Emerging roles of the MAGE protein family in stress response pathways

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The melanoma antigen (MAGE) proteins all contain a MAGE homology domain. *MAGE* genes are conserved in all eukaryotes and have expanded from a single gene in lower eukaryotes to ~40 genes in humans and mice. Whereas some *MAGEs* are ubiquitously expressed in tissues, others are expressed in only germ cells with aberrant reactivation in multiple cancers. Much of the initial research on MAGEs focused on exploiting their antigenicity and restricted expression pattern to target them with cancer immunotherapy. Beyond their potential clinical application and role in tumorigenesis, recent studies have shown that MAGE proteins regulate diverse cellular and developmental pathways, implicating them in many diseases besides cancer, including lung, renal, and neurodevelopmental disorders. At the molecular level, many MAGEs bind to E3 RING ubiquitin ligases and, thus, regulate their substrate specificity, ligase activity, and subcellular localization. On a broader scale, the *MAGE* genes likely expanded in eutherian mammals to protect the germline from environmental stress and aid in stress adaptation, and this stress tolerance may explain why many cancers aberrantly express *MAGEs*. Here, we present an updated, comprehensive review on the MAGE family that highlights general characteristics, emphasizes recent comparative studies in mice, and describes the diverse functions exerted by individual MAGEs.

Introduction: A comparative view of the *MAGE* gene family

Discovery of MAGEs

Classic studies in the 1940s and 1950s provided experimental evidence for the concept that the immune system can recognize and reject tumor cells (1, 2) and opened the floodgates for identifying and characterizing tumor antigens, which could be targeted for cancer therapy. In addition to mutated, fused, overexpressed, and oncoviral proteins (2), male germ cell–specific proteins were added to the inventory in 1991 when melanoma antigen 1 (MAGE-1) was discovered in the melanoma cell line MZ2-MEL (3). MZ2-MEL cells were established from a patient (MZ-2) who had, for 10 years, presented with strong T-cell reactivity against autologous tumor cells in culture (4). This patient had stage IV amelanotic melanoma of an unknown primary tumor and never achieved complete remission despite multiple surgical interventions followed by chemotherapy. Remarkably, continued vaccination with autologous melanoma cell clones that had been mutagenized *in vitro* and lethally irradiated led to the patient surviving for more than 30 years without disease recurrence. To identify the tumor-associated antigens recognized by the cytotoxic T cells in this patient, Boon and his group (3) applied autologous typing and transfection of a cosmid library into the patient-derived MZ2-E cell line. Their efforts led to the discovery of MAGE-1, the first human tumor antigen, which was later renamed MAGE-A1 upon the identification of additional gene family members (3, 5, 6). Namely, subsequent studies (5, 7, 8) identified a whole family of *MAGE* genes, present in all placental mammals. Humans and mice have ~40 *MAGE* genes, which include some designated as pseudogenes, that are further subdivided into two major categories based on their sequence homology, tissue expression pattern, and chromosomal location (Figs. 1 and 2) (5, 8–10). The type I *MAGEs* include the *MAGE-A, -B,* primate-specific *-C,* and mouse-specific *Mage-a–like* (*-al* and *-k1*) subfamily members. Type I *MAGEs* are also called cancer-testis antigens (CTAs) because they are primarily expressed in the testis but are normally silent in other tissues (Fig. 2*A*) (5, 11); however, they are often aberrantly reactivated during oncogenic transformation (Fig. 2*B*) and code for antigens recognized by cytolytic T lymphocytes (5). In contrast, the type II *MAGEs*, consisting of the *MAGE-D*, *-E*, *-F*, *-G*, *-H*, *-L*, and *NECDIN* genes, are more ubiquitously expressed in humans and mice and not typically associated with human cancer (5, 9, 11–13).

Since the discovery of MAGEs, a major research focus has been developing MAGE-targeted immunotherapies. Despite promising results from initial clinical trials (14, 15), MAGE-A3 vaccines ultimately failed in Phase III due to a lack of efficacy (16, 17), which suggested that activation of the T-cell response to MAGE-A3 antigen is not sufficient to inhibit disease progression (18). Furthermore, some patients treated with anti-MAGE therapies developed serious off-target effects, like neuro- and cardiotoxicity (19, 20). The neurotoxicity may have been caused by the anti-MAGE-A3-TCR–engineered T cells recognizing a similar MAGE that is expressed in the brain (*i.e.* MAGE-A12) (11), and the cardiotoxicity was attributed to vaccine recognition of an unrelated peptide (titin) in the heart (19, 20). Besides inefficacy and unexpected side effects, resistance has been another major roadblock. For example, *MAGE-A*

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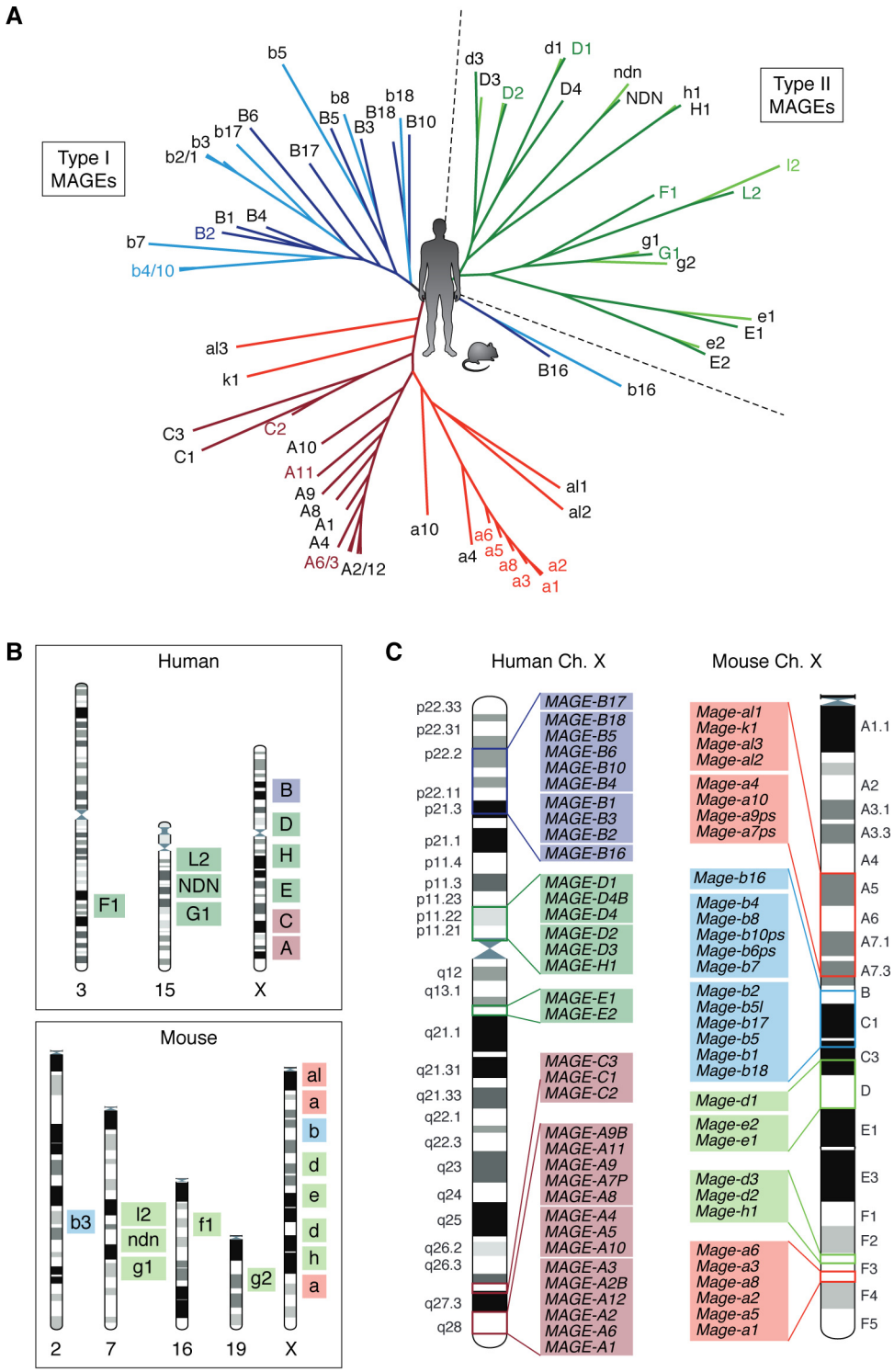

Figure 1. Overview of the MAGE gene family in humans and mice. *A*, phylogenetic tree showing the relationship between human and mouse MAGE proteins. The tree was created by the neighbor-joining construction method using the Jukes–Cantor protein distance measurement from the CLC Main Workbench 20. *B*, chromosomal location of human and mouse MAGE genes. *C*, locations of MAGE genes on the human and mouse X chromosome based on the recent NCBI's genome assembly HRCh38.p13 and GRCm38.p6. For all figures, the type II MAGEs are represented in *green*, MAGE-A and -C subfamilies in *red*, and MAGE-B subfamily in *blue*. *Light colors* indicate mouse Mages and *dark colors* indicate human MAGEs.

expression correlates with poor response to the CTLA-4 checkpoint inhibitors in melanoma patients (21) and faster development of resistance to the epidermal growth factor receptor tyrosine kinase inhibitors and chemotherapy (22–24). Despite these setbacks, research is ongoing to improve clinical outcomes and limit off-target effects of MAGE-based immunotherapies (25, 26). Alternative methods to target MAGE-expressing cancers by utilizing combinations of conventional therapy and immunotherapy are also being investigated (27–30). To successfully and safely target the type I MAGEs, it is

important to understand the mechanisms by which these proteins contribute to oncogenesis, how they are regulated, and what they normally do in physiological contexts. In this review, we dive into what is known about the diverse functions of individual MAGEs, as well as their roles in cancer and other diseases. Although MAGE proteins have diverse functions, emerging studies suggest that responding to stress is a unifying theme of MAGEs.

Genomic organization and structure of human and mouse MAGEs

Most of the *MAGE* genes are located in clusters that are preserved in diverse mammalian species; however, each cluster has undergone a different degree of expansion by duplication or retrotransposition, leading to a number of species-specific genes (13). As shown in Fig. 1, human and mouse genomes encompass different numbers of *MAGE* subfamily members. They also differ in that only humans possess *MAGE-C* genes, and mice possess additional *Mage-a–like* genes that form another subfamily (Fig. 1) (11). Consistent with their classification as CTAs, the type I *MAGE* subfamilies reside in syntenic regions on the X chromosome (Fig. 1, *B* and *C*) (2, 5, 11, 31, 32), where testis-expressed genes are overrepresented (33–35). The autosomal murine gene *Mage-b3* is an exception, as it resides on chromosome 2. Another distinction between humans and mice is that *Mage-a* genes map to two different loci on the murine X chromosome, which could be the result of an interchromosomal recombination event during genome evolution in rodents (Fig. 1, *B* and *C*) (36). In contrast to the uniform genomic location of type I *MAGEs*, type II *MAGE* genes are located on both autosomes and the X chromosome (Fig. 1, *B* and *C*). The type II *MAGEs* also exhibit species-specific copy number variations. For example, the mouse genome has only three *Mage-d* genes and an additional *Mage-g* gene, *Mage-g2* (11, 37). Intriguingly, *Mage-f1* has a point mutation in rodents and is predicted to either be a pseudogene or code for a truncated protein (38); thus, *Mage-g2* may be a rodent adaptation to this *Mage-f1* mutation loss and may serve important functions during germ cell development (37).

Although most MAGE proteins (and their murine homologs) are encoded by a single exon, the coding regions of the MAGE-D subfamily span across more than 11 exons (5, 13). The *MAGE-D* subfamily is also the most conserved subfamily between species, with over 90% identity in the coding sequences (13), and the genomic structure of the murine *Mage-d* genes closely resembles that of humans (39). The majority of the type I genes acquired several 5′ noncoding exons, potentially allowing for differential regulation of expression (6, 40). Some mouse *Mage-b* genes that were originally thought to be pseudogenes (*Mage-b7, -b8*, and *-b17*) because they have the structure of a processed transcript (10) code for full proteins and are expressed on the transcriptional level in a cell-specific manner in the testis, suggesting a functional role in spermatogenesis (11). Furthermore, during primate evolution, human *MAGE-A11* acquired three additional 5′ coding exons that are unique among the type I genes (41). Together, the genomic organization and structure of the MAGE gene family indicate that it has expanded by retrotransposition and local duplication events. After splitting from their phylogenetic ancestor, the MAGE genes independently evolved in each species, with the type I MAGEs evolving most rapidly (13).

Evolution of the MAGE gene family

The *MAGE* gene family is evolutionarily conserved across eukaryotes. Unlike the large multigene family found in placental mammals, earlier eukaryotes, from protozoa to nonplacental mammals like the platypus, possess a single *MAGE* gene (9, 13, 42, 43). The first expansion of the *MAGE* gene family possibly occurred in marsupials, but with the emergence of the placenta and LINE elements in eutherian mammals, the family rapidly expanded (13). During eutherian radiation, the subfamily ancestors were formed by retrotransposition and expanded by gene duplications (5, 12).

Although most of the *MAGEs* that exist today appear to derive from a single ancestral gene, the identity of the founder family member is still a matter of debate. The unique genomic architecture of the *MAGE-D* genes suggests that one of them is the founder (5, 12); however, functional studies of *MAGE-G1* imply that it is most closely related to the ancestral *MAGE* (42, 44). Nevertheless, the type II *MAGEs* clearly appeared earlier, as evidenced by the high homology shared between the human and mouse orthologs (>80% nucleotide sequence identity) (13, 43). In contrast, the type I *MAGE* paralogs within species are more similar to their subfamily members than to their orthologs between species (Fig. 1*A*), suggesting that these duplications occurred after the separation of the species. Mice also lack *MAGE-C* genes, whereas humans lack *Mage-a–like* genes (11), further implying that the type I *MAGE* subfamilies underwent a more recent and rapid evolution.

Within type II MAGE proteins, the N- and C-terminal regions that flank the MAGE homology domain (MHD) are completely different in paralogs but are highly conserved between human and mouse orthologs. This pattern further indicates that the type II genes independently evolved before the phylogenetic separation of the two species, which is also evident by the branching of the human and mouse MAGE phylogenetic tree (Fig. 1*A*) (5). Integrative analysis of genomic structures and codon changes of *MAGEs* and their distinct evolution patterns indicates that negative or purifying selection maintained the established essential, nonredundant functions of type II MAGEs, whereas positive selection allowed the redundant type I MAGEs to diversify or acquire additional functions (13).

The *MAGE* gene family is unique among cancer-testis antigens and the multigenic families of the X chromosome. Although the X chromosome is generally considered to be the most evolutionarily stable chromosome in placental mammals, which is true of the single-copy genes (35, 45), its ampliconic regions are rapidly evolving (46–50). *MAGE* genes fall into both categories of genes, as single-copy type II genes are conserved among mammals, whereas several of the type I genes recently expanded (Fig. 1). The rapid expansion of multicopy/ampliconic genes on the X chromosome is thought to be driven by male X chromosome hemizygosity and the benefits these

genes offer to male reproductive fitness (51). Due to rapid and selective evolution, these genes often lack murine counterparts, barring traditional *in vivo* genetic studies (52–54). Type I *MAGEs* are an intriguing exception, as they are present in all mammals, which enables investigation into their physiological function in animal models (11). Even though several type I genes have diversified in a species-specific manner, they expanded to the same extent in both mice and humans, resulting in a similar number of genes in both species, which suggests that they convergently evolved to serve similar functions.

Comparative MAGE expression

MAGEs in the adult tissues

Upon the initial discovery and characterization of *MAGE* genes, their expression was only detectable in tumor samples and could not be identified in the limited set of normal somatic tissues available to the Boon group (3). Later, mRNA of *MAGE-A*, *-B*, and *-C* subfamily members was discovered in the testis and, in some cases, the placenta, hence their classification as CTAs (5–7, 40, 55–59). Additional studies identified more distant family members that are broadly expressed in normal tissues and are now referred to as type II *MAGEs* (5, 39, 60, 61). Comparative anatomical and developmental gene expression profiling of the entire *MAGE* family revealed five distinct subgroups (Fig. 2*A*) that may predict the functional categories and tissue-specific activities of MAGE proteins (https://mage.stjude.org/) (11).

