



A Comprehensive Guide to the MAGE Family of Ubiquitin Ligases

Anna K. Lee and Patrick Ryan Potts

Department of Cell and Molecular Biology, St. Jude Children's Research Hospital, Memphis, TN 38105-3678, USA

Correspondence to Patrick Ryan Potts: 262 Danny Thomas Pl, Memphis, TN 38105-3678, USA. ryan.potts@stjude.org. <http://dx.doi.org/10.1016/j.jmb.2017.03.005>

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Abstract

Melanoma antigen (MAGE) genes are conserved in all eukaryotes and encode for proteins sharing a common MAGE homology domain. Although only a single MAGE gene exists in lower eukaryotes, the MAGE family rapidly expanded in eutherians and consists of more than 50 highly conserved genes in humans. A subset of MAGEs initially garnered interest as cancer biomarkers and immunotherapeutic targets due to their antigenic properties and unique expression pattern that is primary restricted to germ cells and aberrantly reactivated in various cancers. However, further investigation revealed that MAGEs not only drive tumorigenesis but also regulate pathways essential for diverse cellular and developmental processes. Therefore, MAGEs are implicated in a broad range of diseases including neurodevelopmental, renal, and lung disorders, and cancer. Recent biochemical and biophysical studies indicate that MAGEs assemble with E3 RING ubiquitin ligases to form MAGE-RING ligases (MRLs) and act as regulators of ubiquitination by modulating ligase activity, substrate specification, and subcellular localization. Here, we present a comprehensive guide to MAGEs highlighting the molecular mechanisms of MRLs and their physiological roles in germ cell and neural development, oncogenic functions in cancer, and potential as therapeutic targets in disease.

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Overview of the MAGE Family

Discovery of the MAGEs

During the 1980s, researchers identified a patient, MZ-2, with stage IV amelanotic melanoma of an unknown primary tumor, who had strong T cell reactivity against autologous tumor cells in culture (reviewed in Ref. [1]). Despite surgical intervention and chemotherapy, the patient never achieved a complete remission. However, following multiple vaccinations of patient-derived clones that had been mutagenized *in vitro* and irradiated before being injected intradermally, patient MZ-2 had a remarkable recovery. This dramatic response led researchers to undertake the monumental task of identifying the tumor-associated antigen that allowed for recognition by cytotoxic T cells (CTLs). Through the elegant application of autologous typing and transfection of a cosmid library into the patient-derived MZ2-E cell line, Boon and colleagues discovered and cloned the first human tumor antigen,

melanoma antigen (MAGE)-1 [2]. Subsequent studies and homology searches revealed that MAGE-1, later renamed MAGE-A1, belongs to a larger family of genes that are now known as MAGEs [3].

In humans, the MAGE family consists of about 60 genes (some of which are designated pseudogenes) that are categorized into two classes based on their chromosomal location and expression pattern (Fig. 1a) [3,4]. Collectively, the MAGE-A, -B, and -C subfamily members located on the X chromosome comprise the type I MAGE cancer-testis antigens (CTAs), in that the genes of all three subfamilies are primarily expressed in the testis and are aberrantly expressed in cancers [3]. Conversely, the MAGE-D, -E, -F, -G, -H, -L, and Necdin genes are classified as type II MAGEs, which are not restricted to the X chromosome and are expressed in a variety of tissues [3].

Chromosome organization

Consistent with their classification as type I MAGEs, the MAGE-A genes are clustered in the

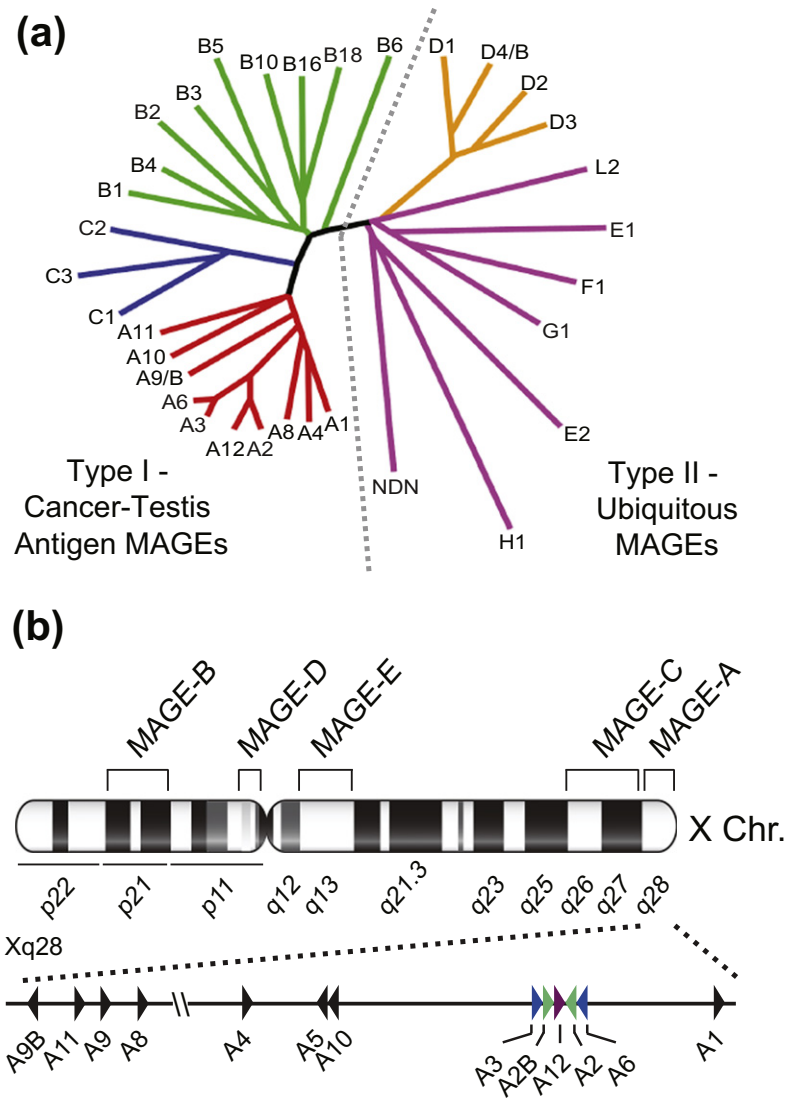


Fig. 1. The MAGE family. (a) Dendrogram tree of the human MAGE protein family. Type I MAGEs include MAGE-A, -B, and -C subfamilies. Type II MAGEs include MAGE-D, -E, -F, -G, -H, -L2, and Necdin families. (b) Chromosomal locations of MAGE subfamilies on the X chromosome. The MAGE-A genes are clustered in the q28 region of the X chromosome. Triangles indicate gene orientation. Colored triangles represent the palindrome arrangement of MAGE-A genes.

q28 region of the X chromosome, the MAGE-B genes at Xp21, and the MAGE-C genes at Xq26–27 (Fig. 1b) [5–11]. This distinct clustering pattern on the X chromosome is not exclusive to the type I MAGE CTAs. In fact, several CTAs, including GAGEs and NY-ESO-1, are also encoded by multigene families on the X chromosome [12]. Interestingly, the X chromosome contains a disproportionately high number of large, highly homologous inverted repeats that predominantly contain genes expressed in the testis [13]. It has been estimated that CTA genes constitute approximately 10% of the DNA sequence on the X chromosome, suggesting that these families are the result of gene duplications [14].

Aside from the four MAGE-D genes located at Xp11 and the three MAGE-E genes at Xq13, the remaining type II MAGEs are single-copy genes and do not exhibit clustering on the X chromosome [15]. Interestingly, MAGE-G1 (15q13.1), MAGE-L2, and Necdin (15q11.2) cluster together on chromosome 15, while MAGE-F1 is located at 3q13 and MAGE-H1 at Xp11.21.

Evolution of the MAGE genes

The MAGE genes are an ancient protein family that can be evolutionarily traced back to a single gene in protozoa that only recently underwent rapid expansion

in placental mammals to create a multigene family (Fig. 2) [16]. Katsura and Satta propose that the evolutionary history of the MAGE gene family can be divided into four phases [17]. In phase I, the ancestral MAGE exists as a single gene, as evidenced by the existence of only a single MAGE homolog in non-mammalian species [3,16,17]. Phase II is characterized by the emergence of eutherian mammals and LINE elements (Fig. 2a). It is during this eutherian radiation that the subfamily ancestors were formed via retrotransposition, with the exception of MAGE-C, which was generated by gene duplication of MAGE-A [3,17]. During phase III, gene duplications occur within the subfamilies, and palindromes form in the MAGE-A subfamily (Fig. 1b) [3,17]. Finally, in phase IV, the human MAGE-A genes undergo sequence divergence, specifically in epitope-coding regions, to allow for the generation of

diverse epitopes that can bind various human leukocyte antigen (HLA) class I molecules [17]. Intriguingly, this co-evolution of HLA and MAGE epitopes may have promoted functional differentiation whereby MAGES acquired a novel human-specific role in cancer immunity in addition to their established function in germ cell development.

While the type I MAGES are composed of three or four exons, with the terminal exon encoding the entire protein, many of the type II MAGES are characterized by a single exon. The MAGE-D genes, however, have a particularly unique genomic structure. Each MAGE-D gene contains 13 exons with the open reading frame split over 11 exons, thus allowing for alternatively spliced mRNAs. Based on their unique, complex genomic structure, it has been proposed that the MAGE-D genes may be closely related to the

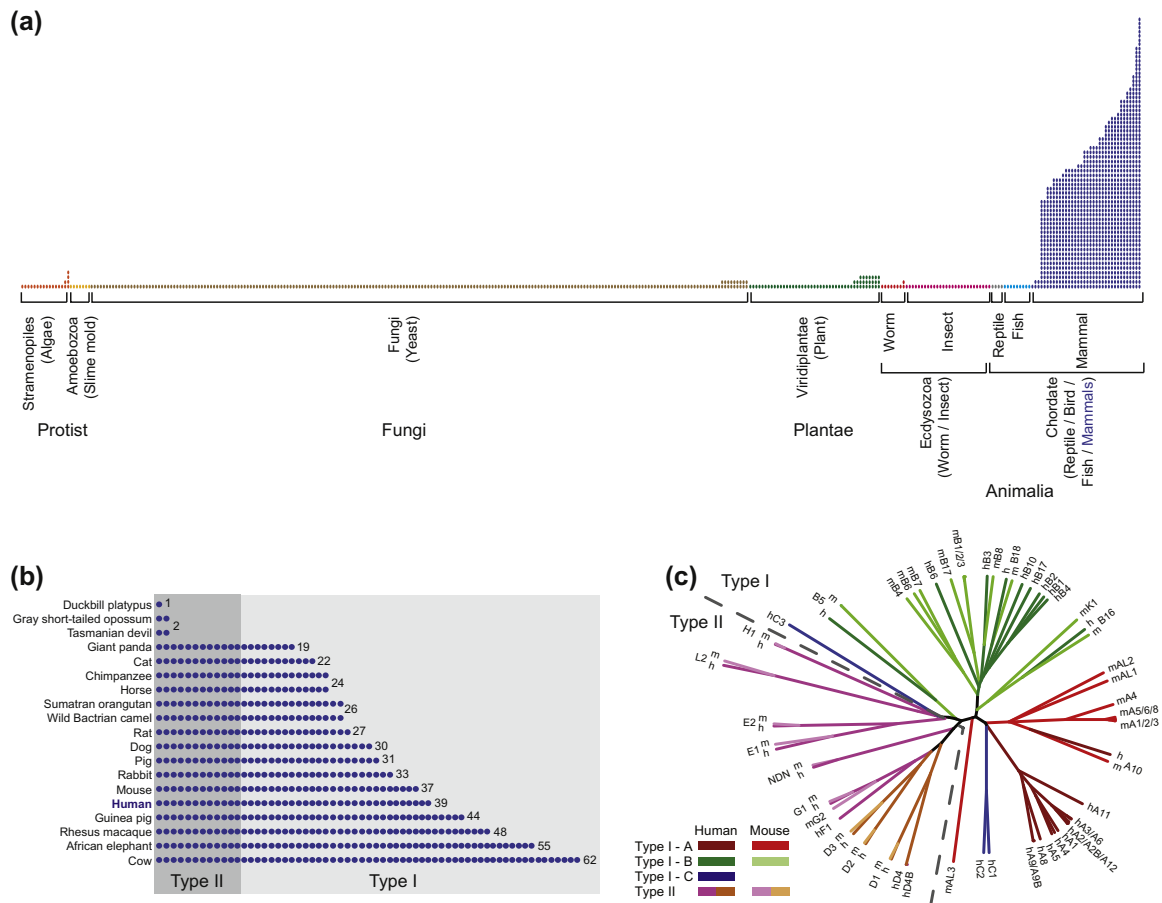


Fig. 2. Evolution of the MAGE genes. (a) The MAGE family is evolutionarily conserved in all eukaryotes. Following the emergence of eutherian mammals (blue), the MAGE family underwent a rapid and dramatic expansion from a single MAGE in lower eukaryotes to a large multigene family. Each column represents an organism with the number of circles denoting the number of MAGE proteins in each organism based on pfam annotation. (b) A detailed view of the recent expansion of MAGES in select mammals. The type II MAGES (designated based on the human MAGES) are more evolutionarily ancient, while the type I MAGES appear to be the result of recent gene duplications. (c) The type II MAGES share high homology with their mouse orthologs, whereas type I MAGES share much higher sequence conservation within their respective subfamilies compared to their mouse orthologs.

ancestral MAGE. In addition, the gene duplication events that generated the MAGE-D genes appear to be much older than the duplication events of other subfamilies. For example, the N and C termini of the MAGE-D proteins are highly conserved between human and mouse orthologs, indicating that these genes evolved independently, long before the phylogenetic separation of the two species [3]. However, it is important to note that the single MAGE genes found in *Entamoeba histolytica* and *Drosophila* are encoded by a single exon, and the number of introns in MAGE genes increases in the different animal phyla as they evolve [16]. Therefore, the ancestral MAGE gene was likely encoded by a single exon and it acquired introns during the course evolution.

