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Melanoma antigen-A11 regulates substrate-specificity of Skp2-mediated protein degradation

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Abstract

Melanoma antigen-A11 (MAGE-A11) is a proto-oncogene involved in androgen receptor signaling and androgen-dependent cell growth. In this report we provide evidence that MAGE-A11 interacts with Skp2 (S phase kinase-associated protein), the substrate recognition protein of the Skp1-Cullin1-F-box E3 ubiquitin ligase, and with Skp2 binding protein, cyclin A. A similar cyclin A binding motif in MAGE-A11 and Skp2 was consistent with a competitive relationship between MAGE-A11 and Skp2 in binding cyclin A. Skp2 inhibited MAGE-A11 interaction with cyclin A. Differential effects of MAGE-A11 on Skp2-mediated protein degradation were also revealed. MAGE-A11 increased Skp2-mediated degradation of cyclin A and retinoblastoma-related protein p130. In contrast, MAGE-A11 decreased Skp2-mediated degradation of E2F1 and Skp2 self-ubiquitination. Stabilization of E2F1 by MAGE-A11 was associated with sequestration and inactivation of Skp2 through the formation of an E2F1-MAGE-A11-Skp2 complex. We conclude that direct interactions of MAGE-A11 with Skp2 and cyclin A regulate the substrate-specificity of Skp2-mediated protein degradation.

Keywords

androgen receptor; melanoma antigen-A11; MAGE-A11; Skp2; cyclin A; E2F1

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1.0. Introduction

Melanoma antigen-A11 (MAGE-A11) is a cancer-testis antigen of the MAGE gene family expressed in primates on the X chromosome (Scanlan et al., 2002). MAGE-A11 functions as a steroid receptor coregulator and proto-oncogenic protein that increases transcriptional activity of the human androgen receptor (AR) and progesterone receptor-B of the steroid receptor family of transcriptional activators (Bai et al., 2005; Su et al., 2012). Lentivirus short hairpin RNA (shRNA) knockdown showed that MAGE-A11 is required for prostate cancer cell growth (Minges et al., 2013). MAGE-A11 contributes to cell cycle progression through interactions with retinoblastoma-related protein p107 and the E2F1 transcription factor important for cell cycle progression and induction of apoptosis (Su et al., 2013; Attwooll et al., 2004).

The ability of MAGE-A11 to stabilize E2F1 (Su et al., 2013) suggested an inhibitory role in S phase kinase-associated protein (Skp2)-mediated protein degradation. Skp2 is the proto-oncogenic F-box domain substrate recognition protein of the Skp1-Cullin1-F-box (SCF) E3 ubiquitin ligase that is frequently overexpressed in cancer and has been linked to prostate cancer progression and metastasis (Bai et al., 1996; Wang et al., 2012a). Skp2 is low during early G1 phase of the cell cycle and increases late in G1 to mediate ubiquitination and proteasomal degradation of substrates (Bassermann et al., 2013). One function of Skp2 is binding cyclin A, a regulatory protein that activates cyclin-dependent kinases (cdk) during the cell cycle (Zhang et al., 1995; Ji et al., 2007; Malumbres et al., 2009). However, regulation of E2F1 by Skp2 and cyclin A is not fully understood (Marti et al., 1999). E2F1 DNA binding and transcriptional activity are regulated by cyclin A-cdk2 (Krek et al., 1994, 1995) and direct interactions between cyclin A and E2F1 have been reported (Krek et al., 1994, 1995; Dynlacht et al., 1994; Xu et al., 1994), but Skp2 did not compete with E2F1 binding to cyclin A (Lu et al., 2014).

In this report we provide evidence that MAGE-A11 is engaged in direct interactions with Skp2 and cyclin A that regulate the target-specific activity of Skp2. MAGE-A11 enhanced Skp2-mediated degradation of cyclin A and retinoblastoma-related protein p130, but inhibited Skp2-mediated degradation of E2F1 by sequestering and inactivating Skp2 through the formation of an E2F1-MAGE-A11-Skp2 complex. A cyclin A binding motif with homology to the cyclin A binding site in Skp2 was identified in MAGE-A11.

2.0. Materials and methods

2.1. DNA vectors

Expression plasmids included pCMV-FLAG-MAGE-2-429, 112-429 and Δ221-249 (Bai et al., 2005; Bai and Wilson, 2008); pSG5-MAGE-2-429 and pSG5-HA-MAGE-2-429, 112-429, 112-307, 112-298, 112-276, 112-268, 112-252 and 112-240 (Bai et al., 2005; Askew et al., 2009, 2010; Su et al., 2012); GAL-MAGE-2-429 and 112-429 (Bai et al., 2005); pCMV-E2F1, pCMV-FLAG-E2F1, pCMV-p107 and pcDNA-p130 (Su et al., 2013; Minges et al., 2015) and FLAG-ubiquitin (Bai and Wilson, 2008). pcDNA3-HA-cyclin A2 and pcDNA3-myc-hSkp2 were provided by Yue Xiong, University of North Carolina at Chapel Hill (Ohta and Xiong, 2001; Andrews et al., 2006). pCMV-FLAG-MAGEΔ151-217

and Δ171-190 were created by PCR mutagenesis using fragments with EcoRI and XhoI or SalI ends inserted into EcoRI and SalI sites in pCMV-FLAG-MAGE. pCMV-FLAG-MAGEΔ314-323 was created by PCR amplification of pSG5-MAGEΔ314-323, digested with EcoRI and XhoI and ligated into EcoRI and SalI sites of pCMV-FLAG. pCMV-FLAG-cyclin A was constructed by PCR amplifying pcDNA3-HA-cyclin A2 with EcoRI and SalI ends and cloned into the same sites of pCMV-Flagb. pVP16-cyclin A was prepared by digesting pCMV-FLAG-cyclin A2 using EcoRI and SalI and the fragment cloned into pVP16 (Clontech Laboratories, Inc.) digested with the same enzymes. pCMV-FLAG-Skp2 was created by PCR amplifying pcDNA3-myc-hSkp2 and the fragment with EcoRI and XhoI ends cloned into EcoRI and SalI sites of pCMV-FLAG. Amplified regions were verified by DNA sequencing.

2.2. Cell culture, transcription assays and immunochemistry

HeLa and HeLa-AR1C-PSA-Luc-A6 human cervical cancer cells were cultured in minimal essential medium containing 10% fetal bovine serum (FBS). Medium for HeLa cells stably expressing AR also contained 0.5 mg/ml geneticin (G418, Gibco) and 0.1 mg/ml hygromycin B (Roche) (Kretzer et al., 2010). LNCaP cells were maintained in RPMI-1640 medium with 10% FBS. CWR-R1 cells were grown in Dulbecco's modified Eagle medium with additives (Karpf et al., 2009). LAPC-4 cells were grown in RPMI-1640 medium with 10% FBS and 1 nM methyltrienolone (R1881), a synthetic androgen agonist. CV1 and COS1 cells were grown in Dulbecco's modified Eagle medium containing 10% bovine calf serum and 20 mM Hepes, pH 7.2. Cell media contained 2 mM L-glutamine (Gibco, Life Technologies), penicillin and streptomycin. EGF was added to the media to promote cell growth and increase phosphorylation of MAGE-A11 (Gregory et al., 2001; Bai and Wilson, 2008).

