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Human MageB2 Expression Enhances E2F Activity, Cell Proliferation And Resistance To Ribotoxic Stress

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Running Title: Targeting E2F activity by human MageB2 expression.

Keywords: cancer biology, cell proliferation, E2F transcription factor, histone deacetylase (HDAC), nucleolus.

CAPSULE

Background: MageB2 is a tumor-specific antigen with unknown function.

Results: Through a mechanism involving histone deacetylases, MageB2 enhances E2F activity and resistance to Actinomycin D.

Conclusion: MageB2 is a protein conferring proliferation properties and resistance to ribotoxic stress to tumor cells.

Significance: Expression of MageB2 in human tumors could be a marker of chemotherapy refraction.

ABSTRACT

MageB2 belongs to the MAGE-I (Melanoma Antigen GEne) family of tumor-specific antigens. Expression of this gene has been detected in human tumors of different origins. However, little is known about the protein function and how its expression impacts on tumor cell phenotypes. In this work we found that human MageB2 protein promotes tumor cell proliferation in a p53-independent fashion as observed both in cultured cells and growing tumors in mice. Gene expression analysis showed that MageB2 enhances the activity of E2Fs transcription factors. Mechanistically, the activation of

E2Fs is related to the ability of MageB2 to interact with the E2F inhibitor, HDAC1. Cellular distribution of MageB2 protein includes the nucleoli; nevertheless ribotoxic drugs rapidly promote its nucleolar exit. We showed that MageB2 counteracts the E2F inhibition by ribosomal proteins independently of Mdm2 expression. Importantly, MageB2 plays a critical role in impairing cell cycle arrest in response to Actinomycin D. Data presented here support a relevant function for human MageB2 in cancer cells both in cycling and stressed conditions, thus presenting a distinct functional feature with respect to other characterized MAGE proteins.

MageB2 (Melanoma Antigen Gene B2) is a member of the Cancer Testis Antigens (CTA), whose expression is restricted to normal testis, but aberrantly expressed in a broad number of human tumors and silent in somatic cells (1). Type I MAGE (MAGE-I) proteins contain an approximately 200 amino acids long conserved domain (MAGE Homology Domain, MHD) that identifies the family. MAGE-I genes are grouped in three clusters of human chromosome X, named MAGE-A, MAGE-B and MAGE-C. In normal cells, repression of MAGE-I gene transcription is mainly maintained by promoter methylation (2).

Since their discovery as tumor antigens (3), MAGE-A genes have been mainly studied as potential targets for cancer vaccines (4). In the last years, our and other groups have started studying the cellular function of different MAGE-A proteins showing their associated pro-oncogenic functions mostly through regulation of specific transcription factors. For example, MageA1 regulates SKIP activity (5), MageA11 expression enhances Androgen Receptor transactivation function (6-9), MageA2 associates to and regulates the p53 tumor suppressor activity (10,11) and to PML causing an impairment in oncogene-induced senescence (12). Overall, emerging data strongly suggest that MAGE-A proteins counteract pro-apoptotic or cell-cycle arresting stimuli (13). Accordingly, MAGE-A expression has been frequently correlated to poor prognosis in cancer patients (14-17).

With respect to MAGE-B, a vaccine raised against murine Mageb2 has been shown to prevent metastasis formation in a mouse model of breast cancer (18). Mouse mageb1, mageb2 and mageb3 genes encode for proteins that are 97-100% identical and therefore almost indistinguishable. siRNA-mediated silencing of those murine mage-b genes reduces cell viability in P815 mast cell line (19) and in S91 melanoma cell line (20). Moreover, murine mage-b silencing was associated to p53-dependent apoptosis (20). More recently, it was shown that murine Mageb18 silencing induces apoptosis and increases the protein levels of p53 (21).

Little is known about human MAGE-B proteins. Human MageB2 presents 50% amino acid identity when compared to mouse Mage-b proteins. MageB2 gene expression starts as early as MAGE-A genes during carcinogenesis (22). Recently, it has been reported a correlation between human MageB2 promoter demethylation and MageB2 gene expression in primary head and neck squamous cell carcinoma (23). In addition, MageB2 overexpression showed growth promoting effect in a transformed oral keratinocytes cell line (23). Even though human MageB2 expression is found in a variety of human cancers such as lung carcinoma (22), head and neck squamous cell carcinoma (23), multiple myeloma (24), renal cell carcinoma (25), its function in tumor cells is mostly unknown.

We report here that human MageB2 expression regulates tumor cell proliferation independently of p53 status. We found that MageB2 is able to enhance E2Fs transcription factors activity through HDACs interaction. MageB2 protein is able to enter the nucleolus

through an N-terminal Nucleolar Localization Signal (NoLS), whereas the ribotoxic drug, Actinomycin D, stimulates its nucleolar exit. We show that MageB2 endogenous expression correlates with a reduced cytostatic effect under ribotoxic stress conditions.

EXPERIMENTAL PROCEDURES

Cell culture and reagents- U2OS, HCT116, H1299, HeK293-T and B16F0 cell lines were obtained from the ATCC and were cultured as recommended. HCT116 $p53^{-/-}$ cells were provided by Bert Vogelstein. MEF DKO $p53^{-/-}$ $mdm2^{-/-}$ were cultured as recommended. U24 cells are HA-MageB2 inducible U2OS cells using the Ecdysone-Inducible Expression System (Invitrogen) and were maintained in medium with additional 1 mg/ml G418 and 250 ug/ml Zeocin (Invitrogen). U24 cells were induced by adding 5uM Ponasterone A (Invitrogen) for 24 hr. TSA and Actinomycin D have been purchased from Sigma-Aldrich.

Growth curves- Cells were fixed in 10% formalin at the indicated times. Crystal violet (0.1%; Sigma-Aldrich) was used to stain cells. Cell-associated dye was extracted in 10% acetic acid and the optical density was measured at 590 nm with each value normalized to day 0. Each point was done in duplicate.

Tumor growth- $1x10^5$ B16F0-GFP or B16F0-GFP-MageB2 cells were resuspended in 0.2ml of PBS and injected subcutaneously into the left flank of 8 weeks old C57BL/6 mice (five mice per group). Mice were visited daily. When tumors reached measurable size their length and width were measured every two days with a caliper and the mean tumor volume was estimated using the formula $(\text{length} + \text{width})^{1/2}$. Mice whose tumors reached a volume of 2 cm^3 were killed by cervical dislocation. The protocol was approved by the Institutional Animal Care and Utilization Committee of School of Exact and Natural Science, University of Buenos Aires.

Plasmids, siRNA and Transfections- U2OS cells were transfected using TransIT-LT1 reagent (Mirus), according to the manufacturer's instructions. HEK293-T, MEF DKO p53-/- mdm2-/- and B16F0 were transfected with PEI (Polysciences). Unless stated otherwise, cells were analyzed 24 hr after transfection. HA-MageB2 was cloned in pCDNA3 (Invitrogen). GFP-MageB2 was obtained by subcloning in pLPC. GFP-MageB2 (1-109), GFP-MageB2ΔNoLS, GFP-mageb2 and GFP-mageb18 were cloned in pEGFP (Clontech). Myc-MageB2 was cloned in pCMV (Clontech). pCDNA3-HA-MageA2, Flag-HDAC1, Flag-HDAC3 and HA-HDAC4 have been previously described (10). MageB2 siRNA was a pool of two different siRNAs (#1 sequence GCGAAGAUCCUCUAACCAGTT, #2 sequence AACUCAGCUACUGAGGAAGAG) and were purchased from Eurofins Genomics. Control siRNA (siC) was the AllStars Negative control siRNA from Qiagen. Cells were transfected with siRNAs using Lipofectamine RNAiMAX Reagent (Invitrogen) as recommended by the manufacturer and analyzed after 72 hr.

Gene reporter assays- 6xE2F-LUC reporter plasmid (pGL3 containing a TATA box and six E2F binding sites)(26) was used to test E2Fs activity. For luciferase normalization, pRL-CMV reporter (Promega), constitutively expressing the Renilla luciferase was cotransfected. After 24 h, cells were lysed and luciferine and coelenterazine-h were used for assayed firefly and Renilla luciferase activity, respectively.

