Selective inhibition of miRNA processing by a herpesvirus-encoded miRNA

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39 **Summary**

40 Herpesviruses have mastered host cell modulation and immune evasion to
41 augment productive infection, life-long latency and reactivation thereof [1,2]. A long
42 appreciated, yet elusively defined relationship exists between the lytic-latent
43 switch and viral non-coding RNAs [3,4]. Here, we identify miRNA-mediated
44 inhibition of miRNA processing as a thus far unknown cellular mechanism that
45 human herpesvirus 6A (HHV-6A) exploits to disrupt mitochondrial architecture,
46 evade intrinsic host defense and drive the lytic-latent switch. We demonstrate that
47 virus-encoded miR-aU14 selectively inhibits the processing of multiple miR-30
48 family members by direct interaction with the respective pri-miRNA hairpin
49 loops. Subsequent loss of miR-30 and activation of the miR-30/p53/Drp1 axis
50 triggers a profound disruption of mitochondrial architecture. This impairs
51 induction of type I interferons and is necessary for both productive infection and
52 virus reactivation. Ectopic expression of miR-aU14 triggered virus reactivation
53 from latency, identifying viral miR-aU14 as a readily drugable master regulator of
54 the herpesvirus lytic-latent switch. Our results show that miRNA-mediated
55 inhibition of miRNA processing represents a generalized cellular mechanism that
56 can be exploited to selectively target individual members of miRNA families. We
57 anticipate that targeting miR-aU14 provides exciting therapeutic options for
58 preventing herpesvirus reactivations in HHV-6-associated disorders.

59

60 **Main Text**

61 MicroRNAs (miRNAs) are important regulators of gene expression implicated in
62 all major cellular processes of life ranging from embryonic development to tissue
63 homeostasis and cancer [5,6]. Accordingly, their biogenesis is tightly regulated at all
64 levels [7]. Shortly after the discovery of cellular miRNAs, a number of viruses,
65 predominantly of the herpesvirus family, were identified to encode and express
66 their own set of viral miRNAs [4,8]. One of these is human herpesvirus 6A (HHV-6A),
67 which has a seroprevalence of >90% in the human population. HHV-6A
68 establishes latency by integrating into the telomeric regions of host chromosomes
69 [9]. Virus reactivation has been associated with cardiac dysfunction, graft rejection
70 as well as neuronal disorders including myalgic encephalomyelitis and chronic
71 fatigue syndrome (ME/CFS) [10]. Here, we discover miRNA-mediated inhibition of
72 miRNA processing as a thus far unknown mechanism that HHV-6A exploits to
73 disrupt mitochondrial architecture, evade the induction of type I interferons and
74 facilitate virus reactivation from latency.

75

76 **HHV-6A induces mitochondrial fission**

77 Mitochondria play a key role in the cell intrinsic defense against viruses. They
78 constantly undergo fission and fusion events that help maintain functional
79 mitochondria in cells under metabolic and environmental stress [11]. To examine
80 whether HHV-6A affects mitochondrial architecture, we infected primary human
81 umbilical vein endothelial cells (HUVEC) with wild-type HHV-6A and imaged
82 mitochondria using a constitutively expressed, mitochondrially targeted GFP
83 (mitoGFP) [12]. Lytic HHV-6A infection resulted in extensive mitochondrial
84 fragmentation by 24 hours post infection (hpi) (Fig. 1a, Extended data Fig. 1a). The

85 same was observed upon reactivation of latent HHV-6A in U2-OS cells induced by
86 Trichostatin-A (TSA) treatment (Extended data Fig. 1b). Mitochondrial fusion-
87 fission dynamics are governed by the activity of Dynamin-related protein 1 (Drp1)
88 [13]. Helical oligomers of Drp1 form a ring around the outer mitochondrial
89 membrane and fragment it [14]. Mitochondrial fragmentation was reflected by
90 increased Drp1 expression during both lytic HHV-6A infection (Fig. 1b) and virus
91 reactivation (Extended data Fig. 1c) as well as colocalization of Drp1 on
92 mitochondrial surfaces in the virus-reactivated cells (Extended data Fig. 1d). Drp1
93 levels are directly controlled at transcriptional level by the p53 tumor suppressor
94 protein [15]. Accordingly, both lytic HHV-6A infection and virus reactivation
95 exhibited increased p53 expression (Fig. 1b, Extended data Fig. 1c) indicating that
96 HHV-6A induces mitochondrial fragmentation via the canonical p53/Drp1 axis [15].
97

98 **HHV-6A inhibits miR-30 processing**

99 Human miR-30 family members regulate mitochondrial fusion and fission by
100 targeting p53 and its downstream target Drp1 [12,15]. Interestingly, Northern blots
101 revealed impaired miRNA processing of various miR-30 family members (miR-
102 30a, miR-30c, miR-30d and miR-30e) upon lytic HHV-6A infection (Fig. 1c,
103 Extended data Fig. 2a, 2b). As such, loss of miR-30c was accompanied by a
104 concomitant increase in pri-miR-30c levels (Fig. 1d) indicating that HHV-6A
105 infection affects pri-miR-30c processing. Similar results were also observed
106 during TSA-induced HHV-6A reactivation (Extended data Fig. 2c-2d).
107 Furthermore, small RNA sequencing data from HHV-6A-infected HSB-2 T-cells
108 confirmed the decrease in miR-30c-5p, miR-30e-5p levels (Extended data Fig. 2e).
109 Additionally, miR-30c-3p and miR-30e-3p levels were also decreased.

110 Quantification of mature miRNA as well as their precursors by qRT-PCR confirmed
111 the decrease in miR-30a, miR-30c and miR-30d levels despite increased levels of
112 their precursors in both lytic HHV-6A infection and reactivation (Extended data
113 Fig. 3a-3b). No significant changes were observed in the levels of the maternal NF-
114 YC transcript under both conditions (Extended data Fig. 3c-3d).
115
116 **miR-aU14 inhibits miR-30c processing**
117 Unlike lytic infection, HHV-6A reactivation in the U2-OS cell model does not
118 progress to fully productive virus replication but is restricted to the expression of
119 some viral miRNAs and mRNAs [16]. We thus asked whether any of the HHV-6A
120 miRNAs might be involved in the observed miR-30 processing defect. Manual
121 sequence inspection revealed an interesting complementarity between HHV-6A
122 miR-U14 and the hairpin loops of pre-miR-30c, pre-miR-30a and pre-miR-30d
123 (Fig. 1e, Extended data Fig. 3e). This viral miRNA is expressed at very high levels
124 during both productive infection [17] and virus reactivation [16]. Because it is encoded
125 antisense to the U14 ORF it was renamed miR-aU14. miR-30c hairpin loop showed
126 the strongest sequence complementarity to miR-aU14 (Extended data Fig. 3f-3g)
127 [18]. Small RNA sequencing of Argonaute (Ago)-bound RNA from HHV-6A-infected
128 HSB-2 cells confirmed this so far incompletely characterized viral miRNA to
129 represent one of the two most abundant viral miRNAs in HHV-6A-infected cells
130 (Extended data Fig. 4a). In addition, transcription start site profiling (TSS) using
131 differential RNA-seq (dRNA-seq; Extended data Fig. 4b) [19,20] identified the pri-
132 miR-aU14 transcript, which initiates 158 nt upstream of the miRNA consistent
133 with the unusual 130 nucleotide pre-miR-aU14 hairpin predicted by mFold [21]
134 (Extended data Fig. 4c). Of note, pre-miR-aU14 is highly conserved between HHV-

135 6A and HHV-6B with the miRNA seed region complementary to the pre-miR-30c
136 hairpin loop showing 100% conservation (Extended data Fig. 4d).
137
138 To assess the role of miR-aU14 in miR-30c processing, we employed a wild-type
139 miR-aU14 miRNA mimic (Wt mimic) and a mutant thereof (Mut mimic) (Fig. 1f).
140 Strikingly, transfection of the wild-type but not the mutant mimic into U2-OS cells
141 reproduced both the loss of mature miR-30c and the concomitant increase in pri-
142 miR-30c (Fig. 1g). To further validate that miR-aU14 was responsible for the miR-
143 30c processing defect, we generated HeLa cells with a doxycycline (dox)-inducible
144 miR-aU14 expressed from a Pol III promoter-driven shRNA and a mutant version
145 (HeLa-Mut) thereof (Extended data Fig. 5a). Dox-induced expression of miR-aU14
146 (HeLa-Wt, Extended data Fig. 5b) but not of its mutant (HeLa-Mut, Extended data
147 Fig. 5c) fully reproduced the miR-30c processing defect.
148

149 **miR-aU14 induces mitochondrial fission**

150 We next asked whether expression of miR-aU14 was sufficient to disrupt
151 mitochondrial architecture via the p53/Drp1 axis. Both transfection and dox-
152 induced expression of Wt but not Mut miR-aU14 induced p53 and Drp1 expression
153 (Extended data Fig. 5d-5e). Furthermore, transfection of the miR-aU14 Wt mimic
154 fully recapitulated the mitochondrial fission phenotype (Extended data Fig. 5f).
155 To validate this effect in the virus context, we generated a mutant virus genome
156 with discrete nucleotide substitutions within miR-aU14 (Extended data Fig. 6a).
157 Mutations were designed to not alter the amino acid sequence of the U14 ORF.
158 Deep sequencing of both the wild-type (HHV-6A-Wt) and mutant (HHV-6A-Mut)
159 bacterial artificial chromosomes (BAC) confirmed the introduced mutations being

160 the only differences between the two viral genomes. In contrast to HHV-6A-Wt,
161 we were unable to reconstitute the miR-aU14 mutant virus despite multiple
162 attempts indicating that the loss of miR-aU14 severely reduced viral fitness.
163 Hence, we generated polyclonal U2-OS cells that either carried chromosomally
164 integrated latent wild-type HHV-6A or its miR-aU14 mutant by selection of cells
165 stably transfected with the respective BACs. Upon virus reactivation with TSA,
166 HHV-6A-Wt but not HHV-6A-Mut impaired pri-miR-30c processing (Fig. 1h),
167 induced Drp1 expression (Extended data Fig. 6b) and triggered mitochondrial
168 fission (Fig. 1i, Extended data Fig. 6c).
169

