

X-box-binding protein 1 (XBP-1) activates lytic Epstein-Barr virus gene expression in combination with Protein Kinase D (PKD)

Prasanna M. Bhende,¹ Sarah J. Dickerson,² Xiaoping Sun,² Wen-hai Feng,¹ and Shannon C. Kenney^{1,2*}

¹Departments of Medicine and Microbiology and Immunology, and Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, North Carolina, and ²Departments of Medicine and Oncology, University of Wisconsin at Madison, Wisconsin

*Corresponding author. Mailing address: Departments of Medicine and Oncology, 421A McArdle, 1400 University Avenue, Madison, Wisconsin, 53706. Phone: 608-261-1196. Fax: 608-262-2824

E-mail: skenney@wisc.edu

Running title: XBP-1 activates lytic EBV expression

Abstract word count: 181

Text word count: 4013

ABSTRACT

1
2 Epstein-Barr virus (EBV) establishes a latent form of infection in memory B cells, while
3 antibody-secreting plasma cells often contain the lytic form of infection. The switch between
4 latent and lytic EBV infection is mediated by the two viral immediate-early proteins, BZLF1 (Z)
5 and BRLF1 (R), which are not expressed in latently-infected B cells. Here we demonstrate that a
6 cellular transcription factor which plays an essential role in plasma cell differentiation, XBP-1,
7 also activates transcription of the two EBV immediate-early gene promoters. In reporter gene
8 assays, XBP-1 alone was sufficient to activate the R promoter, whereas the combination of XBP-1
9 and PKD was required for efficient activation of the Z promoter. Most importantly, expression of
10 XBP-1 and activated PKD was sufficient to induce lytic viral gene expression in EBV-positive
11 nasopharyngeal carcinoma cells and lymphoblastoid cells, while an XBP-1 siRNA inhibited
12 constitutive lytic EBV gene expression in lymphoblastoid cells. These results suggest that the
13 plasma cell differentiation factor, XBP-1, in combination with activated PKD, can mediate
14 reactivation of EBV, thereby allowing the viral life cycle to be intimately linked to plasma cell
15 differentiation.

INTRODUCTION

1
2 Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and is
3 associated with B-cell lymphomas, nasopharyngeal carcinoma, gastric carcinomas and other
4 malignancies (44, 45). EBV undergoes lytic infection in normal oral epithelial cells (31, 51), while
5 usually establishing one of the latent forms of infection in circulating memory B cells. In contrast,
6 tonsillar B cells that express antigens specific for plasma markers commonly contain the lytic form
7 of EBV infection, which results in the production of infectious viral particles (10, 28, 29).

8 The switch from latent to lytic EBV infection is mediated by the immediate-early (IE)
9 protein BZLF1 (Z) and the immediate-early/early (IE/E) protein BRLF1 (R) (1, 16, 57). Z and R
10 are transcription factors which activate one another's transcription, and together are sufficient to
11 activate expression of the entire lytic viral gene expression cascade (17, 49). In latently infected
12 cells, the promoters driving Z and R expression (Zp and Rp) are inactive. Therefore, activation of
13 Zp and Rp by cellular transcription factors is the crucial initial step required for lytic viral gene
14 expression. B-cell receptor engagement activates lytic EBV gene expression in some B-cell lines
15 *in vitro*, and activates both EBV IE promoters in reporter gene assays (23). Although several
16 different individual cellular transcription factors can activate one or both of the two EBV IE
17 promoters in reporter gene assays (23), to date these factors have not been shown to be sufficient
18 for efficient reactivation of lytic viral gene expression from the endogenous viral genome in
19 latently infected cells.

20 While there is a strong correlation between plasma cell differentiation and lytic EBV
21 gene expression in human tonsils, it is not currently understood why this association occurs.
22 Possible explanations would include the ability of one or more lytic viral proteins to induce plasma
23 cell differentiation in B cells. Alternatively, plasma cell differentiation might result in the

1 expression of one or more cellular transcription factor(s) that activate lytic viral gene expression.
2 The human protein XBP-1 is a basic-region leucine zipper transcriptional activator protein
3 belonging to the CREB/ATF (cyclic AMP response element binding protein/activating
4 transcription factor) family that is activated early in the process of plasma cell differentiation and
5 plays an essential role in plasma cell differentiation (32, 42). We therefore hypothesized that
6 XBP-1 might also play a role in mediating reactivation of lytic EBV gene expression during
7 plasma cell differentiation, possibly in conjunction with one or more cellular factors activated by
8 B-cell receptor engagement.

9 In this work, we demonstrate that the combination of the active (spliced) form of
10 XBP-1 (XBP-1s) with activated PKD (a histone deacetylase inhibitor which is activated following
11 antigen receptor engagement in B cells) (36, 52) is sufficient to induce lytic EBV gene expression
12 in lymphoblastoid B cells, as well as nasopharyngeal carcinoma epithelial cells. We show that
13 XBP-1s alone efficiently activates Rp in reporter gene assays, while the combination of XBP-1s
14 and PKD is required for activation of the other EBV IE promoter, Zp. Furthermore, we
15 demonstrate that an siRNA directed against XBP-1 inhibits the low level constitutive lytic EBV
16 gene expression that occurs in lymphoblastoid cells. These results suggest that XBP-1s, in
17 conjunction with activated PKD, induces the switch from latent to lytic EBV infection in plasma
18 cells.

MATERIALS AND METHODS

1

2 **Cell lines.** HONE-1/EBV is a human nasopharyngeal carcinoma cell line, which stably maintains
3 the EBV (Akata strain) genome under G418 selection in a latent form (47). HeLa is a malignant
4 human epithelial cervical cancer cell line. HONE-1/EBV cells were grown in RPMI 1640 medium
5 (Sigma). HeLa cells were grown in Dulbecco's Modified Eagle's Medium (Sigma). Both media
6 were supplemented with 10% fetal bovine serum and penicillin-streptomycin. Early passage
7 lymphoblastoid cells (primary human B cells transformed with the B95-8 strain of EBV) were a
8 gift from Bill Sugden at McArdle Laboratory, University of Wisconsin, Madison and were grown
9 in RPMI medium with 10% fetal bovine serum.

10

11 **Plasmids.** Plasmid DNA was purified through Qiagen columns as described by the manufacturer.
12 Rp-LUC contains the Rp sequences from +37 to -981 relative to the R transcription start site
13 inserted 5' of the luciferase gene in the pGL2-basic vector (Promega). A series of 5' deletion
14 mutants of the Rp-LUC construct (containing promoter sequences from +37 to -30, +37 to -100,
15 +37 to -197, +37 to -634, or +37 to -750) were also constructed. Zp-LUC contains the immediate-
16 early Zp sequence from +28 to -495 relative to the Z transcription start site inserted upstream of
17 the luciferase gene in pGL2-basic. The Zp-CAT construct, containing Zp sequences from -221 to
18 +12 inserted upstream of the CAT gene, or mutations in this construct removing either the ZII
19 (CRE) or ZI (MEF2D) motifs were a gift from Erik Flemington (Tulane University) (15).
20 Plasmids expressing the FLAG-tagged versions of XBP-1s or XBP-1u forms of mouse XBP-1
21 were a generous gift from Laurie Glimcher (Harvard University). A vector expressing the
22 constitutively active form of calmodulin-dependent calcium kinase IV (CaMKIV) was a generous
23 gift from Xiang-Jiao Yang (McGill University). A vector expressing constitutively active PKD,

1 where the serine residues at 738 and 742 have been changed to aspartate, was constructed by Alex
2 Toker (Harvard University) and acquired from Addgene.

3

4 **Luciferase assays.** HeLa cells were transfected using FuGENE 6 (Roche). Luciferase assays were
5 performed 48-72 h after transfection using extracts prepared by freeze-thawing the cell pellet in
6 Reporter Lysis Buffer (Promega) according to the manufacturer's instructions. Luciferase activity
7 was determined with an Auto Lumat LB953 luminometer (EG&G Berthold) in an assay buffer
8 containing 12.5 mM glycylglycine, 2 mM EGTA, 7.5 mM MgSO₄, 7.5 mM K₂HPO₄, 0.5 mM
9 dithiothreitol (DTT), 1 mM ATP, 100 μM luciferin, and 50 mM Tris.

10

11 **CAT assays.** HeLa cells were transfected using FuGene 6 (Roche). Cell extracts were prepared
12 two days post-transfection and incubated at 37 °C with ¹⁴C-labeled chloramphenicol (Amersham)
13 in the presence of acetyl coenzyme A (Roche). Percent acetylation was determined by
14 phosphorimager screening (Molecular Dynamics).