Antibodies- Western blot analysis was performed according to the standard procedures using the following primary antibodies: For MageB2 detection, affinity purified anti-MageB2 raised against a GST-

tagged N-terminal fragment corresponding to amino acids 30 to 116 (GST-MageB2(30-116)). Other primary antibodies: anti-p53 polyclonal (FL-393) antibody, anti-E2F1 monoclonal (KH95) antibody, anti-AR monoclonal (441) antibody and anti-c-myc monoclonal (C-33) antibody were from Santa Cruz Biotechnology; anti-NCL monoclonal antibody was from Zymed Laboratories; anti-vinculin monoclonal antibody and anti-tubulin monoclonal from Sigma-Aldrich. For tags: anti-HA 12CA5 monoclonal antibody (Roche), anti-HA (Y-11) polyclonal antibody (Santa Cruz Biotechnology), anti-Flag M2 monoclonal antibody (Sigma-Aldrich), anti-myc-tag (9B11) monoclonal antibody (Cell Signaling). Anti-GFP was an affinity purified polyclonal antibody raised against GST-GFP.

Immunofluorescence- Immunostaining was performed as previously described (12). Glass slides were analyzed using a laser scan confocal microscope (Zeiss) or an epifluorescence microscope (Leica). Images were obtained at 63X magnification.

Immunoprecipitation- Immunoprecipitation assay was performed as previously described (12).

Quantitative RT-PCR- Total RNA was extracted with Qiazol reagent (Qiagen) and cDNA was transcribed with a QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions. Real-time PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) and StepOnePlus real time PCR machine (Applied Biosystems). Primer sequences are listed here: MageB2 Fw 5'-CCTGACTTCCGCTTTGGAGGCG-3', MageB2 Rev 5'-ATCTCGGGCCTTGCGGCGTT-3'; MCM6 Fw 5'-ATCCCTCTTGCCAAGGATTT-3', MCM6 Rev 5'-GAAAAGTTCCGCTCACAAGC-3'; CycD1 Fw 5'-ACGGCCGAGAAGCTGTGCATC-3', CycD1 Rev 5'-CCTCCGCCTCTGGCATTTTGGAG-3'; CDK1 Fw 5'-CATGGCTACCACTTGACCTGT-3', CDK1 Rev 5'-AAGCCGGGATCTACCATACC-3'; CycE1 Fw 5'-TGAGCCGAGCGGTAGCTGGT-3', CycE1 Rev 5'-GGGCTGGGGCTGCTGCTTAG-3'; MCM7 Fw 5'-CACGGAGTCTCTCAGCACAG-3', MCM7 Rev 5'-AACATCTGTCTGATGGGGGA-3'; B-actin Fw 5'-CCAACCGCGAGAAGATGA-3', B-actin Rev 5'-CCAGAGGCGTACAGGGATAG-3'.

Cellular fractionation- HCT116 cells were fractionated using a hypotonic buffer and series of centrifugations over sucrose cushions according to the following protocol http://www.lamondlab.com/f7nucleolarprotocol.htm

BrdU incorporation assay- Cells were plated in a 96 well plate at a density of 1.2 x 10^5 cells per well in triplicate. 24 hr later cells were treated with 10 nM ActD for 16 hr, or left untreated. Cells were subsequently pulsed with 30 μM bromodeoxyuridine (BrdU, Sigma-Aldrich) for 1 hr, then fixed with 3% PFA in PBS, permeabilized with 0.1% Triton X-100 in PBS, and RNA denatured with 50 mM NaOH for 20 sec. BrdU incorporation was measured by immunofluorescence using an anti-BrdU monoclonal antibody (GE Healthcare Biosciences) and the nuclei were stained with Hoechst. Image acquisition and analysis was performed using an ImageXpress Micro automated high-content screening fluorescence microscope (Molecular Devices) at the Trieste ICGEB High-Throughput Screening Facility. An average of 4,5 x 10^3 cells for each point was scored for BrdU incorporation in at least three independent experiments.

RESULTS

p53-independent effect of MageB2 on cell proliferation- We started studying the requirement of p53 on cell proliferation induced by MageB2 expression, since p53

function has been previously associated to human MageA2 (10) and murine Mageb (20) in apoptosis resistance. To this aim, we knocked-down (KD) MageB2 expression in human colorectal cancer cell lines HCT116, both in their wt-p53 and p53 knockout versions. By following cell proliferation during 7 days, we observed that MageB2 KD similarly affects the proliferation rate in both cell lines (Fig. 1A and 1B). Also, MageB2 KD reduced the number of colonies in both HCT116 cell lines independently of p53 status (Fig. 1C). Consistently, MageB2 KD also correlated with reduced tumor cell proliferation in U2OS cells (wt-p53) (Fig. 1D and 1E). Efficiency of siRNA-mediated KD of MageB2 expression is shown in Fig. 1F. In addition, we observed that opposite to MageA2 (10), MageB2 failed to form protein complex with p53 even if it was overexpressed (Fig. 1G). All these data confirm that MageB2 of human origin regulates tumor cell proliferation and demonstrate that its effect is independent of p53 status.

To corroborate the potential role of human MageB2 in stimulating tumor growth, we generated B16(F0) mouse melanoma cells lines stably expressing GFP or GFP-MageB2 (B16-GFP and B16-GFP-B2). Tumors were generated from B16-GFP and B16-GFP-B2 cells through subcutaneous injection in C57BL/6 mice. Tumor growth was regularly examined until the mass was measurable; from then on we started to follow each mouse independently. As expected, B16-GFP-B2 tumors showed an increased growth rate when compared to B16-GFP tumors (Fig. 2A and 2B), indicating that the growth promoting activity of human MageB2 could be also observed *in vivo*. After animal sacrifice GFP and GFP-MageB2 expression was confirmed in random tumor samples (Fig. 2C).

Human MageB2 Expression stimulates E2F activity- To elucidate the mechanisms by which human MageB2 regulates cell proliferation independently of p53 status, we investigated the E2F pathway, since it has been reported that MAGE-II proteins can target and inhibit E2Fs function (27,28). To this aim, we silenced MageB2 gene expression in U2OS cells by siRNA. We quantified known cell cycle-associated E2F-target genes by RT-qPCR and detected a consistently significant downregulation associated to MageB2 knockdown (Fig. 3A). ChIP-qPCR assay under this conditions indicated that endogenous MageB2 does not affect E2F1 association to chromatin as assessed on E2F1 promoter, a well established E2F1 target (data not shown).

In order to verify the specific effect on E2F transcriptional activity, we analyzed whether MageB2 could regulate E2F activity by reporter gene assay using the 6xE2F-Luc construct. We tested the effect of MageB2 on E2F1, 2 and 3. As shown in Fig. 3B, MageB2 induced all E2Fs tested, suggesting that the effect observed in the RT-qPCR approach could be the result of the regulation of different E2Fs members.

As mentioned above, the highly conserved murine Mageb1, Mageb2 and Mageb3 proteins as well as murine Mageb18 have been associated to cell proliferation. To elucidate whether these related mouse Mage-B proteins could similarly regulate E2F activity, we compared their effect on E2F1 transcriptional activity. Interestingly, only human MageB2 expression up-regulated E2F1 transcriptional activity, while murine proteins did not cause a significantly detectable effect (Fig. 3C). Differently to that reported in mouse cells (19), human MageB2 KD did not result in massive cell death, but in cell growth with limited rate respect to control and independently of p53 status (Fig. 1A, 1B and 1D). These results suggest that even if murine and human MAGE-B gene expression is associated with tumor cell proliferation, they probably make use of different molecular strategies to exert their functions.

MAGE-A proteins have been shown to interact with histone deacetylases (HDACs) (5,10), enzymes known to regulate gene transcription. Regulation of E2F transcriptional activity through HDACs has been extensively studied. Different E2F regulators such as pRb (29) and its family of pocket proteins, Sin3B (30), Kap1 (31), Ebp1 (32) and ELL (33) inhibit E2F activity by recruiting HDACs.

Our next step was to elucidate whether MageB2 could exert its function by targeting HDACs or E2F1 itself. To this aim, we produced GST-MageB2 fusion protein in bacteria and immunopurified HA-tagged HDAC1 and E2F1 from HEK293T cell extracts. Tagged proteins were immunoadsorbed using the anti-HA antibody and protein A sepharose beads. Potential coadsorbed endogenous proteins were dissociated by supplementing the buffer with 500mM NaCl (34). HA-tagged immunopurified proteins were then eluted from the HA-antibody/protein-A sepharose beads using commercial HA peptide. Next, *in vitro* binding assay was performed by incubating immunopurified proteins with GST or GST-MageB2.

Results from *in vitro* binding assay indicate that in these conditions MageB2 strongly interacts with HDAC1 but no interaction is detected between MageB2 and E2F1 (Fig. 4A).

