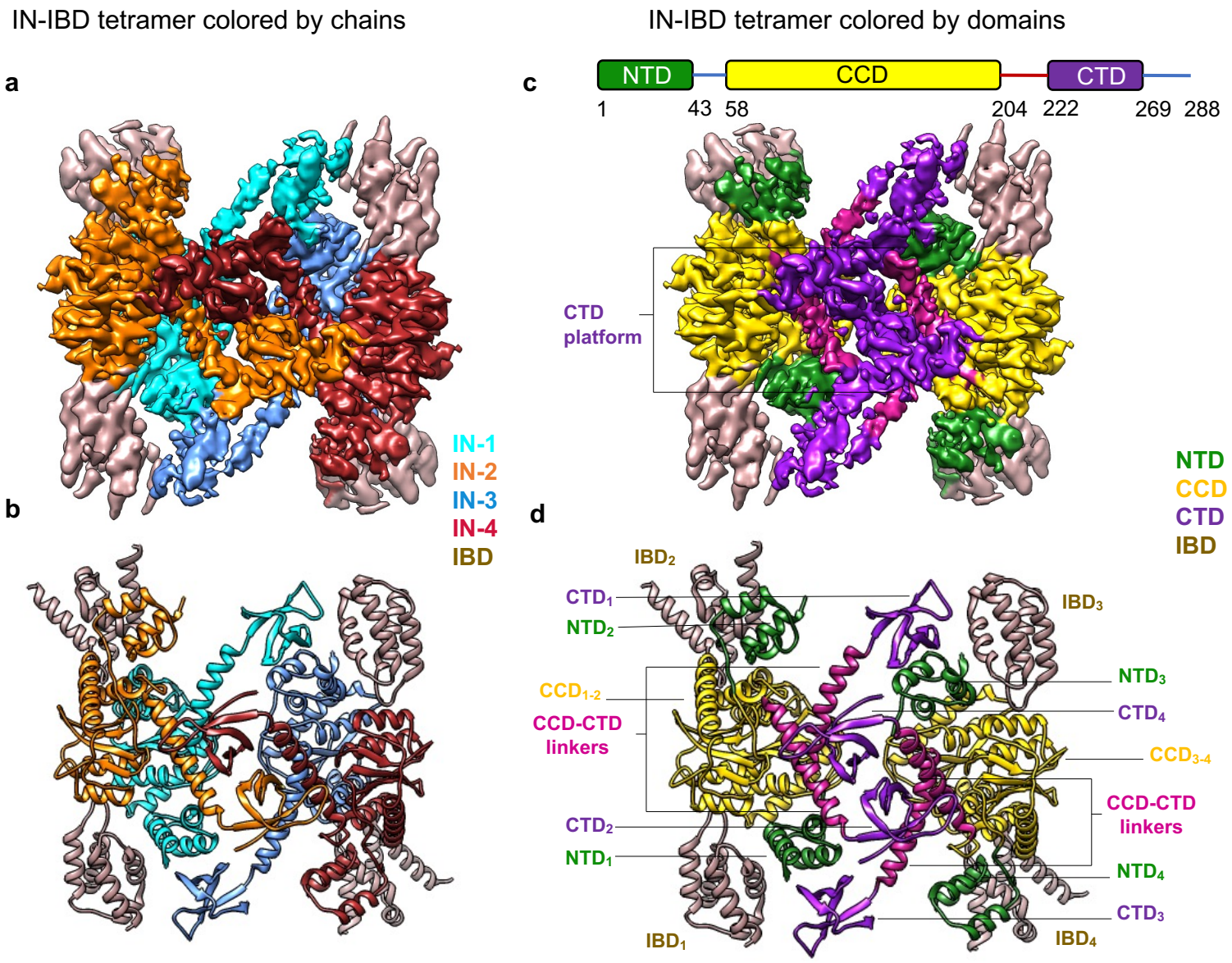

IN-IBD tetramer colored by chains
IN-IBD tetramer colored by domains
a
b
c
d
NTD
CCD
CTD
1
43
58
204
222
269
288
CTD platform
IN-1
IN-2
IN-3
IN-4
IBD
NTD
CCD
CTD
IBD
IBD2
CTD1
NTD2
CCD1-2
CCD-CTD linkers
CTD2
NTD1
IBD1
IBD3
NTD3
CTD4
CCD3-4
CCD-CTD linkers
NTD4
CTD3
IBD4

Figure 2

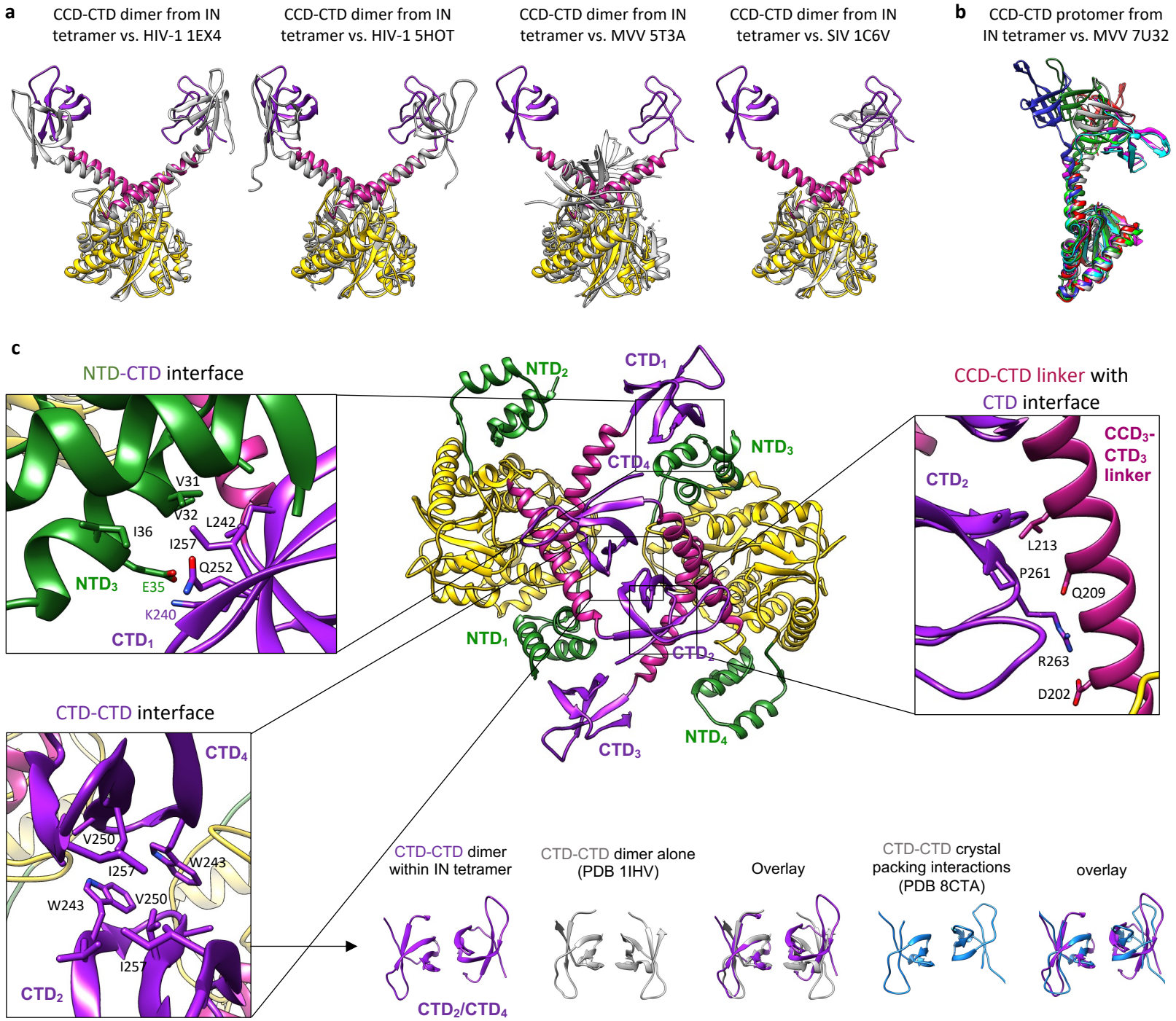

a
CCD-CTD dimer from IN tetramer vs. HIV-1 1EX4
CCD-CTD dimer from IN tetramer vs. HIV-1 5HOT
CCD-CTD dimer from IN tetramer vs. MVV 5T3A
CCD-CTD dimer from IN tetramer vs. SIV 1C6V
b
CCD-CTD protomer from IN tetramer vs. MVV 7U32
c
NTD-CTD interface
V31
V32
L242
I36
I257
Q252
NTD3
E35
K240
CTD1
NTD2
CTD1
NTD3
CTD4
NTD1
CTD2
CTD3
NTD4
CCD-CTD linker with CTD interface
CCD3-CTD3 linker
CTD2
L213
P261
Q209
R263
D202
CTD-CTD interface
CTD4
V250
I257
W243
W243
V250
I257
CTD2
CTD-CTD dimer within IN tetramer
CTD2/CTD4
CTD-CTD dimer alone (PDB 1IHV)
Overlay
CTD-CTD crystal packing interactions (PDB 8CTA)
overlay

