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Epstein-Barr Virus Nuclear Antigen 1 (EBNA1) interacts with Regulator of Chromosome Condensation (RCC1) dynamically throughout the cell cycle --Manuscript Draft--

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Abstract:	<p>The Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1) is a sequence-specific DNA binding protein which plays an essential role in viral episome replication and segregation, by recruiting the cellular complex of DNA replication onto the origin (oriP) and by tethering the viral DNA onto the mitotic chromosomes. Whereas the mechanisms of viral DNA replication are well documented, those involved in tethering EBNA1 to the cellular chromatin are far from being understood. Here, we have identified Regulator of Chromosome Condensation 1 (RCC1) as a novel cellular partner for EBNA1. RCC1 is the major nuclear guanine nucleotide exchange factor (RanGEF) for the small GTPase Ran enzyme. RCC1, associated with chromatin, is involved in the formation of RanGTP gradients critical for nucleo-cytoplasmic transport, mitotic spindle formation, and nuclear envelope reassembly following mitosis. Using several approaches, we have demonstrated a direct interaction between these two proteins and found that the EBNA1 domains responsible for EBNA1 tethering to the mitotic chromosomes are also involved in the interaction with RCC1. The use of an EBNA1 peptide array confirmed the interaction of RCC1 with these regions and also the importance of the N-terminal region of RCC1 in this interaction. Finally, using confocal microscopy and FRET analysis to follow the dynamics of interaction between the two proteins throughout the cell cycle, we have demonstrated that EBNA1 and RCC1 closely associate on the chromosomes during metaphase, suggesting an essential role for the interaction during this phase, perhaps in tethering EBNA1 to mitotic chromosomes.</p>

1 **Epstein-Barr Virus Nuclear Antigen 1 (EBNA1) interacts with Regulator of**
2 **Chromosome Condensation (RCC1) dynamically throughout the cell cycle**

3

4 **Running title:** EBNA1 localizes with RCC1 to chromatin during mitosis

5

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39 **ABSTRACT**

40 The Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1) is a sequence-specific
41 DNA binding protein which plays an essential role in viral episome replication and
42 segregation, by recruiting the cellular complex of DNA replication onto the origin (*oriP*)
43 and by tethering the viral DNA onto the mitotic chromosomes. Whereas the mechanisms
44 of viral DNA replication are well documented, those involved in tethering EBNA1 to the
45 cellular chromatin are far from being understood. Here, we have identified Regulator of
46 Chromosome Condensation 1 (RCC1) as a novel cellular partner for EBNA1. RCC1 is
47 the major nuclear guanine nucleotide exchange factor (RanGEF) for the small GTPase
48 Ran enzyme. RCC1, associated with chromatin, is involved in the formation of RanGTP
49 gradients critical for nucleo-cytoplasmic transport, mitotic spindle formation, and
50 nuclear envelope reassembly following mitosis. Using several approaches, we have
51 demonstrated a direct interaction between these two proteins and found that the EBNA1
52 domains responsible for EBNA1 tethering to the mitotic chromosomes are also involved
53 in the interaction with RCC1. The use of an EBNA1 peptide array confirmed the
54 interaction of RCC1 with these regions and also the importance of the N-terminal region
55 of RCC1 in this interaction. Finally, using confocal microscopy and FRET analysis to
56 follow the dynamics of interaction between the two proteins throughout the cell cycle, we
57 have demonstrated that EBNA1 and RCC1 closely associate on the chromosomes during
58 metaphase, suggesting an essential role for the interaction during this phase, perhaps in
59 tethering EBNA1 to mitotic chromosomes.

60

61 **INTRODUCTION**

62 Epstein-Barr virus (EBV) is a ubiquitous herpesvirus associated with several human cancers
63 (Crawford, 2001). Following primary infection, the virus persists in a life-long, latent state in

64 memory B cells, with intermittent viral production occurring in the oropharynx. *Ex vivo*, EBV
65 has the capacity to induce growth transformation of resting primary human B-lymphocytes,
66 leading to the establishment of lymphoblastoid cell lines (LCLs). In such cell lines, only a
67 small number of viral genes are expressed which act in concert to induce and maintain
68 continuous cell proliferation and survival (Kieff & Rickinson, 2007).

69 In latently infected cells, the EBV genome persists as a multicopy, covalently closed,
70 double-stranded, nuclear episome. When cells proliferate, these episomes replicate once per
71 cell-cycle during S phase, using the cellular DNA-replication machinery and are subsequently
72 equally segregated to the daughter cells, such that a constant copy number of EBV genomes is
73 maintained through cell divisions (Adams, 1987; Nanbo *et al.*, 2007; Yates & Guan, 1991).
74 Both replication and segregation depend on the presence of two viral elements, the EBV *cis*-
75 acting origin of plasmid replication (*oriP*) and the viral protein EBNA1 (Yates *et al.*, 1984,
76 1985). *oriP* is composed of two functional elements: the dyad symmetry (DS) element and the
77 family of repeats (FR) (Reisman *et al.*, 1985). Both contain recognition sites for the EBNA1
78 protein. The DS element, which comprises four EBNA1 binding sites arranged in pairs, is
79 required for DNA replication initiation (Rawlins *et al.*, 1985; Reisman *et al.*, 1985;
80 Wysokenski & Yates, 1989; Chaudhuri *et al.*, 2001; Ritzi *et al.*, 2003; Schepers *et al.*, 2001).
81 The FR element consists of an array of twenty imperfect 30 bp repeats, each one containing
82 an 18 bp EBNA1 binding site (Rawlins *et al.*, 1985; Reisman *et al.*, 1985). FR functions by
83 tethering the viral episomes to human metaphase chromosomes via EBNA1 (Sears *et al.*,
84 2003, 2004; Wu *et al.*, 2000, 2002) and this ensures the stable retention of *oriP*-episomes
85 within the cells (Kirchmaier & Sugden, 1995; Little & Schildkraut, 1995; Nanbo *et al.*, 2007).
86 FR is also required for EBNA1-dependent tethering of EBV genomes to specific
87 perichromatic regions of host chromosomes during interphase (Deutsch *et al.*, 2010) which
88 appears to be essential for efficient replication of the episome (Hodin *et al.*, 2013).

89 EBNA1 is a homo-dimeric DNA-binding protein that recognises an 18 bp palindromic
90 sequence *via* its C-terminal domain (residues 459-607 have been co-crystallised with DNA)
91 (Ambinder *et al.*, 1990, 1991; Bochkarev *et al.*, 1996; Frappier & O'Donnell, 1991; Jones *et*
92 *al.*, 1989; Rawlins *et al.*, 1985; Shah *et al.*, 1992). ChIPseq analyses have shown that, in
93 addition to binding the DS and FR regions of *oriP*, EBNA1 binds multiple sites in the host
94 genome (Lu *et al.*, 2010; Tempera *et al.*, 2015). Independently of its C-terminal-specific
95 DNA-binding domain, EBNA1 can associate with chromatin throughout the cell cycle via its
96 N-terminal half. This N-terminal region carries two domains, called Linking Regions 1 (LR1:
97 aa 40-89) and 2 (LR2: aa 325-379) which confer intramolecular “linking” between EBNA1-
98 DNA complexes as revealed by electrophoretic mobility shift assays (Mackey *et al.*, 1995).
99 Each of these domains consists of a region rich in arginine and glycine (RGG-rich region:
100 GR1 and GR2 respectively) and a unique region (UR1 and UR2 respectively). The RGG-rich
101 regions possess intrinsic AT-hook activity allowing binding to AT-rich DNA (Sears *et al.*,
102 2003, 2004). These regions have been found to be important for EBNA1 replication and
103 transcription activity (Mackey & Sugden, 1999) and to play an essential role in tethering
104 EBNA1 to cellular DNA during interphase (Coppotelli *et al.*, 2011; Coppotelli *et al.*, 2013).
105 EBNA1 attachment to metaphase chromosomes has been mapped to three independent
106 chromosome binding sites (CBS) - CBS-1 (aa 72 to 84), CBS-2 (aa 328 to 365) and CBS-3
107 (aa 8 to 54) - that correlate well with the ability of EBNA1 to confer plasmid maintenance
108 (Kanda *et al.*, 2001; Maréchal *et al.*, 1999; Wu *et al.*, 2002). However, the mechanisms
109 responsible for EBNA1 interaction with mitotic chromosomes are still unclear. It has been
110 proposed that the AT-hook structures within the LR1/LR2 regions could be directly
111 responsible for EBNA1 attachment to the chromosomes (Kanda *et al.*, 2013; Sears *et al.*,
112 2004). Interestingly, HMGA1a a cellular chromatin-binding protein which associates with
113 chromatin through its AT-hook domains, or histone H1, can functionally replace the amino

114 terminus of EBNA1 both in *oriP* plasmid replication and partitioning of the viral episome
115 (Hung *et al.*, 2001; Sears *et al.*, 2003; Thomae *et al.*, 2008). EBNA1 may also interact with
116 chromatin through protein-protein interactions with one or several cellular partners. hEBP2
117 (human Epstein-Barr Binding Protein 2) was the first of the sort identified. (Kapoor *et al.*,
118 2005; Nayyar *et al.*, 2009; Wu *et al.*, 2000). hEBP2 binds to the LR2 region of EBNA1 (Shire
119 *et al.*, 1999), which also corresponds to the CBS-2 region. In a yeast model, hEBP2 was
120 required (in the presence of EBNA1) for the maintenance of a plasmid carrying the EBV FR
121 sequence (Kapoor *et al.*, 2001, 2003). However, a recent study demonstrated that hEBP2 and
122 EBNA1 do not interact during mitosis in living mitotic cells, suggesting that the involvement
123 of hEBP2 might not be direct (Jourdan *et al.*, 2012; Frappier, 2012), or that it might have
124 another role. More recently, HMGB2 (high-mobility group box 2), a well known chromatin
125 component, has been identified as a new partner for EBNA1 (Jourdan *et al.*, 2012). EBNA1
126 interacts with HMGB2 on chromatin during interphase and mitosis, and its depletion partially
127 alters EBNA1 association with the chromosomes. However, HMGB2 depletion is not
128 sufficient to alter EBV episome maintenance in Raji cells (Jourdan *et al.*, 2012). Taken
129 together, these results suggest that several mechanisms cooperate to promote EBNA1
130 association with the chromosomes throughout mitosis and maintenance of the EBV genome
131 within proliferating cells.

132 In order to identify novel proteins that could play a role in EBNA1 chromosomal
133 binding, we performed a yeast two-hybrid screen. From this screen, we identified Regulator
134 of Chromosome Condensation 1 (RCC1), a major nuclear guanine nucleotide exchange factor
135 (RanGEF) for the small GTPase Ran enzyme. In its association with chromatin, RCC1 is
136 involved in the formation of RanGTP gradients critical for nucleo-cytoplasmic transport
137 (Riddick & Macara, 2005), mitotic spindle formation and nuclear envelope reassembly after
138 mitosis (Askjaer *et al.*, 2002; Bamba *et al.*, 2002). RCC1 is a ubiquitous nuclear protein

139 structured as a seven bladed propeller with unstructured small N- and C-terminal tails
140 (Renault *et al.*, 1998). RCC1 directly interacts with histones H2A/H2B (Nemergut *et al.*,
141 2001) and its structure (bound to Ran and the nucleosomes) has been solved: one face of the
142 protein binds to Ran (Renault *et al.*, 2001) whereas binding to chromatin involves the N-
143 terminal tail of the protein as well as loop region in the fourth blade of its β -propeller
144 (England *et al.*, 2010; Makde *et al.*, 2011). RCC1 is modified in cells by removal of the initial
145 N-terminal methionine and mono-, di- or tri-methylation of the new N-terminal residue
146 (serine 2 in human). This modification is present throughout the cell cycle and is necessary
147 for stable chromatin association and normal mitosis (Chen *et al.*, 2007). The association of
148 RCC1 with chromatin in interphase nuclei and mitotic chromosomes is highly dynamic
149 (Cushman *et al.*, 2004; Li *et al.*, 2003) and regulated by its interaction with Ran (Hao &
150 Macara, 2008; Zhang *et al.*, 2002). It is also regulated in a cell cycle dependent manner by
151 various mechanisms including interaction with Ran-GTP-binding protein 1 (RanBP1) (Zhang
152 *et al.*, 2014) and phosphoinositide 3-kinase beta (PI3K β) (Redondo-Muñoz *et al.*, 2015). A
153 role of phosphorylation of serine 2 at the N-terminus of RCC1 has also been suggested but
154 remains controversial (Bierbaum & Bastiaens, 2013; Hutchins *et al.*, 2004; Li & Zheng,
155 2004).

156 Due to its ability to interact with chromatin, especially through mitosis during which the
157 interaction is stabilized, RCC1 appears to be a good candidate to promote the association of
158 EBNA1 with chromatin. We have now confirmed the interaction between EBNA1 and RCC1
159 using various *in vitro* and *ex vivo* assays. We have demonstrated that this interaction is direct
160 and characterized the domains involved. Finally, we found that although the proteins
161 colocalized throughout the cell cycle, they only closely interact during metaphase, strongly
162 suggesting a role for RCC1 in stabilizing the interaction between EBNA1 and the chromatin
163 at this phase of the cell cycle.

