

1 **Human anelloviruses produced by recombinant expression of synthetic genomes**

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19 **ABSTRACT:**

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21 Human anelloviruses are acquired universally in infancy, highly prevalent, abundant in blood,
22 and extremely diverse. Their apparent lack of pathogenicity indicates that they are a major
23 component of the commensal human virome. Despite their being extensively intertwined with
24 human biology, these viruses are poorly understood. A major impediment in studying
25 anelloviruses is the lack of an *in vitro* system for their production and/ or propagation. Here we
26 show that the T cell-derived human cell line MOLT-4 can be transfected with plasmids
27 comprising tandem anellovirus genomes to produce viral particles visualized by electron
28 microscopy. We found that a previously described human anellovirus of the *Betatorquevirus*
29 genus (LY2), as well as a second *Betatorquevirus* detected by sequencing DNA extracted from a
30 human retinal pigmented epithelium (nrVL4619), can be synthesized and produced by these
31 means, enabling further molecular virology studies. Southern blot was used to demonstrate
32 replication, and site-directed mutagenesis of the viral genome was performed to show that the
33 production of anellovirus in this cell line is dependent on the expression of certain viral proteins.
34 Finally, experiments performed in mice using purified nrVL4619 particles produced in MOLT-4
35 cells demonstrated infectivity *in vivo* in the tissue of origin. These results indicate that
36 anelloviruses can be produced *in vitro* and manipulated to improve our understanding of this
37 viral family which is ubiquitous in humans and many other mammals. Applications of this work
38 to gene therapy and other therapeutic modalities are currently under investigation.

39

40 **IMPORTANCE:**

41 Anelloviruses are a major component of the human virome. However, their biology is not well
42 understood mainly due to the lack of an *in vitro* system for anellovirus production and/or
43 propagation. In this study, we used multiple orthogonal measures to show that two different
44 anelloviruses belonging to the *Betatorquevirus* genus can be produced in a T-cell-derived human
45 cell line, MOLT-4, via recombinant expression of synthetic genomes. Additionally, we show that
46 anellovirus particles generated in this *in vitro* system demonstrate infectivity *in vivo*. Our
47 findings enable new molecular virology studies of this highly prevalent, non-pathogenic, and
48 weakly immunogenic family of viruses, potentially leading to therapeutic applications.

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65 INTRODUCTION:

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67 Anelloviruses are small non-enveloped viruses encoding a circular single-stranded,
68 negative-sense DNA genome. Human anelloviruses can be classified into three genera -
69 *Alphatorquevirus* (torque teno virus [TTV]), *Betatorquevirus* (torque teno mini virus [TTMV]),
70 and *Gammatorquevirus* (torque teno midi virus [TTMDV]). Their genome sizes are
71 approximately 3.9 kilobases (kb) for *Alphatorqueviruses*, 2.9 kb for *Betatorqueviruses*, and 3.2
72 kb for *Gammatorqueviruses*. Each contains a non-coding region with an ~100-base pair,
73 guanine-cytosine (GC)-rich sequence and several overlapping open reading frames (ORFs), the
74 largest of which, ORF1 (~700-800 aa), is the putative viral capsid protein¹. Genome replication
75 is thought to occur via a rolling circle mechanism common to other circular DNA viruses and
76 employs host polymerases.

77 Since their discovery in 1997², anelloviruses have been found in many biological samples
78 including blood, nasal secretions, saliva, bile, feces, tears, semen, breastmilk, and urine,
79 suggesting broad tropism for different cell and tissue types³⁻⁶. A study of 44 healthy infants
80 showed that they all acquired anelloviruses within their first year of life⁷. Once acquired,
81 anelloviruses appear to persist in the body and avoid clearance by the immune system; the
82 detection of the same type of anellovirus in samples from an individual dialysis patient collected
83 16 years apart supports the theory that people can remain life-long anellovirus carriers⁸.

84 Human anelloviruses have not been convincingly associated with any disease⁹. In fact,
85 potentially beneficial effects on human health have been suggested for anelloviruses⁴. For
86 instance, acquisition of anelloviruses by newborns^{7,10} could promote the development and
87 maturation of the immune system¹¹. Such results are concordant with a long history of co-
88 evolution between the virus and the host, eventually leading to commensal or even mutualistic
89 relationships¹².

90 Despite their discovery more than 25 years ago and their very high prevalence in the
91 human population, relatively little is known about the biology of anelloviruses because of the
92 lack of an *in vitro* cell culture system and other tools such as reliable serologic assays and animal
93 models^{12,13}. In this study, we demonstrate that a human lymphoblastic cell line, MOLT-4, may be
94 employed for the production of two distinct anelloviruses belonging to the *Betatorquevirus*
95 genus. In addition to characterizing viral gene expression and replication, we provide the first
96 evidence to our knowledge of the production of purified virus particles for analysis by
97 transmission electron microscopy (TEM) as well as infectivity *in vivo*. The *in vitro* production
98 model and related tools described herein promise to advance the study of previously neglected
99 human anelloviruses, including their molecular virology and virus-host interactions.

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101 MATERIALS AND METHODS:

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103 Dissection of human samples

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105 Human eyes were obtained through the National Disease Research Institute (NDRI) and
106 were dissected within 24-48 hours of procurement. Each individual eye was placed on a
107 dissecting plate, and the sclera was incised at a point between the cornea and the optic nerve
108 using a razor blade. From that point, the sclera was cut all the way around. The aqueous humor
109 and vitreous humor were isolated separately. The choroidal layer was then removed, and the

110 retina slowly peeled off and processed. Other compartments in the eye that were isolated and
111 analyzed were the sclera, the iris, the cornea, the conjunctiva, and the optic nerve.

112

113 **DNA extraction and processing**

114

115 Dissected tissue sections were homogenized and DNA was extracted with a PureLink
116 viral DNA/RNA kit from Invitrogen (catalog # 12280050). The samples were processed
117 essentially according to the manufacturer's protocol. The extracted DNA then underwent rolling
118 circle amplification (RCA) following the procedure outlined by Arze *et al* for a final volume of
119 20 μL ¹⁴. The presence of *Anelloviridae* in the samples was tested by PCR with pan-anellovirus
120 primers developed by Ninomiya *et al*.

121

122 **Illumina library preparation and sequencing**

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124 Post-RCA DNA was diluted to a volume of 50 μL to reduce viscosity of the samples and
125 then the concentration of DNA was assessed by Qubit 4 Fluorometer (Thermo Fisher Scientific).
126 Post-RCA DNA was library-prepped using the Nextera DNA kit (Illumina). The samples were
127 prepared following the manufacturer's protocol for 100-500 ng input. Post-RCA DNA was also
128 library-prepped using SureSelect XT HS2 DNA Reagent Kit (Agilent) with target enrichment
129 RNA probes. These target enrichment RNA probes were specifically designed to tile across
130 *Anelloviridae* sequences from our database and were biotinylated to enable capture using
131 streptavidin beads. Library quality control was carried out with D5000 ScreenTape on a 4200
132 TapeStation (Agilent). All libraries were then sequenced on either an iSeq 100 or a NextSeq 550
133 (Illumina).

134

135 **Nanopore library preparation and sequencing**

136

137 Post-RCA DNA was debranched and fragmented to 20 kb-sized fragments following the
138 NanoAmpli-Seq protocol¹⁵. 4.5 μg of RCA material was diluted in 65 μL of nuclease-free water
139 and treated with 2 μL of T7 endonuclease I (New England Biolabs) for 5 minutes at room
140 temperature. The reaction was then loaded in a g-TUBE (Covaris) and centrifuged at 1800 rpm
141 (304 relative centrifugal force [RCF]) for 4 minutes. The g-TUBE was then reversed, and the
142 centrifugation process was repeated. An additional round of T7 endonuclease I and g-TUBE was
143 performed before the mixture was then cleaned up with SPRI beads at a ratio of 1.8 \times with a final
144 elution in 20 μL of nuclease-free water. The concentration of DNA was assessed by Qubit 4
145 fluorometer (Thermo Fisher Scientific). The fragmented samples were then library-prepped with
146 a SQK-LSK109 kit (Oxford Nanopore Technologies) following the manufacturer's protocol.
147 Additionally, post-RCA DNA was debranched and fragmented to 6-8 kb-sized fragments using
148 the above-mentioned protocol with the modification of the g-TUBE (Covaris) being centrifuged
149 at 13200 rpm (16363 RCF) for 30 seconds. The samples were prepared with the SureSelect XT
150 HS2 DNA Reagent Kit (Agilent) with biotinylated target enrichment RNA probes specifically
151 designed for *Anelloviridae* following the manufacturer's protocol with an increased elongation to
152 6 minutes in amplification steps. The samples were then library-prepped with the SQK-LSK109
153 kit (Oxford Nanopore Technologies) following the manufacturer's protocol. Libraries were
154 loaded onto a R9.5 (FLO-MIN107) flow cell and placed onto the MinION Mk1B (Oxford

155 Nanopore Technologies) and run for 48 hours. Only flow cells that passed the manufacturer's
156 flow cell check test were used.

157

158 **Sequence quality control**

159

160 Both Illumina and Nanopore raw sequencing reads were subjected to quality control
161 utilizing FastQC on the sequence datasets derived from each instrument¹⁶. Reports generated by
162 FastQC for each individual sample were then aggregated into a single report using the MultiQC¹⁷
163 utility. Metrics from these reports influenced parameter selection to downstream quality control
164 steps during analysis.

165 Illumina sequence data were filtered to remove low-quality sequences and common
166 adapters using bbdduk with the following
167 parameters: *ktrim=r, k=23, mink=11, tpe=t, tbo=t, qtrim=rl, trimq=20, minlength=50, maxns=2*
168 ¹⁸. The target contaminant file used was assembled by pulling contaminant sequences from NCBI
169 GenBank covering several bacterial and human genetic elements and common laboratory
170 synthetic sequences to be removed.

171 Nanopore sequence data were filtered to remove adapter sequences with porechop using
172 default parameters followed by quality and length filtering using filtlong with parameters --
173 *min_length 2000 --keep_percent 90*^{19,20}. Reads passing quality control were mapped to
174 anellovirus contig sequences with the following parameters: *-cx map-ont*. The resultant PAF file
175 was both visualized in Alvis and parsed to identify best hits to the reference contig sequences,
176 and these reads were further analyzed with pairwise alignments in Geneious (Biomatters) with
177 the MAFFT alignment plug-in with the G-INS-i algorithm²¹. These long reads were used to
178 validate the assembled short-reads and to verify that these contigs were not chimeras.

179 Next, human sequences were removed in two passes with both NextGenMap and BWA
180 against the GRCh37/hg19 build of the human reference genome²²⁻²⁴. NextGenMap was run with
181 parameters *--affine, -s 0.7, and -p*, and BWA was run with default parameters. Mapped reads
182 output in SAM file format were converted to paired-end FASTQ format with both SAMtools and
183 Picard's SamToFastq utility configured with the parameter
184 *VALIDATION_STRINGENCY="silent"*^{24,25}.

185 rRNA contaminants and common laboratory bacterial contaminants were removed with
186 bbmap with the following
187 parameters: *minid=0.95, bwr=0.16, bw=12, quickmatch=t, fast=t, minhits=2*. An accounting of
188 all reference sequences screened against can be found in the provided supplementary data¹⁸.

189 Finally, we de-duplicated the short-read data passing all QC and decontamination steps to
190 speed up and aid in genome assembly quality by using clumpify configured with the
191 parameter *dedupe=t*¹⁸.

192

193 **Genome assembly**

194

195 Short, trimmed, decontaminated, and de-duplicated sequencing reads were assembled
196 with metaSPAdes, with the error correction module disabled via the use of the *--only-assembler*
197 parameter. The resulting contigs were filtered with PRINSEQ lite using the parameters
198 *out_format 1, -lc_method dust, and lc_threshold 20*^{26,27}. Contigs passing this filtering step were
199 then clustered at 99.5% similarity to remove any duplicate sequences via the VSEARCH

200 software's *cluster_fast* algorithm using default parameters. Any putative complete, circular
201 genomes were recovered from contigs using *ccfind*, with all parameters set to defaults^{28,29}.

202

203 **Long-read error correction**

204

205 Nanopore reads classified as anellovirus sequences were error-corrected using paired
206 short-read data utilizing *racon*³⁰. First, short reads classified as anellovirus were mapped to long
207 anellovirus reads using BWA's *mem* algorithm with default parameters²³. The resulting SAM
208 alignment and the short reads and long reads used to produce the alignment were supplied to
209 *racon* for error correction³⁰. Execution of *racon* was conducted using default parameters for three
210 rounds of error correction until the polished product showed no variation from the previous
211 iteration.