Figure 3

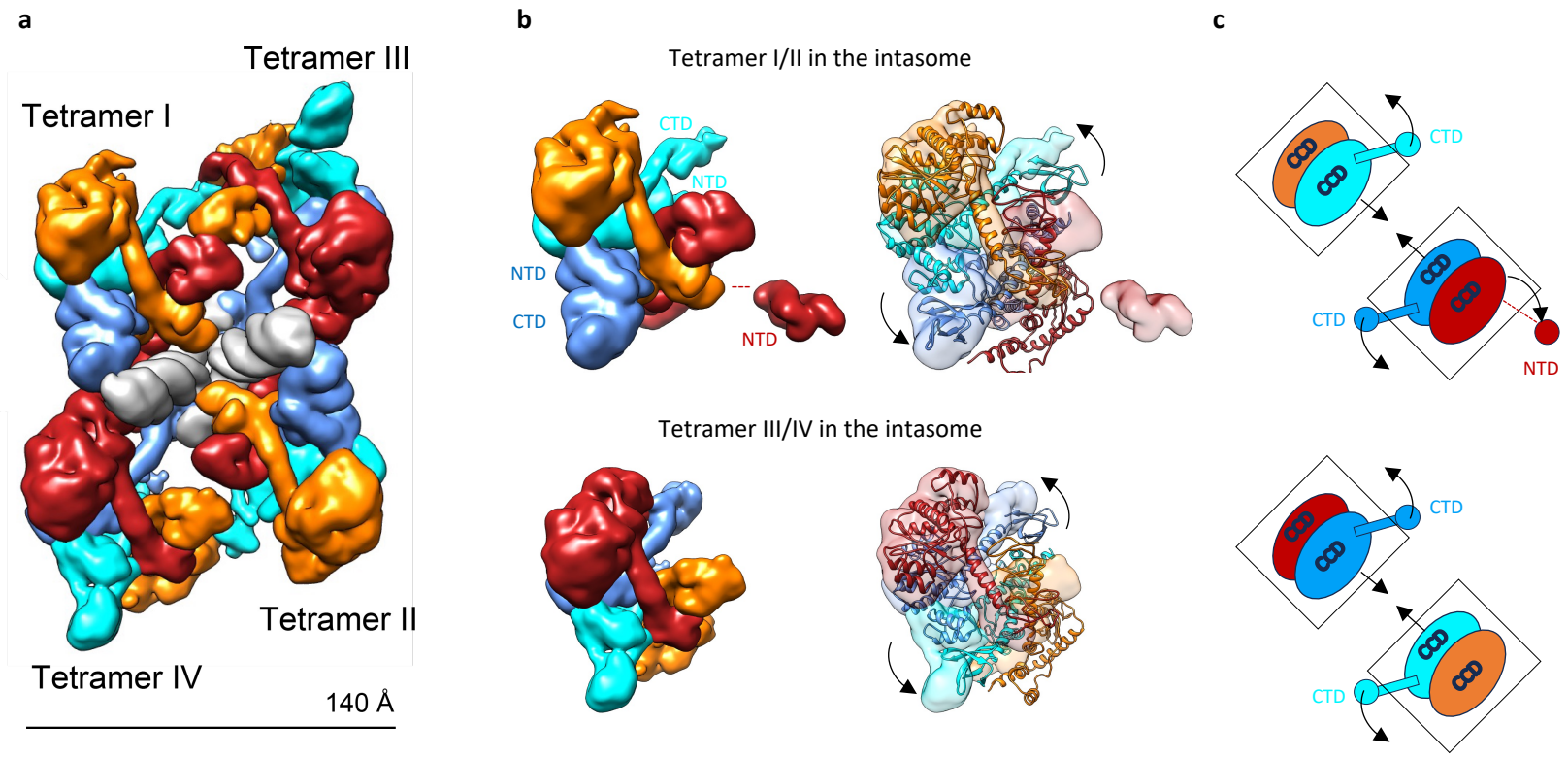

a
Tetramer III
Tetramer I
Tetramer II
Tetramer IV
140 Å
b
Tetramer I/II in the intasome
CTD
NTD
NTD
CTD
NTD
Tetramer III/IV in the intasome
c
CTD
CTD
NTD
CTD
CTD

Figure 4

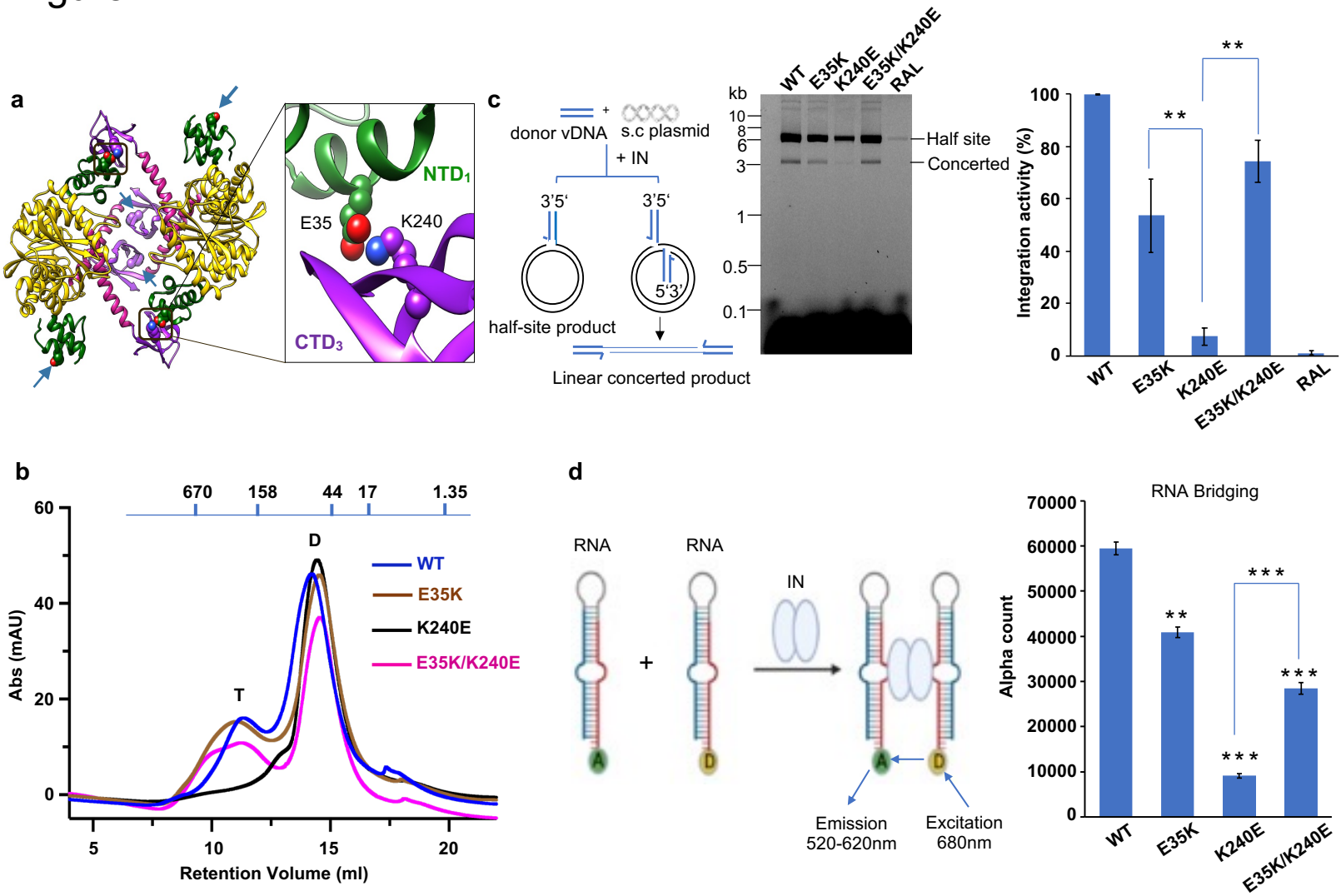

a
NTD1
E35
K240
CTD3
b
670
158
44
17
1.35
D
T
WT
E35K
K240E
E35K/K240E
Abs (mAU)
Retention Volume (ml)
c
donor vDNA
s.c plasmid
+ IN
3'5'
3'5'
5'3'
half-site product
Linear concerted product
kb
WT
E35K
K240E
E35K/K240E
RAL
Half site
Concerted
Integration activity (%)
d
RNA
RNA
IN
Emission
520-620nm
Excitation
680nm
RNA Bridging
Alpha count