164

165 **RESULTS**

166 **Deletion of EBNA1 AT-hook motifs only partially modify its localization to the** 167 **metaphasic chromosomes.**

168 EBNA1 has been suggested to directly bind to AT-rich regions of the chromosomal DNA via
169 AT-hook motifs located within the LR1/GR1 and LR2/GR2 regions (Sears *et al.*, 2004). In
170 particular, fusion proteins between mCherry and various combinations of the EBNA1 regions
171 containing AT-hook motifs, efficiently associated with chromosomes (Kanda *et al.*, 2013).
172 However, the effect of specific deletion of these AT-hook motifs - in the context of the whole
173 protein - on its association with cellular chromosomes has never been tested. We therefore
174 generated derivatives of GFP-EBNA1 with either aa 40 to 53 (deleting most of GR1) or 326
175 to 358 (deleting two thirds of GR2) or both regions deleted (Fig. 1A) and tested their capacity
176 to bind mitotic chromosomes and activate transcription. Association with chromosomes was
177 first analysed by confocal microscopy, following transfection of HeLa cells with expression
178 plasmids for GFP-EBNA1 or the mutated derivatives. Deletion of the GR regions led to the
179 appearance of a faint diffuse staining of the cell nuclei which was more accentuated in the
180 double mutant (Fig. 1B). However, even in the double mutant, a large proportion of the
181 protein remained localized to the metaphasic chromosomes. This suggests that the AT-hook
182 motifs are not the sole domains responsible for EBNA1 attachment to the chromosomes
183 during mitosis. Second, we performed a FRAP analysis during cell interphase to
184 comparatively evaluate the mobility of each protein. The half-time of fluorescence recovery
185 of the EBNA1 GR-deletion mutants, especially that of the double mutant, was strongly
186 diminished as compared with GR-wild type EBNA1, indicating a higher mobility of proteins
187 lacking the AT-hook motifs (Fig. 1C). Finally, to complete the characterization of these
188 mutants, we tested their transactivation ability since LR1 and LR2 regions were previously

189 reported to be important for transcriptional activation (Mackey & Sugden, 1999). The single
190 GR deletion mutants appear to activate LUC expression from the pGL2-FR-TK-LUC reporter
191 construct to similar levels as GR-wild type (Fig. 1D). By contrast the double mutant shows
192 significant reduction in transcriptional activation through FR. Taken together, these results
193 support a role for these two regions in transcriptional activation, but demonstrate that
194 although these regions appear to play an important role in chromatin association during
195 interphase, as could be deduced from the FRAP experiments, they are not absolutely essential
196 for tethering EBNA1 to the mitotic chromosomes.

197 **EBNA1 interacts directly with RCC1**

198 Since the AT-hook motifs, appear not to be essential for EBNA1 association with mitotic
199 chromatin, it is likely that one or more cellular partners are involved in mediating the linking
200 of EBNA1 with the chromosomes. The two cellular proteins which have been previously
201 found to play a role in this process - hEBP2 and HMGB2 - do not appear to be sufficient to
202 account for all the properties of EBNA1 during EBV replication and segregation. In order to
203 identify novel cellular partners of EBNA1, a yeast two-hybrid screen using EBNA1 as bait
204 was performed. From this screen, Regulator of Chromosome Condensation 1 (RCC1) (gene
205 ID: 1104), a guanine-nucleotide releasing factor that promotes exchange of Ran-bound GDP
206 with GTP, was identified. RCC1 plays a key role both in nucleo-cytoplasmic transport and in
207 the regulation of onset of chromosome condensation in S phase (Hadjebi *et al.*, 2008).

208 The interaction between EBNA1 and RCC1 was first examined by co-
209 immunoprecipitation from transfected HeLa cells (Fig. 2A). Myc-tagged RCC1 specifically
210 co-immunoprecipitated with Flag-tagged EBNA1. Consistently, in a reverse experiment,
211 Myc-tagged EBNA1 specifically co-immunoprecipitated with Flag-tagged RCC1.

212 To assess if the interaction is direct, an *in vitro* GST-pulldown assay was performed
213 using both GST-RCC1 and 6xhis-EBNA1 produced in bacteria and purified. 6xhis-EBNA1

214 was incubated with similar amounts of GST or GST-RCC1 proteins bound to glutathione
215 sepharose beads. EBNA1 was efficiently retained on GST-RCC1 beads but not on GST alone,
216 which strongly suggests that a direct interaction occurs between EBNA1 and RCC1 (Fig. 2B).

217 **EBNA1 binds RCC1 via domains previously reported to be essential for chromosome**
218 **binding of the protein**

219 EBNA1 interaction with mitotic chromosomes has been reported to be dependent on three
220 regions: CBS-1 (aa 72 to 84), CBS-2 (aa 328 to 365) and CBS-3 (aa 8 to 54) (Fig. 3A) (Kanda
221 *et al.*, 2001; Maréchal *et al.*, 1999; Wu *et al.*, 2002). We thus investigated the involvement of
222 these regions in the interaction with RCC1. For this, a series of GFP-tagged EBNA1 deletion
223 mutants were expressed in HeLa cells and the lysates incubated with GST-RCC1-bound
224 beads. Deletion mutants containing either CBS-1/CBS-3 (EBNA1 8-92), CBS-2 (EBNA1
225 323-410) or both (EBNA1 8-410) were all able to interact with RCC1, whereas EBNA1 377-
226 641 with both regions deleted, showed considerably reduced interaction (Fig. 3B). This
227 preferential interaction of RCC1 with the CBS domains of EBNA1 supports a putative role
228 for RCC1 in EBNA1's targeting to metaphase chromosomes. Surprisingly however, deletion
229 of region 326 to 376 completely abrogated the interaction with RCC1 (Fig. 3C), even though
230 the CBS-1/-3 domains still present in this mutant were sufficient for interaction with RCC1 in
231 mutant EBNA1 8-92 (Fig. 3B). This suggests that EBNA1 Δ 326-376 protein's general
232 topology may be altered such that the CBS-1/-3 domains are no longer accessible to interact
233 with RCC1.

234 We also tested the capacity of the GR-deleted mutants used in Figure 1, to interact with
235 RCC1 (Fig. 4). Although deletion of each region individually did not prohibit interaction with
236 RCC1, deletion of both motifs had a dramatic effect. This suggests that at least one intact GR
237 sub-region is required for the interaction with RCC1.

238 Taken together, these results indicate that the EBNA1 interaction domains with RCC1

239 overlap closely with the previously characterized EBNA1 chromosome binding regions.
240 However, it is to be noted that mutant $\Delta 40-53/\Delta 326-358$ with GR1 and most of GR2 deleted,
241 was still - at least partially - associated with the mitotic chromosome, whereas no interaction
242 with RCC1 could be detected in the GST-pulldown assay. Thus, although RCC1 is likely to
243 contribute to EBNA1 association with metaphasic chromosomes, it is probably not the only
244 factor involved.

245 **The RCC1 N-terminal tail is essential for RCC1 interaction with EBNA1**

246 RCC1 is composed of small N-terminal and C-terminal unstructured tails surrounding a
247 seven-bladed propeller structure (Makde *et al.*, 2011) (Fig. 5A). Due to the highly structured
248 central domain of the protein, introducing large mutations into this region would likely
249 disorder the entire structure. Therefore, a single RCC1 mutant, RCC1 $\Delta 1-20$, with the N-
250 terminal tail deleted, was generated. Further, the N-terminal tail was cloned in fusion with
251 GST. GST-pulldown using transfected HeLa cell extracts were performed with these proteins
252 (Fig. 5B). Interestingly, we found that EBNA1 interacted strongly with the N-terminal tail of
253 RCC1 and very inefficiently with the rest of the protein. To further map the interaction region,
254 smaller deletions within the N-terminal extremity of RCC1 were introduced. Deletion of aa
255 11-15 or 16-20 did not significantly modify the interaction with EBNA1, in contrast to
256 deletions of aa 1-5 and 6-10 which both impaired the interaction. These results suggest that
257 the 10 first amino acids of the RCC1 N-terminal tail are important for the interaction of RCC1
258 with EBNA1.

259 **Peptide array analysis confirms the RCC1 interaction with the CBS-3/-1 and CBS-2** 260 **domains and also defines potential supplementary RCC1 interaction regions in the C-** 261 **terminal moiety of the protein.**

262 With the aim to delimit the domains of EBNA1 involved in the interaction with RCC1 more
263 precisely, a peptide array analysis was undertaken. A library of overlapping peptides (25-

264 mers), each shifted by 5 amino acids across the entire sequence of EBNA1 (including the
265 GAR) was immobilised onto membranes and probed with recombinant GST-RCC1 full length
266 (FL) and mutants (Fig. 6). Probing the EBNA1 peptide array with FL GST-RCC1 revealed
267 intermediate to strong binding to peptides covering regions that encompass both GR repeats
268 of EBNA1 as well as CBS-1 (aa 72 to 84) and CBS-3 (aa 8 to 54), which is consistent with
269 our GST-pulldown mapping. It is interesting to note that the strongest interacting regions
270 overlap with the AT-hook domains whose deletion in mutant EBNA1 Δ 40-53/ Δ 326-358,
271 completely abrogates binding in our GST-pull down assay (Fig. 4). Thus, there is good
272 agreement between the GST-pull down assay and the peptide array analysis. Moreover, the
273 peptide analysis revealed the presence of unexpected binding regions in the central and C-
274 terminal moiety of EBNA1: a first region between aa G371-E435 lies between the known
275 binding sites for CK2 and USP7 and is well conserved in EBV EBNA1 isolates (Hussain *et*
276 *al.*, 2014); a second region incorporates the C-terminal tail of the protein, rich in negatively
277 charged residues. Reprobing the array with GST, indicated that the binding observed for GST-
278 RCC1 was specific to RCC1.

279 When a second array was probed with GST-RCC1 Δ 1-20, binding was observed for
280 largely the same set of peptides as for full length RCC1, but much weaker, with the notable
281 exception of the C-terminal tail peptides, which showed no binding. Therefore, consistent
282 with our GST-pulldown mapping, RCC1 with its N-terminal 20 aa deleted, only weakly
283 interacts with EBNA1. This suggests that either the N-terminal region of RCC1 is the primary
284 mediator of binding or that it is required for correct folding of full-length RCC1 to enable
285 binding to EBNA1, or possibly both.

286 To distinguish between these possibilities, the second array was stripped and re-probed
287 with just the N-terminal region of RCC1 fused to GST. GST-RCC1 1-20 showed intermediary
288 binding to the C-terminal tail peptides and strong binding to peptides covering region R396 to

289 E435. However, strong binding was not observed with peptides localised in the N-terminal
290 half of EBNA1 although these were strongly bound by full-length RCC1. This suggests that
291 residues 1-20 of RCC1 contribute to the conformation of RCC1, or otherwise promote the full
292 interaction, but may not completely comprise the binding site.

293 Regarding the strongest binding region identified for RCC1 1-20, analysis of the
294 EBNA1 amino acid sequence reveals that the stretch of residues in common between EBNA1
295 peptides interacting strongly with GST-RCC1 1-20 lies between aa 411 to 420
296 (EADYFEYHQE). This region contains 4 negatively-charged residues. By contrast, the N-
297 terminal region of RCC1 (MSPKRIAKRRSPPADAIPKS) contains 6 positively charged
298 residues and one negatively charged residue. It is therefore possible that the interaction of
299 RCC1 with these EBNA1 peptides is largely charge-based and possibly an artefact of the
300 array approach, if these stretches are not normally accessible in the folded protein. To explore
301 this possibility, a new array was generated with a series of mutated peptides spanning residue
302 401 to 430 and probed with GST-RCC1 1-20 (Supplementary Figure 1). This revealed that
303 indeed charge is critical to the binding, such that replacement of the 4 charged residues (E₄₁₁,
304 D₄₁₃, E₄₁₆, E₄₂₀) completely abrogated binding, however F₄₁₅ and Y₄₁₄ were also found to be
305 key for the interaction. This region was thus a candidate for being a core binding site between
306 the N-terminal region of RCC1 and EBNA1. However, an EBNA1 mutant deleted for this
307 region (EBNA1 Δ 411-420) still interacted with RCC1 in a GST-pulldown assay (Fig. 4B, lane
308 5). Therefore, it appears that this region is not required for stabilizing the EBNA1-RCC1
309 interaction *in vitro*. However, it cannot be ruled out that it might play a role in the interaction
310 *in vivo*, in a context where RCC1 is associated with chromatin.

311 **EBNA1 interacts with RCC1 localized to chromatin during mitosis**

312 In order to determine the subcellular localization of the two proteins in living cells,
313 several fluorescent-tagged forms of EBNA1 and RCC1 were expressed in HeLa cells and

314 observed by live cell imaging during interphase and mitosis. The EGFP-RCC1 and EBNA1-
315 RFP proteins colocalize almost perfectly in living cells during interphase and throughout
316 mitosis (Fig. 7): during interphase the proteins colocalize throughout the nucleoplasm, with
317 the exception of the nucleolus from which RCC1 is completely excluded and where weak
318 staining is observed for EBNA1. During prophase and metaphase, both EBNA1 and RCC1
319 appear to be associated with the mitotic chromosomes. Similar observations were made in
320 cells coexpressing other pairs of EGFP- and RFP-tagged forms of the proteins (data not
321 shown).

322 To confirm that EBNA1 interacts with RCC1 in living cells we performed a Förster
323 resonance energy transfer (FRET) analysis. FRET is a nonradioactive energy transfer that can
324 occur when a donor and a compatible acceptor fluorophore are located at less than 10 nm
325 from each other. FRET efficiency relies on the relative position and distance of the donor and
326 acceptor fluorophores, which can be affected by the position of the fluorophore in a fusion
327 protein. Therefore, both the following pairs of fusion proteins: EGFP-RCC1/EBNA1-RFP and
328 EGFP-EBNA1/RFP-RCC1 were tested. No significant FRET was observed with the EGFP-
329 RCC1/EBNA1-RFP pair. However, the EGFP-EBNA1/RFP-RCC1 pair revealed clear FRET
330 activity during both interphase and metaphase (Fig. 8). During interphase, it is interesting to
331 note that although the proteins colocalize throughout the nucleoplasm (Fig. 7), they were only
332 in close interaction at the periphery of the nucleus (Fig. 8). Only very weak interaction was
333 observed during prophase. By contrast, strong interaction was observed between the two
334 proteins on metaphasic chromosomes. Taken together, these results suggest that the
335 interaction between RCC1 and EBNA1 is highly dynamic through the cell cycle. The strong
336 FRET signal observed specifically on metaphasic chromosomes supports a role for RCC1 in
337 stabilizing the EBNA1 interaction with the chromosomes during mitosis.