212

213 **Anellovirus contig identification**

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215 Assembled contigs were screened using NCBI's *blastn* software, with default parameters,
216 to identify putative anellovirus sequences using a custom in-house anellovirus database
217 consisting of 728 curated sequences³¹.

218

219 **Anellovirus genome annotation**

220

221 ORF sequences were identified and extracted from assembled anellovirus contigs using
222 the *OrfM* software with parameters configured to print stop codons (*-p*) as well as ORFs in the
223 same frame as a stop codon (*-s*) and constrained to ORF sequences longer than 50 amino acids (*-m 150*)³².

225 Predicted ORF sequences were further filtered using *seqkit*'s *seq* and *grep* utilities to
226 subdivide ORF sequences into bins corresponding to ORF1, ORF2, and ORF3³³. ORF1
227 sequences were identified by filtering ORF sequences using *seqkit seq* for those no shorter than
228 600 amino acids (*-m 600*) and using *seqkit grep* to search through the ORF sequence data (*-s*) for
229 the conserved motif YNPX²DXGX²N with a regular expression (*-r*)-based pattern (*-p*
230 "*YNP.{2}D.G.{2}*")³⁴. Similarly, ORF2 sequences were recovered using the conserved motif
231 WX⁷HX³CXCX⁵H previously identified in literature through *seqkit*'s *grep* utility (*-p*
232 "*W.{7}H.{3}C.C.{5}H*")³⁵.

233 ORF3 sequences were predicted by utilizing the presence and coordinate positions of
234 predicted ORF1 and ORF2 sequences on the same contig. Predicted ORF3s use a stop codon
235 downstream of those used by ORF1 and their reading frames are different from those of ORF1
236 and ORF2 sequences. Additionally, parsing the ORF3 sequences from internal datasets (median
237 length: 68 aa, minimum length: 50 aa, maximum length: 159 aa through MEME revealed the
238 presence of two previously unknown and highly conserved motifs located near the 3' end of
239 ORF3³⁶. Both novel motifs were also utilized to identify ORF3 sequences using *seqkit*'s *grep*
240 command.

241 Identified ORF sequences required an additional trimming step as *OrfM* produces ORF
242 calls with peptides upstream of canonical start codons. ORF1 sequences were timed to the proper
243 start codon via an in-house written python script that used the presence of the arginine-rich
244 region to identify the first methionine located upstream of it in the direction of the 5' end. In
245 some cases, a non-canonical start codon was predicted as the ORF1 start codon by searching for

246 a threonine-proline-tryptophan or threonine-alanine-tryptophan tripeptide directly upstream of
247 the arginine-rich region. ORF2 and ORF3 sequences were trimmed to the first start codon
248 identified nearest the 5' end of the sequence.

249

250 **Anellovirus genera classification**

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252 Anellovirus contig sequences were identified into one of the three known genera by use
253 of the tblastx software to conduct a homology search against a custom in-house database
254 consisting of 720 curated and classified anellovirus sequences³¹. The top hits that contained
255 suitable coverage across the majority of the contig sequence were then used in genera
256 classification.

257

258 **Primer walking and genome recovery**

259

260 Primers were designed around regions of inconsistencies between the long-read and
261 short-read sequencing data. Post-RCA DNA was amplified using these primers with a Q5 Hot
262 Start polymerase (New England Biolabs). The product was run on a 2% gel to confirm specific
263 binding before sending the PCR product to GeneWiz for Sanger sequencing. Sanger sequencing
264 results were analyzed using Geneious bioinformatics software (Biomatters).

265

266 **Plasmid construction**

267

268 Plasmid containing a single copy of WT LY2, all ORF1 KO LY2 and all ORF2 KO LY2:

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270 The sequence of a previously described anellovirus LY2 (GenBank accession number:
271 JX134045.1) that belongs to the *Betatorquevirus* genus was synthesized by Integrated DNA
272 Technologies into pUCIDT-Kan plasmid (pUCIDT-LY2)³⁷. Esp3I restriction cut sites were
273 added on each side of the genome in this plasmid to enable subcloning, scarless restriction
274 digestion, and ligation of the two ends of the genome to make double-stranded circular genomes.
275 The template plasmid was amplified with the following primers: FWD 5'-
276 ACAGTCTTCAAGGCGTCTCACCTAATAAATATTCAACAGGAAAACCACCTAATT
277 AATTGCC-3' and REV 5'-
278 ACAGTCTTCAAGTGGTCTCATAGGGGGGTGTAAGGGGGCGTAG-3'. PCR reactions (50
279 µl) contained 1.0-unit Phusion DNA polymerase (New England Biolabs catalog # M0419), 1X
280 Phusion HF buffer, 200 µM dNTPs (New England Biolabs # N044), 0.5 µM of each primer
281 (synthesized at Integrated DNA Technologies), 3% DMSO, and 1 ng of template DNA. All PCR
282 reactions were run with the following parameters: initial denaturing at 98°C for 30 seconds
283 followed by 40 cycles of denaturing at 98°C for 15 seconds, annealing at 60°C for 30 seconds,
284 extension at 72°C for 3 minutes, and a final extension at 72°C for 10 minutes.

285

286 Purified PCR product was cloned into a pcDNA 6.2/V5-PL-DEST (Thermo Fisher
287 Scientific catalog # 12537162) destination plasmid in a one-pot reaction containing 50 ng of
288 destination vector, 30 ng of PCR product, 1 × BSA, 1 × T4 DNA ligase buffer (New England
289 Biolabs), 10 units of BspQI (New England Biolabs catalog # R0712), and 400 units of T4 DNA
290 ligase (New England Biolabs catalog # M0202). Cloning reaction was incubated at 50°C for one
291 hour followed by 15 minutes at 16°C.

291 Constructs to knock out the protein expression of either all ORF1 variants (all ORF1 KO LY2)

292 or all ORF2 variants (all ORF2 KO) were designed by inserting a premature stop codon –

293 Cysteine9-STOP and Arginine13-STOP, respectively. These mutations and the surrounding
294 sequences were ordered as gBlocks (Integrated DNA Technologies) for restriction digest cloning
295 into WT LY2. The gBlocks and WT LY2 plasmid were digested with SpeI-HF and SalI-HF
296 (New England Biolands). The plasmid was further treated with QuickCIP (New England
297 Biolands). The digested products were separated by gel electrophoresis, purified and then
298 ligated. All clones were verified through Sanger sequencing at Genewiz.

299 Plasmid containing two copies of LY2 in tandem:

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301 A plasmid harboring two copies of the LY2 genome arranged in a tandem configuration
302 was assembled using a Golden Gate cloning method. The LY2 genome was subcloned into Level
303 1 plasmids as genome 1 (G1) and genome 2 (G2) with PCR primers containing different Esp3I
304 overhangs for later assembly. The plasmids were amplified by PCR with forward G1-F 5'-
305 ACAGCTCTTCAAGGCGTCTCAATGGTAATAAATATTCAACAGGAAAACCACTAATT
306 TAAATTGCC-3' and reverse G1-R 5'-
307 ACAGCTCTTCAGTGCGTCTCATAGGGGGGTGTAAGGGGGCGTAG-3' for G1; and forward
308 G2-F 5'-
309 ACAGCTCTTCAAGGCGTCTCACCTAATAAATATTCAACAGGAAAACCACTAATT
310 AATTGCC-3' and reverse G2-R 5'-
311 ACAGCTCTTCAGTGCGTCTCATTGAGGGGGGTGTAAGGGGGCGTAG-3' for G2. PCR
312 reactions (50 μ l) contained 1.0 unit of Phusion DNA polymerase (New England Biolabs catalog
313 # M0419), 1 \times Phusion HF buffer, 200 μ M of dNTPs (New England Biolabs # N044), 0.5 μ M of
314 each primer (synthesized at Integrated DNA Technologies), 3% DMSO, and 1 ng of template
315 DNA. All PCR reactions were run with the following parameters: initial denaturing at 98°C for
316 30 seconds followed by 40 cycles of denaturing at 98°C for 15 seconds, annealing at 60°C for 30
317 seconds, extension at 72°C for 3 minutes, and a final extension at 72°C for 10 minutes. For
318 assembling the tandem genome plasmid, the destination plasmid, G1 subclone, and G2 subclone
319 were cloned in a one-pot Golden Gate reaction containing 50 ng of the destination plasmid, 30
320 ng of each genome subclone, 1 \times BSA, 1 \times T4 DNA ligase buffer, 10 units of Esp3I (New
321 England Biolabs catalog # R0734), and 400 units of T4 DNA ligase (New England Biolabs
322 catalog # M0202). The cloning reaction was run at 37°C for 15 minutes, 20 cycles at 37°C for 2
323 minutes followed by 15°C for 5 minutes, at 37°C for 15 minutes, at 50°C for 5 minutes, and at
324 80°C for 5 minutes.

325

326 Plasmid containing two copies of nrVL4619 in tandem:

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328 A single copy of the nrVL4619 genome, flanked by BsaI cut sites, was synthesized by
329 GenScript into a pUC57-Kan vector. The nrVL4619 genome was excised and separated from its
330 plasmid backbone using BsaI-HFv2 (New England Biolabs catalog # R3733) and PvuI-HF
331 restriction enzymes (New England Biolabs catalog # R3150); the excised band was purified and
332 ligated to itself to form an *in vitro* circularized (IVC) genome. A plasmid containing tandem
333 copies of nrVL4619 was cloned by linearizing both the IVC genome and a plasmid containing a
334 single copy of nrVL4619 (described above) with NheI-HF restriction enzyme and ligating with
335 T4 DNA ligase (New England Biolabs). All clones were verified through Sanger sequencing at
336 Genewiz.

337

338 **In vitro circularization (IVC) of LY2 genome**

339
340 A circularizable LY2 plasmid was digested with Esp3I (New England Biolabs catalog #
341 R0734) and PvuI-HF (New England Biolabs catalog # R3150), separating the genome and
342 plasmid backbone by gel electrophoresis. To purify the LY2 genome from the excised gel, the
343 gel was placed in 10K MWCO SnakeSkin dialysis tubing (Thermo Fisher Scientific catalog #
344 88242), electroeluting in a 1 × TAE gel box for 18 hours at 40V. Buffer/DNA solution was
345 removed from tubing, incubating mixture with concentrated (2,000,000 units/mL) T4 DNA
346 ligase and ligase buffer (New England Biolabs) at 16°C for 12 hours. Ligated solution was
347 concentrated in 30kD Microsep Advance centrifuge tubes (Pall Corporation).

348 349 **Cell culture**

350
351 MOLT-4 cells were obtained from the National Cancer Institute. Cells were scaled-up
352 and maintained in suspension culture in complete growth medium (Gibco's RPMI 1640 with
353 10% fetal bovine serum [FBS], supplemented with 1 mM sodium pyruvate, Pluronic F-68
354 [0.1%], and 2 mM L-glutamine) at 37°C with 5% CO₂. Cells were seeded into shake flasks (2-L,
355 flat-bottomed, Erlenmeyer flask), each with a working volume of 800 mL, at a density of
356 0.1E+06 viable cells/mL and cultured in an orbital shaker (New Brunswick Innova 2100, 19-mm
357 circular orbit) at 37°C and 100 rpm with >85% relative humidity (RH) for 4 days.

358 359 **Transfection of MOLT-4 cells**

360
361 MOLT-4 cells were transfected with the indicated plasmids either by nucleofection or
362 electroporation.

363 For nucleofection at 25 mL scale, cells were counted using the BioProfile FLEX2
364 analyzer (Nova Biomedical), and 10⁷ cells were pelleted by spinning at 200 × g for 10 minutes.
365 Pelleted cells were resuspended in SF Cell Line Nucleofector Solution with added supplement
366 (Lonza catalog # V4XC2024). 25 µg of the plasmid to be transfected (Aldevron) was added to
367 the resuspended cells and nucleofected using the CM-150 program on the 4D-Nucleofector X
368 Unit (Lonza). Nucleofected cells were allowed to recover in a 37°C incubator with 5% CO₂ for
369 20 minutes, after which they were added to a flask containing pre-warmed complete growth
370 medium.