Alternatively, others have suggested that MAGE-G1 is more functionally related to the ancestral MAGE. The yeast MAGE, Nse3, is a component of the structural maintenance of the chromosome (SMC) 5/6 complex, which plays an essential role in homologous recombination [18–20]. Using proteomics, Taylor *et al.* identified MAGE-G1 as the human ortholog of yeast Nse3 and determined MAGE-G1 and its cognate RING ligase, NSE1, to be essential components of the human SMC5/6 complex [21]. In addition, the *Drosophila* MAGE protein shows highest sequence identity to MAGE-G1 [22]. Moreover, the chicken MAGE protein and human MAGE-G1 interact with E2F1 and the p75 neurotrophin receptor [16]. Therefore, while genomic architecture points to MAGE-D genes, functional studies suggest that MAGE-G1 may be most related to the ancestral MAGE.

Although it is not entirely clear which specific MAGE is the most evolutionarily ancient, it is clear that type II MAGEs appeared earlier than the type I MAGEs (Fig. 2b). Overall, type II MAGEs share high homology with their orthologs, with at least 82% nucleotide sequence identity [23]. Conversely, the mouse Mage-a and -b genes share much higher sequence conservation within their respective subfamilies than with their human orthologs (Fig. 2c) [3,5,24,25]. These discrepancies, in addition to the absence of MAGE-C genes in mice, imply a more recent and rapid evolution of the type I MAGE subfamilies [3].

Zhao *et al.* attribute the distinct evolution of type I and type II MAGEs to differential selection acting on the two classes of genes [23]. Their statistical analyses indicate that type I MAGEs evolved under positive selection while type II MAGEs evolved under purifying or negative selection [23]. Where positive selection allows for diversification or the acquisition of additional functions for the redundant type I MAGEs, purifying selection maintains the established essential, non-redundant functions of the type II MAGEs [23].

MAGE homology domain

The MAGE homology domain (MHD) is a feature common to both type I and type II MAGEs (Fig. 3a).

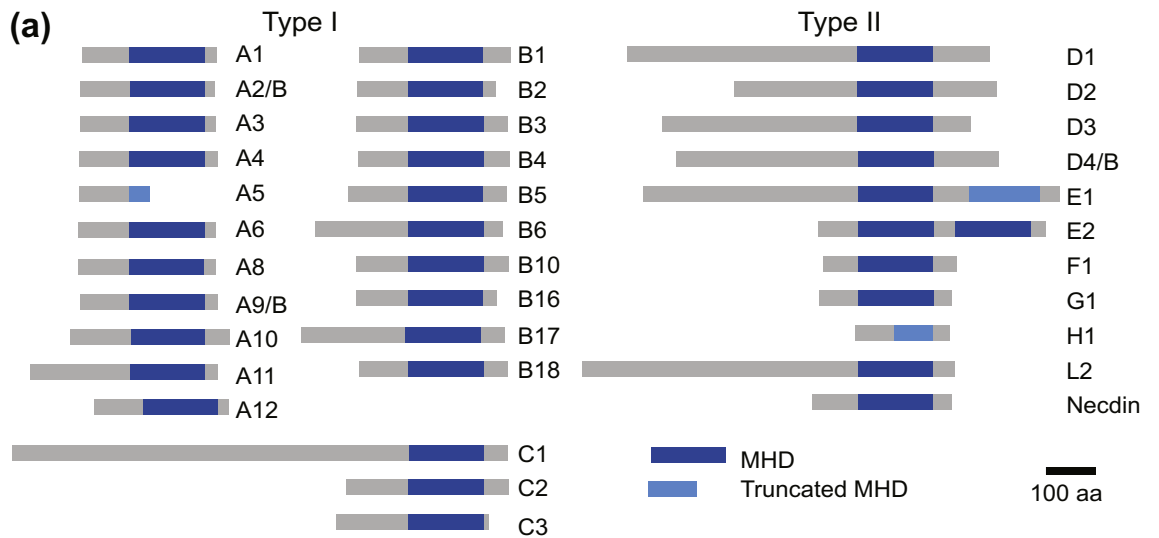
The approximately 170-aa domain is highly conserved, such that all human MHDs share 46% protein sequence identity and most contain a conserved dileucine motif (Fig. 3b) [4,26]. The MHDs within specific subfamilies share even higher conservation; for example, the 12 MAGE-A MHDs and the 4 MAGE-D MHDs are 70% and 75% conserved, respectively [26].

Structural studies have revealed that the MHD consists of two tandem winged-helix (WH) motifs, referred to as WH-A and -B (Fig. 3c and d) [26,27]. Each WH features a characteristic helix-turn-helix motif packed against a three-stranded antiparallel β -sheet “wing”; however, WH-B also contains additional α -helices [26,27]. Overall, the MAGE-A3 and -A4 MHD structures share the same relative orientation and both exhibit a peptide extension binding into the conserved cleft between the two WH motifs; however, the C terminus of MAGE-A4 is more closely associated with the rest of the molecule and forms a longer section of α -helix [27].

Despite the sequence and structural similarities shared among MHDs, mounting evidence suggests that MHDs are more versatile and complex than one might expect. Rather than recognizing and binding a common motif, MHDs confer binding specificity to multiple unique interaction motifs [26]. In addition, biophysical interrogation of MAGE-A4 by native mass spectrometry revealed a broad charge state distribution, indicating that MAGEs are structurally dynamic proteins [28]. Therefore, the flexible MHD may undergo conformational changes that allow for interaction with distinct protein domains, thereby conferring unique functions to individual MAGEs.

Function and Mechanism of MAGE-RING Ligases

Following the initial discovery of MAGEs, the major emphasis, by far, has been on their expression in cancer. While these studies have revealed many valuable aspects regarding the prognostic and therapeutic potential of MAGEs, efforts to characterize their molecular functions in physiology and pathology are limited. However, a growing body of literature has demonstrated that MAGEs assemble with E3 RING ubiquitin ligases to form MAGE-RING ligases (MRLs) that function in a myriad of cellular processes (Fig. 4a). Interaction studies including targeted and global proteomics have reported more than 50 distinct MRLs, including MAGE-A1-TRIM31, MAGE-A2-TRIM28, MAGE-A3-TRIM28, MAGE-A6-TRIM28, MAGE-B18-LNX1, MAGE-C2-TRIM28, MAGE-D1-PRAJA-1, MAGE-G1-NSE1, and MAGE-L2-TRIM27, that have been studied to various degrees. Within these complexes, MAGEs have been shown to regulate their cognate RING proteins by (1) enhancing ligase activity, (2) specifying novel



(b)

MAGE	A1	A2	A3	A4	A6	A8	A9	A10	A11	A12	B1	B2	B3	B4	B5	B6	B10	B16	B18	C1	C2	C3	D1	D2	D3	D4	E1	E2	F1	G1	H1	L2	NDN
A1	100	76	75	80	76	77	71	68	67	77	54	51	55	58	47	49	55	56	53	53	53	55	42	41	42	42	39	30	37	42	21	37	30
A2	76	100	84	71	84	70	65	62	61	91	46	48	45	51	43	42	52	49	48	48	50	51	39	38	38	38	37	28	37	41	22	37	28
A3	75	84	100	69	94	67	64	58	60	84	46	47	45	50	42	43	49	47	47	50	50	51	40	41	40	40	39	29	37	41	25	38	29
A4	80	71	69	100	69	77	72	63	66	71	54	52	53	54	46	48	56	56	51	53	52	49	42	40	42	43	40	29	39	43	23	37	30
A6	76	84	94	69	100	67	63	59	60	84	46	47	45	50	41	42	49	47	47	51	50	51	39	40	40	38	40	30	38	41	25	38	30
A8	77	70	67	77	67	100	75	70	66	71	50	50	52	54	44	47	55	54	53	52	51	57	45	41	43	46	40	31	37	44	22	38	31
A9	71	65	64	72	63	75	100	66	67	64	50	48	53	55	47	46	56	55	52	54	56	56	41	37	41	43	37	29	38	40	20	37	30
A10	68	62	58	63	59	70	66	100	67	61	53	48	54	60	47	46	58	56	53	56	56	57	40	41	40	40	38	31	37	43	22	36	33
A11	67	61	60	66	60	66	67	67	100	60	50	47	54	55	46	47	55	57	54	56	54	51	45	44	45	42	41	33	41	45	24	38	31
A12	77	91	84	71	84	71	64	61	60	100	49	48	47	52	43	44	53	50	47	49	48	49	40	39	40	40	35	30	36	40	23	37	29
B1	54	46	46	54	46	50	50	53	50	49	100	64	59	70	55	59	60	57	58	45	49	41	40	44	44	43	38	33	39	44	23	38	36
B2	51	48	47	52	47	50	48	48	47	48	64	100	57	65	50	51	55	55	46	51	44	39	43	44	42	41	36	45	42	25	38	31	
B3	55	45	45	53	45	52	53	54	54	47	59	57	100	64	61	56	58	61	57	47	48	41	46	44	47	48	38	35	42	45	25	40	34
B4	58	51	50	54	50	54	55	60	55	52	70	65	64	100	60	57	61	60	58	47	51	48	43	48	48	46	43	33	43	47	25	44	37
B5	47	43	42	46	41	44	47	47	46	43	55	50	61	60	100	52	53	53	51	41	41	34	39	40	44	41	39	28	38	42	23	38	33
B6	49	42	43	48	42	47	46	46	47	44	59	51	56	57	52	100	51	52	53	41	43	40	43	45	47	46	40	34	41	42	26	40	37
B10	55	52	49	56	49	55	56	58	55	53	60	55	58	61	53	51	100	64	63	54	51	45	43	45	47	45	42	35	41	46	25	39	36
B16	56	49	47	56	47	54	55	56	57	50	57	55	61	60	53	52	64	100	63	50	51	44	44	47	47	46	44	36	43	49	25	40	37
B18	53	48	47	51	47	53	52	53	54	47	58	55	57	58	51	53	63	63	100	48	53	44	44	43	44	45	40	34	43	42	25	38	34
C1	53	48	50	53	51	52	54	56	56	49	45	46	47	47	41	41	54	50	48	100	69	66	40	42	43	39	38	31	38	40	25	34	33
C2	53	50	50	52	50	51	56	56	54	48	49	51	48	51	41	43	51	51	53	69	100	64	40	40	39	39	39	30	38	41	26	37	32
C3	55	51	51	49	51	57	56	57	51	49	41	44	41	48	34	40	45	44	44	66	64	100	37	42	39	35	36	25	36	38	12	37	25
D1	42	39	40	42	39	45	41	40	45	40	40	39	46	43	39	43	43	44	44	40	40	37	100	78	76	78	46	38	47	43	25	49	37
D2	41	38	41	40	41	37	41	44	39	44	43	44	48	40	45	45	47	43	42	40	42	78	100	79	67	48	34	49	48	30	51	43	
D3	42	38	40	42	40	43	41	40	45	40	44	44	47	48	44	47	47	44	43	39	39	76	79	100	74	47	34	48	47	30	48	43	
D4	42	38	40	43	38	46	43	40	42	40	43	42	48	46	41	46	45	46	45	39	39	35	78	67	74	100	40	38	45	46	25	44	39
E1	39	37	39	40	40	40	37	38	41	35	38	41	38	43	39	40	42	44	40	38	39	36	46	48	47	40	100	38	54	47	29	44	34
E2	30	28	29	29	30	31	29	31	33	30	33	36	35	33	28	34	35	36	34	31	30	25	38	34	34	38	100	34	39	25	36	28	
F1	37	37	37	39	38	37	37	41	36	39	45	42	43	38	41	41	43	43	38	38	36	47	49	48	45	54	34	100	59	27	50	45	
G1	42	41	41	43	41	44	40	43	45	40	44	42	45	47	42	42	46	49	42	40	41	38	43	48	47	46	47	39	59	100	27	50	42
H1	21	22	25	23	25	22	20	22	24	23	23	25	25	25	23	26	25	25	25	26	12	25	30	30	25	29	25	27	27	100	25	26	
L2	37	37	38	37	38	38	37	36	38	37	38	38	40	44	38	40	39	40	38	34	37	37	49	51	48	44	44	36	50	50	25	100	39
NDN	30	28	29	30	30	31	30	33	31	29	36	31	34	37	33	37	36	37	34	33	32	25	37	43	43	39	34	28	45	42	26	39	100

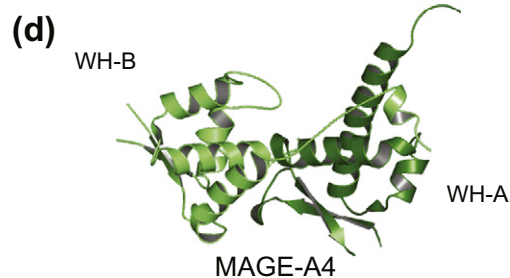
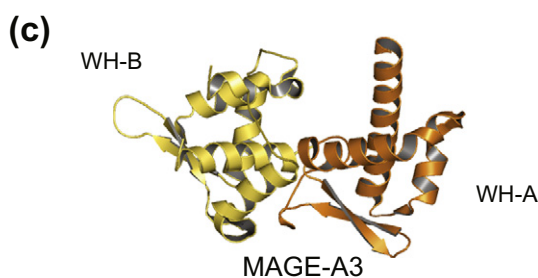


Fig. 3. The MAGE homology domain. (a) List of human MAGE proteins and their conserved MAGE homology domain (MHD) highlighted in blue. (b) The percentage of identical amino acids between the various human MHDs is given and colored on a three-color scale (blue = high, gray = medium, red = low). Crystal structures of the (c) MAGE-A3 and (d) MAGE-A4 MHDs. The two WH motifs (WH-A and WH-B) are noted.