Mammalian two-hybrid transcription assays were performed in HeLa cells (5×10^4/well) in 12-well plates using 0.1 μg 5XGAL4Luc3, 0.05 μg GAL-MAGE, 0.1 μg pVP16-cyclin A and FuGENE 6 transfection reagent (Askew et al., 2007, 2009). Cells were placed in serum-free medium the day after transfection and 24 h later luciferase activity was measured using a Lumistar Galaxy luminometer (BMG Labtech). Luciferase data are representative of three independent experiments and shown as the mean ± S.E.

Immunoblot and coimmunoprecipitation studies were performed by expression in monkey kidney COS1 cells (2×10^6 cells/10 cm dish) using DEAE dextran (He et al., 2002; Askew et al., 2007). The day after transfection, cells were incubated overnight in serum-free medium with 10 ng or 0.1 μg/ml EGF to increase cell growth and promote phosphorylation of MAGE-A11 (Bai and Wilson, 2008). For immunoblot analysis, cells were extracted in buffer containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 2 mM EDTA, 0.05 mM NaF, 2 mM sodium vanadate, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and complete protease inhibitors (Roche Applied Science). For immunoprecipitation studies, cells were extracted in buffer containing 1% Triton X-100, 0.5% deoxycholate, 0.15 M NaCl, 0.05 M NaF, 1 mM EDTA, 50 mM Tris-HCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and complete protease inhibitors and diluted at least 4-fold in the same buffer without deoxycholate before immunoprecipitation

using Anti-FLAG M2 affinity gel (Sigma) (Su et al., 2013; Minges et al., 2015). Cell extracts and immunoprecipitates were analyzed on 10 or 12% acrylamide gels containing SDS. Some immunoblots were stripped and reprobed using a different antibody (Su et al., 2013).

Antibodies used to probe the immunoblots included rabbit MAGE1 and MAGE2 against baculovirus expressed human FLAG-MAGE-A11 (0.5-10 μg/ml) (Su et al., 2013); rabbit Skp2 antibody 4358 (Cell Signaling Technology, 1:50-200); mouse FLAG-M2 antibody F3165 (Sigma-Aldrich, 1:200-2000); rabbit HA antibody ab9110 (1:500-2000) and mouse β-actin ab6276 (1:5000) from Abcam; mouse monoclonal HA-tag 12ca5 antibody from the University of North Carolina Antibody Core Facility (1-2 μg/ml); rabbit Skp2 antibody sc-7164 (1:200), rabbit cyclin A sc-751 antibody (1:200), mouse E2F1 antibody sc-193 (1:200), rabbit p107 antibody sc-318 and rabbit p130 antibody sc-317 (1:200) from Santa Cruz; rabbit AR32 (1 μg/ml) (Quarmby et al., 1990) and AR52 antipeptide antibodies (10 μg/ml) (Lubahn et al., 1988). Precision Plus Dual Stain molecular weight markers were used for calibration (Bio-Rad). Chemiluminescence was detected using SuperSignal West Dura Extended Duration Substrate (Pierce).

2.3. Lentivirus shRNA

Lentivirus coding for MAGE-A11 shRNA-169, 827, 947 and 964, AR shRNA-5, nonspecific 18-bp spacer (NS1) and empty vector (NS2) were prepared from the Open Biosystems TRC1 shRNA library using HEK293 cells (Su et al., 2013). LAPC-4 cells (1.5×10^9/well) plated in 6 well plates in medium containing 1 nM R1881 were treated 24 h later with 0.25 ml virus supernatant/well that contained ~10^6 virus particles/ml. After 48 h cells were passaged to 10 cm dishes and selected over 5 to 6 days in the presence of 3 μg/ml puromycin dihydrochloride (Cellgro). Lentivirus shRNA-947 was most effective in decreasing MAGE-A11 levels, followed by shRNA-169 and shRNA-964, with no effect of shRNA-827 or nonspecific shRNAs (Liu et al., 2011; Minges et al., 2013; Su et al., 2013).

CWR-R1 cell growth assays were performed after selection for lentivirus expression using puromycin. CWR-R1 cells (3×10^6/well) were treated with 0.2 ml/well of HEK-293 cell media (Minges et al., 2013). After 48 h, cells from 2 wells/virus type were passaged into a 10 cm dish and selected using 3 μg/ml puromycin dihydrochloride for 4 days. Selected cells (4×10^5/well) were plated in triplicate in 24-well plates with medium containing 2% charcoal stripped serum without phenol red. The next day and 2 days later, additions were made to achieve 0.1 nM dihydrotestosterone (DHT) and 10 ng/ml EGF. Cell growth was assayed after incubation with or without 0.1 nM DHT and 10 ng/ml EGF using a cell counting kit (Dojindo) (Minges et al., 2013).

3.0. Results

3.1. Competitive relationship between MAGE-A11, Skp2 and cyclin A

Human prostate cancer cells vary in the levels of MAGE-A11 (Karpf et al., 2009) and Skp2 (Fig. 1A). Skp2 was higher in CWR-R1 cells than LNCaP or LAPC-4 cells treated with or without androgen, or in African green monkey kidney CV1 or COS1 cells, but was similar to

human cervical adenocarcinoma HeLa cells (Fig. 1A). A possible influence of MAGE-A11 on Skp2 was investigated initially in LAPC-4 cells using MAGE-A11 specific antibodies and lentivirus shRNAs that decrease endogenous levels of MAGE-A11 to different extents (Liu et al., 2011; Minges et al., 2013; Su et al., 2013). The greatest knockdown of ~58-kDa MAGE-A11 in LAPC-4 cells was seen using shRNA-947 (Liu et al., 2011; Minges et al., 2013; Su et al., 2013). This was supported by studies using MAGE-A11 shRNA-947 in LAPC-4 cells that resulted in lower levels of Skp2 (Fig. 1B, lane 4). Less complete knockdown of MAGE-A11 was seen using shRNA-169 or shRNA-964 (Liu et al., 2011; Minges et al., 2013; Su et al., 2013) and was associated with a smaller or no decrease in Skp2, respectively (Fig. 1B, lanes 2 and 5). MAGE-A11 shRNA-827 or nonspecific shRNAs did not decrease MAGE-A11 or Skp 2 levels (Fig. 1B, lanes 3, 7 and 8). Knockdown of AR using lentivirus shRNA-5 (Liu et al., 2011; Minges et al., 2013; Su et al., 2013) caused a partial decrease in Skp2 and MAGE-A11 (Fig. 1B, lane 6) that could reflect post-translational regulation since neither Skp2 nor MAGE-A11 are classical androgen-regulated genes (Wang et al., 2012b; unpublished studies).

