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# The RNA-sensitive association between nucleolar NPM1/B23 and HTLV-I basic leucine zipper factor (HBZ) potentially acts on HBZ-induced cell transformation

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

### *Author contribution statement*

ZL, EL, GL, SC, ER and BB have designed the research; JMM, HG and YZ have provided material or PBMC samples; ZL, EL, Y Xie, Y Xiao, and JMP have conducted the experiments; ZL, EL, Y Xiao, RL, JMP and BB have analyzed the data; ZL, EL, JMP and BB wrote the paper; ZL, HG, Y Xiao, SZ, GL, JMM, ER, RL, SC, YZ and BB have reviewed and edited the paper.

### *Keywords*

HTLV-1, ATLL, HBZ, NPM1/B23, nucleophosmin, APH-2, nucleolus, RNA binding, cell transformation

### *Abstract*

Word count: 208

Human T-cell leukemia virus type 1 is the causative agent of HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T-cell leukemia-lymphoma (ATL). The HTLV-1 basic leucine zipper factor (HBZ) has been associated to the cancer-inducing properties of this virus, although the exact mechanism is unknown. In this study, we identified (nucleophosmin) (NPM1/B23) as a new interaction partner of the major sHBZ isoform. We show that sHBZ and the less abundant uHBZ isoform interacts with nucleolar NPM1/B23 in infected cells and HTLV-1 positive patient cells, unlike equivalent antisense proteins of related non-leukemogenic HTLV-2, -3 and -4 viruses. We further demonstrate that sHBZ association to NPM1/B23 is sensitive to RNase. Interestingly, sHBZ was shown to interact with its own RNA. Through siRNA and overexpression experiments, we further provide evidence that NPM1/B23 acts negatively on viral gene expression with potential impact on cell transformation. These results suggest that the interaction of NPM1/B23 with sHBZ impact regulation of viral gene expression (including its own) and could contribute to the role of this viral protein in ATL development. Our results hence provide a new insight over HBZ-binding partners in relation to cellular localization and potential function on cell proliferation and should lead to a better understanding of the link between HBZ and ATL development.

### *Contribution to the field*

Several DNA and RNA viruses are known to utilize different compartments of the cells for efficient replication related to viral proteins functions but also in diseases associated to infection. We have been studying the Human T-cell leukemia virus type 1 that is the causal agent of the aggressive adult T-cell leukemia-lymphoma (ATL). Herein, we showed for the first time that a protein named NPM1/B23 was more expressed in ATLL patients and that associated with the viral protein HBZ, which has been known to be crucial for ATL development. Through these results, we are those suggesting that this association between cellular and viral protein could represent a target to block the potential of infected patients to develop this virally induced form of leukemia. Such unique association could also be relevant in other forms of cancers either induced by other viruses or caused by mutations in cellular genes. More studies are currently underway in our laboratory to further explore the mechanism implicating this partnership.

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In review

*Data availability statement*

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In review

1 **A newly identified interaction between nucleolar **NPM1/B23** and the HTLV-I**  
2 **basic leucine zipper factor in HTLV-1 infected cells**

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5 Peloponese<sup>7</sup>, Jean-Michel Mesnard<sup>7</sup>, Éric Rassart<sup>2,3</sup>, Rongtuan Lin<sup>8</sup>, Shuang Zhou<sup>6</sup>, Yiming Zeng<sup>6</sup>,  
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28 Short title: RNA-sensitive interaction between NPM1/B23 and HBZ

29

30 Key words: HTLV-1; ATL; HBZ; NPM1/B23; APL-2; nucleolus; RNA binding; cell transformation

In review

31 **Abstract**

32

33 Human T-cell leukemia virus type 1 is the causative agent of HTLV-1-associated  
34 myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T-cell leukemia-lymphoma (ATL).  
35 The HTLV-1 basic leucine zipper factor (HBZ) has been associated to the cancer-inducing properties  
36 of this virus, although the exact mechanism is unknown. In this study, we identified nucleophosmin  
37 (NPM1/B23) as a new interaction partner of HBZ. We show that sHBZ and the less abundant uHBZ  
38 isoform interact with nucleolar NPM1/B23 in infected cells and HTLV-1 positive patient cells, unlike  
39 equivalent antisense proteins of related non-leukemogenic HTLV-2, -3 and -4 viruses. We further  
40 demonstrate that sHBZ association to NPM1/B23 is sensitive to RNase. Interestingly, sHBZ was  
41 shown to interact with its own RNA. Through siRNA and overexpression experiments, we further  
42 provide evidence that NPM1/B23 acts negatively on viral gene expression with potential impact on  
43 cell transformation. Our results hence provide a new insight over HBZ-binding partners in relation  
44 to cellular localization and potential function on cell proliferation and should lead to a better  
45 understanding of the link between HBZ and ATL development.

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51 **Author Summary**

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53 Several DNA and RNA viruses are known to utilize different compartments of the cells for efficient  
54 replication related to viral proteins functions but also in diseases associated to infection. We have  
55 been studying the Human T-cell leukemia virus type 1 that is the causal agent of the aggressive adult  
56 T-cell leukemia-lymphoma (ATL). Herein, we showed for the first time that mRNA level of *NPM1*  
57 was more abundant in cells from ATL patients when compared to cells from asymptomatic carriers  
58 and that this protein associated with the viral protein HBZ, which has been known to be crucial for  
59 ATL development. Such unique association could also be relevant in other forms of cancers induced  
60 by other viruses. More studies are currently underway in our laboratory to further explore the  
61 mechanism implicating this partnership.

62

63

## 64 **Introduction**

65

66 It is estimated that 5 to 10 million individuals are infected by HTLV-1 worldwide , although most  
67 infected individuals are healthy asymptomatic carriers (AC) (1). Among them, 5% of infected  
68 individuals will eventually develop Adult T-cell leukemia-lymphoma (ATL) (2-4), while 1-2% will  
69 progress toward the neurological disorder, HTLV-1-associated myelopathy/tropical spastic  
70 paraparesis (HAM/TSP)(5). Currently, there are very limited therapeutic treatments available for  
71 these diseases. Interestingly, other members of the HTLV family, known as HTLV-2, -3 and -4, have  
72 not been associated with any human malignancies despite their strong similarity in genomic structure  
73 and viral genes (6-9).

74 Several studies have been devoted to the mechanistic understanding of ATL. One of the  
75 HTLV-1 gene associated to ATL is HTLV-I basic leucine zipper factor (*hbz*), which is encoded by  
76 the antisense strand of the HTLV-1 genome (10-13). Two isoforms (sHBZ and uHBZ) have been  
77 identified and are expressed from spliced and unspliced transcripts, respectively (12, 14-16). The  
78 *shbz* isoform is the most abundant and is expressed at least at four-time higher levels than *uhbz* in  
79 HTLV-1-infected cells (16). Localization studies have further demonstrated differences between  
80 uHBZ and sHBZ: although both HBZ isoforms target the nucleus through three nuclear localization  
81 signals (NLS), most studies have demonstrated that sHBZ also localizes to the nucleolus, while uHBZ  
82 displays a **non-nucleolar** distribution (16, 17). HBZ also contains a Leucine zipper (LZ) motif, which  
83 allows it to interact with several other LZ motif-bearing transcription factors (11, 18). These LZ-  
84 dependent interactions as well as additional interactions with other segments of the proteins are

85 considered important for the different functions attributed to sHBZ, such as maintenance of viral  
86 latency through inhibition of the HTLV-1 transactivator Tax and induced proliferation of infected  
87 malignant cells (18).

88 The association of sHBZ with ATL development has been supported by reports showing that  
89 *shbz* transcripts are detected in most ATL cells from patients and have been shown to be more  
90 abundant in ATL patients versus asymptomatic carriers and HAM/TSP patients, mostly correlating  
91 with proviral load (12, 16, 19). Further support for the contribution of sHBZ in ATL development  
92 has been associated to proliferation studies showing that sHBZ and possibly its transcript could be  
93 essential players in the proliferation of ATL cells (12, 20-22). Reports have also underscored the  
94 oncogenic potential of sHBZ in transgenic mice and in NOD/SCID mice engrafted with HTLV-1-  
95 transformed T cells (20, 21, 23). Several mechanisms have been proposed to account for the capacity  
96 of HBZ to transform cells (24-26). These studies have focused on the sHBZ isoform, while previous  
97 results had suggested that the unspliced HBZ isoform might not be oncogenic, as it is unable of  
98 inducing IL-2-independent proliferation in IL-2-dependent cell lines (12, 27). Of further importance,  
99 the equivalent antisense protein encoded by HTLV-2, APH-2 (Antisense protein of human T-cell  
100 leukemia virus type 2) is also frequently expressed in primary cells from HTLV-2-infected  
101 individuals (28), but does not induce IL-2-independent growth in IL-2-dependent T cell lines and  
102 localizes to the nucleus, but not to the nucleolus, similarly to uHBZ (29).

103 The nucleolus is a dynamic cellular structure and, in addition to its essential role in ribosome  
104 biogenesis, is involved in many biological processes, such as transcription, cell cycle control,

105 genomic stability, cellular aging and viral infection (30). The two most abundant nucleolar  
106 phosphoproteins, B23 (also known as nucleophosmin, NPM1) and C23 (also termed nucleolin) are  
107 implicated in these processes (31-33). Although mainly localized to the nucleolus, these proteins are  
108 known to shuttle constantly and freely between the nucleus and the cytoplasm (34). Important links  
109 between hematological cancers and NPM1/B23 have been highlighted in previous years. Indeed, the  
110 mutation in the *NPM1* gene has been observed in Anaplastic large-cell lymphoma (ALCL), Acute  
111 promyelocytic leukemia (APL) and Acute myeloid leukemia (35-38).

112 Importantly, NPM1/B23 plays functional roles in different stages of viral replication including  
113 nuclear import, viral genome transcription and assembly, as well as final particle formation by  
114 directly interacting with various viral proteins in different cellular compartments during relevant  
115 infections, as exemplified with Chikungunya virus (CHIKV), HIV, HBV, HCV, HDV,HPV and  
116 HSV-1 (39-41). HIV-1 can induce the acetylation of NPM1/B23, which plays a critical role in nuclear  
117 localization of Tat and Tat-mediated transcriptional activation of the integrated provirus (42).  
118 Furthermore, HIV-1 Rev and HTLV-1 Rex are known to be targeted to cell nucleoli to enhance viral  
119 replication (43, 44). In fact, a NPM1/B23 inhibitor, avrainvillamide-analog-3, has in been repurposed  
120 as a broad antiviral replication inhibitor (45, 46).

121 Interestingly, sHBZ interacts with many cellular partners (18), although nucleolar proteins have  
122 never been previously investigated. Based on the nucleolar localization of sHBZ and the implication  
123 of nucleolar proteins in transformation-related processes, we hypothesized that these proteins could  
124 be interacting with sHBZ and might be implicated in properties attributed to the pathogenesis of

125 sHBZ. We report herein that NPM1/B23 is a new potential HBZ-interacting partner, an association  
126 which is not apparent with antisense proteins from other HTLV viruses. Basic regions of sHBZ and  
127 the RNA-binding domain of NPM1/B23 were found to mediate this interaction, while RNA  
128 interfered with sHBZ-NPM1/B23 complex formation. Association of sHBZ with its own transcripts  
129 was further demonstrated. We finally showed that, by silencing approach and overexpression studies,  
130 this association between NPM1/B23 and sHBZ could promote cell transformation. Our results further  
131 suggest that the association of HBZ with NPM1/B23 could partly modulate HBZ expression and  
132 could account for the role of sHBZ in HTLV-1-associated ATL.

In review

133 **Results**

134

135 **Increased expression of *NPM1* in PBMC from ATL patients and association with sHBZ in**  
136 **transfected cells**

137 Previous studies have demonstrated that sHBZ is distributed in the form of nuclear speckles and  
138 that it also localised to the nucleolus (16). The uHBZ isoform shows a similar nuclear pattern,  
139 although it does not predominantly localise to the nucleoli (17). NPM1/B23 being a major nucleolar  
140 protein implicated in hematological malignancies (37), we hypothesized that a functional association  
141 between HBZ and NPM1/B23 could exist. To address this possibility, we first assessed *NPM1*  
142 expression in HTLV-1-infected individuals, using PBMCs isolated from HTLV-1-infected  
143 asymptomatic individuals and from patients suffering from ATL or HAM/TSP. Following qRT-PCR  
144 analyses (Fig 1A), we observed that *NPM1* expression was more pronounced in samples from ATL  
145 patients, while no significant increase was noted in HAM/TSP patients (Fig 1A). Of note, no  
146 correlation could be demonstrated between proviral load of individuals/patients and *NPM1*  
147 expression (data not shown).

148 Since the above results showed an increase in *NPM1* expression in sHBZ-expressing PBMC cells  
149 from ATL patients, we next sought to further examine a potential association between both nucleolar-  
150 located proteins. We initially co-transfected Myc-tagged sHBZ or uHBZ with a DsRed-fused B23  
151 expression vector in COS-7 and HeLa cells (Fig 1B-C). As expected, we first observed that, although  
152 both HBZ isoforms were distributed as nuclear speckles, sHBZ also showed important localization

153 to the nucleolus, while uHBZ was **more non-nucleolar** (Fig. 1B). Furthermore, strong co-localization  
154 between sHBZ and DsRed-B23 was apparent, especially at the nucleolar region, while important co-  
155 localisation could be visualized for uHBZ although slightly different pattern of localisation was noted  
156 at the nucleoli. To further confirm these co-localization data, similar co-transfection experiments of  
157 GFP-HBZ and DsRed-B23 expression vectors were performed in **HeLa cells** (Fig 1D). Higher co-  
158 localisation was more clearly noted for the sHBZ isoform with a predominant nucleolar localisation,  
159 while co-localisation was generally demonstrated for uHBZ, despite being less prominent than for  
160 sHBZ. Similar results were obtained in transfected COS-7 cells (Fig. 1E, Fig, S1). Interestingly, in  
161 these cells, we noted that sHBZ was more prominent in the nucleoli when NPM1/B23 was  
162 overexpressed, while a higher number of uHBZ-expressing cells showed apparent relocation of  
163 uHBZ to the nucleolar NPM1/B23-positive region (Fig 1E).