15

16 **Immunoblot analysis.** HONE-1/EBV cells were transfected using FuGene 6 (Roche);
17 lymphoblastoid cells were transfected with 400 nM of DNA using a nucleofector device (Amaxa)
18 in buffer V with program A30. Cells were harvested 2 days post-infection, washed twice with 1x
19 PBS and resuspended in a 1:3 mixture of SUMO buffer I (5% sodium dodecyl sulfate [SDS], 0.15
20 M Tris-HCl [pH 6.8], 30% glycerol) and SUMO buffer II (25 mM Tris-HCl [pH 8.3], 50 mM
21 NaCl, 0.5% NP-40, 0.5% deoxycholate, 0.1% SDS) and 1x Complete protease inhibitors (Roche).
22 The cells were briefly sonicated and centrifuged. The resulting supernatants were electrophoresed
23 on a 7% SDS-polyacrylamide gel electrophoresis denaturing gel. The proteins were transferred

1 onto a nitrocellulose membrane (Protran), blocked in 1x PBS-5% milk-0.1% Tween 20 and
2 incubated with anti-XBP-1 (rabbit, 1:500, Santa Cruz), anti-BRLF1 (R, mouse, 1:100, Argene),
3 anti-BZLF1 (Z, mouse, 1:100, Argene), anti-EBV EA-D (BMRF1, mouse, 1:250, Vector
4 Laboratories) or anti- β -actin (1:5000, Sigma) antibody for 1 h at room temperature. Vector
5 controls used in these experiments were either the SG5 vector, or a vector expressing an
6 unsplicable form of XBP-1.

7
8 **siRNA experiments.** siRNA against the human XBP-1 message (sense 5'
9 ACAGCAAGUGGUAGA UUUAtt and antisense 5' UAAAUCUACCACUUGCUGUtt) was
10 purchased from Ambion. Negative control siRNA was purchased from Santa Cruz (SC37007). 5
11 $\times 10^6$ lymphoblastoid cells were transfected with 400 nM of XBP-1 siRNA (or control siRNA)
12 using a Nucleofector device (Amaxa) in buffer V with program A30. These conditions allowed at
13 least 75% of cells to be transfected with approximately 50% viability. Two days post-delivery of
14 siRNA, immunoblot analysis was performed to determine the expression level of the early-lytic
15 EBV protein BMRF1 and β -actin, whereas RT-PCR analysis was performed to determine the
16 amount of XBP-1, Z, R, and β_2 -microglobulin transcripts. PCR primers used to detect the XBP-1
17 transcript were 5'CCTTG TAGTTGAGA ACCAGG and 5'GGGGCTTGGTATATATGTGG. RT-
18 PCR analysis of the β_2 -microglobulin, Z and R transcripts was performed as previously described
19 (20).

20

21 **Electrophoretic mobility shift assays (EMSA).** In vitro-translated XBP-1s was made using TNT
22 T7 quick-coupled transcription/translation system (Promega) according to the manufacturer's
23 instructions. EMSA binding reactions were performed in a buffer consisting of 1mM DTT, 10

1 mM HEPES (pH 7.9), 1 mM EDTA, 50 mM KCl, 2 mM MgCl₂ and 5% glycerol(5, 9). 2 μl of in vitro-translated XBP-1s from reticulocyte lysate (or the untranslated reticulocyte lysate), 1 μg dI.dC and 1 mg/ml BSA were added to the binding reaction and incubated for 5 minutes at room temperature before the addition of labeled probe (10,000 cpm) and 20 minutes at room temperature after the addition of the labeled probe. Anti-XBP-1 antibody was added to the reaction and the reaction was further incubated at room temperature for 10 minutes. The reaction mixtures were then loaded onto a 4% polyacrylamide gel and run in 0.5% Tris-borate-EDTA buffer at room temperature. The gel was dried on 3 MM chromatography paper (Whatman) and exposed to autoradiography film (Amersham). The film was developed using Kodak developer.

10 Sequences of oligonucleotide probes used in EMSA are shown below.

11 Positive control: 5' CTCGAGATGGATGACGTGTACAATAAAACGTCAAGCTT 3'(9)

12 Zp(CRE) (-54 to -73): 5' CCTCTGTGATGTCATGGTTT 3'

13 Complementary oligonucleotides were annealed, end labeled with ³²P and used in EMSA.

RESULTS

The combination of XBP-1s and activated PKD is sufficient to induce reactivation of lytic EBV gene expression in latently-infected epithelial cells and lymphoblastoid B cells.

To determine if XBP-1 induces lytic EBV gene expression, a construct expressing the spliced (active) form of the XBP-1 gene product (XBP-1s) was transfected into latently-infected EBV-positive cell lines and lytic viral gene expression was monitored by immunoblot analysis of two different lytic viral proteins (BMRF1 and Z) two days after transfection. In addition, the effect of the activated form of PKD was also examined because antigen receptor engagement in B cells, which stimulates lytic EBV gene expression in certain cell lines, has been shown to convert PKD to its active form (35, 50, 60). PKD directly phosphorylates class II HDAC proteins, causing them to be sequestered in an inactive form with the 14-3-3 protein in the cytoplasm (52).

As shown in Fig. 1, transfection of a latently-infected, EBV-positive nasopharyngeal carcinoma cell line (HONE-1/EBV) (47) with the XBP-1s vector alone resulted in low level expression of the early-lytic viral protein, BMRF1, while the PKD vector alone had no effect. However, the combination of XBP-1s with activated PKD induced much more expression of the BMRF1 and Z lytic viral proteins than did XBP-1s alone. Similar to the results in NPC cells, XBP-1s alone also activated low level expression of the early viral protein BMRF1, in an early-passage EBV-immortalized B cell line (LCL), although the XBP-1s/PKD combination was clearly more effective (Figure 2). There was no detectable level of Z in cells transfected with XBP-1s alone, while XBP-1s and PKD together significantly activated Z expression. In addition, XBP-1s in combination with PKD significantly activated expression of the IE/Early R protein compared with the low level of R induced by XBP-1s alone. These results indicate that the combination of

1 XBP-1 and activated PKD is sufficient to reactivate lytic EBV gene expression in either a B-cell,
2 or epithelial-cell, environment.

3

4 **XBP-1s alone is sufficient to activate the EBV immediate-early promoter Rp in reporter gene**
5 **assays, while the combination of XBP-1 and PKD activates Zp.**

6 To determine if XBP-1s or activated PKD (alone or in combination) can activate either of
7 the two EBV IE promoters, reporter gene constructs containing Rp or Zp driving the luciferase
8 gene were co-transfected into HeLa cells with vector control, the XBP-1s expression vector alone,
9 the PKD expression vector alone, or the combination of both XBP-1s and PKD (Fig. 3). XBP-1s
10 by itself strongly increased the activity of the Rp IE promoter, and this effect was not further
11 enhanced by the addition of PKD. In contrast, the combination of XBP-1s and the constitutively
12 active form of PKD produced more Zp activity than that induced by PKD or XBP-1s alone. A
13 vector expressing the unspliced (inactive) form of the mouse XBP-1 gene product (XBP-1u) did
14 not enhance Rp or Zp activity (data not shown). These results indicate that XBP-1 alone
15 efficiently activates the Rp but not Zp, and that PKD augments XBP-1s-mediated activation of the
16 Zp.

17

18 **XBP-1s activates Rp indirectly.**

19 Neither the Rp, nor Zp, promoter contains a consensus XBP-1 binding motif. To map the
20 XBP-1s-responsive region(s) in Rp, a series of 5' deletion mutants were constructed in the Rp-
21 LUC vector. Rp sequences located between -750 and -634, and between -197 and -100, were both
22 important for XBP-1s activation of the promoter (Fig. 4). In vitro translated XBP-1s did not bind
23 to any of a series of overlapping probes spanning the two XBP-1s responsive Rp elements in

1 electrophoretic mobility shift assays, although it clearly bound to a positive control probe
2 containing the consensus XBP-1 binding motif (data not shown and figure 5B). These data
3 suggest that XBP-1s activates Rp via an as yet unknown indirect mechanism.

4

5 **PKD reverses the inhibitory effect of MEF2D motifs in Zp.**

6 To further examine why PKD is required for efficient XBP-1s activation of the Zp
7 promoter, we examined the effect of mutating the MEF2D (“ZI”) binding motifs in Zp (5, 52),
8 which have previously been shown to inhibit Zp activity when MEF2D interacts with class II
9 histone deacetylases (HDACs) (5, 15). As shown in figure 5A, a vector containing the wild-type
10 Zp (from -221 to +12) linked to the CAT reporter gene responded minimally to XBP-1s or PKD
11 alone, but was efficiently activated by the XBP-1s/PKD combination. In contrast, when two
12 MEF2D binding motifs in Zp-CAT were mutated, the ability of XBP-1s alone to stimulate the
13 promoter was enhanced, and the combination of PKD and XBP-1s together was no more effective
14 than XBP-1s alone. These results indicate that the MEF2D sites act to inhibit XBP-1s activation of
15 Zp, and PKD reverses this effect.