338

339 **DISCUSSION**

340 The mechanisms by which EBNA1 tethers the EBV genome to mitotic chromosomes are far
341 from understood. The AT-hook regions of the protein have been proposed to play a major role
342 in EBNA1 chromosome binding activity and episomal maintenance (Sears *et al.*, 2003, 2004).
343 The use of netropsin, a small molecule that binds to the minor groove of AT-rich DNA, leads
344 to the loss of EBV genomes from cells, supporting the role of the AT-hooks in episomal
345 maintenance (Chakravorty & Sugden, 2015). However, we have found that deletion of the
346 EBNA1 AT-hook regions does not abrogate EBNA1's general targeting to metaphasic
347 chromosomes. This result is consistent with a previous analysis revealing three independent
348 CBS regions (Marechal *et al.*, 1999). In effect, specific deletion of the AT-hook domains
349 leaves CBS-1 (aa 72 to 84) intact. Therefore these data reinforce the idea of alternative or
350 complementary mechanisms of recruitment of EBNA1 to the metaphasic chromosomes,
351 possibly *via* the interaction with cellular chromatin binding factors.

352 Here, we have identified RCC1 as a novel mediator of EBNA1 interaction with
353 metaphase chromosomes. We have characterized the interaction between EBNA1 and RCC1
354 by various methods. Importantly, by performing an *in vitro* assay using both proteins purified
355 from bacteria, we have demonstrated that the two proteins can interact directly. Up to now,
356 however, we have not been able to perform a successful co-immunoprecipitation with
357 endogenous proteins. One explanation, other than a possible interference of antibodies with
358 the interaction and the low level of expression of EBNA1, is the small amount of cells from
359 the total population undergoing mitosis - the phase of the cell cycle in which our FRET
360 experiments demonstrate a close interaction between the proteins.

361 Characterization of the interaction domains revealed that the RCC1 interaction domains
362 of EBNA1 closely overlap with the CBS regions of the protein, known to be important for
363 tethering EBNA1 to the chromosomes (Maréchal *et al.*, 1999). Accordingly, we have found

364 that region 8-92 which includes both CBS-3 and CBS-1, and region 323-410 which includes
365 CBS-2, can interact independently with RCC1. Surprisingly, however, deletion of CBS-2 in
366 mutant Δ 326-376 previously found to impair hEBP2 interaction as well as *oriP* plasmid
367 maintenance and mitotic localization (Shire *et al.*, 1999; Wu *et al.*, 2000), completely
368 abolished EBNA1 interaction with RCC1. This latter result is not consistent with the finding
369 that the CBS-3/-1 region (still present in this mutant) is sufficient alone to mediate the
370 interaction with RCC1. This suggests that the conformation or accessibility of the CBS-3/-1
371 region is compromised in this mutant, affecting various functions of the protein without
372 necessarily reflecting the direct involvement of the deleted region.

373 Since the EBNA1 mutant with the two AT-hook domains deleted, appears to localize to
374 chromatin both during interphase and through mitosis (which had not previously been tested),
375 mechanisms other than the interaction of EBNA1 with AT-rich regions of DNA are likely to
376 be required. However, deletion of the two AT-hook domains also affected the interaction with
377 RCC1, suggesting that still other factors are involved. Another chromatin binding protein,
378 HMGB2, was previously identified as an EBNA1 interacting factor (Jourdan *et al.*, 2012).
379 HMGB2 could thus be responsible for targeting EBNA1 to the chromatin, in the absence of
380 both direct interaction with DNA (via the AT-hooks) and interaction with RCC1. To
381 corroborate such an hypothesis it would be interesting to know more precisely where HMGB2
382 binds within EBNA1. Alternatively, another as yet unidentified partner could be involved in
383 the process. These possibilities are not mutually exclusive, indeed EBNA1 may employ
384 multiple mechanisms to tether the viral genome to chromatin and to associate with the
385 chromatin independently of the viral genome, through the different stages of the cell cycle and
386 under various conditions.

387 Regarding the domains of RCC1 involved in the interaction with EBNA1, the N-
388 terminal flexible region of RCC1 was identified as an essential domain. Interestingly, this N-

389 terminal tail, and in particular the serine at position 2, is the site of post-transcriptional
390 modifications (both α -N-methylation and phosphorylation) that are important for stable
391 chromatin association and regulation of RCC1's NLS interaction with importins α and β
392 (Chen *et al.*, 2007; Li & Zheng, 2004). Such modifications of RCC1 N-terminal tail within
393 mammalian cells could modulate or even prevent the interaction between the two proteins.
394 Conversely, since these modifications have been suggested to play an important role in the
395 mobility of RCC1 during metaphase and in its stabilization on the chromatin (Hutchins *et al.*,
396 2004; Li & Zheng, 2004), EBNA1's interaction with these regions could affect the dynamics
397 of RCC1's interaction with chromatin.

398 Use of EBNA1 peptide arrays permitted a more detailed mapping of the EBNA1
399 interaction regions with respect to full length RCC1 as well as the N-terminal tail. Interaction
400 of full-length RCC1 (and to lesser extent the N-terminal tail of RCC1 alone) with the CBS-1/-
401 3 and CBS-2 regions of EBNA1 was confirmed. In addition, two other regions of EBNA1
402 were identified that might be involved in the interaction: the C-terminal tail, and a negatively
403 charged region located between the previously characterized CK2 and USP7 binding sites.
404 Interestingly, these two regions (particularly the latter) are recognised by RCC1's N-terminal
405 tail. In particular, the N-terminal tail of RCC1 critical for the interaction specifically contacts
406 a region of EBNA1 - DYFEYHQE - located between aa 413 and 420. When set in the context
407 of an *in silico* structural model of full-length EBNA1 (Hussain *et al.*, 2014), this domain
408 appears to be located in a region that resembles a small pocket. This could potentially
409 accommodate the N-terminal region of RCC1, facilitating further interactions between the
410 CBS domains of EBNA1 and the seven-propeller helix of RCC1, and hence stabilising the
411 interaction between the two proteins. However, deletion of this domain does not preclude
412 binding of EBNA1 and RCC1 in the *in vitro* assays used here and we cannot exclude that it
413 may reflect artifactual binding to a site that is not normally accessible to RCC1.

414 With regard to the dynamics of interaction between the two proteins in live cells, the
415 combination of colocalization experiments in live cells and FRET analysis reveals that the
416 two proteins colocalize with the chromatin throughout the cell cycle. However, their
417 proximity varies according to the location within the cell nucleus as well as the phase of the
418 cell cycle: during interphase, although the two proteins appear to be colocalizing throughout
419 the cell nucleus, FRET could only be observed at the periphery of the nucleus, suggesting that
420 close interaction between EBNA1 and RCC1 could be linked to the latter being in a different
421 conformation when actively involved in nucleo-cytoplasmic transport. This result opens up
422 the possibility that the interaction of EBNA1 with RCC1 could play a role in functions other
423 than segregation of the viral episome.

424 Importantly, during mitosis, FRET was mainly observed in metaphase, indicating a
425 more specific role for the RCC1-EBNA1 interaction at this particular stage of mitosis that
426 precedes segregation of sister chromatids. This observation, together with the correlation
427 between EBNA1 regions interacting with RCC1 and EBNA1 domains previously
428 characterized for their role in chromosome binding and episome maintenance, argues for an
429 important role of RCC1 in EBV episome tethering to the chromosomes and subsequent
430 episome maintenance. However, this hypothesis will be difficult to prove directly since RCC1
431 is an essential protein whose downregulation leads to premature chromosome condensation or
432 arrest in the G1 phase of the cell cycle (Uchida *et al.*, 1990). The observation that deletion of
433 the AT-hook domains still permits association of EBNA1 with metaphase chromosomes while
434 it appears to abrogate interaction with RCC1 *in vitro*, does not refute the hypothesis. First, the
435 assay conditions used to detect the interaction *in vitro* may not be optimum and a weak
436 interaction between EBNA1 and RCC1 might nevertheless occur *in vivo* in the absence of the
437 AT-hook domains. Alternatively, in the absence of an interaction between RCC1 and EBNA1,
438 alternative mechanisms tethering EBNA1 to the chromatin may act. Of note, depletion of

439 HMGB2 was found to affect the stability but not to prevent EBNA1 association with
440 chromatin, nor did it impact viral genome maintenance, despite the observed interaction
441 between EBNA1 and HMGB2 on chromatin through mitosis (Jourdan *et al.*, 2012). It is thus
442 likely that several mechanisms are involved in EBNA1 tethering to the chromatin,
443 orchestrated to play a role at different stages of the cell cycle to both bring EBNA1 to the
444 chromatin and stabilize it once there: During interphase, the EBV genomes are distributed to
445 perichromatic regions of the nucleus in a manner dependent on the FR element and EBNA1
446 (Deutsch *et al.*, 2010). It has been suggested that the AT-hook domains of EBNA1 could play
447 an important role in this tethering of the EBV genomes to the chromatin. This hypothesis is
448 strengthened by the demonstration that HMG1Aa, an AT-hook binding protein can
449 functionally replace the N-terminal domain of EBNA1 (Sears *et al.*, 2003; Thomae *et al.*,
450 2008) and by the results of our FRAP analysis showing a higher mobility of EBNA1 with its
451 AT-hook domains deleted; HMGB2 is associated with EBNA1 on the chromatin during
452 interphase and more so during mitosis (Jourdan *et al.*, 2012); RCC1 colocalizes with EBNA1
453 throughout the cell cycle but the interaction appears to be specifically stabilized during
454 metaphase. Moreover, interaction between EBNA1 and the chromatin could be facilitated by
455 a direct interaction through the AT-hook domains with nucleosomal DNA.

456 Finally, it is interesting to note that the ortholog of EBNA1 in KSHV (Kaposi Sarcoma
457 Herpes Virus), LANA (Latency-Associated Nuclear antigen), directly interacts with H2A-
458 H2B dimers to enable its binding to chromosomes (Piolot *et al.*, 2001). The resolution of the
459 crystal structure of the nucleosome complexed with the first 23 amino acids of LANA
460 revealed that the LANA peptide forms a hairpin that interacts with an acidic H2A-H2B region
461 implicated in the formation of higher order chromatin structure (Barbera *et al.*, 2006).
462 Interestingly, RCC1 targets the same region of the nucleosomal H2A-H2B dimer as LANA,
463 and the two proteins have been shown to compete for nucleosome interaction (England *et al.*,

464 2010). Thus, whereas LANA directly contacts the H2A-H2B dimer to enable its binding to
465 the chromosomes, EBNA1 may interact indirectly with the same H2A-H2B dimer through
466 RCC1. Similar to EBNA1, LANA also interacts with several cellular proteins that appear to
467 play a role in the tethering of LANA to chromosomes and/or episomal segregation (Krithivas
468 *et al.*, 2002; Xiao *et al.*, 2010). Thus EBNA1 and LANA have evolved similar but not
469 identical mechanisms to insure anchorage of the viral episomes onto the chromatin at different
470 stages of the cell cycle, allowing efficient replication and segregation of the respective viral
471 genomes.

472

473 **METHODS**

474 **Cell culture and transfections**

475 HeLa and HEK293T cells were grown at 37°C in DMEM, 10% FCS. Plasmid transfection
476 was performed using the PEI transfection reagent (Polysciences).

477 **Plasmids**

478 pEGFP-N1-EBNA1 Δ GA (aa 8 to 641) has been described previously (Jourdan *et al.*, 2012).
479 Unless otherwise indicated, all EBNA1 plasmids used were derived from this plasmid and
480 thus contain EBNA1 deleted for GAR as well as the first 7 N-terminal aa. EBNA1 and RCC1
481 N-terminal tail deletion mutants were generated by site-directed mutagenesis (QuickChange
482 Site-Directed Mutagenesis kit, Stratagene). For the two-hybrid screen, ORFs for EBNA1,
483 EBNA1 8-410 and EBNA1-381Cter were PCR-amplified (KOD Hot Start DNA
484 Polymerase®, EMD Millipore) cloned first in pDONR207 then into pGBKT7, using the
485 Gateway recombinational cloning system (Invitrogen). EBNA1 and deletion mutants cloned
486 into pDEST-Myc, pCI-3xFlag, pDEST15 or pDEST53 were also generated using the Gateway
487 system. A codon-optimised version of EBNA1- Δ GA was cloned into pET22b (Merck
488 Millipore) to generate pET22b-EBNA1. The ORF for full-length RCC1 (alpha isoform) was

489 transferred from pDONR223-RCC1 (obtained from a human ORFeome library) into pDEST-
490 Myc, pCI-3xFlag or pDESTTM15 (Invitrogen) using the Gateway system. pEGFP-C1-EBNA1
491 has been described previously (Jourdan *et al.*, 2012). pRFP and pEGFP fusion proteins were
492 generated by cloning the relevant PCR-amplified ORFs in pRFP-N1, pRFP-C1, pEGFP-N1 or
493 pEGFP-C1, using the In-Fusion®HD cloning kit (Clontech). All oligonucleotides used are
494 listed in Supplementary Table 1.

495 **Luciferase Assays**

496 Renilla or Firefly luciferase activities were measured in a VeritasTM Luminometer (Turner
497 Biosystems) using the Renilla or Firefly Luciferase Assay system (Promega Madison Co).

498 **Yeast two-hybrid screens**

499 The screens were performed as previously described (Bazot *et al.*, 2014) using pGBKT7-
500 EBNA1/-EBNA1-8-410 or -EBNA1-381Cter as bait vectors and a human LCL AD-cDNA
501 library (Invitrogen). Positive clones were sequenced and identified by automatic BLAST
502 (Pellet *et al.*, 2010).