164 To clearly determine if the different HBZ isoforms could associate with NPM1/B23, extracts from  
165 293T cells transfected with Myc-tagged HBZ and DsRed-B23 expression vectors were  
166 immunoprecipitated with anti-Myc antibodies and analysed by Western blot. As depicted in Fig 1F,  
167 sHBZ strongly associated to DsRed-B23 as well as to NPM1/B23. Although levels of uHBZ were  
168 lower than sHBZ levels, due to its inherent instability, **uHBZ nonetheless showed a specific signal**  
169 **for NPM1/B23**. Extracts from HeLa cells **transfected with Myc-tagged HBZ** also revealed association  
170 between sHBZ and NPM1/B23, when immunoprecipitated samples were analysed by Western blot  
171 (Fig 1G).

172 These data hence demonstrated that both isoforms of HBZ associated to NPM1/B23. Although  
173 nucleolar localisation of HBZ was not strictly required, it seemed to favor the interaction between  
174 both proteins.

175

#### 176 **Association between sHBZ and NPM1/B23 in transfected T cell lines and HTLV-1-infected cells**

177 To further validate the association between sHBZ and NPM1/B23, T cell lines were first analysed  
178 in transfection experiments. Hence, SupT1 T cells were transfected with Myc-tagged uHBZ and  
179 sHBZ expression vectors and subsequently analysed by co-immunoprecipitation (Fig 2A). Both HBZ  
180 isoforms indeed formed a complex with NPM1/B23 in this T cell line.

181 To address this interaction with more relevant expression levels of sHBZ, two chronically infected  
182 T cell lines (C8166-45 and MT2), were tested. These experiments were important, as previous studies  
183 had suggested that HBZ did not reside in the nucleolar compartment when analysed in non-  
184 overexpressing HBZ ATL cell lines and in PBMCs from a patient diagnosed with severe acute ATL  
185 (47). Extracts from C8166-45, sHBZ-expressing 293T and uninfected Jurkat cells were  
186 immunoprecipitated with anti-B23 antibodies (Fig 2B). Following Western blot analyses, HBZ-  
187 specific signals were detected in both immunoprecipitated 293T and C8166-45 extracts. No similar  
188 signals were obtained in these experiments with non-HBZ-expressing Jurkat cells or when extracts  
189 were immunoprecipitated with control non-specific IgG antibodies (Fig 2B; Fig S2). Confocal  
190 microscopy analyses of C8166-45 cells demonstrated co-localisation of HBZ and NPM1/B23, while  
191 specificity of the HBZ signal was confirmed by the absence of any signals in uninfected H9 cells

192 (Fig 2C). The interaction between HBZ and endogenous NPM1/B23 was also confirmed by co-  
193 immunoprecipitation experiments in chronically infected MT2 cells (Fig 2D).

194 We further tested the HBZ-NPM1/B23 interaction in freshly infected cells. Using a previously  
195 described protocol (48), HeLa cells were co-cultured with MT2 cells, which were then removed by  
196 thorough washing. As shown in Fig 2E, HBZ was specifically detected in infected HeLa cells. When  
197 infected HeLa cells were further analysed, a clear co-localisation between NPM1/B23 and sHBZ  
198 signals could again be demonstrated. Importantly, following immunoprecipitation with anti-B23  
199 antibodies, HBZ was detected in infected HeLa cells and not in uninfected cells. Furthermore,  
200 immunoprecipitation of infected HeLa cell extract with a control IgG did not lead to the HBZ-specific  
201 signal (Fig 2F). Importantly, HBZ and NPM1/B23 co-localization was confirmed in freshly isolated  
202 CD4+ T cells from a HAM/TSP patient by confocal microscopy (Fig 2G).

203 These results hence clearly demonstrated that sHBZ co-localized and was associated to NPM1/B23  
204 in HTLV-1-infected cells and CD4+ T cells from a HAM/TSP patient and that this interaction  
205 appeared most prominent in the nucleolar-like structure.

206

### 207 **Antisense proteins from other HTLV-1-related viruses do not interact with NPM1/B23**

208 We next sought to determine if NPM1/B23 could interact with antisense-transcript-encoded  
209 proteins of HTLV-2, HTLV-3 and HTLV-4 known as APH-2, APH-3 and APH-4. We have  
210 previously demonstrated that APH-2 is nuclear, but non-nucleolar (28). On the other hand, APH-3  
211 and APH-4 both localizes to the nucleus and the nucleolus, although confocal microscopy analyses

212 did reveal differences in localisation in comparison to sHBZ (49). 293T cells were first transfected  
213 with expression vectors for GFP-sHBZ and GFP-APH-2 fusion proteins, followed by co-  
214 immunoprecipitation of cellular extracts with anti-GFP antibodies. Resulting Western blot analyses  
215 demonstrated that, unlike sHBZ, APH-2 did not co-immunoprecipitate with NPM1/B23 (Fig 3A).

216 We next tested if APH-3 and APH-4 could indeed associate with NPM1/B23. We conducted  
217 confocal microscopy analyses of COS-7 cells co-transfected with DsRed-B23 and Myc-tagged APH-  
218 3 or APH-4 expression vectors. Results confirmed our previous findings that NPM1/B23 co-localized  
219 with APH-3 and APH-4 in nucleoli (Fig 3B). However, when 293T cells were transfected with  
220 expression vectors for Myc-tagged sHBZ, APH-3 and APH-4 and resulting extracts were co-  
221 immunoprecipitated with anti-Myc antibodies, no association between NPM1/B23 and APH-3 or  
222 APH-4 were convincingly demonstrated (Fig 3C). However, as previously reported, in these  
223 analyses, APH-4 and most markedly APH-3 were less abundant in transfected cells and the  
224 interaction between both proteins and NPM1/B23 might have not been detected in these experiments.

225 These results hence demonstrated that APH-2 (and APH-3 and APH-4) are not as potent to  
226 associate with NPM1/B23, even if strong co-localisation with NPM1/B23 is apparent for APH-3 and  
227 APH-4, thereby suggesting that a specific sHBZ region is required for proper association.

228

### 229 **sHBZ associates with C23 without disturbing its interaction with NPM1/B23**

230 We next investigated if the nucleolar C23 protein might also be part of the sHBZ-NPM1/B23  
231 complex. Firstly, the localisation of sHBZ and C23 were examined in COS-7 and HeLa cells co-

232 transfected with Red-C23 and GFP-fused sHBZ or uHBZ expression vectors (Fig 4A-B). Confocal  
233 microscopy analyses indicated co-localisation between C23 and both uHBZ and sHBZ in COS-7 and  
234 HeLa cells, although a more dominant co-localisation was apparent for NPM1/B23. Strikingly,  
235 regions of HBZ-related fluorescence included areas in which C23 was not observed, especially in the  
236 nucleolar region. As co-localisation was detected between sHBZ and C23, co-immunoprecipitation  
237 experiments were next conducted in transfected 293T cells (Fig 4C). When extracts from 293T cells  
238 expressing Myc-tagged sHBZ were immunoprecipitated with an anti-Myc antibody, signals for both  
239 C23 and B23 were detected along with sHBZ (Fig 4C). Similar results were obtained in sHBZ-  
240 expressing HeLa cells showing C23 association to the sHBZ-B23 complex (data not shown).

241 As previous studies have demonstrated that NPM1/B23 interacts with C23 and shuttles C23 to the  
242 nucleolus (50, 51), we were interested in evaluating if sHBZ could impact the interaction between  
243 NPM1/B23 and C23. 293T cells were transfected with increasing amounts of the sHBZ expression  
244 vector and extracts were subjected to immunoprecipitation using an anti-B23 antibody. As shown in  
245 Fig 4D, levels of C23 co-immunoprecipitated with B23 remained steady regardless of the abundance  
246 of sHBZ.

247 These results hence suggested that C23 could associate with the sHBZ-B23 complex but was not  
248 outcompeted from its binding to NPM1/B23 by sHBZ.

249

250 **The HBZ basic regions are necessary for the association between sHBZ and NPM1/B23**

251 HBZ is comprised of several functional domains: the activation domain (AD), nuclear localisation  
252 signals (including basic region BR2, BR1 and DBD), and the Leucine Zipper region (Fig 5A) (13).  
253 The LZ region has been shown to be a crucial region for protein-protein interaction, although other  
254 domains have also been implicated in many protein interactions (52-54). To determine which domain  
255 of HBZ is essential for its binding to NPM1/B23, expression vectors for Myc-tagged sHBZ,  
256 sHBZ $\Delta$ AD and sHBZ $\Delta$ AD $\Delta$ ZIP were transfected in 293T cells. Following immunoprecipitation  
257 with anti-Myc antibodies, Western blot analyses were performed. Both mutants and WT HBZ were  
258 able to associate with NPM1/B23 and C23, despite presenting different intensities. However, the  
259 sHBZ $\Delta$ AD $\Delta$ ZIP mutant seemed to harbour enhanced affinity toward both NPM1/B23 and C23 (Fig  
260 5B). Interestingly, in transfected COS-7 cells, this mutant exhibited a more important nucleolar  
261 localisation than for WT sHBZ with a concomitant higher co-localisation with NPM1/B23 (Fig 5C).  
262 To attempt to refine the region required for NPM1/B23 interaction, we tested two previously  
263 described GFP-fused HBZ mutants containing either BR2-BR1 or BR1-DBD (Fig 5D) (17). Both  
264 mutants showed weaker but specific binding to NPM1/B23 upon analyses of anti-GFP-  
265 immunoprecipitated extracts from transfected 293T cells and suggested that the shared BR1 region  
266 might be important for the association of sHBZ with NPM1/B23.

267 These data thus indicated that the basic regions of sHBZ are required for its association to  
268 NPM1/B23 and that the removal of the AD domain and ZIP region concomitantly induces higher  
269 NPM1/B23 association and nucleolar localisation.

270

271 **The nucleic acid-binding domain of NPM1/B23 is important for its association with sHBZ**

272 NPM1/B23 is characterized by the presence of an homo-oligomerization (HoD), an  
273 heterodimerization (HeD) and a nucleic acid-binding domain (NBD) (Fig 6A) (55). In order to  
274 characterize which domain of NPM1/B23 is important for its association with sHBZ, deletion  
275 mutants were generated and co-localization with sHBZ was assessed by confocal microscopy in  
276 transfected COS-7 cells. As shown in Fig 6B, sHBZ co-localized to nucleoli with HoD- and HeD-  
277 deleted NPM1/B23 mutants. As expected, these deletion mutants co-immunoprecipitated with sHBZ  
278 in transfected 293T cells (Fig 6C). However, in cells expressing the NBD deletion mutant, the  
279 association with sHBZ was strongly reduced, as assessed by co-immunoprecipitation. As expected,  
280 no sHBZ and NPM1/B23-specific signals were detected in anti-Myc immunoprecipitates when sHBZ  
281 was not expressed.

282 To further determine the role of NBD in the sHBZ/NPM1/B23 interaction, NBD fused to DsRed  
283 was expressed in COS-7 cells and, upon analysis by confocal microscopy, shown to strongly co-  
284 localize with sHBZ (Fig 6D). In transfected 293T cells, as opposed to the NBD-deleted form of  
285 NPM1/B23, the NBD only-containing mutant was shown to be associated with sHBZ (Fig 6E). To  
286 validate that nucleolar localisation is important for the association, a DsRed-B23 fusion protein with  
287 a point mutation at position 290 (B23 W290A), known to be non-nucleolar (56, 57), was co-expressed  
288 with sHBZ in 293T cells and analysed by co-immunoprecipitation. As depicted in Fig 6E, association  
289 of this mutant with sHBZ was not observed, despite its successful immunoprecipitation.

290 These data hence provided strong evidence that the NBD domain is responsible for the association  
291 of NPM1/B23 to sHBZ and further confirmed the importance of nucleolar localisation for the  
292 interaction.

293

294 **sHBZ associates with NPM1/B23 in an RNA-sensitive manner and binds to its own mRNA**

295 As the above results demonstrated that the NPM1/B23 NBD domain was sufficient for its  
296 association with sHBZ, we were next interested in determining if DNA or RNA could be involved in  
297 the formation of the sHBZ-NPM1/B23 complex. To address this possibility, extracts from 293T cells  
298 co-transfected with expression vectors for sHBZ and DsRed-B23 or DsRed-B23 $\Delta$ NBD were co-  
299 immunoprecipitated with anti-DsRed followed by DNase or RNase treatment before elution of the  
300 immunoprecipitated complex (Fig 7A). As demonstrated above, the interaction of sHBZ was reduced  
301 with the NBD-deleted NPM1/B23 mutant compared to WT NPM1/B23. After DNase treatment, no  
302 significant change in the intensity of the interaction between sHBZ and NPM1/B23 was apparent, as  
303 revealed by the analysis of the immunoprecipitated samples. Surprisingly, when immunoprecipitated  
304 extracts were treated with RNase, NPM1/B23 was more strongly associated to sHBZ than without  
305 treatment. Unexpectedly, the interaction was also increased with the NBD-deleted NPM1/B23  
306 mutant (see Discussion). A similar important increase in the association between these proteins in  
307 the presence of RNase was revealed in transfected 293T in the absence of overexpressed DsRed-B23  
308 (Fig 7B). We further extended these analyses to C8166-45 cells. Extracts were thus co-  
309 immunoprecipitated with anti-B23 antibodies and treated or not with RNase (Fig 7C). Again, RNase

310 treatment led to stronger association between sHBZ and NPM1/B23. Interestingly, when C23 was  
311 analysed in the complex, comparable levels were detected regardless of the addition of RNase  
312 treatment.

313 Since NPM1/B23 is known to interact with RNA and that RNase treatment increases the  
314 association between sHBZ and NPM1/B23, we thereby suspected that sHBZ might be part of a  
315 complex in which RNA disfavours specific interaction and that sHBZ might in fact be a potential  
316 RNA-binding protein, especially knowing that *its* DBD region has not been shown to bind to DNA.  
317 To test this, we focussed our attention on *shbz* transcripts, as previous studies had suggested that this  
318 viral transcript was more prone to nuclear retention in HTLV-1-infected cells (58, 59). RNA  
319 immunoprecipitation (RIP) was performed with anti-HBZ antibodies on C8166-45 cell extracts.  
320 Isolated RNA was next analysed by RT-PCR using primers specific for the detection of spliced  
321 transcripts (shown in Fig 7D). As depicted in Fig 7D, *shbz* mRNA was specifically detected in anti-  
322 HBZ immunoprecipitates for each tested primer pair, while no equivalent signals were observed in  
323 control immunoprecipitation (IgG). Sequencing confirmed that these signals represented spliced *hbz*  
324 mRNA. Anti-HBZ immunoprecipitated RNA were also negative when analysed for the presence of  
325 *GAPDH* mRNA by RT-PCR.