16 In addition to MEF2D, Zp activity is positively regulated by a CRE motif (“ZII”), and
17 negatively regulated by a ZEB binding site (“ZV”). Mutation of the CRE (“ZII”) site in Zp, which
18 has been previously shown to be bound by ATF-1, ATF-2, CREB, and c-jun (23), decreased Zp
19 activation by the XBP-1s/PKD combination, suggesting that this motif is required for efficient
20 XBP-1s/PKD effect (Fig. 5A). However, we found that XBP-1s binds only very weakly, if at all, to
21 the Zp CRE motif in EMSA assays (Fig. 5B). Mutation of the Zp ZEB binding site (in the context
22 of the Zp-LUC construct) enhanced the ability of XBP-1s/PKD combination to activate Zp (Fig.

1 5C). These results confirm that ZEB binding to Zp, as previously described (27), acts as a
2 negative regulator of Zp activity.

3

4 **CaMKIV and valproic acid also augment the ability of XBP-1s to induce EBV reactivation.**

5 The two isoforms of PKD expressed in B cells are thought to account for the majority of
6 class II HDAC inhibitory activity in uninfected B cells (11, 21, 36). However, as PKD affects a
7 number of cellular pathways in addition to HDAC proteins (3, 8, 19, 34, 38, 48), we determined if
8 other HDAC inhibitors could likewise augment the ability of XBP-1s to induce lytic EBV gene
9 expression. Ca²⁺/Calmodulin-dependent kinase IV (CaMKIV), like activated PKD, directly
10 phosphorylates class II HDAC proteins and inhibits their function (33, 37). Although CaMKIV is
11 not normally expressed in B cells, its transcription is induced by a latent EBV protein, LMP-1 (39),
12 and B-cell receptor engagement converts the CaMKIV protein to its active form (39). Therefore,
13 in B cells with type II or type III latent EBV infection (in which LMP-1 is expressed), CaMKIV
14 (in addition to PKD) could function as a class II HDAC inhibitor.

15 Transfection of HONE-1/EBV cells with a vector expressing a constitutively active
16 form of CaMKIV by itself produced minimal expression of lytic EBV genes BMRF1, R and Z, but
17 greatly augmented the ability of transfected XBP-1s to induce lytic viral gene expression (Fig.
18 6A). In addition, transfected XBP-1s induced expression of lytic EBV genes BMRF1, R and Z in
19 the presence, but not absence, of a pharmacologic HDAC inhibitor, valproic acid (Fig. 6B). These
20 results suggest that it is the HDAC inhibitory activity of PKD and CaMKIV that allows them to
21 complement XBP-1s in reactivating EBV lytic infection.

22

1 **XBP-1s expression contributes to lytic EBV gene expression in early-passage lymphoblastoid**
2 **cells.**

3 EBV-transformed B-cell lines (lymphoblastoid cell lines) usually secrete
4 immunoglobulin (53) and early-passage lines commonly express a small amount of lytic EBV
5 genes (20). XBP-1s may therefore contribute to low-level constitutive lytic EBV gene expression
6 which occurs in some LCLs. To determine if this is the case, we transfected early-passage
7 lymphoblastoid cells with an XBP-1 siRNA or equivalent amounts of a control siRNA (Fig. 7).
8 Cells transfected with the XBP-1 siRNA expressed less XBP-1 RNA than cells transfected with
9 the control siRNA (Fig. 7A). Furthermore, expression of the lytic EBV genes, Z and R, was
10 decreased in cells transfected with XBP-1 siRNA, while β_2 -microglobulin message was not
11 affected (Fig. 7B and C). In addition, transfection of LCLs with XBP-1 siRNA led to a decrease in
12 the expression of the EBV lytic gene BMRF1 as shown by the immunoblot (Fig. 7D). These
13 results indicate that constitutive XBP-1s expression in early-passage EBV-transformed B cells
14 contributes to the constitutive lytic viral gene expression in these cells.

15
16

DISCUSSION

1
2 Tightly latent EBV infection, which cannot easily be recognized and eliminated by
3 the immune response, enables long-term persistence of the virus in the host. Latent EBV infection
4 is established in the long-lived memory B cell compartment, reducing the likelihood that the
5 reservoir of latently infected cells is ever lost. However, to ensure that that the virus is transmitted
6 from host to host, EBV must be periodically reactivated to the lytic form of infection. Increasing
7 evidence suggests that the lytic form of EBV infection in humans occurs in antibody-secreting
8 plasma cells (29) and tonsillar epithelial cells (41). Nevertheless, the molecular mechanism(s)
9 underlying reactivation of lytic EBV infection during plasma cell differentiation have not
10 previously been explained. In this work, we show that X-box-binding protein 1 (XBP-1), in
11 combination with activated PKD, is sufficient to induce lytic EBV gene expression in latently-
12 infected epithelial cells as well as lymphoblastoid cells. Furthermore, we demonstrate that XBP-1
13 is required for the small amount of constitutive lytic viral gene expression that occurs in early-
14 passage lymphoblastoid cells. Since XBP-1 is converted to its active (spliced) form during plasma
15 cell differentiation, while PKD is activated by B-cell receptor engagement, these results help
16 explain why the lytic form of EBV infection is so highly associated with plasma cell differentiation
17 in humans.

18 XBP-1 activation and plasma cell differentiation are intricately linked. On the one
19 hand, the activity of the XBP-1 transcription factor is dramatically enhanced by both
20 transcriptional, and splicing, mechanisms as B cells differentiate into plasma cells. The production
21 of antibodies by plasma cells results in the accumulation of unfolded proteins in the endoplasmic
22 reticulum (ER), leading to ER stress and activation of the unfolded protein response (UPR) (24).
23 UPR then enhances XBP-1 function through at least two different mechanisms. First, UPR

1 induces proteolytic cleaving of the ER protein, ATF6 α , allowing the amino-terminal portion of the
2 protein to transit from the ER to the nucleus, where it binds to the promoter of the XBP-1 gene and
3 activates its transcription (55), (56). Second, UPR induces the homodimerization and
4 transphosphorylation of IRE1 α , converting it to an atypical splicing enzyme which mediates
5 cytoplasmic splicing of XBP-1. Only this spliced form of XBP-1 (XBP-1s) contains a
6 transcriptional activator domain and can activate transcription of downstream target genes (2, 30).

7 On the other hand, XBP-1 is also clearly required for full plasma cell differentiation,
8 although its effect is downstream of another essential plasma cell differentiation factor, Blimp-1
9 (26, 46). B cells derived from XBP-1-knockout mice mature normally but are unable to
10 differentiate into antibody-secreting plasma cells (42). The spliced form of XBP-1 not only
11 activates transcription of numerous ER chaperone genes (55), but also increases the size of the
12 cellular mitochondrial, ER, and secretory apparatus compartments (46). These downstream effects
13 of XBP-1s, which presumably help the plasma cell to efficiently produce and secrete large
14 amounts of antibody (54, 55), may also promote the large scale protein synthesis required for the
15 synthesis of lytic herpesvirus proteins. Interestingly, cytomegalovirus was recently shown to
16 modulate and enhance certain aspects of XBP-1s function (22). By selectively converting to the
17 lytic form of viral infection in plasma cells, EBV has likewise found a mechanism for ensuring that
18 sufficient XBP-1s activity is available in cells with the lytic form of viral replication.

19 XBP-1s is known to activate most downstream target genes, including ER chaperone
20 genes and some MHC class II genes, via direct binding to the promoters of these genes (9, 25, 40,
21 54, 55). XBP-1 may also activate transcription through indirect mechanisms, including enhancing
22 the transcriptional effect of estrogen receptor alpha (12) and inducing expression of
23 CCAAT/enhancer binding protein beta (7). Our EMSA experiments suggest that XBP-1s does not

1 bind directly to Rp, and binds minimally to the CRE motif in Zp. We speculate that XBP-1s
2 indirectly activates Zp and/or Rp by inducing transcription of a cellular factor that activates one or
3 both promoters, or by interacting with one or more cellular proteins that directly bind to Zp and/or
4 Rp. Dissecting the exact mechanism(s) by which XBP-1s increases Z and R transcription will
5 require further study.