170 **Mechanism of the miRNA processing defect**

171 We next tested whether miR-aU14 directly interacts with the pre-miR-30c hairpin
172 loop and thereby interferes with its processing. We consecutitively transfected
173 HeLa cells with equimolar amounts of radiolabeled synthetic miR-aU14, or two
174 mutants thereof, followed by synthetic biotinylated pre-miR-30c 16h later (Fig.
175 2a, b). Affinity purification of pre-miR-30c after 16 h revealed enrichment of wild-
176 type miR-aU14 but not of a control miRNA. Two subtle miR-aU14 mutants showed
177 an intermediate phenotype (Fig. 2c).
178 We next asked whether the presence of the pre-miR-30c hairpin loop was
179 sufficient to mediate its inhibitory effects on miRNA processing. Two artificial
180 target pre-miRNAs were designed that carried the original hairpin loop sequence
181 of pre-miR-30c but contained artificial miRNA stem duplex sequences (termed:
182 miR-A and miR-B) (Fig. 2d). We then generated polyclonal HeLa cells with dox-
183 inducible Pol-III-driven expression of miR-A or miR-B as well as stable
184 transduction with lentiviruses that expressed either wild-type or mutant miR-

185 aU14 (Extended data Fig. 5a). Consistent with the predicted interaction of miR-
186 aU14 with the pre-miR-30c hairpin loop, induction of miR-aU14, but not of the
187 mutant thereof, strongly repressed both miR-A and miR-B processing (Fig. 2e, 2f).
188 To analyze the biochemical basis of miR-aU14-mediated regulation of miR-30
189 biogenesis, we carried out *in vitro* pri- and pre-miRNA processing assays [22]. Total
190 protein lysates from cells exogenously expressing DGCR8 generated pre-miR-30c
191 from radiolabelled *in vitro* transcribed pri-miR-30c. This was inhibited by the
192 miR-aU14 mimic but not the mutant (Extended data Fig. 7a). A more pronounced
193 interference was observed for pri-miR-30a (Extended data Fig. 7b). Moreover, the
194 miR-aU14 mimic, but not the mutant, inhibited processing of radiolabelled *in vitro*
195 transcribed pre-miR-30a and pre-miR-30c by human Ago-associated Dicer
196 complex (Extended data Fig. 7c-7d). Human DGCR8 and Ago-DICER complex
197 purifications were confirmed (Extended data Fig. 7e-7f). These results show that
198 miR-aU14 can interfere with both pri-miRNA and pre-miRNA processing.
199

200 **miR-aU14 inhibits the interferon response**

201 Mitochondria play an important physiological role in intrinsic immunity [23]. Upon
202 activation of toll-like or RIG-I-like receptors, mitochondria serve as antiviral
203 signaling hubs that govern the production of type I interferons (IFNs) [24]. RNA
204 polymerase III can use cytosolic herpesvirus DNA as a template to produce 5'-
205 triphosphate RNAs, which induce type I IFN through the RIG-I pathway [24-26].
206 Enforced mitochondrial fission dampens RIG-I/MAVS signaling and reduces the
207 induction of type I interferons [27]. We thus asked whether miR-aU14-mediated
208 mitochondrial fragmentation impacts on the induction of type I IFN, interferon
209 beta (IFN-β). Exposure of HEK-293 cells, transfected with the miR-aU14 mimic, to

210 the *RIG-I* pathway activator 3p-hpRNA (5' triphosphate hairpin RNA) [28] resulted
211 in reduced mRNA levels of IFN-β (Fig. 3a) as well as the IFN-responsive IFIT1 gene
212 (Fig. 3b) in comparison to cells transfected with the miR-aU14 mutant.
213 We next asked whether miR-aU14 also plays a role in suppressing the production
214 of IFN-β upon HHV-6A reactivation. In addition to inducing virus reactivation by
215 TSA, we treated cells with the JAK/STAT inhibitor Ruxolitinib to prevent
216 secondary IFN-β mediated effects on virus reactivation. The latter was assessed
217 by Northern blot for viral miR-aU14 and sncRNA-U77 (Fig. 3c). Ruxolitinib
218 treatment enhanced TSA-induced virus reactivation resulting in a concordantly
219 greater loss of miR-30c. Expression of viral sncRNA-U77 was significantly reduced
220 for HHV-6A-Mut indicative of impaired virus reactivation. Accordingly, miR-30c
221 levels remained unchanged. Nevertheless, the mutant virus induced significantly
222 greater levels of IFN-β than the wild-type virus (Fig. 3d). Reactivation of wild-type
223 virus reduced IFIT1 mRNA levels by ~5-fold. In contrast, IFIT1 levels increased by
224 1.2-fold for HHV-6A-Mut relative to non-reactivated cells. This was partially
225 inhibited by Ruxolitinib treatment (Fig. 3e). Viral reactivation was confirmed by
226 measuring mRNA levels of the viral immediate early U90 gene (Fig. 3f).
227

228 **miR-aU14 triggers virus reactivation**

229 Considering the observed effects of miR-aU14 on the induction of type I IFNs, we
230 asked whether ectopic expression of miR-aU14 could augment productive wild-
231 type virus infection and rescue reactivation of the mutant virus. Transfection of
232 miR-aU14 mimic but not the control (miRNA) increased the number of cells
233 productively infected with wild-type virus by ~2.5-fold (Fig. 3g). Transfection of
234 miR-aU14 mimic efficiently rescued reactivation of the mutant virus even in the

235 absence of TSA (Fig. 3h). Combination of both TSA and miR-aU14 showed
236 enhanced virus reactivation indicating synergistic effects between the two.
237 Similarly, transfection of miR-aU14 was substantially more effective at inducing
238 reactivation of wild-type virus than TSA (Extended data Fig. 8a). Neither of the
239 two mutant mimics had any effect on virus reactivation (Extended data Fig. 8b).
240 We then asked whether mitochondrial fragmentation, impaired IFN response and
241 HHV-6A reactivation were indeed mediated by the effects of miR-aU14 on miR-30.
242 Both transfection of a miR-30c inhibitor and expression of a miR-30c sponge,
243 decreased mature miR-30c levels, induced p53 and Drp1 expression, and
244 triggered mitochondrial fragmentation (Extended data Fig. 9a-9c). Induction of
245 the miR-30 sponge efficiently reduced the IFN-β response, and enhanced
246 productive infection and viral spread (Extended data Fig. 9d-9f). Finally, it also
247 enhanced virus reactivation upon TSA treatment of the mutant virus (Extended
248 Data Fig. 9g).
249
250 **Targeting human miRNA processing**
251 In principle, miRNA-mediated inhibition of miRNA processing should be
252 applicable to other cellular miRNAs. This is of particular interest as many
253 important cellular miRNAs exist as miRNA families. Targeting hairpin loops rather
254 than the mature miRNA sequences would offer a unique opportunity for the
255 development of more selective miRNA inhibitors. Many of the let-7 family
256 members carry relatively large hairpin loops, which may comprise up to 30 nt.
257 Hence, we designed synthetic miRNA mimics targeting two different regions of the
258 hairpin loop of pre-let-7d (Fig. 4a). Upon transfection into cells, both miRNA
259 mimics efficiently reduced mature let-7d levels consistent with impaired miRNA

260 processing (Fig. 4b). Similar data were obtained for two other miRNA mimics
261 targeting the hairpin loop of let-7f1 (Extended data Fig. 10a-10b).
262 Finally, we speculated that miRNA-mediated inhibition of miRNA processing
263 should also be observable for cellular miRNAs. To identify such regulation, we
264 carried out a systematic blast search of mature human miRNAs against known
265 pre-miRNAs from miRBase. Several search results indicated interesting potential
266 binding sites within pre-miRNAs (Extended data Table 1). However, majority of
267 the respective miRNA pairs were not abundantly expressed in most of the
268 standard human cell lines. We thus focused on one particular candidate pair,
269 namely miR-155 and miR-148b. Our analysis indicated potential binding of miR-
270 155 to pri-miR-148b just 5' of the pre-miR-148b hairpin (Fig. 4c). Transfection of
271 a miR-155 mimic resulted in reduced levels of mature miR-148b (Fig. 4d). miR-
272 155 mimic also strongly interfered with *in vitro* processing of pri-miR-148b
273 (Extended data Fig. 10c-10d). MicroRNA-mediated inhibition of miR-148b
274 processing by cellular miR-155 thus at least partially explains the dichotomous
275 expression of these two human miRNAs [29].
276

277 **Discussion**

278 Regulation of miRNA processing by cellular proteins is well described [7]. The first
279 and best characterized example is the stem cell factor Lin-28, which interacts with
280 the hairpin loop of let-7 family members and blocks their biogenesis [30-32].
281 Recently, a large unbiased screening approach identified ~180 RNA binding
282 proteins that specifically interact with distinct human pre-miRNAs [33]. RBP-
283 mediated regulation of miRNA processing thus constitutes an important
284 regulatory network that shapes miRNA activity and function. Here, we show that

285 miRNA mimics can take over similar functions and selectively inhibit miRNA
286 processing in a sequence-specific manner (Extended data Fig. 11). Interestingly,
287 the interaction of miR-aU14 and miR-30 precursors was not mediated by its seed
288 region but nucleotides 4-9. The miR-aU14-mediated loss of miR-30c was
289 accompanied by a marked increase of pri-miR-30c levels. This implies that the
290 inhibition occurs at the level of pri-miRNA processing within the nucleus,
291 consistent with previous reports that miRNAs may affect pri-miRNA processing
292 by binding to distal sequence elements in the respective pri-miRNAs [34-36]. miR-30c
293 is encoded in an intron of the NF-YC gene [37]. Recognition and cleavage of the
294 intronic pre-miRNA hairpin loop by the RNase III Drosha thus competes with the
295 cellular splicing and RNA degradation machinery. Sterical interference of DGCR8
296 binding to the miR-30c hairpin by miR-aU14 in the nucleus and subsequent
297 degradation of the parental intron upon splicing therefore is the likely explanation
298 for the observed loss of mature miR-30c. Accordingly, inhibition of miR-148b
299 processing by the inflammatory miR-155 also appears to occur in the nucleus as
300 the respective binding site within the pri-miR-148b closely flanks the pre-miR-
301 148b stem. This implies that miR-155 binding sterically inhibits pri-miR-148b
302 cleavage by Drosha. Inhibition of miR-148b processing by miR-155 presumably
303 explains previous reports of dichotomal expression of these two important human
304 miRNAs [29].
305 Interestingly, both lytic HHV-6A infection and virus reactivation also increased
306 pre-miR-30d levels within the cell. This is consistent with data of our *in vitro*
307 processing assay, which indicated inhibition of miR-30 processing at the pre-
308 miRNA level. Accordingly, accumulation of the let-7d pre-miRNA upon exposure
309 to two different artificial miRNAs targeting the hairpin loop indicated impaired

