Regulation of MAGE-A3/6 by the CRL4-DCAF12 ubiquitin ligase and nutrient availability

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Abstract

Melanoma antigen genes (MAGEs) are emerging as important oncogenic drivers that are normally restricted to expression in male germ cells but are aberrantly expressed in cancers and promote tumorigenesis. Mechanistically, MAGEs function as substrate specifying subunits of E3 ubiquitin ligases. Thus, the activation of germline-specific genes in cancer can drive metabolic and signaling pathways through altered ubiquitination to promote tumorigenesis. However, the mechanisms regulating MAGE expression and activity are unclear. Here, we describe how the MAGE-A3/6 proteins that function as repressors of autophagy are downregulated in response to nutrient deprivation. Short-term cellular starvation promotes rapid MAGE-A3/6 degradation in a proteasome-dependent manner. Proteomic analysis reveals that degradation of MAGE-A3/6 is controlled by the CRL4-DCAF12 E3 ubiquitin ligase. Importantly, the degradation of MAGE-A3/6 by CRL4-DCAF12 is required for starvation-induced autophagy. These findings suggest that oncogenic MAGEs can be dynamically controlled in response to stress to allow cellular adaptation, autophagy regulation, and tumor growth and that CRL4-DCAF12 activity is responsive to nutrient status.

Keywords DCAF12; MAGE; starvation; ubiquitin

Introduction

The melanoma antigen gene (MAGE) protein family includes more than 40 genes, many of which are cancer-testis antigens (CTAs) [1,2]. These proteins share a common MAGE-homology domain that is conserved throughout eukaryotes and structurally forms tandem winged-helix motifs [3,4]. The expression of MAGE CTAs is typically restricted to male germ cells, but it becomes aberrantly reactivated in various cancers [5,6]. Mechanisms controlling MAGE expression are not fully understood, but may partly be explained by differential CpG DNA methylation [7,8]. In many cancers, MAGE CTA expression is correlated with advanced disease and poor prognosis, suggesting that these proteins have a role in tumorigenesis and hold promise as new therapeutic targets [5]. For decades, the molecular functions of MAGE CTAs were ignored and research focused on their therapeutic potential in immuno-oncology [9]. An important advance to understanding the function of MAGEs was made when we discovered that MAGE proteins are positive regulators of E3 RING ubiquitin ligases [3]. MAGE proteins bind specific RING proteins to form stable multi-subunit E3 ubiquitin ligase complexes known as MAGE-RING ligases (MRLs); more than 50 MRLs have since been identified [1]. Mechanistic studies of a few MRLs have demonstrated the ability of MAGEs to function as substrate specificity factors [10–14]. Many E3 ubiquitin ligases themselves have been shown to be dynamically regulated in response to different cellular environments [15]. Thus far, very little is known about how MAGEs are post-translationally regulated.

The MAGE-A3 and MAGE-A6 (referred to herein as MAGE-A3/6) CTAs are highly similar (96% identity), functionally redundant, and two of the most frequently reactivated CTAs in human cancer. MAGE-A3/6 forms a stable MRL complex with the TRIM28 E3 RING ubiquitin ligase [3,16,17]. Importantly, MAGE-A3/6 drives cellular transformation, tumor growth, and metastasis [10,16,18]. Recent discoveries revealed that MAGE-A3/6 promotes metabolic reprogramming of cells to drive tumorigenesis [10,19–22]. Specifically, MAGE-A3/6 drives ubiquitination and degradation of two key metabolic enzymes and tumor suppressors, 5’ adenosine monophosphate-activated protein kinase (AMPK) and fructose-1,6-bisphosphatase 1 (FBP1), by the TRIM28 E3 ubiquitin ligase [10,19–22]. FBP1 catalyzes the hydrolysis of fructose-1,6 bisphosphate to fructose-6-phosphate and inorganic phosphate. This reaction is a rate-limiting step in gluconeogenesis and inhibits glycolysis and the Warburg effect [23]. Thus, MAGE-A3/6 promotes glycolysis and the Warburg effect through degradation of FBP1 [19]. AMPK is another key energy homeostasis enzyme that controls a plethora of cellular pathways [24]. AMPK generally represses energy-consuming anabolic processes and promotes energy-producing catabolic processes, such as autophagy [24–28]. Nutrient depletion initiates non-selective macro-autophagy to degrade intracellular macromolecules to allow cells to cope with starvation stress [29]. AMPK represses autophagy through multiple distinct mechanisms, including regulation of
ULK1, a key upstream autophagy kinase [27,28,30]. In line with these findings, we have shown that MAGE-A3/6 inhibits autophagy through degradation of AMPK, and autophagy levels in patient tumors inversely correlate with MAGE expression [10,11]. Consistent with the oncogenic activity of MAGE-A3/6, autophagy has been shown to be tumor suppressive [31,32]. However, under other contexts, autophagy can promote tumor progression [32]. How autophagy is tuned during tumorigenesis and the post-translational regulatory mechanisms controlling MAGE-A3/6 functions remain to be discovered.