6 Although XBP-1s alone activates the EBV Rp IE promoter in reporter gene assays,
7 and high level expression of the R gene product is sufficient to induce lytic EBV infection in many
8 cell types (including Hone-EBV cells) (59), the combination of both XBP-1s and activated PKD is
9 required for induction of lytic EBV infection in latently-infected HONE-1/EBV cells. PKD, which
10 belongs to a group of serine/threonine protein kinases (43), is activated by B-cell receptor
11 engagement via a pathway involving phospholipase C (PLC). PLC hydrolyzes
12 phosphatidylinositol(4,5)-bisphosphate to produce diacylglycerol (DAG), activating novel PKC
13 isoforms, which in turn phosphorylate and activate PKD (50, 52, 60). Activated PKD can then
14 travel to the nucleus and phosphorylate and inactivate class II HDACs.

15 The ability of PKD to disrupt viral latency in conjunction with XBP-1s is likely
16 mediated through inhibition of class II HDAC proteins, since a similar effect was observed using
17 two other HDAC inhibitors, CaMKIV and valproic acid. Inhibition of class II HDAC proteins
18 would be expected to enhance the ability of XBP-1s to activate lytic viral gene transcription in the
19 context of the intact latent genome through at least two different mechanisms. First, Rp is highly
20 methylated with an inactive (unacetylated) chromatin structure in the context of the intact latent
21 EBV genome in many cell lines (4, 6, 58). Chromatin accessibility to transcription factors is
22 regulated by acetylation or deacetylation of nucleosomal histones, and HDAC proteins inhibit the
23 ability of many transcription factors to activate target promoters by converting the chromatin to a

1 less accessible (deacetylated) form. Acetylation of the chromatin around Rp (and possibly Zp) in
2 the context of the intact latent viral genome presumably enhances the ability of XBP-1s to activate
3 the promoter.

4 In addition, certain transcription factors, including members of the myocyte enhancer
5 factor 2 (MEF2) family, directly interact with class II (but not class I) HDAC proteins and tether
6 these HDACs to promoters with MEF2 binding sites. Zp contains a series of MEF2D binding sites
7 (known as ZI motifs), which function as negative regulators when complexed with class II HDAC
8 proteins (5, 13, 15, 18). The finding that removal of the MEF2D sites in Zp allowed XBP-1s alone
9 to activate Zp without PKD strongly suggests that PKD enhances XBP-1s activation of Zp at least
10 partially by inhibiting the interaction between MEF2D and class II HDAC proteins.

11 As XBP-1s can also be activated by the unfolded protein response in non B-cell types, it
12 remains possible that XBP-1s also contributes to induction of lytic EBV infection in other cell
13 types in response to stimuli such as chemotherapy and radiation (13, 14). In any event, activation
14 of the two EBV IE promoters by the combination of two factors (spliced XBP-1 and activated
15 PKD) that are induced by B-cell receptor engagement and plasma cell differentiation helps to
16 ensure that EBV pathogenesis is highly linked to the state of B cell maturation/activation.

ACKNOWLEDGMENTS

We thank Drs. Lawrence Young and Chris Dawson for providing HONE-1/EBV cell line.

This work was supported by National Institutes of Health grants RO1 CA58853 and R01-CA66519.

ACCEPTED

Figure legends

Fig. 1. XBP-1s and PKD synergistically activate lytic EBV gene expression in HONE-1/EBV

cells. HONE-1/EBV cells were transfected with empty vector (-), XBP-1s, constitutively active PKD (PKD), or the combination of XBP-1s and PKD. Immunoblot analysis was performed on cellular extracts 2 days later to quantitate expression of two different lytic EBV proteins (Z and BMRF1), a cellular protein (β -actin), and transfected XBP-1s protein.

Fig. 2. XBP-1s and PKD synergistically activate lytic EBV gene expression in early-passage

lymphoblastoid cells (LCLs). LCLs were transfected with empty vector (-), the XBP-1s expression vector, constitutively active PKD (PKD), the combination of XBP-1s and PKD, or a Z expression vector. Immunoblot analysis was performed on cellular extracts 2 days later to quantitate expression of the lytic EBV proteins (BMRF1, R and Z) and cellular β -actin.

Fig. 3. XBP-1s and PKD activation of Rp versus Zp.

Vectors containing the luciferase (Luc) gene linked to either the R (Rp-Luc) or Z (Zp-Luc) promoters were transfected into HeLa cells in combination with either a control plasmid (-), an expression vector for the spliced form of XBP-1 (XBP-1s), a constitutively active form of PKD (PKD), or both XBP-1s and PKD as indicated.

Luciferase activity was measured two days later. Results are presented as fold-activation in the amount of luciferase activity in conditions transfected with XBP-1s, PKD, or both compared with conditions transfected with the control vector as indicated.

Fig. 4. XBP-1 responsive regions in Rp.

Various 5' Rp-luc deletions were constructed as indicated and transfected into HeLa cells in combination with either empty vector (-) or the vector expressing

XBP-1s (+). Results are presented as fold-activation in the amount of luciferase activity in conditions transfected with XBP-1s versus the vector control for each promoter construct.

Fig. 5. MEF2D and CRE motifs contribute to XBP-1s/PKD activation of Zp. (A) Reporter gene constructs containing wild-type Zp (-221 to +12) inserted upstream of the CAT gene, or containing site-directed mutations in Zp altering the MEF2D or CRE motifs, were co-transfected into HeLa cells with either vector control or the XBP-1s expression vector. CAT activity was quantitated 2 days later. Results are presented as fold-activation in the amount of CAT activity in conditions transfected with XBP-1s compared with conditions transfected with the control vector as indicated for each promoter construct. (B) EMSA was performed using *in vitro* translated XBP-1s (+) or untranslated rabbit reticulocyte lysate (-) in combination with either the positive control oligonucleotide probe containing a consensus XBP-1s-binding motif or a probe containing the Zp CRE motif in the absence (-) or presence (+) of anti-XBP-1 antibody. (C) Zp-Luc constructs with either wild-type Zp, or containing a site-directed mutation in the ZEB binding motif (Δ ZEB), were transfected into HeLa cells either with empty vector or vector expressing mXBP-1s. Luciferase activity was measured two days later.

Fig. 6. XBP-1s and HDAC inhibitors, CaMKIV and valproic acid, activate lytic EBV gene expression. (A) HONE-1/EBV cells were transfected with empty vector (-), XBP-1s or constitutively active CaMKIV either alone or in combination as indicated. Cell extracts were examined by immunoblot for expression of the lytic EBV proteins (BMRF1, R and Z) and cellular β -actin two days later. (B) HONE-1/EBV cells were transfected with empty vector or XBP-1s in the presence or absence of valproic acid (VPA, 1 mM). Extracts were analyzed by immunoblot two days later for the lytic EBV proteins (BMRF1, R and Z) and cellular β -actin.

Fig. 7. XBP-1 is required for lytic EBV gene expression in lymphoblastoid cells. EBV-transformed B cells were transfected with an siRNA directed against XBP-1, or equal amounts of a control siRNA. Results from two separate experiments are shown. (A) The level of XBP-1 expression was examined by RT-PCR analysis two days after siRNA delivery. PCR amplifications were performed using undiluted cDNA, as well as various dilutions of the cDNA, as indicated. (B) The expression level of lytic viral genes, Z and R, was examined by RT-PCR. (C) The expression of the β_2 -microglobulin (β_2 -M) message was examined by RT-PCR. (D) The expression of the early lytic EBV BMRF1 protein, as well as cellular actin, was examined by immunoblot analysis two days after siRNA delivery.