326 These results hence suggested that the association between sHBZ and NPM1/B23 was competed  
327 by RNA and that, like NPM1/B23, sHBZ could associate with RNA, which included its own spliced  
328 transcript.

329

330 ***NPM1/B23* expression alters sHBZ expression with a potential cooperation toward cell**  
331 **transformation**

332 To investigate the potential function of the sHBZ-NPM1/B23 association on HTLV-1 infected  
333 cells, we next sought to repress NPM1/B23 expression. We first attempted to knock-out the *NPM1*  
334 gene in C8166-45 by CRISPR/Cas9: no clones with lack of expression were recuperated, likely due  
335 to the essential role of this protein for cell survival. We thus relied on a silencing approach of *NPM1*  
336 expression in chronically infected cell lines using two different siRNA (Fig 8A). Our data showed  
337 that partial downregulation of *NPM1* was observed in C8166-45 cells, although little effect was noted  
338 on cell viability. Interestingly, *NPM1* silencing led to a reduction in sHBZ expression (mRNA and  
339 protein levels) with a concomitant increase in Tax expression (Fig 8B-C). To determine if  
340 NPM1/B23-sHBZ association could promote cell transformation, a soft agar growth assay was  
341 performed in HeLa cells, which were transfected with expression vectors for GFP-sHBZ, GFP-  
342 uHBZ, DsRed-B23 and/or empty vectors. Resulting clone counts showed that concomitant  
343 expression of either uHBZ or sHBZ with NPM1/B23 led to an increase in cell transformation (Fig  
344 8D), although sHBZ showed a **slightly** higher number of clones (non-significant). Of note,  
345 NPM1/B23 alone did not lead to significant changes in clone numbers.

346 These results thus suggest that nucleolar sHBZ through its interaction with NPM1/B23 could act  
347 upon HBZ expression and subsequently Tax production with a potential link with cell transformation.

348

349 **Discussion**

350

351 Several DNA and RNA viruses are known to make use of the nucleolus for efficient replication.  
352 Several viral proteins in fact transit to the nucleolus, while for certain viruses, infection can lead to  
353 change in the localisation of nucleolar components, or reversibly import cytoplasmic constituents  
354 into the nucleus or nucleolus in order to enhance viral fitness (43, 60). NPM1/B23 has been  
355 previously reported to be a mediator of these associations between the nucleolus and viruses in certain  
356 cases, lending support to replication and acting on functional properties of proteins, such as HIV-1  
357 Rev and Tat, and HTLV-1 Rex (61-63). Herein, we have focussed on HBZ, an HTLV-1 protein  
358 previously shown to localise to the nucleolus. Our findings demonstrate that HBZ associates with  
359 NPM1/B23, that RNA hampers complex formation, and that this association could play a role in  
360 transforming properties of sHBZ.

361 Based on the nucleolar localisation of sHBZ and the previously reported implication of NPM1/B23  
362 in various leukemic cancers (35-38), we focussed our attention on its potential interaction with both  
363 HBZ isoforms. We first determined if NPM1/B23 expression was impacted in PBMCs from ATL  
364 and HAM/TSP patients when compared to asymptomatic HTLV-1-infected individuals. A significant  
365 increase in *NPM1* expression was observed in [PBMCs](#) from ATL patients. Given that expression of  
366 *NPM1* was more important in [PBMCs from](#) ATL patients, we thus speculated that a functional  
367 association could be at play between both nuclear proteins. Hence, this led us to assess the potential  
368 association more precisely between HBZ and *NPM1*. Confocal experiments and co-

369 immunoprecipitation experiments indeed revealed association of NPM1/B23 with both HBZ  
370 isoforms. Since uHBZ does not localize to the nucleolus, these results suggested that NPM1/B23  
371 interaction also occurs externally to the nucleolus, which is reminiscent of the C23-NPM1/B23  
372 interaction also occurring outside of the nucleolus (51). However, of note, when NPM1/B23 was  
373 over-expressed, sHBZ seemed to localize to nucleoli to a greater extent. This was more clearly  
374 observed in COS-7 cells overexpressing NPM1/B23 and uHBZ, the latter being prominently co-  
375 localized with NPM1/B23 to the nucleolus. These results suggest that NPM1/B23, being a shuttling  
376 protein, may help sHBZ to be retained in the nucleoli in a similar manner to other cellular and viral  
377 proteins, such as HIV-1 Tat (43, 62).

378 Our analyses have further validated the association of sHBZ with NPM1/B23 in transfected T cell  
379 lines, chronically infected T cell lines and freshly infected HeLa cells by confocal microscopy and  
380 co-immunoprecipitation experiments. Although the exact isoform detected in infected cells was not  
381 determined in these analyses, previous studies have indicated that the sHBZ isoform was the main  
382 isoform detected in ATL cell lines (16), which is further supported by the nucleolar-like distribution  
383 of most of our HBZ signals. However, recent reports have argued that, in ATL cells (and thereby in  
384 more relevant HBZ-expressing cell context), HBZ did not localise to the nucleolus, as determined by  
385 confocal microscopy analyses and C23 labeling (47). Moreover, a more recent study has suggested  
386 that HBZ only localized in the cytoplasm of infected cells from asymptomatic carriers and HAM/TSP  
387 patients (64). Although it is possible that the HBZ-NPM1/B23 interaction in the context of infected  
388 cells might occur in non-nucleolar compartments, our results nonetheless argue that HBZ in the

389 context of infected cells localizes to the nucleus and the nucleolus, which agrees with previous  
390 studies. Importantly, a previous report has revealed that ATL cells can be devoid of nucleoli (65),  
391 and might not easily lead to a positive nucleolar distribution for HBZ, as those observed in non-ATL  
392 cells. Regardless of these contradictory findings, our results confirmed the association of HBZ with  
393 NPM1/B23 in relevant expression levels.

394 An important finding in this study was that APH-2 and possibly APH-3 and APH-4 were unable  
395 to co-immunoprecipitate NPM1/B23, despite the fact that APH-4 is a nucleolar protein. We and  
396 others have previously reported on functional differences between APH proteins and sHBZ in  
397 addition to differences in subcellular localisation (28, 49, 66-69). These functional differences might  
398 be dependent on the cellular localisation of these proteins and might also be attributed to their varying  
399 affinities toward interacting partners. However, whether the lack of interaction of NPM1/B23 with  
400 antisense proteins of other HTLV viruses is responsible for some of the divergent effects of these  
401 various antisense proteins over the activation potential of different transcription factors remains to  
402 be determined. Further experiments are underway to clarify these results, especially those related to  
403 APH-3 and APH-4, which have strong intrinsic instability and therefore lead to less firm conclusions.

404 An important interacting partner of NPM1/B23 is C23. Among its many functions, C23 plays  
405 a crucial role in cell proliferation and survival (70). In fact, previous studies have used C23 to  
406 demonstrate the nucleolar localisation of sHBZ and deleted mutants of uHBZ (16, 17, 71). Based on  
407 our results showing that C23 only partially co-localized with sHBZ, we suggest that C23 is part of  
408 the sHBZ-associated complex due to its NPM1/B23-interacting properties, which would explain why

409 the NPM1/B23-C23 interaction is not outcompeted by sHBZ. Furthermore, previous studies  
410 indicated that C23 and NPM1/B23 do not completely localize to the same compartment of the  
411 nucleolus (72). Our results showed that the region important for NPM1/B23 interaction with sHBZ  
412 was different from the domain implicated in its association with C23 (50). Indeed, analyses of  
413 deletion mutants of NPM1/B23 allowed us to conclude that the NBD domain was important for its  
414 association with sHBZ. Of note, the residual interaction observed between the NBD-deleted mutant  
415 with sHBZ in some co-immunoprecipitation experiments might be due to its capacity to interact with  
416 endogenous NPM1/B23 through its HoD domain. This could partially redirect this mutant to the  
417 nucleolus and promote association with sHBZ in multimers composed of endogenous NPM1/B23  
418 and the NPM1/B23 mutant. In fact, results from immunoprecipitation experiments indeed argued for  
419 interaction between NBD-deleted B23 mutant and endogenous B23 (data not shown).

420 We have further shown that the association of sHBZ with NPM1/B23 likely depends on the  
421 BR1 and/or BR2 basic regions. Interestingly, these regions are also essential for nuclear and  
422 nucleolar localization and are likely to independently impact the extent of sHBZ-NPM1/B23  
423 association. Deletion of AD and bZIP domains of sHBZ led to stronger interaction with NPM1/B23  
424 with more intense nucleolar localization, as previously demonstrated (17). These results suggest that  
425 interaction with other cellular proteins through these domains may compete for the nucleolar  
426 localization of sHBZ and/or NPM1/B23 interaction. As the two known HBZ isoforms only differ in  
427 their first amino acids (four *vs.* seven for sHBZ and uHBZ, respectively), it is expected that both have  
428 the potential to interact with NPM1/B23, as we have shown in this study. More analyses are needed

429 to precisely decipher the regions and amino acids required from both isoforms for their interaction  
430 and comparison of BR1 and BR2 sequences between sHBZ and antisense proteins from other HTLV  
431 viruses will be helpful.

432 We have further shown that the interaction between the NBD domain of NPM1/B23 and  
433 sHBZ was potentially competed by RNA. As RNase treatment was performed in lysed extracts,  
434 concomitant binding of RNA likely destabilizes NPM1/B23 interaction with sHBZ. The additional  
435 discovered interaction between HBZ and its transcript is of important interest. It is hence possible  
436 that, through this association, HBZ could autoregulate its own expression by modulating nuclear  
437 export of its transcript. Furthermore, the interaction between sHBZ and its own mRNA could be  
438 linked to ATL development. Indeed, *hbz* mRNA was reported to induce IL-2-independent T-cell  
439 growth, but also to enhance transcription of E2F-1 and downstream targets, which has also been  
440 shown to be regulated by NPM1/B23 (12, 22, 73, 74). Hence, [as it has been proposed that NPM1/B23](#)  
441 [association to Rb sequesters it in the nucleolus thereby permitting E2F to be activated, it can be](#)  
442 [speculated that HBZ interaction to NPM1/B23 alters its association to Rb. If \*hbz\* RNA interferes with](#)  
443 [HBZ-NPM1/B23 interaction, then it would modulate the impact of HBZ on E2F-1 activation \(Figure](#)  
444 [9\)](#). In this sense, it is further interesting to note that we have detected higher levels of NPM1/B23  
445 transcripts in PBMC from ATL patients *vs.* other HTLV-1-infected individuals.

446 We have further examined this new association in terms of its potential impact on properties  
447 attributed to sHBZ. Our results demonstrated that silencing of NPM1/B23 expression in the  
448 chronically infected C8166-45 cell line resulted in downregulation of HBZ expression with

449 concomitant increase in Tax level. Of note, no differences in cell viability of *NPM1/B23*-silenced  
450 C8166-45 cells (and MT-2 cells, unpublished result) vs, control siRNA were observed. Although  
451 unexpected, this might be caused by the concomitant increase in Tax expression. Although these  
452 results are not directly assessing the HBZ-NPM1/B23 complex, they do suggest that NPM1/B23  
453 could act upon the stability of sHBZ and limit its relocalisation to nuclear speckles, thereby acting  
454 on the positive autoregulatory loop toward its expression and the negative regulation of Tax  
455 expression (11, 75). On the other hand, as reduced *shbz* RNA is also indicated, NPM1/B23 might  
456 instead be acting directly on the transcript.

457         Given that knock-out of *NPM1* gene was not possible, we instead investigated the potential  
458 implication of NPM1/B23 on sHBZ-mediated transformation in HeLa cells through transfection of  
459 expression vectors. Our results argue that when NPM1/B23 is co-expressed with sHBZ, anchorage-  
460 independent growth shows the highest value, while results showed that *sHBZ* also led to higher level  
461 in the presence of NPM1/B23. This might be in line with our observation that NPM1/B23  
462 overexpression led to higher nucleolar distribution of uHBZ. More experiments are needed to better  
463 understand the importance of the sHBZ-NPM1/B23 complex and the transforming features of sHBZ  
464 in relation to HBZ stability. Furthermore, we need to appropriately distinguish between a direct effect  
465 of the complex on cell transformation *versus* a potential indirect effect via sHBZ-NPM1/B23-  
466 dependent higher sHBZ expression which could also impact this process. The involvement of RNA  
467 in the regulation of this complex will also require further experiments.

468         In conclusion, for the first time, our study demonstrates that NPM1/B23 is a potential

469 interacting partner of HBZ. We speculate that a nucleolar distribution and a strong interaction with  
470 NPM1/B23 might be an important feature of sHBZ to induce IL-2-independent T-cell proliferation,  
471 an attribute, which is not shared with non-proliferation-inducing and non-nucleolar uHBZ and APH-  
472 2. Alternatively, other functional properties of sHBZ might also be attributed or modulated by the  
473 capacity of this viral protein to interact with NPM1/B23 in the nucleolus. Gaining a better  
474 understanding of how sHBZ interacts with NPM1/B23 and the outcome of this interaction at the  
475 cellular level will provide a better understanding of the link between sHBZ and ATL development.

In review

476 **Materials and Methods**

477

478 **Cell lines.** 293T, COS-7 and HeLa cells were cultured in DMEM supplemented with 10% fetal  
479 bovine serum (FBS) (PAA Laboratories Inc., Toronto, Canada). Jurkat, SupT1, H9, MT2 and C8166-  
480 45 cells were maintained in supplemented RPMI-1640 medium. All cell lines were incubated in 5%  
481 CO<sub>2</sub> at 37°C.