503 **Co-immunoprecipitation and western blotting**

504 Cells were lysed in 50 mM Tris-HCl pH 7.5, 150-300 mM NaCl, 1 mM Dithiothreitol (DTT)
505 and 0.5% Nonidet P-40 plus protease inhibitors. For immunoprecipitation of transiently
506 expressed Flag-tagged proteins, extracts were incubated with 20 µl of anti-Flag M2 affinity
507 gel (Sigma) for 4 h at 4°C. After washing, bound proteins were analysed by western blotting,
508 visualised using ECL (Thermo Fisher Scientific). Antibodies: anti-Flag rabbit polyclonal
509 antibody (Sigma), anti-His6 mouse monoclonal antibody (Roche Molecular Biochemicals),
510 anti-c-Myc (9E10) HRP-conjugated antibody (Santa Cruz Biotechnology, Inc). Anti-rabbit
511 and anti-mouse (HRP)-conjugated antibodies (GE Healthcare) were used as secondary
512 antibodies.

513 **Production and purification of the 6xhis-EBNA1 protein**

514 6xhis-EBNA1 was purified from *Escherichia coli* Rosetta (pLysS) strain transformed with
515 pET22b-EBNA1. Cells were lysed in 50 mM NaH₂PO₄, 1 M NaCl, 10 mM imidazole pH 8,
516 protease inhibitors and 1 mg/ml lysozyme. After sonication, the protein was purified by
517 gravity-flow chromatography using Ni-NTA agarose beads. Beads were washed with lysis
518 buffer plus 50 mM imidazole and the proteins eluted in lysis buffer containing 150 mM
519 imidazole.

520 ***In vitro* GST-Pulldowns**

521 Glutathione S-Transferase (GST) and GST-fusion proteins were purified from *Escherichia*
522 *coli* BL21 (DE3) codon plus strain extracts, with glutathione-Sepharose 4B beads (GE
523 Healthcare). Beads carrying the GST or the GST-fusion proteins were equilibrated in MTPBS
524 (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄, 100 mM EDTA, 1% Triton) and
525 incubated with either purified 6xhis-EBNA1 or transfected cell extracts for 4h in MTPBS.
526 Beads were washed 5 times in MTPBS and bound proteins analysed by western blotting.

527 **EBNA1 peptides arrays**

528 25mer peptides comprising the entire sequence of EBNA1 (B95.8 strain), with 5 residue shifts
529 (ie. initiating at residues 1, 6, 11, 16 etc) were synthesized by automatic SPOT synthesis
530 (Kramer and Schneider-Mergener, 1998) directly onto cellulose membranes using Fmoc (9-
531 fluorel methoxycarbonyl) chemistry and Autospot Robot ASS222 peptide synthesizer (Invatis
532 Bioanalytical Instruments AG). Arrays were bathed in ethanol and washed for 10 mins in
533 TBST (50 mM Tris.HCl pH7.5, 150 mM NaCl, 0,05% Tween-20), followed by blocking in
534 TBST, 5% non-fat milk powder (NFM) for 2 hours at RT and washed again with TBST.
535 Arrays were probed with purified GST (as control) or GST fusion proteins, at 2 to 5 ug/mL in
536 TBST, 1% NFM, shaking overnight at 4°C. After washing in TBST, membranes were
537 incubated with rabbit anti-GST-HRP and the array revealed using ECL (Pierce #32106). To
538 strip the array membranes for re-probing, they were covered in 60 mM Tris-HCl pH6.8, 20

539 mM DTT, 70 mM SDS, at 70°C for 30 min.

540 **Confocal microscopy**

541 HeLa cells were plated onto glass-bottomed dishes for confocal microscopy (Ibidi) and
542 transfected with expression vectors coding for EBNA1 and RCC1 fused to either EGFP or
543 RFP. Live cells were analyzed with a Zeiss LSM710 confocal microscope with ZEN software.
544 GFP and RFP signals were acquired using respectively an argon laser at 488nm and a laser
545 diode (DPSS) at 561nm. Z-stack series were also acquired for mitotic cells: the most
546 representative stacks are presented. All analyses were conducted with ImageJ Software.

547 **Fluorescence recovery after photobleaching (FRAP) analysis**

548 Cells used for FRAP acquisition were prepared as for classical microscopy and data collected
549 using a confocal spinning disk microscope. Same parameters were used to acquire all images.
550 Regions of interest were photobleached using a 494-nm laser during 510ms at full power.
551 Images were acquired with an EM gain of 30, 200ms exposure time and a 488-nm laser at
552 9.5% full power. 5 images were acquired before bleaching then 1 image every 0.5s for 5
553 seconds, 1 image per second for 1 minute and 1 image every 5 seconds for 30 seconds.
554 Analysis was performed using ImageJ and EasyFrap software.

555 **Förster resonance energy transfer (FRET) analysis**

556 Cells used for FRET acquisition were prepared as for confocal microscopy and data collected
557 with an LSM-710 confocal microscope. FRET analysis was performed using the FRET
558 Analyzer plugin (<http://rsb.info.nih.gov/ij/plugins/fret-analyzer/fret-analyzer.htm>). Three
559 tracks were used for the acquisition: EGFP (excitation: GFP, reception: GFP range), RFP
560 (excitation: RFP, reception: RFP range) and FRET (excitation: GFP, reception: RFP range).
561 Argon laser and DPSS were used at 2% and 7% power respectively. Gain level was 540 for
562 the GFP signal and 640 or 690 for the RFP signal. Spectral leakage was measured by
563 acquisition of 5 images for each track with EGFP or RFP fusions expressed alone. Double

564 transfected cells were used for FRET acquisition data. In each case EGFP, RFP and FRET
565 fluorescent signals were acquired for each track.

566

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577

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781

782

783 **LEGENDS TO FIGURES**

784 **Figure 1. Deletion of EBNA1 AT-hook domains drastically affects EBNA1-mediated**
785 **transcriptional activation but has only moderate impact on EBV's association with the**
786 **metaphasic chromosomes.** (A) Schematic representation of EBNA1 and AT-hook deletion
787 mutants. Note: deletion coordinates shown are in the context of the GAR deletion (aa 93-325)
788 and deletion of aa 1-7, incorporated in all constructs. GR: glycine/arginine-rich region; UR:
789 unique region; GAR: glycine/alanine-repeat region; DBD: DNA-binding domain; NLS:
790 nuclear localization signal; Ac: acidic. (B) Confocal microscopy analysis of EBNA1 and
791 EBNA1 mutants during metaphase. HeLa cells were transfected with expression vectors for
792 either GFP-EBNA1 or GFP-EBNA1 deletion mutants and the localization of the proteins was
793 assessed in live cells by confocal microscopy. Upper and lower panels correspond to different
794 individual cells. (C) FRAP analysis of EBNA1 and EBNA1 AT-Hooks deletion mutants
795 expressed in HeLa cells. Average $t_{1/2}$ of fluorescence recovery was calculated from a
796 minimum of 6 cells for each protein from a representative experiment. (D) HEK293T cells
797 were transfected with the pGL2-FR-TK-Luc reporter plasmid, which contains the EBV FR-
798 element cloned upstream of the Herpes simplex virus thymidine kinase (TK) promoter, either
799 alone or together with expression vectors for EBNA1 or EBNA1 deletion mutants (as
800 indicated). Luciferase activity was measured for identical amounts of total protein as
801 evaluated by Bradford assay. Relative levels of luciferase activity are shown graphically.
802 Error bars represent standard deviation from three replicate assays. Significant differences
803 were evaluated by a student test (***) indicate a p value < 0,05; ns: non significant).

804

805 **Figure 2. EBNA1 and RCC1 interact directly.** (A) Expression plasmids for Flag-RCC1,
806 Flag-EBNA1 Δ GA, Myc-RCC1 and Myc-EBNA1 Δ GA were transfected into HeLa cells as
807 indicated. Cellular extracts were immunoprecipitated with anti-Flag affinity gel and the

808 immunoprecipitated complexes were analysed by western blotting using an anti-Flag
809 polyclonal antibody or an anti-Myc antibody. Input corresponds to 8% of the cell extract used
810 for immunoprecipitation. (B) 500 ng of purified 6xhis-EBNA1 Δ GA protein were incubated
811 with similar amounts of purified GST or GST-RCC1 bound to glutathione sepharose beads.
812 The EBNA1-bound proteins were analysed by western blotting using an anti-his6 MAb. Input
813 corresponds to 100 ng of purified 6xhis-EBNA1 Δ GA.

814

815 **Figure 3. EBNA1 regions CBS-3, CBS-1 and CBS-2 are involved in the interaction with**
816 **RCC1.** (A) Schematic representation of EBNA1. Abbreviations as in Figure 1 and CBS:
817 chromosome binding sites. (B - C) Expression plasmids for GFP-tagged (B) or Flag-tagged
818 (C) EBNA1 and EBNA1 deletion mutants, as indicated in the figure (note: protein/deletion
819 coordinates shown are in the context of the GAR deletion (93-325) and deletion of aa 1-7,
820 incorporated in all constructs), were transfected into HeLa cells. Cellular extracts were then
821 incubated with similar amounts of GST or GST-RCC1 proteins bound to glutathione
822 sepharose beads. Bound proteins were analysed by western blotting using either an anti-GFP
823 antibody (B) or an anti-Flag antibody (C) as probes. Input corresponds to 10% of cell extract
824 used for the GST-pulldown.

825

826 **Figure 4. Combined deletion of GR1 and GR2 drastically affects interaction with RCC1.**
827 (A) Schematic representation of EBNA1 and EBNA1 deletion mutants. (B) Expression
828 plasmids for GFP-tagged EBNA1 and EBNA1 deletion mutants, as indicated in the Figure,
829 were transfected into HeLa cells. Cellular extracts were then incubated with similar amounts
830 of GST or GST-RCC1 proteins bound to glutathione sepharose beads. Bound proteins were
831 analysed by western blotting using an anti-GFP antibody as probe. Input corresponds to 10%
832 of cell extract used for the GST-pulldown.

833

834 **Figure 5. RCC1 interacts with EBNA1 via its 20 amino acid N-terminal tail.**

835 (A) Schematic representation of RCC1. N- and C- terminal regions of RCC1 surround a seven
836 bladed propeller domain (in grey). Residues 1 to 27 corresponding to the N-terminal tail are
837 detailed below. The bi-partite NLS and phosphorylation sites on Ser 2 and 11 are indicated as
838 well as the α -N-tri-methylation of serine 2 that follows cleavage of the first methionine
839 indicated in brackets. (B) Expression plasmids for Flag-EBNA1 were transfected into HeLa
840 cells and cellular extracts incubated with similar amounts of GST, GST-RCC1 or GST-RCC1-
841 deletion mutants bound to glutathione sepharose beads, as indicated. Bound proteins were
842 analysed by western blotting using an anti-Flag antibody. Input corresponds to 1/40 of the cell
843 extract used for the GST-pulldown.

844

845 **Figure 6. Probing EBNA1 peptide arrays for RCC1 interaction sites.** (A) Arrays of
846 immobilized peptide spots of overlapping 25-mer peptides covering the entire sequence of
847 EBNA1 (including the GA repeat) were probed with recombinant GST-RCC1 (a), GST-
848 RCC1 Δ 1-20 (b), GST-RCC1 1-20 (c) and GST (d) and revealed by incubation with an anti-
849 GST antibody. Light blue, blue and red rectangles respectively indicate low, medium and high
850 affinity binding to contiguous interacting peptides. The limits of the interacting regions are
851 indicated for each rectangle by the positions of the first aa of the first peptide and the last aa
852 of the last peptide. * Note: the GST-RCC1 Δ 1-20 array shown in the figure is the result of a
853 longer exposure compared to the other array images. (B) Schematic representation of EBNA1
854 and its interaction regions with RCC1 as determined by the peptide array. Boxes along the
855 linear depiction indicate binding (strong, intermediate or weak). Abbreviations are similar to
856 those indicated in Figure 1. *indicates the position of the protein kinase 2 (CK2) binding
857 sites. USP7: Ubiquitin Specific Peptidase 7. Positions of the AT-hook regions (between aa 40

858 to 53 and 326 to 358) are indicated in green.