Figure 5

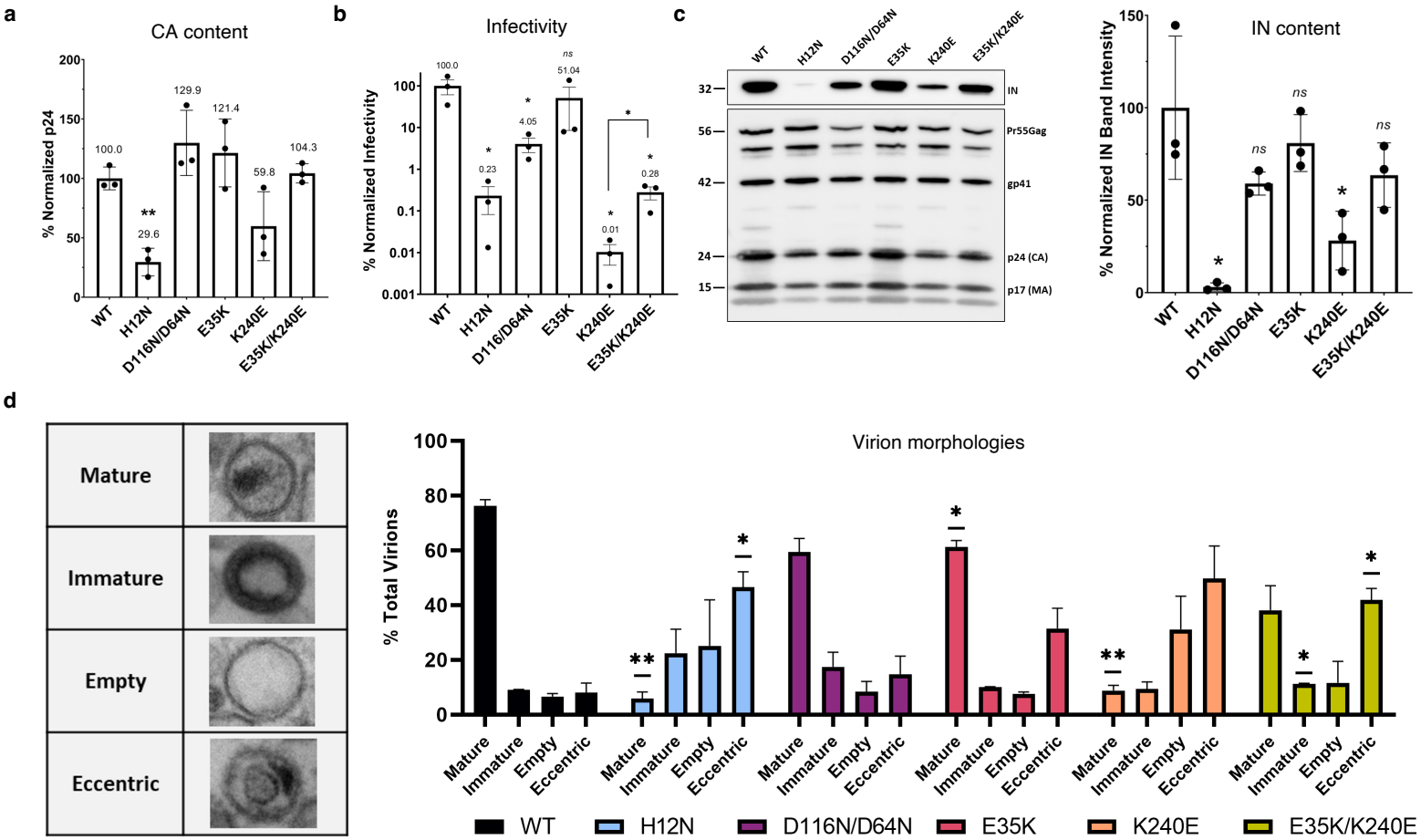
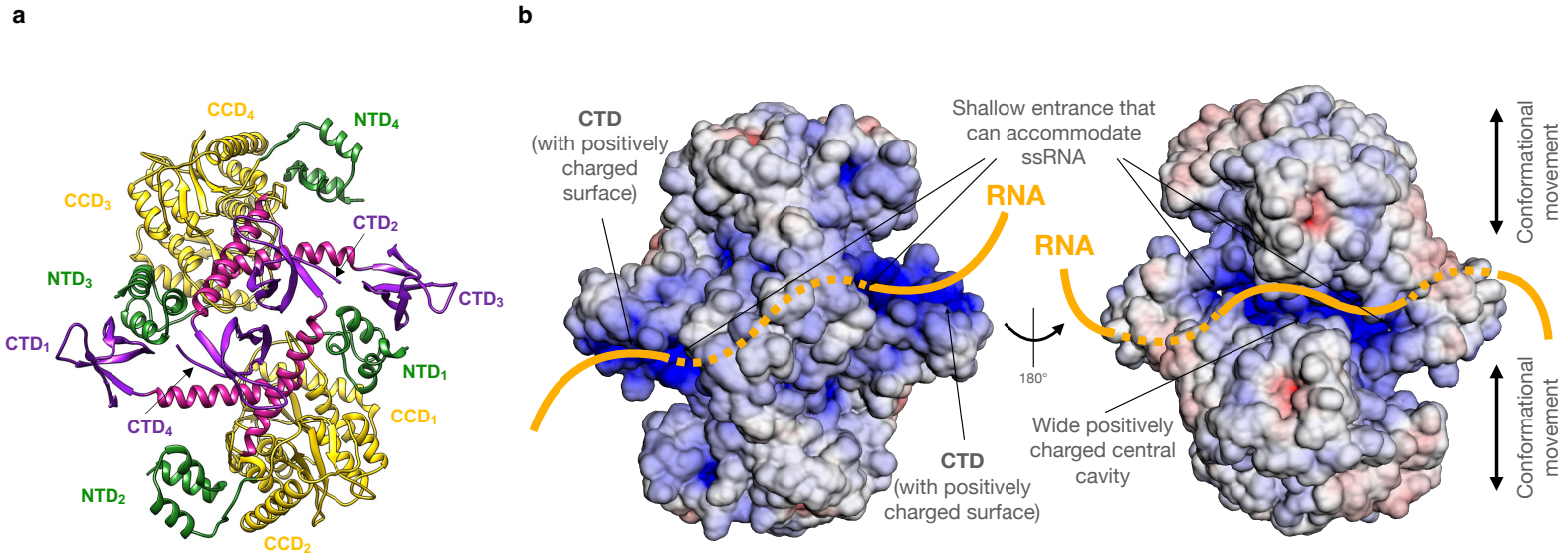
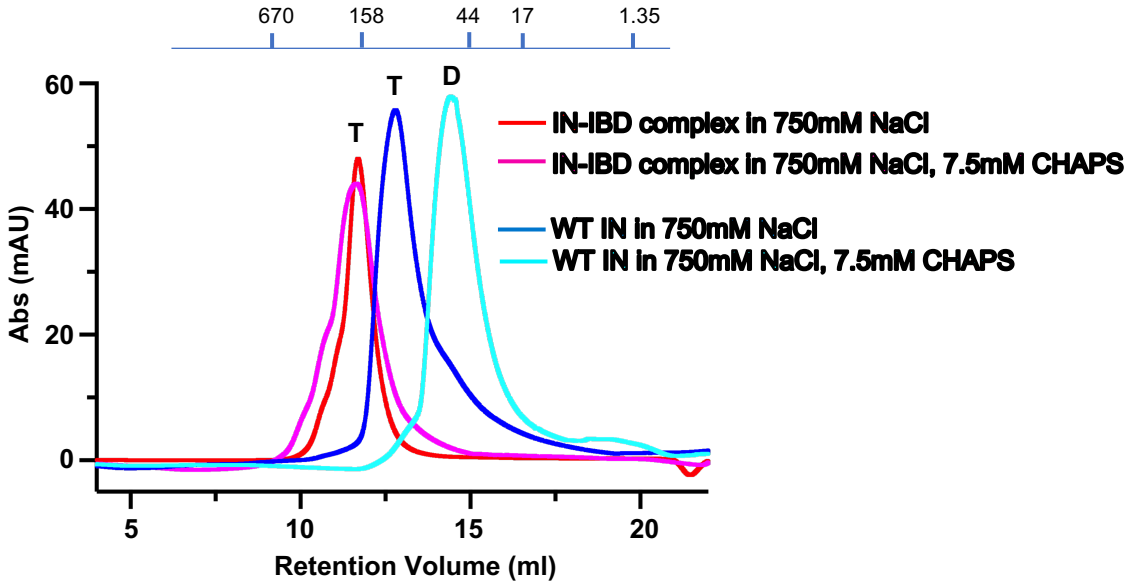

a
CA content
% Normalized p24
100.0
29.6
129.9
121.4
59.8
104.3
WT
H12N
D116N/D64N
E35K
K240E
E35K/K240E
b
Infectivity
% Normalized Infectivity
100.0
0.23
4.05
51.04
0.01
0.28
ns
c
WT
H12N
D116N/D64N
E35K
K240E
E35K/K240E
IN
Pr55Gag
gp41
p24 (CA)
p17 (MA)
IN content
% Normalized IN Band Intensity
ns
d
Mature
Immature
Empty
Eccentric
Virion morphologies
% Total Virions
WT
H12N
D116N/D64N
E35K
K240E
E35K/K240E