Here, we discover that the protein levels of MAGE-A3/6 CTAs are dynamically regulated in cells in response to nutrient availability. We establish through biochemical and proteomics studies that MAGE-A3/6, unlike most proteins, is rapidly degraded by the ubiquitin–proteasome system upon cellular starvation. Furthermore, we establish that the stability of MAGE-A3/6 is in part regulated by insulin signaling. Moreover, we identify the CRL4-DCAF12 E3 ubiquitin ligase as the E3 ubiquitin ligase responsible for the turnover of MAGE-A3/6 upon starvation. These findings provide insights into how MAGE CTAs are post-translationally controlled, provide clues to CRL4-DCAF12 function, and add additional layers to the dynamic regulation of autophagy during tumorigenesis.

Results

Rapid and selective degradation of MAGE-A3/6 upon cellular starvation

While studying how MAGE-A3/6 regulates metabolic pathways and autophagy, we noted that upon short-term starvation of HeLa cells in Earle’s balanced salt solution (EBSS), endogenous MAGE-A3/6 protein levels rapidly decreased within a few hours (Fig 1A and B). Furthermore, this decrease was specific to MAGE-A3/6, as several control proteins showed minimal change under similar conditions (Fig 1B). To fully understand the selectivity of MAGE-A3/6 regulation under these conditions and validate our findings in another cell line, we performed quantitative proteomic analysis of A375 melanoma cells with or without nutrient deprivation, using multiplex tandem mass tag (TMT) labeling, combined with two dimensional liquid chromatography for extensive peptide fractionation, and tandem mass spectrometry for peptide/protein identification and quantification (TMT-LC/LC-MS/MS) [33]. We were able to quantitate the relative abundance of 8,649 proteins (false discovery rate < 1%) and found that only a small number significantly change. Specifically, 130 proteins decreased (log2 fold-change > 0.45, P-value < 0.01) upon starvation, whereas 9 increased (Fig 1C and Dataset EV1). Gene ontology analysis (Dataset EV2) revealed a number of expected proteins altered, including those involved in cellular response to nitrogen starvation, autophagosome assembly, and mitophagy, including LC3/GABARAP and adaptor proteins. In addition, several other expected processes were enriched, such as LDL remodeling, glycoprotein metabolic process, cell surface receptor signaling, response to extracellular stimulus, response to glucocorticoid, sequestering of metal ion, and response to lipid. Thus, this dataset provides insight into how the proteome changes in response to short-term cellular starvation. Importantly, we found that MAGE-A3 and MAGE-A6, as well as the related MAGE-A2 and MAGE-A12, were among the top 130 proteins decreased upon starvation (Fig 1C).

Given that MAGE-A proteins are highly sensitive to starvation across multiple cell lines, we next investigated whether cellular starvation decreases the half-life of MAGE-A3/6. Cells were treated with complete media or EBSS containing cycloheximide to block new protein synthesis, and MAGE-A3/6 protein levels were determined over time. We found that starvation increased the rate of MAGE-A3/6 protein turnover (Fig 1D and E), suggesting that starvation initiates a signaling cascade accelerating MAGE-A3/6 protein turnover and is not simply due to decreased protein synthesis.

Starvation-induced MAGE-A3/6 degradation can be rescued by insulin

To begin to understand the factors controlling MAGE-A3/6 protein stability, we first examined what component in the growth conditions regulates MAGE-A3/6 levels. Cells were treated with EBSS alone or in the presence of non-essential amino acids, glucose, or FBS. We found that MAGE-A3/6 protein levels could be rescued by addition of FBS, but not other supplements (Fig 2A and B). The importance of FBS in regulating MAGE-A3/6 protein stability was further confirmed in non-EBSS conditions by growth of cells in standard media (DMEM) with or without FBS (Fig 2C). We next sought to uncover what factor(s) in FBS were responsible for regulating MAGE-A3/6 protein stability. First, we treated the FBS in various ways to elucidate the nature of the factor. We noted that the factor is heat stable, and separation of small (< 3 kDa) and large (> 3 kDa) components revealed the factor to be < 3 kDa (Fig 2D and E). Given these criteria, we speculated a small molecule or metabolite may be required to stabilize MAGE-A3/6. Consistent with this, FBS in which small lipophilic hormones, growth factors, and metabolites were depleted by charcoal–dextran failed to rescue MAGE-A3/6 degradation upon cellular starvation (Fig 2D and E). To determine the specific heat stable, small, lipophilic factor in FBS regulating MAGE-A3/6 stability, we examined whether known factors depleted by charcoal–dextran were involved. We found that insulin, but not EGF, testosterone, or bovine pituitary extract, could partially rescue MAGE-A3/6 protein levels upon cellular starvation (Fig 2F–H). These data suggest that insulin, and possibly other metabolites in FBS, regulates MAGE-A3/6 protein stability.