Knockdown of MAGE-A11 inhibits the growth of LAPC-4 cells that express higher levels of MAGE-A11 than other prostate cancer cells (Minges et al., 2013). To determine whether MAGE-A11 is also important in prostate cancer cells with lower levels of MAGE-A11, the effect of lentivirus shRNA knockdown of MAGE-A11 was tested in CWR-R1 cells. The combined effects of DHT and EGF (Fig. 1C, solid lines) in promoting CWR-R1 cell growth were inhibited to the greatest extent using shRNA-947 or shRNA-169, which decreased MAGE-A11 levels compared to shRNA-827 or nonspecific shRNA controls that did not decrease MAGE-A11. Inhibition of CWR-R1 cell growth associated with knockdown of MAGE-A11 was also seen in the absence of DHT and EGF (Fig. 1C, dotted lines). The requirement for MAGE-A11 in prostate cancer cell growth and the effects of MAGE-A11 on a cell cycle regulatory protein such as Skp2 raised the possibility that MAGE-A11 interacts with Skp2, or Skp2 binding protein, cyclin A (Lyapina et al., 1998; Michel and Xiong, 1998; Lisztwan et al., 1998).

MAGE-A11 interaction with Skp2 or cyclin A was demonstrated by the coimmunoprecipitation of endogenous Skp2 (Fig. 2A, lane 4) or endogenous cyclin A (Fig. 2B, lane 2) using FLAG octapeptide-tagged MAGE-A11 (FLAG-MAGE). However, demonstration of an interaction between Skp2 and endogenous MAGE-A11 was prohibited by the very low levels of endogenous MAGE-A11. However, an interaction between MAGE-A11 and cyclin A was confirmed in a mammalian two-hybrid assay that resulted in a ~10 fold increase in luciferase activity using a yeast transcriptional activator GAL4 DNA binding domain fusion protein of full-length MAGE-A11 (GAL-MAGE-2-419), herpes virus VP16 activation domain fusion protein of cyclin A (VP-cyclin A), and a GAL4-luciferase reporter gene (Fig. 3A). The carboxyl-terminal region of MAGE-A11 was implicated in cyclin A binding based on a similar ~10 fold increase in luciferase activity using carboxyl-terminal fragment GAL-MAGE-112-429 and VP-cyclin A in the two-hybrid assay. Expression of MAGE-A11 truncation mutants with FLAG-cyclin A showed that carboxyl-terminal fragments that ranged in size from MAGE-112-276 to MAGE-112-429 coimmunoprecipitated with cyclin A, whereas MAGE-112-268 and smaller fragments did not (Fig. 3B). These results suggested that a fragment containing MAGE-A11 amino acid

residues 112-276 was sufficient to interact with cyclin A. It was noteworthy that the F-box region of MAGE-A11 (amino acid residues 329-369) in the carboxyl-terminal MAGE homology domain (Askew et al., 2009) was not required to interact with cyclin A based on evidence that a similar F-box region of Skp2 was not required to interact with cyclin A (Ji et al., 2006).

Examination of the MAGE-A11 amino acid sequence revealed an ^{152}SxxLxxxxLxxLxxxExxS^{170} peptide motif sequence with homology to the Skp2 binding site for cyclin A (Ji et al., 2006) (Fig. 4A). Skp2 amino acid residues 40-60 predict an α-helix that interacts with cyclin A and aligns with MAGE-A11 amino acid residues 150-170 based on the position of serine, glutamic acid and hydrophobic residues. To test whether the 150-170 amino acid region of MAGE-A11 serves as a cyclin A binding site, MAGE-A11 deletion mutants were tested by coimmunoprecipitation with cyclin A (Fig. 4B). MAGEΔ151-217 with a deletion of the putative cyclin A binding site homologous to Skp2 did not interact with cyclin A, whereas other MAGE-A11 deletions did not diminish the interaction with cyclin A (Fig. 4C). Interaction with cyclin A was also not diminished using MAGE-L155A or MAGE-L160A, L163A mutant (data not shown). The inability of single or double amino acid mutants to disrupt MAGE-A11 interaction with cyclin A was consistent with structural flexibility associated with an intrinsically disordered region as predicted by the PONDR algorithm (www.pondr.com/pondr-tut2.html).

The presence of a homologous binding site in MAGE-A11 and Skp2 for interaction with cyclin A raised the possibility of a competitive relationship in cyclin A binding. To test this, coimmunoprecipitation studies were performed using FLAG-cyclin A with or without expression of MAGE-A11 and/or Skp2. The results showed that Skp2 interacted with cyclin A to a similar extent in the presence or absence of MAGE-A11 (Fig 5A, lanes 5 and 7). This result suggested that MAGE-A11 did not compete for Skp2-cyclin A binding. However, the decrease in coimmunoprecipitation of MAGE-A11 by cyclin A when Skp2 was expressed suggested that Skp2 interferes with MAGE-A11 interaction with cyclin A (Fig. 5A, lanes 6 and 7). In addition, while the p27^{Kip1} cyclin-dependent kinase inhibitor inhibited Skp2 interaction with cyclin A (Fig. 5B, lanes 7 and 8) as reported previously (Ji et al., 2006), p27^{Kip1} complexed with cyclin A did not inhibit MAGE-A11 interaction with cyclin A (Fig. 5C, lanes 5 and 7).

The results suggest that MAGE-A11 interacts directly and in a competitive manner with Skp2 and the Skp2 binding partner, cyclin A. The presence of a homologous peptide binding motif in MAGE-A11 and Skp2 that interacts with cyclin A, together with the ability of Skp2 to interfere with the MAGE-A11-cyclin A complex, raised the possibility that MAGE-A11 modulates the substrate activity of Skp2. The results suggest in addition that Skp2-cyclin A forms a higher affinity complex susceptible to inhibition by p27^{Kip1} compared to the MAGE-A11-cyclin A interaction.