310 pre-let-7d processing in the cytoplasm. MicroRNA-mediated inhibition of miRNA
311 processing can thus interfere with miRNA biogenesis at both pri- and pre-miRNA
312 level. Furthermore, these findings demonstrate that miRNA-mediated inhibition
313 of miRNA processing can be readily exploited to specifically target individual
314 miRNAs of large miRNA families that so far could not be individually targeted.
315
316 Viral miR-aU14-mediated inhibition of miR-30 processing explained
317 mitochondrial fragmentation during both lytic HHV-6A infection and virus
318 reactivation via the miR-30/p53/Drp1 axis. This in turn impairs the induction of
319 type I IFN and augments productive virus infection. Interestingly, multiple
320 attempts to reconstitute a miR-aU14 mutant virus from BAC DNA failed, indicating
321 that viral miR-aU14 is crucial for productive virus replication *in vitro*. Similarly,
322 the miR-aU14 mutant virus was severely impaired in its ability to reactivate from
323 latency. It is, however, important to note that reactivation of the miR-aU14 mutant
324 virus by TSA resulted in a significantly stronger type I IFN response than observed
325 for wild-type virus. This indicates that miR-aU14 may not be essential for the
326 desilencing of the latent virus genomes but rather the inhibition of intrinsic
327 cellular defense mechanisms that otherwise efficiently prevent successful virus
328 reactivation. This is in line with the gross disruption of mitochondrial architecture
329 by miR-aU14 via the miR-30/p53/Drp1 axis.
330
331 A peculiar feature of the miR-aU14 locus is that miR-aU14 is expressed antisense
332 to the U14 open-reading frame from a novel pri-miRNA transcript that initiates
333 within the 5' region of the U14 ORF. U14 encodes for a G2/M cell cycle checkpoint
334 regulator of HHV-6 [38], which also interacts with p53 [39]. While miR-aU14 thus has

335 the potential to repress expression of the important U14 protein, mutational
336 analysis indicates that both U14 and miR-aU14 are important for productive HHV-
337 6A infection. Accordingly, transient transfection of miR-aU14 enhanced
338 productive HHV-6A infection and rescued virus reactivation of a miR-aU14
339 mutant virus. The most striking finding, however, was that transfection of miR-
340 aU14 triggered virus reactivation from latency at least as efficiently as the
341 commonly employed histone deacetylase inhibitor TSA. While enhanced
342 mitochondrial fission and impaired intrinsic immunity via the miR-30/p53/Drp1
343 axis augments successful virus reactivation, miR-aU14 may also target other
344 cellular or viral genes that help trigger virus reactivation from latency.

345

346 In summary, our findings reveal a surprising miRNA-mediated mechanism that a
347 prevalent human herpesvirus usurped to interfere with intrinsic immunity,
348 govern the lytic-latent switch and augment productive infection. Importantly,
349 viral miR-aU14 should be readily drugable using antisense approaches
350 (antagomiRs) [40], thereby providing an interesting therapeutic option for
351 preventing herpesvirus reactivation.

352

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Author Contribution:

BKP and LD conceived the idea, developed and supervised the project, and wrote the manuscript. BKP carried out majority of experiments supported by TH and ABP; TH and JT, carried out interferon assays; ABP designed expression constructs

496 and carried out miRNA quantification assays; FK developed mutant virus carrying
497 cells; AE carried out miRNA mimic Northerrn blots; AG carried out
498 Immunoblotting experiments, AWW contributed to miRNA intearaction studies;
499 ML, carried out FACS analysis; CJ, SH carried out dRNA-seq experiment and data
500 analysis; BBK, designed and developed the HHV-6A mutant BAC; TK, MS carried
501 out SIM microscopy and analyzed the SIM data; FE, analyzed the sequencing data;
502 GM, UF, TR contributed essential reagents and analyzed the data. All the authors
503 contributed to manuscript writing.
504

505 **Competing Interests:**

506 The authors declare that they have no competing interests.
507
508

509
510 **Figure legends**

511 **Fig. 1: HHV-6A infection and reactivation induce mitochondrial fission via**
512 **the p53/Drp1 axis.**

513 **a**. Structured illumination microscopy (SIM) images of primary HUVEC cells
514 infected with HHV-6A or mock virus. Magnified images of mitochondria are shown
515 within insets. Mitochondrial numbers and average surface area are presented as
516 scatter plots. n=3. *P = 0.03, ***P = 0.0006.

517 **b**. p53 and Drp1 protein during mock (-HHV-6A) or virus (+HHV-6A) infection.
518 HHV-6A infection was tested by viral glycoprotein gp82/105. GAPDH served as
519 loading control. n=3. Fold change in p53 and Drp1 proteins are shown as mean of
520 three replicates. *P = 0.03.

521 **c.** Mature miR-30 during lytic HHV-6A infection. HHV-6A infection was tested by
522 viral miR-aU14 and sncRNA-U77. n=3.

523 **d.** miR-30c processing defect during lytic HHV-6A infection. n=6. **P = 0.009.

524 **e.** Schematic of putative interaction of miR-aU14 with pre-miR-30c hairpin loop.
525 Predicted sites of interaction are in grey boxes. Mature miR-30c is shown red.

526 **f.** Nucleotide sequences of wild-type (Wt mimic) and mutant (Mut mimic) miR-
527 aU14 mimics. Point mutations are highlighted within red boxes.

528 **g.** Pri-miR-30c processing defect by wild-type or mutant miR-aU14 mimic. n=2.

529 **h.** Pri-miR-30c processing defect during reactivation of wild-type HHV-6A (HHV-
530 6A-Wt) or mutant virus (HHV-6A-Mut). U2-OS cells without HHV-6A served as
531 mock. n=3. ****P ≤ 0.0001.

532 **i.** Average mitochondrial area in wild-type or mutant HHV-6A reactivating cells as
533 a box and whisker plot. *P = 0.02. Data from 3 independent experiments with at

534 least 2 images from each experiment. Box and whiskers (a, i) show minimum to
535 maximum values with all independent replicates, centre denotes median, and the
536 bounds denote the 25th to 75th percentiles.
537 RNA and protein quantification was done by densitometric analysis (b, c, d, g, h).
538 Human U6 served as loading control in Northern blots (c, d, g, h). Data are mean ±
539 SD. Unpaired two-tailed non-parametric t-test (a, b, d, h, i). The uncropped blots
540 are provided in Supplementary Fig. 1.
541
542 **Fig. 2: HHV-6A miR-aU14 inhibits miR-30 processing by direct RNA:RNA**
543 **interaction**
544 **a**. Schematic representation of streptavidin affinity purification for biotinylated
545 pre-miRNA.
546 **b**. Nucleotide sequences of the wild-type miR-aU14 mimic (Wt mimic) and the
547 mutant mimics (Mut and Mut2 mimic). Mutated sequences are in blue within the
548 rectangular boxes.
549 **c**. Affinity purification of biotinylated pre-miR-30c co-precipitates miR-aU14 as in
550 Fig. 2a. A random small RNA was used as a bait for control. Total RNA from input
551 control cells were run in parallel to control for transfection efficiency. n=3.
552 **d.** Putative interactions of the two artificial chimeric pre-miRNAs (pre-miR-A and
553 pre-miR-B) with miR-aU14. The mature miRNA sequences (miR-A and miR-B) are
554 indicated in red. The putative interactions of the transplanted miR-30c hairpin
555 loop with miR-aU14 (blue) are indicated.
556 **e, f**. miR-aU14 impairs processing of artificial miRNAs carrying miR-30c hairpin
557 loop. Polyclonal HeLa cells stably transduced for dox-inducible expression of miR-
558 A or miR-B were re-transduced either with wild-type miR-aU14 (HeLa-Wt),

559 mutant miR-aU14 (HeLa-Mut) or with an empty vector (HeLa-Mock). Total RNA
560 was used in Northern blotting. n=3. *P = 0.04 in 2e, *P = 0.04 in 2f.
561 Human U6 served as loading control in Northern blots (e, f). Data are mean ± SD.
562 Unpaired two-tailed non-parametric t-test (e, f). The uncropped blots are
563 provided in Supplementary Fig. 1.
564
565 **Fig 3: miR-aU14 induced mitochondrial fragmentation suppresses the**
566 **induction of IFN-β.**
567 **a**. Relative IFN-β mRNA levels in hpRNA-stimulated HEK-293 cells in presence of
568 either the miR-aU14 mimic (Wt) or a mutant mimic (Mut) as compared to
569 unstimulated cells. qRT-PCR values were normalized to 5S RNA. n=5. *P = 0.01,
570 **P = 0.002.
571 **b**. Relative IFIT1 mRNA levels quantified as in Fig. 3a.
572 **c**. Pri-miR-30c processing defect upon reactivation of wild-type HHV-6A (HHV-6A-
573 Wt) or the miR-aU14 mutant (HHV-6A-Mut) by TSA alone or together with
574 Ruxolitinib. U2-OS cells without HHV-6A served as mock control. Virus
575 reactivation was studied by measuring the expression of miR-aU14 and sncRNA-
576 U77. n=3. *P <0.04.
577 **d**. Relative IFN-β mRNA levels from similar experiments as in Fig. 3c. n=2.
578 **e**. Relative IFIT1 mRNA levels from similar experiments as in Fig. 3c. n=5. *P =
579 0.03, ***P = 0.0008.
580 **f**. Relative HHV-6 U90 mRNA levels from similar experiments as in Fig. 3c. n=4.
581 **g.** Lytic virus infection in presence of miR-aU14. HSB-2 cells were transfected with
582 either the miR-aU14 mimic or a control mimic. HHV-6A mCherry reporter virus

583 was used to measure cell to cell spread of virus infection, as measured by flow
584 cytometry. n=5. SSC, side scatter. *P = 0.01.
585 **h.** U2-OS cells carrying latent mutant HHV-6A miR-aU14 (HHV-6A-Mut) were
586 transfected with either the miR-aU14 mimic or a control mimic. Cells were treated
587 with TSA to induce virus reactivation, which was analyzed by studying viral miR-
588 U2 and miR-U86 expression, which are both not expressed during virus latency.
589 miR-U86 levels normalized to human U6 are presented as a bar diagram. n=3. **P
590 < 0.008.
591 Data are mean ± SD. Unpaired two-tailed non-parametric t-test (a, b, c, d, e, f, g, h).
592 The uncropped blots are provided in Supplementary Fig. 1. Gating Strategy for
593 Fig.3g is provided in Supplementary Fig. 2.
594
595 **Fig. 4: Human miRNA processing can be selectively inhibited by synthetic**
596 **small RNAs.**
597 **a.** Schematic of putative binding of two different synthetic miRNAs to human pre-
598 let-7d.
599 **b**. let-7d expression in U2-OS cells transfected with miRNA mimics designed
600 against the hairpin loop of pre-let-7d were analyzed by Northern blotting.
601 Precursor/mature let-7d ratios, as determined by densitometry analysis, are
602 shown as bar diagram. n=3. *P = 0.02.
603 **c**. Schematic of the putative binding of miR-155 to pri-miR-148b. Possible
604 sequence interactions between the two RNAs are highlighted in grey boxes.
605 **d**. miR-148b expression in presence of a synthetic miR-155 mimic.
606 Primary/mature miR-148b, as determined by densitometry analysis, are shown
607 as bar diagram. n=4. **P = 0.002.

