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Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated
☐	☒	Clearly defined error bars *State explicitly what error bars represent (e.g. SD, SE, CI)*

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection	No custom software was used.
Data analysis	No custom software was used.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

DNA sequences for expression constructs are as follows: A3A (GenBank accession NM_145699), A3B (GenBank accession NM_004900), A3C (GenBank accession NM_014508), A3D (GenBank accession NM_152426), A3F (GenBank accession NM_145298), A3G (GenBank accession NM021822), A3H (haplotype II; FJ376615), EBV BORF2 (GenBank accession V01555.2), EBV BaRF1 (Genbank accession V01555.2), UGI (GenBank accession J04434.1), RRM1 (GenBank accession BC006498),

EBV BKRF3 (GenBank accession MG021307.1), KSHV ORF61 (GenBank accession U75698.1), HSV-1 UL39 (GenBank accession JN555585.1), HCMV UL45 (GenBank accession FJ527563.1), codon-optimized HIV-1 (IIIB) Vif-Myc (DOI: 10.1128/JVI.00685-10), Akata reference genome (GenBank accession KC207813), ancestral EBV sequence (GenBank accession NC_007605.1).
All raw image files for immunoblots and DNA gel scans are available in the Supplementary Information (Pages 20-32).
Uncropped IF microscopy images available upon request: Fig. 3d-g, Supp. Figs. 2, 7.
Additional nucleotide sequences for Supp. Figs. 9, 11 are available upon request.
Raw data counts for graphs available upon request: Figs. 2c, 3c, 4d, 4e, Supp. Figs. 1b-d, 9b, 11.
Raw data for figures not explicitly stated are available upon request. There are no restrictions on data availability.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample-size calculation was performed. An n=50 was chosen for quantification of Fig. 3c arbitrarily to show trends in A3B localization (no statistics were calculated). An n=12 was chosen for preliminary sequencing of 3D-PCR results (Supp. Fig. 9a, 9b) for ease of pipetting (12-strip PCR tubes) with a few omissions (see below). An n=24 was chosen for secondary 3D-PCR results (Fig. 4 and Ext. Data Figs. 9c, 10) for ease of pipetting (2x 12-strip PCR tubes) with a few omissions (see below). Sequencing data are meant to show trends and no statistics were calculated.
Data exclusions	A few Sanger sequences were omitted from Fig. 4d, Supp. Figs. 9b, 10 due to either: 1) poor sequence quality, 2) incorrectly cloned sequences, or 3) insertions. Reasons 1) and 2) were omitted because data generated from these sequences are irrelevant/not useful. Reason 3) was omitted due to the extreme rarity (<5 total among all samples, n>300) and increased clarity in data presentation. We feel that omission of data due to reason 3) does not impact the results of our study. Omission due to reasons 1) and 2) were pre-established, while reason 3) was not and made only after a careful analysis of the entire data set.
Replication	Unless otherwise noted in figure legends, all experiments were conducted with at least three biological repeats. All replication results were successful with a one exception in Fig. 1c: At least 7 biologically independent co-IP experiments were conducted to identify which A3 family members interact with BORF2. A3B was always the dominant interactor and was 100% reproducible, but there was variation among other family members that was not always reproducible. A3A and A3F were most consistently found to have weak interactions with BORF2. Occasionally, A3G had very weak interactions. A3C, A3D, and A3H was never found to interact with BORF2. Overall, we believe that BORF2 predominantly binds A3B and may also interact with some other A3s due to homology in the A3B C-terminal domain.
Randomization	Randomization was not relevant to our study because there was no experiment that involved allocation of individual samples/organisms/ participants into experiment groups.
Blinding	No blinding was performed on these studies.

Materials & experimental systems

Policy information about availability of materials

n/a	Involved in the study
☐	☒ Unique materials
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Research animals
☒	☐ Human research participants

Unique materials

Obtaining unique materials	All unique materials are readily available from the authors upon request.

Antibodies

Antibodies used	Antibodies used for immunlobot include: mouse anti-FLAG 1:5000 (Sigma F1804), mouse anti-BORF2 1:1000 (Santa Cruz

sc56979), mouse anti-BZLF1 1:3000 (Santa Cruz sc53904), mouse anti-tubulin 1:10,000 (Sigma T5168), mouse anti-β-actin 1:10,000 (Santa Cruz sc47778), rabbit anti-HA 1:3000 (Cell Signaling C29F4), rabbit anti-Strep 1:5000 (Abcam ab76949), rabbit anti-Myc 1:3000 (Sigma C3956), rabbit anti-A3B 1:1000 (5210-87-1327), and rabbit anti-UNG2 1:1000 (kind gift from Sal J. Caradonna, Rowan University).