Type I *MAGEs* show expression restricted to either the testis only (referred to as type Ia *MAGEs*) or to the testis and placenta (type Ib) (Fig. 2*A*). In mice, several type Ib genes are also expressed in the ovary (57, 62). In contrast to the idea that expression of type I *MAGEs* is completely restricted to reproductive organs (5, 6, 8, 55, 63), several type I *MAGEs* (type Ic) are expressed in a variety of organs in both species, including bladder, brain, spleen, small intestine, skeletal muscle, heart, and esophagus (11). Besides indicating potential function(s) outside the gonads, this unexpected expression pattern may also explain some cancer immunotherapy side effects, such as the neurological toxicities observed in patients treated with genetically engineered anti-MAGE-A3 T cells (19, 20). This finding has important implications in cancer vaccine and immunotherapy development because MAGEs are one of the most frequently targeted proteins, and several clinical trials are under way (31, 64, 65).

Type II *MAGEs* display a more ubiquitous pattern of tissue expression and are expressed at higher absolute levels than the type I genes in both species (9, 11, 66–69). The type IIa genes are uniformly and highly expressed in the majority of tissues, and the type IIb *MAGEs* show enriched expression in the brain (Fig. 2*A*) (11, 66–68). Notably, some type IIa genes are also expressed at high levels in the brain, such as *MAGE-D* in the cerebral cortex, medulla, and hippocampus (9, 70). As a type IIb *MAGE*, *MAGE-L2* is widely expressed in various human adult tissues and highly enriched in the brain, particularly in the hypothalamus (11, 67, 68). In mice, *Mage-l2* expression is even more restricted to the brain, and enrichment in the hypothalamus is already detectable in the later embryonic stages (67, 68), suggesting a role for *Mage-l2* during neural development and in the adult brain. Prominent *Mage-l2*–expressing neurons are located in regions (*i.e.* the arcuate nuclei, suprachiasmatic nuclei, paraventricular nuclei, and supraoptic nuclei) involved in appetite and feeding behaviors, underscoring the phenotypes seen in Prader–Willi (PWS) and Schaaf–Yang syndrome (SYS) patients, which will be explained in more detail in later sections of this review (71).

MAGE expression during embryonic development

The expression of type I and II *MAGEs* in placenta and several fetal tissues in human and mouse suggest developmental functions (6, 11, 62, 72, 73). Human placenta expresses several *MAGE-A* genes (6); in contrast, mouse *Mage-a* genes are restricted to expression in the testis, whereas the *Mage-a–like* genes (*Mage-al2* and *-al3*) are highly enriched in the mouse placenta (11). This finding suggests that *Mage-al* genes may be the functional orthologs of human *MAGE-A8, -A10*, and *-A11* in this tissue (11).

Unlike the adult tissues, expression of the type Ia *MAGE* genes is not restricted to the male gonad during embryonic development. Expression in the developing testis and ovary implicates a role for type I *MAGEs* in gametogenesis of both sexes (11, 58, 62, 73). Consistent with mouse expression, human MAGE-A1 and -A4 proteins have been detected in premeiotic germ cells (58) and in fetal ovary (62, 73), suggesting that human and mouse *MAGE-A* genes might share similar functions in premeiotic germ cell development of both species.

Type II *MAGEs* are broadly expressed during embryonic development in humans (69, 74) and mice (Fig. 2*A*) (9, 11, 69, 70). The high expression of type II genes in the brain suggests a role in the development and/or function of the central nervous system (11, 75–77). For example, *MAGE-D1* is highly expressed in the neural tube during early human development and later in the ventricular zone, subplate, and cortical plate (76, 78). Interestingly, several type IIb brain-enriched genes, such as *Ndn* and *Mage-l2,* are more ubiquitously expressed during embryonic development, which implies involvement in a diverse array of biological functions during embryonic development and in pathogenesis of neurodevelopmental disorders (43). In later sections focusing on *MAGE-D1, -D2, -G1,* and *-L2*, we cover these roles in further detail.

Besides expression during late embryonic development, *MAGEs* are also expressed in human and mouse embryonic stem (ES) cells (Fig. 2*A*) (11, 72, 79, 80). Like in adult tissues, *MAGE-D1* and *-D2* are the most highly expressed *MAGEs* in human (76, 77) and mouse ES cells (11, 72, 79, 80), teratocarcinoma cells, and extraembryonic endoderm cells (77). Furthermore, expression of several type II *MAGEs* is increased by retinoic acid–induced differentiation (80, 81). Additional research is warranted to define the contribution of MAGEs in regulation of stemness, differentiation of pluripotent stem cells, and embryonic development.

MAGE expression during spermatogenesis and folliculogenesis

From the early mapping of the *MAGE* gene family (6), it was evident that the majority of type I *MAGEs* exhibit male

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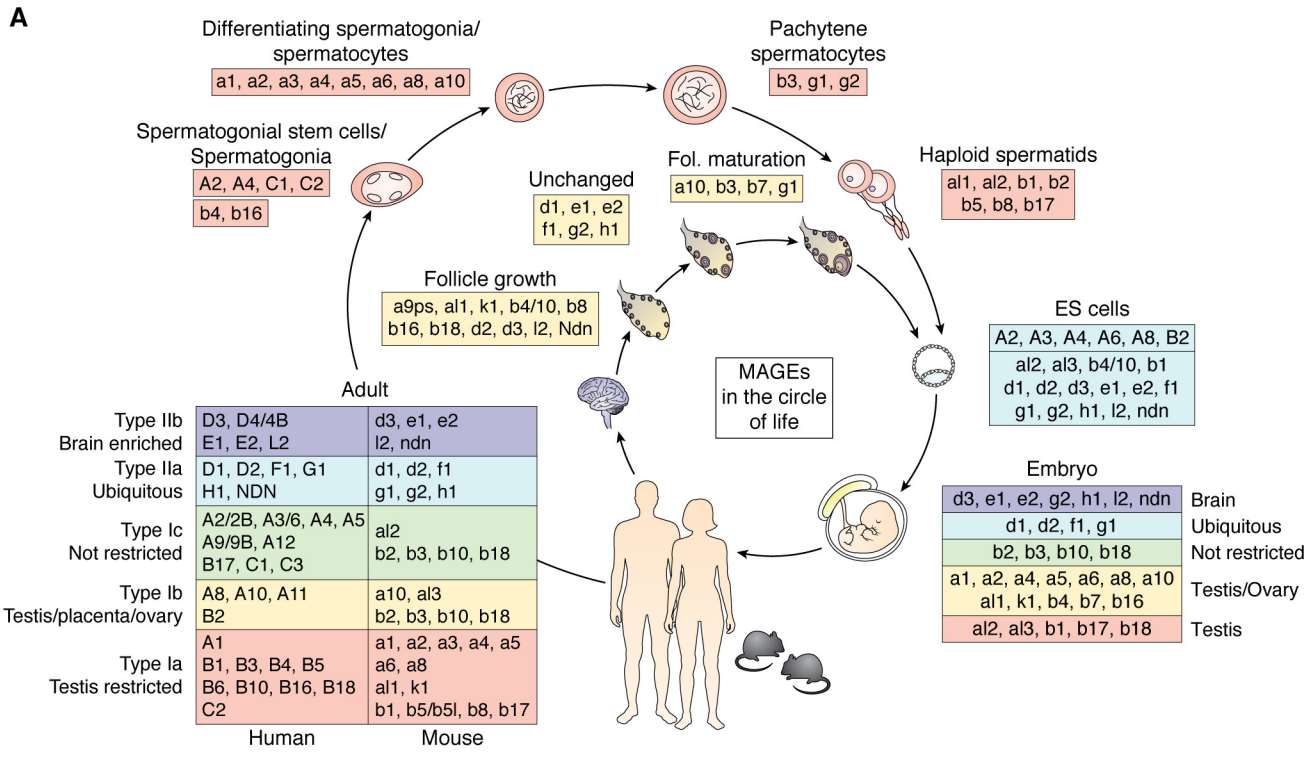

Figure 2. Expression of MAGEs in normal tissues and cancer. *A*, human and mouse MAGE expression during different life stages is indicated. Starting with the *top part* of the *outer circle*, the expression of MAGEs is depicted during spermatogenesis, in ES cells, in an embryo, and finally in adults. *B*, the heatmap displays the percentage of various tumors that express each type I MAGE. The results are based upon data generated by the TCGA Research Network (RRID:SCR_003193).

germline-restricted expression in both humans and mice (7, 11, 56–58, 73, 82–85), which implicated that the potential physiological function of these proteins is related to spermatogenesis. Mammalian spermatogenesis is a highly coordinated and cyclic process of male germ cell generation entailing cell divisions and differentiation to ultimately yield a large number of haploid spermatozoa. Spermatogenesis takes place in the seminiferous tubules of the testis, where somatic Sertoli cells develop an epithelium to support male germ cell proliferation and differentiation (86). In the basal compartment (*i.e.* the gap between the

basement membrane and the Sertoli cell tight junction), spermatogonial stem cells (SSCs) give rise to progenitors, also referred to as undifferentiated spermatogonia, which undergo a series of rapid transit-amplifying mitotic divisions. A surge in retinoic acid signals for progenitors to differentiate and go through a few more rounds of division to ultimately give rise to spermatocytes (87). Spermatocytes then enter meiotic division and cross the blood-testis barrier (BTB) to become pachytene spermatocytes. In the apical compartment of seminiferous tubules, spermatocytes then undergo two meiotic divisions to generate haploid round spermatids that undergo morphological changes to eventually mature into spermatozoa that are released into the lumen. This process takes ~35 days in mice and ~75 days in humans. Cyclic retinoic acid pulsation, which occurs every 8.6 days in the mouse testis, ensures continuity in spermatogenesis and a permanent supply of sperm throughout the life of a male (88–92).

The first round of mouse spermatogenesis is a distinctive program that provides a good model system to study gene expression during sperm development, as specific germ cell types (*i.e.* spermatogonia, spermatocytes, and spermatids) appear postnatally in a well-defined order (93, 94). Analysis of age-dependent *MAGE* expression patterns following initiation of spermatogenesis revealed that the majority of type I *MAGEs* are expressed at distinct stages in premeiotic, meiotic, and postmeiotic cells during sexual maturation (Fig. 2*A*) (11). Specifically, *Mage-b4* and *-b16* are expressed in spermatogonia, including SSCs (11, 57). Prepachytene spermatocytes exhibit peak expression of all *Mage-a* subfamily members, whose expression starts in spermatogonia and hits the highest point just before entry into meiosis and the BTB transition (11, 56). Interestingly, the non-X-chromosome–residing *MAGE* genes, *Mage-g1, -g2,* and *-b3*, are expressed in pachytene spermatocytes during meiosis (Fig. 2*A*) (11). The majority of *MAGE* genes expressed in haploid spermatids are the testis-restricted type Ia *Mage* genes, including *Mage-b1, -b2,* and *-b5* (Fig. 2*A*) (7, 11, 56). Consistent with the broad expression of type II MAGEs in many tissues and somatic cell types, most type II *Mages* are expressed predominantly in the Sertoli cells (11, 95).

Besides the testis, several type I *Mages* are also expressed in the mouse ovary during follicle growth and maturation (Fig. 2*A*) (11). For example, *Mage-b4* is expressed in the first 2 weeks after birth (11), when the rate of primordial follicle recruitment into the growth phase is the greatest, which is in line with immunohistochemistry analysis showing that female germ cells express *Mage-b4* throughout meiosis and in dormant primary oocytes (57). *Mage-a10, -b3,* and *-b7* are enriched later, during follicle maturation (Fig. 2*A*) (11). Intriguingly, the pseudogene *Mage-a9ps*, which is not expressed in any other tissue, is expressed during early ovary development, implying a potential regulatory function of this gene in oogenesis (11). All type II genes are expressed in the ovary, but only a few are regulated during ovary development, such as *Mage-l2*, which is enriched during early follicle growth (Fig. 2*A*). Taken together, these results indicate that *MAGE* genes are expressed in specific cell types and stages during spermatogenesis or folliculogenesis to perform unique and nonoverlapping functions during germ cell differentiation.

Epigenetic and transcriptional regulation of MAGE gene expression

Since first being identified in melanoma, MAGEs have been described in a myriad of tumors of various histological types and stages of progression (Fig. 2*B*) (3, 6, 82–84, 96–101). Given this widespread expression in different cancers, many studies have sought to identify and understand the underlying mechanisms that lead to the ectopic expression of *MAGEs* in cancer. Both the distinct stage-specific expression of MAGE CTAs in the male germline (Fig. 2*A*) (11) and the diverse pattern of activation in specific tumor types (Fig. 2*B*) (43, 101) suggest that a combination of epigenetic alterations with tissue-specific transcription factors is required to permit stable transcriptional activation of *MAGE* expression, although the precise regulatory mechanisms are still not fully understood.

DNA methylation

The discovery that a methyltransferase inhibitor, 5′-aza-2′-deoxycytidine (DAC) was capable of inducing *MAGE-A1* expression indicated that DNA methylation status contributes to *MAGE* silencing in normal tissues and aberrant expression in cancer (96). Accordingly, the level of promoter methylation of various *MAGEs* inversely correlates with their expression in cancers (97, 100, 102–108). The predominant methyltransferase involved in the maintenance of CpG (5′-C-phosphate-G-3′) methylation of *MAGE* promoters is DNMT1 (109). In addition, methyl-CpG–binding domain proteins contribute to the silencing of *MAGE-A* genes (110, 111), further implying the important role of DNA methylation in transcriptional regulation of *MAGE* genes. Although the role of DNA methylation in physiological regulation of *MAGEs* is mostly unknown, the methylation reprograming pattern during gametogenesis (112) suggests that it could contribute to the cell type–specific *MAGE* expression pattern during spermatogenesis.

In line with this idea, BORIS (brother of the regulator of imprinted sites), a demethylation factor involved in regulation of the site specificity and timing of epigenetic reprogramming in germ cells, was recently found to promote aberrant activation of *MAGEs* in human tumors (113, 114). Thus, BORIS—itself a cancer-testis gene—highlights a possible overlap between the regulatory system for induction of *MAGE* genes in both normal male germ cells and cancer cells with respect to CpG methylation. The involvement of BORIS also suggests that aberrant activation of *MAGEs* might not just be a random consequence of genome-wide demethylation in cancer, as previously thought, but rather a process of targeted epigenetic modifications (113). Expression of BORIS in male germ cells overlaps with several *MAGEs* that are expressed from spermatogonia to spermatocytes and also coincides with erasure of the global methylation pattern (11, 113, 115). Furthermore, the illegitimate activation of BORIS also correlates with the up-regulation of several *MAGEs* in cancer (115). However, *MAGE-A1* and other CTA genes are expressed in melanoma in the absence of BORIS activation, suggesting more complex activation of these genes (116).

Altogether, the importance of DNMTs and BORIS in spermatogenesis, their stage-specific expression during male

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germline development and their implication in cancer suggest that DNA methylation impacts *MAGE* expression in germ cells and cancer. Furthermore, the differential acquisition of methylation marks between male and female gametes (112) may also underlie differential expression of *MAGE* genes in male and female gonads, but further studies are required to provide experimental evidence and molecular details of such regulation.

Histone modifications

DNA methylation of *MAGE* promoters is intertwined with post-translational modification of histones, and both work together to enhance *MAGE* gene expression in cancer cells (96, 98, 104, 117). Tumor cells with high expression of *MAGE-A1* and *-A3* exhibit an enrichment in activation marks with a concomitant decrease in the repressive mark (118). Inhibition of DNA methyltransferases and histone deacetylases (HDACs) leads to *MAGE-A11* expression, supporting the idea that DNA methylation and histone modifications play a synergistic role in regulating *MAGE* expression (119). In thyroid cancer and pituitary tumors, reactivation of the fibroblast growth factor receptor 2-IIIb (FGFR2-IIIb) led to repression of *MAGE-A3* and *-A6* by increasing histone deacetylation and histone methylation (120, 121). Histone deacetylation was also shown to be responsible for the silencing of *MAGE-A1*, *-A2*, *-A3*, and *-A12* expression (122), whereas in female pituitary tumors, estradiol promoted H3 acetylation and *MAGE-A3* expression (121). In addition to histone lysine acetylation, histone lysine methylation was also shown to affect *MAGE* gene expression in cancer cells (118). G9A, also known as euchromatic histone lysine *N*-methyltransferase 2 (EHMT2), methylates histone H3 Lys-9 in the *MAGE-A2*, *-A6*, and *-A8* promoter regions, leading to the maintenance of their heterochromatic and silent state (123). Together, a growing body of evidence indicates that diverse epigenetic mechanisms regulate the expression and silencing of *MAGE* genes in cancer cells; however, how these epigenetic mechanisms contribute to their expression in the germline is still mostly unknown. Further studies into epigenetic regulation of *MAGE* gene expression are warranted, in particular, as epigenetic drugs are used in combination with immunotherapy to improve the response of cancer patients (118, 124).