371 For electroporation at 25 mL scale, 10⁷ pelleted cells were resuspended in homemade 2S
372 Chica buffer (5 mM KCl, 15 mM MgCl₂, 15 mM HEPES buffer solution, 150 mM Na₂HPO₄ pH
373 7.2, 50 mM sodium succinate). 100 µg of the plasmid to be transfected (Aldevron) was added to
374 the resuspended cells and electroporated using a NEPA21 electroporator (Bulldog Bio).
375 Electroporated cells were then transferred to a flask containing pre-warmed complete growth
376 medium.

377 Transfected cells were allowed to incubate at 37°C with 5% CO₂ and harvested at the
378 indicated times.

379 380 **Western blotting**

381
382 Cell pellets were resuspended in lysis buffer containing 50 mM Tris pH 8.0, 0.5% Triton-
383 X100, 100 mM NaCl, and 1 × Halt protease inhibitor cocktail (Thermo Fisher Scientific catalog
384 # 78439), followed by two rounds of freeze-thawing. The cell lysates were clarified by

385 centrifugation at $10,000 \times g$ for 30 minutes at 4°C , and the protein concentration was quantified
386 using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific catalog # 23227) according to the
387 manufacturer's protocol. Equal amounts of the cell lysates were mixed with loading dye and Bolt
388 sample reducing agent (Thermo Fisher Scientific catalog # B0009), followed by boiling at 95°C
389 for 5 minutes.

390 For ORF2 and GAPDH, proteins were separated on Bolt 4-12% Bis-Tris gel in $1 \times$ Bolt
391 MOPS SDS running buffer (Thermo Fisher Scientific catalog # B0001). Separated proteins were
392 electro-transferred to nitrocellulose membrane using Trans-Blot Turbo Transfer System (Bio-
393 Rad). For ORF1, proteins were separated on Bolt 12% NU-PAGE gel and transferred to
394 nitrocellulose membrane at 100 volts for 1.5 hours by a wet transfer method using cold $1 \times$ Bolt
395 transfer buffer (Thermo Fisher Scientific catalog # BT0006) supplemented with 20% methanol.

396 After transfer, membranes were blocked in Odyssey blocking buffer (LI-COR) for 1 hour
397 and then incubated with relevant primary antibodies overnight. Anti-ORF2 antibody was
398 generated by immunizing rabbits with purified full-length ORF2 protein expressed in *E. coli*.
399 Anti-ORF1 antibody was generated in mice against the jelly roll domain of the ORF1 protein.
400 Anti-ORF2 and -ORF1 antibodies were used at a concentration of 1:500. Anti-GAPDH antibody
401 (Cell Signaling Technologies, catalog # 97166) was used at a concentration of 1:1000 to detect
402 GAPDH as a loading control.

403 Membranes were washed three times by rocking in a mixture of tris-buffered saline
404 (TBS) and Polysorbate 20 for 10 minutes each. Membranes were then incubated in the relevant
405 secondary antibodies conjugated with fluorescent dyes. Secondary antibodies used were goat
406 anti-mouse IgG paraproteins (IRDye 680RD, LI-COR, catalog # 926-68070, 1:5000 dilution)
407 and goat anti-rabbit IgG (IRDye 680RD, LI-COR, catalog # 926-68071, 1:5000 dilution).
408 Specific immunoreactive proteins were detected using Odyssey DLx imaging system (LI-COR).

409

410 **Reverse transcriptase quantitative PCR (RT-qPCR)**

411

412 Transfected MOLT-4 cells were harvested by centrifugation at $500 \times g$ for 5 minutes.
413 Pelleted cells were lysed using 700 μl QIAzol lysis reagent (Qiagen catalog # 79306), followed
414 by RNA extraction using miRNeasy Mini Kit (Qiagen, catalog # 217004) as per the
415 manufacturer's protocol. Additional DNase treatment was also performed on the harvested RNA
416 using RQ1 RNase-Free DNase (Promega, catalog # M6101) according to the manufacturer's
417 protocol to remove any carryover of double-stranded or single-stranded DNA. cDNA synthesis
418 was performed from DNase-treated RNA with oligo(dT) primer using SuperScript III First-
419 Strand Synthesis System (Invitrogen, 18080-051). qPCR was performed in triplicate using gene-
420 specific primers with SYBR Green PCR Master Mix (Thermo Fisher Scientific catalog #
421 4309155) in QuantStudio 5 Real-Time PCR machine (Applied Biosystems). Relative quantity
422 was calculated using human GAPDH as a loading control. Gene-specific primers with the
423 following sequences were synthesized at Integrated DNA Technologies: For LY2:F:
424 CTTATTACTACAGAAGAAGACGGTAC and R: AAAGGGCGTCTAATCCAACC. For
425 GAPDH, F: ACCACAGTCCATGCCATCAC and R: TCCACCACCCTGTTGCTGTA.

426

427 **Southern blotting**

428

429 Isolation of total DNA from a total of 10^7 transfected MOLT-4 cells was done using
430 DNeasy Blood & Tissue Kit (Qiagen catalog # 69504). Isolated DNA was digested with either

431 NcoI-HF (New England Biolabs # R0193) or NcoI-HF and DpnI restriction enzymes (New
432 England Biolabs catalog # R0176) overnight at 37°C. NcoI-HF cuts the LY2 genome once. The
433 digested samples were separated by gel electrophoresis and subsequently transferred overnight
434 onto a Hybond-N+ membrane. The membrane was hybridized overnight in ULTRAhyb
435 hybridization buffer (Thermo Fisher Scientific catalog # AM8670) and probed using in-house-
436 generated, biotin-labeled oligos to detect the LY2 genome. These LY2-specific probes were
437 made by random priming and labeled with biotin using the BioPrime Array CGH Genomic
438 Labeling System (Invitrogen catalog # 18095012). Membranes were incubated with IRDye800
439 and imaged using Odyssey DLx imaging system (LI-COR).

440

441 **Isopycnic centrifugation**

442

443 Cesium chloride (CsCl) linear gradients:

444

445 Four days after transfection, MOLT-4 cells were harvested by centrifugation, followed by
446 resuspension in lysis buffer containing 50 mM Tris pH 8.0, 0.5% Triton-X100, 100 mM NaCl,
447 and 1 × Halt protease inhibitor cocktail (Thermo Fisher Scientific catalog # 78439). Resuspended
448 cells underwent by two rounds of freeze-thawing and addition of equal volumes of buffer
449 containing 50 mM Tris pH 8.0 and 2 mM MgCl₂. Cell lysates were subjected to treatment with
450 100 U/mL of Benzonase endonuclease (Sigma-Aldrich catalog # E8263) and nutation at room
451 temperature (RT) for 90 minutes. Benzonase-treated cell lysates were clarified at 10,000 × g for
452 30 minutes at 4°C to pellet any cellular debris.

453 CsCl linear gradients were prepared by overlaying 8.5 mL of 1.46 g/cm³ CsCl solution
454 with 8.5 mL of 1.2 g/cm³ CsCl solution in 17 mL Ultra-Clear tubes (Beckman Coulter), which
455 were then spun at a 45-degree angle and a speed of 20 rpm for 13.5 minutes using Gradient
456 Master (BioComp).

457 2 mL of CsCl solution from the top of the tube was replaced with 2 mL of the processed
458 MOLT-4 cell lysates. The sample-containing tube was spun at 31,000 RPM for 18 hours using
459 SW 32.1 rotor (Beckman Coulter). 1-mL fractions were collected from the bottom of the tube.
460 The refractive index of each fraction was measured using Refracto handheld refractometer
461 (Mettler Toledo) to calculate density. Each fraction was desalted using a desalting kit (Thermo
462 Fisher Scientific catalog # 89851) and then subjected to DNase-protected qPCR assay as
463 described below.

464

465 Iodixanol linear gradients:

466

467 MOLT-4 cells were harvested and processed as described above for CsCl linear
468 gradients. To prepare iodixanol linear gradients, 13 mL of 60% OptiPrep (Sigma-Aldrich catalog
469 # D1556) was overlaid with 13 mL of 20% OptiPrep in 26.3-mL polycarbonate tubes, which
470 were then spun at a 46-degree angle and a speed of 20 rpm for 16 minutes using Gradient Master
471 (BioComp). The sample-containing tube was spun at 347,000 × g and 20°C for 3 hours using
472 Type 70 Ti rotor (Beckman Coulter). 1-mL fractions were collected from the top of the tube. The
473 refractive index of each fraction was measured using Refracto handheld refractometer (Mettler
474 Toledo) to calculate density. Each fraction was then subjected to DNase-protected qPCR assay as
475 described below.

476

477 **DNase-protected qPCR assay**

478
479 5 μ l of the sample to be titrated was incubated with 200 U of DNase I endonuclease
480 (Thermo Fisher Scientific catalog # 18047019) in a 20- μ l reaction. The reaction was incubated at
481 37°C for 2 hours, followed by inactivation of DNase I at 95°C for 10 minutes. 4 μ l of the 1:10
482 diluted DNase reaction was subjected to qPCR analysis in a 20- μ l reaction using TaqMan
483 Universal PCR Master Mix (Thermo Fisher Scientific catalog # 4304437) according to the
484 manufacturer's protocol. Primer and probe sequences are listed in Table 1.

485

486 **LY2 scale-up production**

487

488 Nucleofection:

489

490 Cells were counted using BioProfile FLEX2 analyzer (Nova Biomedical), and 2×10^9
491 viable cells were pelleted using Sorvall BIOS A floor model centrifuge (Thermo Fisher
492 Scientific) in 1-L bottles at 500 RCF for 30 minutes. The supernatant was discarded, the pellets
493 were resuspended in 20 mL of P3 solution with added supplement (Lonza), and 2 mg of the
494 plasmid encoding tandem copies of the LY2 genome (Aldevron) was added. The cells were
495 nucleofected using 4D Nucleofector LV Unit (Lonza) and collected in 5 mL of complete growth
496 medium. The nucleofected cells were then transferred to 600 mL of pre-warmed complete
497 growth medium in a shake flask and incubated in a shaker at 37°C and 100 rpm with 5% CO₂
498 and >85% RH for 1 hour.

499 After incubation, the cells were counted using BioProfile FLEX2 analyzer (Nova
500 Biomedical). They were then diluted to 4×10^5 viable cells/mL in pre-warmed complete growth
501 medium in shake flasks (800 mL maximum working volume) and incubated in a shaker at 37°C
502 and 100 rpm with 5% CO₂ and >85% RH for 4 days.

503

504 Harvest and cell lysis:

505

506 Four days after nucleofection, cells were counted using BioProfile FLEX2 analyzer
507 (Nova Biomedical). Cells were then harvested by pelleting using Sorvall BIOS A floor model
508 centrifuge (Thermo Fisher Scientific) at 1000 RCF for 30 minutes, and supernatant was
509 discarded. Cell pellets were resuspended in 30 mL of 20 mM Tris pH 8, 100 mM NaCl, and 2
510 mM MgCl₂ buffer, lysed using LM10 Microfluidizer (Microfluidics) at 10,000 psi, and washed
511 with 30 mL of the same buffer to make a final cell lysate volume of 60 mL. Then the cell lysates
512 were treated with 1 \times Halt protease inhibitor cocktail (Thermo Fisher Scientific) and 100 U/mL
513 Benzonase endonuclease (Sigma-Aldrich) and incubated for 1.5 hours on a stir plate at RT. Next,
514 0.5% Triton X-100 detergent was added to the cell lysates and returned to incubate at RT on the
515 stir plate for 45 minutes. The treated cell lysates were then centrifuged using 5810 R benchtop
516 centrifuge (Eppendorf) at 10,000 RCF for 30 minutes at 4°C to pellet any cellular debris.
517 Cellular debris was discarded, and the supernatant (lysate) was purified using density gradients.

518

519 CsCl step gradient:

520

521 A CsCl step gradient was prepared by underlaying 30 mL benzonase treated and clarified
522 lysate with 3 mL 1.2 g/L CsCl solution and 3 mL 1.4 g/L CsCl solution made in 30 mM Tris and

523 100 mM NaCl (TN) buffer in 38.6 mL Ultra-Clear ultracentrifuge tubes (Beckman Coulter).
524 Next, the tubes were ultracentrifuged using Optima XE (Beckman Coulter) at 31,000rpm and
525 10°C for 3 hours. After the spin, the band at the junction of the 1.2 g/L and 1.4 g/L CsCl was
526 extracted and transferred to 3-12 mL Slide-A-Lyzer dialysis cassettes with a molecular weight
527 cutoff (MWCO) of 10K (Thermo Fisher Scientific). The membranes were placed in 1 ×
528 Dulbecco's phosphate-buffered saline (DPBS) with Mg and Ca salts (Gibco), 0.001% Pluronic
529 F-68 (Gibco), and 100 mM NaCl as a dialysis buffer overnight (O/N) on a stir plate at 4°C.