Antibodies used for immunofluorescence imaging include: rabbit anti-A3B 1:1000 (5210-87-1327), mouse anti-BORF2 1:400 (Santa Cruz sc56979), mouse anti-BMRF1 1:1000 (Millipore MAB8186), mouse anti-FLAG 1:1000 (Sigma F1804), rabbit anti-BiP/GRP-78 1:1000 (Abcam ab21685), rabbit anti-TRAPα 1:1000, goat anti-rabbit AlexaFluor 488 1:1000 (Invitrogen A11034), donkey anti-mouse AlexaFluor 647 1:1000 (Invitrogen A31571), goat anti-mouse AlexaFluor 594 1:1000, goat anti-rabbit AlexaFluor 647 1:1000 (Invitrogen A32733).

Validation

Non-commercial:
5210-87-1327, AIDS Reagent Program: https://www.aidsreagent.org/reagentdetail.cfm?t=monoclonal_antibodies&id=437
anti-UNG2, Muller, S.J. & Caradonna, S. J. Isolation and characterization of a human cDNA encoding uracil-DNA glycosylase. BBA (1991).
anti-TRAPα, Cui, X. A., Zhang, H. & Palazzo, A. F. p180 promotes the ribosome-independent localization of a subset of mRNA to the endoplasmic reticulum. PLoS Biol 10, e1001336 (2012).

Commercial:
Sigma F1804, 377 citations: https://www.sigmaaldrich.com/catalog/product/sigma/f1804?lang=en&region=US
Santa Cruz sc56979, 4 citations: https://www.scbt.com/scbt/product/ebv-ea-r-p85-antibody-6g7
Santa Cruz sc53904, 41 citations: https://www.scbt.com/scbt/product/ebv-zebra-antibody-bz1
Sigma T5168, 840 citations: https://www.sigmaaldrich.com/catalog/product/sigma/t5168?lang=en&region=US
Santa Cruz sc47778, 3579 citations: https://www.scbt.com/scbt/product/beta-actin-antibody-c4
Cell Signaling C29F4, 206 citations: https://www.cellsignal.com/products/primary-antibodies/ha-tag-c29f4-rabbit-mab/3724
Abcam ab76949, 11 citations: https://www.abcam.com/strep-tag-ii-antibody-ab76949-references.html
Sigma C3956, 230 citations: https://www.sigmaaldrich.com/catalog/product/sigma/c3956?lang=en&region=US
Millipore MAB8186, 9 citations: http://www.emdmillipore.com/US/en/product/Anti-EBV-EA-D-p52/50-Antibody-clone-R3,MM_NF-MAB8186#documentation
Abcam ab21685, 147 citations:http://www.abcam.com/grp78-bip-antibody-ab21685.html

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

293T cells: https://www.atcc.org/Products/All/CRL-3216.aspx
293TΔUNG2 cells: Generated using CRISPR/Cas9-mediated deletion of UNG2 from 293T cells, pooled.
HeLa cells: https://www.atcc.org/Products/All/CCL-2.aspx
U2OS cells: https://www.atcc.org/Products/All/HTB-96.aspx
AGS cells: https://www.atcc.org/Products/All/CRL-1739.aspx
AGS-EBV cells: PMID12042810
AGS-EBV(Bx1g) cells: PMID10864642
BORF2-null AGS-EBV cells: Generated using CRISPR/Cas9-mediated deletion of BORF2 from AGS-EBV cells, pooled.
BORF2-null AGS-EBV(Bx1g) cells: Generated using CRISPR/Cas9-mediated deletion of BORF2 from AGS-EBV(Bx1g) cells, pooled.
AGS-EBV(Bx1g)ΔBORF2 cells: AGS cells with infected with BORF2-null EBV(Bx1g) virus and then subject to limiting diluting to generate an isogenic clonal cell line.
Akata cells: https://web.expasy.org/cellosaurus/CVCL_0148
BORF2-null Akata cells: Generated using CRISPR/Cas9-mediated deletion of BORF2 from Akata cells, pooled.
Ramos cells: https://www.atcc.org/Products/All/CRL-1596.aspx
M81 cells: https://www.cell.com/cell-reports/fulltext/S2211-1247(13)00520-2

Authentication

293TΔUNG2 cells were confirmed for deletion of UNG2 by immunoblot and UDG activity assay.
BORF2-null AGS-EBV and AGS-EBV(Bx1g) cells were confirmed for deletion of BORF2 by immunoblot of BORF2 and Sanger sequencing of the BORF2 gene region.
AGS-EBV(Bx1g)ΔBORF2 cells were additionally Sanger sequenced for clonality (n>10).
BORF2-null Akata cells were confirmed for deletion of BORF2 by immunoblot.

Mycoplasma contamination

293T, HeLa, U2OS, AGS, AGS-EBV, AGS-EBV(Bx1g), Akata (and derivative cell lines), Ramos, and M81 cells tested negative for mycoplasma using the commerical Lonza MycoAlert Plus Testing Kit (LT07-710) with Assay Positive Control (LT07-518).

Commonly misidentified lines
(See ICLAC register)

No misidentified cell lines were used in the study according to ICLAC Version 8.0 (Published 1/12/2016).

Method-specific reporting

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ Magnetic resonance imaging