We then performed an assay to verify the MageB2/HDAC1 association in cells. In addition, we compared this association to that of other potential targets of the E2F1 regulating complex, such as the pocket proteins pRb and p107. Immunoprecipitation (IP) assay performed at the same time and conditions confirmed HDAC1 as a strong MageB2 interactor, while pRb or p107 showed low or undetectable interaction with MageB2 (Fig. 4B). Additional IP assay showed that MageB2 can also interact with HDAC3 but not with HDAC4 and HDAC2 (data not shown).

Given that HDAC1 forms part of the E2F1 protein complex and represses E2F1 activity, we assessed the effect of MageB2 in such complex formation.

The results shown in Fig. 4C indicate that in the absence of MageB2 expression, E2F1 and HDAC1 form part of a protein complex. However, MageB2 expression strongly reduced the amount of HDAC1 associated to the E2F1 complex.

The results were confirmed by reverse IP thereby assessing the amount of E2F1 associated to HDAC1 when MageB2 was expressed. Accordingly, immunoprecipitated HDAC1 recruited lower amounts of E2F1 in the presence of MageB2 (Fig. 4D). Of note, MageB2 coimmunoprecipitates with HDAC1 (Fig. 4D, line 3) but is not detectable when E2F1 is immunoprecipitated (Fig. 4C, line 4), thus reinforcing the hypothesis that MageB2 interacts with HDAC1. When the effect of MageB2 expression was tested on the unrelated Androgen Receptor (AR) /HDAC1 complex, no evidence of reduced AR recruitment to HDAC1 (Fig. 4E) was observed, suggesting that MageB2 could preferentially target the E2F/HDAC association. These data suggest that MageB2 can associate to HDACs thus releasing E2F from such interaction and enhancing its free and transcriptionally active fraction.

We reasoned that if MageB2 enhances E2F1 activity by reducing the amount of HDAC1 from the E2F1 protein complex, enhanced expression of HDAC1 should reverse MageB2 effect on E2F1 activity. Then, we assessed the effect of MageB2 on E2F1 activity when high amounts of HDACs were available through ectopic expression. Increased HDAC1 availability clearly reversed the effect of MageB2 on E2F1 activity but not in the absence of MageB2 (Fig. 5A). Since HDACs overexpression could repress general transcription, we further control the specificity of this experiment by transfecting HDAC4, a class II histone deacetylase that does not interact with

MageB2. Results shown in Fig. 5B indicate that HDAC4 does not affect the regulation of E2F1 by MageB2 expression (Fig. 5B).

Finally, to further highlight the relevance of HDAC in our mechanism, we assessed the effect of MageB2 on E2F1 activity when HDACs were inhibited by Trichostatin A (TSA). As expected, inhibition of HDAC activity by TSA enhanced E2F1 activity; however, under this condition E2F1 activity was no longer significantly induced by MageB2 expression (Fig. 5C).

All these data suggest that the mechanism by which MageB2 regulates E2F1 activity could involve the direct interaction between MageB2 and HDAC1. Then, MageB2/HDAC1 association prevents efficient recruiting of HDAC1 to E2F1 complex, thus resulting in enhanced E2F1 activity.

MageB2 cellular localization- MageB2 is a 319 amino acid long protein with a MHD sharing approximately 48% homology with that of MAGE-A proteins (MHD, amino acid 110-290). Previously, it has been reported that MAGE-A proteins can be found in different cell compartments including nucleus (10), cytoplasm (35) and PML-Nuclear Bodies (12). We observed here that endogenous MageB2, in addition to nuclear and cytoplasm fractions, is also present in the nucleolar fraction (Fig. 6A). Accordingly, GFP-tagged MageB2 is observed in the cytoplasm, nucleus and nucleoli, where it colocalizes with nucleolin staining (Fig. 6B left panel). MAGE-I´s MHD domain is conserved throughout all the family members, whereas the N-terminal domain is less conserved and usually unstructured. Studies performed with MageB2 deletion mutants indicate that the region comprised between amino acid 1 and 109 is sufficient to relocalize the GFP protein to the nucleolus (Fig. 6B middle panel). Moreover, by using bioinformatic tools, a 29 amino acid long sequence rich in arginine and lysine was detected as a potential nucleolar localization signal (NoLS) at the MageB2 N-terminal region. To assess its function, we generated the ΔNoLS-MageB2 construct carrying the MageB2 gene with deleted NoLS sequence. We observed that ΔNoLS-MageB2 protein clearly delocalizes from the nucleoli (Fig. 6B right). These results indicate that MageB2 can be driven to the nucleoli depending on a canonical NoLS sequence located at the N-terminus, thus supporting the concept that even if MAGE-I proteins share the conserved MHD, their localization/function is also dependent on short motifs present outside from this domain.

MageB2 Expression and ribotoxic stress- Ribotoxic stress triggers cell-cycle arrest through the regulation of master signaling pathways such as those involving E2F, p53 and c-myc proteins. It has been extensively shown that drugs triggering ribotoxic stress relocalize specific Ribosomal Proteins (RPs) from the nucleoli to the nucleoplasm as a feedback loop to limit high translational demand and consequently cell proliferation. In the nucleus, ribosomal proteins have been found to reduce c-myc activity (36) and target Mdm2, hence enhancing p53 activity (37) and reducing E2F activity (38).

We observed that ribosomal stress as induced by low Actinomycin D (Act-D) concentrations (5-10 nM) does not significantly affect MageB2 protein levels as compared to the established effect on c-myc (Fig. 7A). However, Act-D clearly induces a striking and early exit of MageB2 from nucleoli (Fig. 7B). This behavior is not associated with a general stress response since MageB2 does not change its nucleolar localization after treatments with other types of stressors such as ultra violet radiation (DNA-damage), high temperature (heat-shock) or thapsigargin (Endoplasmic Reticulum stress) (data not shown).

We then evaluated whether MageB2 can enhance E2F activity in the presence of RPs known to inhibit E2F activity. We observed that MageB2 can still enhance E2F1 activity in the presence of RPL11 and RPL23 (Fig. 7C). However, the effect of MageB2 seems to be independent of Mdm2 expression since it is still observed in double knock out (DKO) cells (MEF p53 -/-; mdm2 -/-), where RPL11 and RPL23 failed to regulate E2F activity, as expected (Fig. 7D). These data suggest that upon nucleolar stress, MageB2 expression could counteract the negative regulation of RPs on E2F, thus avoiding part of their cell cycle repressive function.

Act-D treatment promotes a prevalent nucleoplasmic localization of MageB2, but does not significantly affect MageB2 function on E2F1 activity (Fig. 8A) or MageB2 interaction with endogenous HDAC1 (Fig. 8B), suggesting that nucleoplamic MageB2 should still be able to enhance E2F activity. To address the relevance of MageB2 localization on E2F activity, we expressed ΔNoLS-MageB2 missing the nucleolar localization. We observed that ΔNoLS-MageB2 is able to interact with endogenous HDAC1 as the wild-type protein (Fig. 8C) and displays E2F1 activating function similar to wild-type MageB2 (Fig. 8D). These data suggest that nuclear MageB2 is also able to regulate E2F activity.

Our data indicating that under ribotoxic stress conditions MageB2 is mainly localized to the nucleoplasm maintaining E2F activity, prompted us to study whether endogenous MageB2 could enhance cell proliferation under the reported stress condition. To test this relevant biological effect, we assessed BrdU incorporation in U2OS cells with KD endogenous MageB2 in the presence or absence of 10nM Act-D. The analysis was performed by using an automated high-content screening fluorescence microscope. Importantly, MageB2 KD enhanced ribotoxic stress-induced cell cycle arrest as shown in the panel of images in Fig. 8E, left panel. An average of 4.5 x 10^3 cells was automatically analyzed to quantify the percentage of BrdU positive cells in each condition (Fig. 8E, right panel). Obtained data from this experiment indicates that endogenous MageB2 expression plays a role in enhancing cell cycle progression under ribotoxic stress.

DISCUSSION

MAGE-I proteins are expressed in a variety of human tumors and even though they are considered promising antigens to produce anti-tumor vaccines, their expression commonly correlates with poor prognosis in untreated patients. Due to their high sequence homology, it was considered a functionally redundant protein family. However, recently obtained data suggest that specific MAGE-I members (or at least a discrete subset of them) could interfere with normal cell functions by affecting distinct signaling pathways (13).