608 Human U6 served as loading control in Northern blots (b, d). Data are mean ± SD.
609 Unpaired two-tailed non-parametric t-test (b, d). The uncropped blots are
610 provided in Supplementary Fig. 1.
611
612
613 # Online-only Methods

614 **Cell culture**

615 U2-OS (HTB-96), HeLa (CCL-2) and HUVEC (PCS-100-010) cells were purchased
616 from ATCC and cultured in DMEM medium supplemented with 10% (v/v) FBS and
617 200 units/ml penicillin-streptomycin [41,42]. HSB-2 T cells were obtained from HHV-
618 6 Foundation, USA and were maintained in RPMI 1640 media [43]. All cell lines were
619 cultured at 37 °C with 5% CO_2. HEK-293 cells were purchased from ATCC (CRL-
620 1573) and were grown in DMEM medium supplemented with 10% non-heat
621 inactivated FBS and 200 units/ml penicillin-streptomycin. Cells carrying stable
622 GFP expression within mitochondria were developed as mentioned before [12]. Cells
623 stably expressing the mitochondrial targeted GFP were created by cloning the
624 mitochondrial targeted GFP into pLVTHM vector (Supplementary Table S1)
625 backbone and transducing target cells with the lentivirus.
626

627 **Lentivirus generation and cell transduction**

628 The miR-30c sponge was constructed by cloning the necessary miR-30c sponge
629 oligo (Supplementary Table S2) into the pLVTHM vector. For mitochondrial GFP-
630 expressing vectors the original GFP cassette of pLVTHM was replaced with a
631 mitochondria targeted GFP [12]. All the constructs were verified by sequencing. The
632 miR-30c sponge lentivirus were produced in HEK-293 cells as described

633 previously [44,45]. Purified lentiviruses were transduced into target cells in presence
634 of the cationic polymer, Polybrene (Sigma-Aldrich).
635
636 **HHV-6A virus propagation, BAC reconstitution, virus reactivation**
637 HHV-6A virus was grown in HSB-2 cells. For infecting new cells and propagating
638 virus, 10^6 HHV-6A-infected cells were mixed with uninfected HSB-2 cells,
639 pretreated with 2 ng/ml interleukin-2 (IL-2; Sigma) and 5 μg/ml
640 phytohemagglutinin (PHA; Sigma), at a ratio of 10:1 that allowed HHV-6A to infect
641 new cells through cell-to-cell fusion. When more than 80% of cells showed
642 cytopathic effects visible under light microscope, infected cells together with the
643 media were collected to prepare cell-free virus. Infected cells were lysed by
644 repeated freeze-thaw cycles for 3 times in liquid nitrogen. Lysed cells with culture
645 media were centrifuged at 3,500 g at 4 °C for 1 h. The cleared supernatant was
646 filtered through a 0.45 μm filter. Virus particles were pelleted by centrifugation at
647 25,000 g at 4 °C for 3 h. Virus pellet was resuspended in cold IMDM media without
648 antibiotics and frozen at –80 °C until further use. The HHV-6A titers expressed as
649 the 50% tissue culture infective dose (TCID50) were determined by infecting
650 fresh HSB-2 cells at different dilutions and scoring the number of infected cells
651 that exhibited cytopathic effects or by immunostaining the infected cells using an
652 anti-p41 or anti-gB antibody. Viral titers and TCID50 values were calculated using
653 Reed-Münch formula.
654 *E. coli* carrying HHV-6A BAC was obtained from Prof. Yashuko Mori, Japan. Virus
655 reconstitution from BACs was performed as described [17,46]. For generating cells
656 that carried latent HHV-6A genomes, U2-OS cells were transfected with 5 μg of
657 wild-type HHV-6A or mutant BACs using TransIT-X2 transfection reagent (Mirus

658 Bio LLC). Alternatively, the Amaxa 4D nucleofector transfection system was used.
659 48 h after transfection, cells were sorted for green fluorescent protein (GFP)-
660 positive cells and grown until cells turned GFP negative indicative of the
661 establishment of latent infection. HHV-6A genome copy numbers were quantified
662 in each clonal population of cells by qPCR. U2-OS cells carrying latent HHV-6A
663 were tested for virus reactivation by adding Trichostatin A (TSA) to the cell
664 culture medium for 24-48 h. Cells that reactivated efficiently were used for further
665 studies. TSA at a concentration of 80 ng/ml (Sigma, Cat no. T8552)[41] was used for
666 virus reactivation. Primers used for the mutagenesis study are listed in the
667 Supplementary Table 2.
668
669 **Generation of recombinant viruses**
670 U14 mutant viruses were generated using pHHV-6A, an infectious BAC clone of
671 HHV-6A (strain U1102) [46], expressing GFP under the control of the CMV IE
672 promoter, using two-step Red-mediated mutagenesis as described previously [46-
673 49]. Primers used for the mutagenesis are listed in the Supplementary Table 2.
674 Recombinant BAC clones were confirmed by restriction fragment length
675 polymorphism (RFLP), PCR and Sanger DNA sequencing. In addition, Illumina
676 MiSeq sequencing was carried out with ~100-fold coverage to exclude unexpected
677 mutations within the entire viral genome. Reconstitution of both wild-type and
678 miR-aU14 mutant viruses was carried out by nucleofection of BAC DNA into Jjhan
679 cells. The expression of eGFP from the viral backbone allowed direct monitoring
680 of the virus reconstitution process.
681
682 **Average mitochondrial surface area and mitochondrial number analysis**

683 Software and modified algorithm for mitochondrial size and number
684 measurement were previously described by us in detail [12]. All image-processing
685 and analysis steps were performed using Fiji [50]. The numbers of Drp1 fission rings
686 were quantified by processing the confocal micrographs with the Fiji Object
687 Counter plugin after appropriate thresholding. Background was subtracted using
688 the rolling ball background subtraction model. A threshold for detecting Drp1
689 aggregates was applied to the original micrographs by measuring the mean pixel
690 intensity of the control samples in the Drp1 channel and normalizing the pixel
691 intensity of all other samples to this constant. Additionally, several hundred
692 individual Drp1 rings were measured using the Profile function of the ZEN 2012
693 image-processing platform and profile plot plugin (Plot Profile) from Fiji to
694 determine the maximum and minimum ring diameters of constricted and dilated
695 Drp1 rings. Based on this step, the threshold of object detection by the Object
696 Profile algorithm was set to include Drp1 particles that exhibited a diameter
697 between 100 (constricted) and 360 nm [51-54]. Mitochondrial fission sites were
698 defined by profiling regions of low mitochondrial intensity and high Drp1 signals
699 along a linear path through mitochondrial fragments using the Plot Profile plugin
700 from Fiji.
701 The average surface area of mitochondrial fragments was measured by a further
702 modification of the Object Count plugin from Fiji. In brief, while keeping an equal
703 threshold for all images, mitochondrial GFP fluorescence was converted to binary
704 signals, and the algorithm was allowed to numerically categorize the
705 mitochondria as a continuous network or individual fragment and finally
706 determine the area covered by the mitochondrial fragments in micrometers
707 squared. The area was divided by a factor of 0.39 nm and the mean width of

708 HUVEC mitochondria determined by measuring ~300 individual HUVEC
709 mitochondrial fragments using the Profile function of ZEN 2012. Similarly, the
710 average surface area of HeLa mitochondrial fragments was determined by
711 dividing the area by a factor of 0.5 nm. The mitochondrial fragments with no
712 visible connections with neighboring networks were assigned as individual
713 fragments.
714 Drp1 colocalization with the mitochondria was determined using the COLOC2
715 plugin from Fiji. The degree of colocalization was ascertained using Pearson's
716 colocalization coefficient followed by statistical analysis.
717

718 **Immunofluorescence Microscopy**

719 A detailed protocol for standard immunofluorescence microscopy is previously
720 described [55]. The HHV-6A P41 monoclonal antibody (clone 9A5D12) was obtained
721 from Santacruz Biotechnology (SC-65447) (Supplementary Table 3).
722

723 **Immunoblotting**

724 Immunoblotting was carried out as described before [55,56] using rabbit polyclonal
725 anti-Drp1 antibody as well as monoclonal mouse antibodies against p53 and Drp1
726 (Supplementary Table 3). Equal protein loading was confirmed by using
727 antibodies against β-actin or GAPDH. The mouse monoclonal antibody against
728 HHV-6 gp82/105 was a kind gift from HHV-6 Foundation, USA [57]. All the primary
729 antibodies were used at a dilution of 1:1000 and the HRP-conjugated secondary
730 antibodies were used at a dilution of 1:10,000.
731

732 **Structured illumination microscopy (SIM)**

733 Structured Illumination Microscopy (SIM) was performed on the ELYRA S.1
734 system from ZEISS with a Plan-Apochromat 63x/1.40 oil immersion objective.
735 Laser lines 488 nm, 561 nm and 642 nm were used as excitation source with
736 respective filter sets for GFP, mCherry and ATTO643 to separate the fluorescence
737 light. 25 raw images (five rotations with five phases each) were recorded for
738 reconstruction of one final SIM image with the exception of Extended data Figure
739 5f (15 raw images with three rotations). Data processing was done with ZEN
740 software (ZEN 2012 SP5 FP3, ZEISS), including channel alignment by affine
741 transformation for correction of chromatic aberrations using embedded
742 TetraSpeck beads (ThermoFisher, T14792). Brightness and contrast of SIM
743 images were adjusted linearly.

744

745 **Polyacrylamide gel electrophoresis (PAGE) and Northern blotting**

746 Total RNA extraction for Northern blotting was carried out using TRI-reagent with
747 minor modifications. 20 mM $MgCl_2$ was added to the TRI-reagent solubilized
748 lysate before chloroform addition [58]. All the Northern blotting experiments for
749 small RNAs were carried out using 12.5% denaturing Urea-PAGE gels containing
750 0.5X Tris-Borate-EDTA buffer (TBE). Gels were pre-ran for 1 hr at 15 mAmp
751 before sample loading. For every gel, equal amounts of total RNA samples were
752 mixed with 2X RNA loading dye (NEB, B0363S) and were denatured at 95 °C for 3
753 minutes before loading into the gel. 16 X 16 cm gels were used for better
754 separation of primary, precursor and mature miRNAs. After gel running, RNA was
755 transferred to positively charged Nylon membrane using 0.5XTBE and wet
756 transfer apparatus (Biometra Tankblot Eco-Line, Analytic Jena). Membranes were
757 UV-cross linked and pre-hybridized for 1 h before addition of denatured probes.