3.2. Modulation of Skp2 activity by MAGE-A11

We next investigated whether MAGE-A11 differentially affects the substrate-specific activity of Skp2 by testing the effect of MAGE-A11 on several known Skp1-Cullin1-F-box-Skp2 (SCF-Skp2) E3 ubiquitin ligase substrates. These included cyclin A, the transcription

factor E2F1 and retinoblastoma-related protein p130 (Marti et al., 1999; Lyapina et al., 1998; Michel and Xiong, 1998; Lisztwan et al., 1998; Yam et al., 1999; Jiang et al., 2012; Rodier et al., 2005; Claudio et al., 1994; Susini et al., 2001). In a dose-dependent study, increasing levels MAGE-A11 were associated with decreased levels of cyclin A in the presence of Skp2 (Fig. 6A, lanes 4-6). This was in contrast to an increase in cyclin A associated with increasing levels of MAGE-A11 in the absence of Skp2 expression (Fig. 6A, lanes 7-9). These results suggested that MAGE-A11 is involved in the stabilization of cyclin A at low levels of Skp2, but promotes the degradation of cyclin A at elevated levels of Skp2.

E2F1 is a transcription factor that regulates cell cycle progression and is a target of the SCF-Skp2 ubiquitin ligase primarily during S and G2 phases of the cell cycle (Marti et al., 1999). The strong stabilization of E2F1 by MAGE-A11 (Su et al., 2013) raised the possibility that MAGE-A11 does not cooperate with Skp2 in E2F1 degradation. E2F1 levels increased with the expression of MAGE-A11 in agreement with previous studies (Su et al., 2013) and decreased with the expression of Skp2 in the absence of MAGE-A11 (Fig. 6B, lanes 2 and 4). However, unlike the stimulatory effect of MAGE-A11 on Skp2-mediated degradation of cyclin A, E2F1 levels increased when MAGE-A11 was expressed with or without Skp2 (Fig. 6B, lanes 3 and 5).

Retinoblastoma-related proteins p107 and p130 are cell cycle regulators targeted for degradation by different ubiquitin ligases (Claudio et al., 1994; Susini et al., 2001; Rodier et al., 2005; Jiang et al., 2012) and also differ in their interaction with MAGE-A11 (Su et al., 2013). While p107 negatively regulates Skp2 gene expression, p107 itself is not a target of SCF-Skp2 ubiquitin-mediated degradation (Rodier et al., 2005; Jiang et al., 2012) and is stabilized by MAGE-A11 (Fig. 6C, lanes 1 and 3) (Su et al., 2013). In contrast, degradation of p130 is mediated by the SCF-Skp2 ubiquitin complex (Tedesco et al., 2002; Bhattacharya et al., 2003), but p130 does not interact with MAGE-A11 (Su et al., 2013). These findings suggested that MAGE-A11 differentially influences the stability of p107 and p130 through mechanisms that depend on Skp2. Our results showed that the stabilization of p107 by MAGE-A11 was not altered by expression of Skp2 (Fig. 6C, lanes 3 and 4) in agreement with evidence that p107 is not a target of Skp2-mediated degradation. In contrast, while p130 levels were unchanged by Skp2 or MAGE-A11 expression, p130 levels declined with coexpression of Skp2 and MAGE-A11 (Fig. 6C, lanes 8-11).

The results suggest that MAGE-A11 regulates the substrate-specific activity of Skp2. MAGE-A11 enhanced Skp2-mediated degradation of cyclin A and p130, but blocked Skp2-mediated degradation of E2F1.

3.3. A mechanism that protects E2F1 from Skp2-mediated degradation

The apparent stabilization of E2F1 in the presence of MAGE-A11 and Skp2 suggested that MAGE-A11 protects E2F1 from ubiquitination and degradation mediated by Skp2. To investigate the mechanism, studies were performed to determine whether MAGE-A11 influences an interaction between E2F1 and Skp2. Surprisingly, there was no interaction seen between FLAG-Skp2 and E2F1 in the absence of MAGE-A11 (Fig. 7A, lane 6). On the other hand, there was a strong interaction between FLAG-Skp2 and E2F1 with the expression of MAGE-A11 (Fig. 7A, lane 7). Dependence on MAGE-A11 for E2F1

interaction with Skp2 was also seen in the reciprocal experiment where Skp2 coimmunoprecipitated with FLAG-E2F1 only in the presence of MAGE-A11 (Fig. 7B, lanes 6 and 7). The results suggest that the sequestration and inactivation of Skp2 by formation of an E2F1-MAGE-A11-Skp2 complex protects E2F1 from Skp2-mediated degradation.

Protection by MAGE-A11 of E2F1 degradation mediated by Skp2 raised the possibility that MAGE-A11 in some circumstances can inhibit target protein ubiquitination and degradation mediated by Skp2. To test this, we took advantage of evidence that Skp2 itself is subject to Cullin-1-based self-ubiquitination (Wirbelauer et al., 2000). In these studies, p107 served as a negative control based on evidence that p107 is not a target for Skp2-mediated degradation (Rodier et al., 2005). Immunoprecipitation of Skp2 with FLAG-ubiquitin revealed extensive self-ubiquitination of Skp2 that was not altered by the expression of p107 (Fig. 7C, lanes 3-5). On the other hand, MAGE-A11 expressed with Skp2 and FLAG-ubiquitin eliminated the self-ubiquitination of Skp2 in the absence or presence of p107 (Fig. 7C, lanes 9 and 10). The results suggest that MAGE-A11 interaction with Skp2 can block Skp2-mediated ubiquitination.

4.0. Discussion

MAGE-A11 is a low abundance steroid receptor coregulator expressed only in human and nonhuman primates. MAGE-A11 is a member of the type I cancer-testis antigens encoded on the human X chromosome and expressed at greater levels in cancer (Scanlan et al., 2002). During androgen deprivation therapy, MAGE-A11 levels increase in prostate cancer as a result of DNA hypomethylation at the transcriptional start site of the MAGE-A11 gene promoter (Karpf et al., 2009). MAGE-A11 is also expressed in some normal tissues such as human testis, ovary, and in the endometrium in a menstrual cycle-dependent manner (Bai et al., 2005, 2008). MAGE-A11 increases transcriptional activity of human AR and progesterone receptor-B by recruiting p160 and p300 transcriptional coregulators (Askew et al., 2009, 2010; Su et al., 2012) and by linking ligand-activated AR dimers and stabilizing AR at low androgen levels (Bai et al., 2005; Minges et al., 2013). The ability of MAGE-A11 to increase AR transcriptional activity together with inhibition of CWR-R1 (shown here) and LAPC-4 prostate cancer cell growth after shRNA silencing of MAGE-A11 (Minges et al., 2013) indicate an important role in androgen-dependent and castration-resistant prostate cancer. The proto-oncogene activity of MAGE-A11 (Wilson, 2010) is supported by down-regulation of MAGE-A11 by p14-ARF, a tumor suppressor derived from an alternative reading frame of the p16 CDKN2A INK4a locus (Minges et al., 2015).