Mouse mage-b1, -b2, -b3 gene expression has been associated to anti-apoptotic/pro-survival balance (19,20). In addition, mouse Mageb18 protein expression has been reported to have a similar phenotype to that of mouse Mage-b (21). The correlation between human MageB2 expression and cell proliferation has been suggested in normal oral keratinocytes (23). Here we extended the study to human cancer cell lines such as osteosarcoma (U2OS) and colon carcinoma (HCT116) bearing wt or deleted p53 loci. In addition, we assessed the proliferative potential of human MageB2 expression *in vivo* in B16 (F0) melanoma cells. All the reported results are consistent with a significant role of human MageB2 in enhancing cell proliferation independently of p53 status, cell type, or species, thus suggesting its involvement in targeting a general mechanism of cell proliferation.

The first MAGE protein reported to regulate cell proliferation through E2F transcription factors was Necdin, a type-II MAGE protein (27) able to associate and repress E2F1

transcriptional activity. Later, it was reported that another type-II MAGE protein MageG1 (necdin-like 2) also inhibited E2F1 transcription factor through direct association (28). More recently, it has been reported that a type-I MAGE (MageA11) interacts with the pocket protein p107 and enhances E2F1 activity (39) as part of its pro-oncogenic activity in prostate cancer.

As mentioned above, E2F activity is highly sensitive to HDAC activity since a number of proteins inhibit its activity through HDAC recruitment, including pRb (29), Sin3B (30), Kap1 (31), Ebp1 (32) and ELL (33). Our and other groups have demonstrated that MageA1 and MageA2 associate to HDACs to exert their function (5,10). Based on the above mentioned data we investigated whether MageB2 could regulate E2F activity as part of its mechanism responsible for enhanced cell proliferation. We report here that MageB2 interacts with HDAC1 weakening its recruitment to E2F1 protein complex, thus increasing the fraction of free and active E2F. Similar mechanisms have been previously reported for LEF1 (40) and HMGA2 oncoproteins both increasing E2F activity (41). Moreover, potent oncogenes such as Ha-ras, c-myc and E2Fs itself enhance E2Fs mRNA and protein levels (42,43), confirming its key role in cell proliferation.

However, under specific conditions, E2F1 has been involved in triggering cell death by inducing transcription of pro-apoptotic genes (44,45). JAB1 could be a critical protein in this dichotomous role of E2F. It has been reported that JAB1 binds E2F, is found associated to E2F in promoters of apoptotic targets (and not in DNA-replication E2F targets) and is required for E2F-induced apoptosis (46,47). Moreover, hyperactivation of PI3K/AKT pathway specifically abolishes E2F-induced apoptosis (48) in part, by interfering with E2F-JAB1 complex formation (47). Even if E2F expression can promote apoptosis in DNA- damaged cells (49), a number of clinical assays suggest that enhanced E2F activity is associated with invasion (50), metastasis formation (51) and clinically with poor survival (52). Along these lines, induction of E2F activity by MageB2 in a tumor-specific context could be relevant for the final clinical outcome. Other proteins such as EAPP, known to enhance E2F activity are also overexpressed in tumor cells (53,54).

We observed that MageB2 is localized in the nucleus, cytoplasm and nucleoli. This unusual localization for a MAGE protein is quickly lost upon ribotoxic stress as induced by low-concentration Act-D treatment. Contrary to RPs that relocalize from the nucleoli to the nucleus under ribotoxic stress to collaborate with cell cycle arrest, endogenous MageB2 exits the nucleolus but promotes cell cycle progression. Of note, MageB2 is still associated to HDAC1 and enhances E2F activity in Act-D treated cells. Importantly, MageB2 can counteract the repressive effect of RPs on E2F activity. It has been reported that Mdm2 plays a central role in this nucleolar checkpoint since it is targeted by RPs, thus downregulating E2F activity and activating p53. However, MageB2 can regulate E2F activity in the absence of Mdm2 while RPs require its expression, demonstrating that MageB2 and RPs oppositely regulate E2F activity through different molecular mechanisms.

Taking into account all the data presented here, we suggest that human MageB2 behaves as a cell proliferation promoting protein, since it expression enhances the proliferative rate in a variety of cell lines and as well as in a melanoma mouse model. More specifically, we demonstrate that different to MageA2, MageB2 function does not depend on p53 expression. However, MageA2 and MageB2 share the ability to associate to HDACs thus exerting specific regulatory activity on different transcription factors. In this study we observed that MageB2/HDAC1 complex formation enhances E2F activity. In addition, we report for the first time a MAGE protein involved in nucleolar events, a function that has not been reported for any

MAGE-I member. Interestingly, mouse proteins such as Mageb2 or Mageb18 that have been reported to display tumor cell survival properties, are not involved in E2F regulation. The functional homology between human and mouse MAGE proteins should be carefully reconsidered, also in the light of the overall differences in basal metabolic rate between human and mouse (55) possibly linked to the rewiring of both p53 and E2F regulatory networking.

The striking difference between MAGE-II (ubiquitous expression) and MAGE-I (tumor-specific expression) could be the base to propose a novel hypothesis suggesting that enhanced expression of MAGE-I members during tumor cell transformation could play a role in counteracting MAGE-II function. As shown here, MageB2 plays an opposite role to that reported for Necdin and MageG1 on E2Fs activity. Accordingly and with respect to p53, MageA2 represses p53 activity (10,11) while MageD1/NRAGE activates p53 to stop cell proliferation (56). Hence, this hypothesis could promote future experiments to shed light on the basic networking of MAGE gene expression in cancer cells.

Growing body of evidence suggests that different MAGE proteins play distinct roles in cancer cells. Therefore, it should be critical to precisely determine the identity of the different MAGE genes expressed in the different tumor biopsies.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

M.Monte and CS conceived and coordinated the study and M.Monte wrote the paper. LYP and MFL designed, performed and analyzed the experiments shown in Figures 1 to 6. MFT and JEL designed, performed and analyzed the experiments shown in Figure 7. LYP and M.Mano designed, performed and analyzed the experiments shown in Figure 8. All authors reviewed the results and approved the final version of the manuscript.

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ABBREVIATIONS

The abbreviations used are: HDAC, histone deacetylase; NoLS, nucleolar localization signal; BrdU, 5-bromo-2'-deoxyuridine; KD, knock down; TSA, Trichostatin A; RT-PCR retrotranscription followed by polymerase chain reaction; qPCR, quantitative polymerase chain reaction; GFP, green fluorescent protein; IP, immunoprecipitation; AR, androgen receptor; MHD, MAGE Homology Domain; Act-D, actinomycin D; DKO, double knock out; S.D., standard deviation.

FIGURE LEGENDS

FIGURE 1. p53-independent effect of MageB2 expression on tumor cell proliferation. **A**: Determination of cell proliferation in HCT116 cells (HCT116 WT) during 7 days after siRNA transfection for MageB2 KD (siB2) or a control siRNA (siC). **B**: The same protocol of (A) but using HCT116 p53-/- (HCT116 KO). **C**: Quantification of colony formation assay in both HCT116 WT and KO after siRNA transfection for MageB2 KD (siB2) or a control siRNA (siC). * $p < 0.001$. **D**: Determination of cell proliferation in U2OS cells during 7 days after siRNA transfection for MageB2 KD (siB2) or a control siRNA (siC). **E**: Quantification of colony formation assay in U2OS cells after siRNA transfection for MageB2 KD (siB2) or a control siRNA (siC). * $p < 0.001$. **F**: Western blot analysis of siRNA-mediated KD efficiency in HCT116 WT, KO and U2OS cells 48 hours after siRNA transfection for MageB2 (siB2) or a control siRNA (siC). **G**: Immunoprecipitation of HA-tagged MageA2 and MageB2 coexpressed with p53 in H1299, p53 null cells. After transfection, immunoprecipitated material was analyzed by western blot using anti-HA and anti-p53 antibody. Error bars indicate S.D.; a Student's t-test was used for statistical analysis.

FIGURE 2. Overexpression of human MageB2 enhances tumor growth. **A**: Determination of *in vivo* tumor growth after subcutaneous inoculation of melanoma B16(F0) cells stably expressing GFP (GFP) or GFP-tagged human MageB2 (GFP-MageB2). Once the tumors were palpable (P), they were regularly measured (M) each 2 days for 8 days. Each group consisted on 5 C57/B mice. The experiment is representative of 3. **B**: The picture shows a representative mouse belonging to the melanoma B16(F0) cells stably expressing GFP (B16-GFP) or GFP-tagged human MageB2 (B16-GFP-B2). Arrows indicate the two measurements taken from mice to determine tumor volume in (A). **C**: Western blot of B16-F0 tumor extracts expressing GFP (GFP) or GFP-tagged human MageB2 (GFP-B2). To analyze protein expression after tumor growth, a polyclonal anti-GFP antibody was used. Error bars indicate S.D.