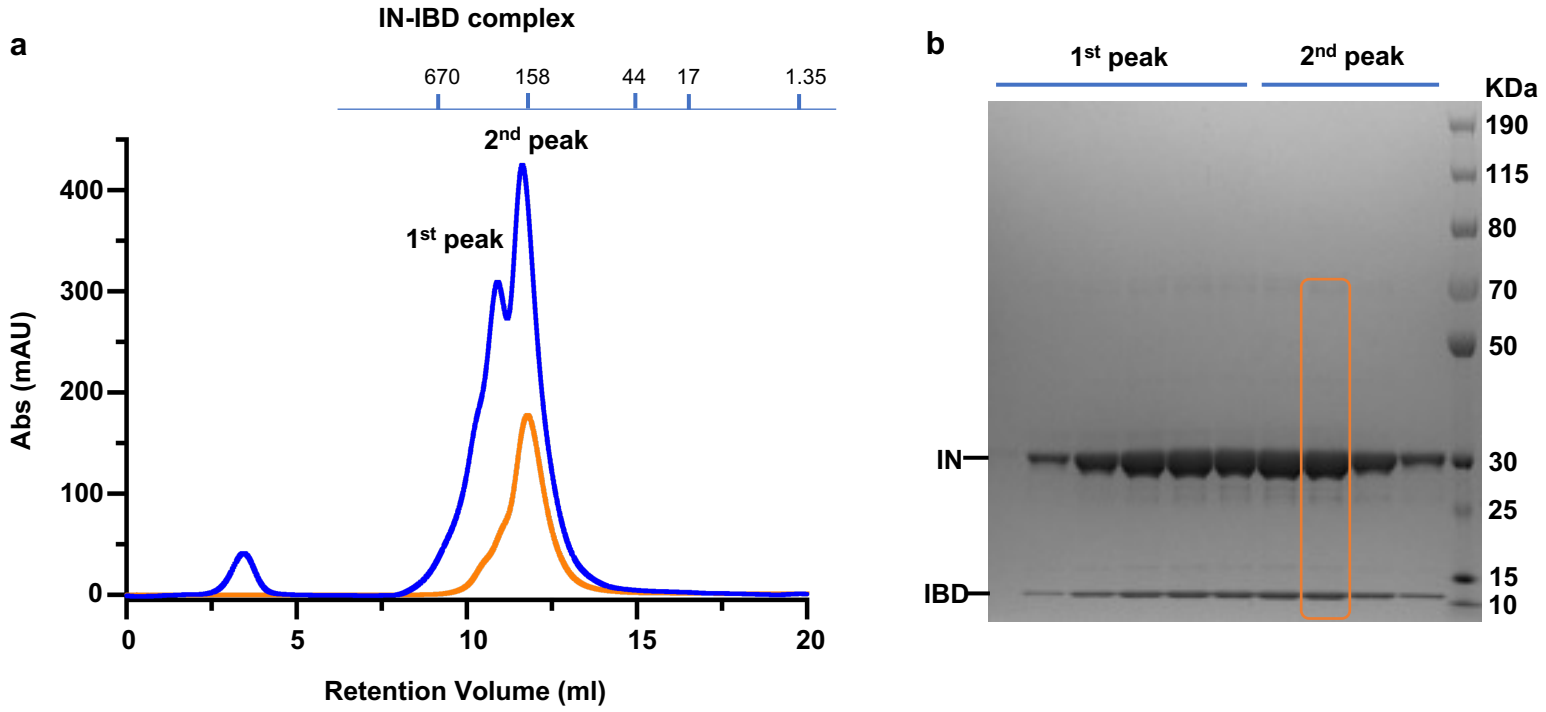
Figure 6


a
CCD4
NTD4
CCD3
CTD2
NTD3
CTD3
CTD1
NTD1
CTD4
CCD1
NTD2
CCD2
b
CTD
(with positively
charged
surface)
Shallow entrance that
can accommodate
ssRNA
RNA
RNA
180°
CTD
(with positively
charged surface)
Wide positively
charged central
cavity
Conformational
movement
Conformational
movement

