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1	Human Cytomegalovirus Immediate-Early 1 Protein Causes Loss of SOX2
2	from Neural Progenitor Cells by Trapping Unphosphorylated STAT3 in the
3	Nucleus
4	Short title: IE1-STAT3 Mediates SOX2 Depletion from NPC
5	
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#### 28 ABSTRACT (141 words)

29

The mechanisms underlying neurodevelopmental damage caused by virus infections remain 30 31 poorly defined. Congenital human cytomegalovirus (HCMV) infection is the leading cause of fetal brain development disorders. Previous work has linked HCMV to perturbations of neural 32 cell fate, including premature differentiation of neural progenitor cells (NPCs). Here we show 33 34 that HCMV infection of NPCs results in the loss of the SOX2 protein, a key pluripotency-35 associated transcription factor. SOX2 depletion maps to the HCMV major immediate-early (IE) transcription unit and is individually mediated by the IE1 and IE2 proteins. IE1 causes 36 SOX2 down-regulation by promoting the nuclear accumulation and inhibiting the 37 phosphorylation of STAT3, a transcriptional activator of SOX2 expression. Deranged 38 39 signaling resulting in depletion of a critical stem cell protein is an unanticipated mechanism by which the viral major IE proteins may contribute to brain development disorders caused by 40 41 congenital HCMV infection.

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## 42 **IMPORTANCE (110 words)**

43

Human cytomegalovirus (HCMV) infections are a leading cause of brain damage, hearing 44 45 loss and other neurological disabilities in children. We report that the HCMV proteins known as IE1 and IE2 target expression of human SOX2, a central pluripotency-associated 46 transcription factor that governs neural progenitor cell (NPC) fate and is required for normal 47 48 brain development. Both during HCMV infection and when expressed alone, IE1 causes the loss of SOX2 from NPCs. IE1 mediates SOX2 depletion by targeting STAT3, a critical 49 upstream regulator of SOX2 expression. Our findings reveal an unanticipated mechanism by 50 which a common virus may cause damage to the developing nervous system and suggest 51 novel targets for medical intervention. 52

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#### 53 INTRODUCTION

54

Congenital human cytomegalovirus (HCMV) infection is the leading cause of birth defects 55 worldwide. Approximately 1% of live newborns are infected in utero with this virus. At the 56 time of birth, 5 to 10% of HCMV-infected newborn infants will exhibit signs of neurological 57 damage, such as microcephaly, cerebral calcification and other abnormal findings (1-3). 58 59 Among infected newborns that have no symptoms at birth, 10 to 15% subsequently develop central nervous system (CNS) disorders, including sensorineural hearing loss, mental 60 retardation and learning disability (4-6). In addition, some authors have suggested that autism, 61 language disorders and other more subtle changes in brain development might be related to 62 congenital HCMV infection (7-9). 63

Although this virus can infect a wide range of organs in vivo, the fetal brain is regarded 64 the principal target of HCMV infection that results in neurological manifestations (10-12). 65 Due to exquisite host-specific tropism of this virus and the lack of animal models that 66 67 faithfully recapitulate major characteristics of human infection, the pathogenesis of HCMV-68 associated disease in the developing CNS is largely unknown. However, recent advances in human neural progenitor cell (NPC) isolation and culture have provided an opportunity to 69 70 study HCMV infection in a cell system relevant to fetal neuropathogenesis. Our previous studies and the work of others have shown that human NPCs are susceptible to HCMV 71 infection and fully permissive to viral replication (13-19). HCMV infection of NPCs affects 72 the cell fate by causing premature differentiation (14, 17, 18). Whole-genome analysis 73 74 demonstrated that HCMV infection modulates the expression of NPC markers (14, 19) 75 including sex-determining region Y (SRY)-box 2 (SOX2), a core transcriptional factor for stem cell self-renewal and pluripotency. 76

SOX2 is widely expressed in early neuroectoderm and neural progenitor cells during 77 78 development (14, 19, 20) as well as in neural stem cells in the adult brain (20, 21). SOX2 missense or heterozygous loss-of-function mutations have been identified to cause ocular 79 malformations, often manifesting as anophthalmia, microphthalmia, or coloboma. These 80 symptoms may be accompanied by hearing loss, learning disability, or brain malformation 81 (22-24). Familial recurrence of SOX2-associated anophthalmia has been observed (24). 82 83 Moreover, the level of SOX2 expression plays an important role in sensory organ, including inner ear and retina, development. Groundbreaking research has demonstrated that forced 84 SOX2 expression in fibroblasts, with or without additional factors, can generate induced 85 86 pluripotent stem cells (25-27). In fact, ectopic SOX2 expression directs reprogramming of fibroblasts into neural stem or precursor cells (25, 28-30). SOX2 is also critical for 87 maintenance of embryonic stem cells (ESCs). The SOX2 levels in ESCs are tightly regulated 88 89 (31) and even small changes in expression can lead to differentiation (32, 33).

STAT3 is a member of the signal transducer and activator of transcription family (34) 90 91 and is expressed in an activated form in the developing CNS as early as during initial NPC proliferation. This protein plays a dichotomous regulatory role in neuro- and gliogenesis (35, 92 36). STAT3 is activated through phosphorylation of tyrosine 705 (Y705) by receptor-93 94 associated kinases in response to various growth factors and cytokines including interleukin 6 (IL-6). Tyrosine phosphorylation leads to the nuclear accumulation of STAT3 homodimers, 95 which act as DNA binding transcriptional activators of numerous target genes including 96 97 SOX2. In ESCs that are differentiated into NPCs, STAT3 promotes cell fate commitment by activating the SOX2 promoter (37). 98

99 Here we investigate the effects of HCMV infection on SOX2 expression in human 100 NPCs. We demonstrate that the HCMV 72-kDa immediate-early 1 (IE1) protein down-101 regulates SOX2 transcription and mediates depletion of the SOX2 protein from HCMV-

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- 102 infected NPCs. IE1 exerts its effect on SOX2 expression by inactivating the upstream
- 103 regulator STAT3.

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#### 104 **RESULTS**

105

HCMV infection of NPCs causes down-regulation of SOX2 mRNA and protein. Previous 106 work has established that human NPCs are fully permissive to HCMV infection (15, 38). As 107 early as 4 h post infection (hpi), a significant decrease of SOX2 mRNA was observed in 108 NPCs, and this decrease became more apparent as infection progressed (Fig 1A). In contrast, 109 110 SOX2 protein levels did not change in these cells at early post-infection times (up to 12 hpi), but a gradual decrease was observed at late times (from 24 hpi) compared with mock-infected 111 cells (Fig 1B). By 48 hpi, the SOX2 protein was clearly down-regulated, and by 96 hpi it was 112 undetectable. The HCMV proteins IE1/IE2, UL44 and gB were also analyzed in this 113 experiment to monitor IE, early, and late viral gene expression, respectively. 114

In addition, the change in SOX2 protein levels was examined in relation to the 115 formation and development of HCMV intranuclear replication compartments visualized via 116 immunofluorescence staining of UL44 (Fig 1C). UL44 is the processivity factor of the 117 118 HCMV DNA polymerase and is first expressed in the early phase of infection. Initially, UL44 was evenly distributed across the infected nucleus, then it formed multiple small foci (36 hpi) 119 representing early viral replication compartments, and finally the small foci merged into 120 121 bipolar foci (48 hpi) or single large late replication compartments (72 hpi, data not shown). In mock-infected NPCs, SOX2 exhibited its typical intense and diffuse nuclear staining. As 122 expected, the SOX2 signal decreased during the course of HCMV infection inversely relating 123 to UL44 foci development. In fact, SOX2 became dispersed and largely disappeared from late 124 HCMV-infected NPCs. 125

126 These observations indicate the presence of a highly effective mechanism for SOX2127 depletion in HCMV-infected NPCs.

128

#### 129 SOX2 depletion from HCMV-infected NPCs requires de novo viral protein synthesis. To

130 determine if SOX2 down-regulation is dependent on HCMV gene products expressed de novo from the infecting viral genome, NPCs were exposed to infectious HCMV or UV-inactivated 131 virus and SOX2 expression was analyzed. As expected, SOX2 expression was suppressed at 132 both the RNA and protein level in infections with the untreated virus (Fig 2A and 2B). In 133 contrast, UV-inactivated virus failed to suppress the expression of SOX2 mRNA (Fig 2A) and 134 protein (Fig 2B). Similar levels of glycoprotein B (gB) derived from the input virus were 135 detected at 12 hpi with both untreated and UV-treated HCMV, confirming that cells had been 136 exposed to equal amounts of infecting virus (Fig 2B). However, de novo synthesis of viral 137 138 proteins was undetectable in infections with UV-treated virus confirming successful inactivation. In another experiment, NPCs were pretreated with cycloheximide (CHX) and 139 then infected with HCMV for 16 h in the presence of CHX. Down-regulation of SOX2 140 141 mRNA induced by HCMV infection was significantly diminished by CHX treatment (Fig. 2C). No obvious change in SOX2 protein levels was observed by 16 hpi (data not shown), 142 143 consistent with the results from Fig 1 and previous reports [14, 19].

These results indicate that one or more viral proteins expressed *de novo* upon HCMV
infection are required for SOX2 down-regulation in NPCs.