Starvation-induced MAGE-A3/6 degradation is proteasome and cullin ligase-dependent

We next determined the molecular mechanisms controlling degradation of MAGE-A3/6 upon cellular starvation. First, we determined whether MAGE-A3/6 mRNA levels were altered in response to EBSS and found no change (Fig 3A). Next, we investigated whether MAGE-A3/6 protein degradation was a result of lysosomal or proteasomal activity. Treatment of cells with EBSS caused rapid degradation of MAGE-A3/6 that could be rescued by the proteasome inhibitor MG132, but not the lysosome inhibitor bafilomycin A1 (Fig 3B and C). These results suggested that starvation promotes degradation of MAGE-A3/6 by the ubiquitin–proteasome system (UPS). Consistently, nutrient
deprivation significantly increased MAGE-A3/6 ubiquitination (Fig 3D).

To understand how MAGE-A3/6 is degraded upon starvation, we sought to identify the E3 ubiquitin ligase responsible. First, we examined the TRIM28 E3 ubiquitin ligase that is known to complex with MAGE-A3/6 [3,10,16]. However, knockdown of TRIM28 in HeLa cells failed to rescue MAGE-A3/6 protein degradation upon starvation (Fig 3E). To identify the ubiquitin ligase responsible, we examined whether inhibiting the largest class of E3 ubiquitin ligases, cullin-RING Ligases (CRLs), could rescue MAGE-A3/6 degradation. Remarkably, inhibition of CRL activity by the NEDD8-activating enzyme E1 (NAE1) inhibitor MLN4924 [34] blocked MAGE-A3/6 degradation upon EBSS treatment (Fig 3F and G). There are seven cullin scaffolds in vertebrates that form hundreds of distinct CRL E3 ubiquitin ligases [35]. To narrow down the specific set of potential CRLs regulating MAGE-A3/6 stability, we inhibited

![Graph](image-url)

**Figure 1. Degradation of MAGE-A3/6 proteins upon nutrient deprivation.**

A, B MAGE-A3/6 protein levels decrease upon EBSS treatment of HeLa cells for the indicated times. Note both bands are MAGE-A3/6. Quantitation represents average of \( n = 3 \) with standard deviation shown.

C Quantitative TMT proteomics of A375 cells grown in complete media (CM) or EBSS for 3 h. Number of proteins affected are shown. Autophagy-related proteins are indicated in gray and MAGE-A proteins in blue.

D, E MAGE-A3/6 protein levels are actively degraded upon nutrient deprivation. HeLa cells were treated for the indicated times with complete media (DMEM + 10% FBS) or EBSS containing 100 \( \mu \)g/ml translation inhibitor cycloheximide (CHX). Protein degradation rates are shown (E). Quantitation represents average of \( n = 3 \) with standard deviation shown.

Data information: Asterisks indicate \( **p < 0.01 \). ns indicates \( p > 0.05 \) using two-way ANOVA.
Figure 2.
the activity of each cullin by expressing its dominant-negative N-terminal fragment. As described previously [36], these dominant-negative cullins retain binding to adaptor molecules, but fail to bind to Rbx1 and E2 ubiquitin-conjugating enzymes, and thus can effectively inhibit endogenous CRLs. Expression of dominant-negative Cul4A or Cul4B, but not Cul1, Cul2, Cul3, or Cul5, partially rescued degradation of MAGE-A3/6 upon starvation (Fig 3H). Furthermore, co-depletion of Cul4A and Cul4B by siRNAs significantly blocked MAGE-A3/6 degradation upon nutrient deprivation (Fig 3I). These data suggest that degradation of MAGE-A3/6 upon cellular starvation is mediated by the UPS and a CRL4 complex.