Supplementary Figure 1

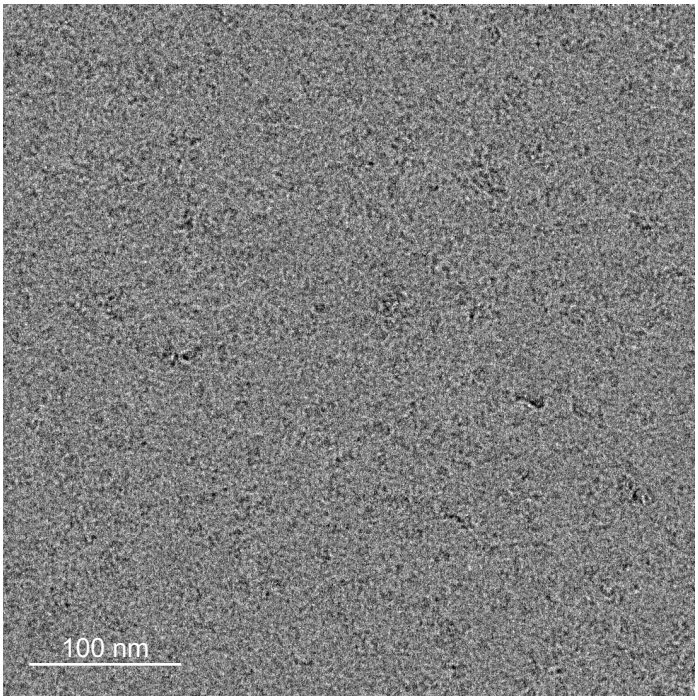


Supplementary Figure 2

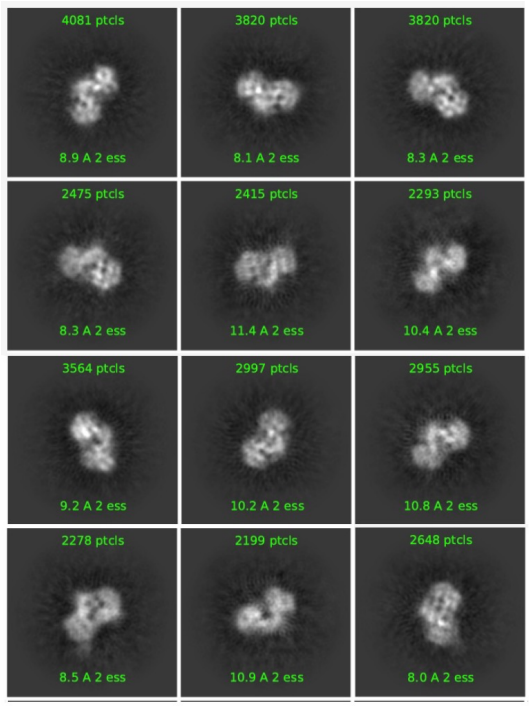


Supplementary Figure 3

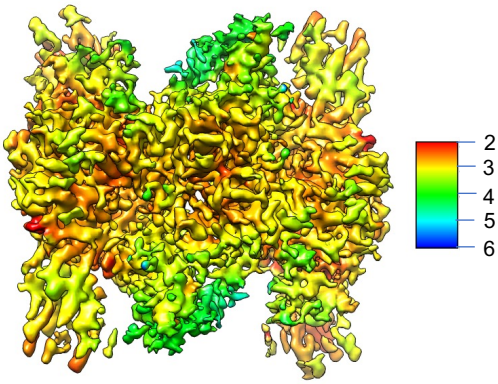
a



b



c



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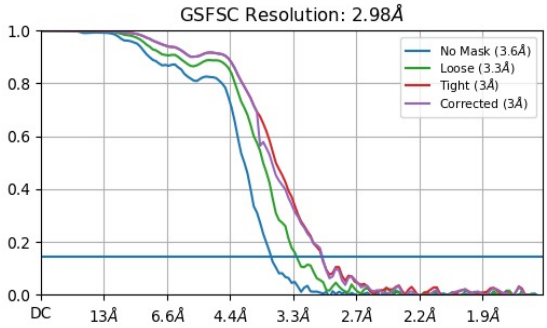
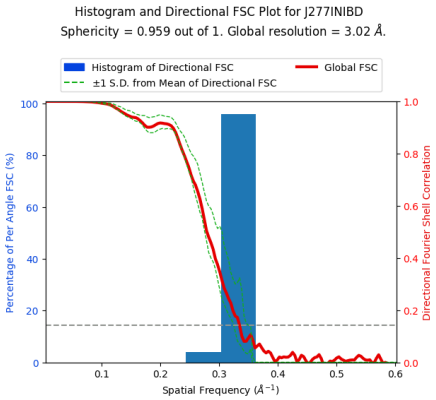
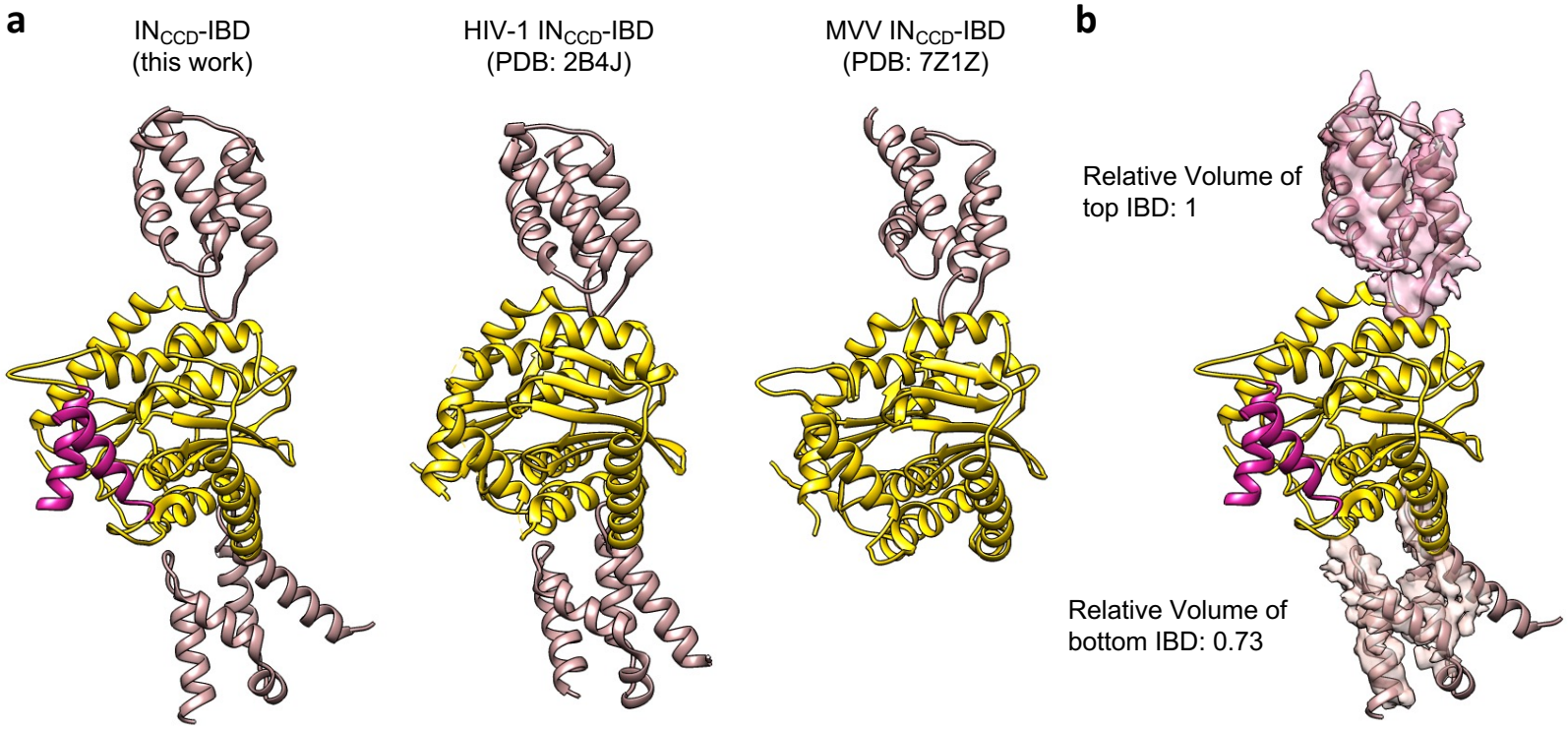


e



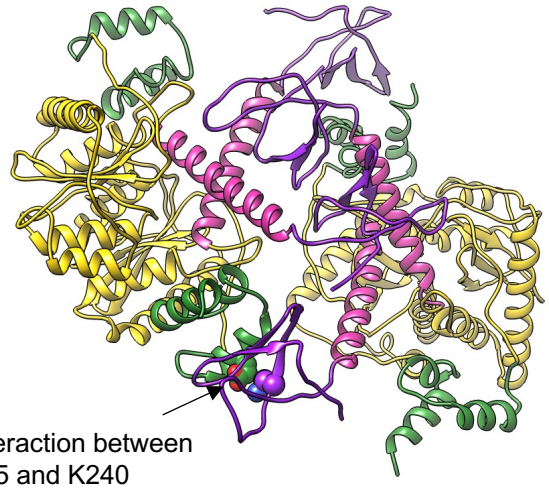
Supplementary Figure 4



Supplementary Figure 5

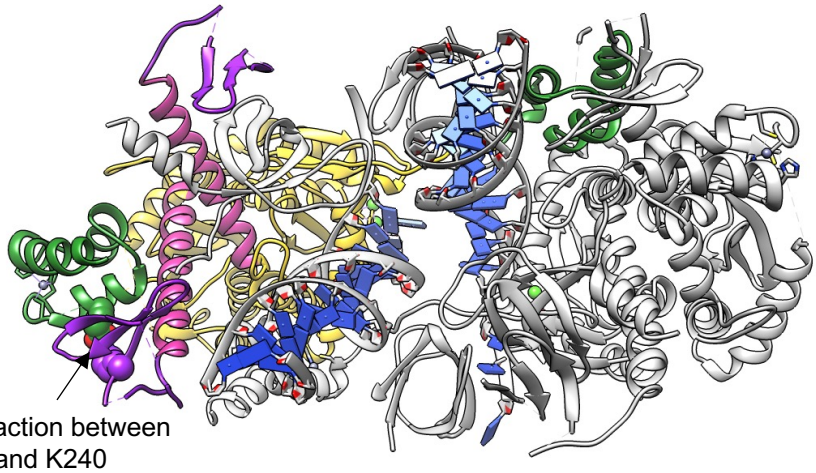
IN tetramer
(this work)

a



HIV-1 conserved intasome core
(6PUT)