Results from the present study expand our understanding of MAGE-A11 to include the regulation of Skp2, an F-box protein and cyclin A binding protein that recruits substrates for degradation by the SCF-Skp2 ubiquitin ligase and contributes to cell proliferation and survival (Xiong et al., 1993; Zhang et al., 1995; Bai et al., 1996). Skp2 is expressed at low levels in normal cells and at higher levels in many cancer cells that include prostate cancer. Increasing levels of Skp2 in prostate cancer correlate positively with shorter survival, clinical staging and metastatic disease (Zhang et al., 1995; Yam et al., 1999; Hershko, 2008). In agreement with reports that androgen withdrawal down-regulates Skp2 (Pernicová et al., 2011), knockdown of MAGE-A11 or AR decreased the levels of Skp2.

Our results show that MAGE-A11 interacts with Skp2 and the Skp2 binding protein, cyclin A, and contains a consensus cyclin A binding motif SxxLxxxxLxxLxxxExxS with homology to the Skp2 binding site for cyclin A. MAGE-A11 interaction with cyclin A stabilizes cyclin A at low Skp2 levels and increases Skp2-mediated degradation of cyclin A when Skp2 levels increase. Notable differences suggested that cyclin A forms a higher affinity complex with Skp2 than with MAGE-A11 based on Skp2 inhibition of the MAGE-A11-cyclin A interaction, but no interference of the Skp2-cyclin A interaction by MAGE-A11. MAGE-A11 and Skp2 interactions with cyclin A were also differentially regulated by the $p27^{Kip1}$ cyclin-dependent kinase inhibitor. Skp2-cyclin A was inhibited by $p27^{Kip1}$ (Ji et al., 2006), whereas Skp2-MAGE-A11 was not inhibited by $p27^{Kip1}$. Phosphorylation of Skp2 by cyclin A-cdk2 stabilizes Skp2 and interferes with inhibition by $p27^{Kip1}$ (Ji et al., 2006). The data suggest that MAGE-A11 interaction with Skp2 and cyclin A regulates the substrate-specificity of Skp2-mediated ubiquitination of target proteins. The ability of MAGE-A11 to increase degradation of cyclin A mediated by Skp2 may explain previous difficulties in demonstrating increased levels of cyclin A with knockdown of Skp2 (Ji et al., 2007). Inhibition of Skp2 self-ubiquitination by MAGE-A11 suggests that MAGE-A11 has a stabilizing effect on Skp2, although this was not always evident in the expression studies. Cell cycle-dependent changes in the levels of endogenous Skp2 or MAGE-A11 may be interrelated.

E2F1 is an essential transcription factor and proto-oncogene that up-regulates the expression of genes that promote cell cycle progression or induce apoptosis. Inhibition of apoptosis by E2F1 involves the up-regulation of p14-ARF (Bates et al., 1998), a tumor suppressor that promotes the down-regulation of MAGE-A11 (Minges et al., 2015). E2F1 levels are often increased in cancer and contribute to up-regulation of Skp2 gene expression (Markey et al., 2002; Vernell et al., 2003; Zhang and Wang, 2006; Yung et al., 2007). Phosphorylation-dependent inactivation of E2F1 by inhibition of DNA binding and transcriptional activity results from the action of cyclin A-cdk2 (Krek et al., 1994). MAGE-A11 increases E2F1 transcriptional activity by interacting with an active hypophosphorylated form of E2F1 (Su et al., 2013). Although previous reports suggest that Skp2 interacts with E2F1 (Krek et al., 1994), we found that MAGE-A11 was required for E2F1 to form a stable complex with Skp2. Stabilization of E2F1 by MAGE-A11 (Su et al., 2013) was associated with the formation of an E2F1-MAGE-A11-Skp2 complex that suggests the sequestration of Skp2 and protection of E2F1 from Skp2-mediated degradation.

Our results suggest that MAGE-A11 regulates the substrate-specificity of Skp2-mediated protein degradation through direct interactions with Skp2, cyclin A and E2F1, and that MAGE-A11 participates in an E2F1-MAGE-A11-Skp2 complex that sequesters and inactivates ubiquitin ligase activity associated with Skp2 to thereby protect E2F1 from Skp2-mediated degradation. Inactivation of Skp2 by stable complex formation was also seen for $p27^{Kip1}$ inactivation of cyclin A-cdk2 (Galea et al., 2008; Dai et al., 2013). Evidence that a complex between MAGE-A11 and Skp2 inhibits the self-ubiquitination of Skp2 suggests that MAGE-A11 can regulate Skp2-mediated ubiquitin ligase activity. The ability of MAGE-A11 to stabilize cyclin A when Skp2 levels are low and increase degradation of cyclin A when Skp2 levels increase suggests an additional regulatory mechanism that controls E2F1 transcriptional activity. Increased degradation of cyclin A mediated by Skp2 and MAGE-

A11 would decrease the phosphorylation of E2F1 by cyclin A-cdk2 and thereby promote activation of E2F1. This is consistent with the ability of MAGE-A11 to interact with and stabilize a hypophosphorylated active form of E2F1 and to increase E2F1 transcriptional activity presumably in association with increased E2F1 interaction with chromatin (Su et al., 2013). The consequences of MAGE-A11 interaction with cyclin A could thereby influence E2F1 activity indirectly since phosphorylation of E2F1 by cyclin A-cdk2 promotes the degradation of E2F1 mediated by Skp2. Similarly, phosphorylation of Skp2 by cyclin A-cdk2 stabilizes Skp2 (Rodier et al., 2008).

Our results support a model (Fig. 8) in which MAGE-A11 regulates the substrate-specificity of Skp2 through interactions with Skp2, cyclin A and E2F1. MAGE-A11 contains a cyclin A binding site homologous to the cyclin A binding site in Skp2. However, despite the similarities in binding sequence, differential regulation was noted by the cyclin-dependent kinase inhibitor $p27^{Kip1}$ inhibition of the Skp2-cyclin A complex that did not inhibit MAGE-A11 interaction with cyclin A. The ability of Skp2 to block the MAGE-A11-cyclin A interaction, whereas MAGE-A11 did not interfere with the Skp2-cyclin A complex suggests that Skp2-cyclin A is a higher affinity complex than MAGE-A11-cyclin A. Formation of an E2F1-MAGE-A11-Skp2 complex protects E2F1 from Skp2-mediated degradation by sequestration and inactivation of Skp2.

Direct interactions of MAGE-A11 with Skp2 and cyclin A may impose conformational constraints that regulate Skp2-mediated E3 ligase activity depending on the substrate. The results suggest additional mechanisms involving MAGE-A11 and Skp2 contribute to prostate cancer growth and support previous evidence implicating MAGE family proteins in specifying novel substrates through direct binding or influencing the subcellular location of E3 ligases (Hao et al., 2013; Weon and Potts, 2015; Pineda et al., 2015). There is evidence that MAGE-G1 and MAGE-C2 regulate RING protein complexes of E3 ubiquitin ligases (Doyle et al., 2010). Evidence for modulation of target substrate degradation by the Skp2 substrate recognition protein reveals a novel role for a MAGE family member in modulating E3 ubiquitin ligase activity.