530

531 CsCl linear gradient and concentration

532

533 A CsCl linear gradient was prepared by underlaying 15 mL 1.2 g/L CsCl solution and 15
534 mL 1.4 g/L CsCl solution in a 30 mL OptiSeal ultracentrifuge tube (Beckman Coulter) and
535 spinning using Gradient Master 108 (BioComp) at a 45-degree angle and a speed of 20 RPM for
536 13.5 minutes. Next, the top 3 mL of CsCl solution was replaced by 3 mL of dialyzed step
537 gradient fraction. The tubes were then ultracentrifuged at 25,000 rpm and 10°C for 18 hours.
538 After the O/N spin, 1 mL fractions were collected in 96 mL-deep well plates from the bottoms of
539 the tubes. Refractive index of each fraction was measured using Refracto handheld refractometer
540 (Mettler Toledo) to calculate density. An aliquot of each fraction was desalted using Zeba 96-
541 well spin desalting plates (Thermo Fisher Scientific) to remove any CsCl and analyzed for LY2
542 titer using DNase qPCR. Fractions of interest were determined based on qPCR titer and density.
543 They were then pooled and transferred to 3-12 mL Slide-A-Lyzer dialysis cassettes with a
544 MWCO of 10K (Thermo Fisher Scientific). The membranes were placed in 1 × DPBS with Mg
545 and Ca salts (Gibco), 0.001% Pluronic F-68 (Gibco), and 100 mM NaCl as a dialysis buffer O/N
546 on a stir plate at 4°C. The dialyzed sample was concentrated ten-fold using Amicon Ultra
547 centrifugal filter units (Sigma-Aldrich, Catalog # Z648043) with a MWCO of 100 kD.

548

549 **nrVL4619 scale-up production**

550

551 Nucleofection, cell harvest, and lysis were performed as described for LY2 above except
552 that the transfected plasmid encoded two copies of the nrVL4619 genome in tandem.

553

554 Iodixanol linear gradient and concentration:

555

556 An iodixanol linear gradient was prepared by overlaying 19 mL of 20% iodixanol
557 solution made in TN buffer with 19 mL of OptiPrep 60% iodixanol solution (Sigma-Aldrich) in
558 38.6 mL Ultra-Clear ultracentrifuge tubes (Beckman Coulter) and spinning on the Gradient
559 Master (BioComp) at a 45-degree angle and a speed of 20 rpm for 16 minutes. Then the top 5
560 mL of iodixanol solution was replaced with 5 mL clarified lysate, and the tubes were
561 ultracentrifuged at 32,000rpm and 20°C for 18 hours. After the O/N spin, 1-mL fractions were
562 collected in 96 mL-deep well plates from the tops of the tubes. An aliquot of each fraction was
563 used to measure refractive index using Refracto handheld refractometer (Mettler Toledo) as well
564 as nrVL4619 titer, as per the protocol described above for the DNase protected qPCR. Fractions
565 of interest were determined based on the viral titer and density measurements. They were then
566 pooled and concentrated ten-fold using the Amicon Ultra centrifugal filter units (Sigma-Aldrich,
567 Catalog # Z648043) with a MWCO of 100 kD.

568

569 Size exclusion chromatography (SEC):

570

571 Prior to SEC, the sample was centrifuged at 12000 rpm for 1 minute. The supernatant
572 was loaded onto a HiPrep 16/60 Sephacryl S-500 HR column (Cytiva) with buffer conditions at
573 50 mM Tris pH 8.0, 150 mM NaCl, and 0.01% poloxamer. The entire purification was
574 performed at 4°C with a 1 mL/minute flow rate. The fractions with significant qPCR numbers
575 were pooled and concentrated using Vivaspin 2, 10,000 MWCO PES concentrator (Sartorius,
576 catalog # VS0201) and Nanosep centrifugal devices with Omega membrane at MWCO of 30K
577 (Pall, catalog # OD030C34).

578

579 **Electron microscopy**

580

581 To visualize virus particles, negative-stained TEM was conducted at Harvard Medical
582 School using Jeol 1200 EX equipped with an AMT 2k CCD camera. 10 µl of sample was blotted
583 on 400-mesh carbon support film (EMS CF400-Cu) for 30 seconds. After washing with double-
584 distilled water for 30 seconds, the grid was stained by 1% of uranyl acetate for 10 seconds before
585 imaging.

586

587 ***In vivo* studies**

588

589 Care and use of animals

590

591 All mouse studies were approved and governed by the Laronde Institutional Animal Care
592 and Use Committee. Female C57Bl/6J mice 8-12 weeks of age were obtained from Jackson
593 Laboratories for use in these ocular studies.

594

595 Subretinal injections

596

597 Pupils were first dilated with one to two drops of 1% tropicamide/2.5% phenylephrine
598 HCl (Tropi-Phen, Pine Pharmaceuticals). The mouse was subsequently anesthetized using an
599 intraperitoneal injection of a ketamine/xylazine cocktail (100/10 mg/kg). One or two drops of
600 0.5% proparacaine (McKesson Corp.) were applied to the eye. An incision approximately 0.5
601 mm in length was made with a micro scalpel 1 mm posterior to the nasal limbus. A 33-g blunt-
602 ended needle on a 5-µl Hamilton syringe was inserted through the scleral incision, posterior to
603 the lens, toward the temporal retina until resistance was felt. 1 µl of either PBS, virus, or vector
604 containing 0.1% of sodium fluorescein (AK-Fluor 10%, Akorn) was then injected slowly into the
605 subretinal space. The eye was examined and the success of the subretinal injection was
606 confirmed by visualizing the fluorescein-containing bleb through the dilated pupil with a Leica
607 M620 TTS ophthalmic surgical microscope (Leica Microsystems, Inc). Eyes with significant
608 hemorrhage or leakage of vector solution from the subretinal space into the vitreous were
609 excluded from the study. After the procedure, 0.3% tobramycin ophthalmic ointment (Tobrex,
610 Alcon) was applied to each treated eye and the mouse was allowed to recover from the
611 anesthesia prior to being returned to its cage in the housing room.

612

613 Intravitreal injections

614

615 Pupils were first dilated with one to two drops of 1% tropicamide/2.5% phenylephrine
616 HCl (Tropi-Phen, Pine Pharmaceuticals). The mouse was subsequently anesthetized using an
617 intraperitoneal injection of a ketamine/xylazine cocktail (100/10 mg/kg). One or two drops of
618 0.5% proparacaine (McKesson Corp.) were applied to the eye. A 34-g beveled needle on a 5- μ l
619 Hamilton syringe was inserted 1 mm posterior to the nasal limbus, taking care not to damage the
620 lens. 1 μ l of either PBS, virus, or vector containing 0.1% of sodium fluorescein (AK-Fluor 10%,
621 Akorn) was then injected slowly into the subretinal space. The eye was examined, and the
622 success of the intravitreal injection was confirmed by visualizing the fluorescein-containing
623 vitreous through the dilated pupil with a Leica M620 TTS ophthalmic surgical microscope
624 (Leica Microsystems, Inc). Eyes with significant hemorrhage, lens damage, or leakage of vector
625 solution outside of the eye were excluded from the study. After the procedure, 0.3% tobramycin
626 ophthalmic ointment (Tobrex, Alcon) was applied to each treated eye and the mouse was
627 allowed to recover from the anesthesia prior to being returned to its cage in the housing room.

628

629 Harvesting and processing of tissue samples for DNA extraction

630

631 Mouse eyes were dissected at indicated time points following subretinal or intravitreal
632 injections (n = 5 for each time point). After enucleation, the retina and posterior eyecup (PEC)
633 were separated and processed individually. These tissues were collected in tubes containing
634 stainless steel beads and flash-frozen immediately. They were stored at -80°C until ready for
635 homogenization. Frozen tissues were homogenized using Geno/Grinder 2010 (SPEX SamplePrep,
636 LLC) at 1250 rpm for 30 seconds. Genomic DNA was isolated from homogenized tissues using
637 the DNEasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions and
638 quantified on Qubit Fluorometer using the Qubit DNA broad range Assay Kit (Thermo Fisher).

639

640 Quantitative PCR analysis

641

642 Genomic DNA was assayed by qPCR on the QuantStudio 5 – Real-Time PCR System
643 (Thermo Fisher) using TaqMan Universal PCR Mastermix (Thermo Fisher). The sequence
644 detection primers and the custom Taqman probe that were used in this study were synthesized by
645 Integrated DNA Technologies (Table 1). All of the reactions including the DNA samples and
646 different dilutions of a known quantity of the linearized mCherry or nrVL4619 plasmid standards
647 were run in triplicate on the same plate. The standard curve method was used to calculate the
648 amount of viral/vector DNA, which was normalized with the total amount of genomic DNA for
649 each sample (quantified using Qubit as described above).

650

Amplicon	Primer/Probe	Sequence (5'>3')
LY2	Forward	GAAGCCACCAAAGCAATT
	Reverse	AGTTCCTGTCTATAGTCGA
	Probe (FAM)	ACTTCGTTACAGAGTCCAGGGG
nrVL4619	Forward	GGATTTTGGGAGGGTCACTC
	Reverse	TACAGTTCCTGGACCTGTGT
	Probe (FAM)	ACACTGGTACCCTAAAATAGATTCA

651

652 **TABLE 1. Primer and probes designed to quantify LY2 and nrVL4619 titers in virus**
653 **preparations produced from MOLT-4 cells.**

654

Target	Label	Sequence 5'>3'
mCherry	Forward Primer	CCGACTACTTGAAGCTGTCC
	Reverse Primer	CGCAGCTTCACCTTGTAGAT
	TaqMan Probe (FAM)	TGATGAACTTCGAGGACGGC
nrVL4619	Forward Primer	GGATTTTGGGAGGGTCACTC
	Reverse Primer	TACAGTTCCTGGACCTGTGT
	TaqMan Probe (FAM)	ACACTGGTACCCTAAAAATAGATTTCA

655 **TABLE 2. Primer and probes designed to quantify AAV2.mCherry and WT nrVL4619 in**
656 **the DNA harvested from *in vivo* study tissue samples.**