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Fig. 1

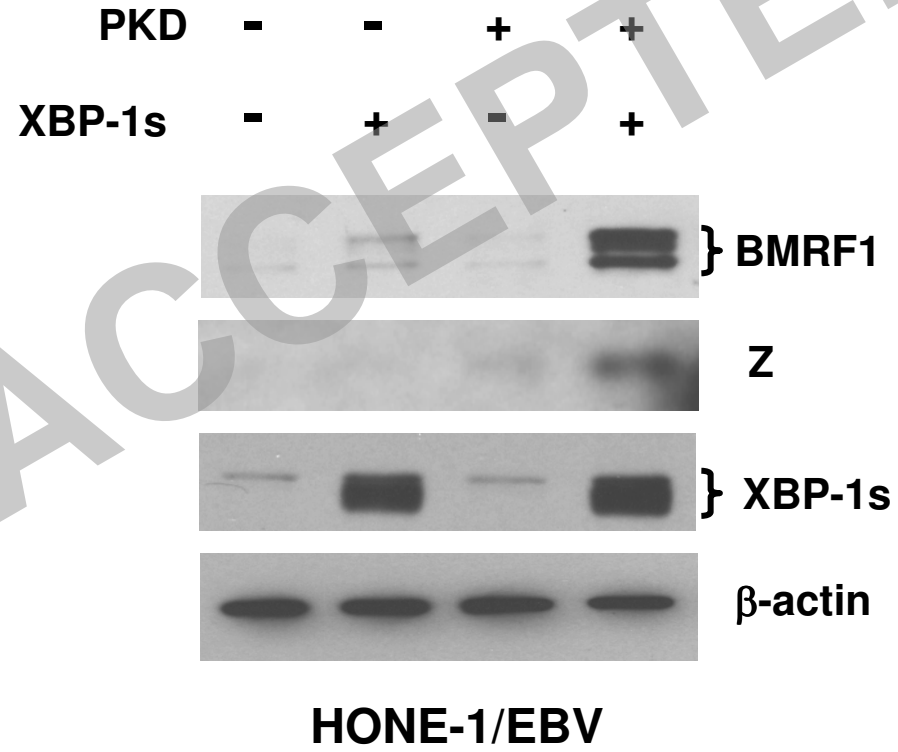


Fig. 2

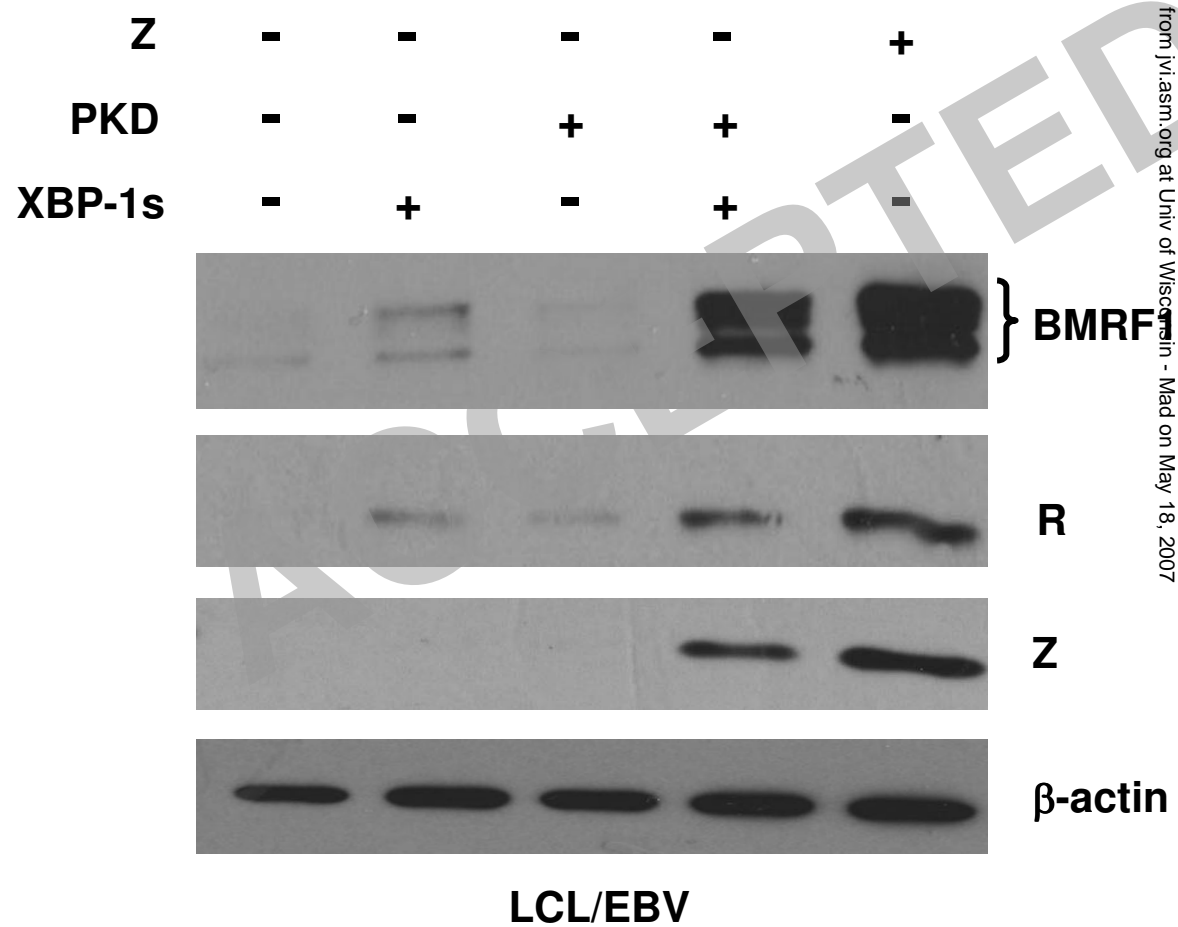
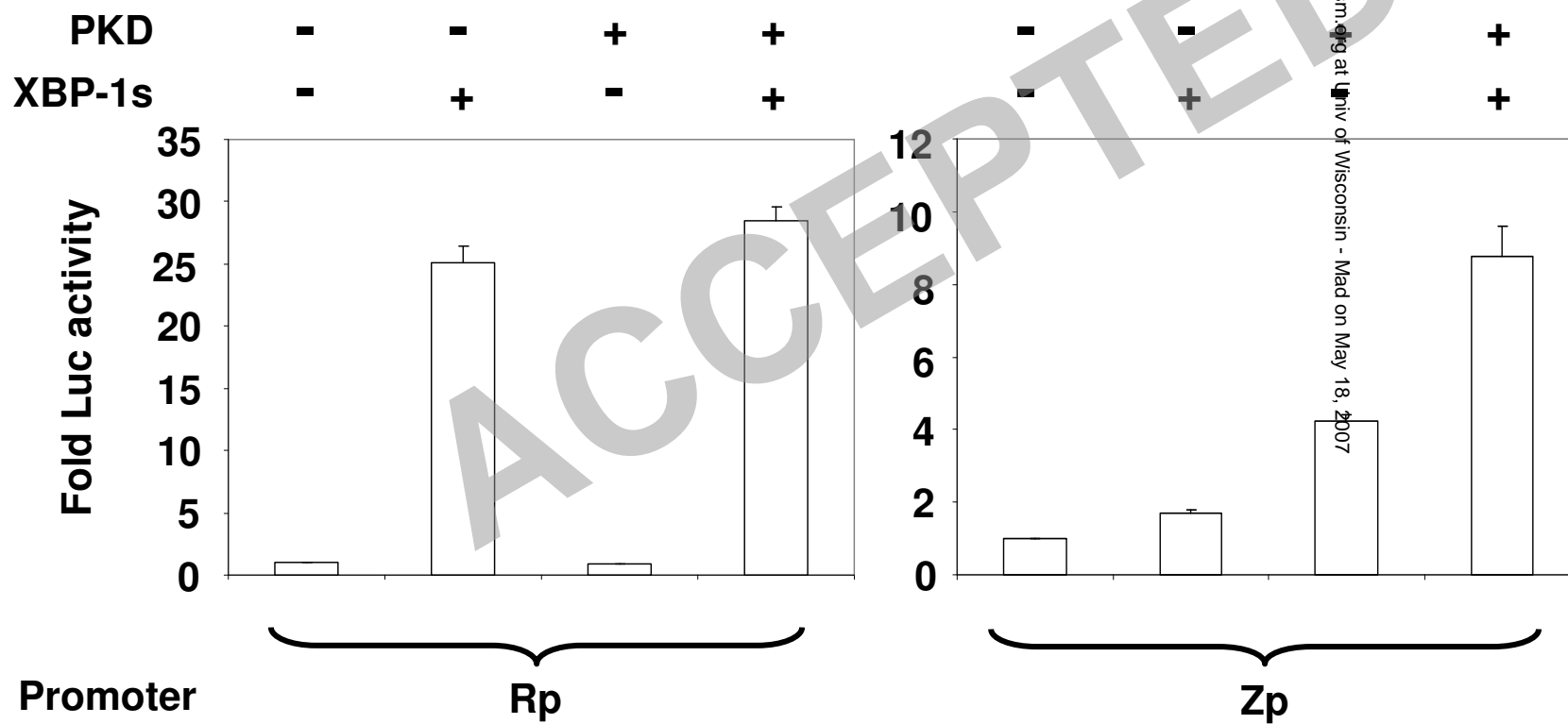