Transcription factors and signal transduction pathways

Both the cell-specific *MAGE* expression during spermatogenesis and their distinct expression in diverse cancers raise questions about the specificity of the regulation of these genes and potential transcription factors involved. In contrast to epigenetic regulation of *MAGE* genes, the transcription factors and upstream activating pathways are still mostly undetermined. Mapping of type I *MAGE* promoter regions using deletional analysis and transcription factor–binding site analysis identified ETS- and SP1-binding elements, which were able to activate *MAGE-A1* expression upon binding ETS transcription factors (125). Methylation of ETS- and SP1-binding sites in several *MAGE-A* promoters was subsequently shown to silence *MAGE-A* expression by preventing transcription factor binding and recruiting methyl-CpG–binding domain proteins (110, 126).

In several cancers, *MAGE-A* and *-C* expression is activated by deregulated proto-oncogenic KIT tyrosine kinase and concurrent promoter DNA demethylation (102, 104, 110, 127, 128). Upon treatment of KIT-dependent mast cells with the tyrosine kinase inhibitor imatinib, the expression of several *MAGE-A* and *-C* genes was inhibited (128). Intriguingly, *Mage-a* gene expression peaks in the seminiferous cycle after the retinoic acid surge that induces Kit signaling and activates differentiation of male gem cells (11, 129, 130). In line with this expression pattern, Mage-a protein expression is the highest in Stra8/Kit-positive spermatogonia and is induced in cultured primary spermatogonia after a retinoic acid spike (11), suggesting that Kit may also regulate expression of *Mage-a* genes during spermatogenesis.

Other signaling pathways may also regulate *MAGE* expression. For example, fibronectin and FGFR2 have been shown to induce expression of *MAGE-A3* in some cancer types (131). *MAGE-A3* expression is also inducible by carcinogens, such as *Helicobacter pylori* (132) or smoking (133), and is affected by miRNAs and lncRNAs (134–138). In all, current data suggest that the MAGE family of genes is repressed in somatic cells by many layers of epigenetic marks and the activities of transcription factors and signaling pathways that become coordinately dysregulated in cancer. However, the exact mechanisms and transcription factors involved in controlling *MAGE* gene expression in cancer and germ cells await discovery.

General characteristics of MAGE proteins

The defining feature of all MAGE proteins is an ~180-amino acid domain known as the MAGE homology domain (MHD). The MHD is present in lower eukaryotes, such as *Saccharomyces cerevisiae, Aspergillus* spp., *Drosophila melanogaster*, and *Arabidopsis thaliana* (5, 9, 44, 139, 140). In the mammalian MAGE family, ~40% of amino acids in the MHD are identical across all the MAGE subfamilies, but higher conservation is evident at the subfamily level, as the MAGE-D and MAGE-A subfamily members share 75 and 70% MHD residues, respectively (141). The MHD is generally positioned near the C terminus of MAGE family proteins and is flanked by short, poorly conserved N- and C termini in type I MAGEs (except human MAGE-C1 and mouse Mage-b4), as well as MAGE-F1, -G1, and NECDIN (Fig. 3*A*) (5, 9). In contrast, the remaining type II MAGEs contain extended N- and C-terminal sequences, but the biological importance of these MHD-flanking regions is unknown (9). Although MAGE family proteins typically have a single MHD, some MAGEs have a duplicated or a truncated MHD (Fig. 3*A*) (5, 9).

The MHD contains two tandem winged-helix (WH) motifs, referred to as WH-A and WH-B (Fig. 3*A*), the latter of which contains a dileucine motif that is important for MAGE biochemical function (141, 142). The crystal structures of MAGE-A3 and -A4 revealed tight binding of an extended peptide sequence within the tandem WH domain of the MHD, suggesting the MHD conveys binding capabilities that may be central to MAGE functionality (142). NMR and native MS of the MAGE-A4 MHD suggest that the domain encompasses compact folded structures and disordered regions with a broad

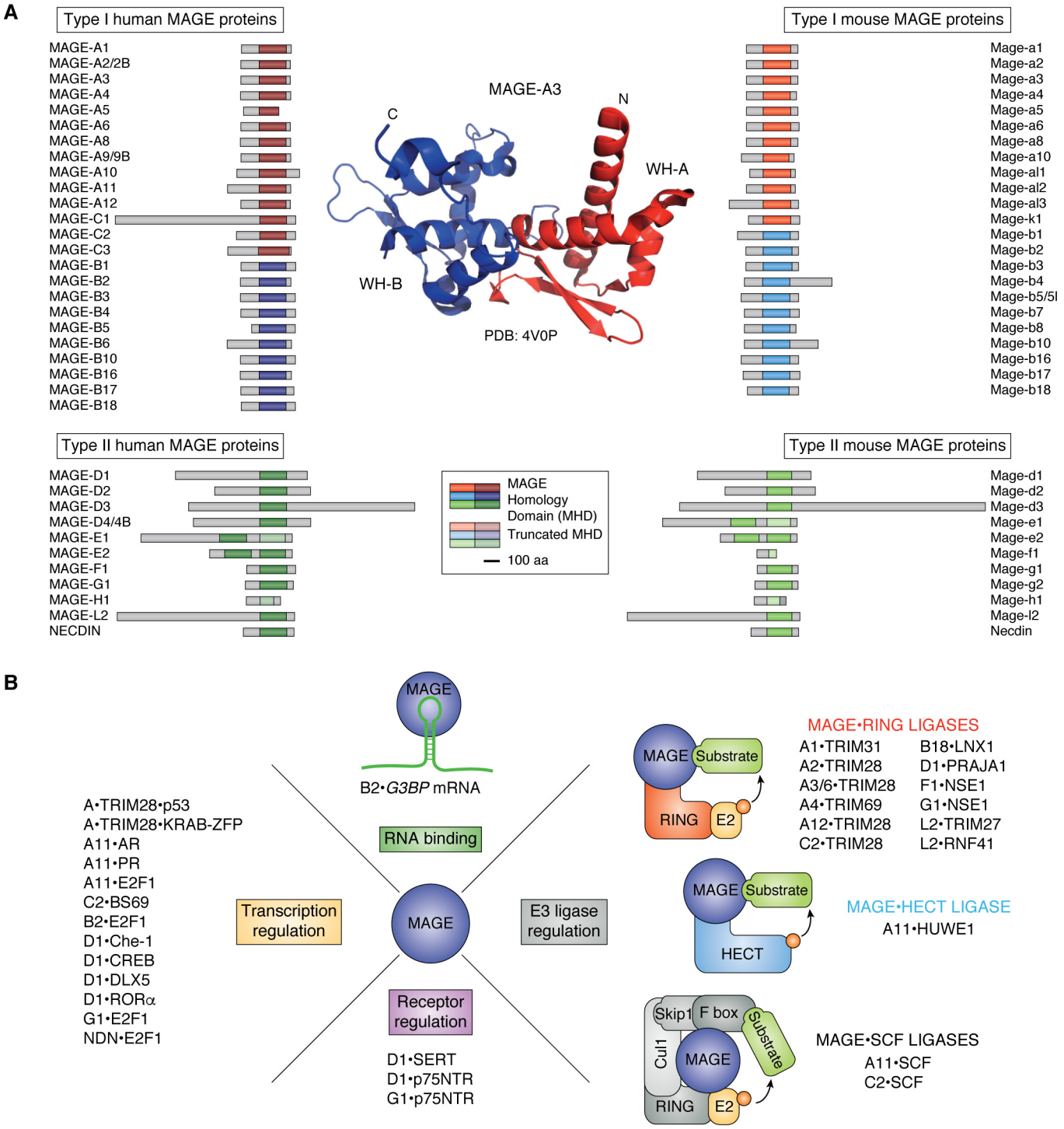

Figure 3. General structure of the MHD and biochemical function of MAGE proteins. *A*, schematic structure of human and mouse MAGE proteins. The MHD for each MAGE is indicated by a *solid colored box*, and the size corresponding to 100 amino acids (*aa*) is shown. The crystal structure of the double winged-helix motif of the MHD of MAGE-A3 (Protein Data Bank entry 4V0P) is shown. The N- and C termini are indicated, and the two WH motifs (WH-A and WH-B) are represented in *red* and *blue*, respectively. *B*, MAGEs bind to and regulate E3 ligases, receptors, transcription factors, and RNA (as an RNA-binding protein) to exert diverse molecular functions (General characteristics of MAGE proteins).

charge state distribution, all of which are suggestive of a dynamic protein (143). This dynamic flexibility of the MHD may confer unique binding preferences and functions to individual MAGEs.

Despite long-lasting interest in MAGE proteins in cancer therapy, the diverse molecular functions of these proteins are just starting to be unraveled. In line with the dynamic nature of the MHD structure, MAGE proteins exert their function through interactions with diverse proteins (Fig. 3*B*). A growing body of evidence suggests that MAGEs assemble with different E3 ligases and, by doing so, modulate ubiquitination of target proteins (43, 141). E3 ligases recognize target substrates and

mediate the transfer of activated ubiquitin from the E2 enzyme to a specific substrate (144). E3 ligases are categorized into four major classes: RING (really interesting new gene) finger, U-box, PHD finger, and HECT (145, 146). Through efforts to identify the function of MAGE proteins, we and others discovered that both type I and II MAGEs bind E3 ubiquitin ligases with RING domains and form MAGE-RING ligases (MRLs) (141, 147–154). Since their identification, several distinct MRLs have been described (Fig. 3*B*). MAGEs recognize their cognate E3 ligase partner through their MHD, and the dileucine motif in WH-B is particularly critical for this interaction (141). In contrast, the region on the RING protein that is recognized by a particular MAGE is variable (141). The disorder and flexibility of the MHD structure likely contributes to the specificity of each MAGE binding to its associated ligase (143). Indeed, the crystal structure of MAGE-G1 in complex with its NSE1 RING ligase demonstrated that the MHD undergoes extensive rearrangements for MRL formation (141).

MAGEs have been shown to regulate their respective E3 ligases through a diverse set of means; of particular interest is their ability to specify novel substrates for ubiquitination (141, 149, 151, 152, 155). For example, MAGE-A3 and -A6 bind the E3 ligase TRIM28 to specify 5′ AMP-activated protein kinase (AMPK) for ubiquitination and subsequent proteasomal degradation (151). AMPK ubiquitination by TRIM28 only in the presence of MAGE-A3 and -A6 implies that MAGEs reprogram ubiquitously expressed E3 ligases, like TRIM28, to serve cell type–specific functions in the germline or promote tumorigenesis in cancer cells (Fig. 4*A*) (151, 156). As another example of MAGEs specifying novel substrates, MAGE-F1 interacts with the E3 ligase NSE1 to target MMS19 for ubiquitination and degradation, which renders cells less competent in repairing DNA damage and predisposes them for oncogenic transformation (38). In another case, MAGE-L2-TRIM27–mediated ubiquitination of WASH protein leads to WASH activation, rather than proteasomal degradation, to facilitate transport of cargo proteins by retromer, a complex involved in endosomal protein sorting (149).

In addition to reprogramming E3 ligases and determining novel substrates, MAGEs often enhance ubiquitination of distinct ligase substrates. Since the initial discovery that MAGE-A3, -A2, -A6, and -C2 all bind TRIM28, subsequent biochemical analysis of the ubiquitin conjugation capacity of TRIM28 *in vitro* and in cells demonstrated that these MAGEs enhance ubiquitination of several TRIM28 substrates and autoubiquitination of TRIM28 (141, 148, 157, 158). The ability to enhance E3 ligase activity seems to be a conserved and shared function of both type I and II MAGEs, as MAGE-G1 also promotes the activity of NSE1 (141, 159). Additionally, MAGE proteins may enhance E3 ligase activity by recruiting E2 ubiquitin-conjugating enzymes to the E3 ligase or by stabilizing the E3-substrate complex (43, 141). Furthermore, MAGEs can also alter E3 ligase subcellular localization to bring the E3 ligase to the substrate. For example, besides specifying WASH for ubiquitination, MAGE-L2 also mediates the localization of TRIM27 to retromer-positive endosomes (149, 160).

Beyond RING ligases, MAGE-A11 and MAGE-C2 have been found to affect Skp1-Cullin-F-box (SCF) cullin-RING ligases (CRLs) (155, 161), but no evidence supports the existence of MAGE-SCF-CRL complexes. CRLs are the largest family of the multicomponent E3 ligases that consist of cullins, RING proteins, adaptor proteins, and substrate recognition proteins (Fig. 3*B*) (162). The cullin-RING module, which structurally resembles MRL, is the catalytic core that recruits E2 ubiquitin-conjugating enzyme and activates the transfer of ubiquitin from the E2 to the substrate (43). MAGE-C2 interacts with the RING protein Rbx1, a component of the SCF ligase (161). In contrast to an activating role in the MRL complex (141), MAGE-C2 inhibits SCF-mediated ubiquitination of cyclin E, preventing its degradation (161). MAGE-A11 binds and regulates the substrate specificity of Skp2, an F-box domain substrate recognition protein in the SCF ligase, and this interaction increases degradation of cyclin A and decreases degradation of the transcription factor E2F1 (155). In addition to MRLs and SCF-CRLs, MAGE-A11 binds HUWE1, a HECT (E6AP type) E3 ligase (Fig. 3*B*). MAGE-A11-HUWE1–dependent ubiquitination of PCF11 promotes alternative polyadenylation and 3′-UTR shortening in cancer cells (163).

In addition to regulation of ubiquitination, a number of MAGE proteins have been implicated in transcriptional regulation, either directly through binding transcription factors or indirectly through regulating their cognate E3 ligases (Fig. 3*B*). For example, MAGE-A2, -A3, and -C2-TRIM28 regulate Krüppel-associated box (KRAB) domain zinc finger protein (KRAB-ZFP) transcription factors and p53 (164, 165). Additionally, MAGE-A11 activates the androgen receptor, and both NECDIN and MAGE-G1 repress E2F1 (43). Interestingly, MAGE-B2 was recently shown to function as an RNA-binding protein (85), further expanding the range of MAGE protein molecular functions that are enabled by flexibility of the MHD.

MAGE proteins exert diverse biological functions

The discoveries over the last decades have provided evidence that MAGEs engage in diverse molecular and cellular functions, but how they contribute to normal physiology and the pathogenesis of cancer and diverse genetic diseases is only beginning to be understood (67, 166–170). In this section, we detail the current understanding of the biological functions of select MAGEs. Although both type I and II MAGEs engage in diverse functions, we are now starting to understand that they commonly converge in providing protection against diverse stressors, suggesting that ever-changing conditions drove MAGE evolution to confer faster adaptation to emerging stressors.

