SUPPLEMENTARY INFORMATION

An acidic patch on EBNA1 coordinates shelterin components TRF2 and Rap1 at the EBV origin of replication

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Supplementary Figure Legends

Supplementary Fig. 1. Structure determination process and results of ½ DS + EBNA1 DBD + TRF2 + Rap1.

a, Silver stain of Native PAGE after Grafix using 10% to 30% glycerol gradient to form protein and DNA complex fixed by 0.125% glutaraldehyde. From left to right, complexes are shown from fractions 11 to 22 with increasing glycerol content representing particles with increasing weight. **b**-**c**, A raw image of the ½DS complex and 2D classes as processed in CryoSparc. **d**, Flow chart showing data processing from 2D classes obtained in **(c)**. 1,631,271 particles from 2D classification were picked to form the ab initio 3D map (bin2), which were divided to 5 classes using the ab initio job in CryoSparc. The best class with 416,816 particles was then picked and submitted to another round of dividing into 3 classes using ab initio. The best class from this classification with 242, 948 particles was put through homogenous refinement to yield a map of roughly 9Å resolution. 2 rounds of 2D classification were used to parse these particles down to 40,377, which were then used for a new initial model and homogenously refined to 7.14Å resolution. **e**, FSC of final map in **(d)**.

Supplementary Fig. 2. Alphafold3 predictions of the 1:4:1:1 ½ DS – EBNA DBD – TRF2 – Rap1 complex do not occlude the TRFH dimerization interface.

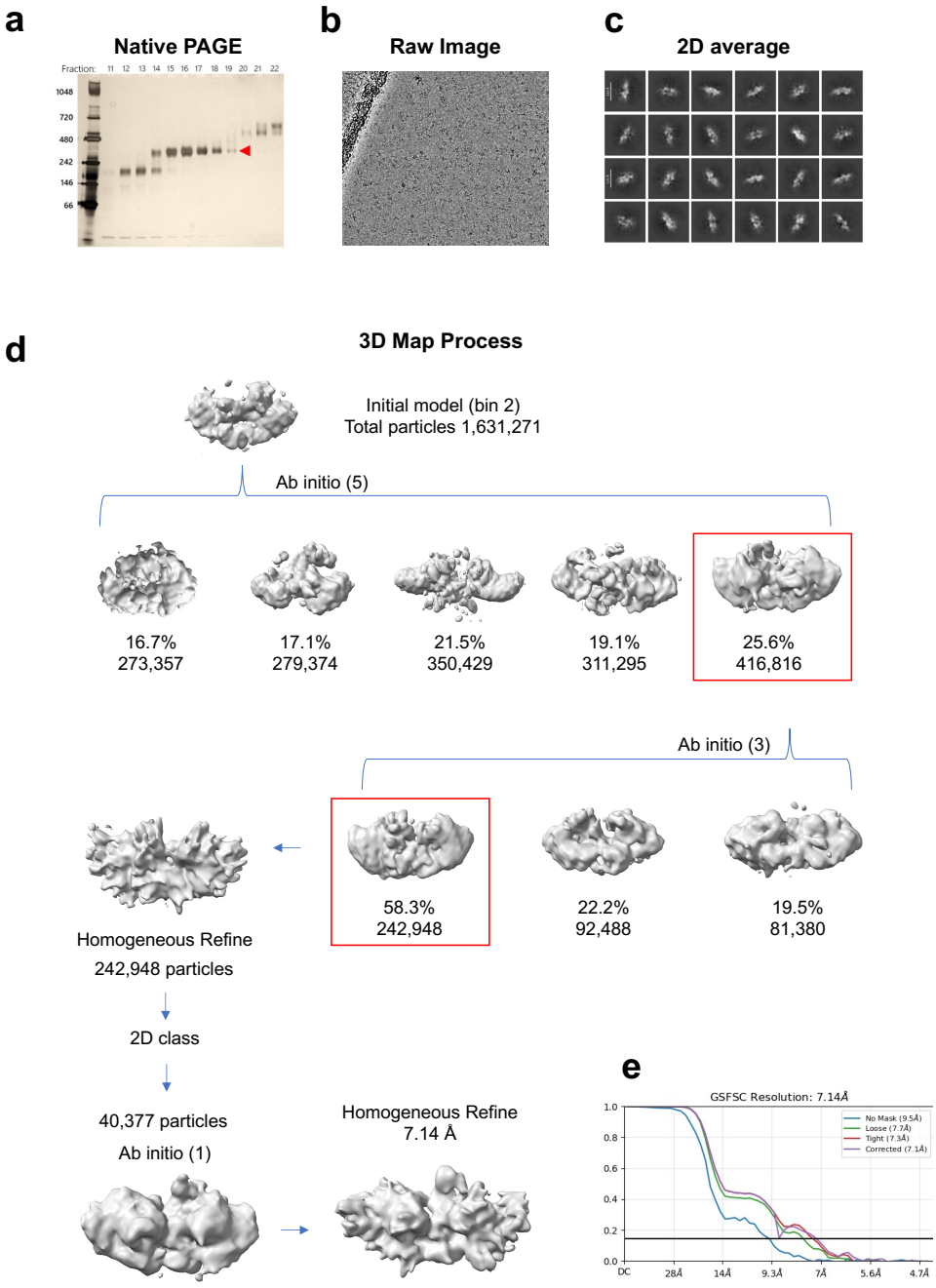
In the Alphafold3 model prediction shown in Figure 2, the orientation of the TRF2 dimerization domain monomer does not occlude the dimerization surface. This allows for the formation of a dimer, the other half of which is likely unresolveable by cryo-EM due to the flexibility of the complex.