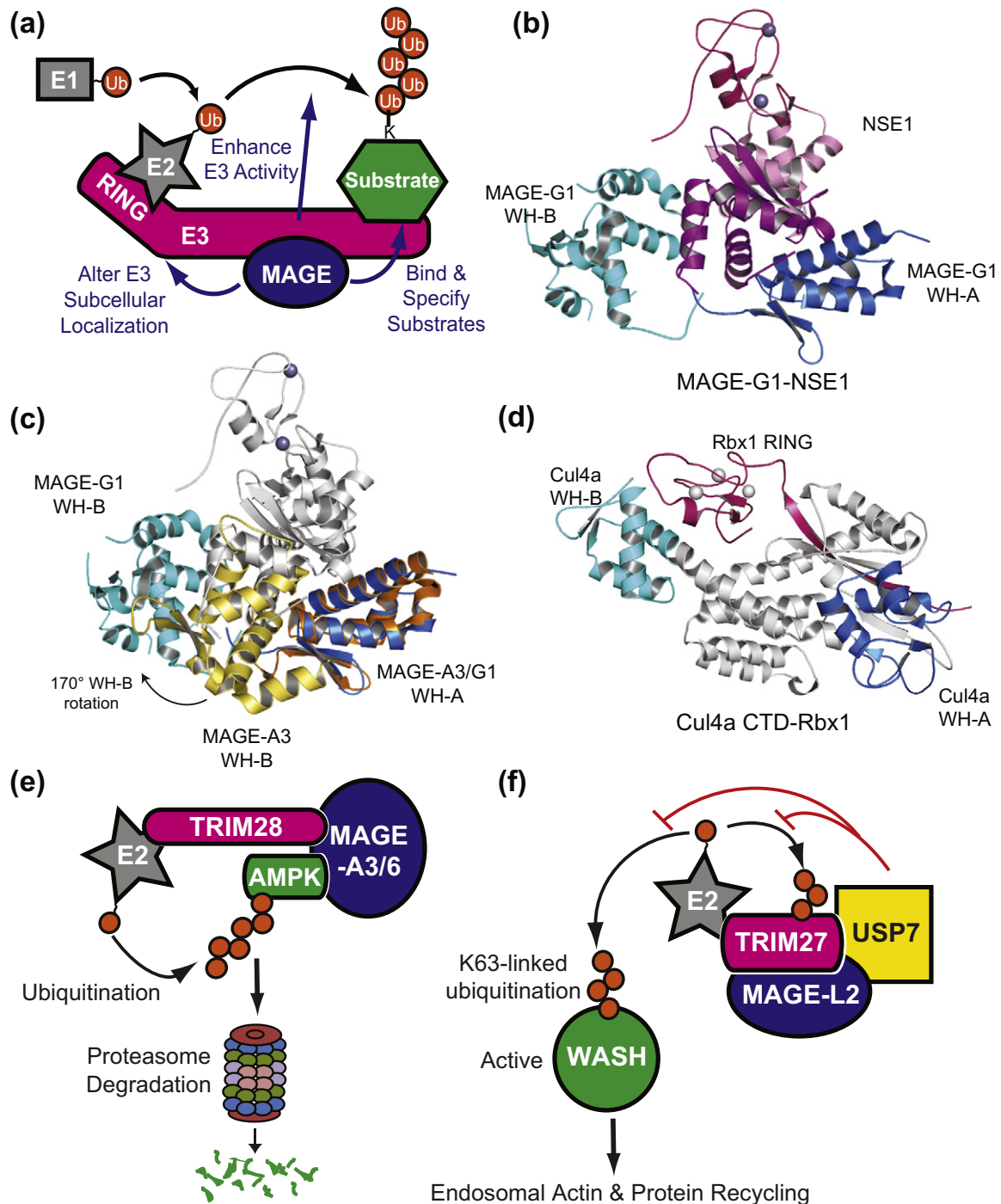


Fig. 4. Structure and function of MAGE-RING ligases. (a) Schematic representing biochemical and cellular functions of MAGE-RING ligases (MRLs). (b) Crystal structure of MAGE-G1-NSE1. (c) Alignment of MAGE-G1-NSE1 and MAGE-A3 based on WH-A. The orientation of the WH motifs differs between unbound MAGE-A3 and NSE1-bound MAGE-G1. (d) Crystal structure of Cul4A C-terminal domain (CTD)-Rbx1 (PDB: 2HYE) shares structural features similar to MAGE-G1-NSE1. (e) Model of MAGE-A3/6-TRIM28 ubiquitination and degradation of the AMPK tumor suppressor. MAGE-A3/6 directly binds AMPK and recruits it to the TRIM28 ubiquitin ligase for ubiquitination and subsequent proteasome-mediated degradation. (f) Model of the MUST complex and K63-ubiquitin regulation of endosomal protein recycling. MAGE-L2, USP7, and TRIM27 form a stable complex where TRIM27 mediates non-degradative K63-linked ubiquitination of WASH. This ubiquitination event leads to WASH activation, generation of F-actin accumulation, and recycling of proteins through the retromer pathway. USP7 functions to fine-tune WASH ubiquitination to allow for precise levels of endosomal F-actin and stabilize the complex through preventing TRIM27 auto-ubiquitination-induced degradation.

substrates for ubiquitination, and (3) altering subcellular location. In the sections below, we discuss in detail specific MRL complexes and the mechanisms by which they function.

Ubiquitination and RING ligases

Ubiquitination is the covalent post-translational modification of lysines on substrate proteins with the small 76-aa ubiquitin protein and regulates nearly all aspects of cell function. The most well-characterized function of ubiquitination is the targeting of proteins for proteasomal degradation; however, ubiquitination also regulates proteasome-independent processes such as endocytosis and lysosomal targeting, nuclear export, DNA repair, and activation of kinases and transcription factors depending on the specific type of ubiquitin chain linkage (reviewed in Ref. [29]). In the ubiquitination cascade, an E1 ubiquitin-activating enzyme activates ubiquitin; this activated ubiquitin is transferred to an E2 ubiquitin-conjugating enzyme and is subsequently ligated to a substrate via an E3 ubiquitin ligase (Fig. 4a) [30]. Therefore, by providing substrate specificity in the ubiquitin pathway, E3 ligases play a critical regulatory role in various cellular pathways.

The majority of known E3 ubiquitin ligases include the RING domain and RING-like proteins, which mediate substrate recognition and subsequent ubiquitin ligation through the activation of the E2 enzyme [29]. The RING domain contains a conserved cysteine- and histidine-rich consensus sequence that coordinates two zinc ions via a cross-brace arrangement [30]. Interestingly, many RING ligases have been implicated in cancer due to their roles in the maintenance of genomic integrity and cellular homeostasis (reviewed in Ref. [31]).

MAGE-RING ligase architecture

Upon determining that MAGE family proteins form complexes with E3 RING ubiquitin ligases, our lab sought to characterize the biochemical and biophysical properties of MRLs. By *in vitro* binding assays, we demonstrated that each MAGE generally binds one specific RING ligase, and highly homologous MAGEs tend to bind the same RING protein [26]. Subsequent mapping of the minimal regions required for MRL complex formation revealed that MAGEs bind their cognate RING ligases via the MHD; however, the region on which MAGEs bind RING ligases varies between different MRLs [26]. For example, MAGE-C2 binds the coiled-coil region of TRIM28, MAGE-B18 binds a basic region between the RING and first PDZ domain of LNX1, and MAGE-G1 binds the WH motifs of NSE1 [26]. These findings further validate the notion that the MHD is flexible and its conformational plasticity allows for unique and complex interactions with specific RING ligases.

In an effort to gain insights into the structural properties of MRLs, we also determined the crystal structure of MAGE-G1 in complex with its cognate NSE1 RING ligase (Fig. 4b) [26]. Consistent with the *in vitro* domain mapping experiments, MAGE-G1 WH-A interacts with both WH motifs of NSE1 via a series of hydrogen bonds and a large hydrophobic interface that includes the conserved dileucine motif [26]. In addition, mutation of the dileucine motif disrupts the binding of not only MAGE-G1 and NSE1 but also complex formation of other MRLs, suggesting that these conserved residues play an important role in the binding interface [26].

Intriguingly, while the WH-A and WH-B motifs of NSE1-bound MAGE-G1 and free MAGE-A4 are similar, with rmsd values of 1.05 Å and 1.07 Å, respectively, their relative orientations are very distinct in the two structures (Fig. 4c) [26,27]. The WH motifs in MAGE-G1 are distantly separated, such that WH-B rotates approximately 170° and translates about 30 Å relative to WH-A [26,27]. Moreover, the peptide extension bound between the two WH motifs in the MAGE-A3 and -A4 structures is not present in the MAGE-G1-NSE1 structure [26,27]. Therefore, the two different conformational states demonstrate that the MHD undergoes extensive rearrangement for MRL complex formation, and the structural changes required to accommodate association with RING ligases may confer binding specificity.

Structurally, MRLs share a number of key features with Cullin-RING ligases (CRLs; Fig. 4d). CRLs, the largest family of multicomponent E3s, generally consist of four subunits: cullins (CUL1–7), RINGs (Rbx1–2), adaptor proteins, and substrate recognition proteins (reviewed in Ref. [32]). The cullin acts as the core molecular scaffold that binds to an adaptor protein and a substrate receptor protein at the N terminus and a RING protein at the C terminus [33]. Like MRLs, these modular CRLs can assemble with multiple substrate recognition proteins to recruit various unique substrates [32]. In addition, the cullin C-terminal domain (CTD) shares marked structural similarities to MAGEs, in that two WH motifs contribute to a groove where the RING domain binds [33]. Interestingly, this region of the cullin CTD, referred to as the cullin homology region, is conserved in several other proteins including the APC2 subunit of the anaphase-promoting complex/cyclosome (APC/c) E3 ligase, suggesting a conserved function that also extends to MRLs [33–36].

MAGEs enhance E3 RING ubiquitin ligase activity

One of the earliest indications that MAGEs form complexes with E3 RING ubiquitin ligases came from the discovery that MAGE-A2, -A3, -A6, and -C2 directly bind and regulate the TRIM28 (also referred to as KAP1) E3 ubiquitin ligase, a multifunctional protein

implicated in transcriptional regulation, cellular differentiation, and DNA damage repair [26,37]. Biochemical analysis of TRIM28 ubiquitin ligase activity revealed that these MAGEs stimulate both TRIM28 auto-ubiquitination and ubiquitination of its substrates, the p53 tumor suppressor and ZNF382, *in vitro* and in cells [26,38,39]. Moreover, the enhanced ubiquitin ligase activity of MAGE-TRIM28 reduced p53 and ZNF382 protein levels in a proteasome-dependent manner [26,38,39]. In addition to MAGEs regulating the TRIM28 ligase, MAGE-A1 has been reported to stimulate the ligase activity of TRIM31 [40]. Furthermore, this ability of MAGEs to enhance E3 ubiquitin ligase activity is not limited to the type I MAGEs. NSE1 normally has weak *in vitro* ubiquitin ligase activity in the presence of the E2 ubiquitin-conjugating enzyme, UbcH13/Mms2; however, upon the addition of MAGE-G1, NSE1 activity is significantly enhanced [18,26]. Therefore, the ability to enhance E3 RING ubiquitin ligases is a feature common to both type I and type II MAGEs.

To gain a better understanding of how MAGEs enhance the ligase activity, we tested four possible mechanisms whereby MAGEs (1) induce a conformational change in the E3 RING ligase, thus promoting increased activity; (2) promote substrate binding to the E2-E3 ubiquitin ligase machinery; (3) stimulate charging of the E2 ubiquitin-conjugating enzyme by the ubiquitin E1; or (4) bind and recruit E2 ubiquitin-conjugating enzymes to the E3 substrate complex. However, we found that MAGE-G1 did not alter NSE1 conformation, neither MAGE-A2 nor -C2 enhanced p53 binding to TRIM28, and MAGE-C2 did not affect UbcH2 charging [26]. Interestingly, we demonstrated that MAGE-A2/C2 specifically bind the UbcH2 E2 ubiquitin-conjugating enzyme, suggesting that MAGEs may enhance ubiquitin ligase activity by recruiting and/or stabilizing the E2 enzyme at the E3-substrate complex [26].

Depending on whether MAGE-A2/C2 can bind UbcH2 and TRIM28 simultaneously, or if the two binding interactions are mutually exclusive, Feng and colleagues propose two mechanistic models by which these MAGEs facilitate TRIM28 E3 ligase activity [41]. In the first model, MAGE-A2/C2 binding to TRIM28 and UbcH2 are mutually exclusive. After transferring one ubiquitin to the substrate, UbcH2 is recharged by an E1 ubiquitin-activating enzyme while remaining in close proximity to the TRIM28 machinery via interactions with MAGE-A2/C2. In this way, MAGEs promote the on-site recharging of the E2 enzyme. In the second model, MAGE-A2/C2 bind to TRIM28 and UbcH2 at the same time. Here, two UbcH2 molecules are recruited to the TRIM28 machinery—with one UbcH2 interacting with the TRIM28 RING domain and the other with MAGE-A2/C2—to promote the sequential assembly of a polyubiquitin chain on the substrate [41]. However, whether MAGE-A2/C2 can bind TRIM28 and UbcH2

simultaneously remains unclear, and further work must be done in order to validate these proposed models and determine whether additional MAGEs bind to their cognate E2 enzymes.

Regulation of AMPK by MAGE-A3/6-TRIM28

MAGE-A3 and the highly similar protein MAGE-A6 (referred to as MAGE-A3/6) also bind TRIM28 [26]. Although multiple studies have highlighted the role of MAGEs and TRIM28 in the regulation of p53 (refer to Regulation of transcription), MAGE-A3/6 expression does not inversely correlate with p53 mutational status, suggesting that this MRL may have additional targets relevant to its function in cancer cells [42]. In an *in vitro* screen to identify direct substrates of MAGE-A3/6-TRIM28, we found that the expression of MAGE-A3/6 enhances the ubiquitination of AMP-activated protein kinase (AMPK) α 1 (Fig. 4e) [42]. Importantly, MAGE-A3/6 not only promotes ubiquitination and subsequent proteasomal degradation of AMPK α 1 but also directly interacts with and specifies AMPK α 1 as a substrate for TRIM28 [42]. Thus, unlike p53, which can be targeted by TRIM28 in the absence of MAGEs, AMPK α 1 is only targeted by TRIM28 in the presence of MAGE-A3/6. Therefore, expression of MAGE-A3/6 in cancer cells reprograms the ubiquitous TRIM28 ubiquitin ligase to degrade a key metabolic regulator and tumor suppressor to enhance tumorigenesis.