758 Hybridization was carried out for 16-20 h using hybridization buffer (7% SDS and
759 3% 20XSSC). Membranes were washed three times for 15 min each at
760 hybridization temperature using wash buffer (1.5% 20XSSC, 0.1% SDS).
761 Membranes were exposed to X-ray films along with an intensifying screen at -80
762 °C for 1-6 days depending upon targets miRNA amounts. Probes for the detection
763 of human miRNAs were developed based on the sequences from miRBase. LNA
764 probes against human miRNA miR-148b, miR-155, Let-7d, Let-7f, miR-30a, miR-
765 30c and miR-30d and miR-30e and U6 were purchased from Exiqon (Qiagen)
766 (Supplementary Table S2). LNA probes were designed against HHV-6A miR-aU14
767 and were synthesized from Exiqon (Qiagen). RNA oligo mimics for miR-aU14 and
768 their mutant counterparts were synthesized from IDT DNA Technologies. miR-
769 155 mimics and inhibitors were purchased from Exiqon (Qiagen). Radiolabeled
770 Decade Marker (Thermo Scientific, AM7778) was used in some of the Northern
771 gels as well as gels for *in vitro* miRNA processing assay.
772

773 **Generation of inducible miR-aU14 cells**

774 For generating miR-aU14 (both wild-type and mutant) expression vectors based
775 on the pLVTHM backbone (Supplementary Table 1), oligos were synthesized from
776 Sigma. Details of oligo sequences are included in the Supplementary Table 2.
777 Detailed methodology for cell line generation is previously described [12]. Briefly,
778 hairpin loop sequences were designed for the respective miRNA sequences,
779 synthesized (Sigma), annealed and cloned into the pLVTHM [45] using MluI and ClaI
780 sites downstream of H1 promoter sequences. Large-scale preparation of DNA
781 required for lentivirus particle formation was carried out using the MACHEREY-
782 NAGEL endotoxin-free plasmid midi prep system. Third-generation lentiviruses

783 carrying pseudotyped VSV were generated by Ca_3PO_4 transfection into HEK-293
784 cells, as previously described [59]. Briefly, supernatants containing lentiviruses
785 were collected, passed through a 0.45 µm filter, and concentrated by
786 ultracentrifugation. Concentrated virus particles were used to infect target cells.
787 After establishment of stable cells carrying the integrated lentiviruses, single cell
788 clones were obtained by cell sorting using FACS.
789

790 **Effects of miR-aU14 on the induction of interferon beta (IFN-β) in HEK-293**
791 **cells**

792 5 x 10^4 HEK-293 cells were seeded per well. After one day of incubation, cells were
793 transfected with the synthetic RNA oligos (250 nM) using TransIT-X2. 48 h later,
794 cells were transfected with 0,5 µg/ml 3P-hpRNA. For mock transfection, cells were
795 treated with only transfection reagent without DNA or miRNA mimic unless
796 indicated otherwise. After 24 h, cells were harvested using 300 µl lysis buffer from
797 the QuickRNA MicroPrep kit (Zymo Research, Germany). Total RNA was extracted
798 according to the manufacturer's instructions. RNA concentrations were measured
799 by NanoDrop (Thermo Scientific). 500 ng total RNA was used for cDNA synthesis
800 with the 5x qRT SuperMix (Bimake). cDNA was then diluted 1:10 and used for RT-
801 qPCR. 2x SYBR Green SuperMix (Bimake) was used for RT-qPCR. The data was
802 evaluated using the $\Delta\Delta C_q$ method. Data were normalized with housekeeping Beta
803 actin mRNAs.
804

805 **Quantitative real time PCR (qRT-PCR) for viral genes**

806 For viral RNA analysis, total cellular RNA extraction was carried out using the
807 Direct-zol RNA purification kit (Zymo Research, Germany) or TRI reagent (Sigma).

808 cDNA synthesis was carried out using the Maxima First Strand cDNA Synthesis kit
809 (Thermo Scientific). qRT-PCRs were performed using the PerfeCTa qPCR
810 SuperMix (Quanta Biosciences) on a ROCHE LightCycler-96 system (Roche Life
811 Sciences, Germany) using SYBR green chemistry. The primer sequences used for
812 HHV-6A p41, U90 are described before [41] and can be found in the Supplementary
813 Table 2. NF-YC mRNA levels were quantified using TaqMan primer based
814 commercial assays (Supplementary Table 2) and were normalized with
815 housekeeping Beta actin mRNAs. NF-YC PCR was carried out in 20 μl reaction
816 using TaqMan fast Advanced master mix (Thermo Scientific, 4444557) and
817 QuantStudio 5 real time PCR machine following manufacturer's instructions.
818

819 **Sequencing of small RNAs**

820 To generate small RNA sequencing libraries, total cellular RNA was isolated using
821 Qiagen miRNeasy kit. Small RNA libraries were prepared using the CleanTag
822 Ligation kit (TriLink BioTechnologies) starting with 1μg of total RNA per library.
823 For quality control, all final libraries were analyzed by Bioanalyzer to check for
824 the expected miRNA fragment peak at 141 bp. Subsequently, a Pippin Prep
825 instrument (Sage Science) was used to isolate fragments between 10 and 35 bp in
826 size. Library concentrations were measured by PicoGreen on an infinite F200
827 instrument (Tecan). Libraries were sequenced on a NextSeq 500 platform
828 (Illumina) using high-output v2 kits with 75 cycles aiming for ~40 million single-
829 ended reads per sample.
830

831 **Analysis of the small RNA sequencing data**

832 Reads that passed the chastity filter on the NextSeq 500 were subject to de-
833 multiplexing and trimming of TriLink adapter residuals using Illumina's bcl2fastq
834 v2 software (v2.19.1). Quality of reads was checked with FastQC software
835 (v0.11.5). Reads shorter than 18 bases were discarded. All reads were mapped to
836 combined index of the human genome (HG38) and the HHV-6A genome (X83413),
837 with the trailing repeat region masked by N. Read mapping was performed using
838 STAR (v2.5.3a) using the parameters suggested by the Encode project for sRNA-
839 seq. Reads were then annotated to mature miRNAs if 5' ends perfectly matched
840 (miRBase 22.1). The raw counts were then normalized and analyzed for
841 differential miRNA expression with the DESeq2 (v1.18.1) package. Statistically
842 significantly differentially regulated genes were called by a Wald test for the
843 interaction of the presence of HHV-6A and activation by TSA with false discovery
844 rate (FDR) 1% (Benjamini-Hochberg corrected p-value).
845
846 **miR-aU14:pre-miR-30c interaction assays**
847 For transfection of radiolabeled miRNA mimics, equimolar amounts of the miR-
848 aU14 mimic and its mutant counterparts were end-labeled separately. After
849 purification through Sephadex G25 column, radioactive counts were measured.
850 Biotinylated synthetic pre-miR-30c-1 was purchased from IDT DNA technologies.
851 For initial experiments, equal counts of each mimic were mixed with biotinylated
852 pre-miR-30c-1 and transfected into HeLa cells using TransIT-X2 reagent.
853 Subsequently, HeLa cells were first transfected with biotinylated pre-miR-30c-1
854 for 16 h and then cells were washed 2 times with 1XPBS followed by transfection
855 of radiolabeled mimics. 16 h post-transfection, total protein lysates were prepared
856 using RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100,

857 1% NP-40, 0.1% SDS, 10% Glycerol and protease inhibitor cocktail (Roche) and
858 10 units/ml RNAse inhibitor (NEB, M0314L). Native cell lysates were incubated
859 with Magnetic Streptavidin beads for 1 h at 4 ^{o}C. Beads were subsequently washed,
860 and bead-bound RNA was eluted in 2X RNA loading dye (Invitrogen). Northern
861 hybridization was carried out to detect bead-bound biotinylated pre-miR-30c-1
862 and miR-aU14. A control bait RNA was used in parallel to check non-specific
863 binding of RNA to beads.

864

865 **Generation of artificial pre-miRNA carrying the miR-30c-1 hairpin loop**

866 Pre-miRNA hairpin of the artificial miR-A or miR-B was expressed in HeLa cells
867 using the pLVTHM lentiviral vector. Two fully artificial hairpin loops were
868 designed for this purpose, which carried a hairpin loop identical to the one in pre-
869 miR-30c (miR-A and miR-B). Oligo sequences for creating the lentiviral vectors
870 are included within the Supplementary Table 2. miRNA expression was induced
871 by three days of doxycycline (1 mg/ml) treatment. Total RNA was isolated using
872 either TRI reagent (Sigma) or miRNeasy kit (Qiagen) and was subject to Northern
873 blotting.

874

875 **Flow cytometry analysis of HHV-6A infected cells**

876 HSB-2 cells were stimulated with 2 ng/ml interleukin-2 (IL-2; Sigma) and 5 mg/ml
877 phytohemagglutinin (PHA; Sigma) for 24 h. Subsequently, pre-stimulated HSB-2
878 cells were washed twice with PBS and seeded in 6-well plates. Cells were
879 transfected with miR-aU14 mimic, miR-30c mimic or control mimics (1-5 nM)
880 using TransIT-X2 (Mirus Bio LLC) transfection reagent. In parallel, cells infected
881 with an HHV-6A mCherry reporter virus were grown in separate plates. 1 day after

882 transfection of miR-aU14, cells were washed, counted and mixed with mCherry
883 reporter virus infected HSB-2 cells at a ratio of 100:1. Infection was allowed to
884 proceed for 2 days. Afterwards, cells were washed two times with PBS, fixed with
885 2% paraformaldehyde (PFA) for 30 min at room temperature. Cells were washed
886 once again with PBS and resuspended in FACS buffer (1X PBS with 0.5% BSA and
887 0.02% sodium azide), passed through a cell sieve and analyzed by Flow cytometry.
888
889 Flow cytometry was performed using the BD Biosciences FACS Calibur and BD
890 CellQuest Pro software. Analysis was performed using FlowJo 10. Briefly, live cells
891 were gated first by SSC vs FSC dot plot analysis followed by visualization of
892 mCherry-positive population using the FL-3 channel (635 nm Red diode laser and
893 670LP filter). The mCherry-positive population was visualized in the third
894 quadrant (Q3), after gating with appropriate experimental controls. Considering
895 the very low rate of cell-to-cell HHV-6A transmission, 60,000 live cell events were
896 collected for each sample.
897

898 **Transcription start site profiling**

899 Transcription start site profiling using differential RNA-seq (dRNA-seq) was
900 performed according to the published protocol [19,20] with some modifications by
901 the Core Unit Systems Medicine (Würzburg). In brief, total RNA was extracted
902 from HHV-6A and mock infected HSB-2 cells. For each sample 3 µg of DNase-
903 digested RNA was treated with T4 Polynucleotide Kinase (NEB) for 1 h at 37 °C.
904 RNA was purified with Oligo Clean & Concentrator columns (Zymo) and each
905 sample was split into an Xrn1 (+Xrn1) and a mock (−Xrn1) sample. The samples
906 were treated with 1.5 U Xrn1 (NEB; +Xrn1) or water (−Xrn1) for 1 h at 37 °C.