657

658 **RESULTS:**

659

660 **LY2 promoter is active in MOLT-4 cells**

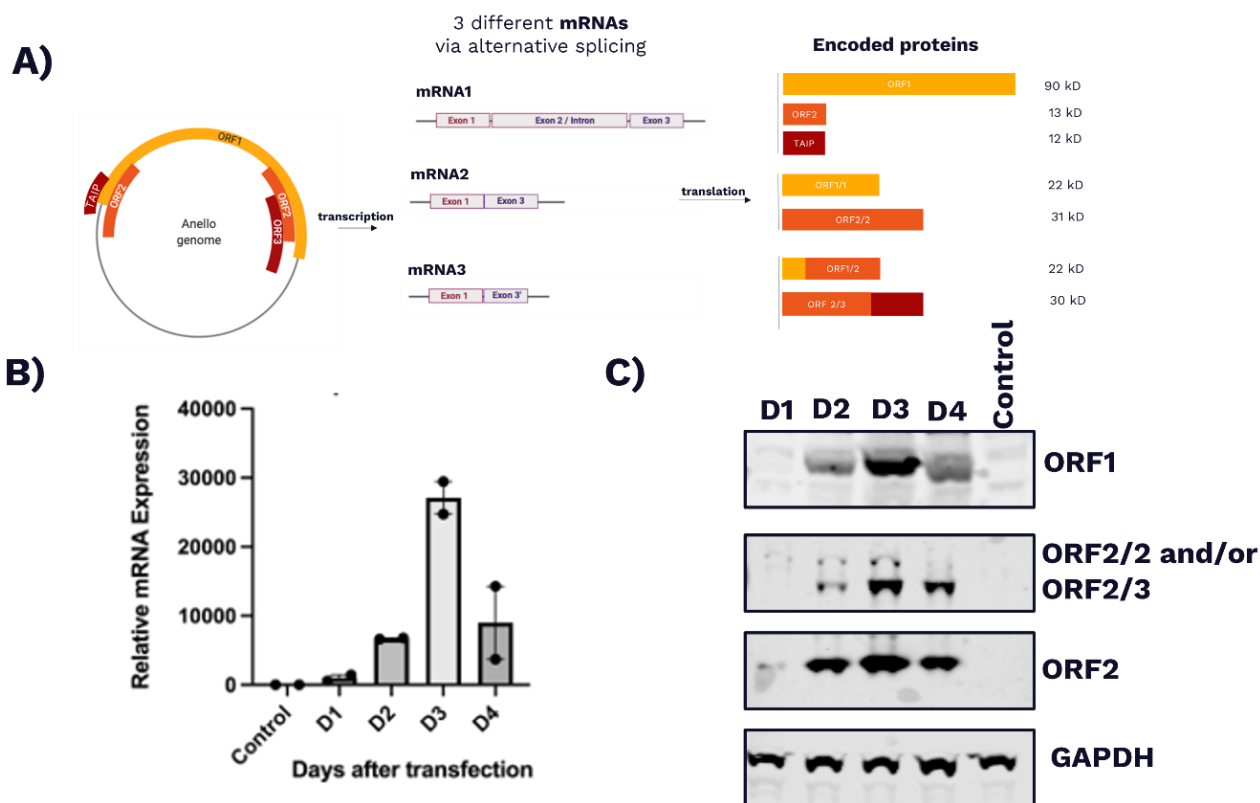
661

662 The viral load of anelloviruses in human plasma has been reported to be a hundred-fold
663 lower than in whole blood, suggesting that cellular components of the blood harbor
664 anelloviruses³⁸, consistent with previous reports of lymphocytes being a major site of anellovirus
665 replication³⁹⁻⁴². Therefore, we examined whether anellovirus genes can be expressed in MOLT-
666 4, a T-cell line derived from a patient with acute lymphoblastic leukemia⁴³. For this, we
667 synthesized a plasmid encoding two copies of the LY2 genome in a tandem arrangement. LY2 is
668 a human anellovirus belonging to the *Betatorquevirus* genus that was previously sequenced from
669 the pleural fluids of children hospitalized in France with parapneumonic empyema³⁷. MOLT-4
670 cells electroporated with this plasmid were harvested at Days 1, 2, 3 and 4 post-transfection and
671 analyzed for the detection of LY2 transcripts by RT-qPCR.

672 Previous anellovirus gene expression studies have described three major mRNA isoforms
673 that are produced as a result of alternative splicing (Fig. 1A)⁴⁴. For our analysis, we used a
674 primer pair that would detect all 3 known isoforms of anellovirus transcripts. Expression of the
675 GAPDH transcript was used for normalization. We were able to detect LY2 transcripts starting at
676 Day 1 post-electroporation. Expression peaked at Day 3 post-transfection and was reduced at
677 Day 4 (Fig. 1B). As expected, we did not detect any LY2 transcripts in untransfected MOLT-4
678 cells.

679 Having detected LY2 transcripts, next we performed a similar time-course experiment to
680 determine LY2 protein expression. Antibodies to detect the putative capsid protein, ORF1, as
681 well as ORF2 and its variants were generated. As shown in Figure 1C, ORF1 is detectable
682 beginning on Day 2 post-transfection, peaks on Day 3, and is reduced beyond Day 3. As the
683 epitope used to generate the anti-ORF1 antibody is specific to the jelly roll domain of ORF1, it
684 cannot detect isoforms such as ORF1/1 and ORF1/2. The anti-ORF2 antibody that we generated
685 can detect all three isoforms of ORF2, including ORF2, ORF2/2, and ORF2/3. The predicted
686 molecular weights for ORF2, ORF2/2, and ORF2/3 are 17, 31, and 30 kDa, respectively. Since
687 ORF2/2 and ORF2/3 are nearly equal in molecular weight, it is challenging to distinguish them
688 based on migration on SDS-PAGE gel in a denatured state. Like ORF1, the expression of ORF2
689 and its isoforms also peaked on Day 3 post-transfection and was reduced thereafter (Fig. 1C).
690 Collectively, these results suggest that the LY2 promoter is active in MOLT-4 cells, enabling
691 transcription and translation of anellovirus genes in this human cell line.

692



693

694 **FIGURE 1. LY2 gene expression in MOLT-4 cells.** A) Schematic of the single-stranded,
 695 circular DNA genome of an anellovirus, alternatively spliced to generate three different mRNAs
 696 encoding seven putative proteins of varying molecular weights as previously described. B) 10^7
 697 MOLT-4 cells were electroporated with 100 μ g of a plasmid encoding two copies of the LY2
 698 genome in tandem. RT-qPCR was performed at Days 1 (D1), 2 (D2), 3 (D3), and 4 (D4) post-
 699 electroporation to study the kinetics of the expression of LY2 transcripts over time.

700 Untransfected MOLT-4 cells (control) were used as a negative control and GAPDH mRNA was
 701 used as a housekeeping gene for normalization. C) 10^7 MOLT-4 cells were electroporated with
 702 100 μ g of a plasmid encoding two copies of the LY2 genome in tandem. Western blot analysis
 703 was performed at D1, D2, D3, and D4 post-electroporation to study the kinetics of the expression
 704 of LY2 proteins (ORF1, ORF2, ORF2/2 and/or ORF2/3) over time. GAPDH protein was used as
 705 a loading control.

706

707 MOLT-4 is permissive for the replication of the LY2 anellovirus

708

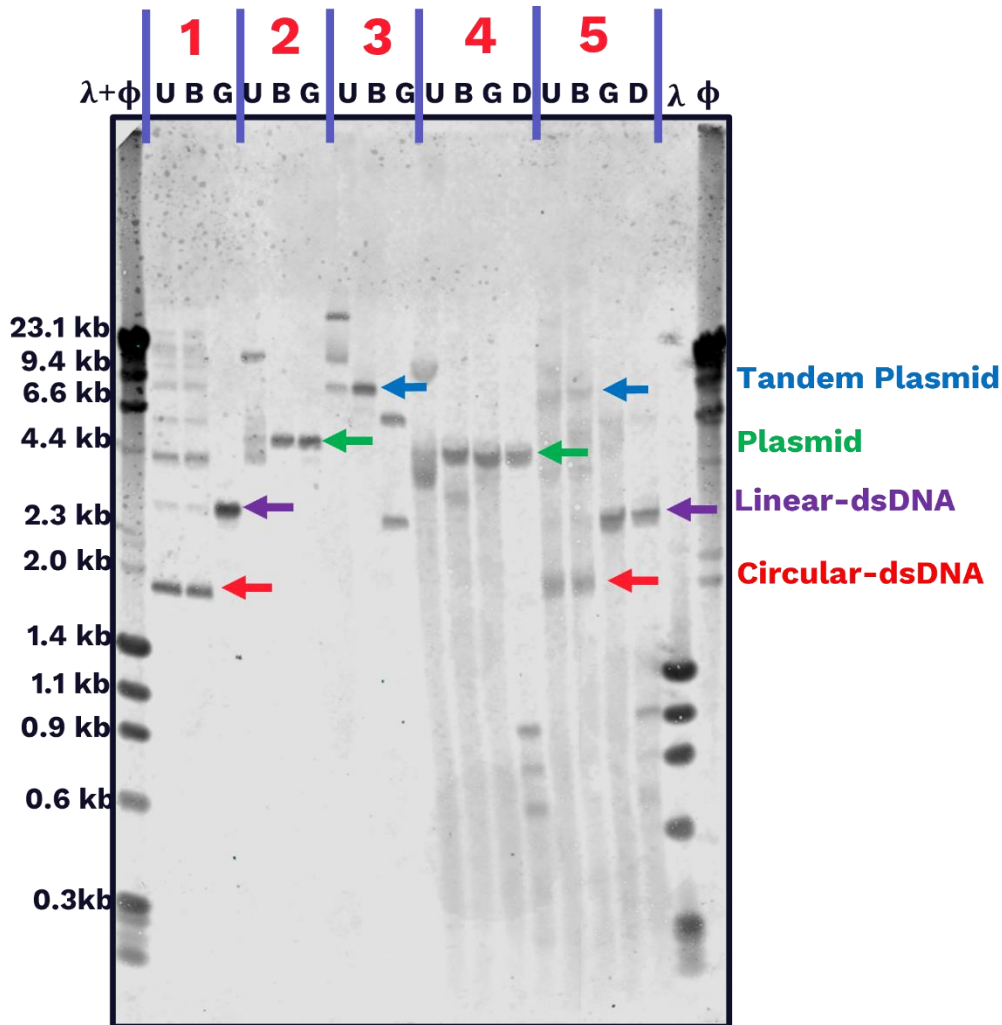
709 Having detected LY2 gene expression in MOLT-4 cells, next we tested whether the cell
 710 line is permissive for replication of the anellovirus genome. We nucleofected plasmids to encode
 711 either a single copy of the LY2 genome or two copies of the LY2 genome in tandem. The cells
 712 were harvested four days post-nucleofection, followed by DNA extraction. Extracted DNA was
 713 either left untreated or treated with a restriction enzyme that digests the plasmid backbone once,
 714 a restriction enzyme that digests the LY2 genome once, or DpnI. DNA replicated in bacterial
 715 cells contains methylated adenine and therefore is sensitive to digestion with DpnI. On the other
 716 hand, DNA that replicates in eukaryotic cells lacks methylated adenine and therefore is resistant
 717 to digestion with DpnI. Hence, DpnI digestion can be used to distinguish between the transfected

718 genome and any genome that may have replicated in MOLT-4 cells. Untreated and treated DNA
719 samples were subjected to Southern blot analysis using probes designed to specifically detect the
720 LY2 genome.

721 For DNA extracted from the sample transfected with a tandem LY2 genome-containing
722 plasmid (Fig. 2, Sample #5), we detected a band of the same size as would be expected for a
723 unit-length, double-stranded LY2 genome. This band was insensitive to digestion with an
724 enzyme that cuts the plasmid backbone but became linear when treated with an enzyme that cuts
725 the LY2 genome once. These observations suggest that this band represents the LY2 genome and
726 not the plasmid backbone. Furthermore, this band was resistant to treatment with DpnI,
727 indicating that the LY2 genome replicated in the transfected MOLT-4 cells. Overall, based on
728 the patterns of banding observed for samples and controls, we conclude that MOLT-4 cells
729 transfected with a tandem LY2 genome-containing plasmid are permissive for replication of the
730 anellovirus and lead to a unit-length, replicated anellovirus genome.

731 A DpnI-resistant band was also detected for the sample transfected with a single LY2
732 genome-containing plasmid (Fig. 2, Sample #4). When this sample was treated with a restriction
733 enzyme that digests either the plasmid backbone once or the LY2 genome once, we detected a
734 band of the same size as would be expected for a linear plasmid containing the entire LY2
735 genome. This result suggests that the single LY2 genome-containing plasmid can also replicate
736 in MOLT-4 cells but does not yield a detectable unit-length anellovirus genome. To our
737 knowledge, this is the first study to conclusively demonstrate the replication of a unit-length,
738 circular, human anellovirus genome in a human cell line using recombinant DNA as an input
739 material.

740



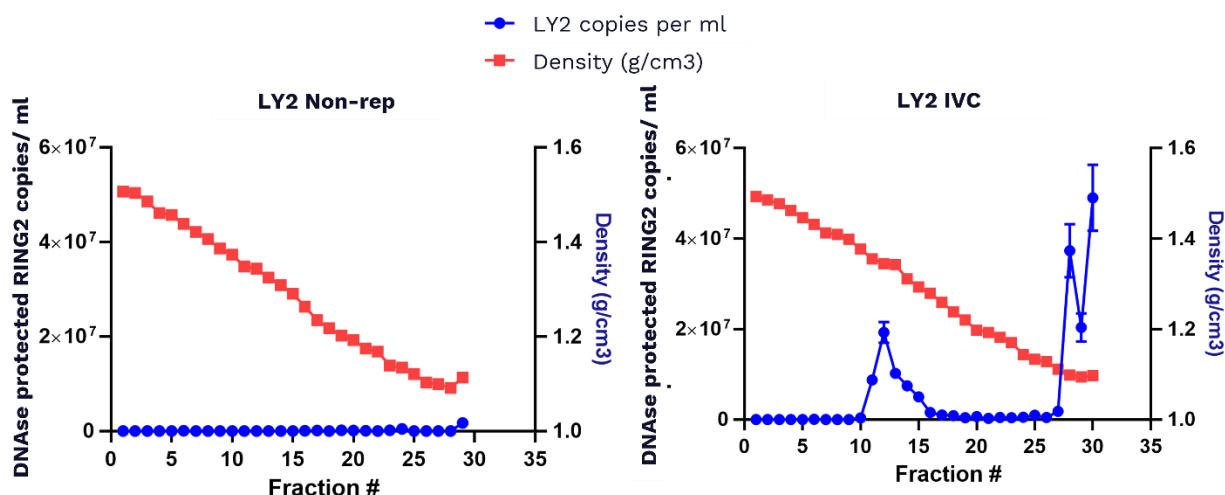
741
742 **FIGURE 2. MOLT-4 cells are permissive for LY2 replication.** 10^7 MOLT-4 cells were
743 transfected with 25 μ g of either a plasmid encoding a single copy of the LY2 genome (Sample
744 #4) or a plasmid encoding two copies of the LY2 genome in tandem (Sample #5). Total genomic
745 DNA was harvested from the cells at four days post-transfection and was either untreated (U) or
746 treated with an enzyme intended to digest the plasmid backbone once (B), an enzyme intended to
747 digest the LY2 genome once (G), or DpnI restriction enzyme (D). As controls, enzyme
748 treatments were also performed in parallel on an in-vitro-circularized LY2 genome (Sample #1),
749 a plasmid containing a single copy of the LY2 genome (Sample #2), and a plasmid containing
750 two copies of the LY2 genome in tandem (Sample #3). Southern blot analysis was performed on
751 the digested samples using probes specific against the LY2 genome. The expected sizes for the
752 plasmid containing tandem LY2 genomes, the plasmid containing a single LY2 genome, a unit-
753 length double-stranded linear genome, and a unit-length double-stranded circular genome are
754 indicated by blue, green, purple, and red arrows, respectively.