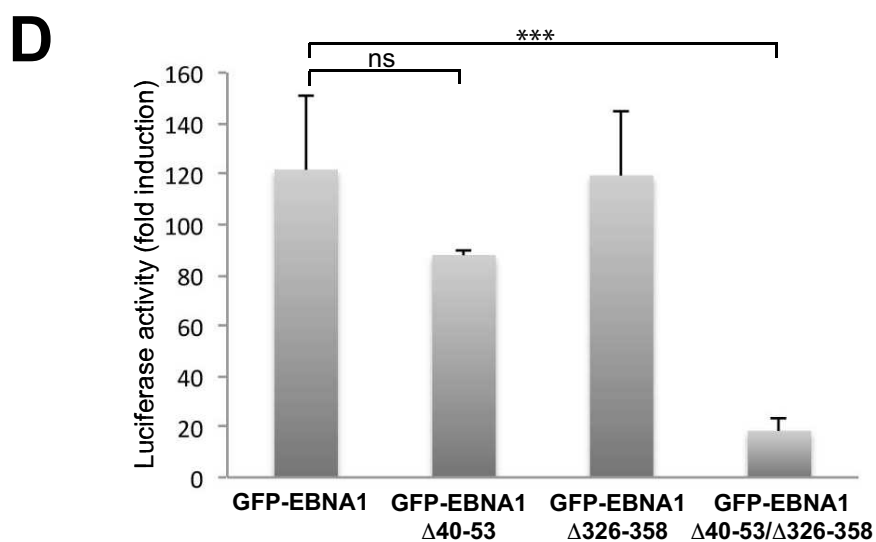
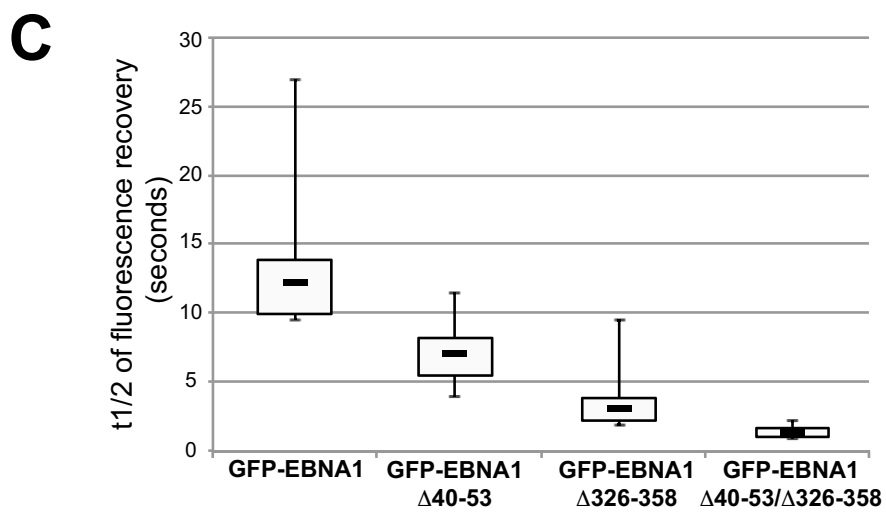
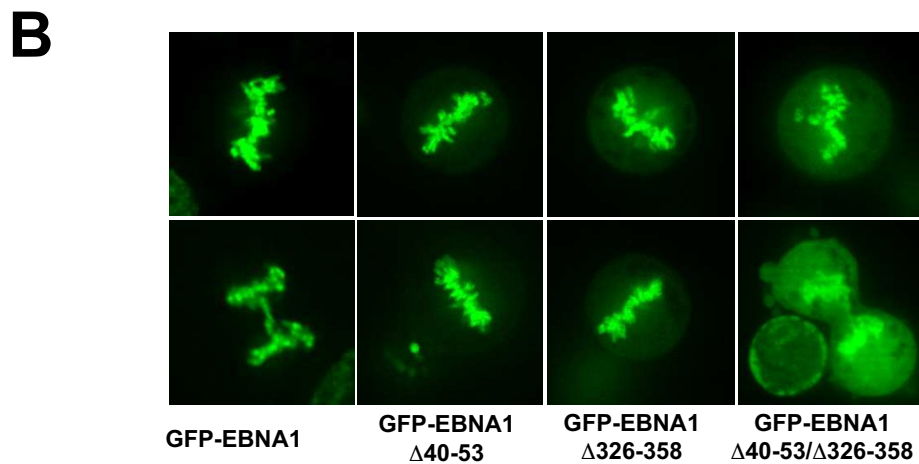
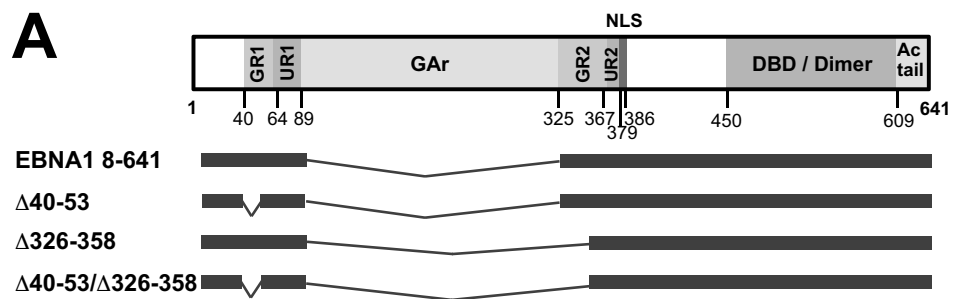
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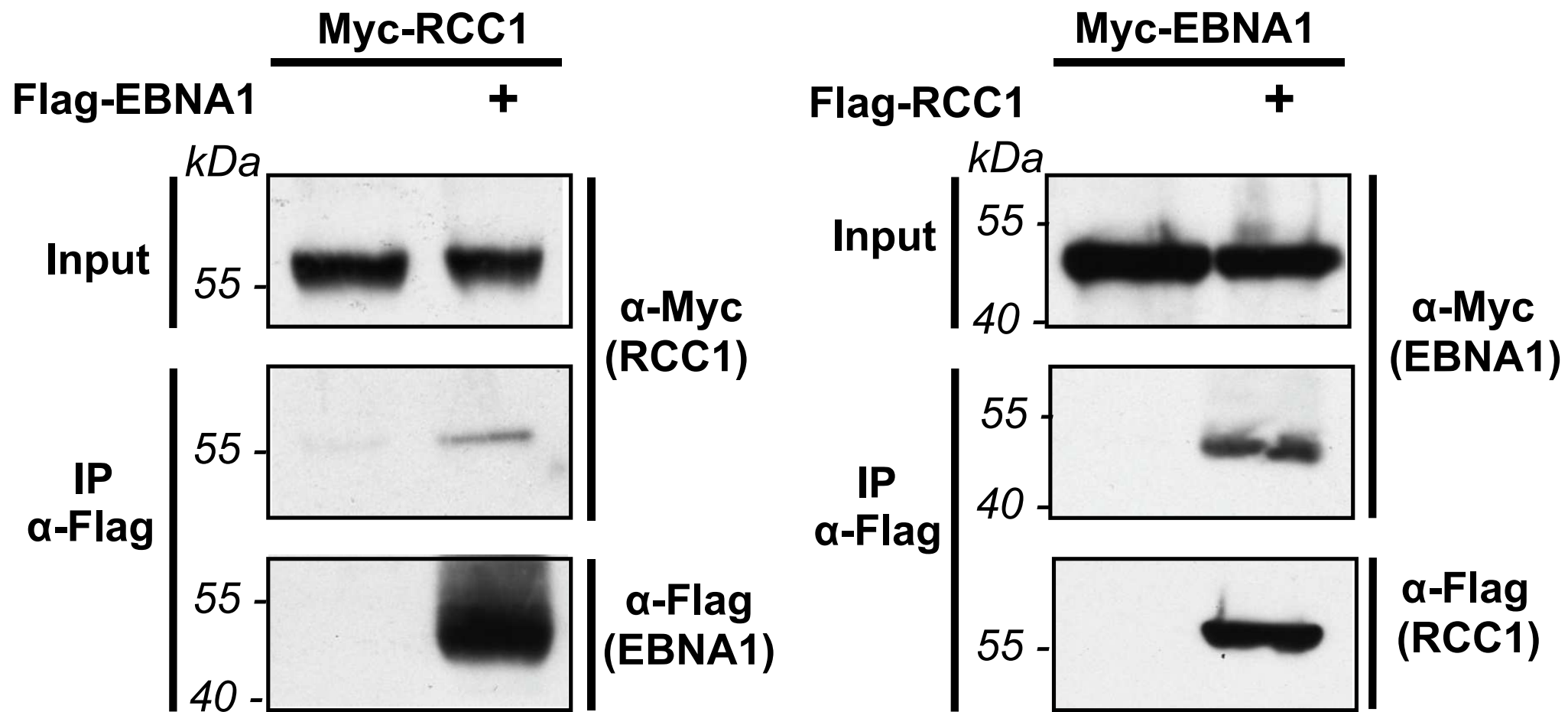
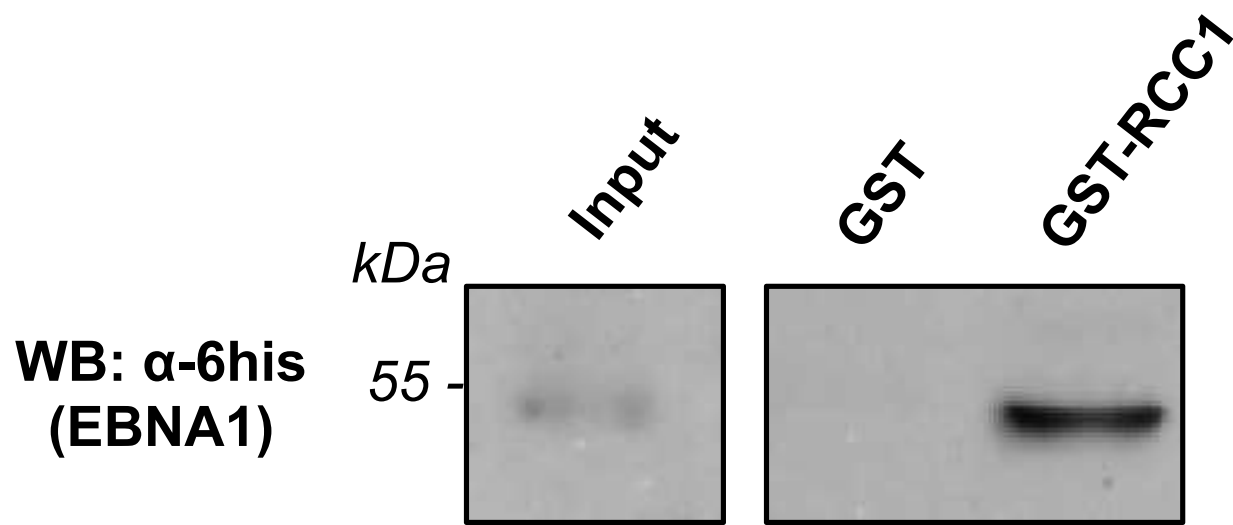
860 **Figure 7. Dynamic localization of EBNA1 and RCC1 throughout the cell cycle.** HeLa
861 cells coexpressing RFP-EBNA1 and EGFP-RCC1 were observed by confocal microscopy at
862 different stages of the cell cycle as indicated. Images show single confocal z-section. Trans:
863 Transmission.