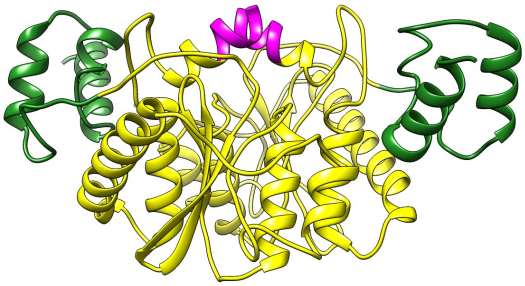
b



Supplementary Figure 6

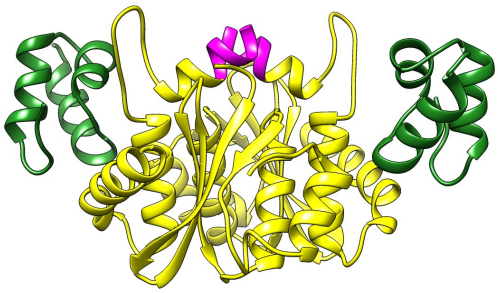
a

HIV-1 IN_{IBD} tetramer



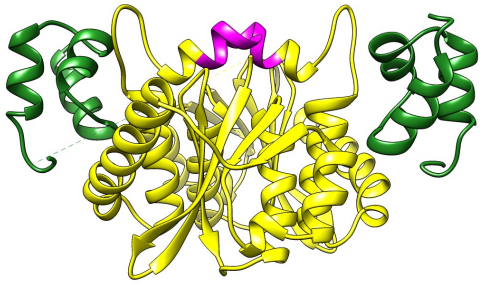
b

HIV-1 two-domain NTD-CCD: PDB 1K6Y



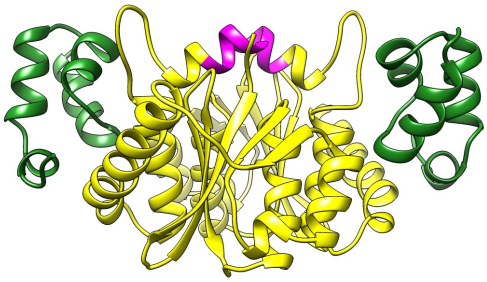
c

MVV two-domain NTD-CCD tetramer (form A): PDB 3HPH