755
756 **MOLT-4 cells are permissive for LY2 packaging**

757
758 Since we demonstrated that MOLT-4 cells are permissive for LY2 replication, we next
759 tested whether LY2 particles can be produced in this human cell line. We nucleofected MOLT-4

760 cells with either a plasmid containing a qPCR amplicon of LY2 (LY2 non-rep) or an *in vitro*
761 circularized, double-stranded LY2 genome (LY2 IVC). Four days after nucleofection, cells were
762 harvested, lysed by two rounds of freeze-thawing, treated with Benzonase, clarified, and
763 subjected to isopycnic ultracentrifugation using CsCl linear gradient. 1-mL fractions of CsCl
764 linear gradient were collected from the bottom of the tube. Each fraction was analyzed for its
765 density as well as for titers of LY2 titer using a DNase-protected qPCR assay.

766 As shown in Fig. 3, the sample transfected with LY2 IVC showed a peak viral titer in
767 fractions with a density of approximately 1.32 g/cm³. This density is consistent with the
768 previously described density of anelloviruses in CsCl^{45,46}. In contrast, the sample transfected
769 with negative control had no detectable viral titer in any fractions. These results conclusively
770 demonstrate that the MOLT-4 cell line is permissive not only for LY2 gene expression and
771 replication, but also for production of LY2 particles. While isopycnic ultracentrifugation to
772 analyze the density of anellovirus particles in CsCl has been previously reported for wild-type
773 anellovirus particles isolated from human specimens, our study is the first to obtain such results
774 for anellovirus particles produced *in vitro* using a recombinant viral genome.
775

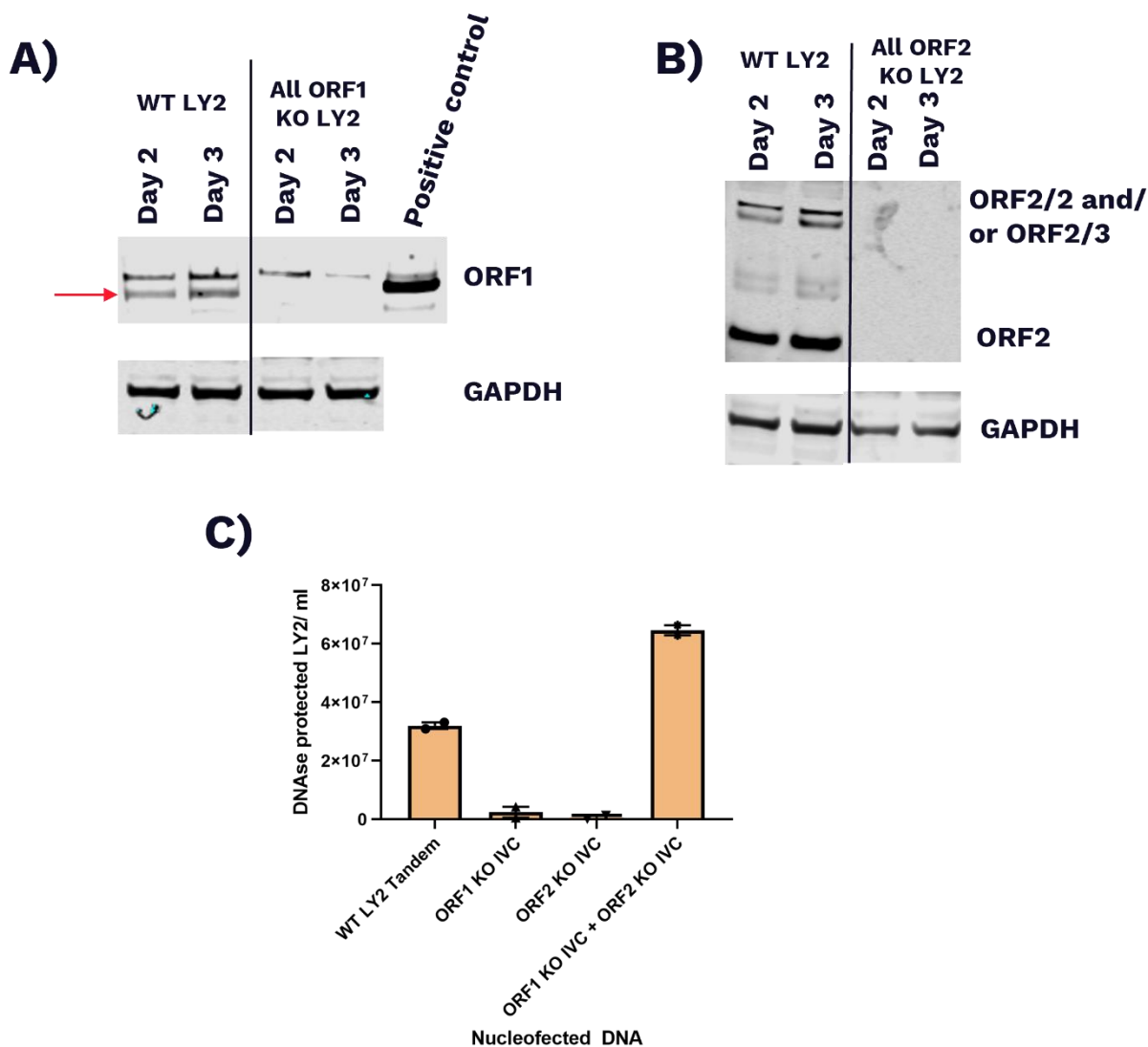


776
777
778 **FIGURE 3. Packaging of LY2 particles in MOLT-4 cells.** 10⁷ MOLT-4 cells were transfected
779 with 25 µg of either a negative control plasmid (non-rep) or an *in vitro* circularized genome of
780 LY2 (IVC). Cells were harvested 4 days post-transfection, lysed, and treated with Benzonase.
781 Clarified lysate was then subjected to isopycnic centrifugation using CsCl linear gradient. Each
782 collected fraction was analyzed for density (plotted in red) and viral titer (plotted in blue). A
783 representative linear gradient profile is shown.

784 **Production of LY2 in MOLT-4 cells is dependent on viral protein expression**

785
786 To assess whether the production of LY2 particles is dependent on viral protein
787 expression, we created LY2 mutant genomes in which either all 3 ORF1 variants including
788 ORF1, ORF1/1, and ORF1/2 were knocked out (ORF1 KO), or all 3 ORF2 variants including
789 ORF2, ORF2/2, and ORF2/3 were knocked out (ORF2 KO). These mutant genomes were
790 generated by inserting premature stop codons into the open reading frames, as described in
791 materials and methods.

792 To confirm successful knockout of the target proteins, plasmids encoding a single copy of
793 either the wild-type LY2 genome, the ORF1 KO genome, or the ORF2 KO genome were
794 transfected into MOLT-4 cells. Western blot analysis was performed at 2 days and 3 days post-
795 transfection. As expected, the ORF1 KO mutant did not express any detectable ORF1 protein
796 (Fig. 4A). Similarly, the ORF2 KO mutant did not express any detectable levels of ORF2 protein
797 or its isoforms (Fig. 4B). To test whether these mutants can produce LY2 virus particles, MOLT-
798 4 cells were transfected with either a plasmid encoding two copies of the wild-type LY2 genome
799 in tandem (WT LY2 tandem), an *in vitro* circularized ORF1 knockout genome (ORF1 KO IVC),
800 or an *in vitro* circularized ORF2 knockout genome (ORF2 KO IVC) or were co-transfected with
801 ORF1 KO IVC and ORF2 KO IVC. Samples were assayed for LY2 production using isopycnic
802 CsCl step gradient. As expected, WT LY2 tandem produced LY2 particles (Fig. 4C). Knocking
803 out the expression of ORF1 and its variants or ORF2 and its variants significantly disrupted the
804 ability to produce the virus in MOLT-4 cells. Interestingly, the mutant genomes were able to
805 trans-complement each other, which was expected assuming co-transfection of the same cells,
806 given that ORF1 KO IVC can produce ORF2 and its variants while ORF2 KO IVC can produce
807 ORF1 and its variants. Overall, these findings demonstrate that the production of LY2 particles
808 in transfected MOLT-4 cells is dependent on viral protein expression.



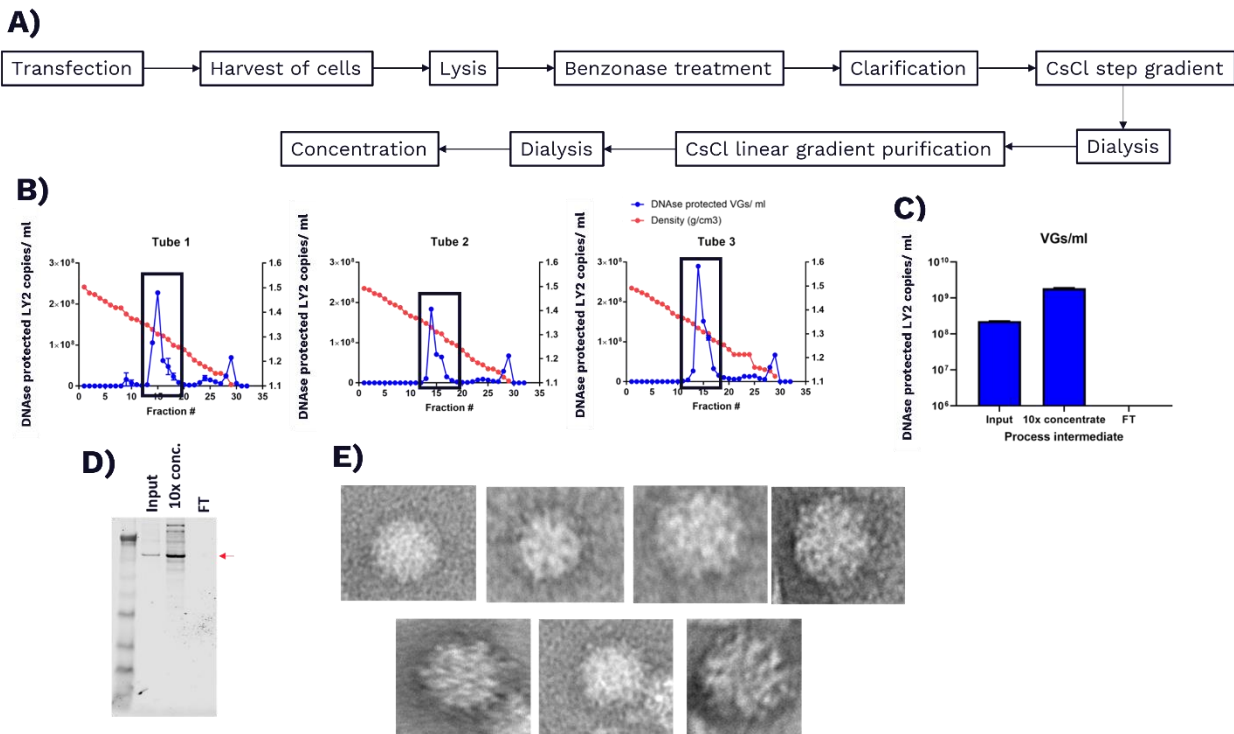
809
 810 **FIGURE 4. Packaging of LY2 particles in MOLT-4 cells is ORF1- and ORF2-dependent.**
 811 10^7 MOLT-4 cells were electroporated with 100 μ g of plasmid encoding either the wild-type
 812 LY2 genome (WT), a LY2 genome in which the expression of all ORF1 variants has been
 813 knocked out (All ORF1 KO), or a LY2 genome in which the expression of all ORF2 variants has
 814 been knocked out (All ORF2 KO). Cells were harvested either 2 or 3 days post-transfection. A)
 815 Western blotting was performed to detect ORF1 protein. Purified ORF1 was used as a positive
 816 control. The ORF1 band is shown using a red arrow. GAPDH was used as a loading control. The
 817 black line denotes where the blot was cut out to remove unnecessary lanes. B) Western blotting
 818 was performed to detect ORF2 and its variants. GAPDH was used as a loading control. The
 819 black line denotes where the blot was cut out to remove unnecessary lanes. C) 10^7 MOLT-4 cells
 820 were transfected with either 25 μ g of a plasmid encoding two copies of the LY2 genome in
 821 tandem (WT LY2 tandem), 25 μ g of an *in vitro* circularized genome of LY2 in which the
 822 expression of all ORF1 variants has been knocked out (ORF1 KO IVC), or 25 μ g of an *in vitro*
 823 circularized genome of LY2 in which the expression of all ORF2 variants has been knocked out
 824 (ORF2 KO IVC), or were co-transfected with 12.5 μ g each of ORF1 KO IVC and ORF2 KO
 825 IVC. Cells were harvested 4 days post-transfection, lysed, and treated with Benzonase. Clarified