146

HCMV major IE proteins mediate SOX2 down-regulation in NPCs. Transcription of SOX2 is suppressed as early as 4 hpi, a time when virion constituents and IE gene products are the only viral components present in the infected cell nucleus. To test whether IE or tegument proteins are sufficient for SOX2 down-regulation, the major IE proteins (IE1 and IE2) and the most abundant tegument protein (pp65) were individually expressed in NPCs using nucleofection. In agreement with the conclusion that newly synthesized viral proteins mediate SOX2 down-regulation (Fig 2), SOX2 levels did not change in NPCs transiently

expressing pp65 compared to control cells (Fig 3A). Thus, pp65 was used as a negative 154 155 control in subsequent assays. Expression of the entire major IE transcription unit, including the IE1 and IE2 proteins, resulted in significant reduction of SOX2 protein levels (Fig 3A). To 156 discriminate between effects on SOX2 expression mediated by IE1 or IE2, each of the two 157 proteins was individually expressed in NPCs. Following transfection of 2 or 5 µg IE1 158 expressing plasmid, the relative SOX2 mRNA levels were reduced to 61 or 23% of control 159 levels, and the corresponding protein levels were reduced to 84 or 33%, respectively (Fig 3B). 160 161 Transfection of 2 or 5 µg IE2 expressing plasmid reduced the relative SOX2 mRNA levels to 71 or 31% of control levels and the protein levels to 88 or 54%, respectively (Fig 3C). 162

These results indicate that the HCMV IE1 and IE2 proteins both play a significant role in down-regulating SOX2 expression. However, IE1 appeared to affect SOX2 more efficiently than IE2. Therefore, and because of the lethal phenotype of IE2-deficient viruses (39, 40), we focused our study on the role of IE1 in SOX2 regulation in present study.

167

HCMV IE1 is required for depletion of SOX2 during infection of NPCs. HCMV infection 168 and IE1 expression were each sufficient to efficiently down-regulate SOX2 at the mRNA and 169 protein level in NPCs. To confirm the requirement of IE1 for SOX2 depletion during HCMV 170 171 infection, the expression of IE1 was knocked down using shRNAs targeting IE1 exon 4 sequences. First, human embryonic lung (HEL) cells were transduced with lentiviruses 172 expressing shRNAs directed against three different IE1-specific sequences, sh-1 to -3, or a 173 174 scrambled shRNA control (scr). The transduced cells were subsequently synchronized and 175 infected with HCMV. Analysis of IE1 protein levels determined at 24 and 48 hpi showed that sh-2 was the most efficient among the three tested shRNAs in reducing the levels of the IE1 176 177 protein (Fig 4A). To confirm the knock-down efficiency of sh-2, another experiment was performed where both IE1 mRNA and protein were analyzed at 24, 48 and 72 hpi. Again, sh-2 178

knocked down IE1 by >60% at the RNA level and 50% at the protein level through the course
of infection (Fig 4B).

Following the successful IE1 knock-down in HEL cells, NPCs were transduced with 181 lentiviruses expressing sh-2 or scr. The transduced NPCs were infected with HCMV and 182 collected at 24 to 60 hpi. Since 24 hpi was previously shown to be the "turning point" of 183 SOX2 protein levels (Fig 1), this time point was chosen as the earliest. As shown in Fig 4C, 184 185 SOX2 protein levels gradually decreased as infection progressed, consistent with Fig 1. Significant knock-down of IE1 was observed at all tested time points in NPCs expressing sh-2 186 compared with scr expressing cells. SOX2 down-regulation was alleviated in HCMV-infected 187 188 NPCs expressing sh-2 compared to cells expressing scr, most notably at 60 hpi.

Although the IE1 knock-down was incomplete and linked to reduced levels of other viral proteins (UL44 and gB), these results support the idea that IE1 contributes to SOX2 down-regulation during HCMV infection of NPCs.

192

193 HCMV infection and IE1 expression inhibit STAT3 tyrosine phosphorylation and relocalize unphosphorylated STAT3 to the nuclei of NPCs. So far, our experiments have 194 195 indicated that HCMV IE1 down-regulates SOX2, but a mechanism through which the viral 196 protein alters expression of the cellular stem cell factor has not been identified. Results from co-immunoprecipitation and yeast two hybrid assays failed to provide evidence for a physical 197 interaction between IE1 and SOX2 (data not shown). Since SOX2 expression was affected at 198 both the protein and RNA level, it also seemed more likely that IE1 interferes with 199 transcription rather than protein stability of SOX2. One key transcription factor regulating 200 SOX2 expression is STAT3 (37), which has been recently identified as a physical interaction 201 partner of IE1 (41, 42). 202

203 To investigate whether HCMV infection and IE1 expression affect SOX2 levels via

modulation of STAT3 activation, the levels of total and tyrosine (Y705)-phosphorylated STAT3 (pSTAT3) were determined in infected NPCs. HCMV infection dramatically suppressed STAT3 tyrosine phosphorylation as early as 4 hpi, and pSTAT3 was maintained at low levels throughout the course of infection as compared to mock-infected NPCs. In contrast, the total STAT3 levels showed little if any changes during infection (Fig 5A).

STAT3 is a nucleocytoplasmic shuttling protein which is efficiently exported from the 209 210 nucleus in its unphosphorylated form (43). Unphosphorylated STAT3 is therefore typically located in the cytoplasm or distributed across both the nuclear and cytoplasmic compartments. 211 However, upon tyrosine phosphorylation pSTAT3 efficiently accumulates in the nucleus (37, 212 213 44, 45). To define the subcellular distribution of STAT3 in HCMV-infected NPCs, we performed immunofluorescence analysis. NPCs on coverslips were infected with HCMV 214 (MOI=1), collected at 8 hpi, and stained for STAT3 and IE1/IE2. STAT3 was mainly confined 215 216 to the nuclei of virus-infected cells, while in mock-infected cells the STAT3 signal was more diffuse and cytoplasmic as well as nuclear (Fig 5B). This observation was consistent with our 217 218 results from HCMV-infected fibroblasts (41, 42). To further investigate STAT3 subcellular localization in HCMV-infected NPCs, the levels of pSTAT3 and total STAT3 in cytoplasmic 219 and nuclear compartments separated by cellular fractionation were analyzed at 4 and 8 hpi. 220 221 GAPDH and lamin B1 served as loading controls for the cytoplasmic and nuclear compartments, respectively, confirming successful fractionation. As expected, pSTAT3 was 222 present predominantly in the nucleus of both mock- and HCMV-infected cells. Compared to 223 mock-infected NPCs, pSTAT3 levels were reduced in both the cytoplasm and nucleus of 224 HCMV-infected cells at 4 and 8 hpi. Total STAT3 resided mainly in the cytoplasm, but the 225 nuclear signal was much stronger in HCMV-infected compared with mock-infected cells at 226 both tested time points (Fig 5B). These results indicate that HCMV infection reduces the 227 levels of activated STAT3 (pSTAT3) and promotes the nuclear accumulation of 228

229 unphosphorylated STAT3 at very early times of infection in NPCs.

230 IE proteins are thought to be the only HCMV gene products expressed at 4 to 8 hpi, when STAT3 is relocalized to the nucleus and its phosphorylation is inhibited. To test whether 231 IE1 was responsible for the observed effects on STAT3, the viral protein was transiently 232 expressed in NPCs. IE1 expression was sufficient to down-regulate pSTAT3 levels and to 233 sequester an unphosphorylated form of the cellular protein in the nuclear compartment (Fig 234 5C). To further confirm the role of IE1 in altering STAT3 phosphorylation and intracellular 235 localization, NPCs were mock-infected or infected with WT, dlIE1, or rvIE1 viruses. The 236 levels of viral proteins (UL44, gB) were similar, except that IE1 was absent and down-237 238 regulation of SOX2 was diminished in the infection with dlIE1 (Fig 5D). Compared with mock-infected cells, the pSTAT3 levels were reduced in WT- and rvIE1-, but not in dlIE1-239 infected cells. Again, the overall steady-state STAT3 levels were rather constant across all 240 241 mock- and HCMV-infected samples (Fig 5E). Finally, based on cellular fractionation analysis, pSTAT3 was located in the nuclei of both mock- and WT- or dlIE1-infected cells. Total 242 243 STAT3 localized mainly in the cytoplasm of mock- and dlIE1-infected cells, while increased nuclear localization was observed in WT-infected NPCs (Fig 5F). 244

These results demonstrate that IE1 is both sufficient and required for the inhibition of tyrosine phosphorylation and nuclear sequestration of unphosphorylated STAT3 observed during HCMV infection of NPCs.

248

## 249 SOX2 down-regulation in HCMV-infected NPCs results from IE1-mediated inhibition of

**STAT3 activation.** The results thus far obtained are all consistent with a mechanism in which IE1 mediates SOX2 down-regulation by limiting STAT3 activation. To further test this possibility, we performed several different experiments. We first treated NPCs with CTS, a chemical inhibitor of STAT3 tyrosine phosphorylation (46). In the presence of CTS, pSTAT3 levels (but not steady-state STAT3 levels) markedly decreased as a function of time of treatment coinciding with gradual loss of SOX2 (Fig 6A). Then, STAT3 expression was silenced using RNA interference. Two different shRNAs, shSTAT3-1 and shSTAT3-2, knocked down STAT3 expression with different efficiencies compared to non-specific shRNAs (shLuci and shDsRed). The extent of STAT3 silencing correlated with differential reduction in pSTAT3 levels, in turn correlating with the levels of SOX2 (Fig 6B).