Fig. 3



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Fig. 4

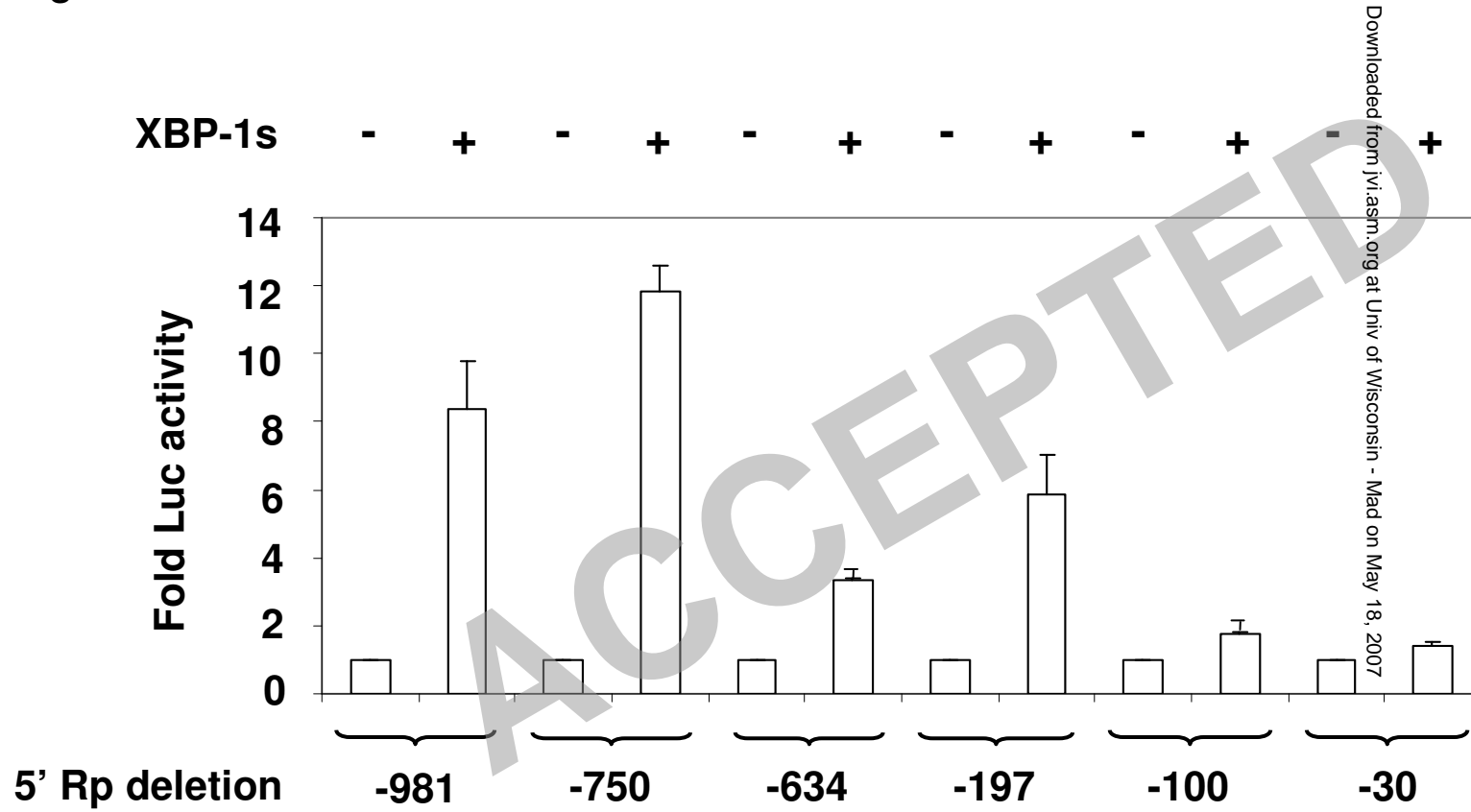


Fig. 5A

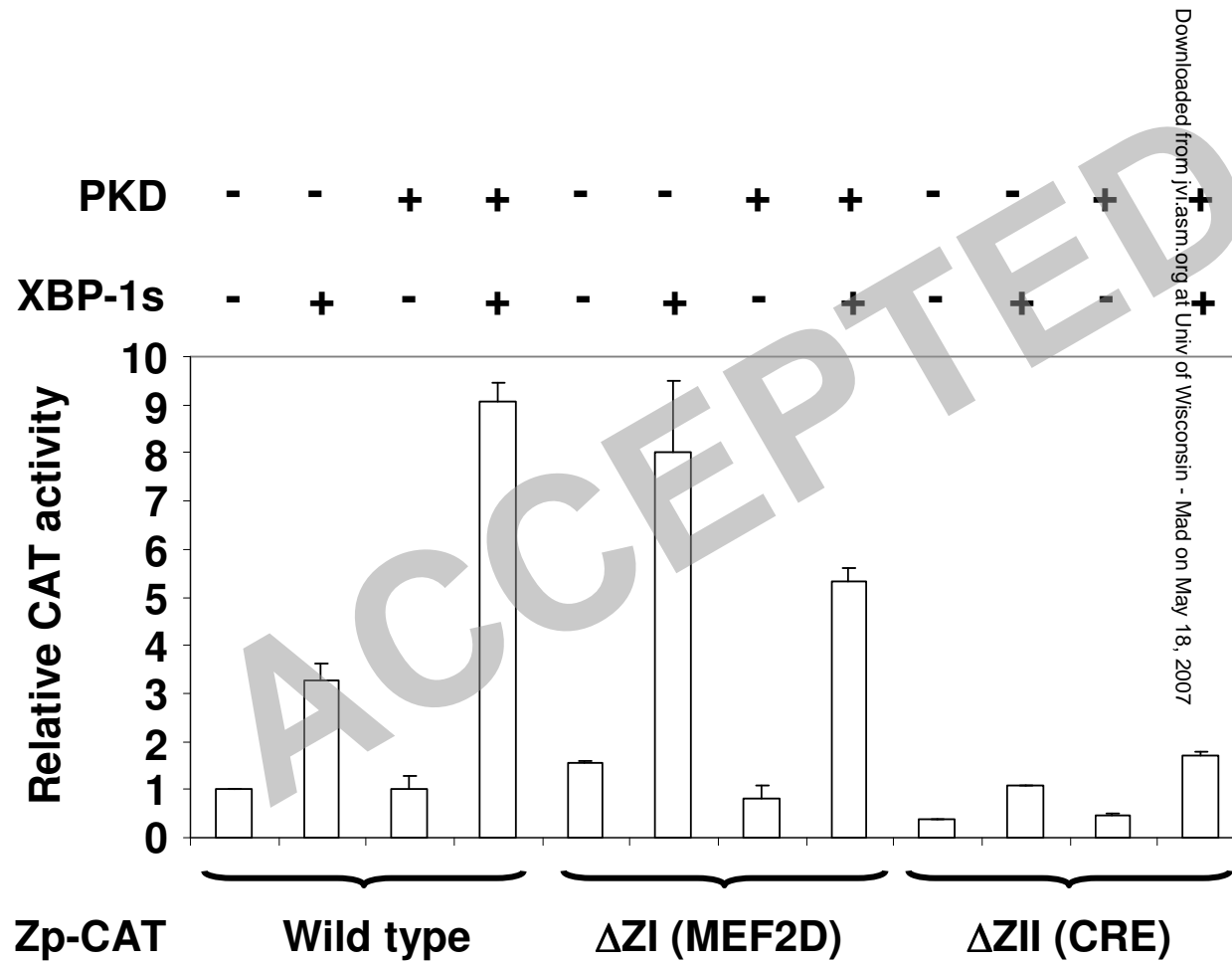