826 lysates were then subjected to isopycnic centrifugation using CsCl step gradient to isolate
827 proteins within a range of density from 1.2 to 1.4 g/cm³. The isolated fraction was dialyzed to
828 remove CsCl and then was used to perform DNase-protected qPCR.
829

830 Transmission electron microscopy (TEM) analysis of LY2 anellovirus

831
832 Visualization of recombinant anellovirus particles has been previously performed only
833 for chicken anemia virus, an avian virus in the *Anelloviridae* family⁴⁷. To further characterize
834 and advance our understanding of the structural biology of human anelloviruses, we analyzed
835 LY2 by TEM. A schematic of the production and purification methodology of LY2 is depicted in
836 Fig. 5A. Briefly, MOLT-4 cells were transfected with a plasmid containing two copies of the
837 LY2 genome in tandem and harvested four days post-transfection. Harvested cells were lysed
838 and treated with Benzonase and detergent. Cell lysates were clarified to remove any cellular
839 debris and subjected to CsCl step gradient to concentrate the virus particles followed by
840 overnight dialysis to remove any CsCl. Dialyzed material was subjected to CsCl linear gradient
841 followed by fractionation. Each fraction was analyzed for density and viral titer.

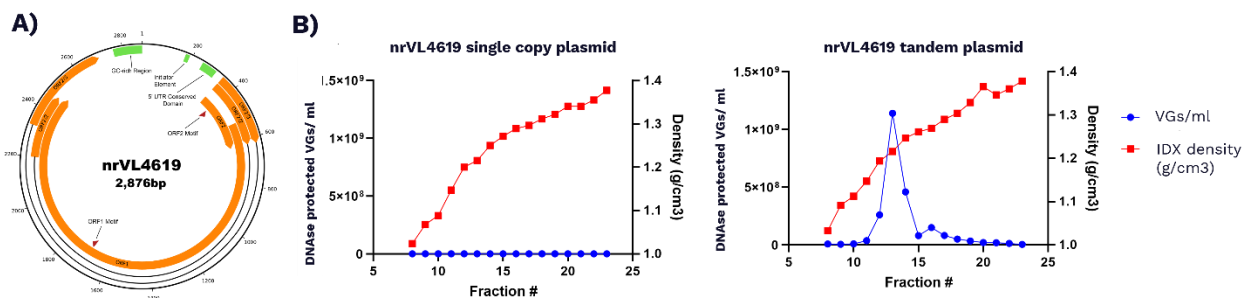
842 As expected, all linear gradient tubes had a peak for DNase-protected LY2 titer at the
843 expected density of 1.32 g/cm^{3.45}. Representative profiles for density against viral titer in each
844 fraction of the linear gradient are shown in Fig. 5B. Next, we pooled the fractions in the peak for
845 all 12 tubes of linear gradient, dialyzed it overnight to remove any CsCl, and concentrated the
846 volume tenfold using diafiltration. As we used 100 kD cutoff diafiltration units, we were able to
847 concentrate the titer of LY2 particles (Fig. 5C). Concomitantly, an increase in the titer of capsid
848 protein ORF1 as assessed by Western blot was observed as expected (Fig. 5D). Negative-
849 staining TEM of this purified virus preparation revealed multiple LY2 particles (Fig. 5E). The
850 observed particles we detected were consistent with the expected ~30nm-diameter viral particles.
851



853
854 **FIGURE 5. Electron microscopy detection of LY2 particles produced in MOLT-4 cells.** A)
855 Schematic of the production and purification of LY2 particles from MOLT-4 cells. B) 2×10^9
856 MOLT-4 cells were transfected with 2 mg of a plasmid containing two copies of the LY2
857 genome in tandem. Cells were harvested 4 days post-transfection, lysed, treated with Benzonase,
858 clarified, concentrated using CsCl step gradient, and dialyzed. Dialyzed material was subjected
859 to isopycnic centrifugation using CsCl linear gradient. Each fraction of linear gradient was
860 analyzed for density and viral titer. Three representative linear gradient profiles are shown.
861 Fractions with the expected density of LY2 (shown with black box) were pooled together and
862 concentrated $10\times$ using Centricon centrifugal filter units. C) The viral titers in the pooled
863 material (input), concentrated material, and flow-through (FT). D) Western blot analysis to
864 detect capsid protein ORF1 in the pooled material (input), concentrated material, and FT. E)
865 Representative TEM images of concentrated LY2 particles.
866

867 **Discovery of an anellovirus in human retinal pigment epithelial (RPE) cells**

868 Anelloviruses have previously been isolated from numerous human non-blood tissues,
869 such as the bone marrow, liver, and conjunctival surface and vitreous fluid of the eye^{48–51}. We
870 investigated the specific anellovirus lineages present in four ocular tissues (cornea, macula,
871 sclera and retina pigment epithelia) from the same subject using our AnelloScope platform¹⁴. We
872 recovered several anellovirus genomes, across all three genera, from half of the investigated
873 ocular tissues and successfully isolated a putative full-length circularized genome designated as
874 nrVL4619 (Fig. 6A).
875



876
877 **FIGURE 6: Recovery of a circularized, complete anellovirus genome from RPE cells and**
878 **its rescue in MOLT-4 cells.** A) Schematic of the fully annotated, circularized genome of
879 nrVL4619 recovered from a dissected RPE tissue. ORF1 and ORF2 were computationally
880 annotated, while ORF2/2 and ORF2/3 were manually curated. B) 10^7 MOLT-4 cells were
881 transfected with 50 μg of either a plasmid encoding a single copy of the nrVL4619 genome or a
882 plasmid encoding two copies of NRVL4619 in tandem. Cells were harvested 4 days post-
883 transfection, lysed, and treated with Benzonase. Clarified lysate was then subjected to isopycnic
884 centrifugation using iodixanol linear gradient. Each collected fraction was analyzed for density
885 (plotted in red as g/cm^3) and viral titer (plotted in blue as viral genomes (vgs)/ml). A
886 representative linear gradient profile is shown.

887

888 To explore the diversity of anelloviruses in the four eye tissues (cornea, macula, sclera
889 and retina pigment epithelia), we conducted two deep short-read sequencing runs (with and
890 without bead-baited target enrichment) and one shallow long-read sequencing run to recover an
891 appropriate amount of genomic anellovirus data. An aggregate of 28.71 Gbp of short-read
892 sequence data and 1.41 Gbp of long-read sequence data were generated across all sequencing
893 runs, of which 3.71 Gbp and 269.3 Mbp of sequence data were classified as anellovirus from
894 short- and long-read sequencing runs, respectively. Strikingly, when examining the two short-
895 read sequencing runs, the run utilizing our bead-baited target enrichment protocol contributed
896 99.9% of those reads identified as anellovirus. These findings highlight the difficulty in isolating
897 anellovirus genome data from non-blood tissue samples and the need for targeted approaches
898 that both amplify the amount of anellovirus in samples and reduce the amount of host
899 background being sequenced.

900 We next attempted to recover complete, circularized, high-quality anellovirus genomes
901 from the RPE genomic data generated. We searched through the existing short-read-assembled
902 contigs to find suitable candidates that were near genome length and contained the expected
903 ORF1, ORF2, ORF3, 5' UTR, and 3' GC-rich region features, producing a *Betatorquevirus*
904 candidate designated nrVL4619-*short*. To confirm the completeness of nrVL4619-*short*, we
905 examined the paired long-read data for any sequences with at least 90% sequence similarity. We
906 found 7 long reads, that were further assembled and error-corrected to produce a contig
907 designated nrVL4619-*long*. When comparing nrVL4619-*short* to nrVL4619-*long*, we observed
908 98.4% similarity, translating to a decrease of 4% in error rate from the pre-corrected long-read
909 sequences. To further improve accuracy, we leveraged the accompanying short-read genomic
910 data by mapping these reads (485,785 mapped reads, 4,275 average coverage) to nrVL4619-*long*
911 over three rounds of additional error-correction, resulting in a final sequence at 99.9% similarity
912 when compared to nrVL4619-*short*.

913 To resolve the 0.1% divergence observed between nrVL4619-*long* and nrVL4619-*short*,
914 found at four positions, we employed high-quality Sanger sequencing. We recovered two
915 overlapping Sanger reads (both forward and reverse) for each of the four sites identified and
916 resolved inconsistencies between nrVL4619-*short* and -*long* at three of the four sites. At the
917 fourth site, located in the ORF1 capsid protein at positions 1330 and 1340, we found no clear
918 consensus sequence established from the Sanger data. Examining all the data generated at this
919 fourth site indicated the existence of an RPE-specific nrVL4619 consensus sequence. In
920 producing a final high-quality sequence of nrVL4619, we pursued the RPE dominant consensus
921 sequence.

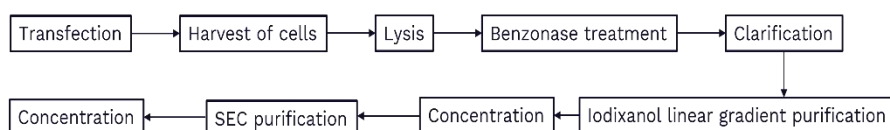
922 923 **Production of nrVL4619 virus particles *in vitro***

924 Whereas LY2 had been previously sequenced from human pleural effusion samples and
925 reported in the literature, nrVL4619 is an anellovirus that we isolated from human retinal
926 pigmental epithelium as described above. To test whether nrVL4619 can be produced *in vitro*
927 like LY2, we electroporated MOLT-4 cells with either a plasmid encoding a single copy of the
928 nrVL4619 genome or a plasmid encoding two copies of the nrVL4619 genome in tandem. Four
929 days after electroporation, cells were harvested, lysed by two rounds of freeze-thawing, treated
930 with Benzonase, clarified, and subjected to isopycnic ultracentrifugation using iodixanol linear
931 gradient. 1-mL fractions of linear gradient were collected from the top of the tube. Each fraction
932 was analyzed for its density as well as for titers of nrVL4619 using a DNase-protected qPCR
933 assay. As shown in Fig. 6B, the sample transfected with nrVL4619 tandem plasmid showed an

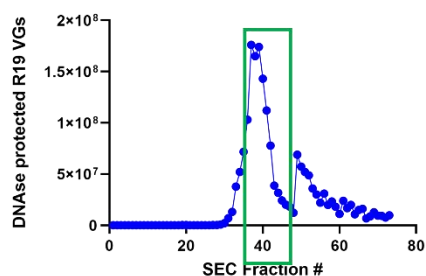
934 enrichment of viral genomes in fractions with a density of approximately 1.21 g/cm^3 , suggestive
935 of successful production of nrVL4619 in MOLT-4 cells. On the other hand, we detected
936 significantly lower to no titers of nrVL4619 in any of the linear gradient fractions tested in the
937 sample transfected with a single copy of the nrVL4619 genome.