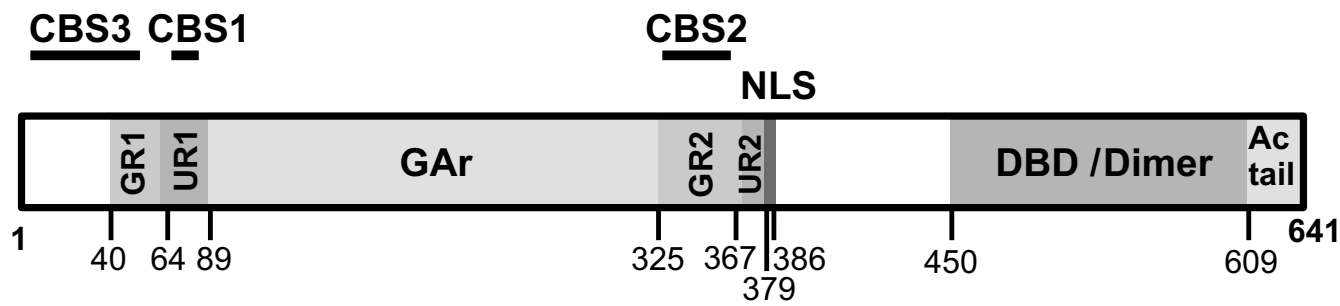
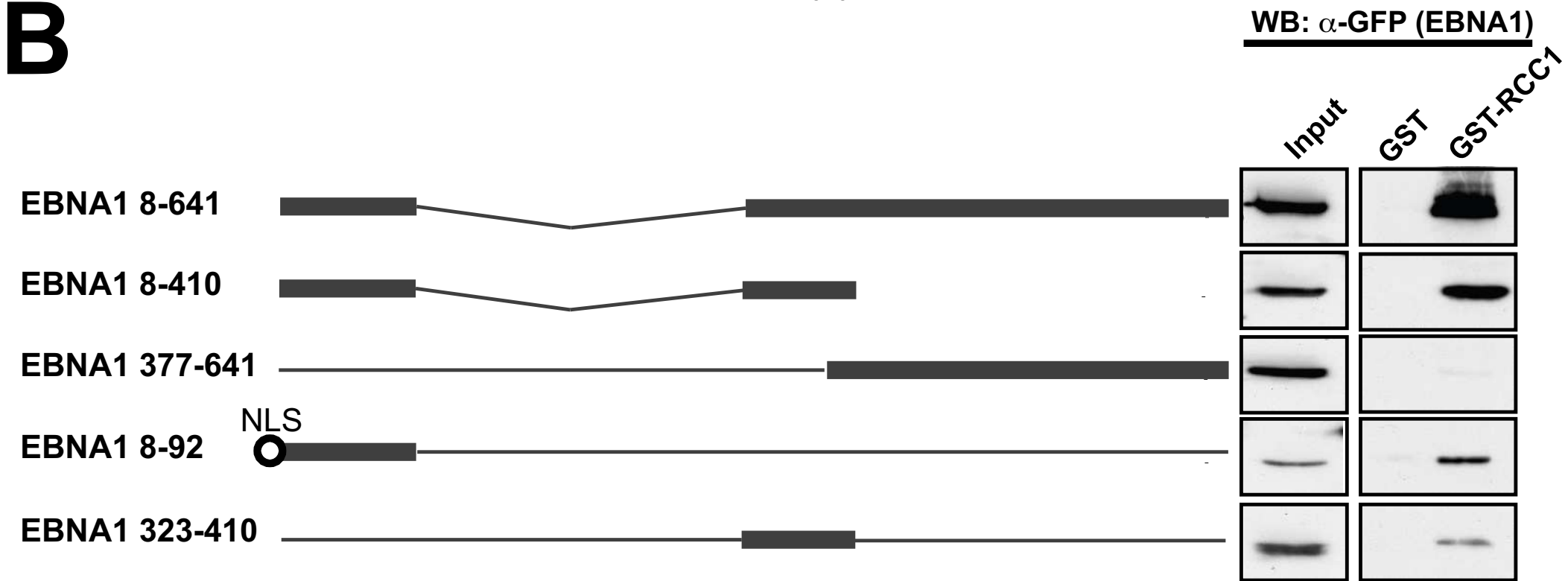
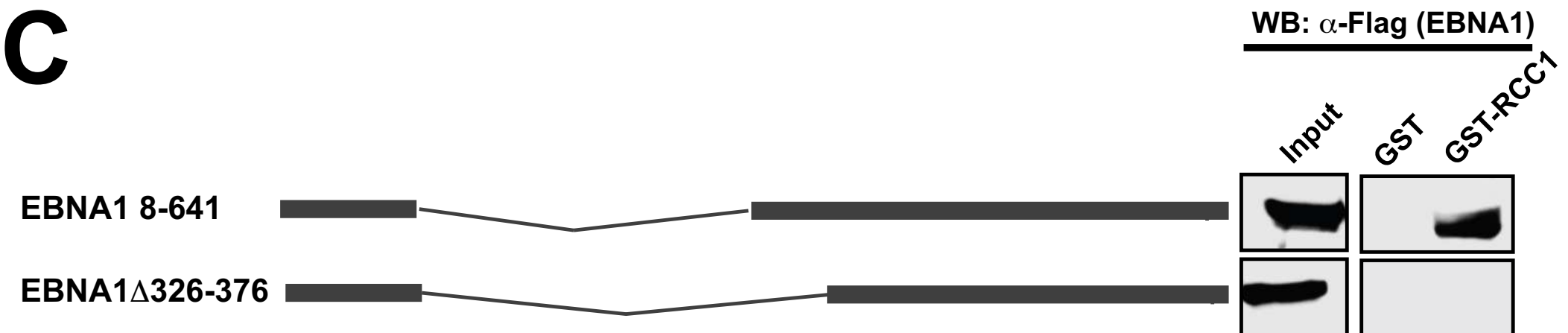
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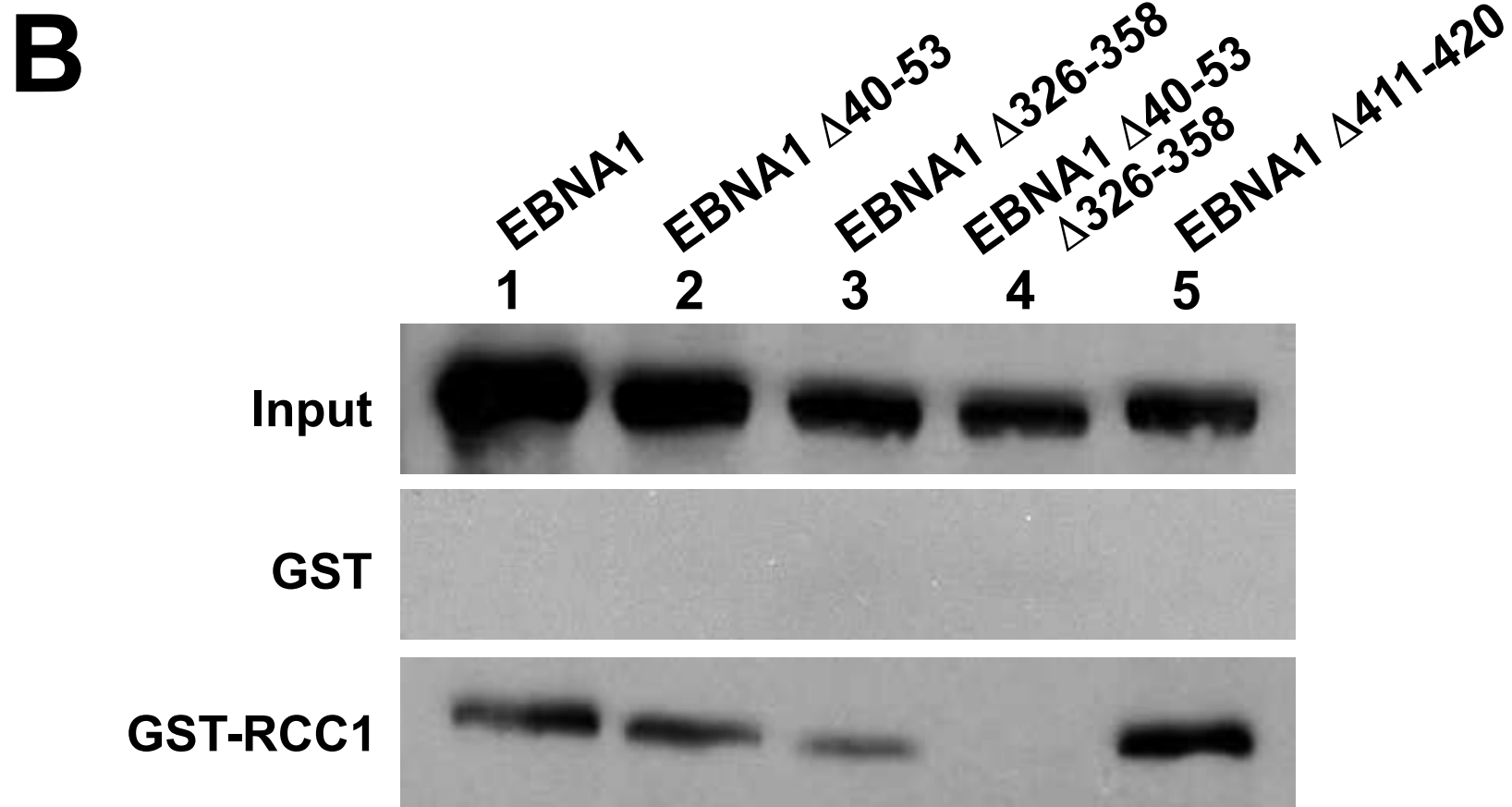
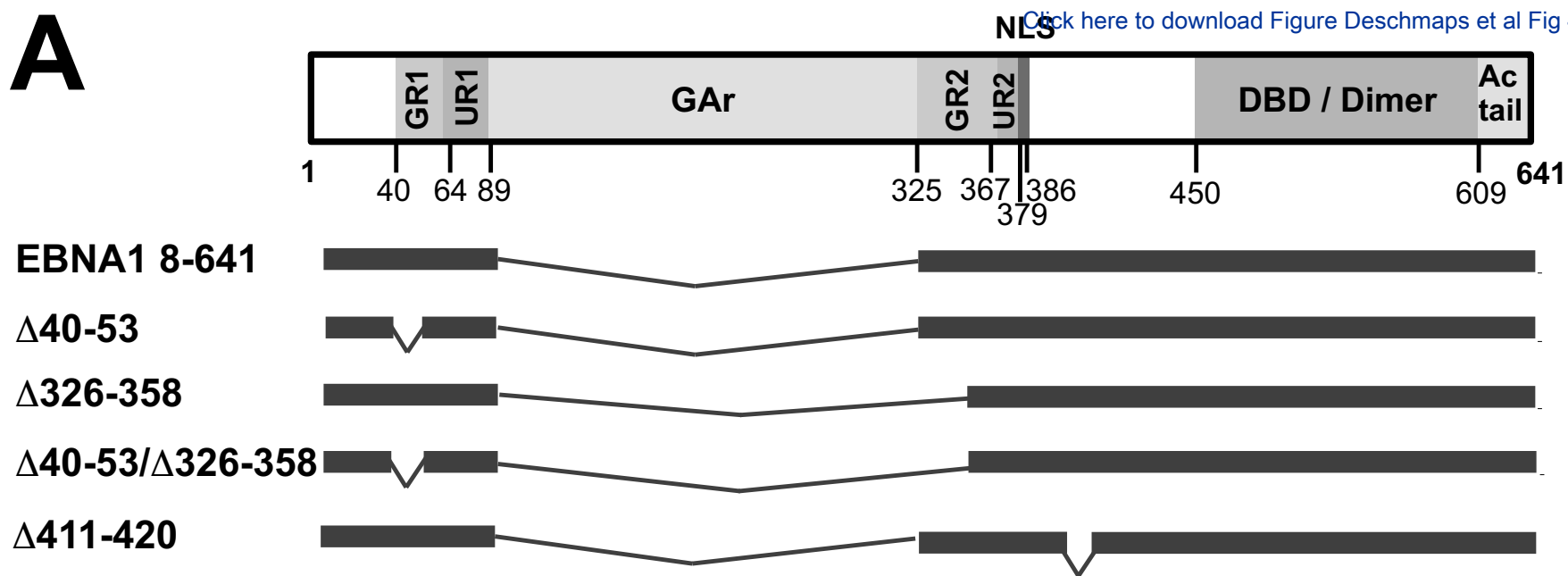
865 **Figure 8. FRET analysis of EGFP-EBNA1 and RFP-RCC1 interaction at different**
866 **stages of the cell cycle.** HeLa cells coexpressing EGFP-EBNA1 and RFP-RCC1 were
867 analysed by Förster resonance energy transfer (FRET) at different stages of the cell cycle as
868 indicated. Analyses were performed using ImageJ software 'FRET Analyzer'. Imaged cells
869 were selected on the basis of both fusion proteins expression levels being similar to that found
870 in single transfected cells used for spectral leakage calculation. Results are presented as three
871 images, considering low, average or high spectral leakage level (noted as low, medium or
872 high cut-off). High cut-off images are the most representative of the FRET signals. The FRET
873 signal is represented using a firescale gradient: blue: no FRET signal, yellow: maximum
874 FRET signal.