AMPK α 1 is the catalytic subunit of the AMPK heterotrimer, a crucial energy sensor in cells [43]. In response to even modest decreases in ATP production, AMPK is activated and promotes catabolic ATP-generating pathways while also inhibiting anabolic ATP-consuming pathways, such as mechanistic target of rapamycin (mTOR) signaling to maintain energy homeostasis [43]. In this way, AMPK and mTOR signaling play opposing functions in the regulation of autophagy, a degradative process important for balancing energy sources during development and in response to nutrient stress [43]. Due to its role as a master regulator of cellular energy, AMPK functions as a critical tumor suppressor to stop cell growth, and its activity is often perturbed in various diseases such as cancer.

Further investigation into the functional consequences of AMPK regulation by MAGE-A3/6-TRIM28 revealed increased glucose consumption and lactate production upon TRIM28 knockdown, suggesting that the inhibition of AMPK by the MRL affects cell metabolism [42]. In addition, we found that MAGE-A3/6-TRIM28 is critical for the maintenance of mTOR activity [42]. Consistent with the role of MAGE-A3/6 in regulating AMPK and mTOR signaling, we demonstrated that the MAGE-A3/6-TRIM28 MRL inhibits autophagy [42,44].

Taken together, these results suggest that the oncogenic MAGE-A3/6-TRIM28 MRL regulates several cellular metabolic regulatory pathways via

ubiquitination and degradation of AMPK α 1 [42]. Interestingly, breast invasive carcinoma, colon adenocarcinoma, and lung squamous cell carcinoma tumors expressing MAGE-A3/6 have significantly reduced total and active AMPK α protein levels and reduced downstream AMPK signaling, indicating that this regulation of AMPK by MAGE-A3/A6-TRIM28 is relevant in human tumors [42]. However, further work will be necessary to examine whether MAGE-A3/6-TRIM28 suppression of autophagy is important for its oncogenic activity and whether MAGE-A3/6-TRIM28 regulates additional AMPK cellular responses. In addition, how this function relates to the physiological role of MAGE-A3/6 in the testis remains unclear. Given that germ cells in the testis change carbon energy sources as they differentiate from spermatogonial stem cells to mature spermatids, it will be intriguing to determine if MAGE-A3/6-mediated AMPK regulation contributes to this metabolic switch during spermatogenesis [45].

Regulation of WASH-mediated endosomal protein trafficking by the MAGE-L2-USP7-TRIM27 (MUST) Complex

Endosomal protein trafficking is an essential process that allows for the delivery of membrane components, receptor-associated ligands, and solute molecules to various intracellular destinations such as the lysosome for degradation, the cell surface, or the *trans*-Golgi network [46]. The primary function of the retromer complex is to select cargo proteins for endosome-to-Golgi transport or retrograde transport. In addition to cargo recognition, the retromer also recruits the Wiskott-Aldrich syndrome protein and scar homolog (WASH) complex, which promotes actin filament (F-actin) nucleation by the Arp2/3 complex [47,48]. The formation of actin patches plays a critical role in endosomal sorting by generating discrete domains into which specific proteins are sorted for transport to their respective destinations [46,49].

Our lab found that MAGE-L2 interacts with the TRIM27 E3 RING ubiquitin ligase and localizes to retromer-positive endosomes through interactions between MAGE-L2 and the VPS35 component of the retromer complex (Fig. 4e) [50]. Importantly, the ubiquitin ligase activity of MAGE-L2-TRIM27 is required for proper recycling of endosomal proteins through the retromer pathway to the *trans*-Golgi network or plasma membrane [50]. Detailed analysis of the retromer pathway demonstrated that MAGE-L2-TRIM27 is required for WASH-mediated F-actin assembly on endosomes. Additionally, more in-depth studies revealed that MAGE-L2-TRIM27, in conjunction with the Ube2O E2 ubiquitin-conjugating enzyme, facilitates the non-degradative K63-linked ubiquitination of WASH [50]. Interestingly, additional mechanistic studies revealed that this ubiquitination of WASH on lysine 220 (K220) by MAGE-L2-TRIM27 destabilizes

autoinhibitory contacts in the WASH complex, thus allowing for its activation and F-actin assembly on endosomes and recycling through the retromer pathway [50].

In a subsequent study, we made the surprising discovery that the USP7 deubiquitinating enzyme (DUB) is an integral component of the MAGE-L2-TRIM27 MRL complex (Fig. 4e) [51]. Through *in vitro* binding experiments, we found that USP7 directly binds both MAGE-L2 and TRIM27 to form an intricate and stable protein complex, referred to as the MUST complex. In the trimeric MUST complex, MAGE-L2 binds the USP7 N-terminal TRAF domain and the C-terminal HUBL1-3 regulatory domains, whereas the CTDs of TRIM27 interact with the catalytic domain of USP7 [51]. Although previous examples of DUBs regulating ligases have been described, the intricate and obligate nature of USP7 for stable formation of the MUST complex suggests an important linkage between conjugating (TRIM27) and deconjugating (USP7) enzymes for proper cellular function.

Functional interrogation of the complex demonstrated that USP7 knockdown or disruption of the MAGE-L2-USP7 interaction impaired endosomal actin accumulation and protein recycling, indicating that USP7 acts in concert with MAGE-L2-TRIM27 and is required for the regulation of WASH-mediated protein trafficking [51]. Interestingly, USP7 performs dual functions in the endosomal protein-recycling pathway: (1) deubiquitination of TRIM27 to protect TRIM27 from auto-ubiquitination-induced degradation and (2) deubiquitination of WASH to precisely regulate WASH activity [51]. Through these seemingly opposing activities, USP7 serves as molecular rheostat to fine-tune endosomal F-actin levels [51]. This buffering capacity of USP7 is critical given that too little or too much F-actin on endosomes can both be detrimental to retromer-mediated recycling [51]. Thus, it is no surprise that nature has elegantly linked the conjugation and deconjugation machinery in a single complex to allow the precise control of WASH ubiquitination and activity. Additional studies will be necessary to determine if MAGE-L2 regulates TRIM27 and USP7 enzymatic functions in addition to acting as a molecular scaffold in the MUST complex and mediating localization to endosomes. Importantly, these findings open up the exciting possibility that the functional cooperativity observed between USP7 and MAGE-L2-TRIM27 may be conserved among other MRLs and DUBs.

Regulation of cyclins by MAGEs and SCF

In contrast to the majority of reports on MRLs, two MAGEs have recently been shown to associate with and regulate CRLs. In the first case, MAGE-C2 was identified as a component of the SCF CRL through interactions with Rbx1 E3 RING ubiquitin ligase [52].

Interestingly, unlike many of the MRL examples described previously, MAGE-C2 stabilized cyclin E by inhibiting SCF-dependent ubiquitination and subsequent proteasomal degradation [52]. This MAGE-C2-mediated stabilization of cyclin E, an essential regulator of cell cycle transition from G1 to S phase, promotes cell cycle progression and cell proliferation [52]. Importantly, cyclin E expression positively correlates with MAGE-C2 expression in melanoma tumor samples, suggesting that this newly identified function of MAGE-C2 may be relevant in tumorigenesis [52].

A subsequent study found that MAGE-A11 interacts with Skp2, an F-box domain substrate recognition protein of the SCF CRL [53]. In this case, MAGE-A11 regulates the substrate specificity of Skp2, such that MAGE-A11 enhances Skp2-mediated degradation of cyclin A and the retinoblastoma (Rb)-related protein p130, but inhibits Skp2-mediated degradation of the E2F1 transcription factor [53]. The authors account the differential effects of MAGE-A11 on Skp2 by proposing a competitive relationship between MAGE-A11 and Skp2 in binding cyclin A [53]. Collectively, these data suggest that MAGEs may regulate cell cycle progression by modulating SCF ubiquitin ligase activity and substrate recognition.

Regulation of transcription

Intriguingly, a number of MAGEs have been implicated in the regulation of various transcription factors. Although some of the means by which MRLs regulate p53 and E2F1 are described in earlier sections, we highlight here the additional mechanisms of MAGE-mediated transcriptional regulation.

p53 is a transcription factor that responds to a variety of stress signals and coordinates a gene expression program that contributes to tumor suppression. In addition to modulating p53 stability through MAGE-TRIM28-induced ubiquitination and proteasome-dependent degradation, several other roles for MAGEs in regulating p53 have been reported. For example, MAGE-A2 may sterically occlude the p53 DNA-binding domain, recruit histone deacetylases (HDACs), or inhibit the MDM2 E3 ligase to repress p53 transcriptional activity [54–56].

E2F transcription factors are key regulators of cell cycle progression, and E2F1 is essential for the transactivation of target genes involved in the G1/S transition [57]. The Rb tumor suppressor is a critical regulator of E2F1 transcriptional activity [57]. During G1 phase, Rb is in a hypophosphorylated state and binds to the transactivating domain of E2F1, thereby repressing E2F1-dependent transcription [57]. As cells progress toward S phase, cyclin-dependent kinases phosphorylate Rb, and E2F1 is released [57]. E2F1 is then free to transactivate cell cycle progression genes [57]. In the same way, Ncdin and MAGE-G1 bind to the transactivation domain of E2F1 and repress E2F1 transcriptional activity [58].

In contrast, investigation into the mechanisms of MAGE-A11 function demonstrated that MAGE-A11 stabilizes the Rb-related protein p107 and promotes p107 binding to E2F1, thus activating E2F1 transcriptional activity [59]. Recent work by Peche *et al.* also showed that MAGE-B2 interacts with HDAC1, an E2F1 repressor, to enhance E2F1 transactivation [60]. Therefore, whereas some type II MAGEs target and inhibit E2F function, other type I MAGEs may stimulate E2F activity to promote tumor cell proliferation.

The androgen receptor (AR), a member of the nuclear receptor superfamily, is a transcriptional regulator that responds to androgens and is particularly important for the growth and progression of prostate cancer [61]. Therefore, androgen deprivation therapy is often the first line of treatment for patients with prostate cancer [61]. However, over time, prostate cancer cells can develop resistance to androgen deprivation; this type of relapse is referred to as castration-recurrent prostate cancer and is associated with high mortality [61].

MAGE-A11 was first identified as an AR coactivator by a yeast two-hybrid screen of a human testis library [62]. Additional studies revealed that MAGE-A11 binds the AR N-terminal FXXLF motif, thereby recruiting the steroid receptor coactivator (SRC)/p160 coactivators and promoting AR transcriptional activity [63]. However, by directly interacting with the p160 coactivator transcriptional mediator protein (TIF2) and the transcriptional regulator p300, MAGE-A11 is also able to enhance AR-mediated gene activation [64]. Subsequent studies demonstrated that epidermal growth factor (EGF), in the presence of dihydrotestosterone, stabilizes the MAGE-A11-AR complex through phosphorylation of MAGE-A11 at threonine 360 and ubiquitination of lysine residues 240 and 245 [65]. Interestingly, during androgen deprivation therapy, MAGE-A11 levels increase in prostate cancer, suggesting that MAGE-A11 plays a key role in the progression of castration-recurrent prostate cancer by enhancing AR transcriptional activity [66].

Summary of MRL function

Since the discovery that MAGEs function in complex with E3 RING ubiquitin ligases, they have been shown to regulate not only E3 enzymatic activity but also substrate recognition and cellular localization. These MRLs act on a diverse array of cellular pathways that have clear implications in various pathologies associated with MAGEs, including transcription, metabolism, protein trafficking, and cell proliferation. However, these mechanistic studies are small in number and further work to determine the functions of additional MAGE members and their direct contributions to disease will be invaluable in our understanding of the MAGE family of proteins. In addition, important challenges in the future include identifying the E3 RING ubiquitin ligases that function

with orphan MAGEs and discovering the substrates of novel and known MRLs, such as MAGE-A1-TRIM31, MAGE-B18-LNX1, and MAGE-G1-NSE1.

Physiological Expression and Function

Type I MAGEs and germ cell development

Many of the type I MAGEs, consistent with their classification as CTAs, are normally expressed only in germ cells and/or placenta. However, due to the high homology between subfamily members and the lack of specific antibodies, determination of the exact spatiotemporal expression profiles of these proteins is not trivial. Initial characterization by Reverse transcription (RT)-PCR revealed that MAGE-A1–A4, -A6, and -A12 are expressed in testis; MAGE-A4 and MAGE-A8–A11 are also expressed in placenta [5]. Like the MAGE-A subfamily, the MAGE-B and MAGE-C genes are also expressed only in testis, with MAGE-B2 expressed in both testis and placenta [3,8,11,67]. This restricted expression to testis suggests a functional role for type I MAGEs in germ cell development.

In males, primordial germ cells (PGCs), the progenitor cells of gametogenesis, are surrounded by somatic Sertoli cells and become prospermatogonia, which proliferate for a few days and then arrest at G0/G1 until birth (Fig. 5) [68]. At puberty, proliferation resumes to initiate spermatogenesis. Spermatogonia are the germ cells of spermatogenesis that remain proliferative throughout life to maintain the pool of stem cells or undergo differentiation to produce spermatozoa. This process occurs through two meiotic divisions, in which tetraploid primary spermatocytes undergo meiosis I to form diploid secondary spermatocytes, which then undergo meiosis II to form haploid spermatids that develop into spermatozoa.

Early immunohistochemical staining demonstrated MAGE-A1 and -A4 expression in spermatogonia and primary spermatocytes but not in spermatids or Sertoli cells of adult testes [69]. Subsequent studies using a different antibody that reacts with MAGE-A1, -A3, -A4, -A6, and -A12 detected MAGE-A expression in migrating PGCs in 5-week-old human embryos and in the nuclei and cytoplasm of spermatogonia and spermatocytes in adult testes [70,71]. Like the human MAGE genes, the murine *Mage-a* genes are expressed in spermatogonia undergoing maturation toward the spermatocyte stage [72,73]. These findings indicate that the expression of these proteins is highly regulated and could play an active role in spermatogenesis. Consistent with this notion, a recent mouse model with the deletion of *Mage-a*1, -a2, -a3, -a5, -a6, and -a8 exhibited reduced size of the testes and diameter of seminiferous tubules [74]. Furthermore, deletion of the *Mage-a* cluster led to an increase in

apoptotic germ cells, primarily in the first wave of testicular apoptosis, and the activation of p53 and induction of Bax in response to genotoxic stress [74]. Moreover, mutations in MAGE-A9B, -C1, and -C3 were identified in a cohort of infertile men [75,76]. Collectively, these results support the hypothesis that these proteins play a critical role in germ cell development.