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Abbreviations

MAGE-A11	melanoma-antigen-A11
AR	androgen receptor
Skp2	S phase kinase-associated protein
SCF	Skp1-Cullin1-F-box
cdk	cyclin-dependent kinase
R1881	methyltrienolone
DHT	dihydrotestosterone

Highlights

- Androgen receptor signaling involves melanoma antigen-A11 (MAGE-A11) coregulator.
- MAGE-A11 interacts with Skp2 (S phase kinase-associated protein) and cyclin A.
- MAGE-A11 and Skp2 interact competitively with cyclin A.
- E2F1 transcription factor is stabilized in an E2F1-MAGE-A11-Skp2 complex.
- MAGE-A11 regulates the substrate-specificity of Skp2-mediated protein degradation.

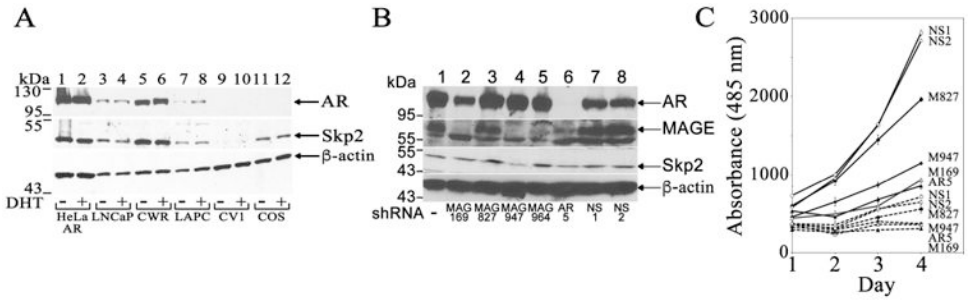

Figure 1. Skp2 levels relative to AR and MAGE-A11 and effects of lentivirus shRNA knockdown of MAGE-A11

[A] HeLa-AR1C-PSA-Luc-A6 (HeLa-AR), LNCaP, CWR-R1, LAPC-4, CV1 and COS1 cells were incubated for 48 h with or without 10 nM DHT in phenol red-free medium containing 10% charcoal stripped serum. Cell extracts (30 μg protein/lane) were probed on the immunoblot using AR, Skp2 and β-actin antibodies. **[B]** Effects of lentivirus shRNA knockdown of MAGE-A11 and AR on MAGE-A11, Skp2 and AR levels were determined in LAPC-4 cells transduced without virus (-) or with lentivirus expressing MAGE-A11 shRNA-169, 827, 947 or 964, AR shRNA-5, nonspecific empty vector shRNA (NS1) or nonspecific 18-bp shRNA (NS2) as described in *Methods*. Cells were incubated for 48 h in serum-free medium containing 50 nM DHT and 0.1 μg/ml EGF. Cell extracts (0.1 mg protein/lane) were probed on immunoblots using AR, MAGE1, Skp2 and β-actin antibodies. **[C]** Effects of lentivirus shRNA knockdown of MAGE-A11 on CWR-R1 cell growth were determined in the absence (dashed lines) or presence of 0.1 nM DHT and 10 ng/ml EGF (solid lines). Cells were selected using puromycin as described in *Methods* for lentivirus shRNA expression of NS1, NS2, MAGE-A11 shRNA-827 (M827) that did not decrease MAGE-A11 levels, or MAGE-A11 shRNA-947 (M947) or 169 (M169) that decreased MAGE-A11 to different extents. Cell growth assays were performed in triplicate over 4 days and show the mean and S.E.

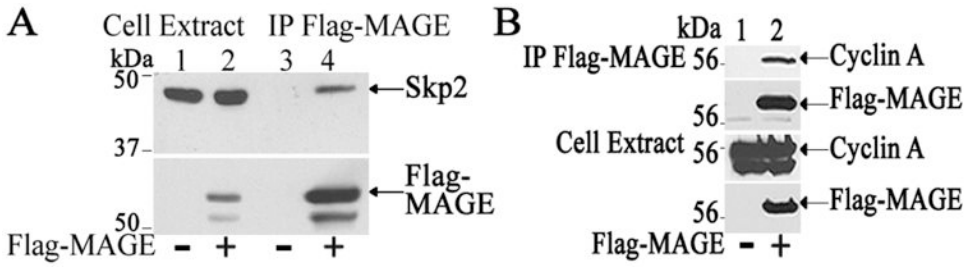

Figure 2. MAGE-A11 interaction with endogenous Skp2 and cyclin A

[A] Interaction of MAGE-A11 with endogenous Skp2 was shown by expressing 5 μg pCMV-FLAG (-) or pCMV-FLAG-MAGE/10 cm dish of COS1 cells treated for 24 h before harvest with 10 ng/ml EGF in serum-free medium. Four dishes were pooled and cell extracts (60 μg/lane) were analyzed on immunoblots using Skp2 and MAGE2 antibodies. Full-length FLAG-MAGE (lower panel, upper band) was also detected in a smaller form (lower band) that likely resulted from partial proteolysis during extraction. **[B]** Interaction of MAGE-A11 with endogenous cyclin A was shown by expressing 3 μg pCMV-FLAG (-) or pCMV-FLAG-MAGE/10-cm dish. Immunoprecipitation was performed using 4 pooled 10-cm COS1 cell dishes as described in *Methods*. Immunoblots of cell extracts (60 μg protein/lane) and immunoprecipitates were probed using cyclin A sc-751 and FLAG-M2 antibodies.