FIGURE 3. Human MageB2 induces E2F activity. **A**: Determination of E2F-target gene expression through RT-qPCR in U2OS cells with normal (siC) or reduced (siB2) MageB2 expression. mRNA levels in siB2 condition have been normalized to siC condition. Each individual mRNA value from the E2F-targets was normalized to actin mRNA value. **B**: Reporter gene assay for E2F activity using 6xE2F-LUC reporter construct and the indicated HA-tagged E2F protein. * $p < 0,05$; ** $p < 0,001$. The assay was performed in U2OS cells. Western blot of expressed proteins is shown below the histogram. # unspecific band. **C:** Reporter gene assay for E2F activity using 6xE2F-LUC reporter construct and GFP-tagged MageB2 from human (MageB2) or mouse (Mageb2) origin and mouse Mageb18. EV: GFP empty vector. Hn: Human, Mo: Mouse. * $p < 0,05$. The assay was performed in U2OS cells. Western blot of expressed proteins is shown below the histogram. # unspecific band. Error bars indicate S.D.; Student's t-test was used for statistical analysis.

FIGURE 4. MageB2 interacts with HDAC1 and interferes with E2F1/HDAC1 complex formation. **A**: In vitro binding assay between HA-tagged proteins produced and purified from HEK293T cells and GST or GST-MageB2 produced and purified from bacteria. **B:** Immunoprecipitation of HA-tagged HDAC1, pRb and p107 in HEK293T cells cotransfected with myc-MageB2. Immunoprecipitated protein complexes obtained with the anti-HA antibody (IP αHA) was assessed by western blot (WB) and probed with the indicated antibodies. **C**: Immunoprecipitation of E2F1 in HEK293T cells cotransfected with Flag-HDAC1 in the presence or not of Myc-MageB2. Immunoprecipitated material obtained when using the anti-E2F1 antibody (IP αE2F1) was assessed

by western blot (WB) and probed with the indicated antibodies. The left panel shows the Total lysates (TL). **D**: Immunoprecipitation of Flag-HDAC1 in HEK293T cells cotransfected with HA-E2F1 and Myc-MageB2. Immunoprecipitated material (IP αFlag) was assessed by western blot (WB) and probed with the indicated antibodies. The left panel shows the Total lysates (TL). **E**: Immunoprecipitation of Flag-HDAC1 in U2OS cells cotransfected with the Androgen Receptor vector (AR) and Myc-MageB2. Immunoprecipitated material (IP αFlag) was assessed by western blot (WB) and probed with the indicated antibodies. The left panel shows the Total lysates (TL). # IgG Heavy chains.

FIGURE 5. HDAC-dependent effect of MageB2 on E2F1 activity. **A**: Reporter gene assay for E2F1 activity using 6xE2F-LUC reporter construct. The effect of MageB2 was assessed with increasing amounts of the Flag-HDAC1. * $p < 0,05$. The assay was performed in U2OS cells. Western blot of expressed proteins is shown below the histogram. EV: empty vector. **B**: Reporter gene assay for E2F1 activity using 6xE2F-LUC reporter construct. The effect of MageB2 was assessed with increasing amounts of the vector expressing HA-HDAC4. * $p < 0,05$. The assay was performed in U2OS cells. Western blot of expressed proteins is shown below the histogram. EV: empty vector. **C:** Reporter gene assay for E2F activity using 6xE2F-LUC reporter construct. The effect of MageB2 was assessed in the presence (TSA) and absence (NT) of 300nM TSA during 24hs. Two different amounts of E2F1 were used as indicated by gray and black bars. * $p < 0,05$. The assay was performed in U2OS cells. Western blot of expressed proteins is shown below the histogram. EV: empty vector. Error bars indicate S.D.; Student's t-test was used for statistical analysis. # unspecific band.

FIGURE 6. Cellular localization of MageB2. **A**: Western blot assay of cytoplasmic (Cytop), Nuclear and Nucleolar fractions obtained from HCT116 cells. Nucleolin (NCL), Tubulin (Tub) and c-myc antibodies were used as markers of nucleolar, cytoplasm and nuclear fractions respectively. **B**: Confocal microscopy images of U2OS cells transfected with GFP-tagged MageB2 constructs (upper panels) containing: wild-type MageB2 (GFP-MageB2 WT), amino acids from 1 to 109 of MageB2 (GFP-MageB2 1-109) and MageB2 with deleted Nucleolar Localization Signal sequence (GFP-MageB2 ΔNoLS). Bottom panels show endogenous nucleolin (NCL) staining as a nucleolar marker.

FIGURE 7. MageB2 expression counteracts ribosomal-protein driven inhibition of E2F1. **A**: Western blot of a time course assay after 5 nM Actinomycin D addition to U2OS cells. Membrane was probed with c-myc, MageB2 and Vinculin (loading control) antibodies. **B**: Confocal microscopy images of HA-MageB2 and Myc-L11 constructs, before (upper panels, untreated) and after (bottom panels, ActD 5nM) 3 hours of Actimomycin D treatment to U2OS cells. Right panel: histogram indicating the percentage of cells displaying MageB2 with nuclear or nucleolar localization before and after the treatment with 5nM ActD. A total of 200 cells were counted in each experimental condition. **C**: Reporter gene assay for E2F activity using 6xE2F-LUC reporter construct. The effect of MageB2 was assessed when cotransfected with Myc-L11 and Flag-L23 ribosomal proteins constructs. *** $p < 0,05$; ** $p < 0,01$; * $p < 0,005$. The assay was performed in U2OS cells. Western blot of expressed proteins is shown below the histogram. EV: empty vector. # unspecific band. **D**: Reporter gene assay for E2F activity using 6xE2F-LUC reporter construct. The effect of MageB2 was assessed when cotransfected with Myc-L11 and Flag-L23 ribosomal proteins

constructs. ** p < 0,05; * p < 0,005. The assay was performed in double knock out (p53-/-; Mdm2-/-) mouse embryo fibroblasts (DKO cells). Western blot of expressed proteins is shown below the histogram. EV: empty vector. # unspecific band. Error bars indicate S.D.; a Student's t-test was used for statistical analysis.

FIGURE 8. MageB2 induces E2F1 activity under ribotoxic condition. **A**: Reporter gene assay for E2F activity using 6xE2F-LUC reporter construct. The effect of MageB2 was assessed in U2OS cells treated or not with 10nM Actinomycin D (ActD) for 24hs. * p < 0,005. **B:** Immunoprecipitation of HA-MageB2 in U2OS cells stably expressing inducible HA-MageB2. Complex formation with endogenous HDAC1 was assessed in the presence (+) or absence (-) of 10nM Actinomycin D (ActD) both with induced (I) and not induced (NI) MageB2 expression. Immunoprecipitated material (IP αHA) was assessed by western blot (WB) and probed with the indicated antibodies. The left panel shows the Total lysates (TL). **C**: Immunoprecipitation of Flag-HDAC1 in HEK293T cells cotransfected with GFP-tagged MageB2 with deleted Nucleolar Localization Signal sequence (GFP-ΔNoLS-MageB2). Immunoprecipitated material (IP αFlag) was assessed by western blot (WB) and probed with the indicated antibodies. The left panel shows the Total lysates (TL). **D**: Reporter gene assay for E2F1 activity using 6xE2F-LUC reporter construct. The effect of GFP-tagged wild-type MageB2 (MageB2) and GFP-tagged MageB2 with deleted Nucleolar Localization Signal sequence (ΔNoLS-MageB2) on E2F1 transcriptional activity was performed in U2OS cells. *** p < 0,01; ** p < 0,05; * p < 0,005. Western blot of expressed proteins is shown below the histogram. EV: GFP empty vector. **E**: BrdU incorporation in U2OS cells previously transfected with siRNA for MageB2 (siB2) or a control siRNA (siC) and treated or not (NT) with 10nM Actinomycin D (ActD 10nM) for 16 hr. Panels show representative merged fluorescence microscope images of BrdU (green) and Hoechst (blue) staining for each experimental condition. Right panel: determination of cell proliferation as the percentage of BrdU with respect to Hoechst stained cells by an automated high-content screening ($4{,}5x10^3$ events/condition aprox). *** p < 0,05; ** p < 0,005; * p < 0,001. Error bars indicate S.D.; a Student's t-test was used for statistical analysis.