Fig. 5B

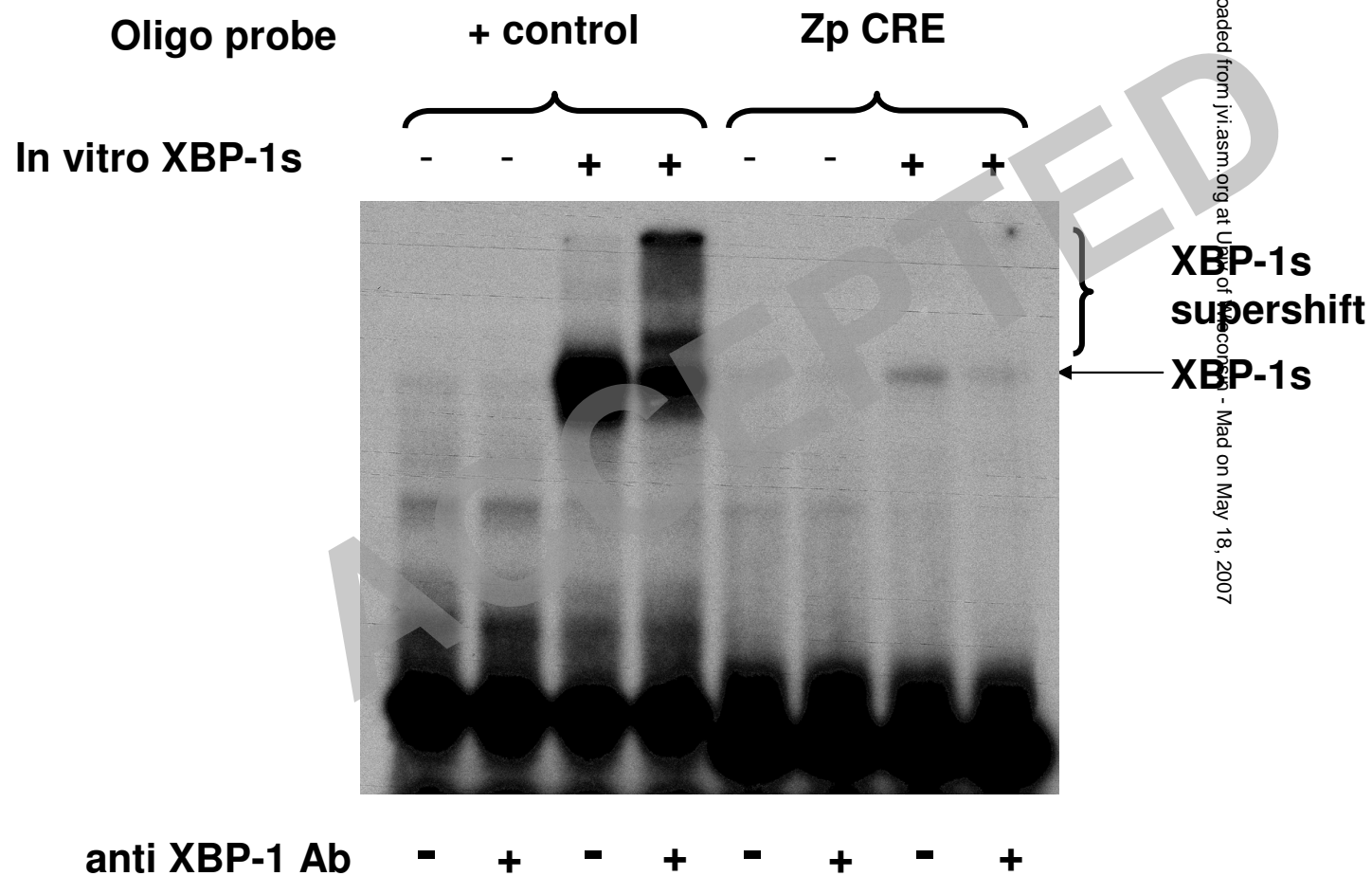


Fig. 5C

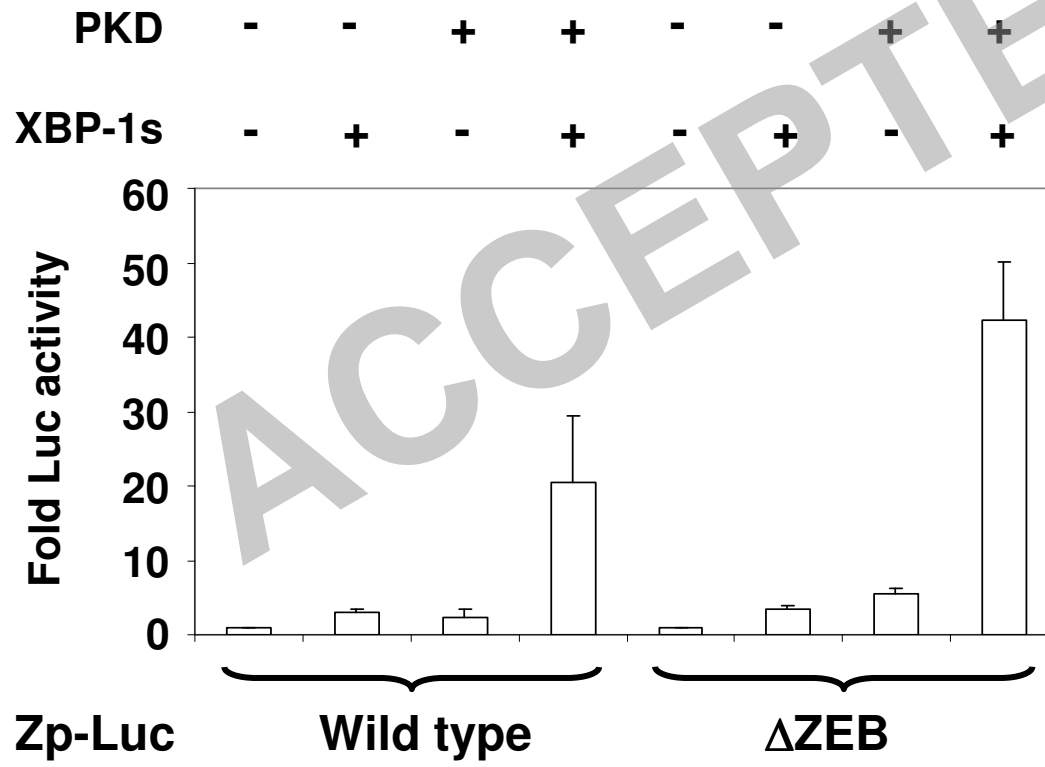


Fig. 6A

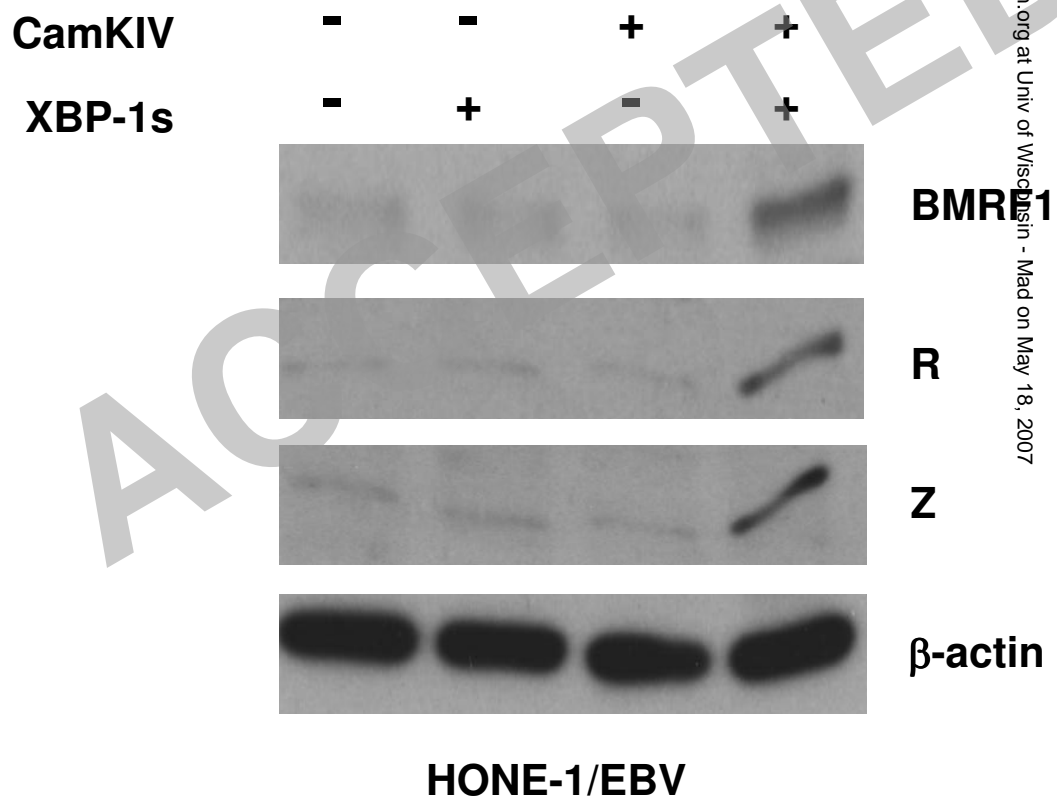