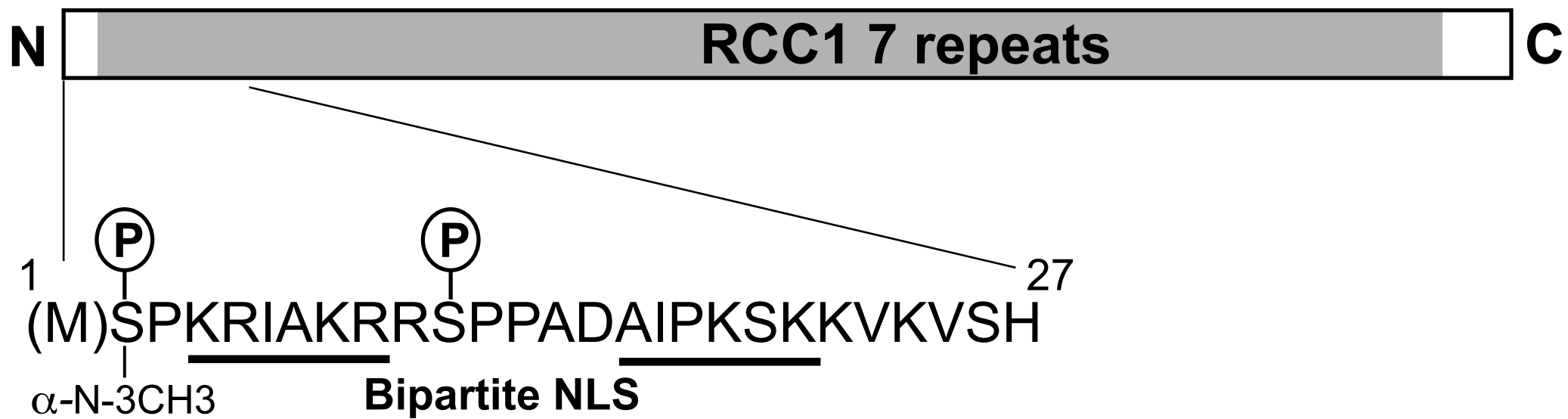
875



A**B**

A**B****C**



A**B**

1 *Input*

2 *GST*

3 *GST-RCC1*

4 *GST-RCC1 Δ 1-5*

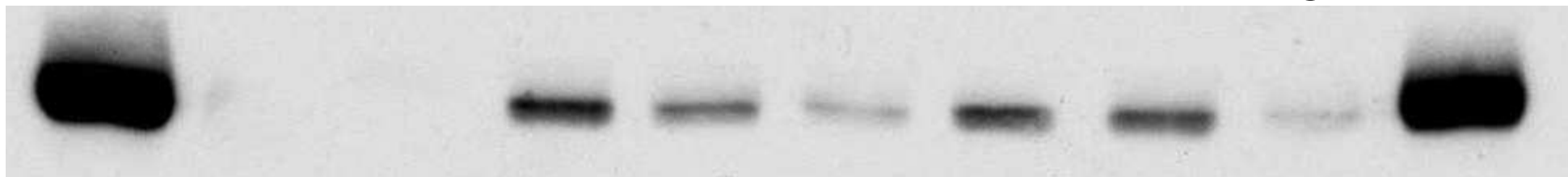
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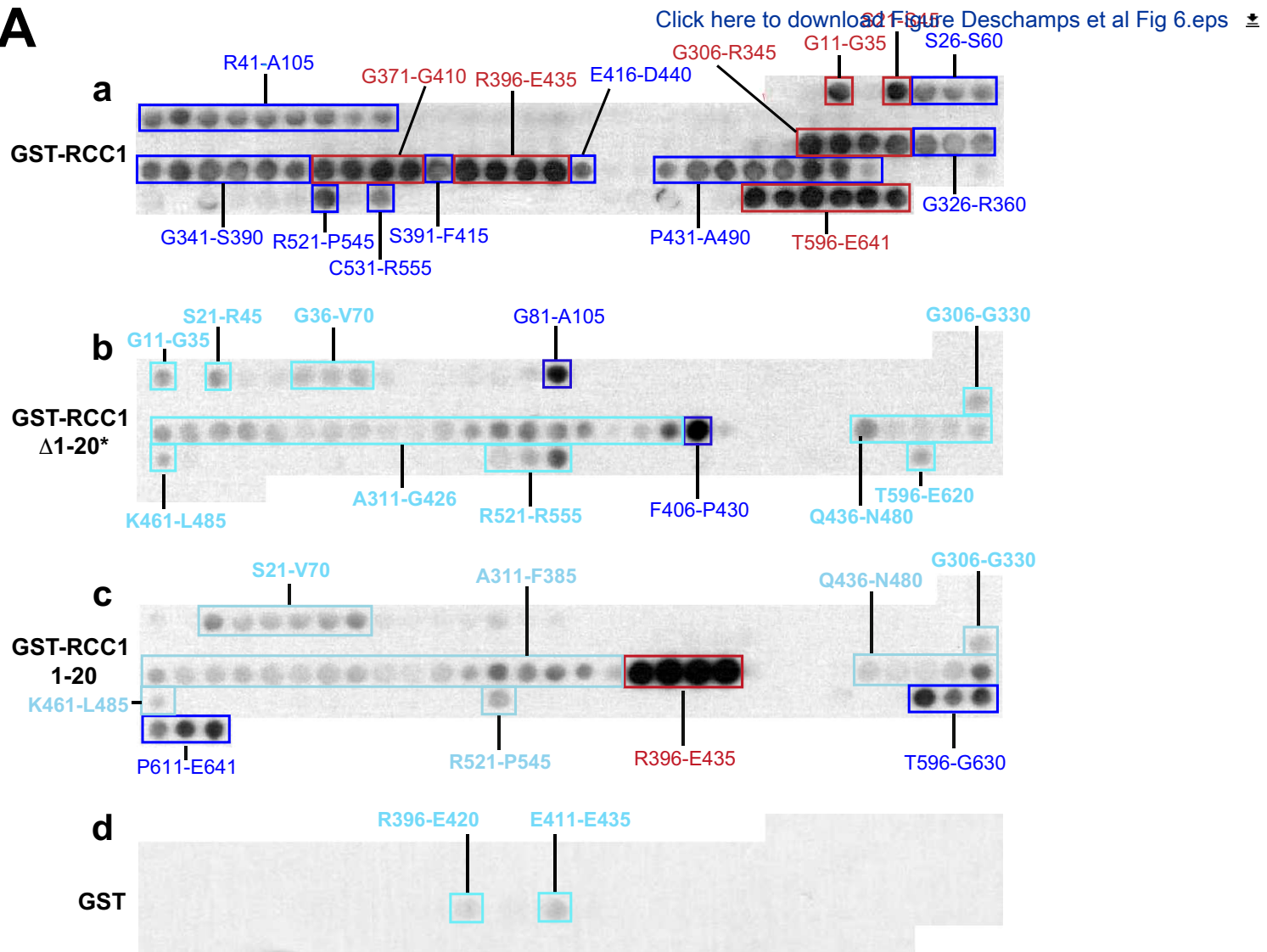
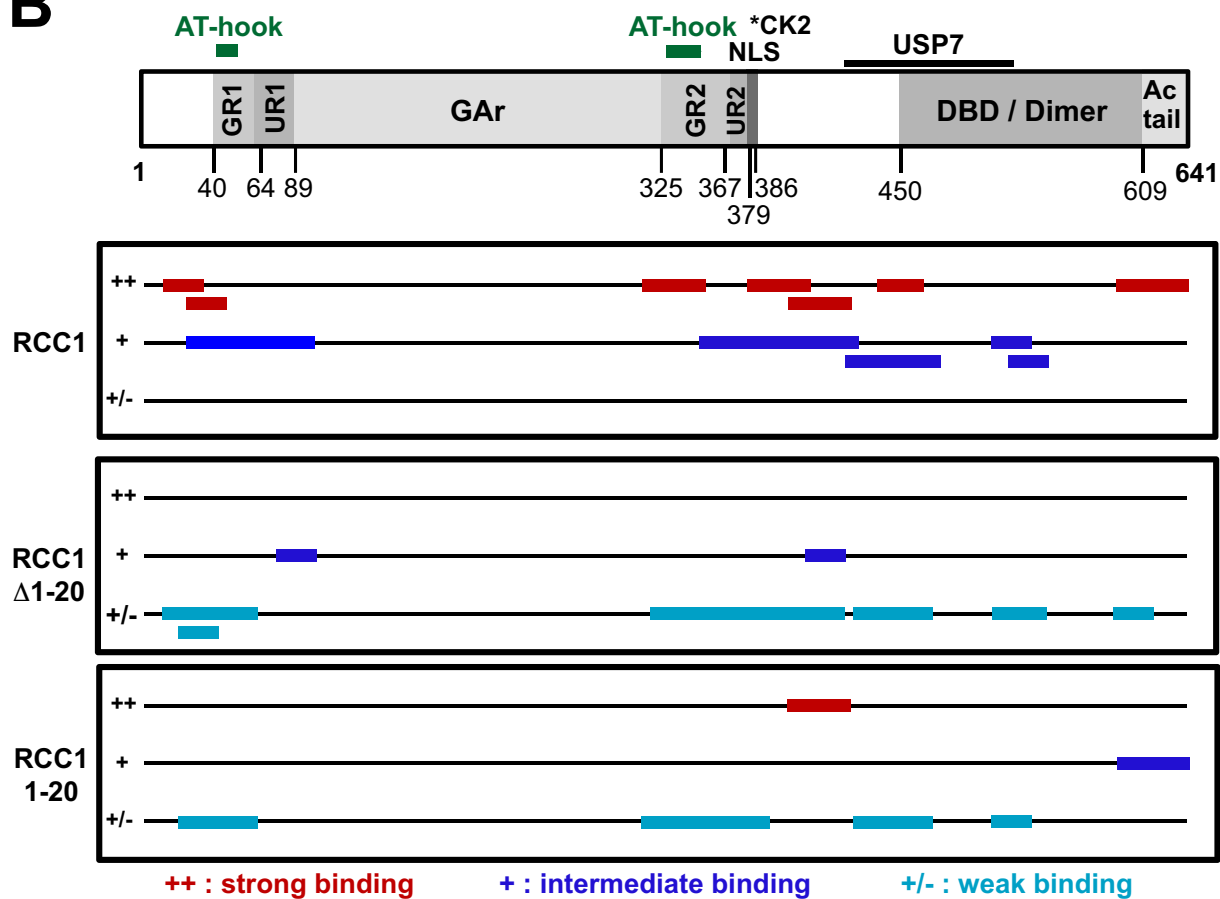
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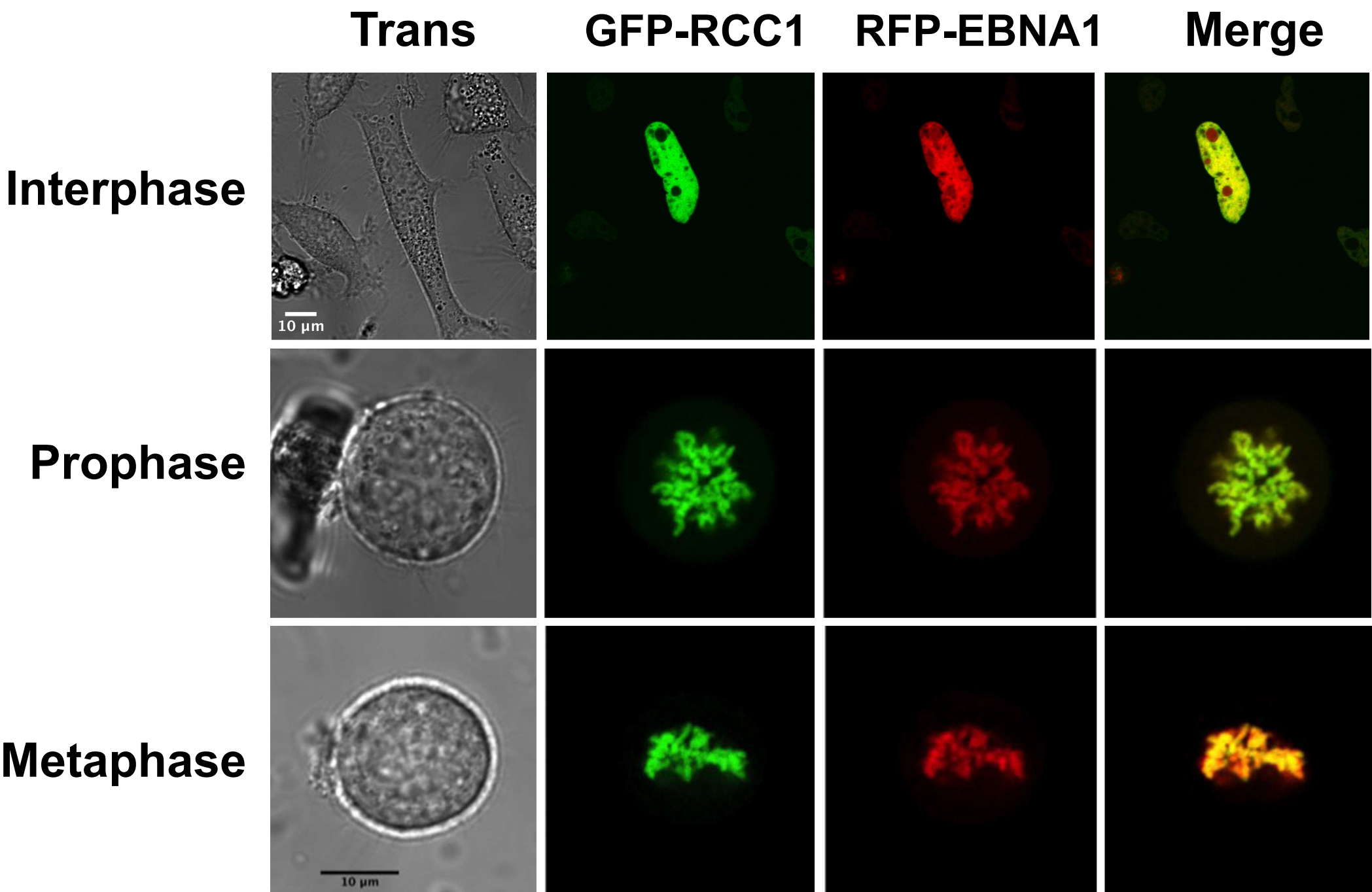
7 *GST-RCC1 Δ 11-20*

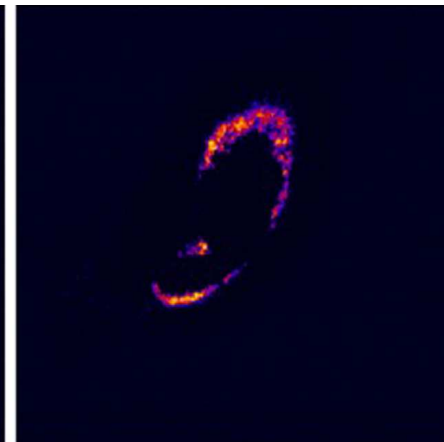
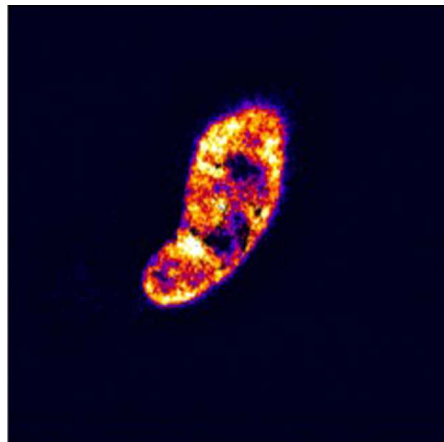
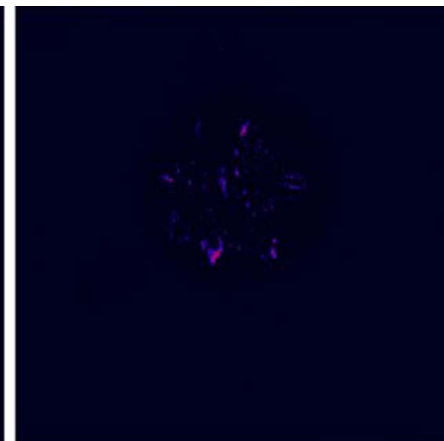
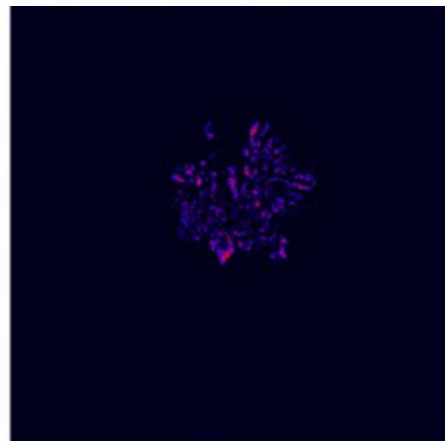
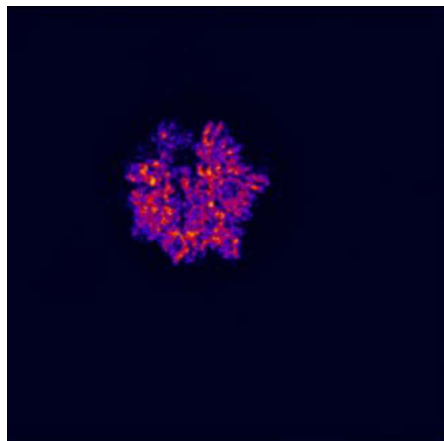
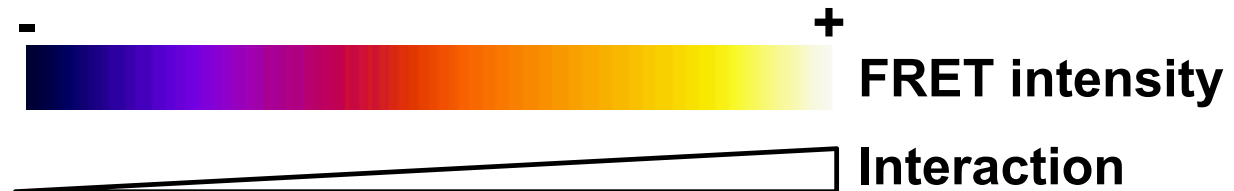
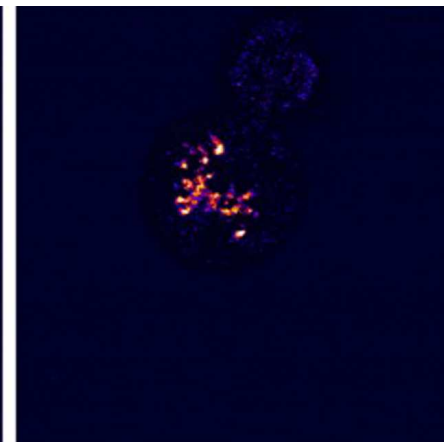
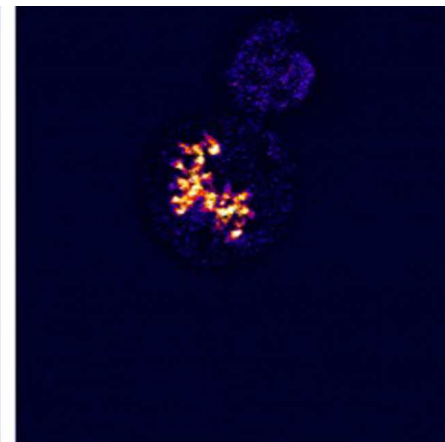
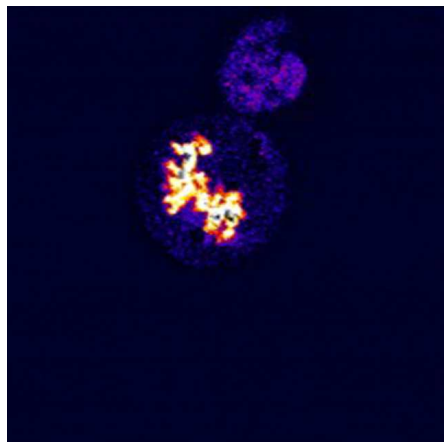
8 *GST-RCC1 Δ 1-20*