938 To test whether we can visualize nrVL4619 virus particles, we scaled up production and
939 purification as summarized in Fig. 7A. Briefly, the processed lysates of the transfected cells were
940 subjected to a two-step purification including isopycnic centrifugation using an iodixanol linear
941 gradient followed by size exclusion chromatography (SEC). A clear peak for the nrVL4619 titer
942 was detected in SEC fractions in which anellovirus particles would be expected to migrate (Fig.
943 7B). When these fractions were pooled and concentrated for TEM analysis, we detected multiple
944 nrVL4619 particles as shown in Fig. 7C and 7D. The morphology of these nrVL4619 particles
945 was consistent with the morphology of LY2 particles (Fig. 6E).
946

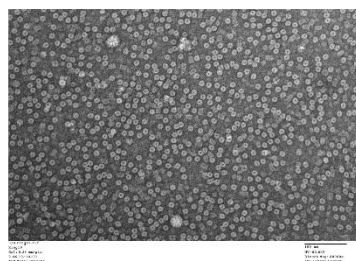
A)



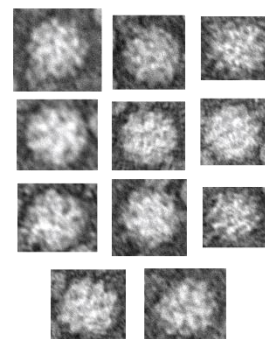
B)



C)



D)



947
948 **FIGURE 7. Electron microscopy detection of nrVL4619 particles produced in MOLT-4**
949 **cells.** A) Schematic of the production and purification of nrVL4619 particles from MOLT-4
950 cells. B) DNase-protected qPCR assay of fractions from SEC of purified nrVL4619. C and D)
951 Representative TEM images of concentrated nrVL4619 particles.

952
953 **nrVL4619 particles demonstrate infectivity *in vivo***

954
955 Since nrVL4619 genome was isolated from human retinal epithelium, we hypothesized
956 that it would have tropism for eye tissue. To examine this, we tested its infectivity and tropism in
957 the eye *in vivo*. Mice were injected either subretinally or intravitreally with PBS, purified WT
958 nrVL4619, or dose-matched AAV2.mCherry as shown (Fig. 8A). Eyes were harvested and
959 separated into the neuroretina (which contains the photoreceptor, bipolar, and ganglion cells) and
960 the posterior eye cup (PEC, which contains the retinal pigmented epithelium, choroid, and
961 sclera). DNA was harvested from these tissues followed by qPCR analysis to detect nrVL4619
962 and AAV2.mCherry genomes. nrVL4619 demonstrated infectivity in both the neuroretina and
963 PEC on Days 7 and 21 following either subretinal or intravitreal injections (Fig. 8B). nrVL4619

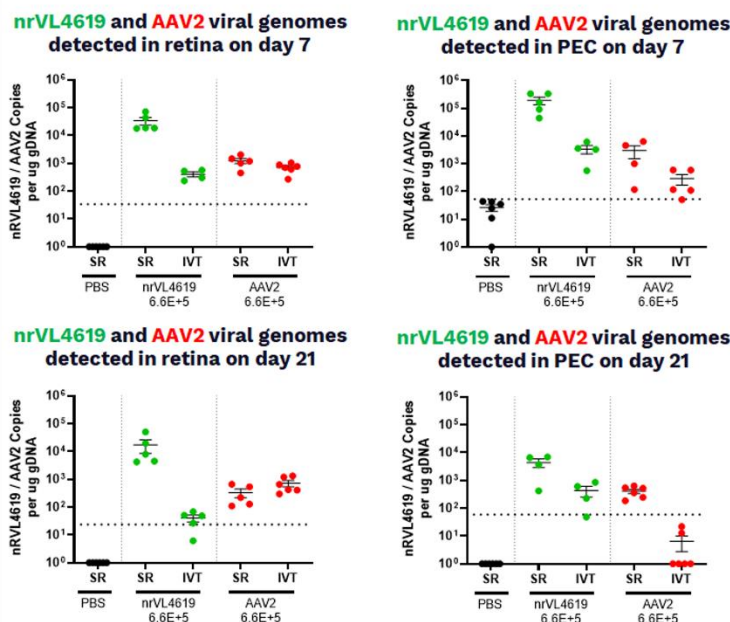
964 demonstrated superior targeting of the neuroretina and PEC following subretinal injection
 965 compared to AAV2.mCherry. This increase in infectivity was observed on both Days 7 and 21.
 966 For intravitreal injections, nrVL4619 also demonstrated superior infectivity in the PEC on Days
 967 7 and 21 compared to AAV2.mCherry. Additionally, we detected nrVL4619 infectivity in the
 968 neuroretina following intravitreal delivery on Days 7 and 21. These data suggest that nrVL4619,
 969 which was isolated from the RPE of a human donor, demonstrates superior targeting of the PEC
 970 than AAV2.
 971

A)

Group	Strain	Treatment Day 0	Dose (vg)	Route	N (eyes)/ Time point	Terminal Day
1	C57BL/6	PBS	0	SR (1ul)	5-6	7 & 21
2	C57BL/6	nrVL4619	6.6E+5	SR (1ul)	5-6	7 & 21
3	C57BL/6	AAV2-mCherry	6.6E+5	SR (1ul)	5-6	7 & 21
4	C57BL/6	nrVL4619	6.6E+5	IVT (1ul)	5-6	7 & 21
5	C57BL/6	AAV2-mCherry	6.6E+5	IVT (1ul)	5-6	7 & 21



B)



972
 973 **FIGURE 8. nrVL4619 infectivity in mouse retina and posterior eye cup (PEC).** A) Table
 974 describing various strain of mouse, treatments, virus/vector doses, routes of administration,
 975 numbers of animals per group, and time points for the *in vivo* study. Bottom panel shows a
 976 schematic of the anatomy of a mouse eye as well as study design. (B) Vector/virus genome
 977 copies present in the neuroretina or PEC, as assessed by qPCR in the harvested DNA from
 978 mouse eyes injected intravitreally (IVT) or subretinally (SR) once with either PBS, 6.6E+5 vg of
 979 Ring 19, or dose-matched AAV2.mCherry. N = 5-6 eyes/group.

980 **DISCUSSION:**

981

982 Anelloviruses are a large and diverse family of viruses comprising the majority of the human
983 virome. Anellovirus infections are acquired during infancy and detected throughout the lives of
984 healthy individuals, suggesting they have evolved to avoid clearance by and live in harmony
985 with the human immune system¹⁰. Previous independent studies as well as our own work have
986 demonstrated the recovery of anelloviral sequences from many types of human tissues and
987 biological samples³⁻⁶. In addition to evading the host immune system, anelloviruses might be
988 leveraging their diversity to achieve broad tropism for a range of tissue and cell types. The non-
989 pathogenic and weakly immunogenic nature of anelloviruses may overcome a major limitation of
990 current vectors by enabling repeat dosing of gene therapies, while specific tropism of the diverse
991 lineages of anelloviruses may enable development of an anellovirus-based gene therapy delivery
992 platform capable of addressing diseases across multiple therapeutic areas.

993

994 Understanding the basic properties of these viruses further will offer new insights into their
995 biology and unlock our ability to harness their biology for therapeutic applications. Previous
996 studies generally have been limited to the detection and sequencing of anellovirus nucleic acids
997 in blood or other specimens. We recently have reported on comprehensive efforts to use
998 genomics, computational biology, and a series of biochemical and biophysical techniques to
999 elucidate the diversity, transmission, structure, and immunogenicity of the anellovirus family.
1000 For example, we developed a proprietary AnelloScope technology to specifically enrich and
1001 recover sequences of anelloviruses from human tissue. We used this technology to demonstrate
1002 in a blood-transfusion cohort that anelloviruses can be transmitted from donors and can persist
1003 for at least nine months in recipients¹⁴. Recently, we used a phage immunoprecipitation
1004 sequencing (PhIP-Seq) assay to comprehensively profile antibody responses to the human
1005 anellome⁵². In this study, we introduce an *in vitro* system for the production of human
1006 anelloviruses, which will enable further basic research into anelloviruses, the knowledge from
1007 which can be used to develop anellovirus-based gene therapies.

1008

1009 As anelloviruses have been previously reported to reside and replicate in immune cells within the
1010 human body, we selected a T-cell-derived cell line of human origin, MOLT-4, and tested its
1011 permissiveness for the production of a *Betatorquevirus* LY2 originally described by a group in
1012 France. LY2 gene expression in MOLT-4 cells was observed, with anellovirus mRNA and
1013 protein levels peaking on the third day after transfection (Fig. 1). When MOLT-4 cells were
1014 transfected with a plasmid encoding tandem copies of the LY2 genome, replication of a unit-
1015 length, circular, double-stranded form of the viral genome was evident (Fig. 2). Notably, a
1016 plasmid encoding a single copy of the LY2 genome also can replicate in MOLT-4 cells but does
1017 not lead to virus rescue, presumably due to lack of the formation of unit-length circular genome
1018 (Fig. 2). The design of the tandem plasmid and its repetitive sequences may lead to genetic
1019 recombination and the generation of some species of the genome that are unit-length, circular,
1020 and double-stranded, which are then replicated by the virus and host replication machinery.
1021 Studies to determine the function of each of the anellovirus-encoded proteins as well as which
1022 host factors are involved in the viral life cycle represent an avenue for additional area of research
1023 that we are currently actively pursuing.

1024

1025 MOLT-4 cells transfected with the tandem plasmid or an *in vitro* circularized, double-stranded
1026 form of the LY2 genome were also permissive for the production of LY2 virus particles, as
1027 shown by peak viral titers in CsCl-based density gradient fractions (Fig. 3, Fig. 5) and reinforced
1028 by TEM analysis showing multiple particles with an icosahedral capsid (Figure 5). Our study is
1029 the first to document the production of a human anellovirus using recombinant viral DNA
1030 genome.

1031
1032 Viral production in MOLT-4 cells was not observed when either ORF1 and its isoforms or ORF2
1033 and its isoforms were knocked out via site-directed mutagenesis of the LY2 genome, implying
1034 that expression of the combination of these proteins is critical for virus production. This
1035 conclusion is supported by the observation that viral production was restored when the cells were
1036 co-transfected with the ORF1 knockout and ORF2 knockout mutant genomes (Figure 4),
1037 indicating successful trans-complementation between the ORF2 proteins expressed by the ORF1
1038 knockout and the ORF1 proteins expressed by the ORF2 knockout.

1039
1040 To assess whether other anelloviruses besides LY2 can be produced in MOLT-4 cells, we
1041 transfected MOLT-4 cells with a plasmid encoding tandem copies of nrVL4619. nrVL4619 is a
1042 separate *Betatorquevirus* that we isolated from the human retinal pigmented epithelium. Similar
1043 to LY2, we were able to detect production of nrVL4619 as assessed by isopycnic centrifugation
1044 and TEM (Fig. 6). When nrVL4619 was intravitreally or subretinally injected into mice, the
1045 anellovirus was shown to infect the retina as well as the posterior eye cup. These findings
1046 demonstrate tropism of nrVL4619 for ocular tissue and provide proof-of-concept for the use of
1047 the AnelloScope technology to discover anelloviruses with tropism for a tissue of interest.

1048
1049 In summary, we have established an *in vitro* cell-based recombinant system that is capable of
1050 producing human anelloviruses, a family of commensal viruses that are detectable in a variety of
1051 human tissues with high prevalence but have been understudied in the past. Using the MOLT-4
1052 cell line to robustly express natural or engineered anelloviruses enables comprehensive
1053 evaluation of their biophysical and biochemical properties, their infectivity *in vitro* and *in vivo*,
1054 and their immune response profile. This study also represents an important step toward the
1055 application of non-pathogenic, weakly immunogenic anelloviruses as vectors for re-dosable gene
1056 therapies targeting a wide array of diseases.

1057 1058 **ACKNOWLEDGEMENTS:**

1059
1060 This study utilized TTVS research materials obtained from the NHLBI Biological Specimen and
1061 Data Repository Information Coordinating Center and does not necessarily reflect the opinions
1062 or views of the TTVS or the NHLBI.

1063 1064 **COMPETING INTEREST STATEMENT:**

1065
1066 DMN, MT, GB, CP, EO, JY, CAA, AB, DV, PT, JC, SL, KS, HS, AM, YC, TO, NLY, RJH, and
1067 SD are employees of and hold equity interests in Ring Therapeutics. TO and RJH are affiliated
1068 with Flagship Pioneering, which also holds equity interests in Ring Therapeutics. KL, CS, NA,
1069 and FD also hold equity interests in Ring Therapeutics.

1070

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1072

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1074

1075

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