260 In addition to the experiments involving inhibition of STAT3, we set out to study the IE1-STAT3-SOX2 axis by activating STAT3 signaling. To this end, we first treated NPCs with 261 IL-6, a major agonist of STAT3 signaling (47). IL-6 treatment led to a marked increase in 262 263 pSTAT3 without significantly affecting the overall STAT3 protein levels. Concurrent with increased STAT3 tyrosine phosphorylation, there was also a rise in SOX2 levels. These data 264 were consistent with the finding that pSTAT3 promotes SOX2 expression in NPCs (Fig 6C). 265 266 Next, we transiently expressed IE1 in NPCs, treated the cells with IL-6 for 4 h or left them untreated, and analyzed the levels of IE1, pSTAT3, total STAT3 and SOX2. Both pSTAT3 and 267 SOX2 were strongly up-regulated by IL-6, but down-regulated by IE1, and IL-6 efficiently 268 counteracted IE1-mediated down-regulation of pSTAT3 and SOX2. Again, the total steady-269 state STAT3 levels did not significantly change upon IL-6 treatment or IE1 expression (Fig 270 6D). Finally, to further investigate the link between IE1 expression, STAT3 activation and 271 SOX2 regulation in the context of HCMV infection, NPCs were mock-infected or infected 272 with HCMV WT or dIIE1. The infected NPCs were treated with IL-6 for 4 h or left untreated 273 prior to collection at 24 and 48 hpi. As expected, IL-6 treatment led to elevated levels of 274 pSTAT3 and SOX2 in mock-, WT- and dlIE1-infected NPCs. HCMV WT infection reduced 275 the pSTAT3 levels slightly at 24 hpi and dramatically at 48 hpi. In contrast, no obvious 276 decrease of pSTAT3 was observed following dIIE1 infection. Concordantly, SOX2 protein 277 levels fell substantially in WT infection at 48 hpi, but remained constant in dIIE1 infection at 278

- the same time point. Again, IL-6 treatment counteracted IE1-dependent down-regulation of
- $280 \qquad both \ pSTAT3 \ and \ SOX2 \ (Fig \ 6E).$
- In summary, these data demonstrate that SOX2 expression in NPCs strictly depends on
- 282 pSTAT3, and that there is a causal link between SOX2 down-regulation by HCMV and
- 283 inhibition of STAT3 activation by IE1, including inhibition of tyrosine phosphorylation and
- trapping unphosphorylated STAT3 in nuclear.

#### 285 **DISCUSSION**

286

Proliferation, differentiation and migration of NPCs as well as synapse formation among 287 mature neurons are all critical factors for fetal brain development and function (48-50). 288 HCMV infection has been shown to induce neural cell loss and abnormal differentiation of 289 NPCs (14, 15, 19). This was not only demonstrated in vitro, but also confirmed in a mouse 290 291 model of congenital infection where the virus appeared to affect NPCs in the subventricular zone (12). Although HCMV is the leading cause of neurological damage in children, the 292 mechanisms by which the virus perturbs NPC proliferation and differentiation have remained 293 unclear. 294

SOX2 is a master controller of stem cells, strikingly illustrated by the fact that its 295 overexpression can reprogram terminally differentiated fibroblasts to induced pluripotent stem 296 cells (25-30). More specifically, SOX2 is a transcription factor essential for maintaining self-297 renewal and pluripotency of ESCs and NPCs. SOX2 mutations cause symptoms resembling 298 299 damage resulting from congenital HCMV infection, including neural development disorders accompanied by ocular malformation (22-24). In this study, we demonstrate that HCMV 300 significantly down-regulates SOX2 mRNA levels in human primary NPCs from as early as 4 301 302 hpi, extending our previous observations (14, 38). The reduced mRNA levels subsequently lead to almost full depletion of the SOX2 protein from HCMV-infected cells at later times (24 303 to 96 hpi). The temporal delay between mRNA and protein down-regulation suggests that the 304 SOX2 protein may be very stable in NPCs, in contrast to what has been reported for ESCs 305 (51). The timing of changes in SOX2 mRNA levels and the observation that the down-306 307 regulation depends on *de novo* viral protein synthesis, pointed us to the HCMV major IE proteins as potential regulators of this stem cell factor. The 72-kDa IE1 and the 86-kDa IE2 308 protein subsequently proved to be sufficient to decrease SOX2 mRNA and protein levels in 309

the absence of other viral proteins, together and individually. IE1 and IE2 are nuclear localized key regulators of viral and cellular transcription during infection (52-54). They have been extensively studied in fibroblasts, but there is little information on how these proteins behave in cell types more relevant to HCMV pathogenesis.

Our results demonstrate that the HCMV IE1 protein is not only sufficient, but also 314 necessary for the reduction of SOX2 levels through the early-late stages of infection. As noted 315 316 above, the down-regulation of SOX2 RNA and protein was also observed with ectopically expressed HCMV IE2, but this protein appeared to be less efficient than IE1 in this respect. 317 We propose that IE2 also contributes to SOX2 down-regulation in the context of HCMV 318 319 infection, although this remains to be formally shown. Due to the difficulties in working with IE2-deficient viruses (IE2 is essential for HCMV replication and difficult to complement), we 320 focused on IE1 in this study. Our findings may come as a surprise in the light of two previous 321 322 reports which concluded that HCMV infection or IE1 expression leads to increased instead of decreased SOX2 levels in human glioma stem-like cells, human glioblastoma cells and mouse 323 324 glioma tissue (55, 56). The seemingly disparate findings might be due to differences in cell types or virus strains. However, in our hands, IE1 induced robust inhibition of STAT3 325 326 activation in all cell types tested so far including glioblastoma-derived cell lines (data not 327 shown). Notably, the previous studies did not discriminate between the levels of SOX2 in IE1 expressing cells and IE1-negative cells in the same culture or tumor. Even if the STAT3 328 pathway is inhibited in HCMV-infected cells or cells ectopically expressing IE1, the 329 secretome associated with these cells may trigger STAT3 signaling in IE1-negative bystander 330 cells. In population analyses, this bystander effect may be detected as an overall increase in 331 STAT3-dependent gene expression (42). 332

Our work also determines the molecular events underlying IE1-dependent SOX2 downregulation, which appear to be intimately linked to JAK-STAT signaling. JAK-STAT (STAT1

- 17 -

and STAT3) signaling pathways play an important role in neural development by regulating 335 336 NPC neurogenesis and gliogenesis (35, 36). IE1 markedly affects the activation state and subcellular localization of STAT3, a key regulatory protein known to affect the pluripotency 337 of ESCs and initiate the commitment to NPC fate via transcriptional activation of SOX2 (37). 338 IE1 inhibits tyrosine (Y705) phosphorylation and promotes nuclear accumulation of STAT3 339 without altering the protein's overall steady-state levels. These observations closely 340 341 recapitulate previous results in fibroblasts and are likely the consequence of a direct physical interaction between IE1 and STAT3 (41, 42). Although paradoxical on the face of it, 342 decreased tyrosine phosphorylation and increased nuclear localization of STAT3 may be 343 344 coupled. STAT proteins, including STAT3, continually shuttle between the cytoplasm and the nucleus. In fact, STAT3 is imported to the nucleus independent of tyrosine phosphorylation, 345 and this is normally followed by export to the cytoplasm. Tyrosine phosphorylation 346 347 transiently increases STAT3 nuclear accumulation due to sequestration by DNA binding or heterodimerization with other phosphorylated STAT proteins (43). Likewise, IE1 may bind to 348 349 STAT3 passing through the nucleus. Nuclear sequestration may reduce the amounts of STAT3 undergoing export to the cytoplasm and, consequently, the pools of cytoplasmic STAT3 350 available for tyrosine phosphorylation by the corresponding (cytoplasmic) JAK family 351 352 kinases. The expected consequences of depleting activatable STAT3 by nuclear sequestration are in line with the reduced pSTAT3 levels, restricted responsiveness to IL-6 and diminished 353 expression of SOX2 we observed in HCMV-infected NPCs. 354

In summary, this study initially links the interaction between IE1 and STAT3 to SOX2 depletion and thereby identifies a novel pathway predicted to contribute to developmental neuropathogenesis caused by congenital HCMV infection.

#### 358 MATERIALS AND METHODS

Ethics statement. Postmortem fetal brain tissues from different gestational age cases were 359 obtained according to the approval notice from the Institutional Review Board 360 (WIVH10201202) and the Guidelines for Biomedical Research Involving Human Subjects at 361 Wuhan Institute of Virology, Chinese Academy of Sciences. The original source of the 362 anonymized tissues was Zhongnan Hospital of Wuhan University (China). The cell isolation 363 procedures and research plans were approved by the Institutional Review Board (IRB) 364 (WIVH10201202) according to the Guidelines for Biomedical Research Involving Human 365 Subjects at Wuhan Institute of Virology, Chinese Academy of Sciences. The need for written 366 or oral consents was waived by IRB (57). 367

368

#### 369 Cells and cell culture

All cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. NPCs 370 were isolated as described previously (38, 58) and cultured in a 1:1 mix of growth medium 371 372 and conditioned medium (14, 15, 19). The NPC growth medium was Dulbecco's Modified Eagle Medium (DMEM)-F12 (Thermo Fisher Scientific) supplemented with 2 mM 373 GlutaMAX (Thermo Fisher Scientific), 100 U/ml penicillin and 100 µg/ml streptomycin 374 (Thermo Fisher Scientific), 50 µg/ml gentamycin (Sigma), 1.5 µg/ml amphotericin B 375 376 (Thermo Fisher Scientific), 10% BIT 9500 (Stem Cell Technologies), 20 ng/ml epidermal growth factor (EGF, Prospec) and 20 ng/ml basic fibroblast growth factor (FGF, Prospec). 377 378 Conditioned medium was collected from cultured NPCs and stored at -20°C after cell debris had been removed by centrifugation. NPCs were maintained as monolayers in fibronectin-379 380 coated dishes and seeded at a defined density into dishes coated with poly-D-lysine (50 µg/ml, Millipore) prior to infection. In order to induce STAT3 activation, NPCs were treated with 381 carrier-free recombinant human IL-6 (183 ng/ml, Biolegend) for the indicated times before 382

being collected for protein analysis. To inhibit STAT3 activation, NPCs were treated for the indicated times with 10  $\mu$ M cryptotanshinone (CTS, Sigma), a small molecular inhibitor of STAT3 tyrosine (Y705) phosphorylation.