MAGE-A3/6 and MAGE-C2 are cancer cell–specific regulators of TRIM28

MAGE-A3, -A6, and -C2 are some of the most commonly expressed CTAs in human cancer (Fig. 2*B*). Given their restricted normal expression (11) and strong antigenic potential (171), these MAGEs attracted a lot of attention as targets for cancer immunotherapy (101). *MAGE-A3* and *MAGE-A6* are highly similar genes that encode proteins with 96% identity (156); we will refer to these as *MAGEA3/6* herein. *MAGEA3/6* are expressed in 30–80% of tumors from diverse types of cancer

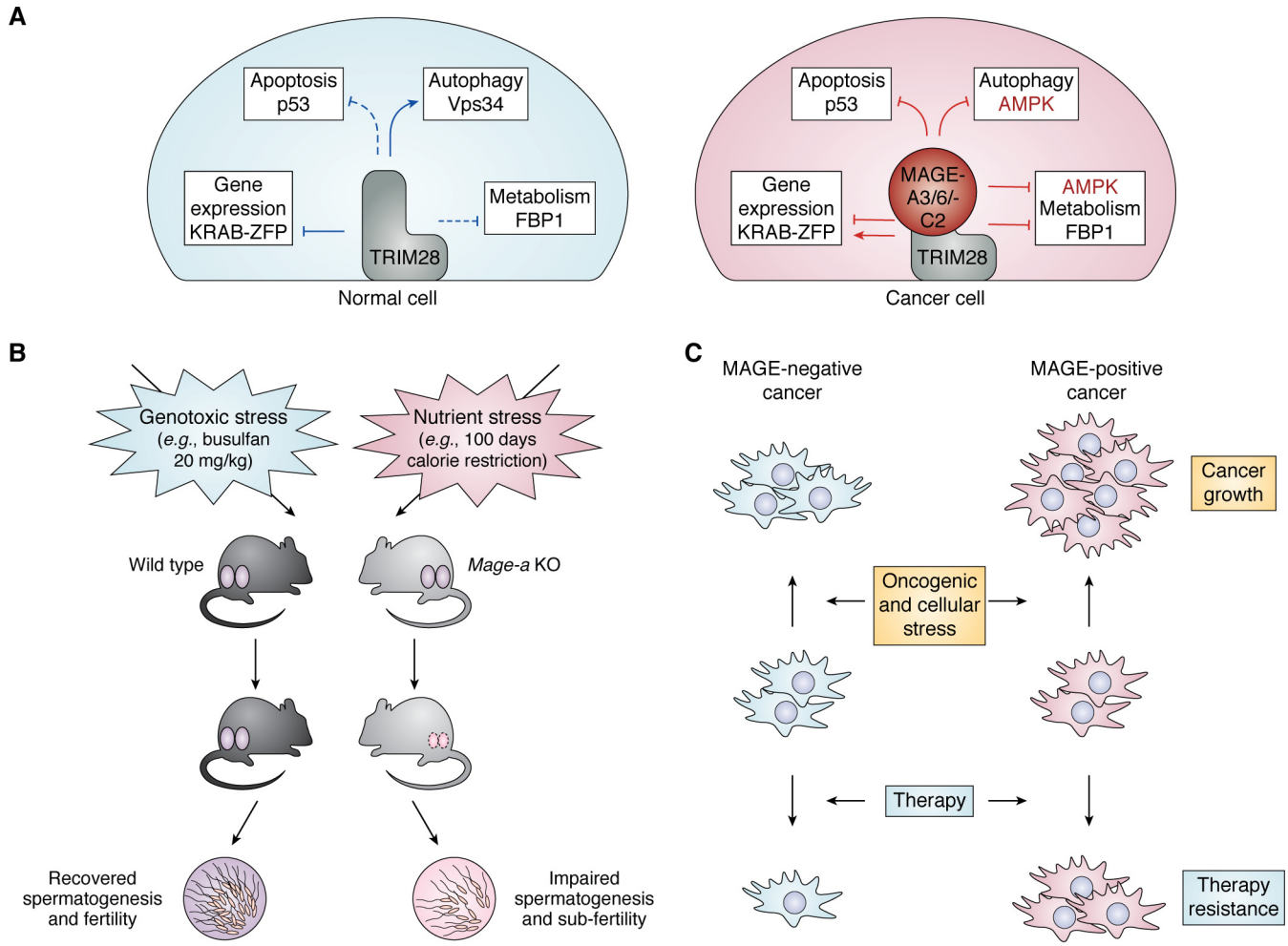

Figure 4. MAGE-A3/6 and -C2 are cell type–specific regulators of TRIM28 that confer stress resistance to male germline and cancer. *A*, MAGE-A3/6 and -C2 act as specific regulators of TRIM28 function in transcriptional regulation, apoptosis, autophagy, and cell metabolism (MAGE-A3/6 and MAGE-C2 are cancer cell-specific regulators of TRIM28). *B*, after genotoxic or nutritional stress, recovery of spermatogenesis in *Mage-a* KO mice is compromised compared with WT mice. *C*, MAGE-A3/6 and -C2 promote cancer growth and enable therapy resistance, likely by protecting cells against diverse stressors they encounter during tumorigenesis and treatment.

(Fig. 2*B*) (101), and expression of *MAGEA3/6* significantly correlates with more aggressive disease progression, poor patient prognosis, and decreased overall survival (151, 172–177). Cancer cells that aberrantly activate *MAGE-A3/6* become dependent on them, as depletion of MAGE-A3/6 leads to decreased cell viability and decreased clonogenicity (151, 178). Furthermore, expression of either MAGE-A3 or -A6 in MAGE-A3/6–negative cancer cells promotes several hallmarks of cancer, all of which suggests that these proteins have a pro-oncogenic function (131, 151, 158). Additionally, MAGE-A3 is enriched in the stem cell population of bladder cancer and in the stem cell-like population of multiple cancer cell lines (179, 180), further indicating a function in the progression of malignancies. MAGE-C2 was first discovered in melanoma cell lines (84, 181) and was then identified as an immunogenic molecule in hepatocellular carcinoma (HCC) (182). *MAGE-C2* expression was subsequently associated with high tumor grade, reduced recurrence- and metastasis-free survival, and decreased overall survival in multiple tumor types (84, 133, 183–195).

Given their importance in cancer, many studies have sought to understand the molecular functions and tumorigenic role of MAGE-A3/6 and -C2. The first insight came with the discovery that MAGE-C2 and MAGE-A proteins bind to TRIM28 (43, 141, 151, 158, 196). TRIM28, also known as KAP1 or Tif1β, is a versatile protein that was first described as a cofactor for transcription factors from the KRAB-ZFP family. Now it is a well-established regulator of gene repression that is particularly important for the silencing of endogenous retroviruses (164, 165). In addition, TRIM28 regulates the activity of transcription factors without a KRAB domain (*i.e.* c-Myc and E2F1), promotes histone deacetylation and methylation, and recruits heterochromatin protein 1 (HP-1) (164, 165). Besides transcriptional and epigenetic regulation, TRIM28 also functions as a signaling scaffold protein and as a SUMO and ubiquitin E3 ligase (197). Furthermore, TRIM28 promotes p53 ubiquitination and degradation (198). Given its versatile molecular functions, TRIM28 is important for many biological processes, several of which are regulated by MAGE proteins (Fig. 4*A*) (199–205).

TRIM28 MRLs regulate transcription—By binding to TRIM28, MAGE-A3 and -C2 regulate KRAB-ZFP transcription factor–mediated gene repression (Fig. 4*A*) (148, 157). With some types of KRAB-ZFPs, MAGEs can relieve the TRIM28-

KRAB-ZFP–mediated repression by promoting ubiquitination and degradation of distinct ZFPs (157). In contrast, MAGEs can also further enhance TRIM28-mediated repression without causing ubiquitination or degradation of the KRAB-ZFPs, whereas ZFPs with a Scan box motif KRAB domain are not affected by MAGEs (148, 157). By modifying TRIM28-KRAB-ZFP function, MAGEs alter the expression of several genes (148, 157), which suggests a potential molecular mechanism underlying the oncogenic role of MAGEs. For example, TRIM28 promotes gene silencing when at the *ID1* promoter, but MAGE-A3 and -C2 are able to relieve the silencing via removal and subsequent proteasomal degradation of ZNF382, the KRAB-ZFP that acts as a tumor suppressor and normally inhibits *ID1* (206). The resulting chromatin relaxation promotes *ID1* expression and unleashes its pro-oncogenic functions (148, 206).

MAGE-C2 has also been implicated in transcriptional regulation, independent of TRIM28. MAGE-C2 interacts with the putative tumor suppressor BS69 (207), which negatively regulates the Epstein–Barr virus (EBV) protein, LMP1, and prevents LMP1-mediated NF-κB activation and IL-6 production (207). MAGE-C2 binding to BS69 mediates the ubiquitination and proteasomal degradation of BS69, thus promoting LMP1-induced IL-6 production, STAT3 signaling, and oncogenic transformation of EBV-infected cells (207, 208). In melanoma cells, MAGE-C2 also interacts with STAT3 and inhibits its degradation to promote amoeboid invasion of cells and potentially confers metastatic potential in tumors (209).

TRIM28 MRLs inhibit p53—TRIM28 regulates p53 protein stability in cells through E3 ligase MDM2 (198, 210); however, TRIM28 MRLs promote p53 degradation independently of MDM2 (Fig. 4*A*) (141, 158). In addition to promoting TRIM28-mediated p53 degradation, MAGE-A proteins directly bind p53 and inhibit its function by recruiting HDAC3 to the promoter binding sites of p53, thereby converting p53 from a trans-activator of *CDKN1A* (p21) into its repressor (211). MAGE-A proteins also interfere with the DNA-binding surface of the p53 core domain and prevent p53 from interacting with its cognate binding sites in chromatin, further inhibiting the expression of p53-responsive genes (212). In multiple myeloma, MAGE-A3 was predominantly detected in relapsed patients, where its expression correlated with higher proliferation status (213). Further investigation revealed that MAGE-A3 is required for the survival of proliferating myeloma cells through both p53-dependent and -independent mechanisms (213).

MAGE-C2 may also regulate tumor growth by enhancing cellular proliferation and DNA damage apart from TRIM28-mediated regulation of p53. Through binding to another RING domain protein Rbx1 (Fig. 3*B*), MAGE-C2 inhibits the E3 ligase activity of the SCF complex and prevents ubiquitination and proteasomal degradation of cyclin E; thus, MAGE-C2 increases the levels of cyclin E, which promotes G_1-S transition and cell proliferation (161). In addition, binding of MAGE-C2 to TRIM28 increases ataxia-telangiectasia-mutated (ATM)-dependent phosphorylation of TRIM28 Ser-824, which facilitates the repair of DNA double-strand breaks (214).

Together, these findings indicate that MAGE-A3/6 and -C2 inhibit apoptosis, promote cell growth and tumor survival, and likely confer resistance to antitumor drugs, such as etoposide (Fig. 4*C*) (158, 198, 211, 213, 215). MAGE-mediated p53 inactivation might be particularly important in cancers with low mutation rates, like in melanomas and cervical carcinomas, where WT p53 is often present (216, 217). Interestingly, several cancer cell lines with p53 deletion are dependent on MAGEs for viability, and p53 mutation status does not correlate with *MAGE-A3/6* or *-C2* expression, which led to the finding that these MAGEs have p53-independent functions that contribute to their pro-oncogenic activity (151).

TRIM28 MRLs inhibit autophagy and rewire cancer metabolism—In addition to regulating p53 and cell viability, MAGE-A3/6 and -C2 also act as oncogenes by inhibiting autophagy and enabling metabolic flexibility in cancer cells (Fig. 4, *A* and *C*) (151, 196). MAGE-A3/6 and -C2 accomplish the metabolic rewiring by specifying two major metabolic proteins, AMPK and fructose-1,6-biphosphatase (FBP1), for TRIM28-mediated ubiquitination and subsequent proteasomal degradation (151). Unlike the targeting of p53 (141), AMPKα1 is targeted by TRIM28 only in the presence of MAGE-A3/6 or -C2 (151). Through binding to AMPKα1 and TRIM28, these MAGE proteins enable the ubiquitination and subsequent proteasomal degradation of AMPKα1 that leads to a reduction in overall AMPK protein levels (151). In line with MAGE-A3/6 regulating AMPK stability, MAGE-A3/6 mRNA expression inversely correlates with AMPK activity and protein levels in diverse cancer patient samples, including breast, lung, and colon cancer (134, 151). In colon cancer, MAGE-A3/6 mRNA levels inversely correlate with the expression of miR-1273g-3p, which silences MAGE-A3/6 expression and inhibits human colorectal cancer cell growth via AMPK activation (134). Likewise, in hepatocellular carcinoma, another miRNA, miR-448, was shown to inhibit expression of MAGE-A6, thereby activating AMPK signaling and inhibiting tumor growth, as well as inhibiting stemness maintenance and self-renewal of cancer stem cells (137). In glioma and renal cell carcinoma, MAGE-A6 was also shown to promote cell survival by targeting AMPKα1 (135, 138). Furthermore, MAGEA6-AMPK signaling was activated by lnc-THOR silencing, which inhibited human glioma cell survival (136), further corroborating an important role of AMPK regulation in the oncogenic function of MAGE-A3/6.

As a master sensor of cellular energy that is activated in response to energy stress, AMPK promotes catabolic processes, such as autophagy, while inhibiting anabolic processes and cell growth to restore energy balance (218–220). Accordingly, depletion of MAGE-A3/6 and -C2 or TRIM28 in several MAGE-positive cancer cells not only increases the levels of both total AMPK and the active form, but also leads to concomitant suppression of the mTOR signaling pathway (134–138, 151, 156). These results suggest that MAGE-A3/6 and MAGE-C2 can rewire cancer metabolism toward dependence on mTOR signaling for survival (221). Given that inhibition of MAGE-A3/6 expression and consequent activation of AMPK signaling inhibits cell growth in several cancer cell types (101, 151), MAGE-A3/6 may serve as biomarkers for effective use of AMPK agonists (*e.g.* metformin) and mTOR inhibitors (*e.g.* everolimus) that are already used in the clinic (134–138, 222, 223).

By inhibiting AMPK, MAGE-A3/6 and -C2 also act as molecular switches that convert TRIM28 from a pro-autophagic to

an anti-autophagic factor (Fig. 4*A*) (151, 156). In the absence of MAGEs, TRIM28 functions as a pro-autophagic factor through its SUMO ligase activity, where it SUMOylates PIK3C3/VPS34 to promote formation of the PIK3C3-BECN1 complex and autophagy (156). However, in cells that express MAGE-A3/6 or MAGE-C2, TRIM28 MRLs target AMPK for degradation and, by doing so, inhibit autophagy and promote mTOR signaling, which may provide the optimal environment for early tumor formation and growth (151, 224).

In addition to regulating AMPK and mTOR signaling, MAGE-A3/6 and -C2-TRIM28 also impact glucose metabolism (151). In HCC, MAGE-A3 and -C2 enhanced TRIM28-mediated degradation of FBP1, a rate-limiting enzyme in gluconeogenesis (196). By promoting FBP1 degradation, TRIM28 MRLs increased glucose consumption and lactate production, promoted the Warburg effect, and reprogrammed cancer cell metabolism to support HCC progression (196). Thus, inhibiting MAGE-TRIM28–mediated degradation of substrates, such as FBP1, could be a therapeutic option for the treatment of advanced HCC. Intriguingly, the FBP1 substrate, fructose 1,6-bisphosphate (FBP), has been recently identified as a glucose sensor and inhibitor of AMPK activation (225), suggesting that TRIM28 MRLs may inhibit AMPK activity by several mechanisms. However, additional studies are necessary to understand the underlying mechanistic details and the specific contributions of MAGE-A3/6 suppression of AMPK through MAGE-mediated AMPKα1 ubiquitination compared with indirect regulation by degradation of FBP1 and subsequent increase in FBP.

Beyond controlling cell metabolism, MAGE-A3/6 themselves are also regulated in response to nutrient availability (226). Upon short-term cellular starvation, MAGE-A2, -A3/6, and -A12 are rapidly degraded by the ubiquitin–proteasome system, suggesting dynamic regulation of these proteins in different metabolic states (226). Starvation-induced degradation of these MAGE-A proteins is controlled by the CRL4-DCAF12 E3 ligase and depends on two glutamate residues (–EE*) at the extreme C terminus of the proteins that serve as a degron signal for CRL4-DCAF12 targets (226, 227). Intriguingly, CRL4-DCAF12 likely does not degrade MAGE-C2 because it lacks these C-terminal glutamates, which is indicative of diverse regulation and functional complementarity of seemingly redundant MAGE proteins.

The regulation of MAGE-A3/6 by CRL4-DCAF12 is important to achieve robust autophagy induction during nutrient starvation (226). Although inhibition of autophagy is often critical for tumor initiation, the reinstitution of autophagy promotes tumor progression (224). Thus, MAGE-A3/6 may suppress autophagy during early phases of tumor initiation, but as nutrient stress on tumors emerges, this brake may be relieved through CRL4-DCAF12–mediated down-regulation of MAGE-A3/6 to allow autophagy induction and tumor progression (226). Besides nutrient deficiency, acquisition of cancer-associated mutations in MAGE-A6 was also found to release autophagy inhibition in pancreatic cancer (228), in which MAGE-A6 is among the top 16 most commonly mutated genes (229). The identified MAGE-A6 mutations lead to its proteasomal degradation, suggesting that pancreatic cancer progression depends on the release of autophagy inhibition through degradation of MAGE-A6, induced either by nutrient deficiency or acquisition of mutations (228). Further studies will show how these processes are fine-tuned during tumorigenesis and the relevance to their physiological function in germ cells. Because glycolysis is important for stemness maintenance in SSCs, MAGE-A3/6 expression in spermatogonia implies a role in SSC biology and spermatogenesis regeneration (11, 83, 99, 230).

Mage-a proteins enable robust spermatogenesis under genotoxic and nutritional stress in mice

In primary cultures of undifferentiated spermatogonia, Mage-a proteins are important for maintaining the stemness of SSCs as knockdown of Mage-a proteins leads to the loss of ID4-positive cells and lower transplantation efficiency (11, 231). Intriguingly, the depletion of two (*Mage-a4* and *-a10*), six (*Mage-a1*, *-a2*, *-a3*, *-a5*, *-a6*, and *-a8*), or all eight *Mage-a* genes does not affect male reproduction in mice (11, 232), suggesting that Mage-a proteins are dispensable for unperturbed male spermatogenesis when animals are kept under normal laboratory conditions.