The CRL4-DCAF12 E3 ubiquitin ligase regulates MAGE-A3/6 protein stability

CUL4 forms a molecular scaffold in which the Rbx1 E3 binds its C-terminus and recruits E2 ubiquitin-conjugating enzymes. The DDB1 linker protein binds CUL4 N-terminus and mediates recruitment of substrate adaptor proteins called DCAFs (DDB1 and CUL4 associated factors) [37–40]. There are approximately 100 DCAFs that each recognizes specific proteins to target them for degradation by CRL4. Therefore, we sought to determine which specific DCAF specifies MAGE-A3/6 degradation by CRL4. To do so, we performed tandem affinity purification of stably expressed MAGE-A3 from HEK293 cells. This resulted in identification of the CRL4 linker, DDB1, and two specific DCAF substrate adaptors, DCAF5 and DCAF12 (Fig 4A; Dataset EV3). To determine whether DCAF5 or DCAF12 plays an important role in regulating MAGE-A3/6 stability, we first confirmed their interaction with MAGE-A3 by co-immunoprecipitation studies. HA-DCAF5 and HA-DCAF12 were co-expressed with Myc-MAGE-A3 and were immunoprecipitated by anti-Myc. Analysis of the immunoprecipitated revealed that DCAF12, but not DCAF5, robustly bound MAGE-A3 (Fig 4B). To determine if starvation increases DCAF12 binding to MAGE-A3, we examined MAGE-A3 binding to endogenous DCAF12 in complete media or EBSS. We observed increased interaction of MAGE-A3 with endogenous DCAF12 upon nutrient deprivation (Fig 4C). Furthermore, this interaction was diminished by treatment with insulin (Fig 4C). We were unable to perform all endogenous co-immunoprecipitation experiments due to technical limitations of the antibodies available.

To assess whether DCAF12 is required for starvation-induced degradation of MAGE-A3/6, we knocked down DCAF12 (or DCAF5 as a control) and examined MAGE-A3/6 protein levels after EBSS treatment. Consistent with our co-immunoprecipitation data, knockdown of DCAF12, but not DCAF5, diminished MAGE-A3/6 degradation to a similar level as MLN4924 treatment (Fig 4D and E). This result was confirmed using two individual DCAF12 siRNAs (Fig 4F). Furthermore, nutrient deprivation-induced MAGE-A3/6 ubiquitination is significantly decreased in DCAF12 KO cells (Fig 4G). These

Figure 2. MAGE-A3/6 proteins are stabilized by insulin in FBS.

A, B FBS (10%), but not non-essential amino acids (NEAA; 10 mM) or glucose (4.5 g/l), rescues MAGE-A3/6 protein levels upon EBSS treatment. HeLa cells were treated with EBSS and the indicated supplements for the indicated times. Quantitation represents average of n = 3 with standard deviation shown.
C Removal of FBS from normal growth conditions (DMEM + 10% FBS) results in MAGE-A3/6 protein degradation in HeLa cells.
D, E MAGE-A3/6 protein stability is controlled by a < 3 kDa, heat stable, lipophilic molecule present in FBS. HeLa cells were treated with EBSS alone, or EBSS containing 10% complete FBS, 10% charcoal–dextran-stripped FBS, 10% boiled FBS, 10% dialyzed > 3 kDa FBS, or 10% dialyzed < 3 kDa FBS for the indicated times. Quantitation represents average of n = 3 with standard deviation shown.
F, H Insulin partially rescues MAGE-A3/6 protein levels upon nutrient deprivation. HeLa cells were treated for the indicated times with EBSS alone or EBSS containing NEAA (10 mM) or insulin (10 μg/ml). Quantitation represents average of n = 3 with standard deviation shown.

Data information: Asterisks indicate ***P < 0.001; ns indicates P > 0.05 using two-way ANOVA.

Figure 3. MAGE-A3/6 is degraded by the proteasome upon nutrient deprivation.

A MAGE-A3/6 mRNA levels do not change upon nutrient deprivation. RNA was isolated from HeLa cells treated with EBSS for the indicated times. RT-qPCR analysis was performed to detect both MAGE-A3/6 transcript using a common primer set. Data were normalized to 18S rRNA levels. Quantitation represents average of n = 3 with standard deviation shown.
B, C Proteasome inhibition rescues MAGE-A3/6 protein levels upon nutrient deprivation. HeLa cells were treated for the indicated times in EBSS alone or EBSS containing lysosome inhibitor bafilomycin A1 (BafA1, 50 nM) or proteasome inhibitor MG132 (10 μM). Quantitation represents average of n = 3 with standard deviation shown.
D, E Nutrient deprivation increases ubiquitination of endogenous MAGE-A3/6. HeLa cells were treated with MG132 (10 μM) in complete media or EBSS for 6 h. Cell extracts were incubated with control agarose or agarose-TUBE2 to isolate ubiquitinated proteins. Input and pulldown samples were probed for the indicated proteins.
F, G MAGE-A3/6 protein instability upon nutrient deprivation is not rescued by knockdown of its associated E3 ubiquitin ligase, TRIM28. HeLa cells were treated with control or TRIM28 siRNAs for 6 days before incubation in EBSS for the indicated times.
H, I Degradation of MAGE-A3/6 upon nutrient starvation is dependent on a cullin E3 ubiquitin ligase complex. HeLa cells were transfected with vector control or the indicated dominant-negative (D/N) cullin constructs for 48 h before harvesting cells after 0 or 4 h EBSS treatment.