Human embryonic lung fibroblasts (HEL cells, maintained in the laboratory) and HEL 386 cells immortalized by transfection with pCI-neo-hTERT (HELf cells, kindly provided by Dr. 387 Chen, Columbia University), were cultured in Minimal Essential Medium (MEM, Thermo 388 Fisher Scientific). Human embryonic kidney (HEK) 293T cells (CRL-11268) were grown in 389 DMEM. Both MEM and DMEM were supplemented with 10% fetal bovine serum (FBS, 390 Thermo Fisher Scientific), penicillin and streptomycin, and 2 mM L-glutamine (Thermo 391 392 Fisher Scientific). For the establishment of an IE1 expressing HELf cell line, a lentivirus stock was prepared from plasmid pCDH-puro-IE1. A volume of 2 ml from this stock was used 393 to transduce  $1 \times 10^6$  HELf cells. Transduced cells were cultured in normal medium for 2 days 394 395 to allow for transgene expression, and then switched to selection medium containing 8 µg/ml puromycin (Sigma) for 2 weeks with medium changes every other day. Expression of IE1 was 396 397 confirmed by Western blotting. The resulting HELf cell line stably expressing IE1 was designated HELf-IE1 and maintained in medium containing puromycin (4 µg/ml). 398

399

#### 400 Plasmids and nucleofection

Plasmids pcDNA3-IE1 and pcDNA3-IE2 were constructed by subcloning a *Bam*HI-*Bam*HI fragment containing the full-length coding sequence of the HCMV 72-kDa IE1 or 86-kDa IE2 protein, respectively, from pSG-IE1 or pSG-IE2 (kindly provided by Dr. Fortunato, University of Idaho), respectively, into the backbone of pcDNA3.0. Plasmid pcDNA3-pp65 was generated by cloning a fragment PCR-amplified from HCMV (Towne) cDNA between the *Bam*HI and *Eco*RI sites of pcDNA3.0 (57). Plasmid pSVH (kindly provided by Dr. Tang, Howard University) containing the entire HCMV major IE transcription unit (including IE1,

408 IE2 and the major IE promoter-enhancer) was also used.

409 Plasmids expressing short hairpin RNAs (shRNAs) directed against HCMV (Towne) IE1 sequences were constructed based on lentiviral vector pLKO.1 puro (Addgene plasmid 410 #8453). Three shRNAs targeting IE1 exon 4 and a scrambled shRNA not targeting any viral 411 or human gene were designed (http://jura.wi.mit.edu/bioc/siRNAext), synthesized, and 412 inserted between the AgeI and EcoRI sites of pLKO.1 puro to generate pLKO.1-shRNA-IE1-1 413 (sh-1), pLKO.1-shRNA-IE1-2 (sh-2), pLKO.1-shRNA-IE1-3 (sh-3), and pLKO.1-scramble 414 (scr), respectively. Sequences are listed in Table 1. For the establishment of HELf-IE1 cells 415 (described above), a fragment containing the IE1 coding sequence was recovered from pSG-416 417 IE1 and cloned into the BamHI site of pCDH-CMV-MCS-EF1-puro (System Biosciences). The resulting construct was designated pCDH-puro-IE1. 418

Plasmids expressing shRNAs directed against human STAT3 sequences were 419 420 constructed based on lentiviral vector Tet-pLKO-puro (Addgene plasmid #21915). Two shRNA plasmids targeting STAT3, Tet-pLKO-puro-shSTAT3-1 and Tet-pLKO-puro-421 422 shSTAT3-2, were generated by inserting annealed oligonucleotides #1102 and #1103 or #1134 and #1135, respectively, between the EcoRI and AgeI sites of Tet-pLKO-puro. Likewise, 423 plasmids Tet-pLKO-puro-shLuci and Tet-pLKO-puro-shDsRed expressing shRNAs directed 424 425 against *Photinus* luciferase or *Discosoma* red fluorescent protein sequences, respectively, not present in human cells were constructed by inserting annealed oligonucleotides #1098 and 426 #1099 or #1038 and #1039, respectively, between the EcoRI and AgeI sites of Tet-pLKO-427 puro. Oligonucleotide sequences are listed in Table 1. 428

To transiently overexpress individual viral proteins, NPCs were transfected with plasmids pSVH, pcDNA3-IE1, pcDNA3-IE2, pmaxGFP (provided by Lonza to assess transfection efficiency), pcDNA3-pp65, or empty vector (pcDNA3.0) using Nucleofector technology (Lonza) according to the manufacturer's instructions. In brief,  $5 \times 10^6$  NPCs were mixed with 100  $\mu$ l Mesenchymal Stem Cell (MSC) Nucleofector Solution (82  $\mu$ l Nucleofector Solution with 18  $\mu$ l Supplement 1) and 5  $\mu$ g pSVH, pcDNA3-IE1, pcDNA3-IE2, pmaxGFP, pcDNA3-pp65, or pcDNA3.0. The cell-DNA suspension was transferred to certified cuvettes, which were inserted into a Nucleofector II, and program A-033 was applied. Immediately following nucleofection, 500  $\mu$ l NPC growth medium was added to the cuvette, and the sample was gently transferred to poly-D-lysine-coated dishes. After 24 h, the culture medium was replaced, and cells were analyzed at 48 h post transfection.

440

#### 441 **HCMV preparation and infection**

Enhanced green fluorescent protein (EGFP) expressing bacterial artificial chromosome-442 derived wild-type (T-BAC, herein referred to as TNWT), IE1-deficient (TNdIIE1), and 443 revertant (TNrvIE1) variants of the HCMV Towne strain (ATCC-VR977) and the parental 444 445 virus HCMV Towne strain (ATCC-VR977) were used in this study. The construction of TNdIIE1 and TNrvIE1 was described previously (59). The TNWT and TNrvIE1 viruses were 446 447 propagated in HEL cells and titrated by plaque assay as described previously (60, 61). The TNdIIE1 mutant was propagated and titrated in HELf-IE1 cells. HCMV particles from 448 infected cell supernatants were concentrated by ultracentrifugation, after removing the cell 449 debris by high speed centrifugation, and resuspended in NPC growth medium to avoid any 450 potential undesired effects induced by components (including FBS) in the medium used for 451 virus preparation (61). UV-inactivated HCMV was prepared by exposure to 6000 J/cm<sup>2</sup> in a 452 CL-1000 Ultraviolet Crosslinker (UVP), sodium pyruvate was added to a final concentration 453 of 5 mM to prevent damage from free radicals induced by ultraviolet radiation (62). 454

For NPC infections, confluent cell monolayers on fibronectin-coated dishes were dissociated using Accutase (Millipore),  $3 \times 10^6$  cells were reseeded in poly-D-lysine-coated 100-mm dishes or uncoated dishes with poly-D-lysine-coated coverslips, and cells were

allowed to attach overnight. Cells were exposed to HCMV at the indicated multiplicities of 458 459 infection (MOIs). For the evaluation of IE1-directed shRNAs, NPCs or HEL cells were infected with HCMV at an MOI of 1. To overcome the delay of infection process of TNdlIE1, 460 MOI of 10 was used. After incubation for 3 h to allow for virus adsorption, the inoculum was 461 removed and cells were refed with fresh medium. Cells were collected and analyzed at the 462 indicated times post infection. To study infection in the absence of *de novo* protein synthesis, 463 NPCs were pretreated with 10 µg/ml cycloheximide (CHX, Sigma) for 1 h prior to infection, 464 infected with HCMV in the presence of CHX (10 µg/ml), and collected at 16 hpi for RNA and 465 protein analysis. 466

467

#### 468 Lentivirus preparation and transduction

Stocks of replication-defective lentiviruses were prepared as described previously (57, 63). 469 Briefly,  $1.5 \times 10^6$  HEK 293T cells were seeded in a 100-mm dish. On the next day, calcium 470 phosphate precipitation was applied to cotransfect the cells with packaging plasmids pML-471 472  $\Delta 8.9$  (12 µg) and pVSV-G (8 µg) (System Biosciences) along with 15 µg of one of the following expression plasmids: pCDH-puro-IE1, pLKO.1-scramble, pLKO.1-shRNA-IE1-1, 473 pLKO.1-shRNA-IE1-2, pLKO.1-shRNA-IE1-3, Tet-pLKO-puro-shDsRed, Tet-pLKO-puro-474 shLuci, Tet-pLKO-puro-shSTAT3-1, or Tet-pLKO-puro-shSTAT3-2. Following a medium 475 change, the lentivirus containing supernatants were collected 72 h after transfection and stored 476 at -80°C. 477

The lentivirus stocks were used to transduce HEL cells, HELf cells, or NPCs. To this end,  $5 \times 10^6$  NPCs were seeded in fibronectin-coated dishes and infected with equal volumes (2 ml) of lentivirus stock on the following day. Lentivirus stock was added to the NPCs again on the next day. The inoculum was replaced with fresh culture medium 3 h after each transduction. Likewise,  $5 \times 10^5$  HEL cells were infected with 2 ml lentivirus stock on the day following seeding, and the inoculum was replaced with fresh culture medium 5 h after infection. The transduced cells were cultured for 3 days to allow for transgene expression, before they were subjected to HCMV infection and/or RNA or protein analysis. The transduction procedure used to establish HELf-IE1 cells is described above.