Interestingly, *Mage-b*4, like *Mage-a* genes, is preferentially expressed in spermatogonia, whereas *Mage-b*1 and -b2 are found in postmeiotic spermatids [25,72,73]. In male germ cells, *Mage-b*4 is preferentially expressed during cell cycle arrest. When cells resume mitosis and enter meiosis, *Mage-b*4 protein levels decrease and are hardly detectable in pachytene cells, suggesting that *Mage-b*4 may be important for the cell cycle arrest of male germ cells. This differential expression of MAGEs led investigators to hypothesize that *Mage-a* and *Mage-b*4 might be involved in germ cell differentiation while *Mage-b*1 and -b2 regulate spermiogenesis.

In female germ cells, MAGE-A1 is expressed in the human oogonia prenatally, and MAGE-A4 is expressed in some migrating PGCs and early oogonia in female human embryos [77,78]. In addition, *Mage-b*4 is expressed in premeiotic germ cells and during the pachytene and telophase portions of meiosis, suggesting that MAGEs might also function in developing oocytes [25].

In addition to their role in germ cells, MAGE-A proteins may also be involved in neuronal development. By immunohistochemistry, MAGE-A reactivity was detected in the spinal cord and brain stem of the early developing CNS and in peripheral nerves. Investigators also report MAGE-A-positive PGCs in the adrenal cortex of early fetuses [71]. Therefore, MAGE-A might also function during embryonic development. However, additional mouse models and mechanistic studies are required to demonstrate the functional relevance of MAGEs in germ cells and to determine how their physiological roles may be co-opted in the context of cancer.

Type II MAGEs and neural development

Type II MAGEs include the MAGE-D, -E, -F, -G, -H, -L, and *Necdin* genes. In contrast to the type I MAGEs, type II MAGE genes are ubiquitously expressed at various levels in many tissues. Intriguingly, a number of these type II MAGEs are enriched in the brain and have been implicated in various neural processes.

MAGE-D1, also referred to as NRAGE or Dlxin, is a type II MAGE that has been implicated in multiple pathways including apoptosis, cell cycle progression, and differentiation (reviewed in Ref. [4]). Initial analysis showed that MAGE-D1 is highly expressed in the brain but is also detected in most embryonic and adult tissues [3]. Additional investigation into MAGE-D expression showed that the members of this subfamily are widely expressed throughout the

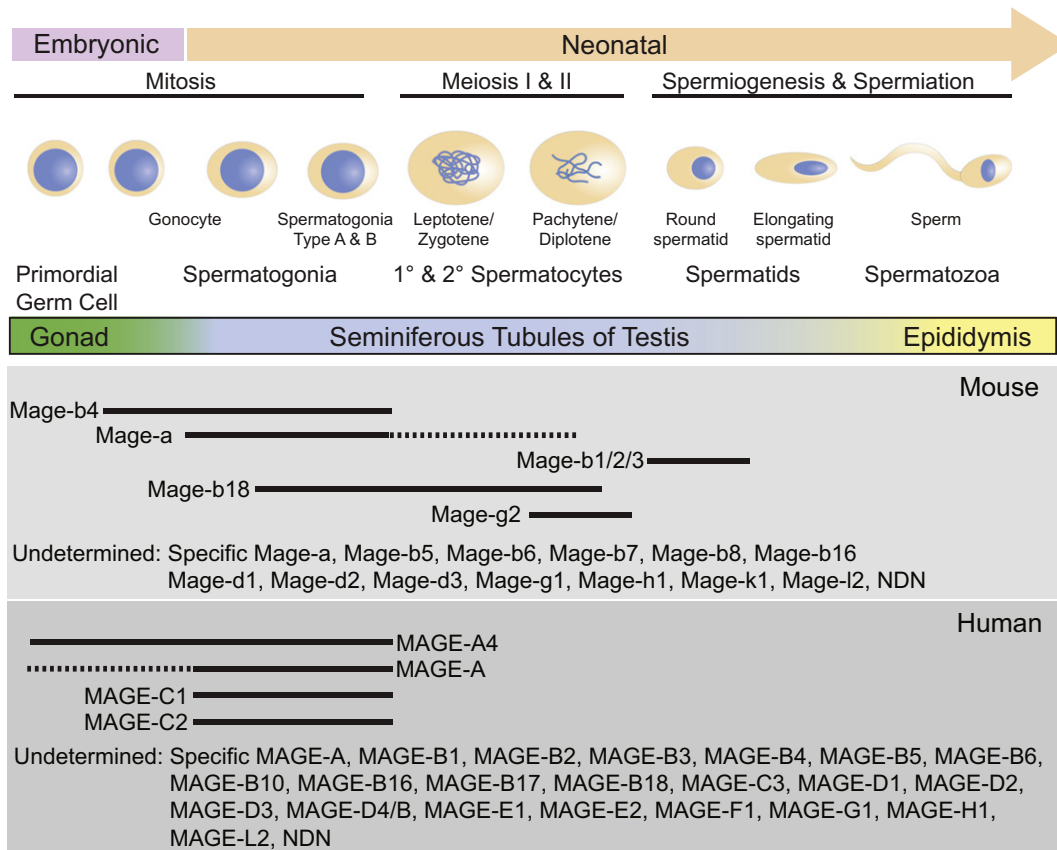


Fig. 5. MAGEs are differentially expressed during spermatogenesis. Schematic diagram of germ cell development and where specific MAGE genes are expressed. The expression profiles of most MAGE genes are unknown.

human adult brain, with strongest signals in the cerebral cortex and medulla [79]. Based on the widespread distribution of MAGE-D genes in the brain, it is likely that this MAGE subfamily plays a general role in neural differentiation and maintenance. Interestingly, the MAGE-D genes are located in a chromosomal region associated with many monogenic X-linked neurodevelopmental disorders [80]. Therefore, the enrichment of MAGE-D in the cerebral cortex and hippocampus—the structures involved in higher function—suggests that these genes can be candidates for such disorders.

Consistent with this, *Mage-d1* knockout mice demonstrate symptoms of depression such as decreased locomotor activity, social interaction, and reward responsiveness, as well as increased anxiety and immobility time; and treatment with antidepressants attenuated some of these behavioral changes related to depression [81]. Notably, *Mage-d1*-null mice display decreased extracellular serotonin levels and increased serotonin transporter (SERT) protein levels, suggesting that deficiency in MAGE-D1 induces both behavioral and neurological phenotypes of depression [81]. Further investigation revealed that MAGE-D1 regulates ubiquitination and proteasomal degradation of SERT, consistent with previous reports

associating MAGE-D1 with PRAJA-1 E3 ligase and the modulation of *Msx2*- and *Dlx5*-dependent transcription and neuronal differentiation [81–83]. In an alternative *Mage-d1*-deficient (hemizygous) mouse model, loss of *Mage-d1* results in reduced social interactions, decreased sexual activity leading to infertility in males, reduced motor activity, late-onset obesity associated with hyperphagia, and increased anxiety-like behaviors [84]. Several of these phenotypes can be explained by significantly reduced levels of mature oxytocin in the brain of *Mage-d1*-deficient mice and suggest that the combined effects of reduced SERT and oxytocin can contribute to an altered serotonergic system and the observed phenotypes in the *Mage-d1* knockout model [81,84].

Intriguingly, several of the phenotypes reported in the *Mage-d1*-deficient mice, including hyperphagia and reduced sociability, mimic symptoms of individuals with Prader–Willi syndrome (PWS) and autism spectrum disorder (ASD). In fact, one of the earliest insights into the physiological function for MAGE genes came from the genetic analysis of PWS, which showed that individuals with PWS bear deletions or mutations within a specific chromosomal region containing *NECDIN* and *MAGEL2* and with MAGE-G1 in close proximity [85,86].

Necdin mRNA is ubiquitously expressed and is detected in all developing neurons in the central and peripheral nervous systems in early development [87,88]. After E13, Necdin is enriched in discrete regions of the nervous system, such as the hypothalamus, thalamus, and pons, suggesting a specific spatial and temporal function therein [89]. Like MAGE-D1, various studies have implicated Necdin in neuronal differentiation and survival [90–92].

In two independent Necdin-deficient mouse models, mutant mice exhibited respiratory distress and death [87,93]. Strikingly, individuals with PWS exhibit similar breathing defects with irregular rhythm and often manifest sleep apneas [94]. Investigation into the observed respiratory failure in mice demonstrated that Necdin is expressed in medullary serotonergic neurons, and Necdin deficiency alters the serotonergic metabolism, thereby contributing to abnormal respiratory rhythmogenesis [94,95].

Although the surviving Necdin-deficient mice showed a normal growth pattern, the mutant mice exhibited altered behavioral phenotypes reminiscent of PWS patients, such as increased skin scraping and enhanced spatial learning and memory [87]. In addition, immunohistochemistry revealed a significant decrease in the number of oxytocin- and luteinizing hormone-releasing hormone (LHRH)-producing neurons in the hypothalamus—the primary region of the brain involved in PWS.

MAGE-L2 is widely expressed in fetal tissues and is enriched in various parts of the brain [96]. Unlike its human counterpart, mouse *Magel2* is almost exclusively expressed in the hypothalamus and peaks during neurogenesis during E15–17; however, it is also detected in non-neuronal tissues such as the genital tubercle, midgut region, and placenta [51,96].

Loss of MAGE-L2 is also implicated in neurodevelopmental disorders, and multiple mouse models have been generated to determine its physiological function. *Magel2*-null mice, generated by *lacZ* knock-in allele, exhibited 10% postnatal lethality, normal birth weights, and slightly decreased food intake [97]. However, close observation of their growth pattern revealed that the *Magel2*-deficient mice displayed a two-phase weight curve as seen in PWS patients, such that mutant mice showed delayed growth and decreased weight gain in the first 4 weeks of life, followed by increased weight gain, higher fat mass, and elevated leptin, insulin, and cholesterol levels [98]. It was later discovered that *Magel2*-null mice have reduced muscle mass and increased expression of atrophy genes, indicating that the loss of *Magel2* contributes to hypotonia and related musculoskeletal abnormalities such as scoliosis and digital contractures [99].

Consistent with PWS phenotypes and cellular evidence that MAGE-L2 modulates the activity of circadian rhythm proteins, *Magel2*-null mice exhibit reduced daytime activity and disrupted circadian regulation [97,100,101]. In addition, the amounts of

orexins (the neuropeptides that regulate wakefulness), orexin-positive neurons, and orexin-2 receptors were all reduced in the hypothalamus of *Magel2*-deficient mice [97]. Taken together, these data suggest that *Magel2* is required for proper hypothalamic function and maintains circadian rhythm potentially through the regulation of orexin levels.

Additional behavioral assays reported increased anxiety-like behavior in *Magel2*-null mice. Magnetic resonance imaging (MRI) analysis found that regions of the brain with moderate to high *Magel2*, such as the amygdala, hippocampus, and the nucleus accumbens, but not the hypothalamus, were significantly smaller in *Magel2*-deficient mice [102]. In addition to reduced brain volume, the *Magel2*-mutant mice have reduced serotonin and its metabolite 5-HIAA and reduced dopamine. Both serotonergic and dopaminergic pathways are implicated in various neurobehavioral disorders typically seen in PWS patients, including anxiety, depression, and obsessive behavior. Administration of selective serotonin reuptake inhibitors in PWS mitigates aggressive and compulsive behaviors, further substantiating the notion that altered serotonergic pathway contributes to some of the behavioral aspects of PWS [103,104].

Investigation into the reproductive functions of MAGE-L2 showed early-onset reproductive decline in *Magel2*-null mice [105]. In males, testosterone, but not luteinizing hormone or follicle-stimulating hormone levels, were reduced; however, despite reduced testosterone, the male reproductive organs and sperm show no overt differences. Interestingly, the male *Magel2*-deficient mice had altered olfactory preference. Female mice had delayed and lengthened puberty and were infertile by 24 weeks. Although there were no differences in the gross anatomy of ovaries and uteri collected from the infertile 26-week-old *Magel2*-null females, the ovarian histology of these mice showed an absence of corpora lutea, suggestive of normal folliculogenesis with missed ovulations. In fact, *Magel2*-null females exhibited abnormal estrous cycles, similar to other circadian mutant female mice and women with PWS.

In a more recent *Magel2* mouse model, *Magel2*-deficient mice exhibited 50% postnatal mortality and the impaired suckling and subsequent feeding deficits seen in PWS newborns [106]. In the hypothalamus, *Magel2* mutant neonates had reductions in the hypothalamic neuropeptides oxytocin, orexin-A, and arginine-vasopressin. Interestingly, injection of oxytocin just after birth rescued the suckling initiation defects and the neonatal lethality.

These observations from multiple mouse models, and complementary cellular studies, draw striking parallels to neurological disorders. Taken together, the data suggest that many type II MAGEs play critical roles in differentiation and neural development, such that loss of function leads to a spectrum of cognitive, behavioral, and developmental deficits.

MAGEs in Disease

MAGE-L2 in Prader-WilliWS and Schaaf-Yang syndromes

Prader-Willi syndrome (PWS OMIM 176270) is a complex neurodevelopmental disorder that was first described in the medical literature in 1956 [107]. It is characterized by infantile hypotonia with poor suck and failure to thrive, often necessitating assisted feeding [108]. Beginning in early childhood and overtime, individuals with PWS exhibit hyperphagia, rapid weight gain, developmental delay, intellectual disability, hypogonadism, and short stature [108]. In addition to these cardinal features of PWS, a characteristic behavior and cognitive profile, including reduced activity, obsessive-compulsive traits, and temper outbursts (often associated with food and eating), has also been ascribed to this multisystem syndrome [108,109].