Data information: Asterisks indicate ***P < 0.001; ns indicates P > 0.05 using two-way ANOVA.
Figure 3.
data suggest that CRL4-DCAF12 controls the degradation of MAGE-A3/6 upon nutrient deprivation. Additionally, it should be noted that we see increased levels of MAGE-A3/6 upon DCAF12 knockdown when cells are grown in complete media (Fig 4E). This result suggests that CRL4-DCAF12 likely plays a role in regulation of MAGE-A3/6 protein levels under basal cellular growth conditions.
In vitro reconstitution of CRL4-DCAF12 ubiquitination of MAGE-A3/6 will provide further evidence for direct regulation of MAGE-A3/6 by CRL4-DCAF12.

Specific MAGE-A proteins are regulated by the nutrient-sensitive CRL4-DCAF12 ligase

Relatively, little is known about the CRL4-DCAF12 E3 ubiquitin ligase. In Drosophila, it has been reported to be required for apoptosis in response to specific stimuli [41]. To identify proteins regulated by CRL4-DCAF12, we performed quantitative TMT isobaric labeling proteomics on control or DCAF12 knockout A375 cells. We found a small number of proteins, 33, whose abundance increased upon DCAF12 knockout (Fig 5A; Dataset EV1). Importantly, five of these 33 proteins were MAGE-A proteins: MAGE-A2, MAGE-A2B, MAGE-A3, MAGE-A6, and MAGE-A12. These results confirm our previous findings and identify potentially novel DCAF12 targets.

Next, we performed quantitative proteomics on DCAF12 knockout cells upon starvation. Consistent with expectation, we found a large number of autophagy proteins downregulated (Fig 5B). More importantly, we found that degradation of MAGE-A proteins upon starvation was blunted in DCAF12 knockout cells (Fig 5B; Dataset EV1). Intriguingly, on average the 33 DCAF12 targets showed a more substantial decrease in protein levels upon starvation as compared to the remainder of the proteome (Fig 5C). This result suggests that starvation, and specifically, loss of FBS components like insulin, stimulates the CRL4-DCAF12 pathway. We did not observe changes in DCAF12 expression in response to EBSS (Fig 4C). Thus, the signal transduction pathways and molecular mechanisms of this observation remain to be elucidated.

Recently, it has been reported that CRL4-DCAF12 targets proteins with two glutamates at their extreme C-terminus (−EE*) [42]. Interestingly, the MAGE-A proteins regulated by CRL4-DCAF12 (MAGE-A2, MAGE-A2B, MAGE-A3, MAGE-A6, and MAGE-A12) all contain an −EE* motif (Fig 5D). Consistent with previous results [42], mutation of the DCAF12 degron in MAGE-A3 by addition of DNYNEPKANQ* to the C-terminus blocked DCAF12-induced degradation (Fig 5E). Interestingly, the related MAGE-A1, MAGE-A4, MAGE-A9, MAGE-A10, and MAGE-A11 proteins all have C-terminal extensions (typically 2 amino acids) and, in some cases, mutations of the EE motif (Fig 5D). Consistent with −EE* motifs in MAGE-A3 being important for CRL4-DCAF12, the levels of MAGE-A1, MAGE-A4, MAGE-A10, and MAGE-A11 that lack −EE* motifs are not altered by starvation or DCAF12 knockout (Fig 5F and G). These results suggest that CRL4-DCAF12 is a nutrient-sensitive E3 ubiquitin ligase that regulates specific MAGE-A proteins.

Degradation of MAGE-A3/6 by DCAF12 is important for autophagy induction upon nutrient deprivation

The results described above suggest that CRL4-DCAF12 decreases MAGE-A3/6 protein stability in response to nutrient deprivation.
Intriguingly, we have previously shown that MAGE-A3/6 inhibits autophagy through degradation of AMPK [10,11,17]. Thus, we examined whether autophagy induction upon cellular starvation is dependent on DCAF12-mediated MAGE-A3/6 degradation. The formation of cellular LC3 puncta in response to EBSS is a classical measure of autophagy [43]. Thus, we determined whether DCAF12 is required for LC3 puncta formation in response to EBSS. Indeed, knockdown of DCAF12 significantly decreased the number...
Figure 6.
of GFP-LC3 puncta in U2OS cells after EBSS treatment (Fig 6A and B). Furthermore, DCAF12 KO A375 cells had reduced endogenous LC3 puncta in response to EBSS compared with A375 parental control cells (Fig 6C and D). To determine if these effects are due to the failure to degrade the MAGE-A3/6 autophagy inhibitory proteins, we co-depleted MAGE-A3/6 with DCAF12 in U2OS and A375. Strikingly, depletion of MAGE-A3/6 rescued LC3 puncta formation in response to EBSS in DCAF12 knockdown U2OS or knockout A375 cells (Fig 6A–D). These findings were confirmed by examining the levels of the p62/SQSTM1 autophagy adaptor protein that is often turned over during autophagy (Fig 6E). These results suggest that autophagy induction upon cellular starvation requires MAGE-A3/6 degradation by DCAF12 and thus provide biological significance to this regulation.