482

483 **HTLV-1 infection of HeLa cells.** Infection of HeLa cells was performed, as previously described  
484 (48, 76). HeLa cells were first seeded at 5 x 10<sup>5</sup>/ml, incubated overnight and then co-cultured with  
485 Jurkat or MT2 cells (1.5 x 10<sup>6</sup>/ml) for 16 h. MT2 cells were subsequently removed by several washes  
486 with supplemented RPMI-1640. HeLa cells were then cultured for 5 days and used for further  
487 analyses.

488

489 **PBMC and CD4<sup>+</sup> T cell isolation.** PBMCs were isolated from blood samples of non-infected donors  
490 and HTLV-1-infected patients (asymptomatic (AC)), ATL, and HAM/TSP patients, including a  
491 Chinese patient (77) using the MACSprep™ PBMC Isolation Kit according to manufacturer's  
492 instructions (Miltenyi Biotec Inc). CD4<sup>+</sup> T cells were further isolated from PBMC samples from  
493 non-infected and the HAM/TSP Chinese patient with the CD4<sup>+</sup> T Cell Isolation Kit (Miltenyi Biotec  
494 Inc) and used for confocal microscopy. All experiments and analyses using the patient samples were  
495 approved by the Medical Research Ethics Committee of the Second Affiliated Hospital of Fujian

496 Medical University and Fujian Province Government (2016-SAH-FMU-No.48). Additional blood  
497 samples from HTLV-1-infected patients and non-infected donors were obtained from the CHU of  
498 Martinique. Isolated PBMC samples (11 from AC individual, 11 from HAM/TSP patients and 11  
499 from ATL patients) were stored for research purpose at the Center of Biological Resources of  
500 Martinique (CeRBiM). HTLV-1 AC patients were recruited according to World Health Organization  
501 (WHO) criteria. AC had no neurologic or hematological symptoms. According to the French  
502 Bioethics laws, the collection of samples from AC, and ATL has been approved by the ethic  
503 committee of the French Ministry of Research. Because the protocol is non-interventional, no  
504 informed consent was required, as stated by the French Public Health code and therefore the study  
505 was conducted anonymously.

506  
507 **Plasmid constructs.** Expression vectors for sHBZ and uHBZ (provided by Dr. J.M. Mesnard,  
508 Université de Montpellier 1, Montpellier, France), APH-2 (pGFP-APH-2; provided by Dr. R.  
509 Mahieux, ENS Lyon, France), APH-3 and APH-4 have been previously described and express GFP  
510 fusion or Myc-tagged proteins (pmyc/his-spHBZ, pmyc/his-uHBZ, pmycAPH-3, pmycAPH-4,  
511 pAPH-3-GFP and pAPH-4-GFP) (28, 49, 71). The pmyc/his-HBZ $\Delta$ AD and pmyc/his-  
512 HBZ $\Delta$ AD $\Delta$ ZIP vectors have also been previously reported and express different deletion mutants of  
513 HBZ (71). The pDsRed-nucleolin and pDsRed-B23 vectors were generous gifts from Dr. D.  
514 Archambault (Université du Québec à Montréal, Montréal, Canada) and respectively express C23  
515 and NPM1/B23 fused to DsRed (78). Deletions and point mutations in the pDsRed-B23 vector were

516 generated using the Phusion™ Site-Directed Mutagenesis Kit (Thermofisher Scientific Inc., Waltham  
517 MA) according to manufacturer's instructions and the following primer pairs: 5'-  
518 GCTGTGGAGGAAGATGCAGA-3' and 5'-GTACCGTCGACTGCAGAATTC-3' for pDsRed-  
519 B23-ΔHoD; 5'-TCTCTTCCCAAAGTGGAAGCC-3' and 5'-  
520 AGCTTCCTCATCATCAAAATCATCA-3' for pDsRed-B23-ΔHeD; 5'-  
521 TAATCTAGAGGATCCACCGGATCT-3' and 5'-ACCACCTTTTTCTATACTTGCTTGC-3' for  
522 pDsRed-B23-ΔNBD; 5'-TCTCTTCCCAAAGTGGAAGCCAAATTC-3' and 5'-  
523 CATGGTACCGTCGACTGCAGAATTCG-3' for pDsRed-NBD (B23); 5'-  
524 GGCTATTCAAGATCTCTGGCAGGCGAGGAAGTCTCTTTAA-3' and 5'-  
525 TTAAAGAGACTTCCTCGCCTGCCAGAGATCTTGAATAGCC-3' for pDsRed-B23-W290A.

526  
527 ***siRNA transfection***

528 Control siRNA (#SIC001, Sigma) and siRNAs targeting NPM1/B23 (siRNA21 (SI049500010):  
529 CACCAGTGGTCTTAAGGTTGA and siRNA22  
530 (SI04949994): AAGGACAAGAATCCTTCAAGA) were ordered from Qiagen and transfected into  
531 MT-2 and C8166-45 cells with Neon Transfection System (Invitrogen), according to manufacturer's  
532 instructions. After 24 h, cells were harvested for further analyses.

533  
534 ***qRT-PCR analysis.*** Total RNA was isolated using the Trizol reagent (Invitrogen), as previously  
535 described (79). After reverse transcription (RT, High-Capacity cDNA Reverse Transcription,  
536 Thermofisher Scientific Inc.), transcript levels were assessed by qPCR analysis using the SYBR

537 green PCR master mix (#04707516001, Roche Diagnostics) and gene-specific primer sets. PCR  
538 cycles were set as follow: 95°C, 5 min, followed by 40 cycles at 95°C for 5 sec, 60°C for 10 sec,  
539 72°C for 30 sec, 95°C for 5 sec). Data were analyzed using the LightCycler®480 Software (Roche  
540 Diagnostics). The relative expression levels were calculated for each gene using the  $\Delta\Delta C_t$  method  
541 and normalized to the house-keeping gene HPRT-1. The following forward/reverse primers  
542 purchased from BioRad were used for these analyses: NPM1/B23 5'-  
543 GCTGGTGCAAAGGATGAGTT-3' and 5'-AAGGGAAACCGTTGGCTGTA-3'; Tax 5'-  
544 CCGGCGCTGCTCTCATCCCGGT-3' and 5'-GGCCGAACATAGTCCCCCAGAG-3'; HBZ: 5'-  
545 AGAACGCGACTCAACCGG-3' and 5'-TGACACAGGCAAGCATCGA-3'; HPRT-1  
546 (housekeeping gene): 5'-ATGGGGAAGGTGAAGGTCGG-3' and 5'-  
547 TGGAGGGATCTCGCTCCTGG-3'.

548  
549 **Cell transfection.** Cells ( $5 \times 10^4$  COS-7 and  $5 \times 10^6$  293T) were plated for 24 h prior to transfection  
550 using the polyethylenimine (PEI) reagent with 0.25  $\mu$ g and 6  $\mu$ g of DNA, respectively at a 1:9  
551 DNA:PEI ratio (Polysciences Inc). HeLa cells and SupT1 cells were transfected with Lipofectamine  
552 2000 (Invitrogen) following the manufacturer's protocol. After 6 to 12 h, cells were washed and  
553 replenished with fresh supplemented media. Cells were analysed from 24 to 48 h post-transfection.

554  
555 **Immunoprecipitation experiments.** Cellular extracts were prepared by resuspending cells in lysis  
556 buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA, 1% Triton). Immunoprecipitation was  
557 performed using Dynabeads Protein G (Invitrogen) according to manufacturer's instructions. Briefly,  
558 40  $\mu$ l of Dynabeads Protein G were incubated 2 h at room temperature with 5  $\mu$ g anti-Myc (#sc-40,

559 Santa Cruz Biotechnology Inc., Santa Cruz CA), 4 µg anti-B23 (#sc-5564, Santa Cruz Biotechnology  
560 Inc.), 4 µg anti-DsRed (Santa Cruz Biotechnology Inc.) or 4 µg anti-GFP antibodies. Cell extracts  
561 were then incubated overnight at 4°C with the antibody-bead complex. For certain experiments,  
562 RNaseA (100 µg/mL) (#RB0473, Biobasic Inc., Markham, Canada) or DNaseI (10 µg/ml)  
563 (#10104159001, Sigma-Aldrich Canada), was added to the antibody-bead complex and incubated for  
564 20 min at room temperature. Bound fractions were eluted with 20 µl of loading buffer and then  
565 analysed by Western blot.

566

567 **Western blot.** Immunoprecipitated or total extracts were separated on a 12% SDS- PAGE and  
568 transferred to a PVDF membrane (Millipore, Mississauga, Canada). Membranes were blocked in  
569 PBS/5% milk or PBS/0.3% BSA and incubated with anti-HBZ (1:2000) (11), anti-B23 (1:500,  
570 Sigma-Aldrich Canada Co., Oakville, Canada), anti-C23 (1:500, #sc-13057, Santa Cruz  
571 Biotechnology Inc.), anti-DsRed (1:1000,#sc-101526, Santa Cruz Biotechnology Inc ), anti-GFP-  
572 HRP (1:1000, #sc-9996, Santa Cruz Biotechnology Inc.), anti-Tubulin (1:5000, #T5168, Sigma-  
573 Aldrich Canada Co.), anti-β-Actin (1:5000, #sc-47778, Santa Cruz Biotechnology Inc.), anti-Tax  
574 (1:2000, #sc-57872, Santa Cruz Biotechnology Inc), or anti-Myc (1:250) antibodies overnight. After  
575 several washes, membranes were incubated with HRP-conjugated sheep anti-rabbit IgG antibodies  
576 (1:5000, #5220-0336, Seracare, Milford MA) or anti-mouse IgG antibodies (1:5000, #5220-0338,  
577 Seracare) for 2 h, washed several times and incubated with the BM Chemiluminescence Blotting  
578 Substrate. Membranes were analysed using the Fusion FX7 device.

579

580 **Confocal microscopy.** Cells were washed three times with PBS and fixed with PFA 4% followed by  
581 permeabilization with 0.5% Triton. Infected HeLa, C8166-45 and CD4<sup>+</sup> T cells were additionally  
582 treated with trypsin (2.5 µg/ml) for 5 min at 37°C prior analyses for endogenous NPM1/B23, as  
583 previously performed (80). Cells were then washed with cold PBS three times and incubated  
584 overnight in the presence of mouse anti-HBZ (1:100), anti-Myc (1:100) or anti-B23 (1:250)  
585 antibodies followed by three washes in cold PBS and subsequent incubation with goat anti-mouse  
586 IgG coupled to Alexa Fluor 488 or goat anti-rabbit IgG coupled to Alexa Fluor 594 (1:500)  
587 (ThermoFisher Scientific Inc.) for 1 h at room temperature. After three washes in cold PBS, cells  
588 were incubated with DAPI for 5 min and coverslips were then mounted in ProLong Antifade reagent.  
589 Samples were observed at room temperature with a 60X objective under oil immersion and with a  
590 numerical aperture (NA) of 1.4 with a Nikon A1 laser scanning confocal microscope (Nikon Canada,  
591 Mississauga, Canada).

592

593 **RNA immunoprecipitation (RIP).** C8166-45 cells were lysed in a solution composed of 50 mM Tris–  
594 HCl pH 8, 100 mM NaCl, 1 mM EDTA, 1% Triton, and RNase inhibitor. Immunoprecipitation was  
595 carried out by adding 100 µl Dynabeads Protein G (Invitrogen) followed by incubation with 10 µg  
596 control non-specific IgG or anti-HBZ antibody for 2 h at room temperature. Cell extracts were then  
597 incubated with the antibody-bead complex overnight at 4°C. After three washes with cold PBS–  
598 Tween (2%), beads were treated with 1 ml TRIzol followed by DNase (2 U) treatment for 10 min at

599 37°C (Biobasic Inc.). RT was next performed on 2 µg RNA in the presence of High Reverse  
600 Transcriptase (Biobasic Inc) and oligo(dT)18 for 2 h at 37°C (Thermofisher Scientific Inc.). After  
601 cDNA synthesis, PCR amplification was conducted with the Vent DNA Polymerase (New England  
602 Biolabs, Whitby, Canada) in the presence of the following forward/reverse primer pairs: 5'-  
603 AACTGTCTAGTATAGCCATC-3' (primer 21-5) and 5'-CGCAGAGTTGAACAAGCAGG-3'  
604 (primer 20-19); 5'-CAGGCAAGCATCGAAACAGC-3' (primer Exon-1-3) and primer 20-19 (14).  
605 PCR products were run on a 1.5% agarose gel and resulting amplicons were sequenced.

606

607 ***Anchorage-independent growth assay.*** HeLa cells ( $1.2 \times 10^6$ /well) were transfected with pEGFP-c1,  
608 sHBZ, uHBZ and/or DsRed-B23 expressing vectors (total of 2 µg DNA/well). After 24 h, for each  
609 transfection condition, cells were counted, and 100 cells were mixed in 0.3% agar-1X DMEM  
610 medium solution and seeded in each well of a 6 well-plate precoated with 0.6% agar-1X medium  
611 solution. Cells were covered with 0.5 ml of medium, which was replenished every three days. At 15-  
612 day post-transfection, plates were read and analyzed with a GloMax plate reader.

613

614 ***Cell viability assay.*** MT2 and C8166-45 cells ( $1 \times 10^6$ ) were transfected with 50nM siNC, or  
615 NPM1/B23-specific siRNA-21 and siRNA-22 using the Neon® Transfection System and  
616 transfection kit (Thermofisher Scientific Inc., MPK5000 and MPK1096). Cells were next seeded into  
617 triplicate wells on 96-well plate. After 48 h, 10µl Cell counting KIT-8 buffer (CCK-8, Sigma-

618 Aldrich) was added into each well and incubated for 4 h at 37°C. Absorbance was measured at 450  
619 nm with the EnSpire 2300 Multilabel Reader (PerkinElmer) at room temperature.

620

621 **Statistical analysis.** All statistical analyses were conducted with the Graphpad Prism 9. [Comparisons](#)  
622 [between groups were carried out using one-way ANOVA for comparison tests.](#) In all cases, a P value  
623 less than 0.05 was considered significant.