9 *GST-RCC1 1-20*



A**B**



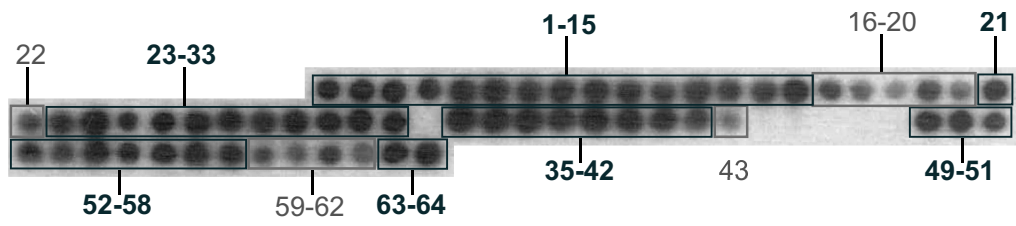
Cut-off level**Low****Medium**[Click here to download Figure Deschamps et al Fig 8.eps](#)**High****Interphase****Prophase****Metaphase**

SUPPLEMENTARY MATERIAL

Legend to supplementary Figure 1.

The N-terminal region of RCC1 (MSPKRIAKRRSPPADAIPKS) contains 6 positively charged residues and one negatively charged residue. The C-terminal tail of EBNA1, from residue 610 to 641 has 14 negatively charged residues, interspersed with glycines and no positively charged residues. The stretch of residues in common between the EBNA1 peptides that span R396 to E435, which interact strongly with both RCC1 and RCC1 1-20, are aa 411 to 420 (EADYFEYHQE) and referred to below as the core region. This region contains 4 negatively-charged residues but is surrounded by residues of negative and positive charge.

To determine the key residues in this binding site, a new array was generated spanning the EBNA1 sequence from residue 401 to 430 and including a series of mutated peptides comprising: sequential Ala replacement peptides (Ala scanning); N- and C-terminal deletion peptides; Ala replacement of the four negatively-charged residues of the core region; and the core region alone (as shown). This array was probed with GST-RCC1 1-20. Sequential replacement of residues with Ala showed no (or little) reduction in binding until residue 413 (peptide 16). Ala replacement of DYFEY and Q of the core all showed substantial reduction in binding by GST-RCC1 1-20, with replacement of F₄₁₅ showing the weakest binding with these single replacements (peptides 16 to 20 and 22). Further Ala replacement to residue 430 (peptides 23 to 33) showed no reduction in binding, confirming the span of the core binding region. Replacement of the four charged residues (E₄₁₁, D₄₁₃, E₄₁₆, E₄₂₀) in the core completely abrogated binding (peptide 34), while binding to the 10 residue core alone (peptides 63 and 64) was as strong as binding to the full-length 25mer peptides incorporating this region. Sequential N-terminal deletion of the 25mer peptide revealed reduced binding upon deletion of Y₄₁₄ of the core and no binding with further N-terminal deletion, again revealing F₄₁₅ to be a critical residue in binding (peptides 43 to 48). It is noteworthy that this 15mer deleted up to F₄₁₅ still has 3 glutamate (negative) residues, but does not bind GST-RCC1 1-20. Sequential C-terminal deletion of the 25mer peptide (peptides 59 to 62) revealed reduced binding upon deletion of E₄₂₀, confirming the C-terminal limit of the core region. These data show that residues 413 to 420 (DYFEYHQE) of EBNA1 represent a tight binding region of the first 20 residues of RCC1, that the negatively charged residues in this region are essential for the interaction, and that F₄₁₅ and Y₄₁₄ are also key in the interaction.



1	GRRPFFHPVGEADYFEYHQEGGPDG	++	35	HPVGEADYFEYHQEGGPDGEPDVP	++
	ARRPFFHPVGEADYFEYHQEGGPDG	++		PVGEADYFEYHQEGGPDGEPDVP	++
	GARPFFHPVGEADYFEYHQEGGPDG	++		VGEADYFEYHQEGGPDGEPDVP	++
	GRAPFFHPVGEADYFEYHQEGGPDG	++		GEADYFEYHQEGGPDGEPDVP	++
5	GRRAFFHPVGEADYFEYHQEGGPDG	++		EADYFEYHQEGGPDGEPDVP	++
	GRRPAFHPVGEADYFEYHQEGGPDG	++	40	ADYFEYHQEGGPDGEPDVP	++
	GRRPFAHPVGEADYFEYHQEGGPDG	++		DYFEYHQEGGPDGEPDVP	++
	FHPVGEADYFEYHQEGGPDGEPDVP	++		YFEYHQEGGPDGEPDVP	++
	AHPVGEADYFEYHQEGGPDGEPDVP	++		FEYHQEGGPDGEPDVP	+/-
10	FAPVGEADYFEYHQEGGPDGEPDVP	++		EYHQEGGPDGEPDVP	-
	FHAVGEADYFEYHQEGGPDGEPDVP	++	45	YHQEGGPDGEPDVP	-
	FHPAGEADYFEYHQEGGPDGEPDVP	++		HQEGGPDGEPDVP	-
	FHPVAEADYFEYHQEGGPDGEPDVP	++		QEGGPDGEPDVP	-
	FHPVGAADYFEYHQEGGPDGEPDVP	++		EGGPDGEPDVP	-
15	FHPVGEADYFEYHQEGGPDGEPDVP	++		FHPVGEADYFEYHQEGGPDGEPDV	++
	FHPVGEAAIFYEYHQEGGPDGEPDVP	+	50	FHPVGEADYFEYHQEGGPDGEPD	++
	FHPVGEADAFEYHQEGGPDGEPDVP	+		FHPVGEADYFEYHQEGGPDGEP	++
	FHPVGEADYAEYHQEGGPDGEPDVP	+/-		FHPVGEADYFEYHQEGGPDGE	++
	FHPVGEADYFAIYHQEGGPDGEPDVP	+		FHPVGEADYFEYHQEGGPDG	++
20	FHPVGEADYFEAHQEGGPDGEPDVP	+		FHPVGEADYFEYHQEGGPD	++
	FHPVGEADYFEYAQEGGPDGEPDVP	++	55	FHPVGEADYFEYHQEGGP	++
	FHPVGEADYFEYHAEGGPDGEPDVP	+		FHPVGEADYFEYHQEGG	++
	FHPVGEADYFEYHQAGGPDGEPDVP	++		FHPVGEADYFEYHQEG	++
	FHPVGEADYFEYHQEAGPDGEPDVP	++		FHPVGEADYFEYHQE	++
25	FHPVGEADYFEYHQEGAPDGE PDVP	++		FHPVGEADYFEYHQ	+/-
	FHPVGEADYFEYHQEGGADGEPDVP	++	60	FHPVGEADYFEYH	+/-
	FHPVGEADYFEYHQEGGPA GEPDVP	++		FHPVGEADYFEY	+
	FHPVGEADYFEYHQEGGPAEPDVP	++		FHPVGEADYFE	+/-
	FHPVGEADYFEYHQEGGPDGAPDVP	++		EADYFEYHQE	++
30	FHPVGEADYFEYHQEGGPDGEADVP	++		FHPVGEADYFEYHQEGGPDGEPDVP	++
	FHPVGEADYFEYHQEGGPDGEPAVP	++			
	FHPVGEADYFEYHQEGGPDGEPDAP	++			
	FHPVGEADYFEYHQEGGPDGEPDVA	++			
	FHPVGAAYFAYHQAGGPDGEPDVP	-			

Supplementary Figure 1. Characterization of amino acids within EBNA1 region 401 to 430 that show strong interaction with the RCC1 N-terminal 20 amino acid tail.

Construction name	Primers for gateway cloning
pDONR-EBNA1	5'-GGGGACAACCTTTGTACAAAAAAGTTGGCATGACAGGACCTGCAAATGGCC-3' 5'-GGGGACAACCTTTGTACAAGAAAGTTGGTCACTCCTGCCCTTCCTCAC-3'
pDONR-EBNA1 8-410	5'-GGGGACAACCTTTGTACAAAAAAGTTGGCATGACAGGACCTGCAAATGGCC-3' 5'-GGGGACAACCTTTGTACAAGAAAGTTGGTACCCTACAGGGTGGAAAAATGG-3'
pDONR-EBNA1 381-Cter	5'-GGGGACAACCTTTGTACAAAAAAGTTGGCATGCCCAGGAGTCCCAGTAGTCAG-3' 5'-GGGGACAACCTTTGTACAAGAAAGTTGGTCACTCCTGCCCTTCCTCAC-3'
pDONR-RCC 1-20	5'-GGGGACAACCTTTGTACAAAAAAGTTGGCATGTCACCCAAGCG-3' 5'-GGGGACAACCTTTGTACAAGAAAGTTGGTTAGCTTTTGGGGATGGC-3'
pDONR-EBNA1 8-92nls	5'-GGGGACAACCTTTGTACAAAAAAGTTGGCATGCCTAAGAAGAAGCGCAAAGTC GGACCTGGAAATGGCC-3' 3'-GGGGACAACCTTTGTACAAGAAAGTTGGTCACTCCTGCTCCTGTTCCACCG-5'
pDONR-EBNA1 323-410	5'-GGGGACAACCTTTGTACAAAAAAGTTGGCATGGAGCAGGAGGTGGAGGC-3' 5'-GGGGACAACCTTTGTACAAGAAAGTTGGTACCCTACAGGGTGGAAAAATGG-3'
	Primers for Infusion cloning
pRFP-N1-EBNA1	5'-GGCAGGAGAAGGATCCGGCCTCCTCCGAGGACGTCATC-3' 5'-TCTAGAGTCGCGGCCGCGCAGAATTCTTAGGCGCCGGTGGGA-3'
pRFP-C1-EBNA1	5'-CGCTAGCGCTACCGGTGAGCTCGGATCCATGGCCTCC 3' 5'-TCCAGGTCTGTATCTGCAGTTCTATAGGCGCCGGTGGAGTG 3'
peGFP-N1-RCC1	5'-CTCAAGCTTCGAATC ATGTCACCCAAGCGCATAG-3' 5'-GGCGACCGGTGGATCC GCTCTGTTCTTTGTCCTTGACTAA-3'
peGFP-C1-RCC1	5'-TCAAGCTTCGAATCTGCAGGGACTTTCCCCAGCACAGCCAGCATGT-3' 5'-GCACGCATGATGTCTACTCACTCGGCAGGCGGGGGACATTC -3'
pRFP-N1-RCC1	5'-GGACTCAGATCTCGAGATGTCACCCAAGCGCATAGC-3' 5'-GGAGGAGGCCGGATCCCCGCTCTGTTCTTTGTCCTTGAC-3'
pRFP-C1-RCC1	5'-CGCTAGCGCTACCGGTATGGCCTCCTCCGAGGACG-3' 5'-GAAGCTTGAGCTCGAGCGCCGGTGGAGTGGCG
	Primers for site directed mutagenesis
peGFP-N1-EBNA1 Δ40-53	5'-GGGGCTCCTGGATGGTTATCACCCCCTCTT-3'

	5'-AAGAGGGGGTGATAACCATCCAGGAGCCCC-3'
peGFP-N1-EBNA1 Δ326-358	5'-CACGGTGG AACAGGAGAAAGAGCCAGGGGG-3' 5'-CCCCCTGGCTCTTTCTCCTGTTCCACCGTG-3'
peGFP-N1-EBNA1 Δ411-420	5'-CCACCCTGTAGGGGGTGGCCCAGATGGTG-3' 5'-CACCATCTGGGCCACCCCCTACAGGGTGG-3'
pDEST15-RCC1 Δ1-20	5'-GTACAAAAAAGTTGGCATAGCTAAAAGAAGGTCCC-3' 5'-GAGACCTTCACCTTCTTATCTGCTGGGG-3'
pDEST15-RCC1 Δ1-5	5'-GTACAAAAAAGTTGGCATAGCTAAAAGAAGGTCCC-3' 5'-GGGACCTTCTTTTAGCTATGCCAACTTTTTGTAC-3'
pDEST15-RCC1 Δ6-10	5'-GCATGTCACCCAAGCGCTCCCCCAGCAGATGC-3' 5'-GCATCTGCTGGGGGGAGCGCTTGGGTGACATGC-3'
pDEST15-RCC1 Δ11-15	5'-GCATAGCTAAAAGAAGGGCCATCCCCAAAAGC-3' 5'-GCTTTTGGGGATGGCCCTTCTTTTAGCTATGC-3'
pDEST15-RCC1 Δ16-20	5'-CCCCAGCAGATAAGAAGGTGAAGGTCTC-3' 5'-GAGACCTTCACCTTCTTATCTGCTGGGG-3'

Supplementary table : Oligonucleotides used for PCR-amplifications, Infusion cloning and Site-Directed mutagenesis.