487

#### 488 Gene silencing with shRNAs

To determine the IE1-specific silencing efficiency, equal amounts of shRNA expressing 489 lentiviruses (sh-1, sh-2, sh-3, and scr) were used to transduce HEL cells in parallel. The 490 resulting HEL cells were cultured for 2 days to allow for shRNA expression, and this was 491 492 followed by 2 days serum starvation with serum free medium to synchronize the cells. The synchronized cells were reseeded in 60-mm dishes  $(1 \times 10^6 \text{ cells/dish})$ , allowed to attach, and 493 infected with HCMV (MOI = 1). The infected cells were collected at 24 and 48 hpi, and the 494 495 levels of IE1 were determined by protein analysis. After selection of the most efficient shRNA (sh-2), NPCs were transduced with sh-2 and scr lentiviruses in parallel. Transduced NPCs 496 497 were cultured for 48 h to allow for shRNA expression, reseeded in poly-D-lysine-coated dishes  $(3 \times 10^6 \text{ cells/dish})$ , and infected with HCMV (MOI = 1) on the following day. Cells 498 were collected at the indicated times post infection and subjected to protein and RNA 499 analysis. 500

501 NPCs transduced with shRNA expressing lentiviruses Tet-pLKO-puro-shDsRed, Tet-502 pLKO-puro-shLuci, Tet-pLKO-puro-shSTAT3-1, or Tet-pLKO-puro-shSTAT3-2 (described 503 above) were treated with 1  $\mu$ g/ml doxycycline hyclate (Dox, Aladdin) for 48 h, with a medium 504 change after 24 h, to induce shRNA expression. Cells were collected and analyzed at the 505 indicated times.

506

#### 507 **Quantitative reverse transcriptase PCR (qRT-PCR)**

Transfected or infected NPCs were collected at the indicated times. Total RNA was isolated 508 509 using the RNAiso Plus reagent (Takara) followed by RNase-free DNase I treatment (Thermo Fisher Scientific) to remove residual genomic DNA. Equal amounts (500 ng) of DNA-free 510 RNA were reverse transcribed using the PrimeScript RT Reagent Kit (Perfect Real Time; 511 Takara) according to the manufacturer's instructions. Then, qPCR was performed in a CFX96 512 Connect Real-Time PCR Detection System (Bio-Rad). Each 20-µl qPCR reaction contained 2 513 µl RT product, 10 µl 2× iTaq Universal SYBR Green Supermix (Bio-Rad), and 200 nM 514 forward (F) and reverse (R) primers. Primer sequences are shown in Table 1. Amplification 515 was performed by denaturation at 95°C for 5 min, followed by 35 two-step cycles of 95°C for 516 517 10 s and 60°C for 30 s. Melting curve analysis was carried out at 95°C for 1 min, 55°C for 1 min, and 55 to 95°C for 10 s. Each reaction was performed in triplicate, and results for the 518 target gene mRNA were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 519 using the  $2^{\Delta\Delta CT}$  method. Three independent experiments were performed, and results are 520 presented as the means  $\pm$  one standard deviation (SD). Data were statistically evaluated using 521 the Student's t-test. A *P*-value of ≤0.05 was considered statistically significant. 522

523

#### 524 Western blotting

525 At the indicated times, cells were washed in phosphate-buffered saline (PBS), detached with Accutase, collected, counted, and centrifuged. Cell pellets were snap-frozen in liquid nitrogen 526 and stored at -80°C until completion of the time course. Then, cell pellets were lysed in 527 radioimmunoprecipitation assay (RIPA) buffer. Equal amounts of cell lysates were separated 528 529 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). After incubation with the indicated 530 531 primary and corresponding secondary antibodies, signals were detected using a Chemiluminescence machine, and analyzed by densitometry program (Image J). At least three 532

sets of independent experiments were performed and representative results were shown. 533 534 HCMV proteins were detected using mouse monoclonal antibodies to IE1 (clone p63-27, IgG2a), IE1/IE2 (CH16), UL44 (IgG1, Virusys), glycoprotein B (gB; clone 27-156, IgG2b), 535 or pp65 (IgG1, Virusys). Cellular proteins were detected using a goat polyclonal antibody to 536 SOX2 (clone L1D6A2, IgG1), a mouse monoclonal antibody to STAT3 (clone 124H6, IgG2a, 537 Cell Signaling Technology), a rabbit polyclonal antibody to pSTAT3 (Y705) (IgG, Cell 538 Signaling Technology), a mouse monoclonal antibody to  $\beta$ -actin (IgG, Santa Cruz 539 Biotechnology), a rabbit polyclonal antibody to GAPDH (IgG, Proteintech), or a rabbit 540 541 polyclonal antibody to lamin B1 (IgG, Proteintech). Secondary antibodies used were horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Bioscience), donkey 542 anti-rabbit IgG (Amersham Bioscience), or donkey anti-goat IgG (Proteintech). 543

544

#### 545 **Immunofluorescence assay**

546 Viral and cellular proteins in cells grown on coverslips were detected by indirect immunofluorescence analysis as described previously (64). Briefly, NPCs were seeded on 547 poly-D-lysine-coated coverslips in uncoated dishes and mock- or HCMV-infected (MOI = 3). 548 549 Coverslips were collected at the indicated times post infection. UL44, SOX2 or STAT3 were stained with the respective primary antibodies: mouse monoclonal anti-UL44 (IgG1, Virusys), 550 goat polyclonal anti-SOX2 (Santa Cruz Biotechnology), or mouse monoclonal anti-STAT3 551 (clone 124H6, IgG2a, Cell Signaling Technology). The secondary antibodies included 552 (FITC)-conjugated 553 fluorescein-isothiocyanate donkev anti-goat IgG (Jackson 554 Immunoresearch), Alexa Fluor 488-conjugated goat anti-mouse IgG1 (Molecular Probes), and Alexa Fluor 488-conjugated goat anti-mouse IgG2a (Molecular Probes). Nuclei were 555 counterstained with Hoechst 33342 dye, and coverslips were mounted with anti-fade 556 mounting solution containing paraphenylenediamine (65). Images were obtained using a 557

Nikon Eclipse 80i or Nikon Eclipse Ti-S epifluorescence microscope equipped with a Nikon
DS-Ri1 camera and processed using the NIS-Elements F3.0 software.

560

#### 561 Cellular fractionation

Nucleofected or infected NPCs were washed in pre-cooled PBS and collected by scraping. 562 Cytosolic and nuclear fractions were prepared using the Nuclear-Cytosol Extraction Kit 563 (Applygen Technologies) following the manufacturer's instructions. Briefly, cell pellets were 564 lysed in pre-cooled Cytosol Extraction Buffer A (CEB-A), vortexed, reacted with Cytosol 565 Extraction Buffer B (CEB-B), vortexed, and centrifuged. The resulting supernatant contained 566 the cytosolic fraction. The precipitate was washed with CEB-A, lysed in Nuclear Extraction 567 Buffer (NEB), vortexed, and centrifuged. The resulting supernatant contained the nuclear 568 fraction. 569