Discussion

These findings provide insights into the post-translational regulation of specific MAGE-A CTAs. In particular, our results are intriguing given the role of MAGE-A3/6 in regulation of cellular metabolic processes, including autophagy suppression in cancer [10,11,17]. Autophagy plays a complicated role in tumorigenesis, initially acting as a tumor suppressor, but later being required for tumor progression as cells encounter nutrient stress [31,32,44]. Our findings suggest that MAGE-A3/6 may suppress autophagy early to circumvent tumor suppressive pathways and promote tumor initiation. However, as tumors progress and nutrients become limiting, autophagy may be unleashed by the activation of CRL4-DCAF12 pathway to downregulate MAGE-A3/6. Further work on defining the precise contribution of MAGE-A3/6 (and other CTAs) to tumor initiation versus tumor progression is warranted.

In addition, our results begin to elucidate signaling pathways, such as those triggered by insulin, and molecular mechanisms controlling the stability of oncogenic MAGE-A3/6 proteins. These findings suggest novel, unanticipated therapeutic strategies to target cancer growth through MAGE-A3/6 inhibition. Our results suggest that targeting MAGE-A3/6 during early stages of tumorigenesis, when autophagy is tumor suppressive, will have maximal therapeutic potential. The identification of MAGE-A3/6 degraders would be of utmost interest as cancer-specific therapeutics. Further understanding of how DCAF12 recognizes MAGE-A3/6 C-terminal –EE* degron will be pivotal in the future.

Finally, we illuminate cellular functions for the enigmatic CRL4-DCAF12 complex, including identification of a number of potential substrates. Understanding its substrate selection and the mechanisms controlling its activity in response to starvation will be important. For example, several putative substrates we identified by proteomics do not conform to the reported –EE* C-terminal degron motif [42]. Whether these are true DCAF12 targets with alternative degron motifs remains to be determined. Additionally, the molecular mechanisms by which insulin signaling inhibits CRL4-DCAF12 need investigation. Notably, we observed minimal changes in total DCAF12 protein levels under various growth conditions, suggesting potential post-translational modification of DCAF12 itself or its substrates may play key roles in tuning this pathway in response to changing cellular environments.

In summary, this study provides the first insights into the molecular mechanisms controlling the stability of oncogenic MAGE CTAs and provides a biological basis for this regulation in the control of autophagy.

Materials and Methods

Cell culture, transfection, and treatments

HeLa TetON cells (Clontech), A375 melanoma cells (ATCC), and U2OS (ATCC) were grown under standard laboratory conditions in DMEM high glucose (Thermo Fisher; 11-965-092), 10% FBS (HyClone; SH30910.03), 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B. A375 DCAF12 knockouts were previously described [42] and generously provided by Dr. Stephen Elledge (Harvard Medical School). Earle’s balanced salt solution (EBSS) was obtained from Thermo Fisher (24010-043). Charcoal–dextran-treated FBS was obtained from HyClone (SH30068.01). Fractionation of FBS into < 3 and > 3 kDa fractions was performed using snake skin dialysis tubing 3.5 kDa molecular weight cutoff (Thermo Fisher 88244) or Amicon Ultra-15 centrifugal filter unit with Ultracel-3 kDa membrane (Millipore UFC900324) according to manufacturer’s instructions.

siRNA transfections were performed using Thermo Fisher Lipofectamine RNAiMAX according to the manufacturer’s instructions. Cells treated with siRNA were cultured for 72 h before treatment. Plasmid transfections were performed using Thermo Fisher Lipofectamine 2000 according to the manufacturer’s instructions. Cells were treated as indicated 48 h after transfection.

Immunoblotting

Samples were prepared directly in hot 1× SDS sample buffer, resolved on Mini-PROTEIN TGX precast protein gels (Bio-Rad), and
transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked in TBST containing 5% milk powder (w/v) for 1 h at room temperature (RT) before primary antibody (indicated below) incubation in TBST [25 mM Tris pH 8.0, 2.7 mM KCl, 137 mM NaCl, 0.05% Tween-20 (v/v), and 10 mM 2-mercaptoethanol] containing 5% milk powder (w/v) or 5% bovine serum albumin (w/v) overnight at 4°C. Membranes were washed three times for 5 min each in TBST before incubation in secondary antibody diluted in TBST containing 5% milk powder (w/v) for 1 h. Membranes were then washed three times for 10 min each before probing with ECL detection reagent (GE Healthcare; RPN2209 or RPN2236) or detection by LiCor Odyssey software. Quantitation was done using densitometry analysis (ImageJ) or LiCor Odyssey software.