Given the very recent evolutionary appearance of *Mage-a* genes and the astonishingly conserved basic processes of spermatogenesis, the lack of a phenotype in these *Mage-a* KO mice in an optimal environment suggests that Mage-a proteins may provide an advantage when animals endure stress (Fig. 4*B*) (11, 232). In support of this idea, short-term treatment of *Mage-a* KO mice with the genotoxic agent *N*-ethyl-*N*-nitrosourea results in higher germ cell apoptosis (11, 232). Interestingly, unchallenged *Mage-a* KO mice exhibit increased p53 protein levels, indicative of increased apoptosis and conservation of p53 regulation by human and mouse MAGE-A proteins within both germ cells and cancer (11, 141, 233). This result is consistent with increased survival of SSCs after irradiation in p53 KO mice (234). Furthermore, exposure to genotoxic stress by treatment with the chemotherapeutic busulfan, which completely ablates the germline, impairs the recovery of spermatogenesis in *Mage-a* KO mice, likely a result of both greater germ cell damage during the treatment and lower stem cell regenerative capacity after treatment (Fig. 4*B*) (11).

In accordance with MAGE-A3/6 regulation of AMPK and metabolism in human cancer cells and nutrient availability being one of the major drivers of evolution, mouse Mage-a proteins are also involved in metabolic adaptation of germ cells. Inducing nutrient stress with caloric restriction impairs spermatogenesis in *Mage-a* KO mice, indicating that Mage-a proteins provide an advantage to spermatogenesis when food is limited (Fig. 4*B*) (11). Interestingly, the peak expression of *Mage-a* genes just before male germ cells start transitioning through the BTB (Fig. 2*A*) suggests that *Mage-a* genes may have evolved to provide protection to the germline during metabolic stress, either arising from the environment or due to the metabolic switch during spermatogenesis. Accordingly, spermatogonial cells from *Mage-a* KO mice are more sensitive to 2-deoxy-D-glucose (2DG)-induced glycolysis inhibition than WT cells (11). Intriguingly, MAGE-A6–expressing human

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pancreatic cancer cells hijack this protective function when treated with 2DG, as they adapt faster to the induced metabolic stress and develop resistance against 2DG (Fig. 4*C*) (11).

MAGE-A11 regulates androgen receptor signaling and alternative polyadenylation

MAGE-A11 is a unique, primate-specific member of the MAGE-A subfamily that acts as a steroid hormone receptor transcriptional coregulator and proto-oncogenic protein implicated in prostate cancer (41). Another molecular function for MAGE-A11 in promoting mRNA alternative polyadenylation (APA) was recently discovered, defining an additional oncogenic function of this enigmatic member of the MAGE-A subfamily (163).

MAGE-A11 is normally expressed in the testis (Fig. 2*A*), in syncytiotrophoblasts of the placenta (11, 163), and cyclically in human endometrium (235). Like other type I MAGEs, MAGE-A11 is often aberrantly activated in human tumors (Fig. 2*B*) (43, 101, 105, 111, 163, 236–238). Not only is MAGE-A11 aberrantly expressed in cancer, but its expression is necessary and sufficient to drive tumorigenesis (163). Additionally, MAGE-A11 has been associated with worse disease progression (239–241) and therapy resistance (242, 243).

Initially identified through a yeast two-hybrid screen using the androgen receptor (AR) F*XX*LF motif as bait, MAGE-A11 was subsequently shown to act as a coregulator that stabilizes ligand-free AR (Fig. 3*B*) (244). The AR F*XX*LF motif interacts with a putative F-box motif located in the MHD of MAGE-A11, and this interaction is modulated by checkpoint kinase 1 and MAPK phosphorylation and ubiquitination (245, 246). MAGE-A11 promotes AR transcriptional activity though F-box–mediated interactions with coactivators, such as steroid receptor coactivator (SRC)/p160, transcriptional intermediary factor 2 (TIF2), and histone acetyltransferase p300 (244, 246, 247). MAGE-A11 expression has also been shown to increase during androgen deprivation therapy in prostate cancer, suggesting that the capacity of MAGE-A11 to activate AR in an androgen-independent fashion may play a critical role in the development of castration-recurrent prostate cancer (43, 105).

In addition to AR, MAGE-A11 interacts with the progesterone receptor (PR). MAGE-A11 specifically binds the PR-B isoform, which positively regulates the effects of progesterone and induces transcription via interactions with p300 during the cyclic development of the human endometrium (235). The highest levels of MAGE-A11 occur in the mid-secretory stage, coincident with the window of uterine receptivity to embryo implantation; however, the role of MAGE-A11 during decidualization and pregnancy establishment (248) has not been fully explored. The cellular functions of MAGE-A11 in female reproduction are almost completely unknown. Intriguingly, MAGE-A11 expression correlates with delayed endometrial decidualization and infertility in polycystic ovary syndrome, a common gynecological disorder that affects up to 12% of women of reproductive age (249). This association suggests that MAGE-A11 may also have an important role in female reproduction.

Besides interacting with steroid hormone receptors, AR, and PR, MAGE-A11 has also been shown to function as an E3 ligase substrate adapter to regulate protein ubiquitination and stability (Fig. 3*B*) (150). MAGE-A11 interacts with Skp2, the substrate recognition protein of the SCF E3 ligase, as well as with cyclin A, a target of Skp2; the presence of MAGE-A11 leads to enhanced E2F1 transcription activity by increasing Skp2-mediated degradation of cyclin A and decreasing degradation of E2F1 (155). MAGE-A11 also interacts with and stabilizes RB family proteins, including p107 and RB (237). Remarkably, MAGE-A11 binding to p107 flipped p107 from a transcriptional repressor to a transcriptional activator of AR and E2F1 to promote tumorigenesis in prostate cancer (237). Interestingly, the tumor suppressor p14-ARF has been demonstrated to target MAGE-A11 for degradation in a lysine-independent fashion and prevent MAGE-A11 activation of E2F1 (250).

Beyond the function of MAGE-A11 in conjunction with AR, PR, and E2F1, MAGE-A11 also regulates APA—the process by which the mRNA 3′-end processing complex utilizes one of several possible polyadenylation sites within the UTRs of a gene and, thus, regulates the length of the 3′-UTR of an mRNA transcript (Fig. 5*A*) (163). At least 70% of mammalian transcripts are alternatively polyadenylated (251, 252). APA-mediated 3′-UTR shortening (3′-US) can affect mRNA transcript stability, translation efficiency, nuclear export, and cellular localization (253). APA often occurs in a tissue– or developmental stage–specific manner and is associated with diverse biological processes, including T lymphocyte activation, brain development and function, and male germ cell differentiation (254–256).

Despite its integral function in normal biology, aberrant APA, including 3′-US, is often associated with cancer as a hallmark of most tumors (257, 258). An effort to uncover the molecular mechanisms of MAGE-A11 oncogenic activity identified PCF11, the poly(A) cleavage factor of the mRNA 3′-end processing complex, as a MAGE-A11 binding partner (163). The direct interaction of MAGE-A11 with PCF11 leads to the ubiquitination and degradation of PCF11 by recruiting the E3 ligase HUWE1 (Fig. 5*A*) (163). This regulation of PCF11 by MAGE-A11 drives APA and contributes to 3′-US in human tumors (163). Consistent with previous findings that depletion of CFIm25, another component of the mRNA 3′-end processing complex, induces 3′-US (259–261), MAGE-A11-HUWE1 ubiquitination of PCF11 inhibits the association of CFIm25 with RNA polymerase II, leading to remodeling of the mRNA 3′-end processing complex and inducing 3′-US (163).

Several *bona fide* oncogenes and tumor suppressor genes have altered 3′- UTRs associated with MAGE-A11-HUWE1–mediated ubiquitination of PCF11, which directly links MAGE-A11 function to tumorigenesis (Fig. 5*A*) (163). Analysis of ovarian and lung squamous carcinomas from TCGA data sets shows that MAGE-A11–expressing tumors have a significantly higher number of transcripts with 3′-US than MAGE-A11–negative tumors, implying that MAGE-A11 drives 3′-US in human cancer (163). MAGE-A11–induced 3′-US has both *cis* and *trans* effects on oncogenes (*i.e.* cyclin D2) and tumor suppressors (*i.e.* PTEN), either by increasing protein levels through loss of miRNA repression or down-regulating competing

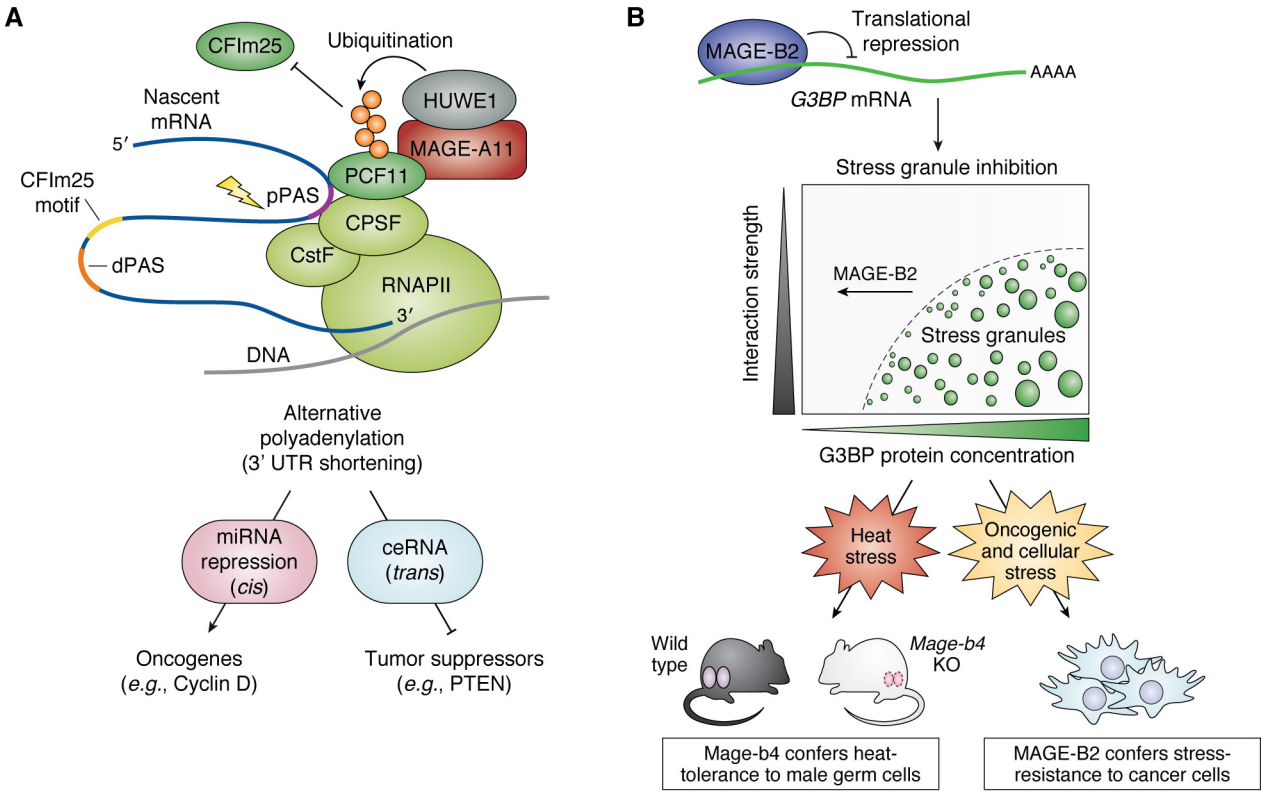

Figure 5. MAGE-A11 and -B2 affect transcription and translation, respectively. *A*, by binding to the E3 ligase HUWE1, MAGE-A11 specifies PCF11 for ubiquitination, which displaces CFIm25 from the mRNA 3′-end processing complex. The subsequent remodeling of the complex leads to 3′-UTR shortening, which leads to increased levels of oncogenes through loss of miRNA repression. Additionally, down-regulation of ceRNAs inhibits tumor suppressors. Thus, MAGE-A11 function in APA contributes to tumorigenesis. *B*, MAGE-B2 binds to the *G3BP* mRNA transcript to repress its translation and decrease G3BP protein concentration. As a result, MAGE-B2/-b4 inhibit stress granule formation and promote cellular stress tolerance, giving a growth advantage to cancer cells and heat tolerance to male germ cells.

endogenous mRNAs (ceRNAs), respectively, to eventually stimulate key progrowth pathways (163).

Interestingly, polyadenylation site choice in germ cells differs from somatic cells and often results in 3′-US, which generates germ cell–specific transcripts and protein isoforms (255, 256, 262). The mechanism underlying widespread APA in germ cells leading to 3′-US is not well-understood, but changes in composition of the polyadenylation machinery have been proposed (263). Given their molecular function in cancer, MAGE-A11 and HUWE1 may be important factors in promoting APA in male germ cells. Consistently, HUWE1 has been shown to be important for spermatogonial differentiation and entry into meiosis (264).

MAGE-B2/Mage-b4 regulate stress tolerance in cancer and germ cells

Like other type I MAGE CTAs, MAGE-B2 is primarily expressed in the testis and is aberrantly expressed in various cancers, where it has been implicated in tumor growth and progression (Figs. 1 and 2). More specifically, MAGE-B2 overexpression promotes cell proliferation in transformed oral keratinocytes, whereas MAGE-B2 depletion reduces proliferation in osteosarcoma cell lines (106, 265). Moreover, subcutaneous injection of mouse melanoma cell lines expressing human MAGE-B2 enhances tumor xenograft growth in mice (265). Interestingly, MAGE-B2 is thought to be activated early during carcinogenesis and is also expressed in the cancer stem cell–like population derived from colon adenocarcinoma cells (180). Despite the mounting evidence indicative of MAGE-B2's oncogenic potential, little was known about its molecular function until a recent study revealed a theme analogous to MAGE-A function—stress tolerance (85).

MAGE-B2 enhances the cellular stress threshold by suppressing stress granule (SG) assembly (Fig. 5*B*) (85). SGs are conserved ribonucleoprotein membraneless organelles that form in response to a variety of stress stimuli (266, 267). Upon exposure to stress, translation stalls, polysomes disassemble, and a number of proteins and mRNAs condense into cytoplasmic SGs via liquid–liquid phase separation (LLPS) (267–270). Although the specific proteins and mRNAs that localize to SGs are stress-dependent, G3BP1 and its paralog G3BP2 (collectively referred to as G3BP) are uniquely critical for SG core assembly, as overexpression of G3BP induces spontaneous SG formation and deletion ablates SGs in response to sodium arsenite (271–279). Whereas the exact molecular features that drive SG formation are still being elucidated, G3BP is predicted to promote LLPS of SGs due to its ability to bind RNA and form higher-order oligomers (272).

MAGE-B2 regulates SG dynamics, such that depletion of MAGE-B2 leads to increased SG formation and overexpression of MAGE-B2 has the opposite effect (85). MAGE-B2 depletion in U2OS osteosarcoma cells was previously shown to decrease

cell number and colony formation capacity without changing BrdU incorporation (265), suggesting that MAGE-B2 depletion does not affect cell proliferation (85). However, upon exposure to prolonged oxidative stress by a low dose of sodium arsenite, MAGE-B2 KO cells exhibit reduced cell viability that can be rescued by re-expression of MAGE-B2 (85, 265). Furthermore, low-dose treatment with the ribotoxic agent actinomycin D also leads to reduced proliferation of MAGE-B2 KO cells compared with WT MAGE-B2–expressing cells (265). Together, these findings suggest that MAGE-B2 increases cellular stress tolerance and provides a growth advantage in nonoptimal conditions.

Further investigation into the mechanism by which MAGE-B2 alters SG assembly revealed that MAGE-B2 reduces G3BP protein levels (85). Because *in vitro* LLPS is highly dependent on protein concentration (266), MAGE-B2-mediated regulation of G3BP protein levels alters SG formation and cell viability under prolonged stress (85). In a surprising deviation from the prototypical MAGE-RING ligase complex, MAGE-B2 works as an RNA-binding protein that directly binds the *G3BP* transcript to inhibit its translation and, thus, alters G3BP protein levels (85). In addition, MAGE-B2 binding to the 5′-UTR of *G3BP* displaces the translational activator DDX5, indicating that MAGE-B2 and DDX5 act in a competitive manner to fine-tune G3BP concentration and to regulate SG dynamics (Fig. 5*B*) (85).