624

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633

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- 844

In review

845 **Supporting information captions**

846

847 **Fig 1. Expression of *NPM1* in PBMC from infected individuals and association with sHBZ in**  
848 **transfected cells. A.** RNA from PBMCs of asymptomatic individuals (AC) or HAM/TSP and ATL  
849 patients of Caribbean origin was analyzed by qRT-PCR for *NPM1* expression (\*\*  $p < 0.01$ ). COS-7  
850 (B) and HeLa (C) cells were co-transfected with expression vectors for Myc-tagged sHBZ or uHBZ  
851 (vs. pcDNA3.1) alone or in the presence of pDsRed-B23. Fixed cells were incubated with anti-Myc  
852 antibodies followed by goat anti-mouse IgG coupled to Alexa Fluor 488 and stained with DAPI. Cells  
853 were then analyzed by confocal microscopy. HeLa (D) and COS-7 (E) cells were co-transfected with  
854 pEGFP-C1-sHBZ, pEGFP-C1-uHBZ or pEGFP-C1 (CTL) and pDsRed-B23. Cells were fixed and  
855 stained with DAPI prior to confocal analyses. F. Expression vectors of Myc-tagged sHBZ or uHBZ  
856 (vs. pcDNA3.1) and pDsRed-B23 were co-transfected in 293T. Cells lysates were  
857 immunoprecipitated with an anti-Myc antibody. Immunoprecipitates and cell extracts were analyzed  
858 by Western blot using anti-B23, anti-DsRed or anti-Myc antibodies. G. Expression vectors of Myc-  
859 tagged sHBZ or uHBZ (vs. pcDNA3.1) were transfected in HeLa cells. Cells lysates were  
860 immunoprecipitated with an anti-Myc antibody. Immunoprecipitates and cell extracts were analyzed  
861 by Western blot using anti-B23 or anti-Myc antibodies. The experiments were repeated three times.

862

863 **Fig 2. sHBZ interacts with NPM1/B23 in transfected and infected T cells. A.** SupT1 cells were  
864 transfected with the Myc-tagged sHBZ or uHBZ expression vector (vs. empty vector pcDNA3.1) and

865 extracts were next immunoprecipitated with anti-Myc antibodies. Western blot analyses were  
866 conducted with anti-Myc, anti-B23 or anti-tubulin antibodies. **B.** Extracts from Jurkat, C8166-45 and  
867 293T cells transfected with the Myc-tagged sHBZ expression vector were immunoprecipitated with  
868 anti-B23 antibodies. Immunoprecipitates and total cellular extracts were subsequently analyzed by  
869 Western blot using anti-HBZ, anti-B23 or anti-tubulin antibodies. **C.** C8166-45 and uninfected H9  
870 cells were analyzed for NPM1/B23 and HBZ localization by confocal microscopy. **D.** MT2 cell  
871 extracts were immunoprecipitated with anti-HBZ antibodies (vs. non-specific IgG) and total and  
872 immunoprecipitated extracts were analyzed by Western blot using anti-B23 or anti-HBZ antibodies.  
873 **E.** HeLa cells were co-cultured with Jurkat or MT2 cells for 5 days and HeLa cells were analyzed by  
874 confocal microscopy for HBZ and NPM1/B23. **F.** Extracts from HeLa cells co-cultured with Jurkat  
875 or MT2 were immunoprecipitated with anti-B23 or non-specific IgG antibodies and analysis of  
876 immunoprecipitated and total extracts was performed by Western blot using anti-HBZ or anti-B23  
877 antibodies. **G.** CD4<sup>+</sup> T cells from an uninfected individual and an HTLV-1-infected patient were  
878 analyzed by confocal microscopy for NPM1/B23 and HBZ. For confocal microscopy experiments  
879 (**C, E, G**), cells were incubated with anti-HBZ and anti-B23 antibodies, followed by goat anti-mouse  
880 IgG coupled to Alexa Fluor 488 and goat anti-rabbit IgG coupled to Alexa Fluor 594, respectively  
881 and final DAPI staining. These experiments were repeated three times

882

883 **Fig 3. Comparison between sHBZ, APH-2, APH-3 and APH-4 for their association with**  
884 **NPM1/B23.** 293T cells were transfected with expression vectors for EGFP-fused sHBZ or APH-2

885 (vs. empty vector) (A) or for Myc-tagged sHBZ, APH-3 and APH-4 (vs. empty vector) (C). Cell  
886 lysates were immunoprecipitated with anti-GFP or anti-Myc antibodies and analyzed by Western blot  
887 using anti-B23, anti-GFP or anti-Myc antibodies. **B.** COS-7 cells were co-transfected with expression  
888 vectors for Myc-tagged sHBZ, APH-3 or APH-4 and pDsRed-B23. Cells were fixed and were  
889 analyzed by confocal microscopy with an anti-Myc antibody and stained with DAPI. The  
890 experiments were repeated three times.

891

892 **Fig 4. sHBZ is associated with C23 but does not impact its interaction with NPM1/B23. A-B.**

893 HeLa (A) and COS-7 cells (B) were co-transfected with expression vectors for GFP-fused sHBZ or  
894 uHBZ (vs. pEGFP-C1; CTL) and pDsRed-C23. Fixed cells were analyzed by confocal microscopy  
895 following staining with DAPI. **C.** 293T cells were transfected with expression vectors for Myc-  
896 tagged sHBZ (vs. empty vector pcDNA3.1). Cell extracts were immunoprecipitated with anti-Myc  
897 antibodies. Immunoprecipitates and cell extracts were analyzed by Western blot using anti-C23, anti-  
898 B23 or anti-Myc antibodies. **D.** 293T cells were co-transfected with increasing concentrations of the  
899 Myc-tagged sHBZ expression vector. Cells were lysed and lysates were used for  
900 immunoprecipitation with anti-B23 antibodies. Immunoprecipitates and cell extracts were analysed  
901 by Western blot using anti-C23, anti-Myc or anti-B23 antibodies. The experiments were repeated  
902 three times.

903

904 **Fig 5. The basic regions of sHBZ are sufficient for its association with NPM1/B23. A.** Important  
905 domains/regions of sHBZ. **B.** 293T cells were transfected with Myc-tagged sHBZ, sHBZ $\Delta$ AD, or  
906 sHBZ $\Delta$ AD $\Delta$ ZIP expression vectors. Cellular extracts were immunoprecipitated with anti-Myc  
907 antibodies. Immunoprecipitates or total cellular extracts were subsequently analysed by Western blot  
908 using anti-Myc, anti-C23 or anti-B23 antibodies. **C.** COS-7 cells were co-transfected with sHBZ or  
909 sHBZ $\Delta$ AD $\Delta$ ZIP expression vectors and pDsRed-B23. Cells were fixed, stained with DAPI and  
910 analyzed by confocal microscopy. **D.** 293T cells were transfected with expression vectors for EGFP-  
911 fused sHBZ, sHBZ-BR1DBD or sHBZ-BR2BR1 (vs. pEGFP-C1). Cellular extracts were  
912 immunoprecipitated with anti-GFP antibodies. Immunoprecipitates or total cellular extracts were  
913 subsequently analyzed by Western blot using anti-GFP or anti-B23 antibodies. The experiments were  
914 repeated three times.

916 **Fig 6. The RNA-binding domain of NPM1/B23 is required for its association with sHBZ. A.**  
917 Domains of NPM1/B23 and deletion/point mutants. **B, D.** COS-7 cells were co-transfected with  
918 expression vectors for GFP-fused sHBZ and DsRed-fused B23 WT, B23 $\Delta$ HoD, B23 $\Delta$ HeD (**B**) or  
919 NBD (**D**) (vs. empty vector pEGFP-C1: CTL). Cells were fixed, stained with DAPI and analyzed by  
920 confocal microscopy. **C-E.** 293T cells were co-transfected with expression vectors for Myc-tagged  
921 sHBZ and wild-type or deletion/point mutants of NPM1/B23 fused to DsRed. Cellular extracts were  
922 immunoprecipitated with anti-Myc (**C**) or anti-DsRed antibodies (**E**). Immunoprecipitates and total

923 extracts were analyzed by Western blot with anti-Myc, anti-DsRed, anti-B23 or anti-tubulin  
924 antibodies. The experiments were repeated three times.

925

926 **Fig 7. Association of RNA to the HBZ-NPM1/B23 complex hampers their association. A-B.** 293T

927 cells were transfected with the expression vector for Myc-tagged sHBZ alone (**B**) or in the presence  
928 of pDsRed-B23, pDsRed-B23 $\Delta$ NBD or pDsRed (**A**). Cell extracts were immunoprecipitated with  
929 anti-DsRed (**A**) or anti-B23 (**B**) antibodies, and next treated with DNaseI or RNaseA before elution.

930 Immunoprecipitated and total extracts were analysed by Western blot using anti-Myc, anti-DsRed,  
931 anti-B23 or anti-tubulin antibodies. **C.** Extracts from Jurkat and C8166-45 cells were

932 immunoprecipitated with anti-B23 antibodies and treated or not with RNaseA before elution.

933 Immunoprecipitated and total extracts were analysed by Western blot using anti-HBZ, anti-B23 and  
934 anti-C23 antibodies. **D.** C8166-45 cell lysates were immunoprecipitated with either control IgG or

935 anti-HBZ antibodies. Isolated RNA from immunoprecipitated samples were analyzed for *shbz*  
936 transcripts by RT-PCR with indicated primers in the top of the panel (size of expected amplicons are  
937 also indicated) (negative control H<sub>2</sub>O). The experiments were repeated twice.

938

939 **Fig 8. NPM1 expression modulates viral gene expression and alters cell transformation. A.**

940 C8166-45 cells were transfected with *NPM1*-specific siRNA (si21 and si22) vs. control siRNA  
941 (siCTRL). After 72 hours, cell viability was determined by Cell Counting Kit-8 and *NPM1* expression

942 levels were quantified by qRT-PCR. **B.** C8166-45 cells transfected with *NPM1* siRNAs or scrambled

943 siRNA were analyzed for *NPM1*, *hbz* and *tax* expression by qRT-PCR at 72h post-transfection. The  
944 data are presented as mean  $\pm$  SEM (n=3). Values for siCTRL-transfected cells were set as 100%. **C.**  
945 NPM1/B23, Tax and HBZ protein levels were analyzed by Western blot analyses 72h after siRNA  
946 transfection.  $\beta$ -actin was analyzed as a loading control. **D.** HeLa cells were transfected with sHBZ,  
947 uHBZ and/or DsRed-B23 expressing vectors (vs. corresponding empty vectors). After 24h, cells were  
948 counted, and 100 cells were seeded into each well of 6 well-plates for each group in soft agar solution.  
949 At 15-day post-transfection, plates were read and analyzed with a GloMax plate reader. Experiments  
950 were performed in triplicates and repeated twice. The data are presented as mean  $\pm$  SEM (n=3), ns=  
951 non-significant, \*p<0.05 and \*\*p<0.001(ANOVA comparisons tests).

952  
953 **Fig 9. Proposed model of the association between NPM1/B23 and sHBZ.** Based on our results,  
954 sHBZ would interact with NPM1/B23 in the nucleolar region of HTLV-1-infected cells. **A.** In a non-  
955 infected cell, the activation of the E2F-1 transcription factor involves phosphorylation of Rb and its  
956 sequestration in the nucleolus. NPM1/B23 helps in this sequestration by direct interaction with pRb.  
957 **B.** In HTLV-1 infected cells, we speculate that sHBZ forms a ternary complex with NPM1/B23 and  
958 Rb (potentially phosphorylated) and leads to sequestration of Rb in the nucleolus through an  
959 alternative B23-based mechanism. **C.** As our results further lead to the speculation that this ternary  
960 complex could be outcompeted by bound RNA, we propose that binding of *shbz* transcripts to HBZ  
961 could interfere with the formation of the HBZ-NPM1/B23 complex, subsequently leading to  
962 modulation of E2F-1 activation via alteration of Rb, although the outcome remains unknown.

963

964

965 **Fig S1. Co-localization of NPM1/B23 and HBZ in COS-7 cells.** COS-7 cells were co-transfected  
966 with expression vectors for GFP-fused uHBZ or sHBZ and DsRed-B23. Cells were analyzed by  
967 confocal microscopy for HBZ and NPM1/B23.

968

969 **Fig S2. Absence of HBZ-specific signals in immunoprecipitation experiments in control T cells.**  
970 Extracts from 293T cells transfected with pMyc-sHBZ and from C8166-45 were immunoprecipitated  
971 with anti-B23 or control IgG antibodies. Immunoprecipitated and total extracts were analyzed by  
972 Western blot with anti-HBZ or anti-B23 antibodies.