The genetic causes of PWS include deletion of paternal 15q11-q13 (65–75% of cases), maternal uniparental disomy (20–30%), and imprinting defects (1–3%) [85]. Although the deletion sizes can be variable, most individuals with PWS lose the expression of genes in the PWS locus (15q11-q13), which comprises MKRN, MAGEL2, NDN, NPAP1, SNURF-SNRPN, a family of six small nucleolar RNA (snoRNA) genes, and several long non-coding RNAs (lncRNAs) (Fig. 6a) [85]. This locus has also been associated with general neuropsychiatric illness and ASD [110,111]. In fact, 10–40% of individuals with PWS meet the criteria for ASD [112]. Genotypic and phenotypic correlations have revealed that individuals with PWS caused by maternal uniparental disomy are more commonly affected with ASD (38%) than those with PWS caused by microdeletion (18%) [113].

Despite the evident importance of genes in the PWS locus, the individual phenotypic contribution of each gene is not entirely clear. Previous reports have shown that individuals with deletions of the SNORD116 snoRNA cluster presented with key characteristics of PWS; however, an individual with paternal deletion of MKRN3, MAGEL2, and NDN exhibited obesity and intellectual disability but not the typical PWS phenotype [114–117]. This indicates that although the loss of multiple genes may be required to produce the syndrome, the loss of individual genes could contribute to the various distinct phenotypes of this complex disorder.

Schaaf *et al.* initially identified four individuals with truncating mutations on the paternal allele of MAGEL2—the first individuals reported as having point mutations in a protein-coding gene within the PWS locus (Fig. 6b) [118]. All four individuals presented with ASD, intellectual disability, and varying degrees of PWS phenotype. For example, while subject 2 exhibited classic PWS according to the

diagnostic criteria established by Holm *et al.*, the other three subjects did not meet the full clinical criteria [108,118]. Due to the phenotypic overlap with PWS, the condition was initially considered a PWS-like syndrome. However, as the cohort of individuals with truncating MAGEL2 mutations grew, it became more apparent that the clinical condition caused by these mutations manifests specific phenotypes distinct from PWS. For example, hyperphagia and subsequent obesity—hallmarks of PWS—were either absent or only mildly present in individuals with MAGEL2 mutations [118,119]. In addition, ASD was overrepresented among individuals with molecularly confirmed mutations in MAGEL2 [118,119]. Moreover, joint contractures, a phenotype rarely reported in PWS, were found in 23 of the 28 cases with MAGEL2 mutations [118–120]. To highlight these phenotypic differences, the clinical condition caused by truncating MAGEL2 mutations was renamed Schaaf-Yang syndrome (SHFYNG; OMIM 615547).

In determining the molecular function of MAGE-L2, our lab demonstrated that MAGE-L2, in complex with the TRIM27 E3 RING ubiquitin ligase and USP7 deubiquitinating enzyme, regulates WASH-dependent actin polymerization and protein trafficking (refer to Regulation of WASH-mediated endosomal protein trafficking by the MAGE-L2-USP7-TRIM27 (MUST) complex), Fig. 4f) [50,51]. These findings, taken together with the characterization of MAGE-L2 truncating mutations in individuals with SHFYNG, led us to hypothesize that similar phenotypes may also present in individuals with USP7 mutations. We identified seven cases with either heterozygous deletion or mutation of USP7 that resulted in phenotypes similar to those seen in SHFYNG and PWS, including intellectual disability, ASD, hypotonia, and hypogonadism [51]. Interestingly, these individuals with USP7 haploinsufficiency did not present with the typical PWS phenotypes of infantile feeding difficulties, hyperphagia, excessive weight gain, and characteristic craniofacial features [51]. They did, however, exhibit phenotypes specific to USP7 mutation or deletion, such as seizures and aggressive behavior, suggesting that specific genes in the PWS locus contribute to a spectrum of shared and independent phenotypes [51].

Intriguingly, individuals with mutations in WASH complex components also exhibit neurological pathologies. For example, autosomal dominant mutations in spastic paraplegia (SPG8), the gene encoding Strumpellin, result in hereditary spastic paraplegia [121,122]. Likewise, recessive mutations in KIAA1033, the gene encoding SWIP, are associated with autosomal recessive intellectual disability and late-onset Alzheimer's disease [123,124]. In addition, TRIM27 has been implicated in ASD and Parkinson's disease, suggesting that alterations in protein recycling may contribute to neurological disorders [125,126].

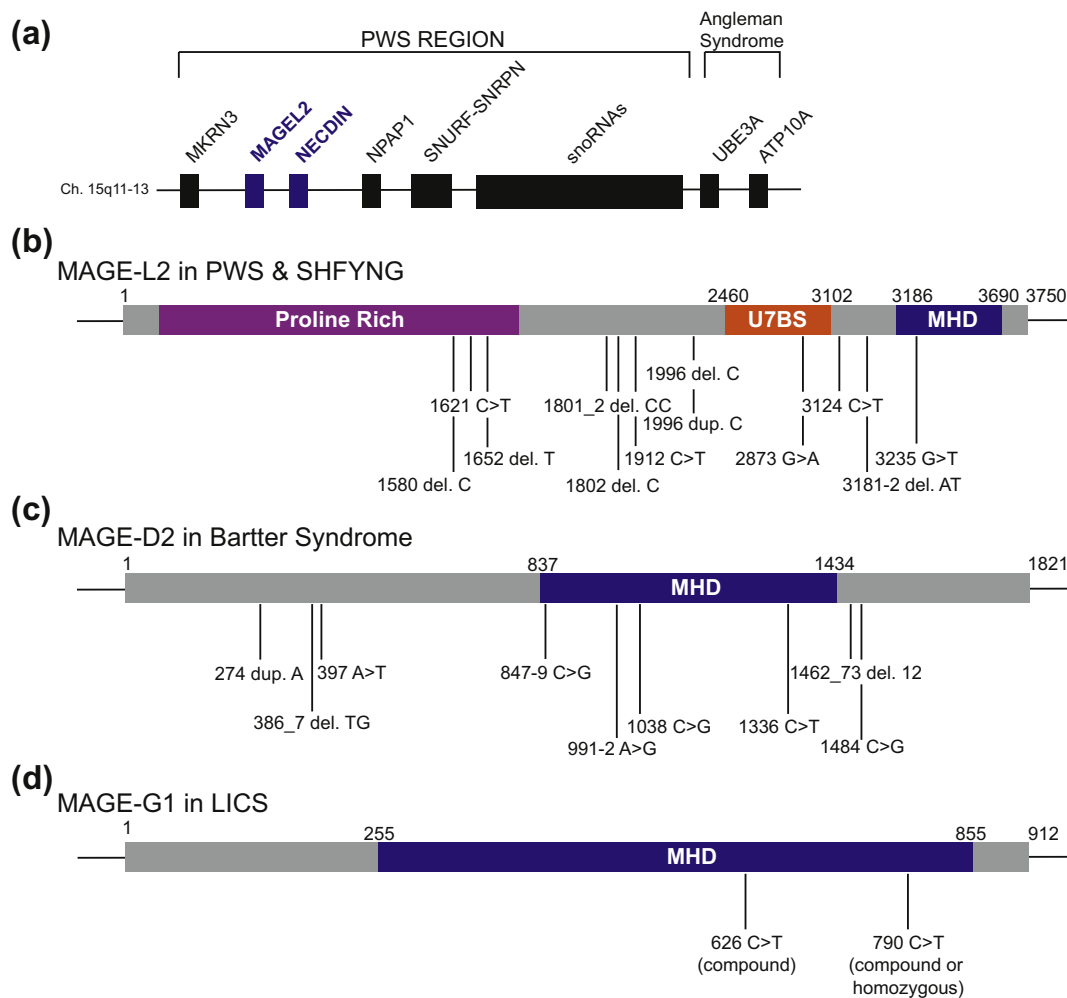


Fig. 6. Type II MAGEs are associated with various diseases. (a) Schematic representation of the PWS locus. Identified mutations in (b) MAGEL2, (c) MAGE-D2, and (d) MAGE-G1 associated with PWS and SHFYNG, Bartter syndrome, and lung disease immunodeficiency and chromosome breakage syndrome (LICS), respectively.

MAGE-D2 in Bartter syndrome

Polyhydramnios is the excessive accumulation of amniotic fluid caused by an imbalance between production and removal the fluid [127]. While most cases of polyhydramnios are mild and result from the gradual increase of amniotic fluid, severe polyhydramnios may result in preterm birth and an increased risk of other perinatal complications [127]. Some of the known causes of polyhydramnios include fetal esophageal atresia and maternal diabetes; however, in 30–60% of cases, the cause remains unknown [127–129]. Antenatal Bartter syndrome (OMIM 300971), one of the few Mendelian diseases associated with polyhydramnios, is a rare, often life-threatening autosomal recessive renal tubular disorder characterized by fetal and postnatal polyuria, renal salt wasting, hypercalciuria, and hypokalemia [130].

Previously it was known that mutations in SLC12A1 (encoding NKCC2), KCNJ1, CLCNKA,

CLCNKB, or BSND impair the kidneys' ability to reabsorb salt and can cause Bartter syndrome [131]. Recently, Laghmani *et al.* identified mutations in MAGE-D2 that cause X-linked polyhydramnios with prematurity and a transient but severe form of antenatal Bartter syndrome (Fig. 6c) [129]. These patients initially exhibited a more severe presentation of antenatal Bartter syndrome with earlier onset of polyhydramnios and preterm labor for male offspring [132]. Immediately after birth, the infants developed progressive polyuria and severe hypercalciuria; however, within weeks, clinical symptoms spontaneously resolved [132]. Through genetic sequencing of nine families with transient antenatal Bartter syndrome and idiopathic polyhydramnios, the authors identified seven truncating mutations (two nonsense, two frameshift, and three splice-site mutations) and two nontruncating mutations (one missense and one in-frame deletion) in MAGE-D2 [129].

Further investigation revealed that MAGE-D2 promotes the expression and activity of the two crucial sodium chloride cotransporters (NKCC2 and NCC) necessary for proper ion reabsorption in the thick ascending limb of the loop of Henle and distal tubules [129]. Additionally, Laghmani and colleagues demonstrate that MAGE-D2 interacts with HSP40, a cytoplasmic chaperone that interacts with both NKCC2 and NCC and has been shown previously to regulate the biogenesis of NCC [129,133]. Collectively, these findings indicate that MAGE-D2 plays a key role in fetal renal salt absorption, amniotic fluid homeostasis, and maintenance of normal pregnancy. However, further work will be important to determine how MAGE-D2 regulates NKCC2 and NCC and if this regulation is dependent on ubiquitin-mediated trafficking.

MAGE-G1 in lung disease immunodeficiency and chromosome breakage syndrome (LICS)

Members of the SMC family of proteins form three highly conserved heterodimeric complexes that regulate mitotic proliferation, meiosis, and DNA repair to support genomic stability [19]. One such complex, the SMC5/6 complex, consists of SMC5 and SMC6 in addition to non-SMC elements including the MAGE-G1-NSE1 MRL [18,21]. The SMC5/6 complex promotes homologous recombination-mediated DNA repair and is essential for DNA damage response and telomere lengthening by recombination [19,20,134].

Recently, a report associated missense mutations in MAGE-G1 with an autosomal recessive chromosome breakage syndrome that leads to severe lung disease in early childhood (referred to as LICS; Fig. 6d) [135]. Two sisters (subjects A and B) with homozygous MAGE-G1 mutations (c.790C > T) exhibited B and T cell abnormalities, increased infection susceptibility, and eczema. In addition, they experienced feeding difficulties, failure to thrive, weight loss, psychomotor retardation, and axial hypotonia—phenotypes reminiscent of PWS and SHFYNG. After the onset of pediatric acute respiratory distress syndrome, these individuals also manifested respiratory complications such as pneumomediastinum, pneumothorax, and subcutaneous emphysema. Following multiple episodes of virus-induced pneumonia, the affected individuals experienced severe progressive irreversible lung damage and died at 12 and 14 months. An affected brother and sister (subjects C and D, respectively) from a second family also developed a similar clinical history of progressive and severe pediatric acute respiratory distress syndrome and infectious pneumonia. Exome sequencing of the second family revealed compound heterozygous mutations in MAGE-G1 with a maternally inherited c.790C > T mutation (identical to the first family) and a paternally inherited c.626C > T mutation [135].

In the SMC5/6 complex, MAGE-G1-NSE1 and NSMCE4 form a molecular bridge between SMC5 and SMC6 and are essential for complex formation [136,137]. Interestingly, the two identified variants of MAGE-G1 result in p.Leu264Phe (L264F) and p.Pro209Leu (P209L) and were both shown to disrupt interactions with NSMCE4 and destabilize the SMC5/6 complex [135]. Consistent with this, SMC5 and SMC6 protein levels were significantly reduced while MAGE-G1 protein was not detectable in fibroblasts from affected individuals [135]. Moreover, cells from subject B exhibited increased numbers of micronuclei, a hallmark of genome instability [135]. These cells also demonstrated hypersensitivity to various DNA-damaging agents and defective homologous recombination. In addition, cells from subject B displayed defects in recovery from replication stress, similar to MMS21/NSMCE2-defective cells, and could be rescued by expression of wild-type MAGE-G1 [135].

Therefore, the identified mutations in affected individuals alter the stability of the SMC5/6 complex and result in faulty homologous recombination and impaired recovery from replication stress. Although the affected individuals manifest clinical features similar to those seen in Nijmegen breakage and AT chromosomal breakage syndromes, the severe and ultimately fatal pulmonary disease is unique to this novel chromosome breakage syndrome [135].

Type I MAGEs in cancer

In addition to their physiological expression in reproductive tissues, the type I MAGE CTAs are re-activated in a wide variety of tumors (Table 1). This aberrant expression of type I MAGEs and their prognostic value in various cancers have been extensively documented (Table 2).

Given that the MAGE CTAs were originally identified in melanoma cells, it is not surprising that a number of MAGEs are expressed at high frequencies in melanoma. However, it is interesting that MAGE-A1–4 and -C1 expression changes over the course of cancer progression, such that these MAGEs express at higher frequencies in metastases compared to primary melanoma samples [138–140]. In addition, expression of these MAGE-A genes associates with thicker tumors and ulcerated melanomas, supporting the notion that high MAGE CTA expression correlates with advanced tumor grade [138,139].