907 Digestion efficiency was checked on a 2100 Bioanalyzer (Agilent) and 5′ caps were
908 removed by incubation with 20 U of RppH (NEB) for 1 h at 37 °C. Afterwards, RNA
909 was purified and eluted in 7 µl of nuclease free water and 6 µl was used as input
910 material for the NEBNext Multiplex Small RNA Library Prep Set for Illumina
911 sequencing. Library preparation was performed according to the manufacturer's
912 instruction with the following modifications: 3′ adapter, SR RT primer and 5′
913 adapter were diluted 1:2, 13 cycles of PCR were performed with 30 sec of
914 elongation time. No size selection was performed at the end of library preparation.
915 Concentrations of libraries were determined using the Qubit 3.0 (Thermo
916 Scientific) and their fragment sizes were determined using the Bioanalyzer.
917 Libraries were pooled at equimolar concentrations. Sequencing of 75 bp single-
918 ended reads was performed on a NextSeq 500 (Illumina) at the Core Unit Systems
919 Medicine in Würzburg (dRNA-seq). Reads were mapped using the same settings
920 as for miRNA sequencing. TSS were called using iTiSS version 1.2 [60].
921

922 **Ago immunoprecipitation for miRNA enrichment and sequencing**

923 Ago immunoprecipitation was carried out using a previously published protocol
924 [61] with minor modifications. 5 million HSB-2 cells were infected with HHV-6A for
925 3 days as described before. Afterwards, native cell lysates were prepared from
926 mock-infected and HHV-6A infected cells using lysis buffer (50 mM Hepes, pH 7,5;
927 150 mM NaCl; 0.5% NP-40; 1 mM NaF; 10% Glycerol, 2.5 mM $MgCl_2$; protease
928 inhibitor cocktail (Roche), 0.5 mM DTT; 10 units/ml RNAse inhibitor (NEB,
929 M0314L)). Anti-flag magnetic beads were washed with PBS and were incubated
930 with TNR6B-flag peptide [61] overnight at 4 °C on a head-over-tail rotor. Beads were
931 washed once with PBS and once with lysis buffer. Afterwards, half of the beads

932 were incubated with cell lysates overnight at 4 °C on a head-over-tail rotor. The
933 remaining beads were stored at 4 °C. The day after, beads were separated from
934 the lysate on a magnet. Collected cell lysates were then mixed with the remaining
935 TNR6B-flag beads and were allowed to incubate overnight at 4 °C on a head-over-
936 tail rotor for further protein-antibody binding. Collected beads were washed 4
937 times with wash buffer (50 mM Hepes, pH 7,5; 150 mM NaCl; 1 mM NaF; 10%
938 Glycerol, 2.5 mM $MgCl_2$; protease inhibitor cocktail (Roche), 0.5 mM DTT; 10
939 units/ml RNAse inhibitor (NEB, M0314L)). Finally, beads were resuspended in
940 PBS containing 0.5 mM $MgCl_2$. A small fraction of these beads was used for
941 denatured protein lysate preparation (eluate 1) for immunoblotting. The
942 remaining beads were lysed in TRI-reagent and used for RNA isolation.
943 Next day, re-incubated beads were processed as described above to obtain the
944 second eluate (eluate 2) and the second batch of Ago-bound RNA. Total RNA from
945 both batches were pooled together and processed for miRNA sequencing. miRNA
946 sequencing was carried out at CeGaT GmbH, Tübingen using NEXTflex Small RNA-
947 seq kit v3 (Bioo Scientific, Germany).
948

949 **miRNA *In vitro* processing assay**

950 miRNA *in vitro* processing assay was carried out following a protocol from Narry
951 Kim lab [22] with some minor modifications. For generation of *in vitro* transcribed
952 radiolabeled pri-miRNAs, respective DNA sequences flanking 100 nt on both 5'
953 and 3' side to pre-miRNA (from miRbase 22.1) were cloned into pcDNA3 vector in
954 front of T7 promoter. A XhoI restriction site was included immediately at the 3'
955 end of the pri-miRNA sequence. Primer sequence details for generation of pri-
956 miR-30a, -30c, -30d, -148b are provided in Supplementary Table 2. For generation

957 of *in vitro* transcribed radiolabeled pre-miRNAs, primers were designed to allow
958 direct *in vitro* transcription from a T7 promoter (Supplementary Table 2). An
959 additional 'G' nucleotide was added at 5' end wherever necessary to allow efficient
960 *in vitro* transcription (IVT) of pre-miRNAs. Pri-miRNA carrying plasmids were
961 linearized with XhoI and purified before *in vitro* transcription. For pre-miRNA
962 transcripts, T7-carrying primers were used to amplify pre-miRNA sequences
963 using 2X Phusion High-Fidelity PCR Master Mix with HF buffer (Thermo Scientific,
964 F531L) and were agarose gel purified before use in IVT reaction. 1 μg of linearized
965 plasmid or 30 ng purified pre-miRNA PCR product was used for each 30 μl of IVT
966 reaction. Each IVT reaction was carried out using 4U/μl of RNAsin, 0.3 U/μl of T7-
967 RNA polymerase, 1 mM each of G, A and C, 0.2 mM U, 3 μCi/μl of α-32P UTP. After
968 4 h of IVT reaction at 37 °C, reaction was stopped by addition of 30 μl of 2X RNA
969 loading dye. Samples were denatured at 95 °C for 5 minutes and were loaded to a
970 4% Urea-PAGE gel (for pri-miRNA) or a 6% Urea-PAGE gel (for pre-miRNA) and
971 were allowed to be separated for 2-3 h. Gels were wrapped in plastic wrap and
972 were exposed to X-ray films for 2-3 min. After careful alignment with the X-ray
973 films, gel bands carrying the radiolabeled pri-miRNA or pre-miRNA transcripts
974 were cut out of the gel and were eluted overnight in AES buffer (Acetate-EDTA-
975 SDS buffer) at 4 °C. RNA was precipitated with 100% ethanol in presence of 15 μg
976 of Glycoblue (Thermo Scientific, AM9515) as a coprecipitant and was washed in
977 80% Ethanol, air dried and was dissolved in DEPC water. Purified IVT transcripts
978 were aliquoted and stored at -80 °C till further use.

979 For pri-miRNA processing assay, HEK-293 cells were transfected with either a
980 mock or flag-DGCR8 constructs (20 μg/15 cm plate) (Supplementary Table 1) 2

981 days prior to the experiments. Additionally, Flag-tagged Drosha construct (2
982 μg/15 cm plate) were also transfected together with flag-DGCR8 for better protein
983 expression and purification. For pre-miRNA processing assay, HeLa cells were
984 seeded to be used for Ago Immunoprecipitation. On the day of experiment, cells
985 were scraped out, washed in cold 1XPBS and were lysed in 20 mM Tris-HCl (pH
986 8.0), 100 mM KCl, and 0.2 mM EDTA. Cell lysate was sonicated for three cycles
987 pulsed at 50% duty cycle for 30 sec and incubated on ice for 30 sec. Cell lysate was
988 centrifuged at 16000g, 4 °C and the purified lysate was collected for further use.
989 HeLa cells were also processed similarly. After removing an aliquot of total lysates,
990 Flag-magnetic beads were added to the lysate (20 μl slurry) and were incubated
991 on a head to tail rotor for 1h at 4 °C. For Ago immunoprecipitation, Flag-magnetic
992 beads were incubated with TNR6B peptides beforehand and then bead-bound
993 TNR6B peptides were then added to the total lysate. As a mock, empty Flag-
994 magnetic beads were added to total HeLa lysate and were processed similarly.
995 After 1 h incubation, beads were washed 5 times with lysis buffer. Bead bound
996 proteins were stored on ice and were used in *in vitro* processing assay within 3 h.
997 An aliquot of total lysate and bead-bound proteins were collected in 1X Laemmli
998 buffer for Immunoblotting.

999 For *in vitro* processing assay, 30 μl reaction was set up containing 6.4 mM $MgCl_2$,
1000 10^4 cpm of radiolabeled pri-miRNA transcript or pre-miRNA transcript, 1U/μl of
1001 RNAsin, 15 μl of total protein lysate or bead-bound IP proteins. To test effects of
1002 the miR-aU14 mimics on *in vitro* miRNA processing, equimolar amounts of mimics
1003 were included in the reaction before addition of the protein lysate. After 90 min of
1004 incubation at 37 °C, reaction was stopped by adding 1ml of Tri reagent. RNA was

1005 purified in presence of Glycoblue and was finally dissolved in 15 ul of 2X RNA
1006 loading dye. RNA samples were denatured and loaded onto a 12.5% Urea-PAGE
1007 gel for separation together with RNA decade marker. Gel was wrapped in plastic
1008 wrap and was directly exposed to X-ray films at -80 °C for few hours to overnight.
1009

1010 **TaqMan Real time PCR assay for pri-/pre-miRNA and mature miRNA**

1011 1–10 ng of total RNA per reaction was used for cDNA synthesis for mature miRNA
1012 using TaqMan Advanced miRNA cDNA synthesis kit (Thermo Scientific, A28007),
1013 which involved 4 steps: Poly(A) tailing, Adaptor ligation, Reverse transcription
1014 and Amplification (miR-Amp). cDNA synthesis was carried out using
1015 manufacturer's instructions. For pri-/pre-miRNA assay and NF-YC mRNA
1016 quantification, 1 μg of total RNA was used for cDNA synthesis using Maxima H-
1017 minus cDNA synthesis kit (Thermo Scientific, K1651). cDNA was stored at -80 °C
1018 till further use. Ready-made commercial TaqMan PCR primers were purchased
1019 from Thermo Scientific (Supplementary Table 2) and PCR was carried out in 20 μl
1020 reaction using TaqMan fast Advanced master mix (Thermo Scientific, 4444557)
1021 and QuantStudio 5 real time PCR machine following manufacturer's instructions.
1022 Data was evaluated using the $\Delta\Delta C_q$ method. Cq values for mature miRNA
1023 amplification were normalized with U6 snRNA. Cq values for pri-/pre-miRNAs
1024 were normalized with beta actin. Furthermore, Cq values of virus infected samples
1025 were normalized against the same from Mock infected samples and Cq values of
1026 TSA treated samples were normalized against Cq values of Mock (vehicle control)
1027 treated samples for final figures.
1028