### Antibodies, siRNAs, and plasmids

Primary antibodies used in this study are as follows: anti-MAGE-A3 [3], anti-NSE1 [12], anti-Myc (Roche; 9E10), anti-HA (Roche; 12), donkey anti-rabbit IRDye 680RD (LiCor, 926-68073), and HRP (GE Healthcare, NA931V), donkey anti-mouse (LiCor, 926-2775S), Cul4A (Bethyl; A300-739A), and Cul4B (Bethyl; A303-863A). Secondary antibodies used in this study are as follows: donkey anti-rabbit HRP (GE Healthcare, NA934V), sheep anti-mouse HRP (GE Healthcare, NA931V), donkey anti-mouse (LiCor, 926-32212), donkey anti-rabbit IRDye 680RD (LiCor, 926-68073), and goat anti-rabbit Alexa 488 (Thermo Fisher, A32723).

The following siRNAs were purchased from Sigma: siControl (5'-ACUCACUUCGUAUCUCAUUdTdT-3'), siDCAF5 (5'-CAGUAUACCUUUUGCGCAdTdT-3'), 5'-GCAUGAUGACGAGCCAUIdTdT-3', and 5'-CAUAGAUAUCUGUGGCGAdTdT-3'), siDCAF12 (5'-CUCA CAAGGCCGAAGGGAdTdT-3'), 5'-CAUCUAGCGUGUUGUAUAdTdT-3', and 5'-CAUCUUCGACUGACGAdTdT-3'), siTRIM28 (5'-GC AUGAACCCUGUGUUGAdTdT-3'), siCUL4A (5'-CAAGCAGCGUG CUGAUIdTdT-3'), 5'-CUACUAGUGUGAGAACAGAdTdT-3', 5'-CCUA UAGUCUCAAGCGAdTdT-3'), siCUL4B (5'-GGAUCAUUGUGGUGA CGUIdTdT-3'), 5'-CAUUAGAAGCACGGUGUGAAdTdT-3', 5'-CAUU GCCAGCUCAGUUGIdTdT-3', and siMAGE-A3/6 (5'-GAUGGUGUGA UAGGCUGCAdTdT-3').

Dominant-negative cullin expression constructs were a kind gift from Wade Harper (Harvard Medical School).

### Proteomics profiling by TMT-LC/LC-MS/MS

Protein Digestion and Labeling: Cell pellets were lysed in lysis buffer (50 mM HEPES, pH 8.5, 8 M urea, and 0.5% sodium deoxycholate). Protein concentration of the lysates was determined by a Coomassie stained short gel [45] with bovine serum albumin (BSA) as a standard. 100 µg of protein for each sample is digested with LysC (Wako) at an enzyme-to-substrate ratio of 1:100 (w/w) for 2 h [46] in the presence of 1 mM DTT. Following this, the samples were diluted to a final 2 M urea concentration with 50 mM HEPES (pH 8.5). The samples were further digested with trypsin (Promega) at an enzyme-to-substrate ratio of 1:50 (w/w) for 3 h. The peptides were reduced by adding 1 mM DTT for 30 min at RT followed by alkylation with 10 mM iodoacetamide (IAA) for 30 min in the dark at RT. The untreated IAA was quenched with 30 mM DTT, and the digest was incubated overnight at RT. The digestion was terminated by adding trifluoroacetic acid (TFA). The acidified peptide mixture was desalted using C18 ultra microspin column (Harvard Apparatus). The eluent from the column was dried and re-suspended in 50 mM HEPES (pH 8.5). The samples were labeled with Tandem Mass Tag (TMT 11-plex) (Thermo Fisher) following manufacturer’s recommendations.

The pooled TMT-labeled sample was desalted using C18 microspin column (Harvard Apparatus) and vacuum dried. The dried TMT sample was re-suspended in 10 mM ammonium formate, fractionated by offline basic pH reverse phase LC on an XBridge C18 column (3.5 µm particle size, 2.1 mm × 25 cm, Waters) into 160 fractions over 160-min gradient by 20–50% buffer B (95% acetonitrile, 10 mM ammonium formate, pH 8.0, 0.4 ml/min flow rate). These 160 fractions were concatenated into 40, which were analyzed by acidic pH reverse phase LC-MS/MS analysis [47].

Each fraction was dried and reconstituted in 5% formic acid, loaded on a reverse phase column [75 µm × 25 cm, 1.9 µm C18 resin (Dr. Maisch GmbH, Germany)] interfaced with a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific). Peptides were eluted by ~15-50% buffer B gradient over 70 min (buffer A: 0.2% formic acid, 3% DMSO; buffer B: buffer A plus 70% acetonitrile, 0.25 µl/min flow rate). The column was heated at 65°C by a butterfly portfolio heater (Phoenix S&T) to reduce backpressure. The mass spectrometer was operated in data-dependent mode with a survey scan in Orbitrap (60,000 resolution, 1 × 105 AGC target and 50 ms maximal ion time) and 20 MS/MS scans (60,000 resolution, 1 × 105 AGC target, 150 ms maximal ion time, 35 normalized collision energy in HCD, 1.0 m/z isolation window, and 20 s dynamic exclusion).