FIGURE 1

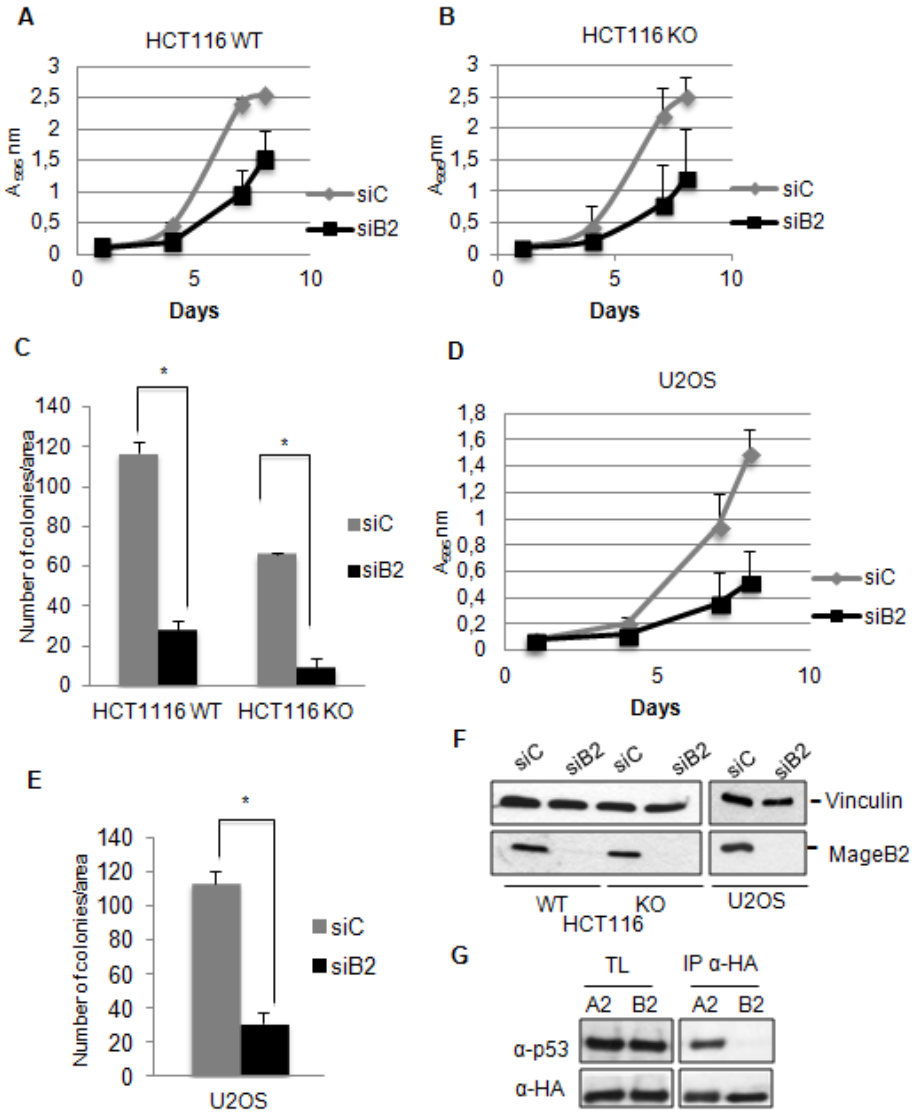


FIGURE 2

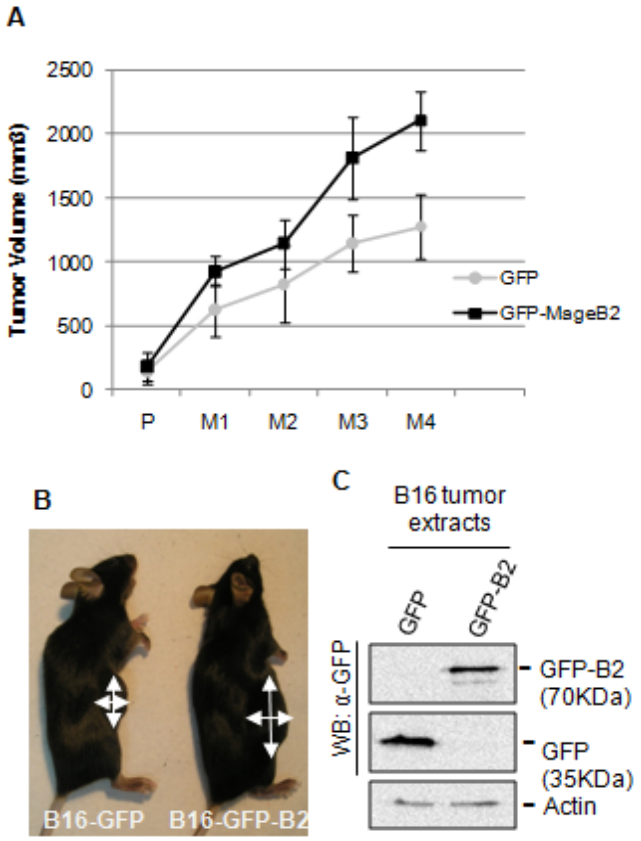


FIGURE 3

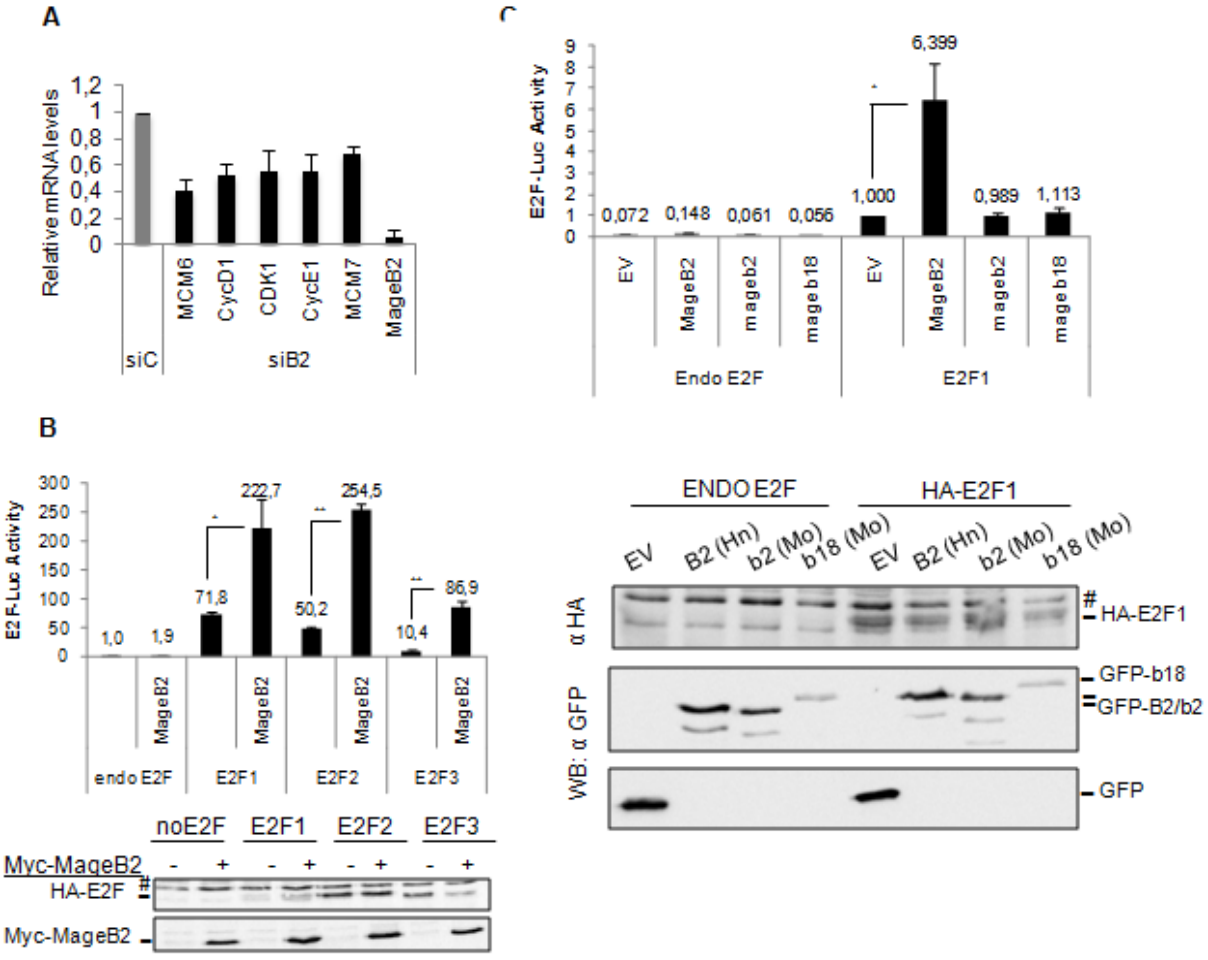


FIGURE 4

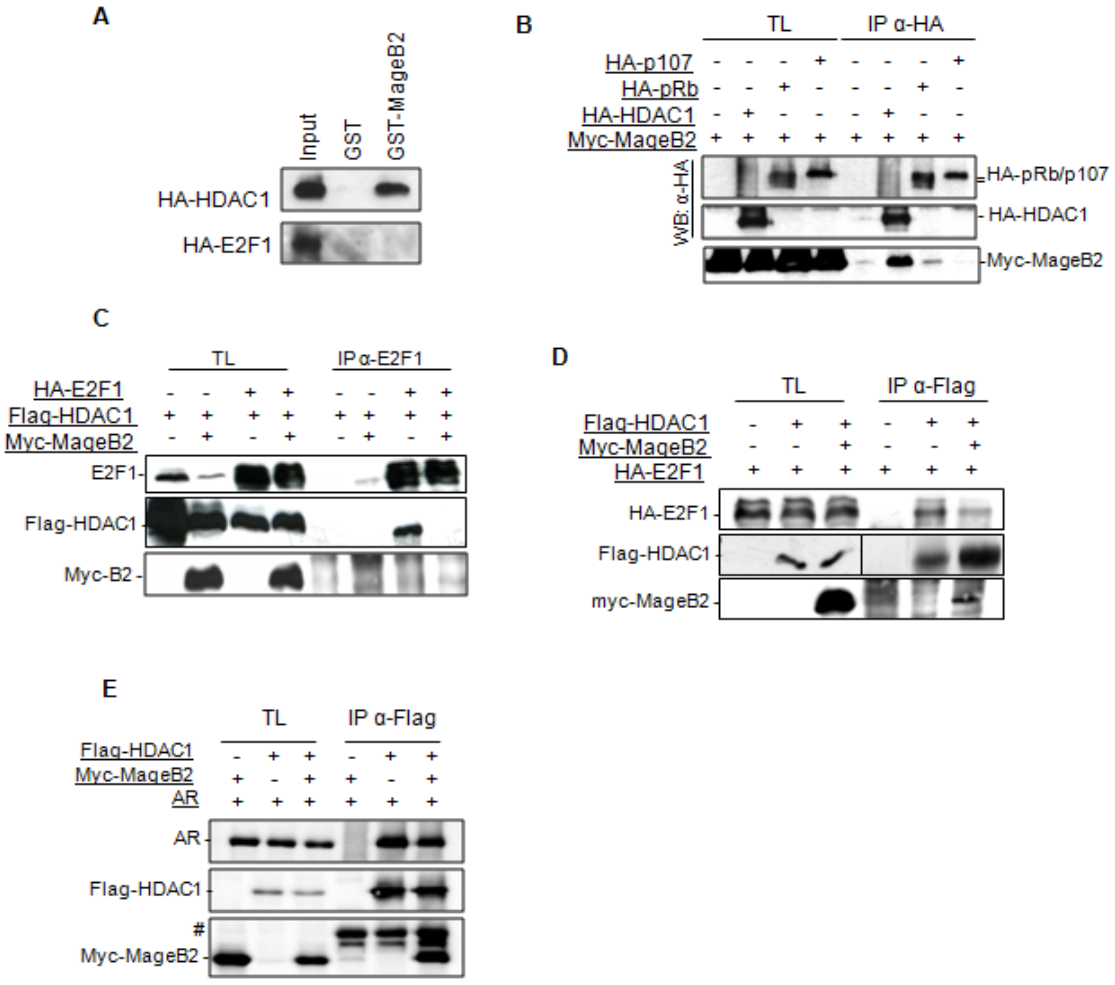