1029 **Statistics**

1030 All statistical calculations were performed using GraphPad Prism 9.0. Error bars
1031 displayed on graphs represent the means ± SD of three or more independent
1032 replicates of an experiment. Statistical significance was calculated separately for
1033 each experiment and are described within individual figure legends. For image
1034 analysis, six or more biological replicates per sample-condition were used to
1035 generate the represented data. qPCR and qRT-PCR data are representative of 2-4
1036 independent experiments. The results were considered significant at $P \leq 0.05$.
1037

1038 **Reporting summary**

1039 Further information on research design is available in the Nature Research
1040 Reporting Summary linked to this paper.
1041

1042 **Materials availability:**

1043 Further information and requests for resources and reagents should be directed
1044 to Bhupesh K Prusty (bhupesh.prusty@uni-wuerzburg.de) and Lars Dölken
1045 (lars.doelken@uni-wuerzburg.de).
1046

1047 **Data availability:**

1048 The sequencing datasets produced in this study are deposited at GEO with the
1049 accession number GSE179867. BAC sequencing results are deposited at
1050 BioProject database with the BioProject ID PRJNA792929. Raw experimental data
1051 are deposited to Mendley (doi:10.17632/grnz4krxp2.3).
1052
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1054

1055

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1140
1141 **Legends for Extended Data Figures:**

1142 **Extended data Fig. 1: HHV-6A reactivation induces mitochondrial fission via**
1143 **the p53/Drp1 axis.**

1144 **a.** Mitochondria in lytic HHV-6A infected primary HUVEC cells expressing soluble
1145 GFP within mitochondrial lumen (mitoGFP). Expression of HHV-6 p41 protein was
1146 tested for virus infection. Representative images from 3 independent
1147 experiments. n=3. **b.** HHV-6A reactivation in U2-OS cells expressing mitoGFP and
1148 carrying latent HHV-6A genome was stimulated for 24 or 48 h using trichostatin
1149 A (TSA) (T) or DMSO as solvent control (C). Cells were either fixed after one (C1
1150 or T1) or two (C2 or T2) days of treatment and studied using confocal microscopy.
1151 Representative confocal images of cells two days post treatment are shown.

1152 Mitochondrial number and average surface area as quantified from confocal
1153 images are presented as box and whiskers plot. Immunostaining against HHV-6
1154 p41 protein was carried out to identify virus reactivation. n= 9 images per
1155 conditions. **P = 0.005, ****P < 0.001. **c.** Virus reactivation in U2-OS cells with or
1156 without latent HHV-6A was induced for 48 h using TSA (T) or solvent control (C).
1157 Changes in Drp1 and p53 protein expression were quantified using densitometric
1158 analysis of immunoblots. GAPDH normalized Drp1 protein levels are presented as
1159 bar diagram. n=3 per condition. *P = 0.04. **d.** Structured illumination microscopy
1160 (SIM) was used to quantify Drp1 rings around mitochondria. Cells from the above
1161 experiment were processed for SIM after Drp1 immunostaining. Enlarged Drp1
1162 rings are shown within insets. Drp1 rings per cell together with Pearson's overlap
1163 co-efficient were quantified from the obtained images. *P = 0.01, **P = 0.005. Box
1164 and whisker plots (b, d) show minimum to maximum values with all independent
1165 replicates, centre denotes median, and the bounds denote the 25th to 75th
1166 percentiles. Data are mean ± SD. Two-tailed non-parametric t-test (b-d). The
1167 uncropped blots are provided in Supplementary Fig. 1.
1168
1169 **Extended data Fig. 2: HHV-6A lytic infection and reactivation impairs miR-**
1170 **30 processing.**
1171 **a.** HSB-2 cells were infected for 48 h with HHV-6A for lytic virus infection. Total
1172 RNA from mock- and HHV-6A-infected cells were analyzed by Northern blotting.
1173 n=3. **b.** Experiment similar to 'a' from three independent replicate experiments
1174 are shown. RNA decade marker (DM) and in vitro transcribed (IVT) RNA (300 nt)
1175 were run as size marker. **c.** HHV-6A reactivation in U2-OS cells carrying latent
1176 HHV-6A was induced for 48 h using TSA. Cells without HHV-6A served as mock.

1177 The pri-miR-30c to mature miR-30c ratios, as determined by densitometric
1178 analysis, are shown as bar diagrams. n=3. *P = 0.04. **d.** Virus reactivation of U2-OS
1179 cells carrying latent HHV-6A was induced for 48 h using TSA. RNA from three
1180 independent replicates are shown. Cells without HHV-6A served as mock. Blots for
1181 human U6, miR-aU14 and sncRNA-U77 are shown only once as the same blot was
1182 stripped and used for reprobing against multiple miR-30 family members. All the
1183 images shown are from a single gel. HHV-6 miR-aU14 and sncRNA-U77 were
1184 probed to confirm virus reactivation. A probe against human U6 was used as
1185 loading control. n=3. **e.** MA plot showing normalized log2 fold changes in miR-30
1186 family members from HSB-2 cells. Upper panel shows read counts of major 5'
1187 miRNAs. The lower panel shows read counts of minor miR-30 members 3'
1188 miRNAs. n=3. A prominent band of 80-90 nt was detected on miR-30d blots using
1189 LNA probes and are marked as ? as no known miR-30d products are known to
1190 have such sizes. Data are mean ± SD. two-tailed non-parametric t-test (c). The
1191 uncropped blots are provided in Supplementary Fig. 1.
1192

1193 **Extended data Fig. 3: HHV-6A infection and impairment of miR-30**
1194 **biogenesis.**

1195 **a.** HHV-6A lytic infection impairs miR-30 biogenesis. HSB-2 cells were infected for
1196 48 h with HHV-6A for lytic virus infection. Total RNA from mock- and HHV-6A-
1197 infected cells were analyzed for pri-miRNA/pre-miRNA and mature miRNA
1198 expression by qRT-PCR using TaqMan primers. n=5 independent experiments. *P
1199 < 0.05, ***P < 0.004. **b.** HHV-6A reactivation impairs miR-30 biogenesis. HHV-6A
1200 reactivation in U2-OS cells carrying latent HHV-6A was induced for 48 h using TSA.
1201 Solvent control (DMSO) treated cells served as mock. n=3 independent

1202 experiments. *P < 0.04, **P < 0.08, ***P < 0.0001. pri-/pre-miR values represents
1203 the sum of primary (pri-) and precursor (pre-) miRNAs as TaqMan qPCR primers
1204 can't differentiate between the two miRNA intermediates. **c** and **d.** Human NF-YC
1205 mRNA levels were studied in the same samples as in Fig. a and b respectively. Cq
1206 values for mature miRNA amplification were normalized with U6 snRNA. Cq
1207 values for pri-/pre-miRNAs were normalized with beta actin. Furthermore, fold
1208 change values of virus infected samples were normalized against the same from
1209 mock infected samples and TSA treated samples were normalized against mock
1210 (solvent control) treated samples. NF-YC Cq values were normalized aginst beta
1211 actin. Data are mean ± SD. Two-tailed non-parametric t-test (a-d).
1212 **e.** Schematic of potential binding site of miR-aU14 in the hairpin loop of different
1213 miR-30 family members. Sequence complementarity between miR-aU14 and pre-
1214 miR-30a, pre-miR-30d. Potential RNA-RNA interaction regions are highlighted in
1215 grey. Mature miR-30 sequences are indicated in red. miR-aU14 is shown in blue. **f,**
1216 **g.** Two different possible conformations (f and g) for the interaction between miR-
1217 aU14 and pre-miR-30c were predicted by VfoldCPX 2D RNA analysis. Free binding
1218 energies for respective conformations as predicted by the software are indicated.
1219

1220 **Extended data Fig. 4: Characterization of HHV-6A encoded miR-aU14.**
1221 **a.** Identification of Ago-bound viral miRNAs during lytic HHV-6A infection. HSB-2
1222 cells were infected for 72 h with wild-type HHV-6A followed by Ago
1223 immunoprecipitation and small RNA sequencing. n=2 independent experiments.
1224 **b.** dRNA-seq normalized read counts from HSB-2 cells infected with HHV-6A (n=2)
1225 are shown for both the sense (upper panel) and antisense (lower panel) strand.
1226 Location of miR-aU14 and U14 ORF as well as the identified transcription start

1227 sites (TSS) of the U14 mRNA (highlighted as ‘A’ in blue) and of pri-miR-aU14
1228 (highlighted as ‘B’ in red) are indicated against the GenBank accession number
1229 X83413.2. TSS identified pri-miR-aU14 to initiate 158 nt upstream of miR-aU14
1230 within the first third of the U14 ORF. The TSS that we identified for the U14 mRNA
1231 indicates that translation of the U14 ORF only initiates at a second AUG (indicated)
1232 that is located 15 nt downstream of the reference AUG. **c.** Predicted folding of pre-
1233 miR-aU14. The mature miR-aU14 sequence is highlighted in red. **d.** Putative miR-
1234 aU14 sequences from two different strains of HHV-6A (HHV-6A U1102, GenBank
1235 X83413.2; HHV-6A GS, GenBank KC465951.1) and one strain of HHV-6B (HHV-6B
1236 Z29, GenBank AF157706.1) were aligned. Sequence homology of mature miR-
1237 aU14 region is highlighted with red rectangles.
1238

1239 **Extended data Fig. 5: Viral inhibition of miR-30c processing triggers**
1240 **mitochondrial fragmentation.**
1241 **a.** Doxycycline-(Dox)-inducible wild-type and mutant miR-aU14 shRNAs were
1242 expressed in HeLa cells under control of a dox-inducible Pol III promoter. Mature
1243 miR-aU14 sequence are indicated in red. Point mutations are highlighted in blue
1244 within a black rectangle. **b.** HeLa cells were stably transduced to express dox-
1245 inducible miR-aU14. miR-30c and miR-aU14 expression was probed by Northern
1246 blotting. Probe against human U6 served as loading control. Ratio of pri-miR-
1247 30c/miR-30c, as determined by densitometric quantification, are shown. n=3. *P
1248 = 0.04 **c.** Polyclonal HeLa cells stably transduced to express either wild-type
1249 (HeLa-Wt1, 2 and 3) or mutant (HeLa-Mut1 and 2) miR-aU14 were induced. miR-
1250 30c and miR-aU14 expression was probed by Northern blotting. **d.** miR-aU14
1251 mimic induces Drp1 protein expression. Total protein lysates from cells