In the context of normal physiology, the enriched expression of *MAGE-B2* and its mouse orthologs, *Mage-b4* and *Mage-b10* (referred to herein as *Mage-b4* due to highly similar sequence identity), in undifferentiated spermatogonia and SSCs (Fig. 2*A*) suggested that MAGE-B2 functions in stem cell maintenance and differentiation (11, 57, 85, 280–283). In support of this idea, Mage-b4 depletion in *in vitro* primary cultures of undifferentiated spermatogonia from Id4-eGFP reporter mice demonstrated that Mage-b4 plays a key role in SSC maintenance and promotes recovery after *in vivo* transplantation (85). Because male germ cells are very sensitive to heat (284–286), most mammals maintain testes in scrotum outside the body, thereby sustaining spermatogenesis at temperatures 5–7 °C lower than the core body temperature (287). In line with MAGE-B2 inhibition of SG formation being important for increasing the cellular threshold against stress, Mage-b4 provides thermotolerance for the male germline (85). *Mage-b4* KO mice exposed to testicular heat stress exhibit increased SG formation in spermatogonia and severely impaired recovery of spermatogenesis with significantly reduced fertility and increased damage within the seminiferous tubules (Fig. 5*B*) (85). Together, these data suggest that, like *Mage-a* genes, *Mage-b4* evolution was driven by protecting the male germline and preserving fertility in more extreme conditions.

MAGE-B2 also enhances the activity of E2Fs (265), which are transcriptional regulators of cell cycle progression (288). Elevated expression of E2F target genes in tumors is thought to induce aberrant cell proliferation and increase cell cycle–generated genomic errors (288). MAGE-B2 overexpression enhances E2F reporter activity, whereas depletion of MAGE-B2 reduces transcript levels of known cell cycle–associated E2F target genes, such as MCM6, CyclinD1, and CDK1 (265). Through binding the E2F1 repressor HDAC1, MAGE-B2 reduces the inhibitory E2F1-HDAC1 interaction and promotes E2F1 function (265). Interestingly, both MAGE-A11 and -B2 promote E2F transcriptional activity and cell growth, whereas the type II MAGE NECDIN inhibits E2F function and induces growth arrest (66, 155, 289). Whether MAGE-B2–mediated activation of E2F contributes to stress tolerance warrants further investigation.

Curiously, whereas MAGE function is typically assessed in the context of spermatogenesis or tumorigenesis, MAGE-B2 was originally identified in pediatric systemic lupus erythematosus (SLE) patients (290). SLE is a chronic autoimmune disease that causes widespread inflammation and tissue damage in affected organs (OMIM #152700). Because genome-wide methylation abnormalities are present in SLE patients (291, 292), it is plausible that disrupted DNA methylation allows for aberrant MAGE-B2 expression and the presentation of normally hidden antigens to provoke an autoimmune response and inflammation. Whereas the pathogenic role of MAGE-B2 in SLE remains unknown, the presence of MAGE-B2 protein and autoantibodies in patients with active lupus nephritis and the ability of MAGE-B2 to stimulate an immune response when it is presented by the MHC suggest a potential role in immune activation (290, 293–296).

Since its identification in SLE, MAGE-B2 autoantibodies have also been detected in patients with autoimmune polyendocrine syndrome type 1 (APS1), a monogenic autoimmune disorder that is caused by loss-of-function mutations in the autoimmune regulator (*AIRE*) gene (297, 298). *AIRE* encodes a transcription factor that plays an essential role in establishing self-tolerance in the thymus by driving promiscuous expression of tissue-restricted antigens (299, 300). This AIRE-driven antigen display allows naive T cells to be exposed to tissue-specific antigens and for the subsequent elimination of autoreactive T cells. In patients with APS1, defective AIRE allows autoreactive T cells to survive, thereby creating an autoimmune response. Intriguingly, infertility is a common manifestation of APS1 in both male and female patients. Whether MAGE-B2 antigens play a role in APS1 infertility remains unknown. Interestingly, two variant alleles of the rs1800522 AIRE SNP were shown to differently modulate MAGE-B2–specific T-cell survival and *in vivo* susceptibility to melanoma in mice (301); however, whether this finding translates to cancer predisposition in humans is yet to be determined.

MAGE-D1 fine-tunes apoptosis and differentiation during neurogenesis and oncogenesis

The MAGE-D subfamily, comprised of four genes in humans and three highly homologous orthologs in mice (Fig. 1), was identified through sequence homology with the initially discovered MAGE-A genes (39, 60). Among all of the MAGEs, *MAGE-D1* (also referred to as *NRAGE* and *Dlxin-1*) is expressed at the absolute highest level across diverse tissues (11). In the central nervous system, *MAGE-D1* is expressed throughout the neural tube during the early stage of neurogenesis and becomes restricted within the ventricular zone, subplate, and cortical plate during the later stage (69). High

expression in the brain during development and in adults suggests a functional relevance in neurogenesis and brain physiology (70, 302–304)

In addition to the MHD, MAGE-D1 protein contains a unique WQ*XP*X*X* hexapeptide repeat domain that confers specific interactions. Both the hexapeptide repeat domain and the MHD are important for MAGE-D1's molecular functions (75, 305–308). The cytosolic region of p75 neurotrophin receptor (p75NTR) was identified as the first binding partner of MAGE-D1 that promotes neuronal apoptosis during development (75, 308). Further studies not only uncovered additional MAGE-D1 interacting partners, but also expanded its diverse biological functions to include apoptosis, cell cycle progression, cell adhesion, angiogenesis, and developmental morphogenesis (75, 76, 78, 305, 309, 310).

MAGE-D1 is required for apoptosis during embryonic development and neurogenesis

MAGE-D1 regulates several apoptotic pathways, each caused by distinct input signals to ultimately drive neurogenesis (311). By binding to p75NTR, MAGE-D1 triggers cell cycle arrest and mediates neurotrophin-dependent apoptosis that requires JNK activation (75, 308). Following nerve growth factor treatment, MAGE-D1 accumulates at the plasma membrane to prevent p75NTR from binding to the receptor tyrosine kinase TrkA, which normally blocks p75NTR-dependent apoptotic signaling (75). *In vitro*, MAGE-D1 also promotes neurodifferentiation of PC12 cells by interacting with TrkA and by early activation of the MEK and Akt signaling pathways (312).

In addition, MAGE-D1 promotes apoptosis through interaction with the axon guidance receptor UNC5H1 (313) and different antiapoptotic proteins, like members of the inhibitors of apoptosis protein (IAP) family (314). In neural progenitor cells, MAGE-D1 interacts with the RING domain of X-linked IAP (XIAP), promoting its caspase-mediated cleavage and degradation (314) to transmit proapoptotic signals and NF-κB activation via the BMP alternative pathway (315). MAGE-D1 can also interact with CHE-1, an apoptosis-antagonizing transcription factor, which inhibits apoptotic signaling by binding RB and removing HDAC1 from E2F target promoters (78). MAGE-D1 sequesters CHE-1 in the cytoplasm, thereby promoting its ubiquitination and proteasomal degradation to further promote apoptosis (78).

Interestingly, MAGE-D1 itself is also regulated by ubiquitination. Bone morphogenetic protein 4 (BMP4) enhances Lys-63–linked polyubiquitination of MAGE-D1 by the SCF^{FBXO7} E3 ligase complex and facilitates formation of the MAGE-D1–TAK1–TAB1 complex, which up-regulates NF-κB and p38 and promotes caspase-dependent apoptosis (316, 317). BMP4-mediated activation also leads to formation of the MAGE-D1-TAK1-TAB1-XIAP complex, which has been shown to stimulate p38 in renal branching morphogenesis (310), IKK-α/β in macrophage migration inhibitory factor production (315), and G_0/G_1 arrest and apoptosis in dental pulp cells (318).

Together, the pleiotropic molecular functions of MAGE-D1 all converge on promoting apoptosis and cell cycle exit of neural progenitors to promote neuronal differentiation (316). To balance proliferation and differentiation during neurogenesis, MAGE-D1 protein level is controlled by PRAJA1, a RING E3 ligase that ubiquitinates MAGE-D1 and promotes its degradation by the proteasome (319).

Beyond neurogenesis, MAGE-D1 is also implicated in the differentiation of other cell types through regulation of the DLX/MSX family of homeodomain proteins, which MAGE-D1 binds via its hexapeptide repeat domain (320). MAGE-D1 binds to and enhances the activity of DLX5, a homeodomain-containing transcription factor that is expressed in the forebrain, limbs, and branchial arches during embryonic development and is important for digit formation in mice (306). Besides DLX5, MAGE-D1 also interacts with other homeodomain proteins, DLX4 and MSX2 (306), the latter of which is targeted for ubiquitination and proteasomal degradation by the MAGE-D1-PRAJA1 MRL (141, 320). Additionally, MAGE-D1 cooperates with the type II MAGE NECDIN in the developing brain and skeletal muscle, where MAGE-D1-NECDIN heterodimer inhibits MSX2 to promote terminal differentiation of postmitotic cells (320, 321). Besides PRAJA1, MAGE-D1 levels are also controlled by ROR2 receptor tyrosine kinase, which binds and sequesters MAGE-D1 at the cell membrane to prevent it from interacting with MSX2 (322).

In addition to expression and function during embryonic development, MAGE-D1 can be induced by muscle injury and is required for p21 induction and muscle regeneration (323). Furthermore, MAGE-D1 was implicated in regulation of the cytoskeleton and cell adhesion, in part by inhibiting hypoxia-induced HIF-1 activation (324, 325). However, further study is needed to understand the molecular mechanisms and the biological significance of MAGE-D1 functions in these processes.

Mage-d1 KO mice exhibit defects in neurogenesis and brain function

Based on its significance in facilitating neuronal apoptosis and neurogenesis during embryonic development, a *Mage-d1* KO mouse model was generated to further explore MAGE-D1 functions (5). Although *Mage-d1* KO mice are normal in terms of gross morphological and histopathological features, developmental apoptosis of the sympathetic neurons of the superior cervical ganglia is defective, similar to that observed in p75NTR KO mice (326). In line with *in vitro* data (75), primary cultures of sympathetic neurons derived from *Mage-d1* KO animals are resistant to BDNF-p75NTR–mediated apoptosis. Further, *Mage-d1* KO mice show defects in hair follicle catagen phase, another p75NTR-dependent apoptotic process, where hair follicles regress following morphogenesis (326). *Mage-d1* KO mice also present with defects in motor neuron apoptosis that are not perturbed in p75NTR KO mice, supporting the role of Mage-d1 in p75NTR-independent apoptosis (76, 313, 326).

These KO mice also revealed the role of Mage-d1 in other brain functions, including regulation of mood, behavior, memory, body weight, and circadian rhythm (70, 302–304). Although not cyclic by itself, Mage-d1 regulates the circadian rhythm by binding to nuclear receptor RORα (303). Further, Mage-d1 promotes ubiquitination and proteasomal degradation of serotonin transporter (SERT), which prevents serotonin

ASBMB

uptake, leading to lower serotonin levels in the prefrontal cortex, which, ultimately, results in symptoms of depression (70). Further analysis of KO mice implicated Mage-d1 in a complex behavioral syndrome that includes anxiety, decreased social interaction, memory loss, and obesity. These symptoms are explained by a reduction of mature oxytocin, a neuropeptide produced within the hypothalamus and released into the blood from the posterior pituitary that stimulates bonding and prosocial behavior (302), but the molecular mechanism underlying Mage-d1 regulation of oxytocin levels warrants further investigation. *Mage-d1* KO mice also exhibit impaired cognitive functions, suggesting that Mage-d1 is involved in synaptic transmission and hippocampus-dependent learning and memory formation (304). By interacting with cAMP-response element–binding protein (CREB) transcription factor, Mage-d1 regulates the expression of BDNF, which is instrumental in hippocampus-dependent learning and memory formation (304).

By interacting with a range of proteins, from transcription factors, like CREB and RORα, to transmembrane receptors, like SERT and p75NTR, MAGE-D1 modulates diverse physiological pathways to fine-tune neurogenesis and brain functions. Intriguingly, despite these extensive functions, mice can survive without Mage-d1. Yet because Mage-d1 exhibits many diverse functions in the brain, we speculate that it enabled animals to better adapt to challenges and provided an evolutionary advantage.

MAGE-D2 in cellular stress response and kidney function

The function of MAGE-D2 remained mysterious for a long time, but discovering the cellular localization of MAGE-D2 provided the first insights into its biological role. MAGE-D2 may be found within the cytoplasm and the nucleolus; however, the location of MAGE-D2 changes throughout the cell cycle (327). Whereas MAGE-D2 is cytoplasmic and nucleolar in G_1 phase, it becomes progressively more nucleoplasmic upon the entrance into S phase (327). In prophase before the disassembly of the nucleus, MAGE-D2 leaves the nucleolus and becomes completely cytoplasmic during mitosis, until it eventually reenters the nucleolus in early G_1 (327).

MAGE-D2 regulates DNA damage response

The main function of the nucleolus is the rapid production of small and large ribosome subunits, a process that must be highly regulated to achieve proper cellular proliferation and growth, as well as to respond quickly to stress (328). Interestingly, more than half of the nucleolar proteome represents proteins, including MAGE-D2, that are transiently stored in the nucleolus and can be rapidly released to control cellular stress responses (327, 328). Upon genotoxic and nucleolar stress, MAGE-D2 is shuttled from nucleoli to the nucleoplasm, leading to a G_1/S block in cell cycle progression (327). DNA damage triggers MAGE-D2 phosphorylation by ATM/ATR, which is required to maintain proper levels of p21 and p27 to facilitate cell cycle arrest (329). Depletion of MAGE-D2 leads to reduced CHK2 phosphorylation and increased CHK1 phosphorylation, an indication of sustained ATR activation, suggesting a role for MAGE-D2 in maintaining genomic stability (329). Conversely, MAGE-D2 can also regulate p53 by decreasing p53 transcriptional activity and inhibiting TRAIL-induced apoptosis (330, 331). Altogether, the storage of MAGE-D2 in nucleoli appears to be a way to harbor MAGE-D2 until cellular stressors are encountered, allowing for rapid cell cycle arrest (327).

Antenatal Bartter's syndrome and MAGE-D2's role in embryonic kidney function

The discovery of another important MAGE-D2 function stemmed from the identification of *MAGE-D2* mutations in patients with antenatal Bartter's syndrome (OMIM #300971), which confers an increased risk of adverse perinatal outcome, especially preterm delivery. The *MAGE-D2* mutations identified in infants with transient antenatal Bartter's syndrome (332) account for 9% of all cases of antenatal Bartter's syndrome and explain 38% of patients who lack other characterized mutations (333). This syndrome is normally caused by loss-of-function mutations in the proteins that mediate renal salt reabsorption in the thick ascending limb of the loop of Henle, where MAGE-D2 is specifically expressed in fetal and adult kidneys (11, 332, 334).

Follow-up studies revealed that MAGE-D2 affects the expression and function of sodium chloride cotransporters, Na-K-Cl cotransporter (NKCC2) and Na-Cl cotransporter (NCC), which are important regulators of salt reabsorption (332). Rather than being properly embedded in the apical cell membrane of tubular epithelial cells, NKCC2 and NCC were predominantly cytoplasmic in patients and co-localized with endoplasmic reticulum markers (332). Loss-of-function and gain-of-function studies revealed that MAGE-D2 protects NKCC2 and NCC from Hsp40-mediated endoplasmic reticulum–associated degradation, indicating that MAGE-D2 is essential for fetal renal salt reabsorption, amniotic fluid homeostasis, and the maintenance of pregnancy (332). Given that MAGE-D2 mutant–associated antenatal Bartter's syndrome self-resolved postnatally, MAGE-D2 is likely less important for postnatal sodium homeostasis mechanisms (5, 332, 335). Intriguingly, patients with MAGE-D2 mutations also presented with lower plasma bicarbonate, implying that MAGE-D2 potentially also affects sodium bicarbonate transport (333).

In adult kidneys or renal cells, MAGE-D2 expression is inducible after acute kidney injury or stress caused by cisplatin, folic acid, the inflammatory cytokine TWEAK, or serum deprivation (336). Future studies are needed to elucidate the molecular mechanisms behind stress-induced MAGE-D2 expression and the role of MAGE-D2 in stress response within the kidney.

MAGE-F1 regulates the cytosolic iron-sulfur (Fe-S) assembly (CIA) pathway

Unlike all of the other MAGE genes, *MAGE-F1* is uniquely located on chromosome 3 (Fig. 1*B*). As expected for a type II MAGE, *MAGE-F1* expression is ubiquitous across human and murine normal tissues (Fig. 2*A*) and is found in many tumor types, such as ovarian, breast, cervical, melanoma, and leukemia (11, 337). Beyond initial characterization of the *MAGE-F1* gene and its expression, MAGE-F1 had no known functions

until a 2018 study from our laboratory reported a role in the CIA pathway (38).