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## 581 **REFERENCES**

- Bale JF, Jr. 1984. Human cytomegalovirus infection and disorders of the nervous system. Arch Neurol
   41:310-320.
- Stagno S, Pass RF, Cloud G, Britt WJ, Henderson RE, Walton PD, Veren DA, Page F, Alford CA.
   1986. Primary cytomegalovirus infection in pregnancy. Incidence, transmission to fetus, and clinical outcome. JAMA 256:1904-1908.
- 587 3. Bale JF, Jr. 2014. Congenital cytomegalovirus infection. Handb Clin Neurol 123:319-326.
- Conboy TJ, Pass RF, Stagno S, Britt WJ, Alford CA, McFarland CE, Boll TJ. 1986. Intellectual development in school-aged children with asymptomatic congenital cytomegalovirus infection. Pediatrics 77:801-806.
- 591 5. **Pass RF, Stagno S, Myers GJ, Alford CA.** 1980. Outcome of symptomatic congenital cytomegalovirus infection: results of long-term longitudinal follow-up. Pediatrics **66**:758-762.
- 5936.Rosenthal LS, Fowler KB, Boppana SB, Britt WJ, Pass RF, Schmid SD, Stagno S, Cannon MJ.5942009. Cytomegalovirus shedding and delayed sensorineural hearing loss: results from longitudinal595follow-up of children with congenital infection. Pediatr Infect Dis J 28:515-520.
- 596 7. Sweeten TL, Posey DJ, McDougle CJ. 2004. Brief report: autistic disorder in three children with
   597 cytomegalovirus infection. J Autism Dev Disord 34:583-586.
- 5988.Yamashita Y, Fujimoto C, Nakajima E, Isagai T, Matsuishi T. 2003. Possible association between<br/>congenital cytomegalovirus infection and autistic disorder. J Autism Dev Disord 33:455-459.
- 600
  9. Zhang XW, Li F, Yu XW, Shi XW, Shi J, Zhang JP. 2007. Physical and intellectual development in children with asymptomatic congenital cytomegalovirus infection: a longitudinal cohort study in Qinba mountain area, China. J Clin Virol 40:180-185.
- 60310.Boppana SB, Pass RF, Britt WJ, Stagno S, Alford CA. 1992. Symptomatic congenital<br/>cytomegalovirus infection: neonatal morbidity and mortality. Pediatr Infect Dis J 11:93-99.
- 60511.Dahle AJ, Fowler KB, Wright JD, Boppana SB, Britt WJ, Pass RF. 2000. Longitudinal investigation606of hearing disorders in children with congenital cytomegalovirus. J Am Acad Audiol 11:283-290.
- Tsutsui Y, Kosugi I, Kawasaki H. 2005. Neuropathogenesis in cytomegalovirus infection: indication of the mechanisms using mouse models. Rev Med Virol 15:327-345.
- 609 13. Cheeran MC, Hu S, Ni HT, Sheng W, Palmquist JM, Peterson PK, Lokensgard JR. 2005. Neural
   610 precursor cell susceptibility to human cytomegalovirus diverges along glial or neuronal differentiation
   611 pathways. J Neurosci Res 82:839-850.
- Luo MH, Hannemann H, Kulkarni AS, Schwartz PH, O'Dowd JM, Fortunato EA. 2010. Human
   cytomegalovirus infection causes premature and abnormal differentiation of human neural progenitor
   J Virol 84:3528-3541.
- Luo MH, Schwartz PH, Fortunato EA. 2008. Neonatal neural progenitor cells and their neuronal and
   glial cell derivatives are fully permissive for human cytomegalovirus infection. J Virol 82:9994-10007.
- McCarthy M, Auger D, Whittemore SR. 2000. Human cytomegalovirus causes productive infection
   and neuronal injury in differentiating fetal human central nervous system neuroepithelial precursor
   cells. J Hum Virol 3:215-228.
- 620 17. Odeberg J, Wolmer N, Falci S, Westgren M, Seiger A, Soderberg-Naucler C. 2006. Human
   621 cytomegalovirus inhibits neuronal differentiation and induces apoptosis in human neural precursor cells.
   622 J Virol 80:8929-8939.
- 18. Odeberg J, Wolmer N, Falci S, Westgren M, Sundtrom E, Seiger A, Soderberg-Naucler C. 2007.
   Late human cytomegalovirus (HCMV) proteins inhibit differentiation of human neural precursor cells into astrocytes. J Neurosci Res 85:583-593.
- Pan X, Li XJ, Liu XJ, Yuan H, Li JF, Duan YL, Ye HQ, Fu YR, Qiao GH, Wu CC, Yang B, Tian XH, Hu KH, Miao LF, Chen XL, Zheng J, Rayner S, Schwartz PH, Britt WJ, Xu J, Luo MH.
  Later passages of neural progenitor cells from neonatal brain are more permissive for human cytomegalovirus infection. J Virol 87:10968-10979.
- 630 20. Wegner M. 2011. SOX after SOX: SOXession regulates neurogenesis. Genes Dev 25:2423-2428.
- Brazel CY, Limke TL, Osborne JK, Miura T, Cai J, Pevny L, Rao MS. 2005. Sox2 expression
   defines a heterogeneous population of neurosphere-forming cells in the adult murine brain. Aging Cell
   4:197-207.
- Fantes J, Ragge NK, Lynch SA, McGill NI, Collin JR, Howard-Peebles PN, Hayward C, Vivian
  AJ, Williamson K, van Heyningen V, FitzPatrick DR. 2003. Mutations in SOX2 cause anophthalmia. Nat Genet 33:461-463.
- Ragge NK, Lorenz B, Schneider A, Bushby K, de Sanctis L, de Sanctis U, Salt A, Collin JR, Vivian
  AJ, Free SL, Thompson P, Williamson KA, Sisodiya SM, van Heyningen V, Fitzpatrick DR. 2005.
  SOX2 anophthalmia syndrome. Am J Med Genet A 135:1-7; discussion 8.

- Schneider A, Bardakjian TM, Zhou J, Hughes N, Keep R, Dorsainville D, Kherani F, Katowitz J,
  Schimmenti LA, Hummel M, Fitzpatrick DR, Young TL. 2008. Familial recurrence of SOX2
  anophthalmia syndrome: phenotypically normal mother with two affected daughters. Am J Med Genet
  A 146A:2794-2798.
- Ring KL, Tong LM, Balestra ME, Javier R, Andrews-Zwilling Y, Li G, Walker D, Zhang WR,
  Kreitzer AC, Huang Y. 2012. Direct reprogramming of mouse and human fibroblasts into multipotent
  neural stem cells with a single factor. Cell Stem Cell 11:100-109.
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. 2007.
  Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861-872.
- Takahashi K, Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult
   fibroblast cultures by defined factors. Cell 126:663-676.
- Kim J, Efe JA, Zhu S, Talantova M, Yuan X, Wang S, Lipton SA, Zhang K, Ding S. 2011. Direct
   reprogramming of mouse fibroblasts to neural progenitors. Proc Natl Acad Sci U S A 108:7838-7843.
- Lujan E, Chanda S, Ahlenius H, Sudhof TC, Wernig M. 2012. Direct conversion of mouse
  fibroblasts to self-renewing, tripotent neural precursor cells. Proc Natl Acad Sci U S A 109:2527-2532.
- Maucksch C, Firmin E, Butler-Munro C, Montgomery J, Dottori M, Connor B. 2012. Non-Viral
  Generation of Neural Precursor-like Cells from Adult Human Fibroblasts. J Stem Cells Regen Med
  8:162-170.
- Rizzino A. 2013. Concise review: The Sox2-Oct4 connection: critical players in a much larger interdependent network integrated at multiple levels. Stem Cells 31:1033-1039.
- Kopp JL, Ormsbee BD, Desler M, Rizzino A. 2008. Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem Cells 26:903-911.
- Masui S, Nakatake Y, Toyooka Y, Shimosato D, Yagi R, Takahashi K, Okochi H, Okuda A,
  Matoba R, Sharov AA, Ko MS, Niwa H. 2007. Pluripotency governed by Sox2 via regulation of
  Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol 9:625-635.
- 665 34. Imada K, Leonard WJ. 2000. The Jak-STAT pathway. Mol Immunol 37:1-11.
- Bonni A, Sun Y, Nadal-Vicens M, Bhatt A, Frank DA, Rozovsky I, Stahl N, Yancopoulos GD,
  Greenberg ME. 1997. Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science 278:477-483.
- Sun Y, Nadal-Vicens M, Misono S, Lin MZ, Zubiaga A, Hua X, Fan G, Greenberg ME. 2001.
   Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. Cell
   104:365-376.
- Foshay KM, Gallicano GI. 2008. Regulation of Sox2 by STAT3 initiates commitment to the neural precursor cell fate. Stem Cells Dev 17:269-278.
- Bern W, Jin Y, Chen J, Rottier RJ, Steel KP, Kiernan AE. 2013. Ectopic expression of activated notch or SOX2 reveals similar and unique roles in the development of the sensory cell progenitors in the mammalian inner ear. J Neurosci 33:16146-16157.
- Heider JA, Bresnahan WA, Shenk TE. 2002. Construction of a rationally designed human cytomegalovirus variant encoding a temperature-sensitive immediate-early 2 protein. Proc Natl Acad Sci U S A 99:3141-3146.
- 40. Marchini A, Liu H, Zhu H. 2001. Human cytomegalovirus with IE-2 (UL122) deleted fails to express early lytic genes. J Virol 75:1870-1878.
- 41. Reitsma JM, Sato H, Nevels M, Terhune SS, Paulus C. 2013. Human cytomegalovirus IE1 protein disrupts interleukin-6 signaling by sequestering STAT3 in the nucleus. J Virol 87:10763-10776.
- 42. Harwardt T, Lukas S, Zenger M, Reitberger T, Danzer D, Übner T, Munday DC, Nevels M,
  Paulus C. 2016. Human Cytomegalovirus Immediate-Early 1 Protein Rewires Upstream STAT3 to
  Downstream STAT1 Signaling Switching an IL6-Type to an IFNgamma-Like Response. PLoS Pathog
  12:e1005748.
- 688 43. Reich NC. 2013. STATs get their move on. JAKSTAT 2:e27080.
- Fan G, Martinowich K, Chin MH, He F, Fouse SD, Hutnick L, Hattori D, Ge W, Shen Y, Wu H,
  ten Hoeve J, Shuai K, Sun YE. 2005. DNA methylation controls the timing of astrogliogenesis
  through regulation of JAK-STAT signaling. Development 132:3345-3356.
- Miller FD, Gauthier AS. 2007. Timing is everything: making neurons versus glia in the developing cortex. Neuron 54:357-369.
- 69446.Shin DS, Kim HN, Shin KD, Yoon YJ, Kim SJ, Han DC, Kwon BM. 2009. Cryptotanshinone695inhibits constitutive signal transducer and activator of transcription 3 function through blocking the696dimerization in DU145 prostate cancer cells. Cancer Res 69:193-202.
- 697 47. Onishi K, Zandstra PW. 2015. LIF signaling in stem cells and development. Development 142:2230698 2236.
- 699 48. Biran J, Tahor M, Wircer E, Levkowitz G. 2015. Role of developmental factors in hypothalamic