1252 transfected with either a control mimic, miR-aU14 mimic (Wt mimic), or the
1253 mutant miR-aU14 mimic (Mut mimic) were analysed by immunoblotting for Drp1
1254 and p53 protein levels. *P = 0.04. **e.** Total protein lysates from polyclonal HeLa
1255 cells with dox-inducible wild-type (HeLa-Wt1) or mutant miR-aU14 (HeLa-Mut1)
1256 were subjected to immunoblot analysis. Drp1 protein levels were quantified by
1257 densitometric analysis and were normalized against GAPDH. *P = 0.01. **f.**
1258 Mitochondrial architecture was studied in U2-OS cells transfected either with
1259 miR-aU14 mimic (Wt mimic) or the mutant miR-aU14 mimic (Mut mimic). In
1260 upper panel, fixed cells were used for confocal microscopy. n=4. In lower panel,
1261 SIM images co-stained with Drp1 are shown. Average mitochondrial area and
1262 mitochondrial numbers per cell graphs were generated from confocal images.
1263 Drp1 aggregates per cell and associated Pearson's overlap co-efficient were
1264 generated from SIM images. *P < 0.04. Magnified areas of mitochondria are shown
1265 within insets. Box and whisker plots (f) show minimum to maximum values with
1266 all independent replicates, centre denotes median, and the bounds denote the 25th
1267 to 75th percentiles. Data are mean ± SD. Two-tailed non-parametric t-test (b, d, e,
1268 f). The uncropped blots are provided in Supplementary Fig. 1.
1269

1270 **Extended data Fig. 6: Characterization of miR-aU14 mutant HHV-6A virus.**
1271 **a.** The miR-aU14 sequence was altered in the HHV-6A BAC without disturbing the
1272 amino acid (aa) sequences of the U14 ORF on the opposing strand. Sequence
1273 information at the site of mutation is shown. **b.** HHV-6A reactivation of U2-OS cells
1274 carrying latent wild-type (HHV-6A-Wt) or miR-aU14 mutant (HHV-6A-Mut) HHV-
1275 6A genomes was stimulated for 48 h using TSA. U2-OS cells without latent HHV-
1276 6A served as mock. Total protein lysates were subjected to immunoblot analysis.

1277 GAPDH was used as loading control. Normalized fold change in Drp1 protein levels
1278 are presented as a bar diagram. n=2. **c.** Mitochondrial architecture in wild-type or
1279 mutant HHV-6A reactivating cells (green arrowheads) carrying stable RFP within
1280 mitochondria (mitoRFP). The BAC-derived HHV-6A backbone carries a GFP
1281 expression cassette facilitating expression of GFP upon virus reactivation. DAPI
1282 served as counterstaining in confocal imaging. n=3 independent experiments with
1283 at least 2 images per condition. Data are mean ± SD. two-tailed non-parametric t-
1284 test (b). The uncropped blots are provided in Supplementary Fig. 1.
1285
1286
1287 **Extended data Fig. 7: miR-aU14 mimic interferes with *in vitro* processing of**
1288 **pri-miR-30 and pre-miR-30.**
1289 **a.** *In vitro* pri-miR-30c processing is inhibited by wild-type miR-aU14 mimic. HEK-
1290 293 cells were transfected with Flag-DGCR8 or a mock construct. Total cell lysate
1291 from both mock and Flag-DGCR8 expressing cells were used to process *in vitro*
1292 transcribed radiolabelled pri-miR-30c. Immunoprecipitated Flag-DGCR8 protein
1293 complex or mock IPs lysates were used in parallel. Either wild-type miR-aU14
1294 mimic or a mutant mimic (Mut2 mimic) was used in the reaction to see their effect
1295 on miRNA biogenesis. **b.** Similar assays were carried out using *in vitro* transcribed
1296 pri-miR-30a. Immunoprecipitated human Ago protein complex was added to the
1297 reaction separately to check if it can help miR-aU14 to bind to pri-miR-30a and
1298 interfere with DGCR8 binding. **c.** miR-aU14 was able to interfere with pre-miR-30c
1299 processing by Ago:Dicer complex. **d.** miR-aU14 was able to interfere with pre-miR-
1300 30a processing by Ago:Dicer complex. **e.** Flag-DGCR8 immunoprecipitation was
1301 verified by immunoblotting. **f.** Ago immunoprecipitation was verified by

immunoblotting. DM, Decade RNA marker. n=2 (a-f). Same IP lysates were used for both replicates. The uncropped blots are provided in Supplementary Fig. 1.

Extended data Fig. 8: miR-aU14 regulates virus lytic/latent switch.

a. U2-OS cells carrying wild-type HHV-6A miR-aU14 (HHV-6A-Wt) were transfected with either the miR-aU14 mimic or a control mimic. Cells were induced with TSA and the extent of virus reactivation was analyzed by Northern blotting for viral miR-U2, miR-U86 and sncRNA-U77. miR-30c levels were checked for validating effects of miR-aU14 mimic on miR-30c processing. Human U6 normalized miR-U86 levels from six independent experiments are presented in a bar diagram. n=6 independent experiments. *P < 0.02. **b.** U2-OS cells carrying latent wild-type HHV-6A were transfected with miR-aU14 mimic or the two mutant miRNA mimics. Cells were induced with TSA and the extent of virus reactivation was analyzed by Northern blotting for viral miR-U86, miR-U2. n=2. A probe against human U6 was used as loading control. Data are mean ± SD. Two-tailed non-parametric t-test (a). The uncropped blots are provided in Supplementary Fig. 1.

Extended data Fig. 9: miR-30c inhibition induces p53/Drp1 axis and allows better HHV-6A growth.

a. miR-30c sponge efficiently downregulates miR-30c. U2-OS cells were exposed to miR-30c inhibitor or miR-30c sponge lentivirus. 3 days later miR-30c levels were analyzed by Northern blotting. n=3. **b.** Both miR-30c sponge and 30c inhibitor induces p53/Drp1 axis. U2-OS cells were exposed to miR-30c sponge

1327 lentivirus or miR-30c inhibitor in presence of mock or p53 siRNA. 3 days later, p53
1328 and Drp1 levels were studied by immunoblotting. GAPDH was used as a loading
1329 control. Drp1 levels are shown as a scatter plot. n=3. *P = 0.04. **c.** Mitochondrial
1330 architecture was compared in U2-OS cells in response to miR-aU14 Wt mimic or
1331 miR-30c sponge or miR-30c inhibitor. Average mitochondrial area are presented
1332 as a scatter plot. Magnified images of mitochondria are shown within insets. *P =
1333 0.01. **d.** miR-30c sponge decreases Interferon beta (IFN-β) response. U2-OS cells
1334 transduced with miR-30c sponge were transfected with hpRNA and the induced
1335 IFN-β response was analyzed after normalization against IFN-β mRNA in cells
1336 without hpRNA transfection. *P = 0.03. **e.** Effect of miR-30c sponge and HHV-6A
1337 infection on miR-30c biogenesis. HEK-293 cells were transduced with miR-30c
1338 sponge and were infected with cell free HHV-6A at MOI of 5 for 48 h. Total RNA
1339 was used for Northern blotting. U6 was used as loading control. n=2. **f.** Effect of
1340 miR-30c mimic on HHV-6A lytic infection. HSB-2 cells were transfected with either
1341 a control or the miR-aU14 mimic (Wt mimic) together with miR-30c mimic. HHV-
1342 6A mCherry reporter virus was used to measure cell to cell spread of virus
1343 infection by flow cytometry. n=2. *P = 0.03. **g.** miR-30c sponge alone reactivates
1344 mutant HHV-6A. U2-OS cells carrying no virus (mock) or miR-aU14 mutant HHV-
1345 6A (HHV-6A-Mut) are transduced with miR-30c sponge lentivirus with or without
1346 TSA. Virus reactivation was assessed by measuring virus sncRNA-U77. n=1. Data
1347 are mean ± SD. Two-tailed non-parametric t-test (b, c, d, f). The uncropped blots
1348 are provided in Supplementary Fig. 1. Gating Strategy for Extended data Fig.9f is
1349 provided in Supplementary Fig. 2.

1350

1351 **Extended data Fig. 10: Human miRNA processing can be selectively inhibited**
1352 **by synthetic small RNAs.**
1353 **a.** Schematic of putative binding of two different synthetic miRNAs to human pre-
1354 let-7f1. **b.** Targeting the hairpin loop of pre-let-7f1 with synthetic miRNAs
1355 interferes with miRNA processing. Two different miRNA mimics designed against
1356 the hairpin loop of pre-let-7f1 or control mimic were transfected into U2-OS cells.
1357 let-7d miRNA levels were quantified by Northern blotting. A probe against human
1358 U6 was used as loading control. Pre-let-7f1 / let-7f1 ratio, as determined by
1359 densitometric analysis, are shown in the bar diagram. n=4 independent replicates.
1360 Data are mean ± SD. *P = 0.03, two-tailed non-parametric t-test (b). **c.** Sequence
1361 details of miR-155 mimic and the mutant miR-155. Altered sequences are
1362 highlighted within a yellow box and are marked in red font. **d.** *In vitro* processing
1363 of pri-148b is inhibited by wild-type miR-155. HEK-293 cells were transfected
1364 with flag-DGCR8 or a mock construct. Total cell lysate from both mock and flag-
1365 DGCR8 expressing cells were used to process in vitro transcribed radiolabelled
1366 pri-miR-148b. Immunoprecipitated flag-DGCR8 protein complex or mock IPs
1367 lysates were used in parallel. Either wild-type miR-155 mimic (155 mimic) or a
1368 mutant mimic (155 mut mimic) was used in the reaction to see it's effect on miRNA
1369 biogenesis. n=2 independent experiments. DM, Decade RNA marker. Predicted
1370 pre-miR-148b size is 99 nt, which was barely detectable in our assay.
1371 Interestingly, in this assay, the miR-155 mimic resulted in the appearance of an
1372 alternative processing intermediate of about 100 nt, which matched the 99 nt
1373 predicted miR-148b pre-miRNA included in miR-Base. While this 99 nt processing
1374 intermediate does not match to the actual cleavage site of mature miR-148b, this
1375 finding provides additional evidence that miR-155 inhibits pri-miR-155

1376 processing resulting in alternative pri-miR-148b processing. The uncropped blots
1377 are provided in Supplementary Fig. 1.
1378

1379 **Extended data Fig. 11: Graphical abstract of regulation of miRNA-mediated**
1380 **miRNA processing.**
1381 Schematic of regulation of miRNA processing by other miRNAs.
1382
1383

1384 **Extended Data Table 1:**
1385 List of human miRNAs that can potentially bind to other human pre-miRNAs
1386 either within the hairpin loop or within the stem loop region.
1387
1388