MS/MS raw files were processed by tag-based hybrid search engine JUMP [48]. The data were searched against the UniProt mouse database concatenated with a reverse protein sequence decoy database. Searches were performed using a 10 ppm mass tolerance for precursor ions and fragment ions, fully tryptic restriction with two maximal missed cleavages, three maximal modification sites, and the assignment of a, b, and y ions. TMT tags on lysine residues and N-termini (+229.16293 Da) and carbamidomethylation of Cys residues (+57.02146 Da) were used for static modifications, and Met oxidation (+15.99491 Da) was considered as a dynamic modification. MS/MS spectra were filtered by mass accuracy and matching scores to reduce protein false discovery rate to <1%. Proteins were quantified by summing reporter ion counts across all matched PSMS using an in-house program in the JUMP software suite [49].

### Tandem affinity purification

Tandem affinity purification was performed as described previously [3]. Ten 15-cm² dishes of HEK293/TAP-MAGE-A3 or HEK293/TAP-vector stable cells were harvested in TAP-lysis buffer [10% glycerol, 50 mM HEPES-KOH pH 7.5, 100 mM KCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.1% NP-40, 10 mM NaF, 0.25 mM Na3VO4, 50 mM β-glycerophosphate, 2 mM dithiothreitol (DTT), and 1× protease inhibitor cocktail]. Cleared lysates were bound to IgG-Sepharose beads (GE Amersham) for 4 h at 4°C. Beads were washed three times in lysis buffer and TEV buffer (10 mM HEPES-KOH pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM DTT, and 1×...
protease inhibitor cocktail). Protein complexes were cleaved off the beads by 70 μg TEV protease in TEV buffer overnight at 4°C. Supernatants were diluted in calmodulin binding buffer (10 mM HEPES-KOH pH 8.0, 150 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 0.1% NP-40, 6 mM CaCl₂, and 10 mM 2-mercaptoethanol) and incubated with calmodulin-Sepharose beads (GE Amersham) for 90 min at 4°C. Captured protein complexes were washed three times in calmodulin binding buffer and calmodulin rinse buffer (50 mM ammonium bicarbonate pH 8.0, 75 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, and 2 mM CaCl₂). Proteins were eluted in 2× sodium dodecyl sulfate (SDS) sample buffer, boiled for 10 min, concentrated in microcon concentrators (Millipore), and subjected to SDS–polyacrylamide gel electrophoresis (PAGE). Gels were stained with colloidal Coomassie blue stain (Peirce) according to manufacturer’s protocol. Unique bands were excised, and in-gel proteolysis was performed using modified porcine trypsin digestion overnight. The resulting peptide mixture was dissolved and subjected to nano-LC/MS/MS analysis on a ThermoFinnigan LTQ instrument, coupled with an Agilent 1100 Series HPLC system. Peptide sequences were identified using the Mascot search engine (Matrix science).

Gene expression analysis
RNA was extracted using RNAStat60 (TelTest) according to the manufacturer’s directions.
cDNA for qPCR assays was prepared from DNase-treated RNA using High Capacity cDNA Reverse Transcription kit (Life Technologies). Gene expression levels were measured in triplicate on an Applied Biosystems 7900HT with SYBR Green chemistry (384-well format) using the following primers: MAGE-A3/A6 forward: 5′-GTGAGGGCCAGGTCTGA-3′, MAGE-A3/A6 reverse: 5′-GGGC AATTGAGCCACT-3′, 185 rRNA forward: 5′-ACCCAGCTTGG AATAATGG-3′, and 185 rRNA reverse: 5′-GCCTCAGTTCCGAAA CCA-3′.

Immunoprecipitation and ubiquitin pulldown assays
For immunoprecipitation assays, cells (6-cm² dish) were washed and collected in ice cold 1× PBS. Cells were lysed in NP-40 lysis buffer [50 mM Tris–HCl, pH 7.7, 150 mM NaCl, 0.5% NP-40 (v/v), 1 mM dithiothreitol (DTT), and 1× protease inhibitor cocktail] for 30 min on ice followed by centrifugation at 15,000 × g for 15 min at 4°C. Cleared supernatant was applied to pre-washed protein A beads (Bio-Rad) covalently coupled to anti-Myc antibody isolated from the DCAF3 knockout cell line. This work was partially supported by NIH grant R01GM134260 (JP), Worldwide Cancer Research grant 15-O177 (PRP), and American Cancer Society Research Scholar Award 181691010 (PRP).

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Conflict of interest
The authors declare that they have no conflict of interest.

References