Although MAGE-F1 is capable of interacting *in vitro* with two RING domain proteins, NSE1 and TRIM27, the interaction with NSE1 is stronger (141). Through binding to the NSE1 E3 ligase, MAGE-F1 specifically targets MMS19 for ubiquitination and subsequent degradation by the proteasome (Fig. 6*A*) (38). MMS19, together with CIA1 and CIA2B, is part of the CIA-targeting complex that transfers Fe-S clusters to client Fe-S proteins, many of which are involved in DNA repair processes (*e.g.* FANCJ, POLD1, XPD, and RTEL1) (338–340). The synthesis and insertion of Fe-S clusters into apoproteins is a highly coordinated process among several proteins that are members of the mitochondrial iron-sulfur cluster assembly and export system and the CIA machinery (341, 342). Following generation of iron- and sulfur-containing cofactors from a precursor product by the mitochondrial iron-sulfur cluster machinery, components of the CIA pathway assemble the Fe-S cluster and incorporate the Fe-S cluster into cytosolic and nuclear apoproteins (342, 343).

By regulating MMS19 protein levels, MAGE-F1 controls flux through the CIA pathway (38). MAGE-F1-NSE1–mediated ubiquitination and degradation of MMS19 leads to decreased Fe-S incorporation into downstream targets, such as DNA repair enzymes, and sensitizes cells to DNA-damaging agents due to their reduced DNA repair capacity (Fig. 6*B*) (38). Given that the efficiency of cytosolic Fe-S cluster assembly regulates intracellular iron homeostasis, as iron regulatory protein 1 (IRP1) itself contains an Fe-S cluster that is lost in iron-replete conditions, we speculate that MAGE-F1-NSE1 ubiquitination and degradation of MMS19 also alters iron homeostasis regulation (38, 342).

MAGE-F1 expression in several tumors suggested that it may play a role in cancer. In line with this idea, MAGE-F1 overexpression in lung squamous carcinoma cell lines is necessary and sufficient to drive xenograft tumor growth (38). Furthermore, *MAGE-F1* is amplified along with known oncogenes on chromosome 3q (*e.g.* PIK3CA, SOX2, and TP63) in several cancers (38). Consistent with MAGE-F1 down-regulating the MMS19 CIA pathway and reducing cellular DNA repair capacity, patient tumors with MAGE-F1 amplification have greater mutation burdens (38). In head and neck squamous carcinoma tumors, increased expression of MAGE-F1 and NSE1 correlates with decreased patient survival (38), further implying clinical relevance of MAGE-F1-NSE1 MRL.

Interestingly, MAGE-F1 amplification is found in cancers that are frequently associated with smoking, like lung squamous cell carcinomas (344, 345). Given that MMS19 is important for nucleotide excision repair, which is involved in the repair of smoking-induced lesions, the down-regulation of MMS19 by increased MAGE-F1 levels may foster tumorigenesis through promoting replication stress and increasing mutational burden (38, 339). Thus, by promoting replicative stress and suppressing DNA repair pathways, MAGE-F1 amplification and down-regulation of the CIA pathway may contribute to genomic instability and oncogenesis.

Intriguingly, MAGE-F1 is only conserved in placental mammals, and many mammalian species, including rodents, have acquired insertions, deletions, or mutations in the *MAGE-F1* gene that lead to in-frame stop codons (12, 13, 335). Although the reason for MAGE-F1 pseudogenization in specific mammalian lineages awaits future determination, it is possible that MAGE-F1 pseudogenization prevented excessive genomic instability in distinct species or is a consequence of differential regulation of iron and oxygen homeostasis.

MAGE-G1 is a component of the SMC5/6 complex involved in promoting genome stability

MAGE-G1, also referred to as NDNL2 or NSMCE3, is the most closely related to MAGE-F1 with 59% identical amino acids within the MHD (43, 337). Human *MAGE-G1* and its murine ortholog *Mage-g1* are broadly expressed (Fig. 2*A*), with the highest levels found in the testis, ovary, and brain (11, 66, 67). Although *MAGE-G1* is closely located to the imprinted region on chromosome 15 that is implicated in neurodevelopmental disorders and autism susceptibility, including PWS (OMIM #176270) and Angelman syndrome (OMIM #105830), *MAGE-G1* is not imprinted and is not known to be involved in these disorders (67, 346, 347).

MAGE-G1 role in maintaining genome stability

A proteomic approach identified MAGE-G1 as a subunit of the SMC5/6 complex (139, 140) that is critical for chromosome replication and DNA repair in somatic cells (348, 349) and meiotic recombination in yeast and mammals (350–352). The structural maintenance of chromosome (SMC) heterodimers, in association with non-SMC element (NSE) subunits, perform important roles in the organization, packaging, and repair of chromosomes. SMC5/6 associates with six NSE subunits, NSE1–6, among which MAGE-G1 represents NSE3 (139, 140, 353–357). The SMC5/6 complex is crucial for maintenance of genomic stability, as it regulates DNA replication, checkpoint responses, and DNA repair processes, including telomere maintenance and repair pathways for double-strand breaks and homologous recombination (349, 358–362). Accordingly, cells with mutations in SMC5/6 complex subunits are sensitive to genotoxic stressors (363). The role of MAGE-G1 in SMC5/6 function and DNA damage repair is still enigmatic; however, depletion of MAGE-G1 by siRNA results in degradation of all of the other complex subunits, suggesting that it is a critical component of the complex like the other members (140). Namely, NSE1, NSE3, and NSE4 form a trimeric complex that bridges the globular heads between SMC5 and SMC6 (364, 365). In both *S. pombe* and human cells, MAGE-G1 strongly interacts with NSE1 not only within the SMC5/6 complex, but also independently (141), which indicates that MAGE-G1-NSE1 MRL may have additional functions besides those mediated by the SMC5/6 complex (140, 357).

Given their expression in meiotic male germ cells during the pachytene stage (Fig. 2*A*) (11, 351) and their crucial role in yeast meiosis (139), MAGE-G1 and other components of the SMC5/6 complex are predicted to have an important function during mammalian meiosis. MAGE-G1 was also suggested to be the closest ortholog of the first autosomal MAGE gene in marsupials, implying that the autosomal transposition of the ancestral MAGE was driven by its important function in meiosis and the

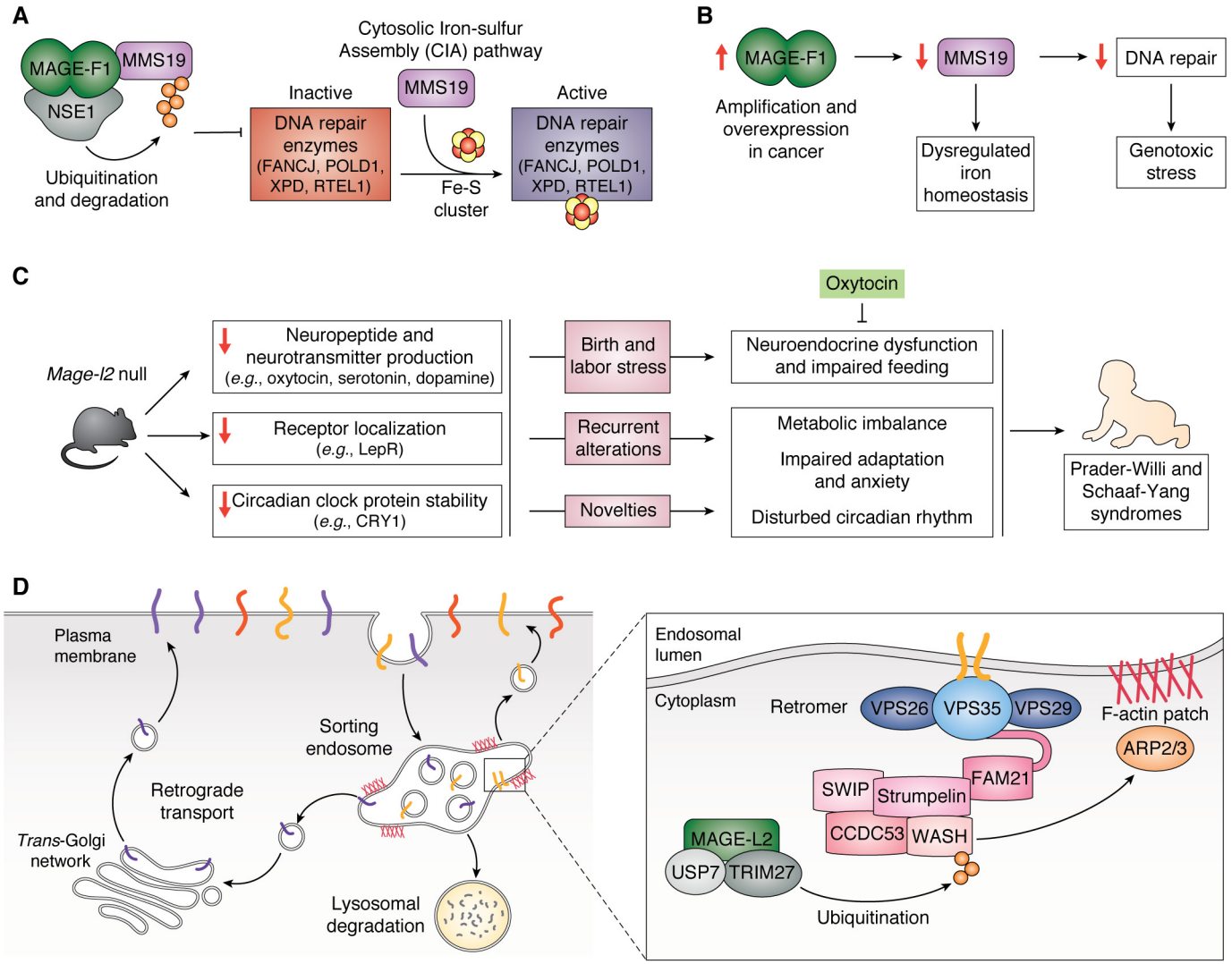

Figure 6. Functions and pathways of the type II MAGEs, MAGE-F1 and -L2. *A*, MAGE-F1 controls flux through the CIA pathway by regulating MMS19 protein levels. MAGE-F1 interacts with the E3 ligase NSE1 to form an MRL that ubiquitinates MMS19, promoting its degradation. Decreased MMS19 protein levels lead to decreased iron-sulfur (Fe-S) cluster incorporation into downstream targets, like DNA repair enzymes. *B*, increased levels of MAGE-F1 contribute to dysregulated iron homeostasis by degrading MMS19 and genotoxic stress by suppressing DNA repair pathways. *C*, MAGE-L2 contributes to the neurodevelopmental disorders PWS and SYS. *Mage-l2*–null mice exhibit decreased levels of mature neuropeptides, transmembrane receptors, like LepR, and circadian rhythm proteins, all of which may contribute to impaired adaptation to recurring and acute changes in the environment and contribute to the phenotypes seen in KO mice and PWS and SYS patients. Administration of oxytocin immediately after birth or during the first postnatal week rescues survival and normal adult social behavior of *Mage-l2*–null mice. *D*, MAGE-L2 is involved in endosomal protein trafficking. Cargo proteins on endosomes are trafficked to either the plasma membrane, the *trans*-Golgi network, or the lysosome for degradation. The retromer complex (*blue*) recognizes cargo proteins and, based on their destination, sorts them into endosomal tubules reshaped by localized F-actin patches. VPS35 interacts with the WASH regulatory complex (*pink*). The MUST complex (MAGE-L2-USP7-TRIM27) activates WASH by adding a Lys-63–linked polyubiquitin chain, which recruits ARP2/3 and promotes downstream F-actin nucleation.

appearance of sex chromosome inactivation (366). Interestingly, although current data suggest that the SMC5/6 complex is not acutely required for premeiotic DNA replication and meiotic progression during mouse spermatogenesis, the SMC5/6 complex ensures genome integrity and, thus, fertility when germ cells are challenged by exogenous DNA damage (367).

Notably, the mouse genome contains the additional, related *Mage-g2* gene, which also exhibits increased expression at the pachytene spermatocyte stage (Fig. 2*A*) (11, 37). Proteomic and genomic analyses suggest that Mage-g2 does not engage with the Smc5/6 complex but, rather, binds independently to the testicular germ cell-specific serine/threonine-protein kinase 31 (Stk31) and heat shock protein 9 (Hspa9) (37). Although further analysis showed that Mage-g2 reduced the kinase activity of Stk31, the functional relevance of binding to Hspa9 is currently unknown (37). Because various stressors up-regulate Hspa9 to suppress the engagement of apoptosis and regulate the functions of p53 (368), Mage-g2 binding to Hspa9 may be related to stress response in the testis, which further supports the emerging concept that MAGE genes evolved to provide an advantage in stressful circumstances.

MAGE-G1 in lung disease immunodeficiency and chromosome breakage syndrome

Interestingly, missense mutations in *MAGE-G1* have been identified in children with lung disease immunodeficiency and chromosome breakage syndrome (LICS; OMIM #617241)

(369). All four affected children manifested clinical features similar to those seen in Nijmegen breakage and ataxia-telangiectasia chromosomal breakage syndromes, yet LICS patients were uniquely predisposed to severe and ultimately fatal pneumonia during early childhood (369).

The mutations in Pro-209 and Leu-264 abolish formation of the trimeric MAGE-G1-NSE1-NSE4 complex, which facilitates the bridging of SMC5 and SMC6 (365, 370), thereby disrupting the whole SMC5/6 complex (369). Furthermore, patient-derived fibroblasts containing the homozygous L264F *MAGE-G1* mutation showed multiple chromosomal rearrangements and increased levels of micronuclei (369), indicative of genome instability during mitosis, similar to those seen in cells with mutations in *NSE2* (371). Also similar to cells with *NSE2* mutations, these fibroblasts displayed an increased sensitivity to genotoxic treatment, defects in the homologous recombination repair pathway, and delays in recovery from hydroxyurea-induced replication stress (369). These results imply that the MAGE-G1 mutant proteins destabilize the SMC5/6 complex, resulting in incoherent homologous recombination and impaired recovery from replication stress. The impact of the identified MAGE-G1 mutations in LICS further underscores the importance of MAGE-G1 in maintaining chromosome stability.

MAGE-dependent switching of NSE1 function

As mentioned earlier, NSE1 also functions outside the SMC5/6 complex through binding to MAGE-F1 to regulate the CIA pathway during DNA damage response (38). Although similar MAGEs have been reported to bind the same RING protein with redundant activity, like MAGE-A3/6 and -C2 binding TRIM28, the interaction of MAGE-F1 or MAGE-G1 with NSE1 is novel because these MRLs exhibit nonoverlapping functions (38, 140, 141, 151, 156, 357). Whereas it is not known how the binding of MAGE-F1 or MAGE-G1 to NSE1 may mechanistically switch its function, we have a few possible explanations. Because the N and C termini of these two MAGEs differ, it is plausible that these regions impart different substrate specificity on NSE1. Another possible explanation is that the binding of MAGE-F1 or MAGE-G1 leads to different NSE1 conformations that promote different activities. Additionally, the binding of MAGE-G1-NSE1 to the SMC5/6 complex or other proteins may hinder interaction with MMS19. Yet another possibility is that MAGE-G1-NSE1 and MAGE-F1-NSE1 localize to distinct subcellular compartments.

Whether one or more of these proposed mechanisms is responsible for switching the function of NSE1 warrants further investigation. However, it is clear that MAGE proteins enable diversification of E3 ligase function by expanding their regulatory potential and allowing contextual changes in multiple pathways through altering formation of distinct MRLs. The differential binding of MAGE-G1 and MAGE-F1 to NSE1 not only diversifies function but also promotes opposing activities, as MAGE-G1-NSE1 promotes DNA repair through the SMC5/6 complex (369), whereas MAGE-F1-NSE1 inhibits DNA repair through degradation of MMS19 (38).

MAGE-L2 regulates protein recycling and hypothalamic functions

MAGE-L2 is one of the largest MAGE proteins (Figs. 3*A*) with an N-terminal region that is highly proline-rich (>30%) (372), but the functional significance of this domain is still unknown. The human *MAGE-L2* gene is one of ~150 imprinted genes with monoallelic expression (373) located within the imprinted domain on the chromosome 15 that is critical for the manifestation of two distinct neurodevelopmental disorders, the PWS and Angelman syndrome (372). In addition to *MAGE-L2*, the PWS region contains five other protein-coding genes, including *NECDIN*, also a *MAGE* gene, and six small nucleolar RNA genes.

MAGE-L2 in PWS, SYS, and other genetic disorders

PWS is a multigenic disorder that affects one in 15,000 children and results either from the deletion of paternal 15q11-q13 (65–75% of cases), from maternal uniparental disomy (20–30%), or from imprinting defects (1–3%) (374). The major clinical features of PWS include intellectual and physical disabilities, endocrine dysfunctions, obesity, and maladaptive behaviors (170, 374–377), all implying hypothalamic dysfunction. At birth, PWS is characterized by neonatal hypogonadism, hypotonia with feeding difficulties, and failure to thrive (374). Later, PWS children develop intellectual disability, hypothyroidism, short stature, maladaptive social behaviors, and, most notably, hyperphagia leading to childhood obesity and type II diabetes (374).