Supplementary Fig. 3. Interaction of TRF2 TRFH dimerization domain with EBNA1 is not dependent on regions outside the EBNA1 DBD.

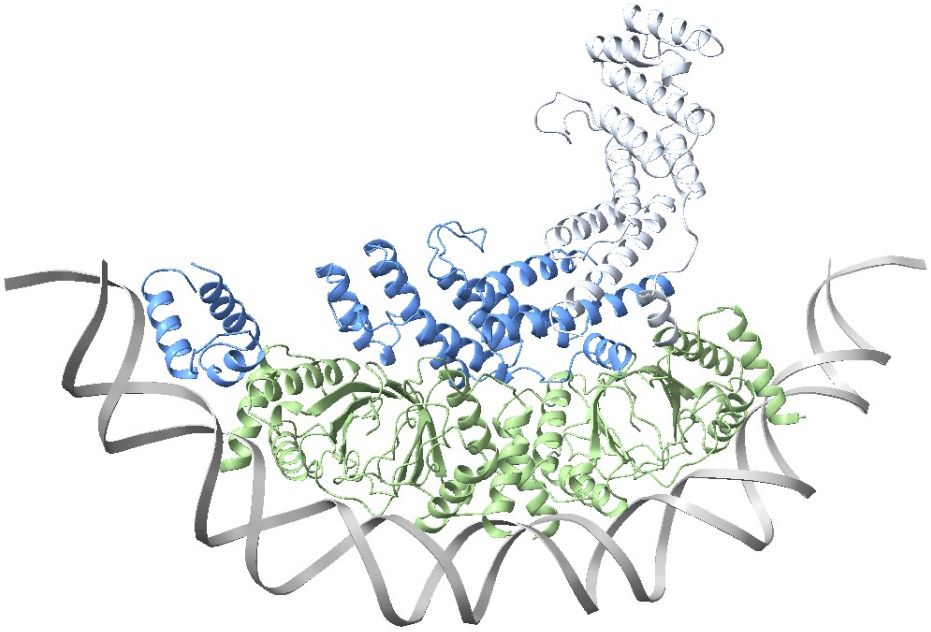
EMSA shows binding of TRF2 homodimerization domain (“TRFH,” residues 85-287) to the ½ DS EBNA1 complex forms whether EBNA1 is near full length (Δ90-325), includes only the construct used in cryo-EM experiments (401-607), or includes only the structurally well-defined portion of the DNA binding domain (459-607).

Supplementary Fig. 4. 8x mutant EBNA1 shows similar stability to the WT.
a, Cyclohexamide treatment was used to determine the stability of the WT versus the 8x mutant EBNA1 over a period of 24 hours, and stability was found to be comparable for both, indicating that the drop in 8x mutant EBNA1 as shown in Figure 5C is not due to stability problems, and more likely due to a lack of EBNA1-encoding plasmid being replicated by the DNA replication machinery. **b**, Quantification of the Western blot shown in (A), performed in triplicate.

Supplementary Figure 1.



Supplementary Figure 2.

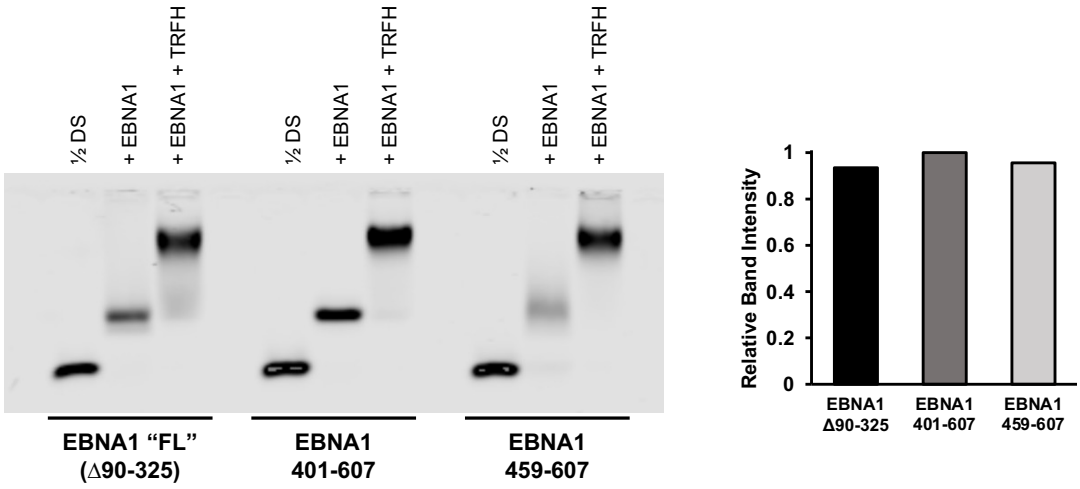


EBNA1

TRF2 (monomer 1)

TRF2 (monomer 2)

Supplementary Figure 3.



Supplementary Figure 4.