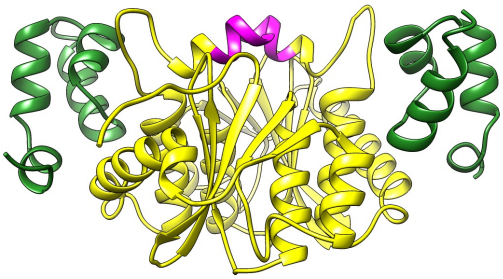
d

MVV two-domain NTD-CCD tetramer (form B): PDB 3HPG



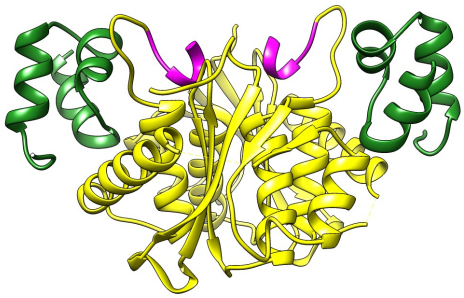
e

MVV NTD-CCD dimer from intasome: PDB 7U32

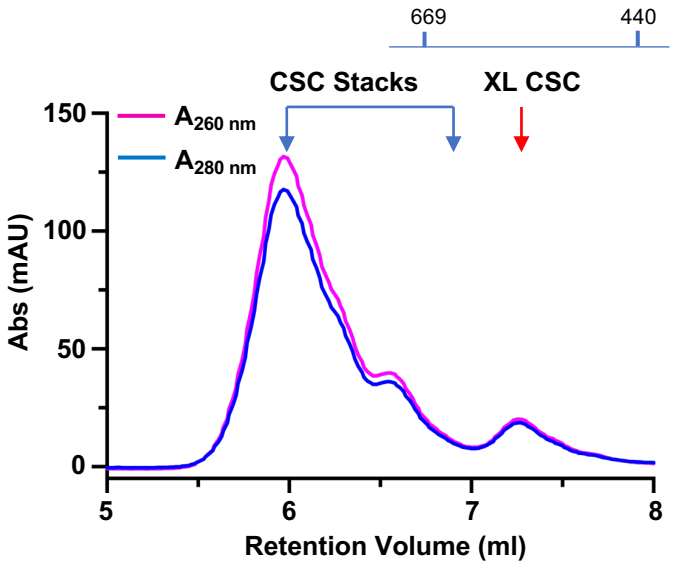


f

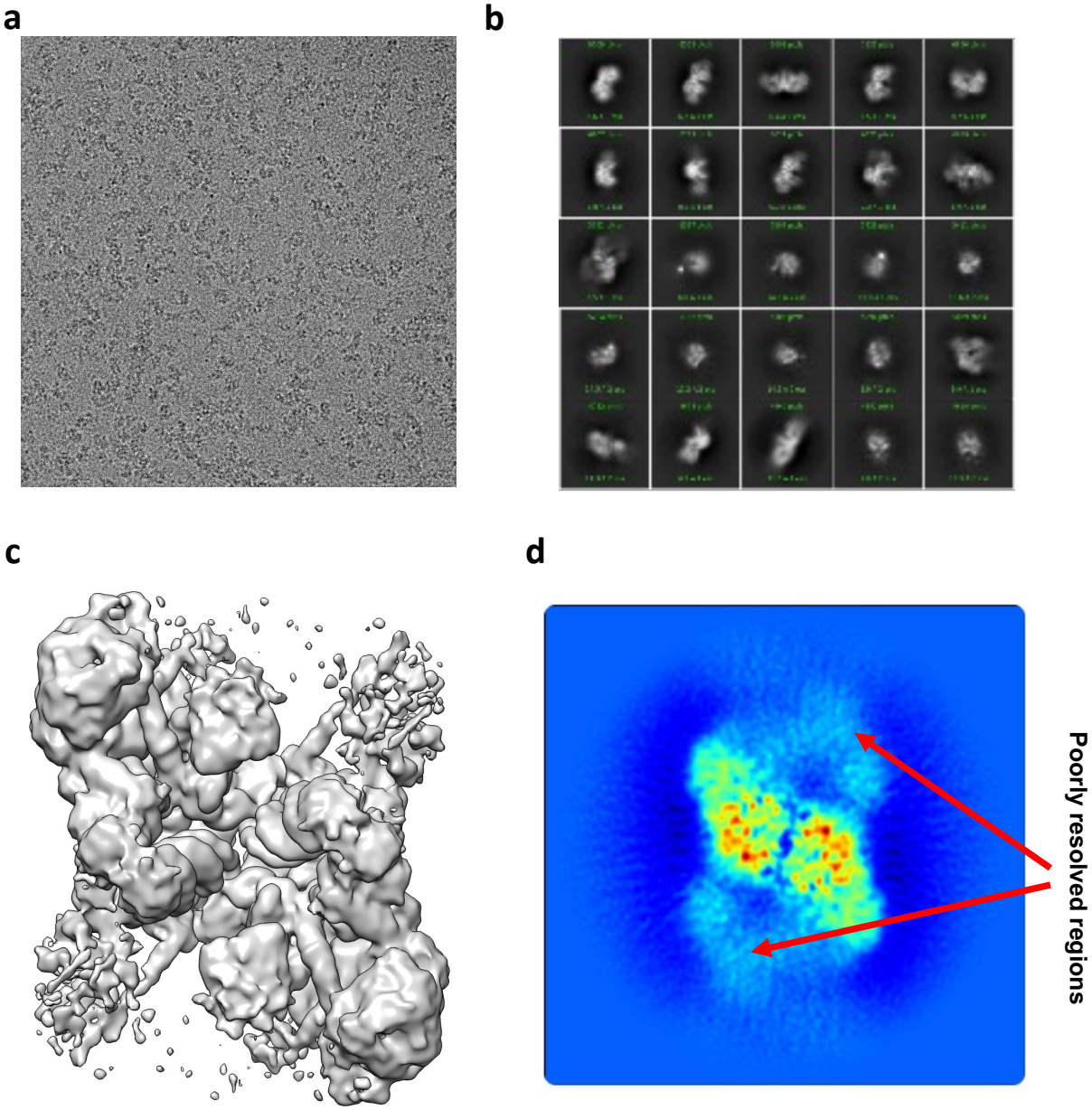
MMTV NTD-CCD dimer: PDB 5CZ2



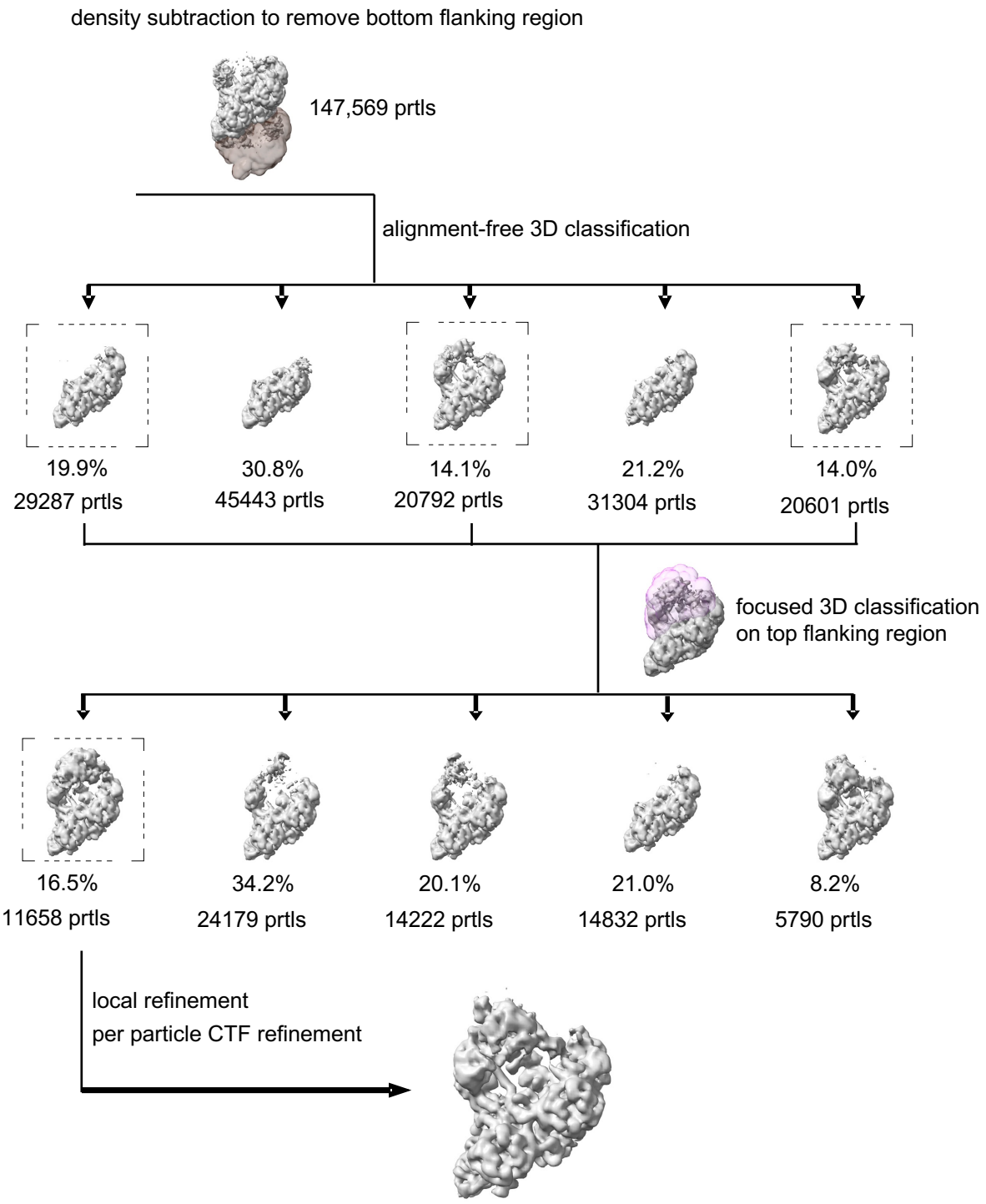