Fig. 6B

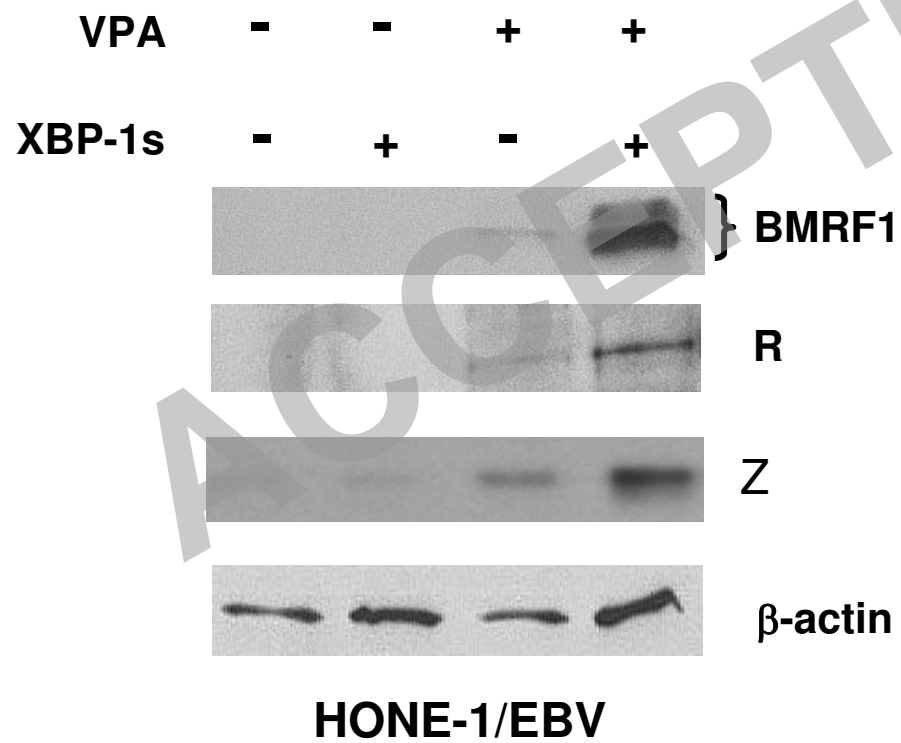


Fig. 7A

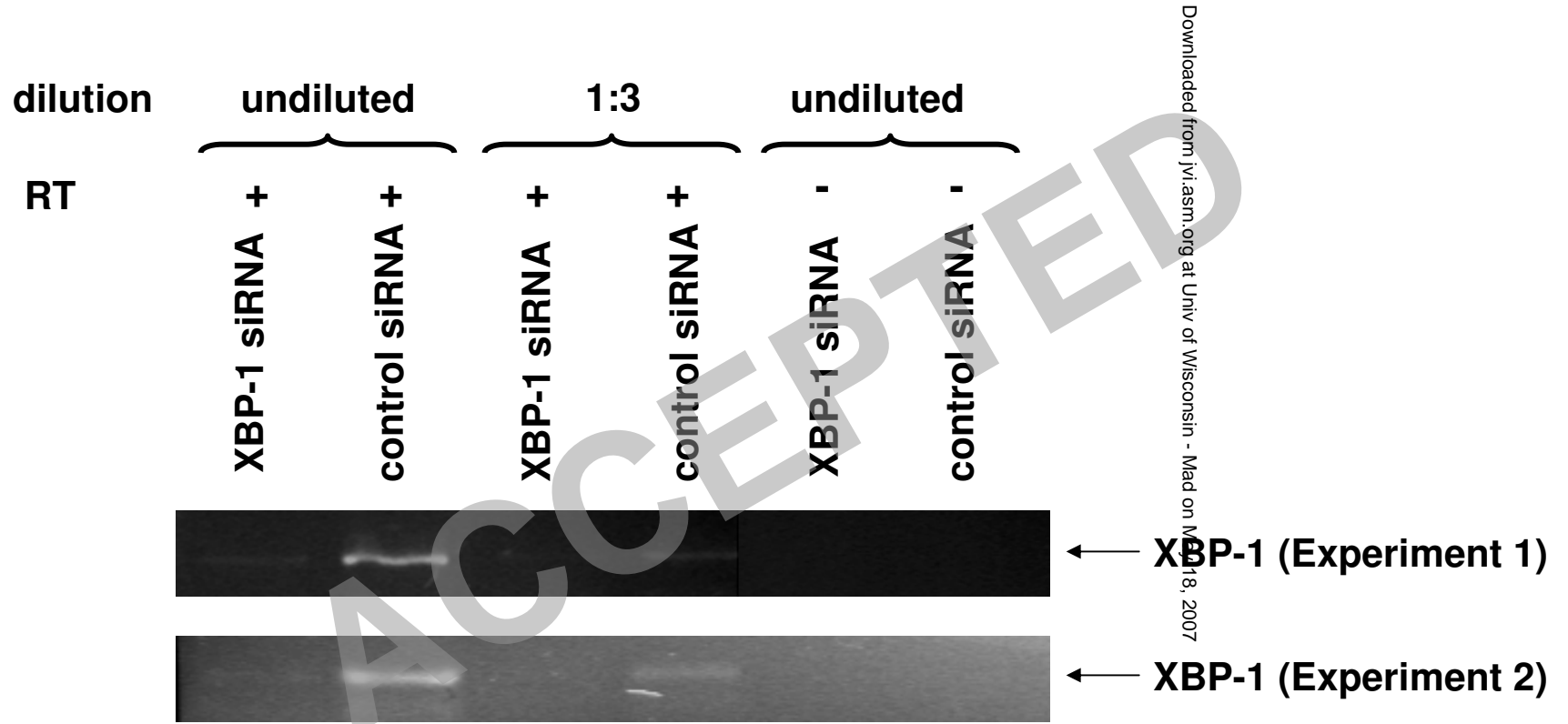


Fig. 7B

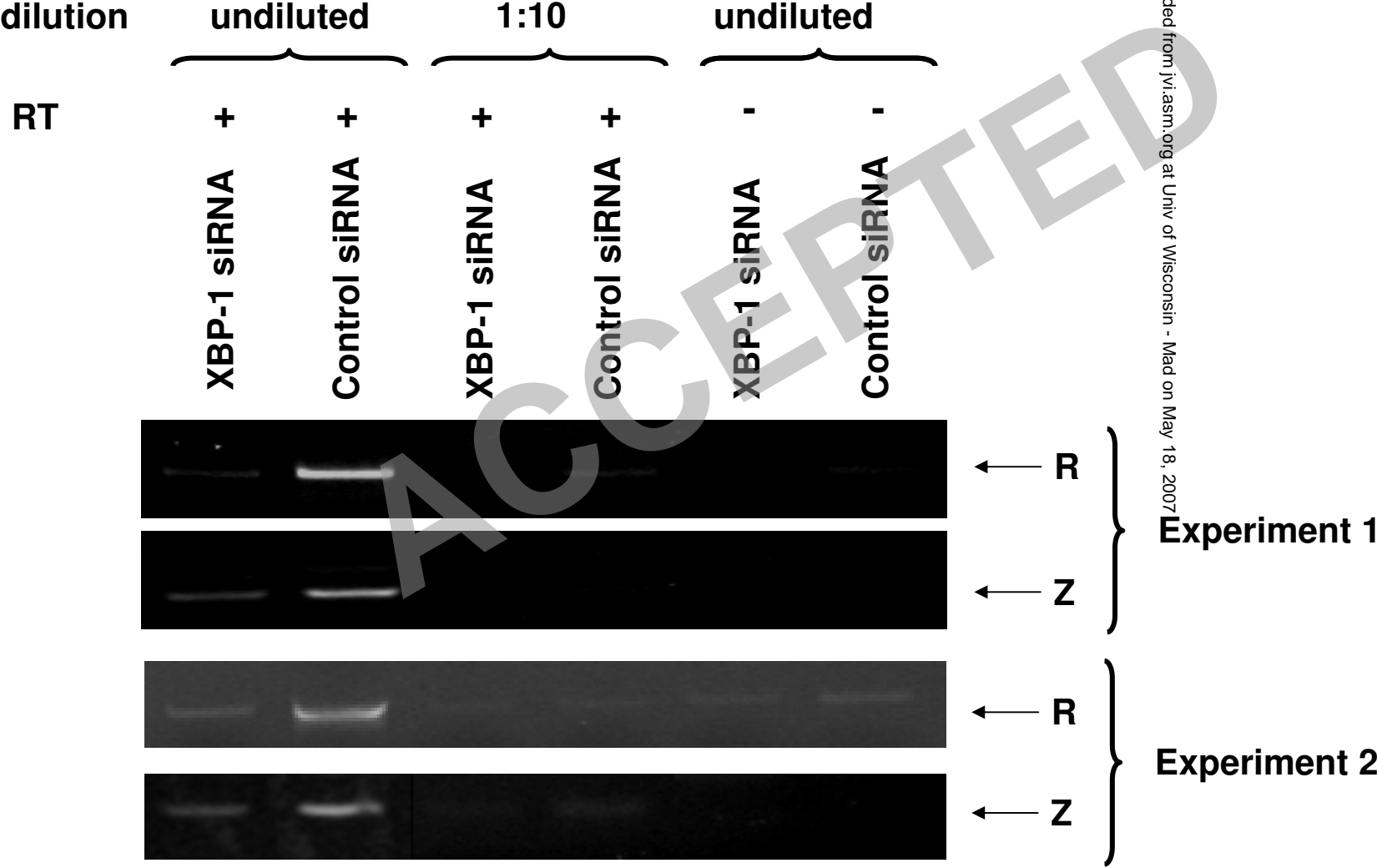


Fig. 7D