973

**Figure 1**

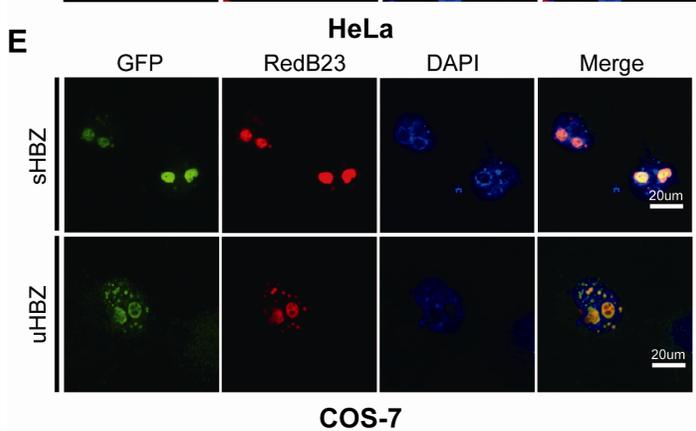
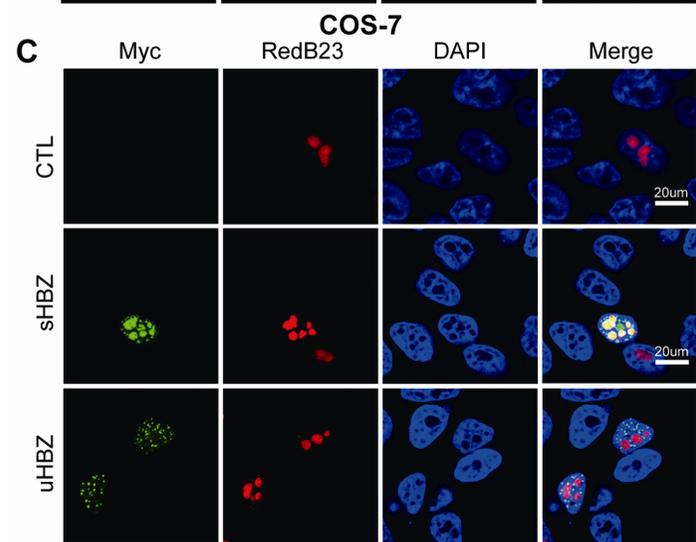
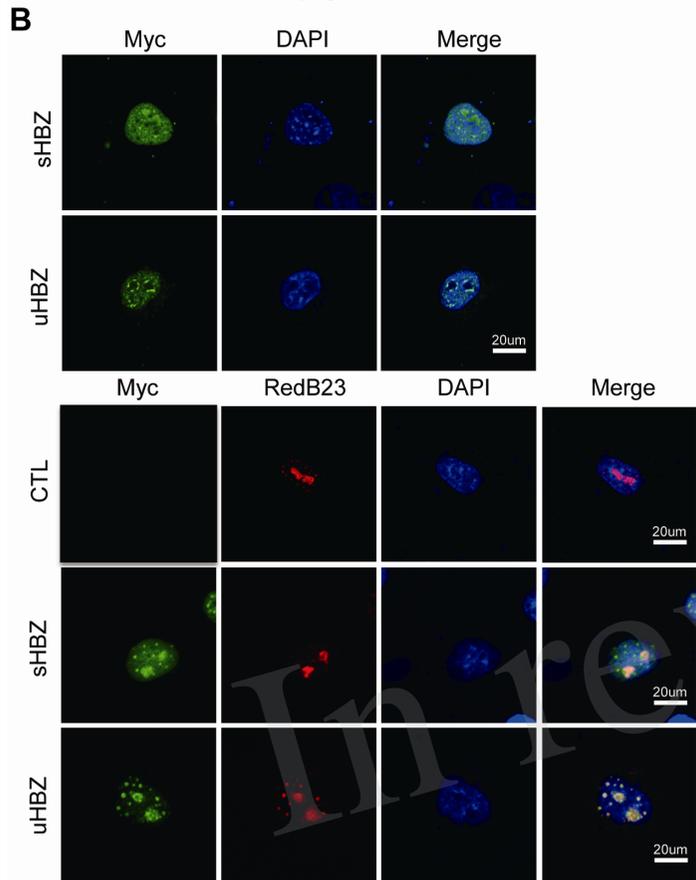
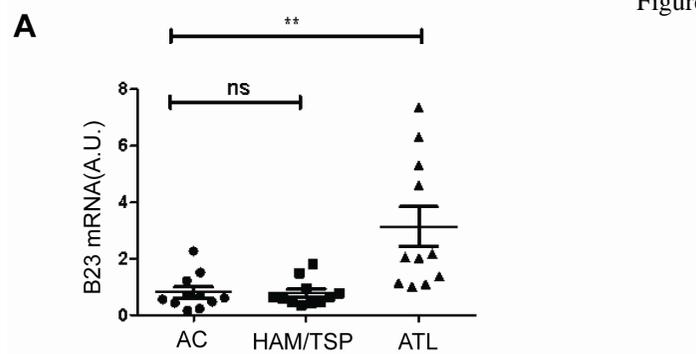


Figure 1.TIF

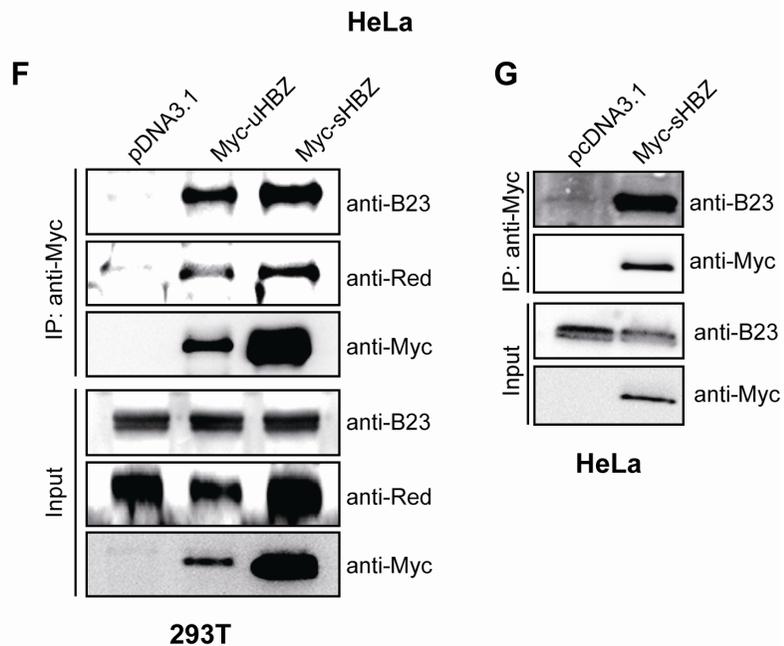
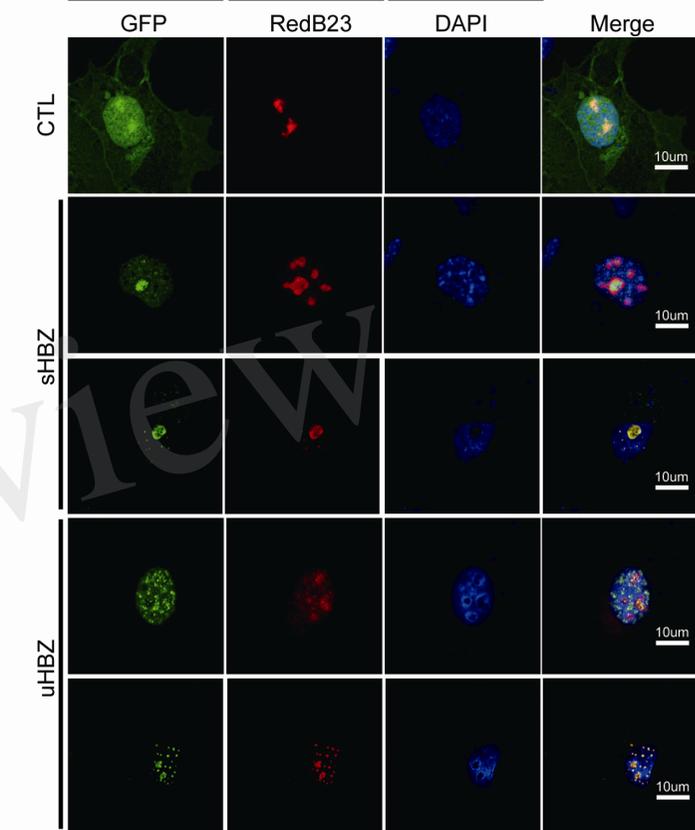
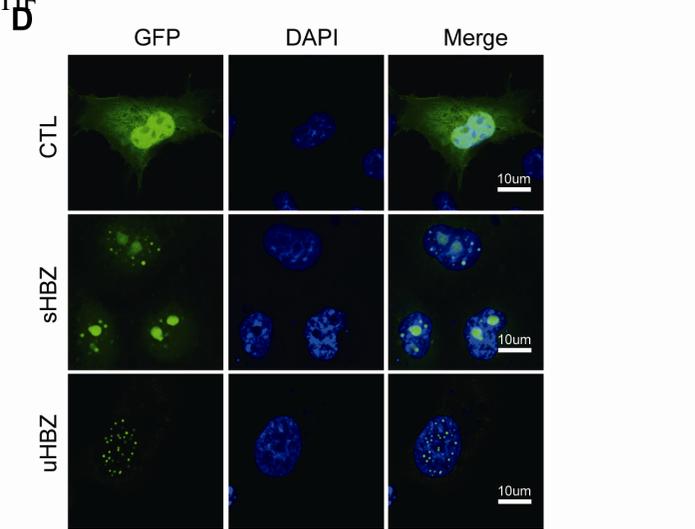
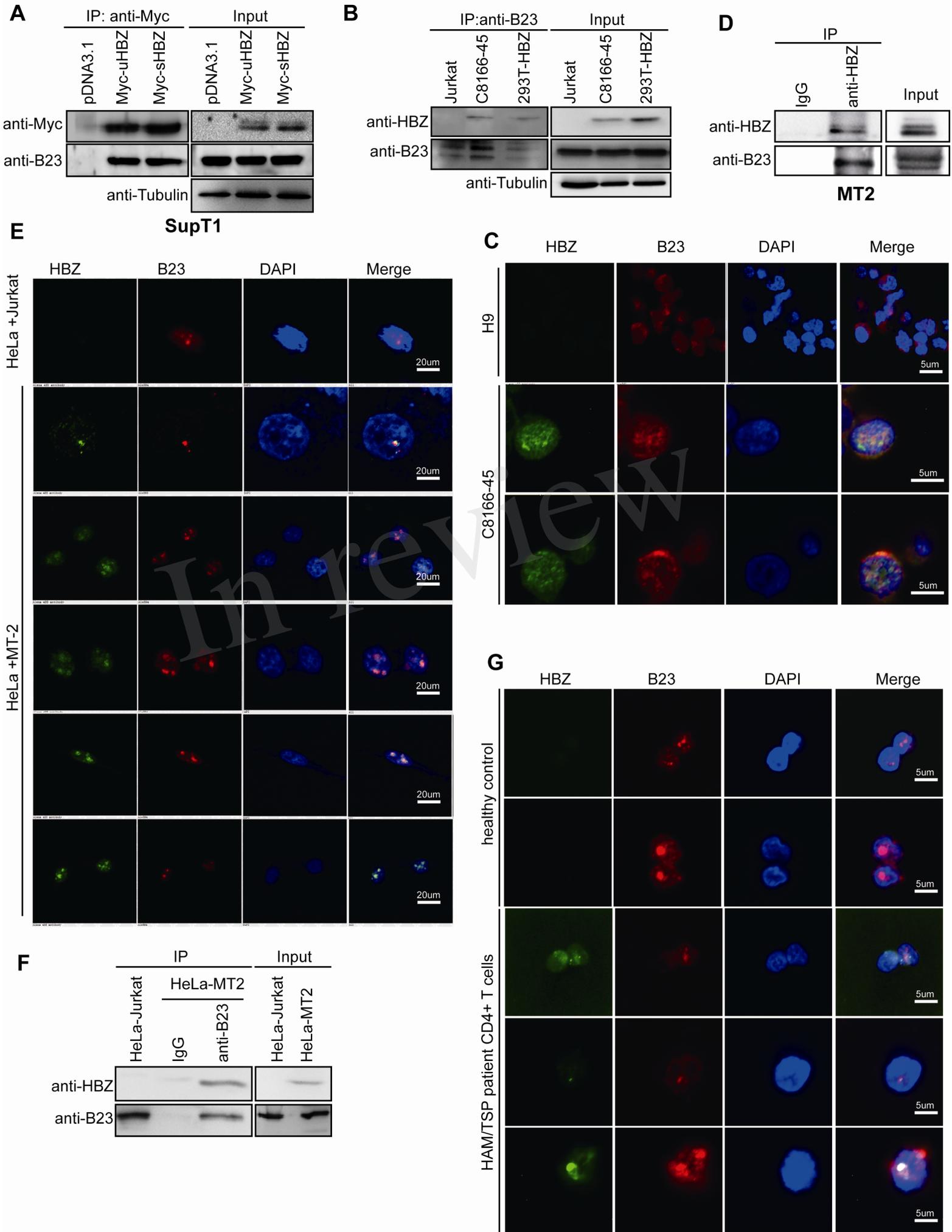
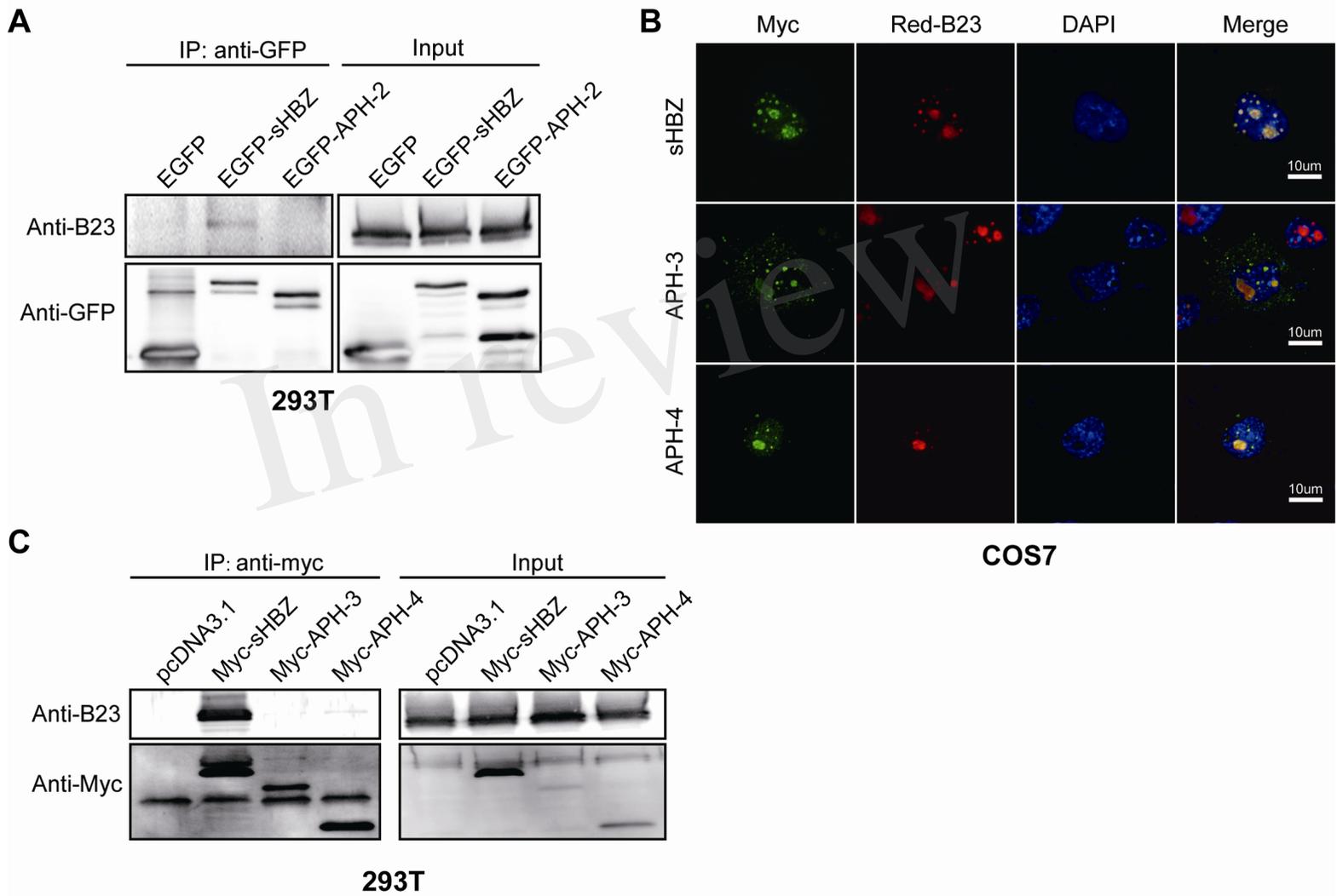