Besides PWS, *MAGE-L2* has also been implicated in the PWS-like neurodevelopmental disorder Schaaf–Yang syndrome (SYS, OMIM #615547) (372), where patients harbor diverse truncating pathogenic variants of the paternal *MAGE-L2* gene (170, 376, 378). The most pathogenic variant, c.1996delC, resulted in lethal arthrogryposis in several patients (376, 378, 379). Although many SYS symptoms overlap with those of PWS, the lack of hyperphagia in SYS distinguishes it from PWS (374). In addition, SYS patients have a higher prevalence of autism spectrum disorder and display joint contractures that range in severity from mild contractures of the distal phalanges to lethal arthrogryposis (170, 376, 378–380). The severity of many SYS features varies based on mutation location, yet it is interesting that deletion of the entire *MAGE-L2* gene in PWS results in a milder phenotype than the *MAGE-L2* truncating mutations in SYS. However, this genotype-to-phenotype correlation needs further investigation to discover the molecular underpinnings that drive these differences.

MAGE-L2 has also been implicated in other developmental syndromes. Truncating mutations of *MAGE-L2* have been identified in patients with arthrogryposis multiplex congenita (OMIM #208100) (379) and Chitayat–Hall syndrome (OMIM #208080) (381), which are characterized by multiple severe joint contractures that result in minimum fetal movement, global developmental delays, and growth hormone deficiency. Although the genetic background for most patients with Opitz trigonocephaly C syndrome (OTCS, OMIM #211750) is still enigmatic, the *de novo* nonsense *MAGE-L2* mutation c.1912C > T (p.Q638) was identified in one of 10 OTCS

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patients (382). Given the phenotypic overlap between SYS and OTCS, the promising candidates for OTCS causative genes include those encoding MAGE-L2 molecular partners that form the MUST complex (MAGE-L2-TRIM27-USP7) (43, 68, 149, 372), as well as proteins implicated in MUST-regulated retrograde transport (43, 68, 372, 382). Analogously, components of the MUST-regulated WASH-retromer protein recycling pathways have been implicated in various neurological disorders, including SWIP mutations in intellectual disability (383), strumpellin in hereditary sporadic paraplegia (384), and USP7 variants in pediatric neurodevelopmental disorders like hypotonia, seizures, and autism spectrum disorder (68). These disorder associations suggest an important role for MAGE-L2–regulated molecular pathways, which are described in the following paragraphs, in neurodevelopment and central nervous system functions. All studies so far are based on nonsense frameshifting mutations that result in truncated *MAGE-L2* (170, 376, 378, 379, 382, 385), yet the pathogenic mechanism of missense *MAGE-L2* mutations remains completely unstudied.

Mage-l2–null mice yield insights into PWS and SYS

Two mouse models with targeted deletion of *Mage-l2* recapitulate several fundamental aspects of PWS and SYS (372). Similar to the phenotypes seen in children with PWS and SYS, these animals display growth retardation at early stages of life, followed by weight gain after weaning, increased adiposity, and unbalanced metabolism (Fig. 6*C*) (168, 169, 386, 387). Additionally, *Mage-l2*–null mice exhibit abnormal circadian rhythm and feeding behaviors, similar to the hyperphagia present in PWS patients (168, 169, 386–388). In line with predicted hypothalamic defects and disturbed endocrine phenotypes seen in patients, *Mage-l2*–null mice show diverse neuroendocrine dysfunctions, including decreased levels of the neuropeptides and biogenic amines, which may account for maladaptive behaviors, like increased anxiety and deficit in preference for social novelty (386, 389).

The mouse model developed by Muscatelli and her group (390), where the *Mage-l2* promoter and most of the coding region was deleted—in contrast to the lacZ insertion that replaces the C-terminal domain of *Mage-l2* in the first model developed by Wevrick's group—but leaving intact the last 1165 base pairs that code for the MHD, also showed a significant reduction in oxytocin level, and, impressively, a single bolus of oxytocin immediately after birth rescued the suckling deficit and neonatal survival (Fig. 6*C*) (390). Moreover, daily administration of oxytocin in the first postnatal week restored normal social behavior and learning abilities in adult animals (391), further implicating oxytocin as a critical mediator of phenotype development. Accordingly, *Mage-l2*–null mice have a significantly decreased level of mature oxytocin in the hypothalamus (391), which is similar to the orexin maturation defects observed in the Wevrick model (168, 169). Although the mechanism leading to neuropeptide reduction in PWS patients and *Mage-l2*-null mice is still not clear, increased pro-hormone levels suggest defective processing into bioactive circulating hormones (386).

MAGE-L2 regulates endosomal protein recycling

Consistent with MAGEs regulating E3 ligases (43), MAGE-L2 directly binds RING E3 ligase TRIM27 (also known as Ret finger protein, or RFP) through its MHD (68, 149). In addition to TRIM27, MAGE-L2 binds the deubiquitinating enzyme USP7 to form the MUST complex that controls WASH activity to facilitate endosomal protein recycling (Fig. 6*D*). USP7 stabilizes this complex by interacting with both MAGE-L2 and TRIM27 (68, 141, 149). MAGE-L2-TRIM27 mediates Lys-63–linked ubiquitination of the protein WASH (149), which, unlike most ubiquitin linkage forms that target proteins for degradation, activates WASH and enables WASH-mediated endosomal protein recycling (Fig. 6*D*) (149). USP7-mediated deubiquitination prevents WASH overactivation and also counteracts TRIM27 autoubiquitination to prevent its proteolytic degradation, thereby stabilizing the MUST complex (68).

Endosomal protein recycling is an essential process that enables proper sorting of membrane proteins from endosomes to either the plasma membrane (*e.g.* diverse transporters, signaling receptors, and cell adhesion molecules (392–398)) or the *trans*-Golgi network (*e.g.* intracellular sorting receptors, transmembrane peptidases, and SNAREs (399–402)). Given the diversity of cargo proteins dependent on the endosomal protein recycling pathway for their proper localization and function, dysfunction of MAGE-L2 or the MUST complex can impact a range of physiological and pathophysiological processes.

On a mechanistic level, MUST-dependent WASH activation enables F-actin nucleation to provide the initial force needed for membrane invagination and subsequent vesicle scission that triggers retrograde transport in the early endosome (160, 384, 403–406). In the early endosomes, cargo proteins originating either from the plasma membrane or the biosynthetic pathway are sorted and then dissociated through the interconnected network of endosomal membranes. The master regulator of cargo protein recognition and sorting is the retromer complex (composed of VPS35, -26, and -29 (407, 408)), which also recruits the WASH regulatory complex (SHRC; composed of WASH, FAM21, CCDC53, SWIP, and strumpellin) to start the transport after cargos are selected. MAGE-L2 directly interacts with VPS35 to recruit the MUST complex to endosomes for WASH activation (Fig. 6*D*) (149).

Upon its activation, WASH then activates the ARP2/3 actin-nucleating complex and triggers F-actin nucleation at the endosomal surface, a necessary step for proper protein trafficking (160, 384, 403). WASH, like all WASP family members, contains a C-terminal VCA (verprolin homologous or WH2, central hydrophobic, and acidic) domain that binds both actin and the ARP2/3 complex to trigger actin filament nucleation (160). Because the VCA domain is autoinhibited through intra- and intermolecular interactions, activating signals expose the motifs to enable F-actin nucleation (160, 372, 384, 403, 404). Whereas the exact mechanistic role of the Lys-63–linked polyubiquitin chains on WASH is unclear, this modification may physically disrupt the autoinhibitory contact between WASH and other components of the SHRC, thus exposing the WASH VCA domain for interactions with ARP2/3 (149, 409). Alternatively, the Lys-63–linked polyubiquitin chain on WASH may

also recruit additional factors to promote ARP2/3 complex-mediated actin nucleation on endosomes. Given that MAGE-L2 is mammalian-specific and highly enriched in the hypothalamus (11, 68), whereas WASH and retromer are conserved in all eukaryotes (407, 408), MAGE-L2 may represent a tissue-specific regulator of WASH that is critical for protein trafficking, localization, and function in the mammalian hypothalamus.

Interestingly, MAGE-L2 also engages another E3 ligase, RNF41, and deubiquitinating enzyme, USP8, to regulate endosomal protein recycling of the leptin receptor (LepR) (154). LepR activity in the hypothalamus is vital for the regulation of appetite and energy balance (410, 411). Compared with WT littermates, *Mage-l2*–null mice display reduced levels of LepR and Rnf41, indicating a defect in endosomal recycling, which is rescued by Mage-l2 expression (154). Concurrently, these mice exhibit increased levels of Usp8 and Stam1, a component of another sorting complex called ESCRT (endosomal sorting complexes required for transport) (154). Stam1 was previously shown to stabilize the ESCRT complex and push trafficked cargo proteins like LepR into multivesicular bodies for subsequent lysosomal degradation (412). Thus, the altered abundances of Usp8 and Rnf41 may increase Stam1 and promote LepR degradation, ultimately contributing to the obesity phenotype observed in PWS (154). This study suggests an additional MAGE-L2 complex, which resembles MUST, that plays a role in hypothalamic protein recycling; however, further studies are required to determine the exact molecular mechanisms involved.

The day-night routine disruptions commonly present in PWS and SYS patients and the circadian rhythm defects observed in *Mage-l2-null* mice implicated another role for MAGE-L2 in the circadian clock (Fig. 6*C*) (168, 372). Indeed, MAGE-L2 manipulates the ubiquitination and stability of proteins that regulate circadian rhythm, in particular the key circadian rhythm protein cryptochrome 1 (CRY1) (388, 413). Furthermore, *Mage-l2* has circadian expression and is highly expressed in the SCN, the area of the brain that establishes and controls circadian rhythm (71, 168). Through anticipating recurring changes in the environment to appropriately adjust behavior and physiology, the internal circadian clock confers an evolutionary advantage to most species on Earth.

Taken together, the brain- and hypothalamus-enriched MAGE-L2 regulates different E3 ligases to control sorting and proper localization of proteins, as well as the stability of neuropeptides and circadian proteins. Identifying which cargo proteins are aberrantly trafficked due to the loss of MAGE-L2 will be imperative for teasing out its contribution to the behavior, feeding, circadian rhythm, and endocrine homeostasis anomalies seen in PWS and SYS, which will lead to developing better therapeutic strategies. Although the exact molecular mechanisms underlying MAGE-L2 function need further investigation, current data suggest that MAGE-L2 evolved to enable better adaptation to recurrent and unanticipated changes in the environment.

Conclusions and future perspectives

Investigation of human and mouse MAGE proteins has uncovered many of their diverse cellular functions, but we are just starting to understand how these functions contribute to normal physiological processes and the pathogenesis of cancer and other genetic diseases. At the molecular level, the dynamic nature of the MHD structure enables individual MAGE proteins to preferentially bind different proteins, including E3 ligases, to exert their functions. MAGEs regulate their respective E3 ligases through enhancing ligase activity, specifying novel substrates for ubiquitination, and altering ligase subcellular localization. As a result, MAGEs regulate many biological pathways, including metabolism and autophagy, DNA repair, cell cycle progression, apoptosis, mRNA polyadenylation and stability, stress granule formation, and membrane protein recycling.

Through in-depth examination of the diverse functions exhibited by individual MAGEs, the overarching theme of stress tolerance has emerged as a unifying thread that further ties the MAGE family together. Based on thorough characterization of *Mage* expression in spermatogenesis and the location of many *MAGE* genes on the X chromosome, where the rapid expansion of multicopy/ampliconic genes is likely driven by their beneficial roles in male reproductive fitness, the type I *MAGE* genes presumably expanded in eutherian mammals to protect the germline from environmental stress and aid in stress adaptation to preserve fertility. This theory is supported by the detrimental effects on spermatogenesis reported in *Mage-a* and *-b4* KO mice exposed to genotoxic, metabolic, and heat stress. Many type II MAGE proteins, like MAGE-G1 and Mage-g2, also play a role in protecting the genomic integrity and fitness of the germline. Additionally, ubiquitously expressed MAGE proteins respond to stressors in other tissues, like MAGE-D2 after kidney injury, and may have provided an evolutionary advantage, as is likely the case for MAGE-D1 and MAGE-L2 in the brain. Interestingly, analysis of novel eutherian genes, many of which reside on the X chromosome like *MAGEs*, suggests extensive genetic modification to pathways involved in testis and brain function during eutherian mammal evolution, indicating that these genes provided an evolutionary advantage (414). In all, our findings evoke the conclusion that MAGEs provide a selective advantage by enabling better stress adaptation, either on a cellular or an organismal level.

Intriguingly, the protective responses of MAGEs to stress in the germline are likely hijacked by cancer cells that aberrantly express MAGEs, to foster a tumorigenic environment. For example, MAGE-A11's ability to induce APA and result in 3′-US, typical for germ cell–specific transcripts, appears to be co-opted by cancer cells to promote tumor growth. Furthermore, MAGE-A3/6 confer resistance to metabolic stress, and MAGE-B2 increases cellular stress tolerance in cancer cells by suppressing stress granule formation. Given that MAGE expression often correlates with resistance to checkpoint inhibitors, targeted therapy, and chemotherapy (21–24), we speculate that MAGEs may contribute to therapy resistance by activating diverse stress-adaptation pathways.

As highlighted throughout this review, many aspects related to MAGE family expression and function remain enigmatic. Although the expression patterns of MAGE CTAs in male germ cells and various cancers indicate that epigenetic alterations work in concert with tissue-specific transcription factors

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to transcriptionally activate MAGEs, we do not yet fully grasp which mechanisms control expression of MAGEs within germ, somatic, and cancer cells. Beyond these epigenetic mechanisms, defining the contribution of MAGEs to regulation of stemness, differentiation of pluripotent stem cells, and embryonic development is also warranted. Similarly, the possible roles of MAGEs in female reproduction have not been studied, despite the fact that some MAGEs, like MAGE-A11, are expressed in the placenta and uterus. Ongoing efforts to successfully and safely target the type I MAGEs will greatly benefit from understanding the mechanisms by which these proteins contribute to oncogenesis, their regulation, and their normal physiological functions. Furthermore, the recently published expression analysis of MAGEs (11) in various tissues and life stages will aid in predicting and avoiding potential off-target effects of immunotherapies that target a single MAGE. In addition, elucidating which molecular mechanisms are responsible for poor response to immunotherapy and chemotherapy resistance development in MAGE-positive tumors will be critical.

In summary, the MAGE family comprises an exciting group of proteins with diverse functions that contribute to stress tolerance in germ and cancer cells. Addressing the open questions mentioned in this review will deepen our understanding of MAGEs and reveal strategies for treating cancer and other diseases.

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Abbreviations—The abbreviations used are: MAGE, melanoma antigen; CTA, cancer-testis antigen; ceRNA, competing endogenous mRNA; BDNF, brain-derived neurotrophic factor; MHD, MAGE homology domain; PWS, Prader–Willi syndrome; SYS, Schaaf–Yang syndrome; ES cells, embryonic stem cells; SSC, spermatogonial stem cell; BTB, blood-testis barrier; DAC, 5′-aza-2′-deoxycytidine; HDAC, histone deacetylase; MRL, MAGE-RING ligase; AMPK, AMP-activated kinase; SCF, Skp1-Cullin-F-box; CRL, cullin-RING ligase; KRAB, Krüppel-associated box; ZFP, zinc finger protein; HCC, hepatocellular carcinoma; SUMO, small ubiquitin-like modifier; EBV, Epstein–Barr virus; ATM, ataxia-telangiectasia-mutated; FBP1, fructose-1,6-biphosphatase; FBP, fructose 1,6-bisphosphate; 2DG, 2-deoxy-d-glucose; AR, androgen receptor; PR, progesterone receptor; SG, stress granule; LLPS, liquid–liquid phase separation; SLE, systemic lupus erythematosus; APS1, autoimmune polyendocrine syndrome type 1; IAP, inhibitors of apoptosis protein; XIAP, X-linked IAP; SERT, serotonin transporter; LepR, leptin receptor; CREB, cAMP-response element–binding protein; CIA, cytosolic iron-sulfur (Fe-S) assembly; SMC, structural maintenance of chromosome; NSE, non-SMC element; LICS, lung disease immunodeficiency and chromosome breakage syndrome; OTCS, Opitz trigonocephaly C syndrome; SHRC, WASH regulatory complex; KO, knockout.

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