This trend is not limited to melanoma. For example, MAGE-A3/6 and -C2 expression in breast cancer associates with tumor estrogen receptor- and progesterone receptor-negative status, high histologic grade, and worse survival [141,142,209]. In ovarian cancer, MAGE-A1, -A9, and -A10 expression also correlate with poor survival [143,144]. Likewise, MAGE-A1, -A2, -A3/6, -A12, -B2, and -C1 are expressed in non-small cell lung cancer (NSCLC) where MAGE-A3/6 and -A9 expression are

Table 1. Summary of MAGE expression in select cancer types

MAGE ^a	Percentage of MAGE-positive patient tumors					References
	Melanoma	Lung	Breast	Ovarian	HNSCC	
A1	16–61%	24–49%	6%	11–54%	16–23%	[138, 139, 143, 146, 147, 149, 207]
A2	44–84%	40%	20%	3%	20–31%	[138, 149]
A3	48–80%	38–55%	10–26%	3–37%	29–41%	[42, 138, 141, 143, 147–150, 207]
A4	9–34%	19–65%	13–19%	32–47%	23–47%	[138, 139, 143, 147, 149, 207]
A6	48–82%	26–50%	28%	5%	31–43%	[42, 141, 148–150]
A8	4–9%	7%	4%	3%	1–3%	[208]
A9	8–23%	69%	16–45%	25–37%	25–33%	[144, 207]
A10	13–61%	43%	8%	19–52%	13–19%	[147, 207]
A11	15–41%	61%	8–67%	33%	18–38%	[208]
A12	45–77%	27%	9–15%	5%	13–28%	[208]
B1	4–23%	9%	25%	8%	0–7%	[208]
B2	10–27%	41%	3%	8%	19–35%	[151]
B3	0%	0%	1%	5%	1%	[208]
B4	0%	0%	3%	0%	0%	[208]
B6	18–25%	55%	1%	5%	20–27%	[150]
B16	6–21%	6%	21%	1%	1–4%	[208]
B18	9–20%	9%	4%	18%	1–5%	[208]
C1	24–62%	28%	5–38%	16–35%	9–13%	[138, 140, 143, 153, 207, 209]
C2	33–67%	21%	8–58%	13%	4–13%	[142, 209]

^a MAGE expression correlates with DNA methylation status (highlight)

associated with advanced tumor type and decreased survival [145–147,207]. Similarly, expression of MAGE-A1-6, -B2, and -B6 in patients with head and neck squamous cell carcinoma (HNSCC) correlates with advanced clinical stage of cancer and poor oncologic outcomes [148–151]. Furthermore, MAGE-A1, -A6, -A8, -A9, and -A11 are expressed at significantly higher levels at the tumor front of advanced stages of HNSCC, suggesting that these MAGEs contribute to malignancy [152].

Indeed, a growing body of evidence supports the notion that MAGEs function as oncogenic drivers, rather than simple biomarkers or passengers of global genomic dysregulation [208]. Consistent with this idea, some studies have reported that MAGEs can be turned on early during the process of tumorigenesis even before the clinical signs of the disease [145]. Furthermore, investigation into the oncogenic potential of these genes has demonstrated that MAGE-A3/6 is required for the viability of patient-

Table 2. Correlation between MAGE expression and overall survival in select cancer types

Cancer type	MAGE	Overall survival			References
		HR	CI (95%)	P-value	
Lung	A3	3.226	1.446–7.918	0.004	[42,146,210]
	A9	2.334	1.664–3.274	0.001	
	C2	1.909	0.954–3.821	0.068	
Breast	A1	2.285	0.910–5.732	0.078	[211]
	A3	3.446	1.00–11.77	0.049	
	A9	3.702	1.392–9.845	0.009	
Ovarian	C2	3.07	1.47–12.01	0.003	[142]
	A9	2.271	1.372–3.761	0.001	
	A9	2.376	1.380–4.089	0.002	
Colorectal	A9	3.57	1.457–8.762	0.005	[214]
Laryngeal	A9	2.17	1.121–4.205	0.022	[215]
Liver	A11	2.689	1.434–5.040	0.002	[216]
Esophageal	A12	1.78	1.23–2.58	0.002	[217]
Gastric					

HR, hazard ratio; CI, confidence interval

derived breast, colon, lung cancer, and multiple myeloma cells, whereas MAGE-A3/6 does not significantly alter the viability of MAGE-A3/6-negative cells [42,153]. This specificity suggests that upon the expression of MAGE-A3/6, these cancer cells become dependent on or addicted to MAGE-A3/6 expression for viability [42]. Consistent with these findings, MAGE-A3/6 expression drives several hallmarks of cancer such as cell proliferation, cell migration, invasion, and anchorage-independent growth (Fig. 7a) [42,154]. Remarkably, expression of MAGE-A3/6 is sufficient to stimulate foci formation in fibroblasts and promote anchorage-independent growth in non-transformed human colonic epithelial cells [42]. In an orthotopic xenograft mouse model for thyroid cancer, MAGE-A3/6 expression results in significantly increased tumor growth and larger, more numerous lung metastases [154]. These results are consistent with a model wherein MAGE-A3/6 functions as a potent driver of tumorigenesis.

While most MAGE-related studies focus on the MAGE-A subfamily and their involvement in cancer, the MAGE-B and -C subfamilies are also associated with tumor growth and progression (Fig. 7a). For example, knockdown of mouse *Mage-b* genes reduces cell viability in melanoma and mast cell lines [37,155]. Similarly, MAGE-B2 promotes cell proliferation in transformed oral keratinocytes, osteosarcoma, and colon cancer cell lines [60,151]. Moreover, *Mage-b* knockdown suppresses growth in a syngeneic mouse model, whereas overexpression of MAGE-B2 enhances tumor growth in mice [37,60]. Likewise, MAGE-C2 has also been shown to promote cell proliferation in malignant melanoma cells and tumor metastasis *in vivo* by enhancing STAT3 signaling [52,156]. These findings indicate that MAGE CTAs have oncogenic functions. Additional studies are needed, especially with regard to the MAGE-B and -C subfamilies, to determine the specific activities and mechanisms by which MAGE CTAs promote cancer development.

Interestingly, MAGE CTAs exhibit preferential expression in cancer stem cells (CSCs), a small population of cancer cells that have the ability to self-renew, differentiate, and initiate tumor growth [157]. Analysis of the MAGE CTA expression profiles in cancer stem-like cells demonstrated that MAGE-A2 and -A3/6 are enriched in the CSC-like side population cells derived from lung and colon adenocarcinoma cells, while MAGE-A4 and -B2 are preferentially expressed in the CSC-like side population derived from colon adenocarcinoma cells [158]. Additionally, MAGE-A3 exhibits enriched expression in the CSC-like side population in bladder cancer [159]. Consistent with these findings, MAGE-C1 expresses in stem cells and immature B cells [160]. This preferential expression of MAGE CTAs in CSCs is reminiscent of their physiological enrichment in spermatogonial stem cells. The marked similarities

between these cells types, such as immortalization, immune evasion, and induction of specific differentiation pathways, have led investigators to speculate that the expression of MAGEs and activation of their physiological functions in cancer initiate a gametogenic program that might contribute to tumor formation, progression, and CSC maintenance (Fig. 7b).

Transcriptional Regulation

Although there is an abundance of evidence for the deregulated expression of type I MAGE CTAs in cancer, the precise mechanisms regulating their aberrant expression are only beginning to be elucidated. While some cancers such as melanoma and lung cancers frequently express MAGEs, others including leukemia and uveal melanoma rarely do. In addition, some tumors tend to express a single or few MAGEs, while others express multiple MAGEs simultaneously [161]. Although the details of how specific MAGE genes get turned on in cancer are not fully understood, it is apparent that epigenetic events, including DNA methylation and histone modifications, contribute to the regulation of MAGE expression in both normal and neoplastic cells (Fig. 7c).

DNA methylation

DNA methylation of promoter CpG dinucleotides by DNA methyltransferases (DNMTs) exerts a repressive effect on transcription by preventing transcription factor binding and by recruiting methyl-CpG-binding domain proteins, which in turn bind chromatin remodeling co-repressor complexes to repress gene expression. A growing body of literature has shown that DNA methylation is the primary regulation of CTA expression in normal and cancer cells. In fact, Weber and colleagues provided the first insights into the transcriptional regulation of MAGEs by demonstrating that 5-aza-2'-deoxycytidine (decitabine), a DNMT inhibitor, induces MAGE-A1 expression in cultured melanoma cells [162]. Subsequent analysis revealed that the expression of several MAGE-A genes correlates with the methylation status of its promoter in various types of neoplastic cells [163,164]. Importantly, an unmethylated MAGE-A1 promoter was shown to drive the expression of a reporter gene in MAGE-A1 nonexpressing cells, indicating that the methylation status of the MAGE CTA promoter is the leading mechanism for regulating expression [165,166]. Likewise, downregulation of DNMT1 results in the activation and stable hypomethylation of a methylated MAGE-A1 transgene in melanoma cells [167]. Interestingly, Sigalotti *et al.* reported an association between DNA hypomethylation of CTA promoters and CTA expression in populations of putative melanoma stem cells, suggesting that this form of epigenetic regulation is potentially an important

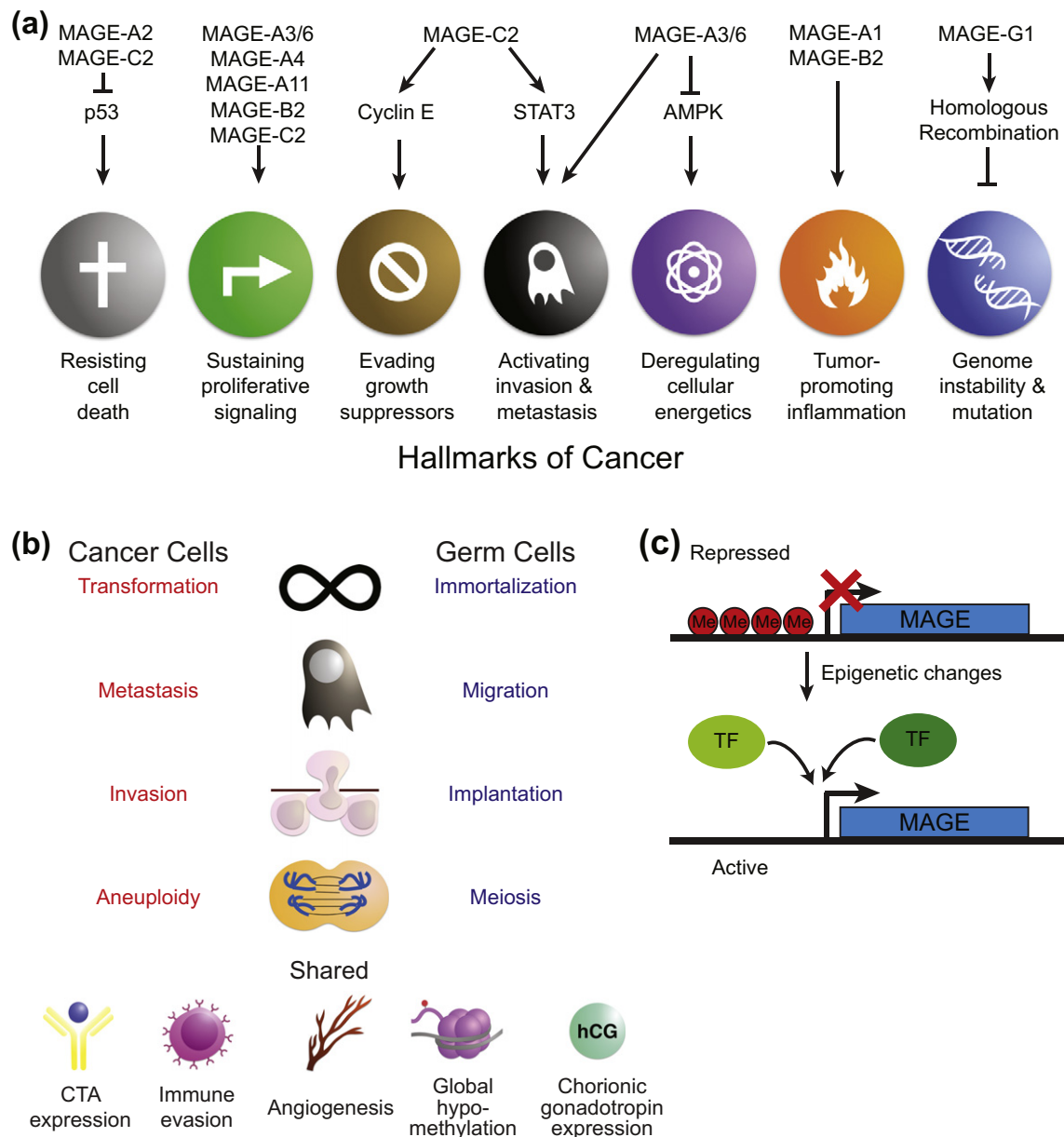


Fig. 7. Type I MAGEs promote tumorigenesis. (a) Specific functions of several type I MAGEs in driving the hallmarks of cancer are shown. (b) The shared phenotypes between cancer cells and germ cells suggest that the activation of the gametogenic program might contribute to tumorigenesis. (c) Transcriptional activation of MAGEs. Type I MAGEs are typically silenced but can be activated by epigenetic changes (including DNA CpG demethylation) and specific transcription factors.

mechanism of CTA gene regulation in cancer stem cells [168]. Therefore, methylation-specific PCR can be used to evaluate the hypomethylation status of CpG sites in the promoter regions of MAGEs as a method for early cancer detection [145]. However, while DNA methylation clearly plays an important role in regulating MAGE expression, there is mounting evidence that demethylation is not sufficient to drive the expression of all MAGEs and that additional non-epigenetic mechanisms are required [162,169,170].

Histone modifications

Histone modifications have also been shown to function in the regulation of CTA expression. Histones assemble with DNA into nucleosomes, the basic unit of chromatin. Their flexible N-terminal tails protrude from the nucleosomes and are targeted for post-translation modification, including acetylation and methylation (reviewed in Ref. [171]). Histone acetylation by histone acetyltransferases results in chromatin decompaction and gene transcription. Conversely,

HDACs serve the opposite function and promote the compaction of chromatin to prevent the accessibility of DNA to transcription factors and RNA polymerase. In the case of MAGE-A2 and MAGE-A12, treatment with HDAC inhibitors results in the upregulation of transcriptional activity [172].