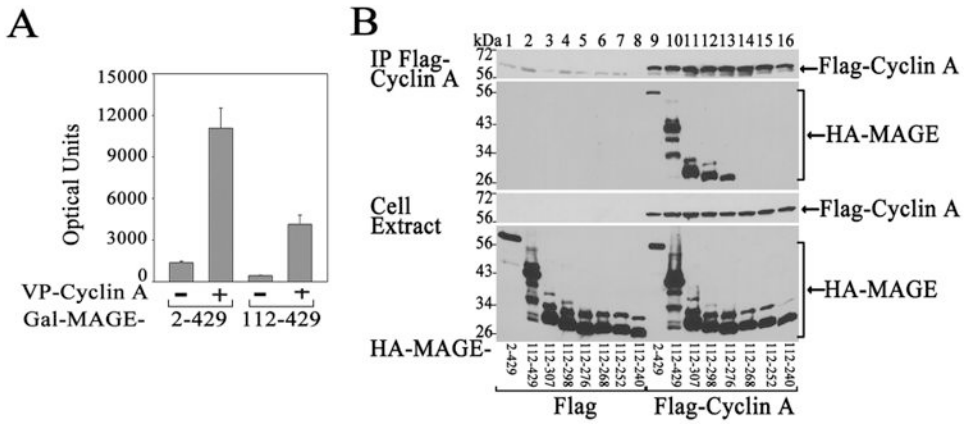

Figure 3. Interaction between MAGE-A11 and cyclin A

[A] Mammalian two-hybrid interaction between GAL-MAGE and VP-cyclin A was demonstrated by expressing in HeLa cells 0.1 μg 5XGAL4Luc3 with 50 ng full-length GAL-MAGE-2-429 and 0.1 μg pVP16 (-) or pVP16-cyclin A, or 50 ng GAL-MAGE-112-429 and 0.1 μg pVP16 (-) or pVP16-cyclin A. **[B]** Requirement for MAGE-A11 amino acid residues 112-276 to interact with cyclin A was shown by expressing 4 μg/dish pCMV-FLAG or pCMV-FLAG-cyclin A with the following pSG5-HA-MAGE vectors/dish of 2 pooled 10 cm COS1 cell dishes: 4 μg full-length 2-429, 0.4 μg 112-429, 2 μg 112-307, or 6 μg 112-298, 112-276, 112-268, 112-252 or 112-240. The day after transfection cells were incubated overnight and again for 1 h before harvest in serum-free medium containing 0.1 μg/ml EGF and 1 μM MG132 proteasome inhibitor. Immunoprecipitation was performed as described in *Methods*. Immunoblots of cell extracts (60 μg protein/lane) and immunoprecipitates were probed using HA ab9110 and FLAG-M2 antibodies.

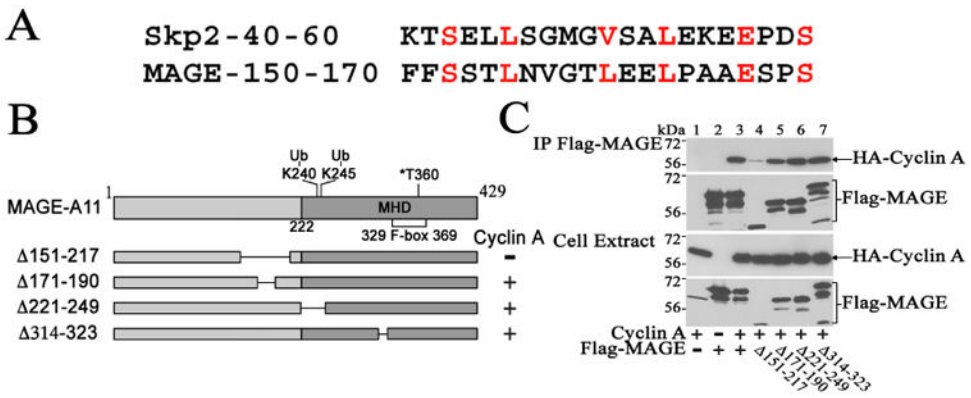

Figure 4. Homologous cyclin A binding site in MAGE-A11 and Skp2

[A] Sequence alignment of Skp2 amino acid residues 40-60 cyclin A binding region (Ji et al., 2006) with MAGE-A11 amino acid residues 150-170. Homologous amino acid residues are shown in red. **[B]** Schematic of MAGE-A11 deletion mutants used to test for interaction with cyclin A included MAGEΔ151-217, Δ171-190, Δ221-249 and Δ314-323. Also shown are MAGE-A11 monoubiquitination sites Lys-240 and Lys-245 (Ub-K240 and Ub-K245), checkpoint kinase Chk1 phosphorylation site Thr-360 (Bai and Wilson, 2008), F-box amino acid residues 329-369 (Askew et al., 2009) and MAGE homology domain (MHD, shaded). − and + indicate interaction with cyclin A. **[C]** The interaction region in MAGE-A11 for cyclin A was shown by immunoprecipitation by expressing 2 μg pcDNA3 (-) or pcDNA3-HA-cyclin A with 2 μg pCMV-FLAG (-), full-length pCMV-FLAG-MAGE or pCMV-FLAG-MAGEΔ151-217, Δ171-190, Δ221-249 or Δ314-323 in COS1 cells. The day after transfection cells were incubated for 18 h in serum-free medium containing 0.1 μg/ml EGF. Immunoblots of cell extracts (50 μg protein/ml) and immunoprecipitates were probed using HA ab9110 and FLAG-M2 antibodies.

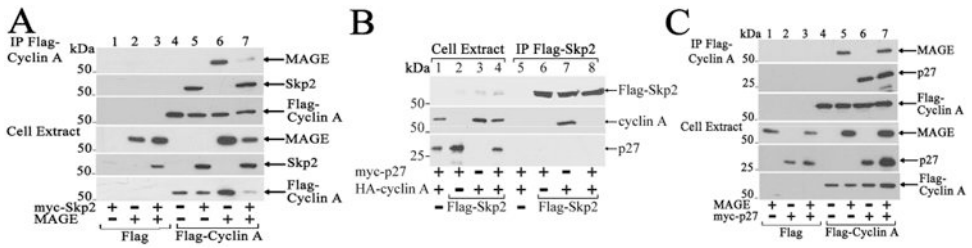

Figure 5. Competitive inhibition of MAGE-A11, Skp2, cyclin A and p27^{Kip1} complexes

[A] Inhibition of the MAGE-A11-cyclin A interaction by Skp2 was shown by immunoprecipitation by expressing 6 μg pCMV-FLAG or pCMV-FLAG-cyclin A/10 cm COS1 cell dish with or without 3 μg pSG5-MAGE and/or 4 μg pcDNA3-myc-Skp2. The day after transfection cells were incubated for 18 h in serum-free medium containing 10 ng/ml EGF. Immunoblots of cell extracts (60 μg protein/ml) and immunoprecipitates were probed using MAGE2, Skp2 and FLAG-M2 antibodies. **[B]** Inhibition of the Skp2-cyclin A interaction by p27^{Kip1} (p27) was shown by expressing 3 μg pCMV-FLAG (-) or pCMV-FLAG-Skp2 with or without 2 μg pcDNA3-HA-cyclin A alone or together with 3 μg pcDNA3-myc-p27 in COS1 cells. The day after transfection cells were incubated in serum-free medium containing 0.1 μg/ml EGF. Immunoblots of cell extracts (50 μg protein/lane) and immunoprecipitates were probed using HA ab9110, p27 and Skp2 antibodies. **[C]** Absence of inhibition of the MAGE-A11-cyclin A interaction by p27 was shown by expressing 5 μg pCMV-FLAG or pCMV-FLAG-cyclin A in 10 cm COS1 cell dishes with 3 μg pSG5-MAGE alone or together with 3 μg pcDNA-myc-p27. The day after transfection cells were placed in serum-free medium containing 10 ng/ml EGF. Cell extracts (60 μg protein/lane) and immunoprecipitates were probed on the immunoblots using MAGE2, FLAG-M2 and p27 antibodies.