FIGURE 5

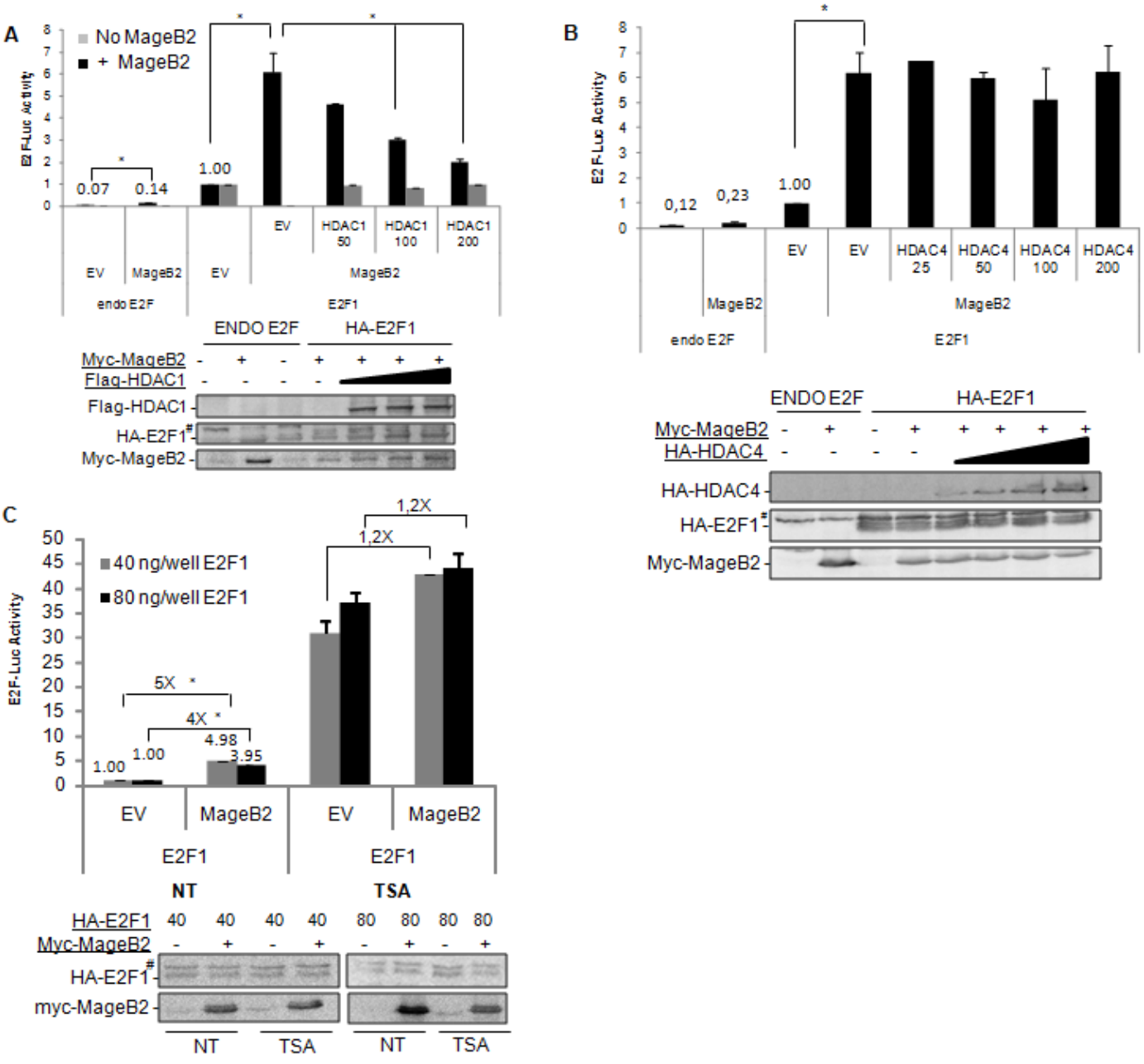


FIGURE 6

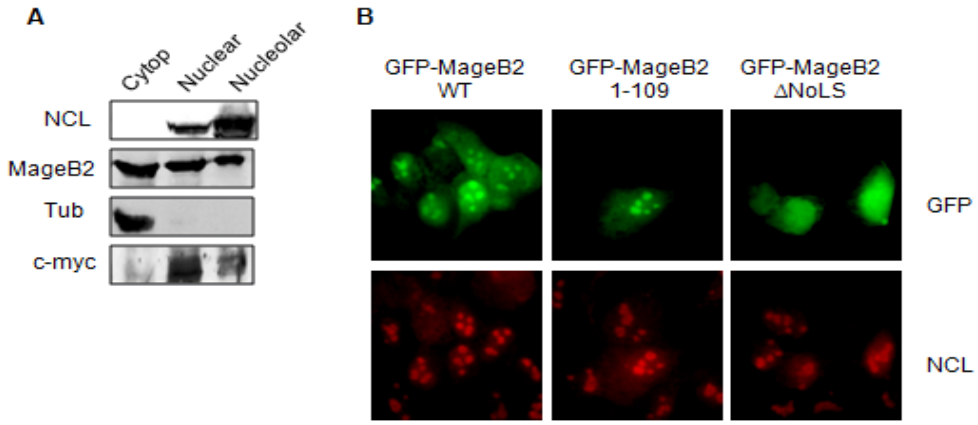


FIGURE 7

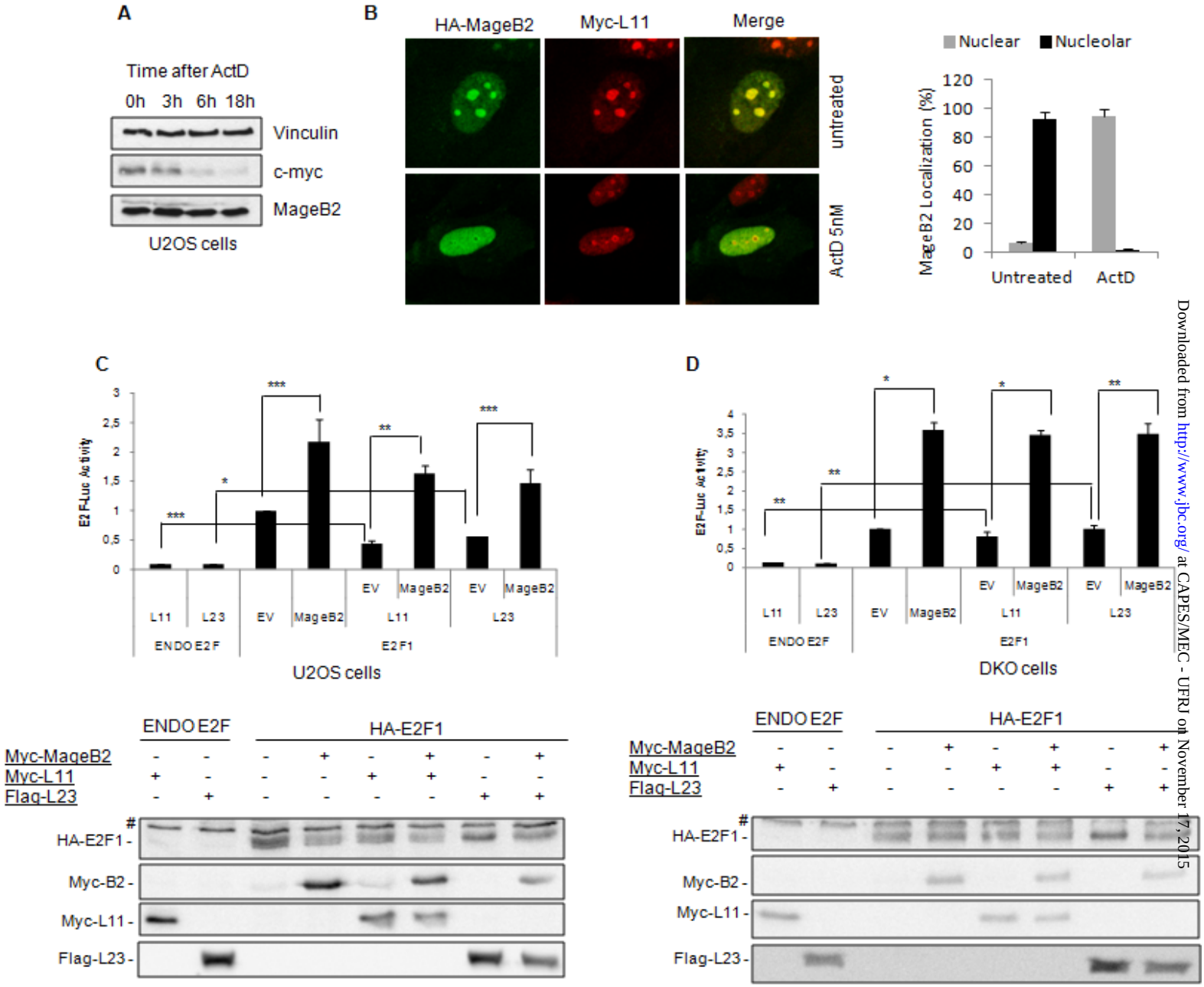