700		function. Front Neuroanat 9:47.
701	49.	Guerrini R, Parrini E. 2010. Neuronal migration disorders. Neurobiol Dis 38:154-166.
702	50.	Hofman MA. 2014. Evolution of the human brain: when bigger is better. Front Neuroanat 8:15.
703	51.	Fang L, Zhang L, Wei W, Jin X, Wang P, Tong Y, Li J, Du JX, Wong J. 2014. A methylation-
704		phosphorylation switch determines Sox2 stability and function in ESC maintenance or differentiation.
705		Mol Cell <b>55:</b> 537-551.
706	52.	Noris E, Zannetti C, Demurtas A, Sinclair J, De Andrea M, Gariglio M, Landolfo S. 2002. Cell
707		cycle arrest by human cytomegalovirus 86-kDa IE2 protein resembles premature senescence. J Virol
708		<b>76:</b> 12135-12148.
709	53.	Spector DH. 1996. Activation and regulation of human cytomegalovirus early genes. Intervirology
710		<b>39:</b> 361-377.
711	54.	Stinski MF. Meier JL, 2007. Immediate-early viral gene regulation and function. In Arvin A.
712	• • •	Campadelli-Fiume G Mocarski E Moore PS Roizman B Whitley R Yamanishi K (ed.) Human
713		Herpesviruses: Biology Therapy and Immunoprophylaxis Cambridge
714	55	Soroceanu I. Matlaf I. Khan S. Akhavan A. Singer F. Bezrookove V. Decker S. Ghanny S.
715	55.	Hadaczek P Bengtsson H Ohlfest I Luciani-Torres MC Harkins I Perry A Guo H
716		Sateronoulos P Cabbs CS 2015 Cytomegalovirus Immediate-Farly Proteins Promote Stemness
717		Properties in Clichlastoma Cancer Des 75:2065-2076
719	56	Formare O Partol: I. Ir. Dahbar A. Odabarg I. Khan 7. Davida I. Hamarlik D. Davida I.
710	50.	Formara O, Dartek J, JL, Kanbar A, Oueberg J, Khan Z, Fereuo I, Hamerik F, Dartek J, Stragliotta C, Londomuri N, Sodorborg Nouslar C, 2016, Cutomogolovinus infaction induces a storm
719		Stragnotto G, Landazuri N, Soderberg-Naucier C. 2010. Cytomegalovirus infection induces a stem
720		cell phenotype in numan primary ghobiastoma cells: prognostic significance and biological impact. Cell
721	<i>с</i> <b>л</b>	Death Differ $23:201-209$ .
122	57.	Fu YR, Liu XJ, Li XJ, Shen ZZ, Yang B, Wu CC, Li JF, Miao LF, Ye HQ, Qiao GH, Rayner S,
123		Chavanas S, Davrinche C, Britt WJ, Iang Q, McVoy M, Mocarski E, Luo MH. 2015. MicroRNA
724		miR-21 attenuates human cytomegalovirus replication in neural cells by targeting Cdc25a. J Virol
725		<b>89:</b> 1070-1082.
726	58.	Schwartz PH, Bryant PJ, Fuja TJ, Su H, O'Dowd DK, Klassen H. 2003. Isolation and
727		characterization of neural progenitor cells from post-mortem human cortex. J Neurosci Res 74:838-851.
728	59.	Knoblach T, Grandel B, Seiler J, Nevels M, Paulus C. 2011. Human cytomegalovirus IE1 protein
729		elicits a type II interferon-like host cell response that depends on activated STAT1 but not interferon-
730		gamma. PLoS Pathog 7:e1002016.
731	60.	Shen ZZ, Pan X, Miao LF, Ye HQ, Chavanas S, Davrinche C, McVoy M, Luo MH. 2014.
732		Comprehensive analysis of human cytomegalovirus microRNA expression during lytic and quiescent
733		infection. PLoS One 9:e88531.
734	61.	Liu XJ, Yang B, Huang SN, Wu CC, Li XJ, Cheng S, Jiang X, Hu F, Ming YZ, Nevels M, Britt
735		WJ, Rayner S, Tang Q, Zeng WB, Zhao F, Luo MH. 2017. Human cytomegalovirus IE1
736		downregulates Hes1 in neural progenitor cells as a potential E3 ubiquitin ligase. PLoS Pathog
737		<b>13:</b> e1006542.
738	62.	Fortunato EA, Dell'Aquila ML, Spector DH. 2000. Specific chromosome 1 breaks induced by human
739		cytomegalovirus. Proc Natl Acad Sci U S A 97:853-858.
740	63.	Tiscornia G. Singer O. Verma IM. 2006. Production and purification of lentiviral vectors. Nat Protoc
741		1:241-245.
742	64.	Duan Y. Miao L. Ye H. Yang C. Fu B. Schwartz PH. Ravner S. Fortunato EA. Luo MH. 2012. A
743		faster immunofluorescence assay for tracking infection progress of human cytomegalovirus. Acta
744		Biochim Biophys Sin (Shanghai) 44:597-605.
745	65	Luo MH. Rosenke K. Czornak K. Fortunato EA 2007 Human cytomegalovirus disrupts both ataxia
746	55.	telangiectasia mutated protein (ATM)- and ATM-Rad3-related kinase-mediated DNA damage responses
747		during lytic infection I Virol <b>81</b> ·1934-1950
7/8		anng 1700 milouon, 9 + noi 010175 + 1750.
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#### 749 FIGURE LEGENDS

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Figure 1. HCMV infection down-regulates SOX2 at the mRNA and protein level in 751 NPCs. NPC monolayers were mock (M)- or virus (V)-infected with HCMV (TNWT) at an 752 MOI of 3 and collected at the indicated times post infection for mRNA or protein analyses. 753 (A) SOX2 mRNA levels during HCMV infection of NPCs. The levels of SOX2 mRNA 754 normalized to GAPDH were determined by qRT-PCR at 4 to 120 hpi. Log<sub>10</sub> values of virus-755 to-mock (V/M) ratios are given for each time point. Results shown are average ± standard 756 deviation (SD) of data from three independent experiments, each conducted in triplicate. (B) 757 SOX2 and viral protein levels during HCMV infection of NPCs. SOX2, IE1/IE2, UL44, and 758 759 gB steady-state protein levels were determined by Western blotting at 4 to 96 hpi. Actin served as a loading control. The values listed below the blots indicate the relative SOX2 760 protein levels compared to corresponding mock controls following actin normalization. ND, 761 not detectable. (C) Cellular distribution of SOX2 in relation to viral replication compartments 762 during HCMV infection of NPCs. The distributions of SOX2 and UL44 were determined by 763 indirect immunofluorescence assay at 12 to 48 hpi. NPCs grown on poly-D-lysine-coated 764 coverslips were stained with antibodies against SOX2 (green) and UL44 (red), nuclei were 765 counterstained with Hoechst 33342 (blue). Phase contrast images are also shown. Scale bars, 766 5 μm. 767

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**Figure 2.** *De novo* synthesis of HCMV proteins is required for SOX2 down-regulation in NPCs. Comparison of HCMV infections with active virus (V) and UV-inactivated virus (UV). NPCs were mock-infected (M) or infected with active or UV-irradiated TNWT at an MOI of 3 and collected at the indicated times post infection. (A) SOX2 mRNA levels normalized to GAPDH were determined by qRT-PCR. Log<sub>10</sub> values of inactivated virus-to-

mock (UV/M) and active virus-to-mock (V/M) ratios are shown. Data from three independent 774 experiments were analyzed by one way ANOVA, and results are presented as average  $\pm$  SD. 775 \*\*,  $p \le 0.01$ . (B) Levels of SOX2 and representative viral proteins (IE1/IE2, UL44, and gB) 776 were determined by Western blotting. Actin served as a loading control. The values listed 777 778 below the blots indicate the relative SOX2 protein levels compared to corresponding 779 mock controls following actin normalization. Data were from three independent experiments, results are presented as average  $\pm$  SD. \*,  $p \le 0.05$ ; \*\*,  $p \le 0.01$ . (C) Effect of protein synthesis 780 inhibition by CHX treatment on SOX2 mRNA levels. NPCs were pretreated with CHX for 1 h 781 782 prior to infection, and then mock (M)- or virus (V)-infected with HCMV (TNWT) at an MOI of 3. Cells were collected at 16 hpi for analysis of SOX2 mRNA by qRT-PCR. Data from 783 three independent experiments were analyzed by one way ANOVA, and results are presented 784 785 as average  $\pm$  SD. \*\*, p  $\leq$  0.01.

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Figure 3. HCMV major IE proteins down-regulate SOX2 mRNA and protein in NPCs. 787 HCMV pp65, IE1, IE2 or IE1 and IE2 combined were transiently expressed in NPCs 788 789 following nucleofection. Samples were collected at 48 h post transfection for mRNA (qRT-PCR) or protein (Western blotting) analysis. Actin served as a loading control. The relative 790 level of SOX2 protein compared to corresponding controls following actin normalization. 791 Data from three independent experiments were analyzed by one way ANOVA, and results are 792 presented as average  $\pm$  SD. \*, p < 0.05; \*\*, p < 0.01. (A) Effect of pp65 and major IE proteins 793 794 (IE1 and IE2) on SOX2 protein levels. Left: NPCs were transfected with 5 µg pcDNA3.0 (vector) or pcDNA3-pp65. Right: NPCs were transfected with 5 µg pcDNA3-pp65 as control 795 796 (vector) or pSVH (IE1 and IE2). (B) Effect of IE1 on SOX2 mRNA and protein levels. NPCs were transfected with 5 µg pcDNA3-pp65 (vector), or 2 to 5 µg pcDNA3-IE1. (C) Effect of 797 IE2 on SOX2 mRNA and protein levels. NPCs were transfected with 5 µg pcDNA3-pp65 798

799 (vector), or 2 to 5  $\mu$ g pcDNA3-IE2.