Depending on the lysine residue that is modified, histone methylation is associated with both transcriptional activation and repression. For example, methylation of histone H3 lysine 9 or lysine 27 (H3K9 or H3K27, respectively) are repressive marks, whereas methylation of histone H3 lysine 4 (H3K4) is an active mark. In a study from Tachibana *et al.*, knockout of the histone methyltransferases that catalyze H3K9me2, G9a, and/or GLP induced the expression of MAGE-a genes in mouse embryonic stem cells [173]. In addition, in G9a- or GLP-null mouse ES cells, MAGE-a genes are hypomethylated [173]. These results suggest that histone modifications are also involved in the repression of MAGE CTAs.

Transcription factors

Although less is known about the non-epigenetic mechanisms of MAGE gene regulation, the E26 transformation-specific (ETS) transcription factor sites have been shown to function in the regulation of MAGE-A genes. The ETS protein family is one of the largest families of transcription factors, and members of this family are implicated in the development of various tissues and cancer progression. Work from de Smet and colleagues revealed that two inverted ETS motifs near the transcriptional start site of MAGE-A1 drive the transcriptional activity of the unmethylated promoter [165,174]. In cells where the endogenous MAGE-A1 promoter is methylated and inactive, MAGE-A1 promoter transgenes are highly active and dependent on ETS sequences [165,174]. Further studies have shown that the methylation of a CpG in the ETS consensus sequence inhibits ETS binding [165,174]. Although the identity of the exact ETS factor that drives MAGE-A expression remains unclear, one potential candidate is ETS1, which was shown to activate MAGE-A genes when overexpressed.

BORIS, a paralog of the imprinting regulator and chromatin insulator protein CTCF, has also been implicated in promoting the expression of MAGE-A1. In fact, several MAGE-A genes contain BORIS binding sites, and the proteins have been shown to coexpress in head and neck cancer [175]. Moreover, BORIS overexpression in immortalized oral keratinocytes led to MAGE-A induction and DNA hypomethylation [175].

Signal transduction pathways

In addition to transcription factors, signal transduction pathways, such as activated tyrosine kinases, have been implicated in MAGE CTA expression. The KIT

receptor tyrosine kinase is a proto-oncogene that binds to c-kit ligand, also known as steel factor or stem cell factor, to activate its tyrosine kinase activity and signal transduction pathway. Yang *et al.* reported that the treatment of mast cells with the tyrosine kinase inhibitor imatinib results in the downregulation of MAGE-A, -B, and -C and potentially alters DNA methylation levels [176]. In addition to KIT, others have reported that fibroblast growth factor receptor 2 (FGFR2) activation downregulates MAGE-A3 expression [177]. In addition, FGFR2 and MAGE-A3 promoters show reciprocal DNA methylation patterns [178]. Therefore, FGFR2 and KIT appear to have opposing effects of MAGE-A expression. Interestingly, FGFRs are known to play a critical role in a variety of processes including tissue repair and angiogenesis [179]. Given that MAGE-A1 is expressed during wound healing and in the joints of patients with juvenile arthritis, MAGE-A1 may have a role in inflammation [180,181]. Furthermore, MAGE-B2 was originally identified as being expressed in patients with systemic lupus erythematosus where MAGE-B2 autoantibodies can be found [182,183]. Thus, additional investigation of roles for type I MAGE CTAs in other disease contexts besides cancer is warranted in the future.

Therapy

Cancer immunotherapy

Since the identification of MAGEs, significant effort has gone into the development of CTA-based immunotherapeutic strategies (Table 3). Compared to chemotherapy, which often has limited efficacy in patients with relapsed cancer or advanced disease, immunotherapy has the potential to provide long-lasting responses by modulating the immune response against specific cancer proteins.

From an immunological perspective, MAGE CTAs are ideal target molecules for cancer immunotherapies due to their widespread, prominent expression in various cancers but restricted normal expression to the immune-privileged testis, thus limiting the possibility of an autoimmune response but maximizing their potential for broad application to large cohorts of patients [184–186]. MAGE-A3, which codes for an antigenic nonapeptide that is recognized by CTLs on the HLA-A1 molecule, garnered particular interest as an immunotherapeutic. In a pivotal clinical trial where tumor-bearing HLA-A1-positive patients with metastatic melanoma were treated with subcutaneous injections of MAGE-A3 peptide, 7 out of 25 patients displayed significant tumor regression, including 3 complete responses [187]. However, MAGE-A3-specific CTL responses were not detected during the course of vaccinations, even in patients with positive clinical responses [187]. Subsequent studies confirmed these immunologic and clinical responses

Table 3. Clinical trials with MAGEs

MAGE	Tumor type	Phase	Intervention	Identifier	Status
A3	Melanoma	I/II	MAGE-A3.A1 peptide and CpG7909	NCT00145145	Terminated
A3	Melanoma	II	GSK1203486A recombinant MAGE-A3 ASCI	NCT00706238	Terminated
A3	Melanoma	II	GSK2132231A, dacarbazine	NCT00849875	Terminated
A3	Melanoma	III	GSK 2132231A	NCT00796445	Terminated
A3	Lung	I	GSK1572932A, cisplatin, vinorelbine, radiotherapy	NCT00455572	Terminated
A3	Lung	III	GSK1572932A	NCT00480025	Terminated
A3, A12	Metastatic	I/II	anti-MAGE-A3/12 TCR, aldesleukin, cyclophosphamide, fludarabine	NCT01273181	Terminated
A3	Melanoma	I/II	Enhanced TCR Transduced Autologous T Cells	NCT01350401	Ongoing
A3	Multiple myeloma	I/II	Enhanced TCR Transduced Autologous T Cells	NCT01352286	Ongoing
A3, C2	Melanoma	II	TriMix-DC and ipilimumab	NCT01302496	Ongoing
A3, C1	Multiple myeloma	I	CT7, MAGE-A3, and WT1 mRNA-electroporated Langerhans cells (LCs)	NCT01995708	Recruiting
A3	Metastatic	I/II	Anti-MAGE-A3-DP4 TCR, cyclophosphamide, fludarabine, aldesleukin	NCT02111850	Recruiting
A3	Metastatic	I/II	MG1MA3 (MG1 Maraba/MAGE-A3 adenovirus vaccine)	NCT02285816	Recruiting
A10	Urinary bladder, head and neck, melanoma	I	Autologous genetically modified MAGE A10 ⁷⁹⁶ T cells	NCT02989064	Recruiting
A10	Lung	I/II	Autologous genetically modified MAGE A10 ⁷⁹⁶ T cells	NCT02592577	Recruiting
A3	Lung	I/II	Ad-MAGEA3, MG1-MAGEA3, pembrolizumab	NCT02879760	Recruiting

in melanoma patients treated with MAGE-A3-pulsed DCs [188,189].

Additional MAGE CTA-based vaccines have utilized recombinant proteins to (1) induce both CD8+ and CD4+ immune responses, thereby magnifying the CTL response; (2) generate responses against multiple epitopes; and (3) avoid specific HLA-type requirements for patients [190]. In a phase II clinical trial where 36 patients with stage III or IV M1a melanoma were treated with MAGE-A3 combined with AS15 immunostimulant, 3 patients exhibited complete responses [191].

Based on these preliminary data, two large clinical trials were set forth—PREDICT and DERMA. PREDICT was a phase II study with the goal of evaluating the clinical activity of MAGE-A3 antigen-specific cancer immunotherapeutic (ASCI) in patients with MAGE-A3-positive unresectable metastatic melanoma [192]. The goal of DERMA (phase III) was to evaluate the benefit of MAGE-A3 ASCI in melanoma patients after surgical tumor removal. Unfortunately, the objective response rate was lower than in previous studies [192]. Consistent with these findings, DERMA was terminated early following the assessment showing the lack of efficacy of the treatment.

Similarly, MAGE-A3 ASCI showed promising results in phase II clinical trials of patients with NSCLC and inspired the largest phase III therapeutic trial in lung cancer—MAGRIT [193–196]. In the case of patients with MAGE-A3-positive and surgically resected NSCLC, MAGE-A3 ASCI failed to increase disease-free survival compared with placebo, and further development of the MAGE-A3 ASCI was stopped [197].

More alarming than the lack of efficacy, however, are the unexpected deaths associated with MAGE-based immunotherapies. In order to overcome the complications associated with low frequency of

cancer antigen-specific cells and low avidity of expanded effector T cells, T cells can be genetically engineered to express T cell receptors (TCRs) that have high affinity and specificity. Unfortunately, in a study utilizing anti-MAGE-A3 TCR gene therapy as a treatment for metastatic cancers, one patient developed transient Parkinson-like symptoms, while two patients lapsed into comas and died [198]. Further investigation revealed that T cells transduced with the generated TCR also recognized MAGE-A12, which is expressed in the brain [198]. Therefore, treatment resulted in neuronal cell destruction that manifested as necrotizing leukoencephalopathy [198]. In a separate study using anti-MAGE-A3 TCR therapy, two patients developed progressive cardiogenic shock and died within a week of infusion. In this case, the generated TCR recognized another peptide derived from titin, a component of striated muscle, and the off-target reactivity resulted in severe myocardial damage with T cell infiltration [199]. These studies demonstrate the need for more rigorous studies of MAGE expression and stringent analysis of engineered TCRs.

Combination therapy

In an effort to develop alternative methods to target MAGE-expressing cancers and improve clinical outcomes, investigators are utilizing combinatorial approaches including conventional therapy, immunotherapy, and molecular targeting.

One obstacle to overcome in the development of immunotherapies is T cell recognition of cancer cells expressing MAGE CTAs. In cancer cells, promoter hypermethylation suppresses MHC expression, thereby impeding antigen presentation [200]. Therefore, several groups have indicated that treatment with demethylating agents such as decitabine can

potentially enhance antigen presentation and cancer cell recognition by upregulating both MHC and MAGE expression [162,201–203]. A phase I clinical trial combining dectabine and DC vaccine targeting MAGE-A1 and -A3 for patients with relapsed neuroblastoma demonstrated a response in six of nine patients with complete response in one patient [204]. Although the preliminary data of this combination therapy look promising, future trials are needed to better determine the efficacy of this treatment.

A major obstacle to the practical application of cancer immunotherapy is the ability of tumor cells to evade the immune system by promoting an immunosuppressive tumor microenvironment. Therefore, investigators have turned to immune checkpoint therapies, which target regulatory pathways in T cells, to enhance the antitumor immune response. Rather than activating the immune system to directly target tumor cells, immune checkpoint therapies remove inhibitor pathways that block effective antitumor T cell responses (reviewed in Ref. [205]). Therefore, combination approaches with immune checkpoint therapies may be beneficial in the treatment of MAGE expression cancers and are currently ongoing (Table 3).

In addition to these proposed treatments, molecular targeting of specific protein-protein interactions may prove to be a powerful therapeutic strategy. A prime candidate is targeting specific MRLs. For example, Bhatia and colleagues screened a library of compounds and identified three potential compounds that interfere with MAGE-C2-TRIM28 binding, promote death in MAGE-positive cells, and activate p53 [206]. These data support the therapeutic value of inhibiting MAGE binding to cognate E3 ligases to block the oncogenic functions of MAGE proteins. Furthermore, by knowing the precise cellular targets and functions of MRLs, novel drug susceptibilities can be predicted based on MAGE expression status in individual tumors. For example, considering the interplay among MAGE-A proteins, p53, and HDAC, the use of HDAC inhibitors in combination with other therapeutic approaches could help restore p53 tumor suppressor activity. Furthermore, utilization of AMPK agonists (such as metformin) or mTOR inhibitors may be an effective future treatment for MAGE-A3/6-positive cancers. Future studies validating these approaches in cell and animal models will be important.

Conclusions and Perspectives

Since their initial discovery, the MAGEs have gained much attention and interest as cancer biomarkers and targets of cancer immunotherapies. Despite the fact that initial attempts to develop MAGE-based immunotherapies have not been entirely successful, recent studies highlighting a role for MAGEs in the regulation of E3 RING ubiquitin ligases have greatly expanded our understanding of the diverse functions of MAGE

family members and their impact on various molecular processes (such as p53 signaling, cell metabolism, and protein trafficking) and have opened up novel avenues for personalized cancer-specific therapies targeting MRLs.

However, many unanswered questions remain. How do the cellular functions attributed to MAGEs contribute to disease progression? How do MAGEs regulate pathways that affect tissue-specific outcomes? What transcriptional programs lead to the aberrant re-expression of type I MAGEs in cancer? Do type I MAGEs have similar functions in their physiological context of germ cells as they do in cancer cells? What are the cellular and physiological functions of the relatively unexplored MAGEs? What are the substrates of many of the uncharacterized MRLs?

Although our understanding of the molecular mechanisms by which MRLs function is still developing, continued identification and characterization of the functional interplay among MAGEs, RING ligases, and deubiquitinating enzymes will provide valuable insights into the role of MAGEs. In addition, structural studies may help us not only to understand the biophysical underpinnings of MRLs but also design specific MAGE inhibitors for therapeutic purposes. The continued study of MAGEs clearly holds great promise for answering fundamental biological questions and may reveal new ways to target them for the treatment of disease.

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Abbreviations used:

CTL, cytotoxic T cell; MAGE, melanoma antigen; CTA, cancer-testis antigen; HLA, human leukocyte antigen; MHD,

MAGE homology domain; SMC, structural maintenance of the chromosome; WH, winged-helix; MRL, MAGE-RING ligase; CRL, Cullin-RING ligase; CTD, C-terminal domain; AMPK, AMP-activated protein kinase; F-actin, actin filament; DUB, deubiquitinating enzyme; Rb, retinoblastoma; HDAC, histone deacetylase; AR, androgen receptor; PGC, primordial germ cell; SERT, serotonin transporter; PWS, Prader-Willi syndrome; ASD, autism spectrum disorder; SHFYNG, Schaaf-Yang syndrome; CSC, cancer stem cell; DNMT, DNA methyltransferase; FGFR2, fibroblast growth factor receptor 2; ASCI, antigen-specific cancer immunotherapeutic; TCR, T cell receptor; mTOR, mechanistic target of rapamycin; WASH, Wiskott-Aldrich syndrome protein and scar homolog.

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