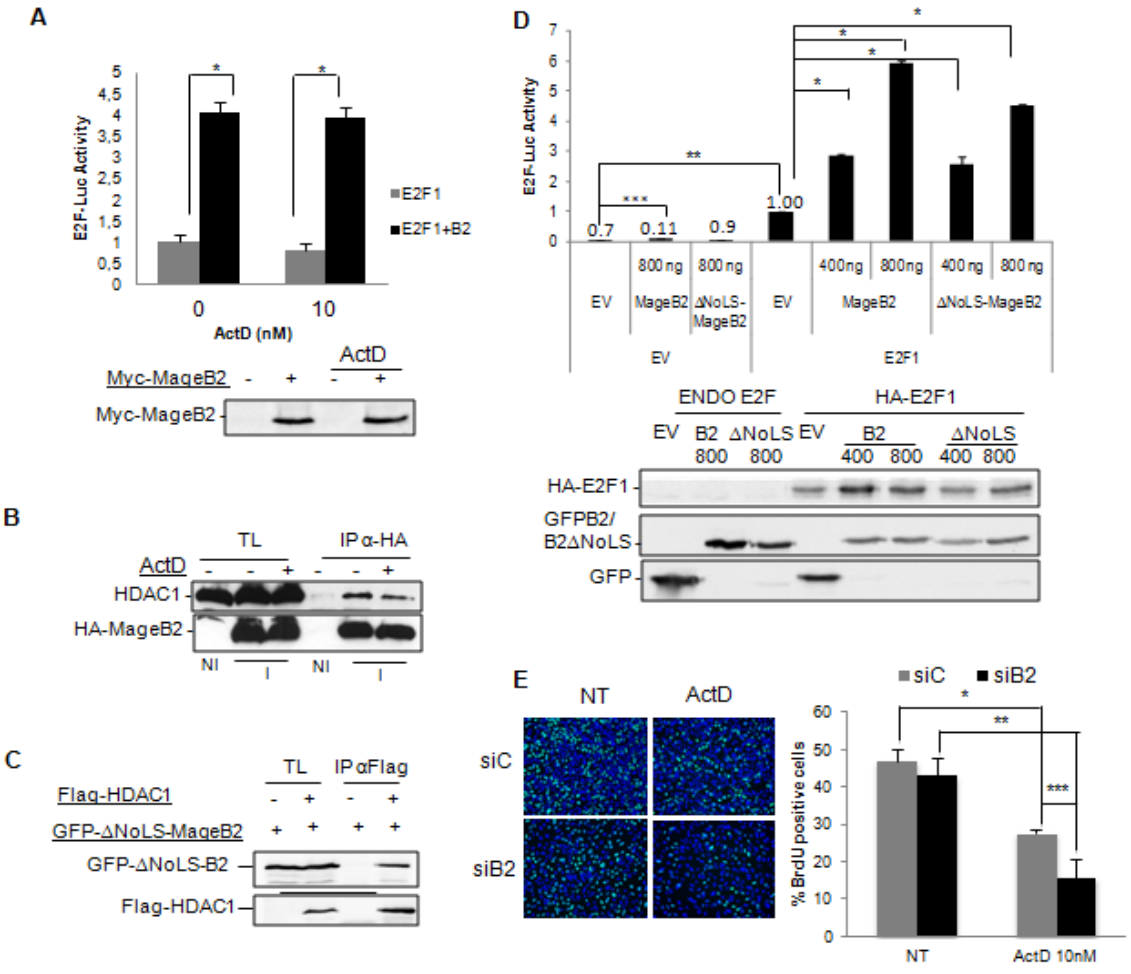


FIGURE 8



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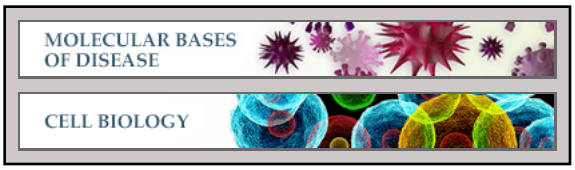

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