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Figure 4. IE1 knock-down attenuates HCMV-induced SOX2 down-regulation in NPCs. 801 (A) IE1-directed knock-down efficiency of candidate shRNAs in HEL cells. HEL cells were 802 transduced with lentiviruses expressing shRNA-IE1-1 (sh-1), shRNA-IE1-2 (sh-2), shRNA-803 IE1-3 (sh-3), or shRNA-scramble (scr). At 48 h post transduction, cells were infected with 804 HCMV (TNWT) at an MOI of 1 and collected at 24 or 48 hpi. IE1 protein levels were 805 determined by Western blotting. Actin served as a loading control. (B) IE1-directed knock-806 down efficiency of sh-2 in HEL cells. HEL cells transduced with sh-2 or scr expressing 807 808 lentiviruses were infected with HCMV (TNWT) as in (A). Left: IE1 mRNA levels were determined by qRT-PCR at the indicated times post infection; data from two independent 809 experiments were analyzed by one way ANOVA, and results are presented as average  $\pm$  SD. 810 \*,  $p \le 0.05$ ; \*\*,  $p \le 0.01$ . Right: protein levels were determined accordingly at the indicated 811 times by Western blotting. (C) Effect of sh-2 on IE1 and SOX2 expression in NPCs. NPCs 812 were transduced with sh-2 or scr expressing lentiviruses, cultured for 48 h, reseeded at a 813 density of  $3 \times 10^6$  cells/dish, and mock (M)- or virus (V)-infected with TNWT at an MOI of 1. 814 Protein levels of IE1, UL44, gB and SOX2 at the indicated times post infection are shown. 815 816 Actin served as a loading control.

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Figure 5. HCMV infection or IE1 expression inhibit STAT3 tyrosine phosphorylation and promote nuclear accumulation of unphosphorylated STAT3 in NPCs. (A) Inhibition of STAT3 tyrosine (Y705) phosphorylation by HCMV infection. NPCs were mock (M)- or virus (V)-infected with TNWT at an MOI of 3 and collected at the indicated times post infection. The protein levels of IE1, pSTAT3 and total STAT3 were determined by Western blotting. Actin served as a loading control. (B) Trapping STAT3 in nuclear by HCMV infection. NPCs were mock (M)- or virus (V)-infected with TNWT at an MOI of 1. Left: For

indirect immunofluorescence analysis, NPCs on coverslips collected at 8 hpi were stained 825 826 with antibodies against STAT3 (green) or IE1/IE2 (red), and nuclei were counterstained with Hoechst 33342 (blue). Infected (IE1/IE2 positive) cell is indicated with a white arrow. Scale 827 828 bar, 10 µm. Right: For cellular fractionation analysis, fractions enriched in cytoplasmic (Cyt) or nuclear (Nuc) proteins were prepared from cells collected at 4 or 8 hpi. Protein levels of 829 830 pSTAT3 and total STAT3 in each fraction were determined by Western blotting. GAPDH and 831 lamin B1 served as controls for the Cyt and Nuc fraction, respectively. (C, D and E) Inhibition of tyrosine phosphorylation and nuclear sequestration of unphosphorylated STAT3 by IE1. 832 833 Fractions enriched in cytosolic (Cyt) or nuclear (Nuc) proteins or total cell extracts were prepared. (C) For transfection analysis, NPCs were transfected with pcDNA3-IE1 or 834 empty vector (Ctrl). Protein levels of IE1, pSTAT3, and total STAT3 were determined by 835 Western blotting. GAPDH and lamin B1 served as controls for the Cyt and Nuc fraction, 836 respectively. For HCMV infection analysis, NPCs were mock-infected (M) or infected with 837 838 TNWT, TNdIIE1, or TNrvIE1 viruses at an MOI of 10. The levels of the indicated viral and cellular proteins in whole cell extracts (D, E) or in the Cyt and Nuc fractions (F) were 839 determined by Western blotting. Actin, GAPDH and lamin B1 served as controls for total 840 841 extracts or Cyt and Nuc fractions, respectively.

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Figure 6. SOX2 expression strictly depends on pSTAT3, and IE1 mediates SOX2 depletion by inhibiting STAT3 activation. (A) Inhibition of STAT3 correlates with suppression of SOX2 expression. NPCs were treated with the chemical inhibitor cryptotanshinone (CTS) for the indicated times. The protein levels of total STAT3, pSTAT3, and SOX2 were determined by Western blotting. Actin served as a loading control. Und, undetectable. (B) Silencing of STAT3 correlates with suppression of SOX2 expression. NPCs were transduced with lentiviruses Tet-pLKO-puro-shLuci (shLuci), Tet-pLKO-puro-shDsRed

(shDsRed), Tet-pLKO-puro-shSTAT3-1 (shSTAT3-1) Tet-pLKO-puro-shSTAT3-2 850 or (shSTAT3-2) and treated with doxycycline for 48 h to induce shRNA expression. The protein 851 levels of total STAT3, pSTAT3, and SOX2 were determined by Western blotting. Actin served 852 as a loading control. (C) IL-6-mediated activation STAT3 correlates with induction of SOX2 853 expression. NPCs were treated with IL-6 for 2 or 24 h. The protein levels of pSTAT3, total 854 STAT3 and SOX2 were monitored by Western blotting. Actin served as a loading control. 855 856 Data from three independent experiments were analyzed by one way ANOVA, and results are presented as average  $\pm$  SD. \*, p  $\leq$  0.05; \*\*, p  $\leq$  0.01. (D) IL-6 counteracts IE1-dependent 857 858 SOX2 down-regulation in transiently transfected NPCs. NPCs were transfected with pcDNA3-IE1, treated with IL-6 for 4 h or left untreated and collected at 48 h post 859 transfection. The protein levels of IE1, total STAT3, pSTAT3, and SOX2 were determined by 860 Western blotting. Actin served as a loading control. Data from three independent experiments 861 were analyzed by one way ANOVA, and results are presented as average  $\pm$  SD. \*, p  $\leq$  0.05; 862 \*\*, p ≤ 0.01. (E) IL-6 counteracts IE1-dependent SOX2 down-regulation in HCMV-infected 863 NPCs. NPCs were infected with TNWT or TNdlIE1 viruses at an MOI of 10, treated with IL-864 6 for 4 h or left untreated, and collected at 48 hpi. The protein levels of IE1/IE2, total STAT3, 865 866 pSTAT3, and SOX2 were determined by Western blotting. Actin served as a loading control. Data from three independent experiments were analyzed by one way ANOVA, and results are 867 presented as average  $\pm$  SD. \*, p  $\leq$  0.05; \*\*, p  $\leq$  0.01. 868

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Purpose	Name	Sequence (5'-3')
IE1	scr F	CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTT
knock-do		AACCTTAGGTTTTTG
wn	scr R	AATTCAAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAG
		CGACTTAACCTTAGG
	sh-1 F	CCGGGCATGTATGAGAACTACATTGCTCGAGCAATGTAGTTCTC
		ATACATGCTTTTTG
	sh-1 R	AATTCAAAAAGCATGTATGAGAACTACATTGCTCGAGCAATGT
		AGTTCTCATACATGC
	sh-2 F	CCGGGCTGTGCTGCTATGTCTTAGACTCGAGTCTAAGACATAGC
		AGCACAGCTTTTTG
	sh-2 R	AATTCAAAAAGCTGTGCTGCTATGTCTTAGACTCGAGTCTAAGA
		CATAGCAGCACAGC
	sh-3 F	CCGGGCCTGAGGTTATCAGTGTAATCTCGAGATTACACTGATAA
		CCTCAGGCTTTTTG
	sh-3 R	AATTCAAAAAGCCTGAGGTTATCAGTGTAATCTCGAGATTACA
		CTGATAACCTCAGGC
STAT3	#1102	CCGGGCCTCAAGATTGACCTAGACTCGAGTCTAGGTCAATCTTG
knock-do		AGGCTTTTT
wn	#1103	AATTAAAAAGCCTCAAGATTGACCTAGACTCGAGTCTAGGTCA
		ATCTTGAGGC
	#1134	CCGGAGTCAGGTTGCTGGTCAAACTCGAGTTTGACCAGCAACC
		TGACTTTTTT
	#1135	AATTAAAAAAGTCAGGTTGCTGGTCAAACTCGAGTTTGACCAG
		CAACCTGACT
	#1098	CCGGGTGCGTTGCTAGTACCAACCTCGAGGTTGGTACTAGCAA
		CGCACTTTTT
	#1099	AATTAAAAAGTGCGTTGCTAGTACCAACCTCGAGGTTGGTACT
		AGCAACGCAC
	#1138	CCGGGTGGGAGCGCGTGATGAACCTCGAGGTTCATCACGCGCT
		CCCACTTTTT
	#1139	AATTAAAAAGTGGGAGCGCGTGATGAACCTCGAGGTTCATCAC
		GCGCTCCCAC
PCR	pp65 F	CGCGGATCCATGGAGTCGCGCGGTCGCCGT
	pp65 R	CCGGAATTCTCAACCTCGGTGCTTTTTGGG
qRT-PC	SOX2 F	GCCGAGTGGAAACTTTTGTCG
R	SOX2 R	GCAGCGTGTACTTATCCTTCTT
	GAPDH F	GAGTCAACGGATTTGGTCGT
	GAPDH	GACAAGCTTCCCGTTCTCAG
	R	

Table 1. Oligonucleotides used in this study.













Α

































Nuc Et l Ш Lamin B1

Ε



D



F



# Figure 6 A