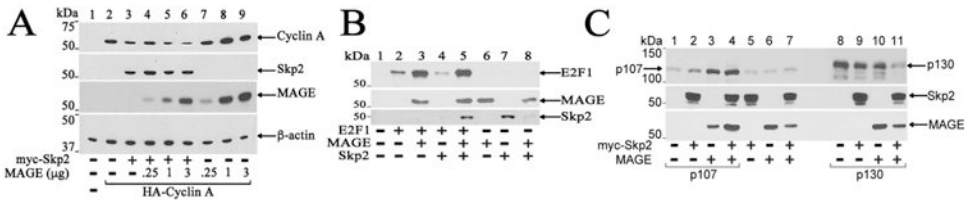

Figure 6. Substrate-specific effects of MAGE-A11 on Skp2-mediated degradation of cyclin A, E2F1 and retinoblastoma related protein p130

[A] Effects of MAGE-A11 on cyclin A were tested by expressing 3 μg pcDNA/10 cm dish of COS1 cells (lane 1) or 3 μg pcDNA-HA-cyclin A alone or with 0.25, 1 or 3 μg pSG5-MAGE with or without 4 μg pcDNA-myc-Skp2 as indicated. The day after transfection cells were incubated in serum-free medium containing 10 ng/ml EGF. Cell extracts (60 μg protein/lane) were probed on immunoblots using HA, Skp2, MAGE2 and β-actin antibodies. **[B]** Inhibition of Skp2-mediated E2F1 degradation by MAGE-A11 was shown by expressing 2 μg pCMV5 (-) or pCMV-E2F1 with 2 μg pSG5-MAGE and 2 μg pcDNA3-myc-hSkp2 alone or together in COS1 cells. After transfection cells were incubated overnight with 10 ng/ml EGF in serum-free medium. The immunoblot of cell extracts (40 μg protein/lane) was probed using E2F1, MAGE1 and Skp2 antibodies. **[C]** Increased degradation of retinoblastoma-related protein p130 but not p107 by Skp2 and MAGE-A11 was shown by expressing 2 μg CMV-p107 (lanes 1-4) or pcDNA-p130 (lanes 8-11) with or without 1 μg pcDNA-myc-Skp2 and/or 1 μg pSG5-MAGE in COS1 cells as indicated. The day after transfection cells were incubated in serum-free medium containing 0.1 μg/ml EGF. Cell extracts (40 μg protein/lane) were probed on immunoblots using p107, p130, Skp2 sc-7164 and MAGE1 antibodies.

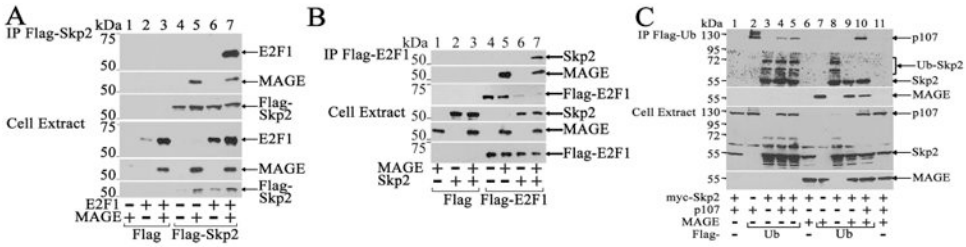

Figure 7. E2F1-MAGE-A11-Skp2 complex and inhibition of Skp2 self-ubiquitination by MAGE-A11

[A] An E2F1-MAGE-A11-Skp2 complex was shown by expressing 2 μg pSG5-MAGE with or without 2 μg pCMV-E2F1 and 3 μg pCMV-FLAG or pCMV-FLAG-Skp2 in 10 cm COS1 cell dishes. The day after transfection cells were incubated with 10 ng/ml EGF in serum-free medium. Immunoblots of cell extracts (40 μg protein/lane) and immunoprecipitates were probed using E2F1, MAGE2 and FLAG-M2 antibodies. **[B]** An E2F1-MAGE-A11-Skp2 complex was also shown by expressing 2 μg pSG5-MAGE with or without 3 μg pcDNA-myc-hSkp2 and pCMV-FLAG or pCMV-FLAG-E2F1 in COS1 cells. The day after transfection cells were incubated with 10 ng/ml EGF in serum-free medium. Immunoblots of cell extracts (60 μg protein/lane) and immunoprecipitates were probed using Skp2, MAGE2 and FLAG-M2 antibodies. **[C]** Effect of MAGE-A11 on Skp2 self-ubiquitination was investigated by immunoprecipitation after expressing 4 μg pcDNA3 (-) or pcDNA3-myc-hSkp2 with 3 μg pSG5-MAGE or 3 μg pCMV-p107 alone or together with 6 μg pCMV-FLAG (-) or pCMV-FLAG-ubiquitin (Flag-Ub) in COS1 cells. The day after transfection cells were incubated with 0.1 μg/ml EGF in serum-free medium. Immunoblots of cell extracts (40 μg protein/ml) and immunoprecipitates were probed using p107, Skp2 and MAGE1 antibodies.

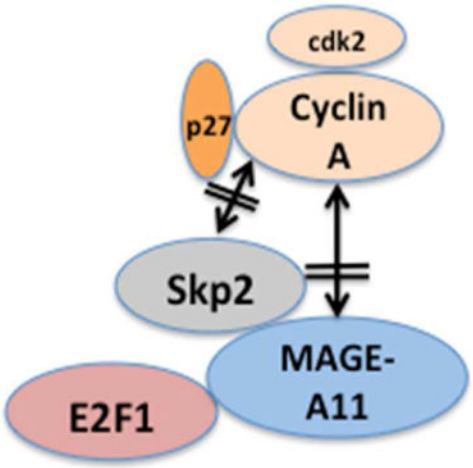

Figure 8. Model of MAGE-A11 interactions with Skp2, cyclin A and E2F1

Skp2-mediated protein degradation involves a cyclin A-cdk2-p27^{Kip1} complex that is regulated by MAGE-A11 interaction with Skp2, cyclin A and E2F1. MAGE-A11 contains a cyclin A peptide binding motif homologous to the cyclin A binding site in Skp2. MAGE-A11 interaction with cyclin A stabilizes cyclin A at low Skp2 levels, and promotes cyclin A degradation at higher levels of Skp2. MAGE-A11 interaction with cyclin A is not inhibited by the p27^{Kip1} cyclin-dependent kinase inhibitor that inhibits Skp2 interaction with cyclin A. However, MAGE-A11 interaction with cyclin A is inhibited by Skp2. MAGE-A11 mediates a stable complex between Skp2 and E2F1 that sequesters Skp2 and blocks Skp2-mediated down-regulation of E2F1.