Figure 2.TIF

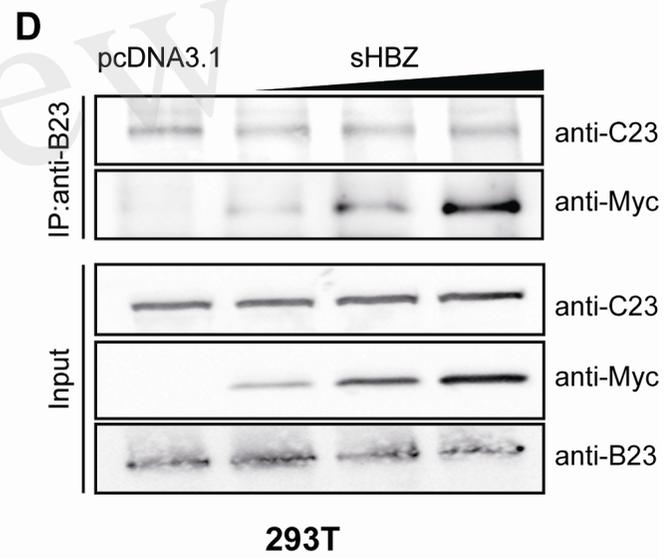
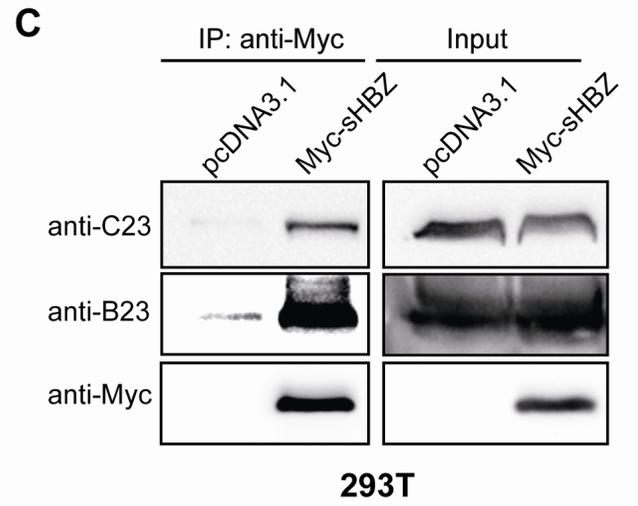
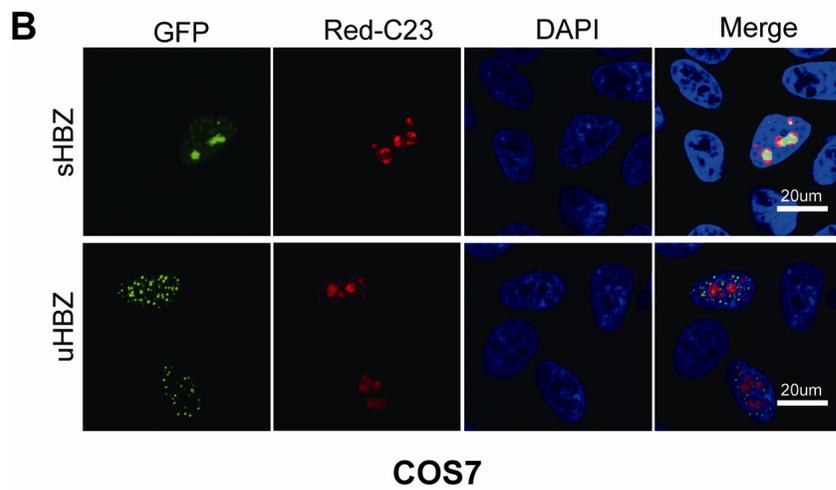
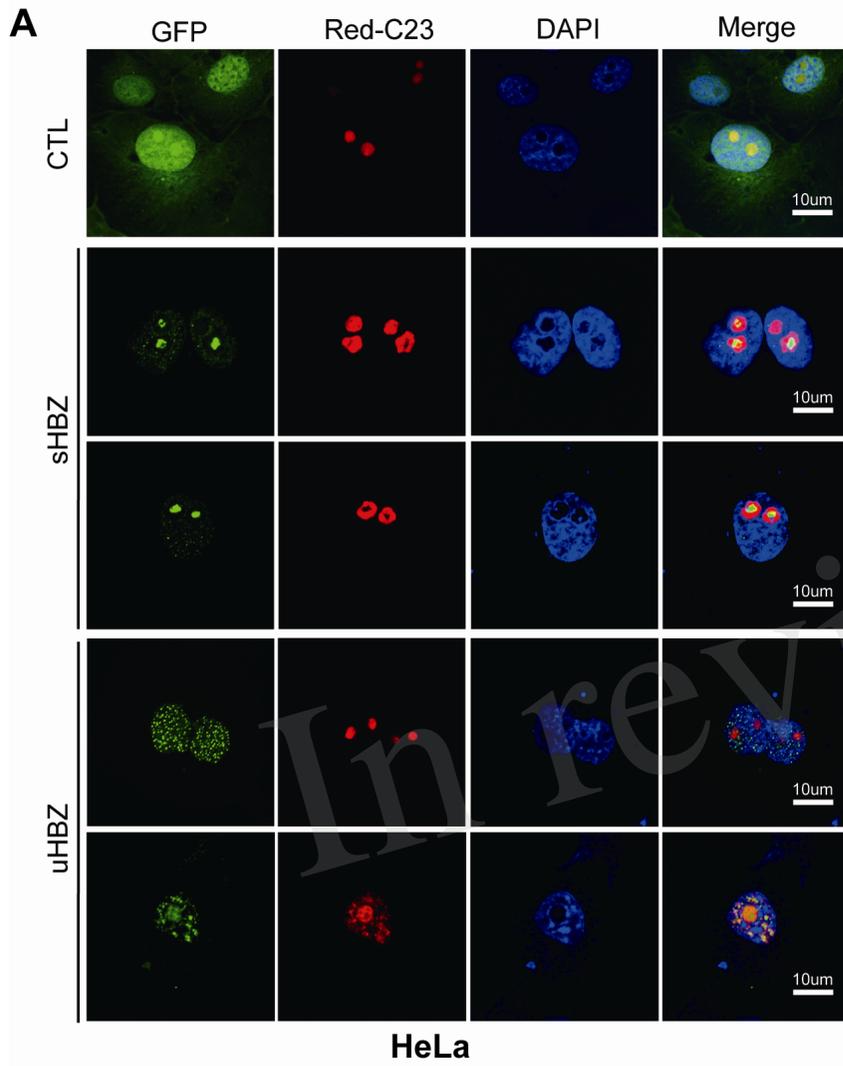
**Figure 2**



## Figure 3

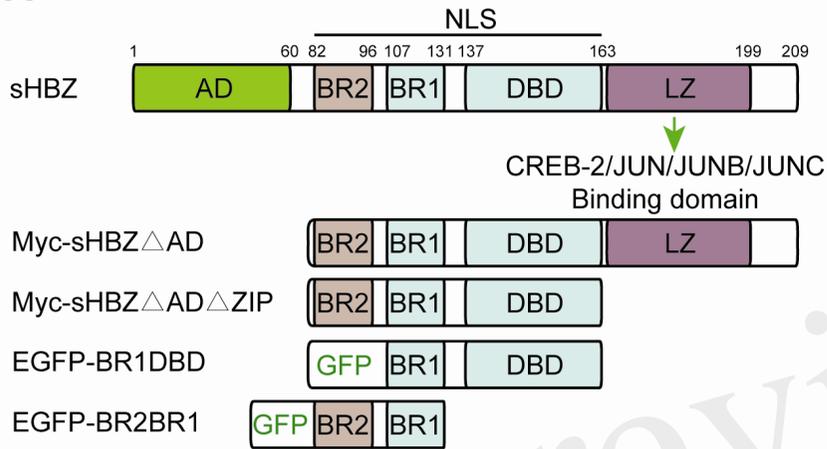


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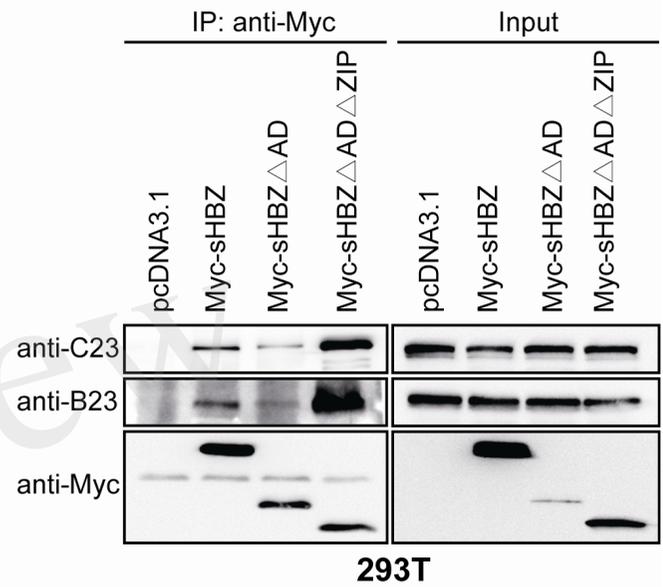