Supplementary Figure 7



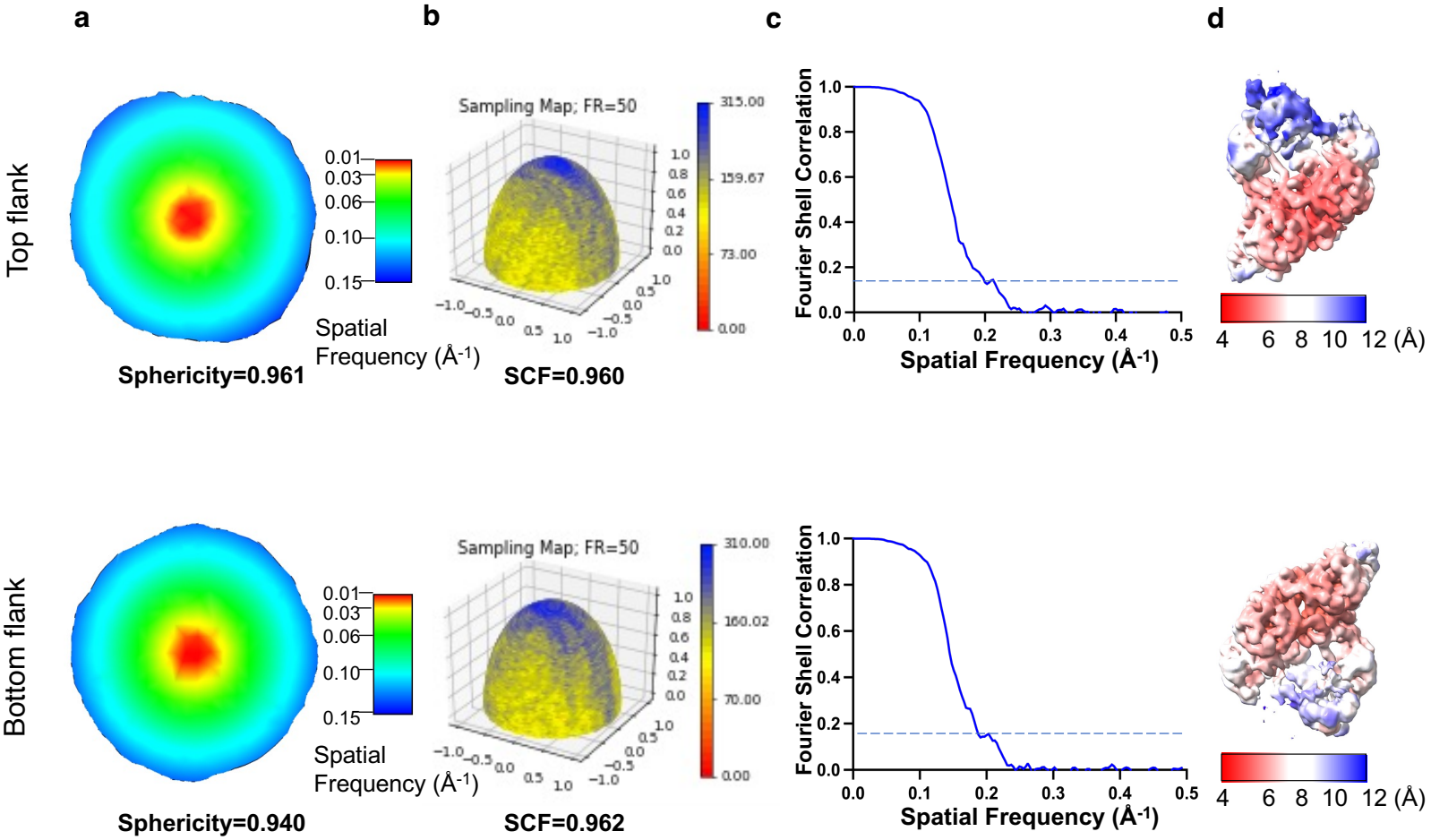
Supplementary Figure 8



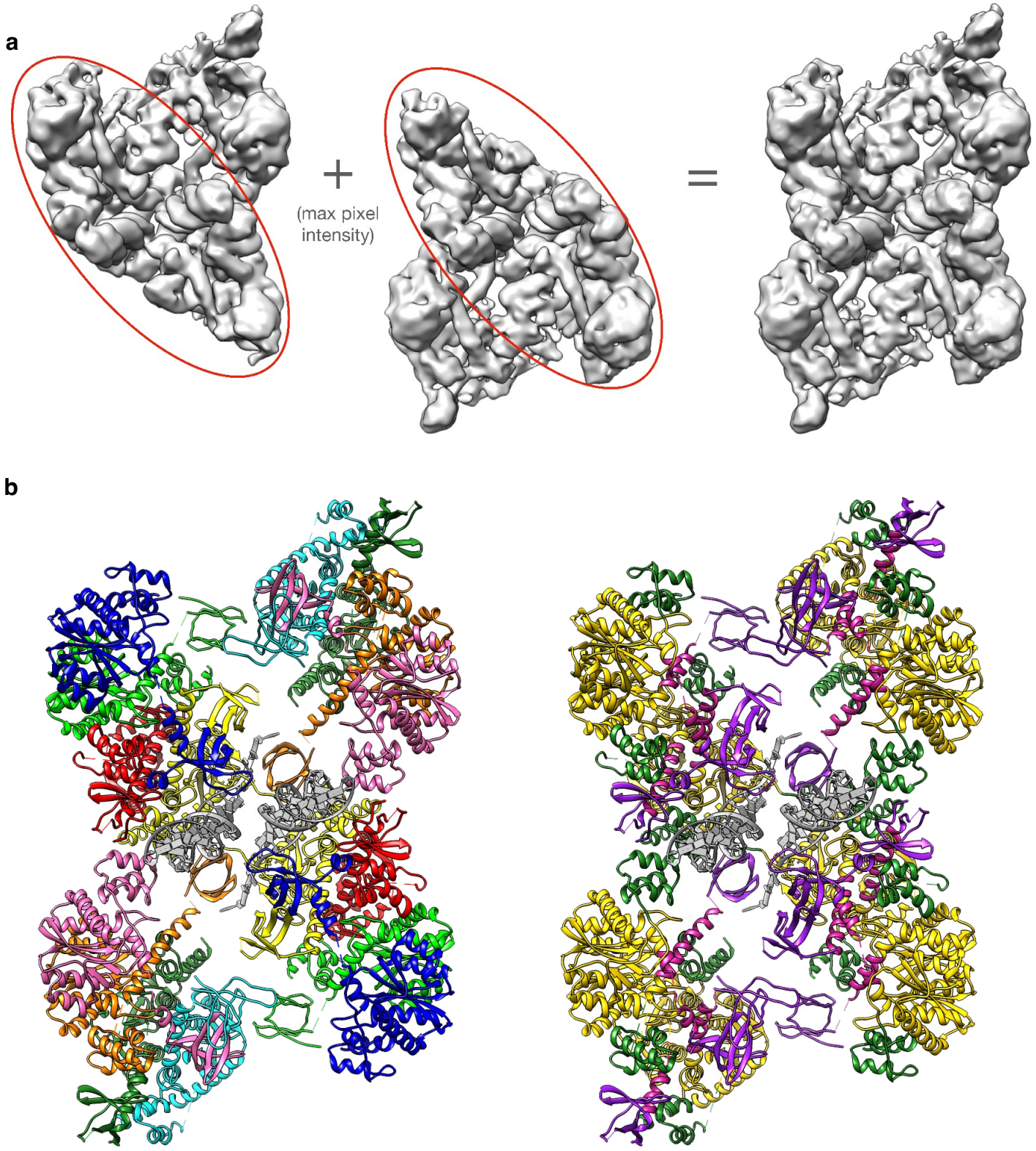
Supplementary Figure 9



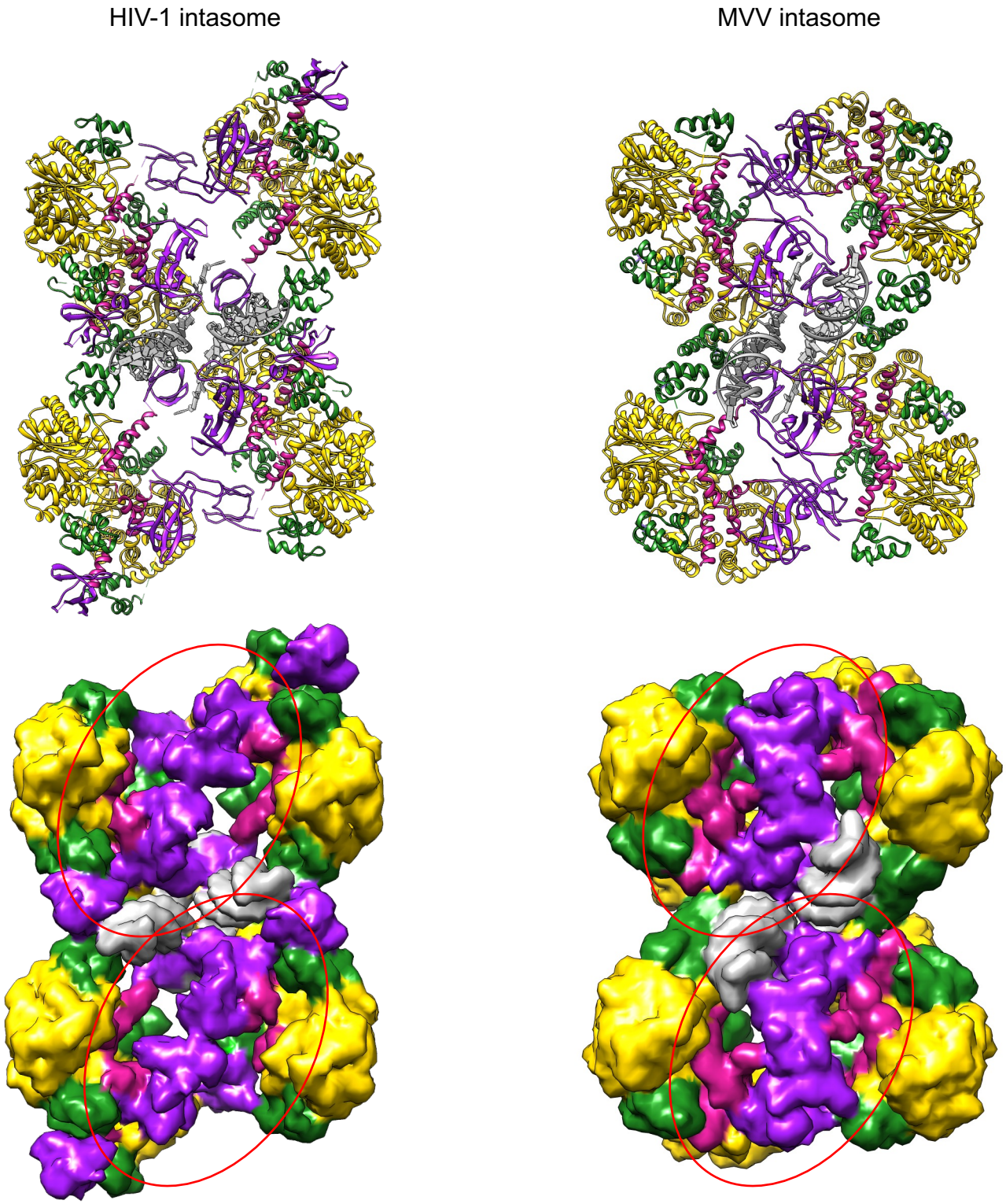
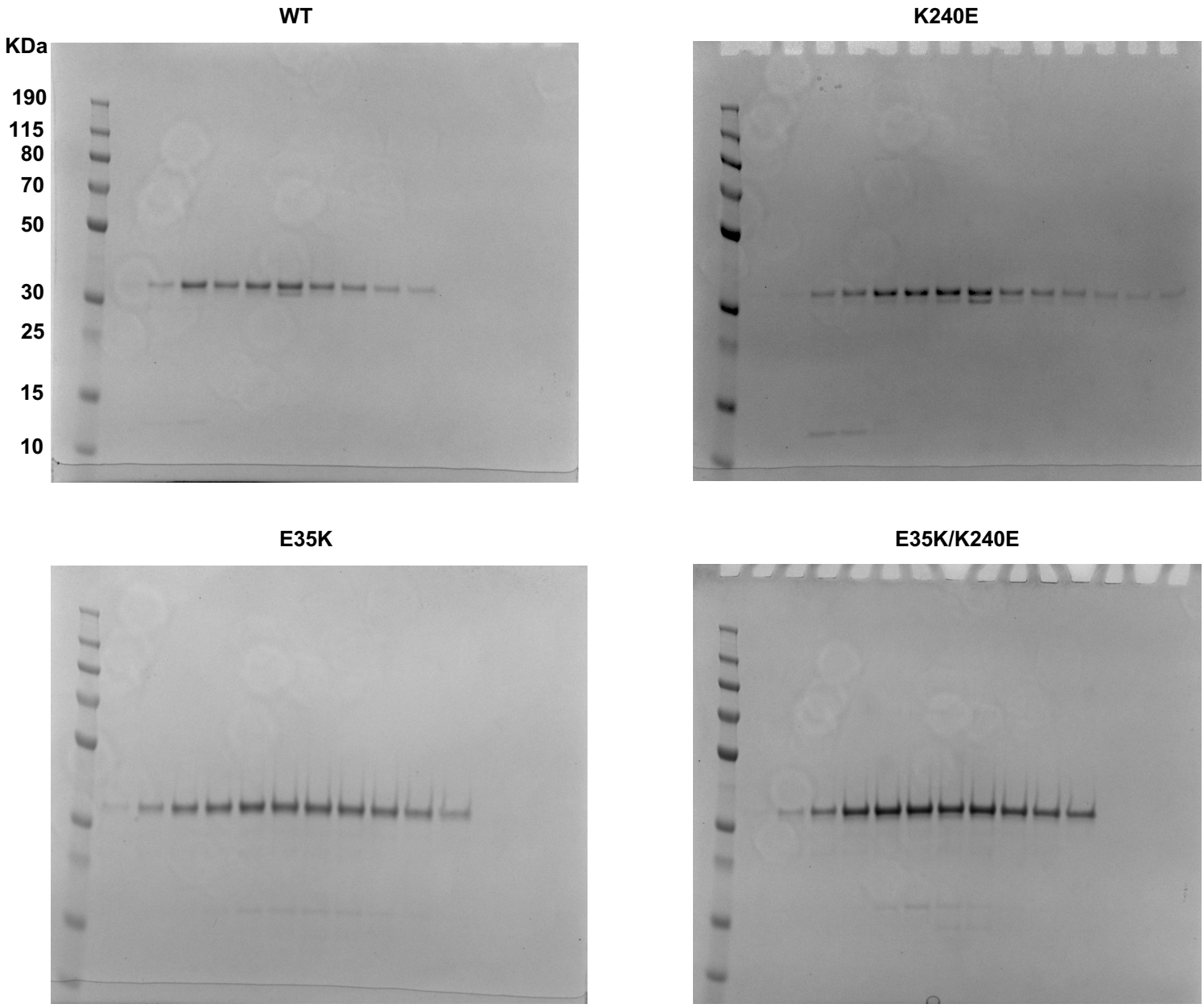
Supplementary Figure 10



Supplementary Figure 11



Supplementary Figure 12



Supplementary Figure 13