## Figure 5

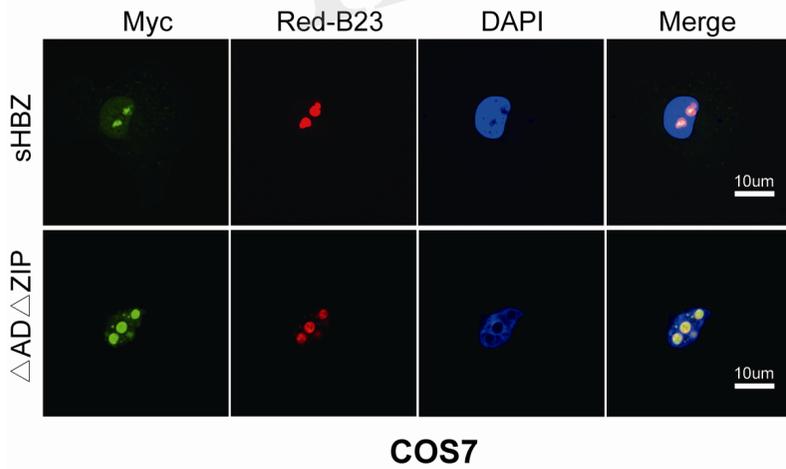
A



B



C



D

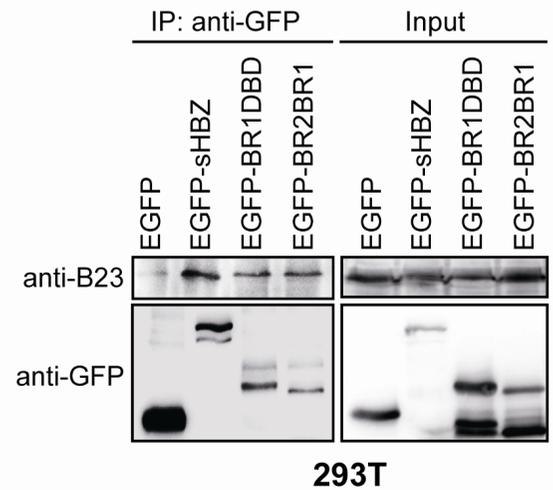
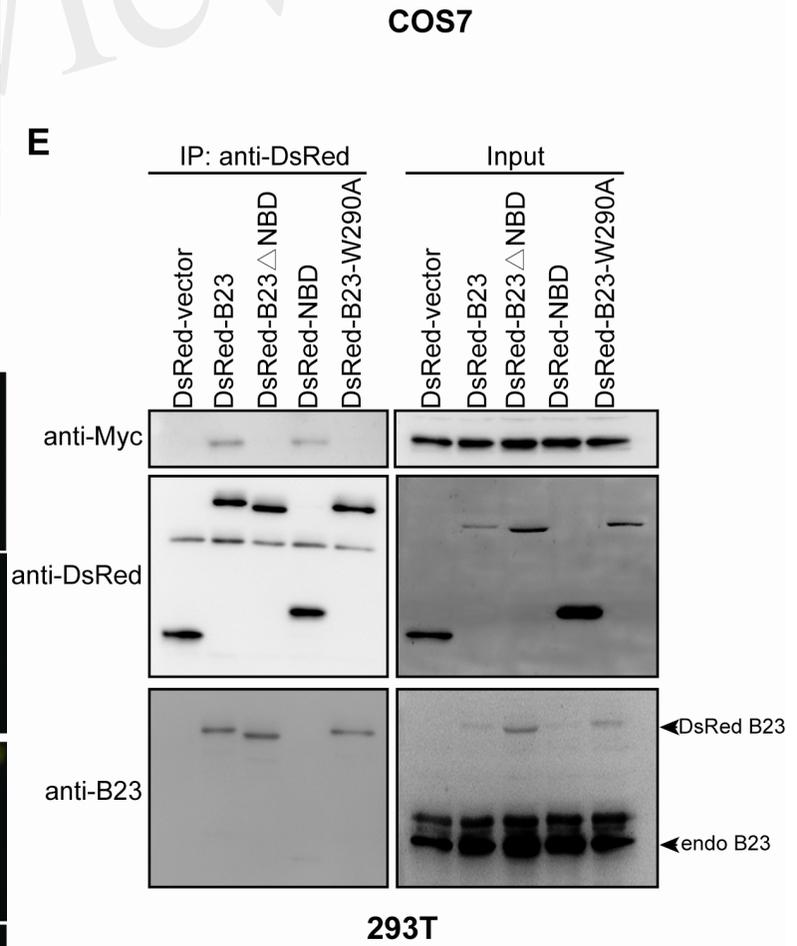
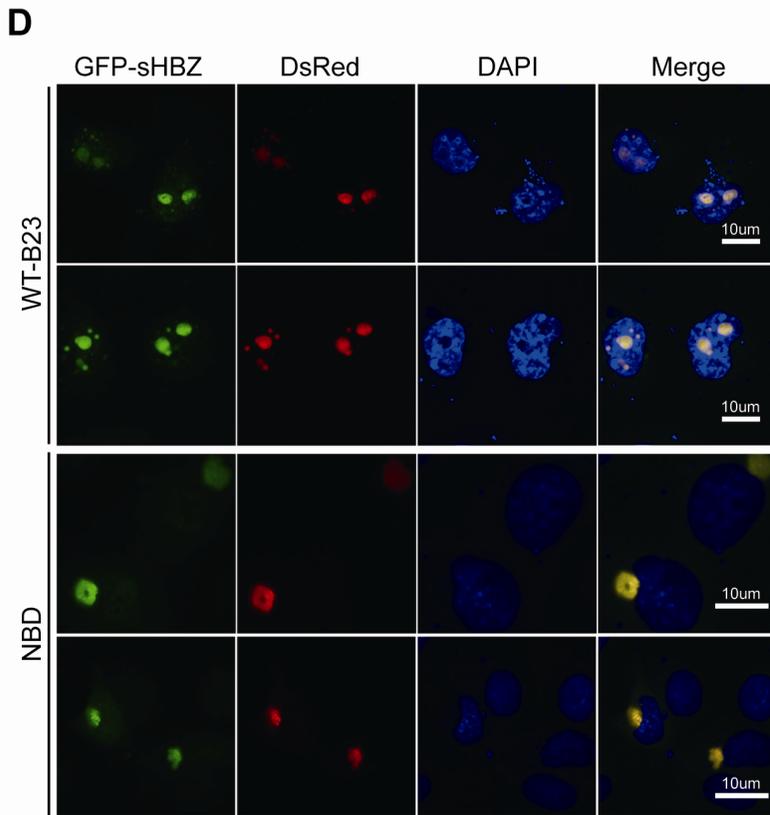
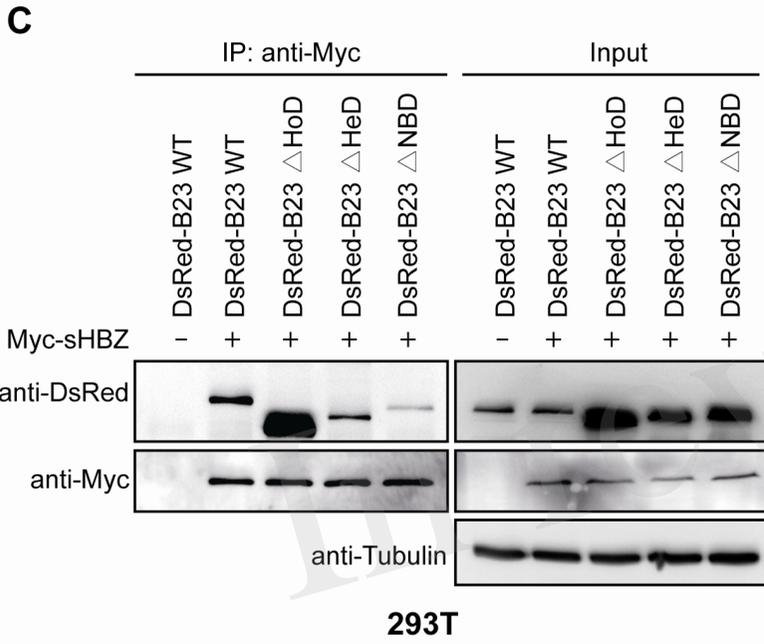
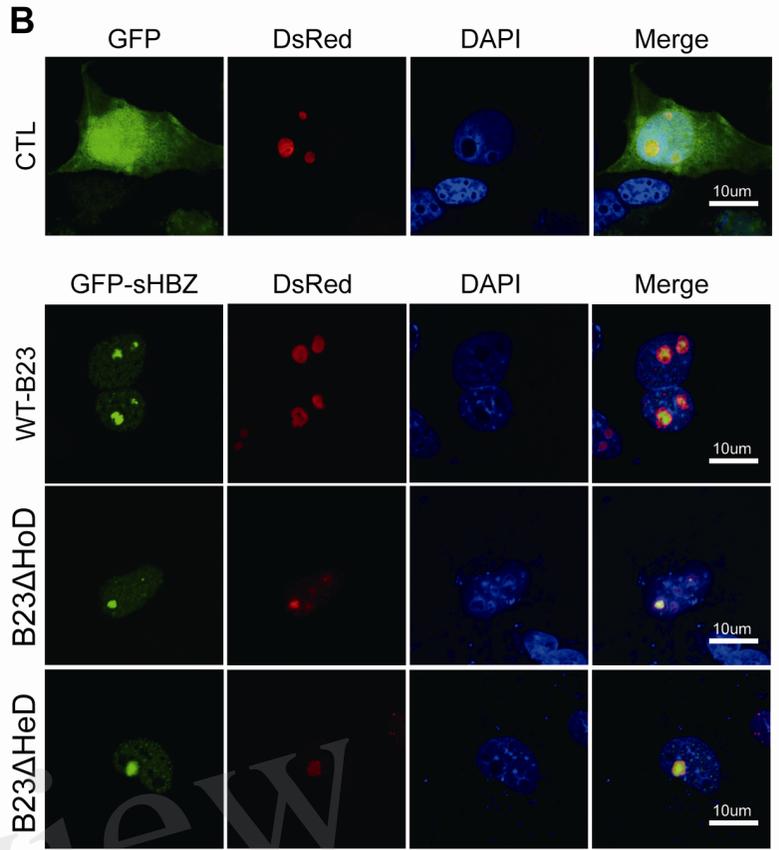
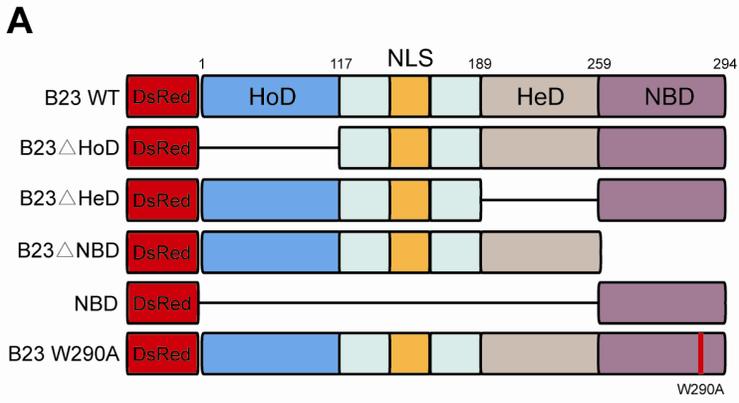


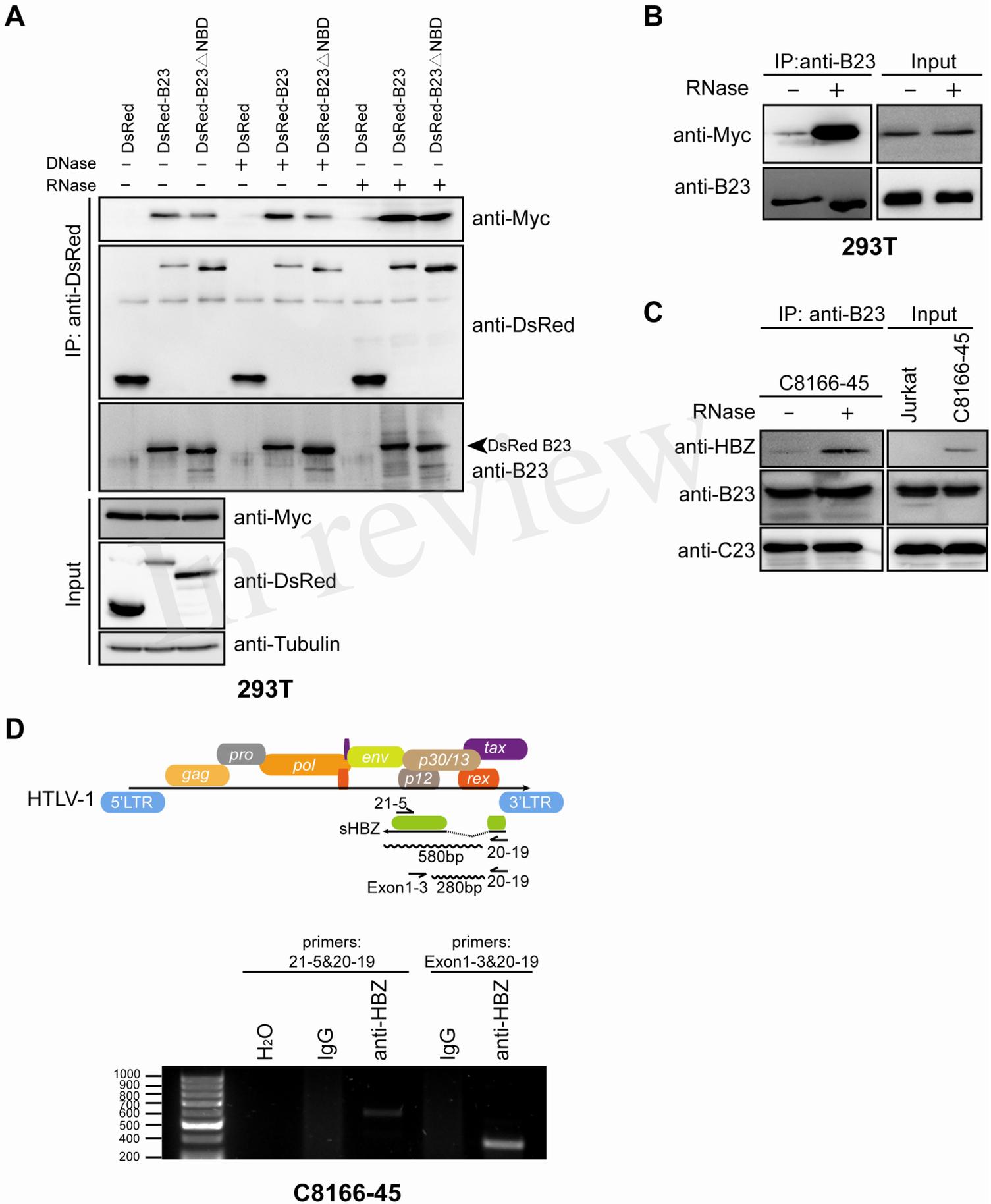
Figure 6.TIF

**Figure 6**



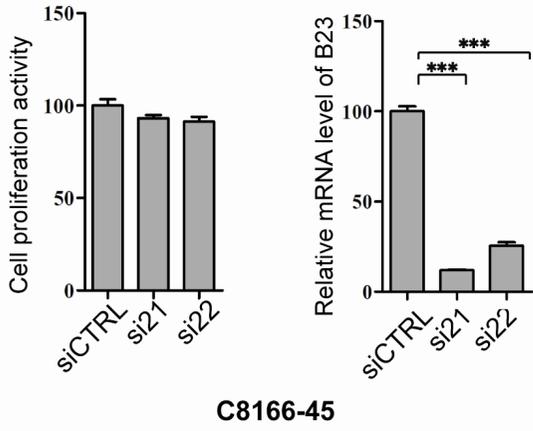
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**Figure 7**

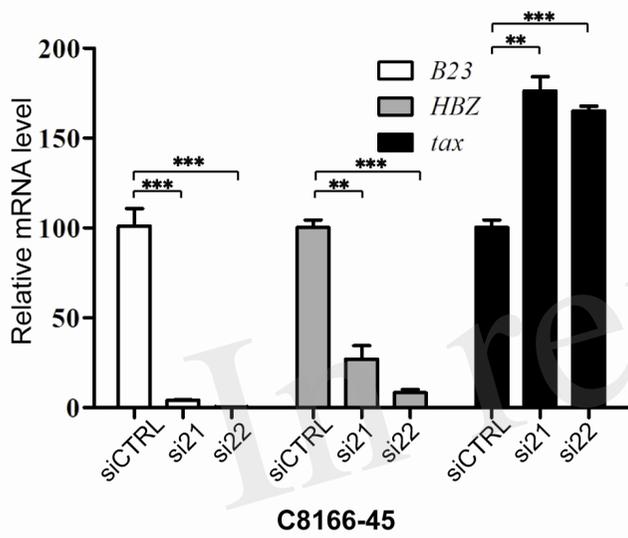


**Figure 8**

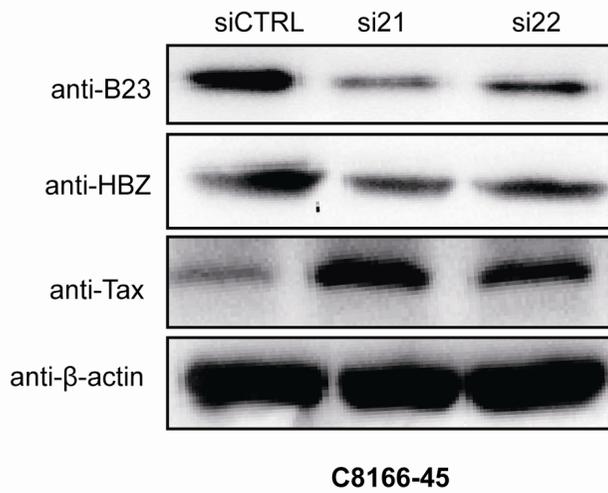
**A**



**B**



**C**



**D**

