Structural basis for substrate recognition and chemical inhibition of oncogenic MAGE ubiquitin ligases

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Testis-restricted melanoma antigen (MAGE) proteins are frequently hijacked in cancer and play a critical role in tumorigenesis. MAGEs assemble with E3 ubiquitin ligases and function as substrate adaptors that direct the ubiquitination of novel targets, including key tumor suppressors. However, how MAGEs recognize their targets is unknown and has impeded the development of MAGE-directed therapeutics. Here, we report the structural basis for substrate recognition by MAGE ubiquitin ligases. Biochemical analysis of the degron motif recognized by MAGE-A11 and the crystal structure of MAGE-A11 bound to the PCF11 substrate uncovered a conserved substrate binding cleft (SBC) in MAGEs. Mutation of the SBC disrupted substrate recognition by MAGEs and blocked MAGE-A11 oncogenic activity. A chemical screen for inhibitors of MAGE-A11:substrate interaction identified 4-Aminoquinolines as potent inhibitors of MAGE-A11 that show selective cytotoxicity. These findings provide important insights into the large family of MAGE ubiquitin ligases and identify approaches for developing cancer-specific therapeutics.
Melanoma antigens (MAGEs) are a large (>40 in humans) and highly conserved family of proteins present in all eukaryotes. MAGEs can be classified into two categories based on their tissue expression pattern. Two-thirds of MAGEs, including MAGE-A, -B, and -C subfamilies are considered type I MAGEs due to their restricted expression in the testis and other reproductive tissues. Conversely, MAGE-D, -E, -F, -G, -H, -L and Necdin subfamilies are type II MAGEs that have broad expression in many tissues. Both type I and II MAGEs share a conserved domain known as the MAGE homology domain (MHD) involved in protein–protein interactions. MHDs consist of tandem winged-helix motifs (WH-A and WH-B) that feature a helix-turn-helix packed against a three-stranded antiparallel beta-sheet. The overall structures of MHDs are similar between MAGEs, but the relative orientation of WH-A to WH-B can differ.

Although type I MAGEs are typically restricted to expression in the testis, they are often aberrantly expressed in many cancer types and are often referred to as cancer-testis antigens. The aberrant expression of MAGEs in tumors is not simply an inert consequence of genomic dysregulation, but instead MAGEs play active roles in driving tumorigenesis. For example, MAGE-A3, -A6, and -A11 are aberrantly expressed in many cancer types, including melanoma, breast, colon, and lung cancers, where they are required for cancer cell viability and promote multiple hallmarks of cancer, such as anchorage-independent growth and xenograft tumor growth. Recent studies have highlighted an important role of MAGEs in controlling core oncogenic and tumor suppressor pathways in cells, including metabolic rewiring by MAGE-A3/6 through modulation of AMPK and autophagy and promotion of alternative polyadenylation by MAGE-A11. In addition, MAGE-F1 is highly amplified in multiple cancer types leading to reduced DNA repair capacity and increased mutational burden in tumors through downregulation of the cytosolic iron-sulfur assembly pathway.

Thus, given the prominent, cancer-selective expression of MAGEs and their oncogenic functions, MAGEs have emerged as prime therapeutic targets. However, this effort has been impeded by our limited understanding of how to target these enigmatic proteins.

Growing evidence suggests that MAGE functions as substrate adapters for E3 ubiquitin ligases (reviewed in Lee and Potts). MAGEs bind to specific single-subunit E3 ubiquitin ligases, both RING and HECT type ligases, including MAGE-A1-TRIM31, MAGE-A3-TRIM28, MAGE-A11-HUWE1, MAGE-B18-LNX1, MAGE-D1-PRAJA1, MAGE-F1-NSE1, MAGE-L2-TRIM27, and MAGE-A3-TRIM28. Importantly, MAGEs alter the function of these ligases by recruiting novel substrates to the ligases for ubiquitination, including MAGE-A3 mediating the ubiquitination of AMPK by TRIM28, MAGE-A11 mediating ubiquitination of PCF11 by HUWE1 and MAGE-F1 mediating ubiquitination of MMS19 by NSE1. In each of these cases, the MAGE functions as protein glue that mediates recruitment of the substrate to the ligase complex for ubiquitination. Thus, MAGEs reprogram single-subunit ubiquitin ligases. Notably, the specific MAGE bound to a ligase can dictate distinct substrates and functions. For example, both MAGE-F1 and MAGE-G1 bind the NSE1 ligase. However, these MAGEs have distinct substrates with opposing cellular functions in modulating DNA repair pathways.

In many ways MAGEs have similar properties to Cullin-RING Ligase (CRL) adapter protein families, such as F-box, BTB, DCAF, and SOCS box proteins, which allow modularity, functional diversification, and adaptability of CRLs. However, unlike CRL adapters, the molecular and structural basis of how MAGEs recognize substrates has been unknown.

Here, we determine how the MAGE-A11 oncogene recognizes its substrate, PCF11. Biochemical analysis uncover a consensus degron motif sequence recognized by MAGE-A11 in PCF11 and other putative substrates. The crystal structure of MAGE-A11 bound to its substrate PCF11 is determined revealing a conserved substrate binding cleft (SBC) in the MHD. Importantly, mutational analysis of this region across MAGEs reveals its importance for MAGE:substrate recognition with sequence diversity around the SBC driving MAGE substrate selectivity. Disruption of the MAGE-A11 SBC blocks MAGE-A11 oncogenic activity suggesting an avenue for discovery of MAGE-directed cancer therapeutics. An in vitro biochemical assay is first developed to monitor and map the protein–protein interaction between MAGE-A11 and PCF11, which is then used in a high-throughput chemical screen to discover sub-micromolar small molecule inhibitors of MAGE-A11 that lead to MAGE-dependent cytotoxicity. These findings advance our understanding of MAGE ubiquitin ligases and provide proof of concept for chemical inhibition of MAGEs in the pursuit of cancer therapeutics.

**Results**

**Characterization of MAGE-A11 interaction with PCF11.**

Recently, we reported that MAGE-A11 drives tumorigenesis through ubiquitination of the 3′-mRNA processing factor PCF11 resulting in alternative polyadenylation (APA) of transcripts and 3′-UTR shortening (3′-UTR)10. To understand how MAGE-A11 recognizes its substrate, we mapped the degron motif in PCF11 required for MAGE-A11 binding. We found that deletion of PCF11 amino acids 653–702 completely blocked MAGE-A11 binding by co-immunoprecipitation (co-IP) (Fig. 1a). To narrow down the minimal degron motif within these 50 amino acids, we developed a time-resolved fluorescence resonance energy transfer (TR-FRET) assay to monitor binding of a series of PCF11 peptides to GST-MAGE-A11 in vitro (Fig. 1b and Supplementary Fig. 1a). Notably, of the eight PCF11 peptides tested, three overlapping peptides centered on PCF11 amino acids 682–696 showed robust binding with IC_{50} of 59–77 nM (Fig. 1c). These findings were confirmed by an orthogonal assay showing changes in the thermal stability of MAGE-A11 by the PCF11 degron peptide (Fig. 1d and Supplementary Fig. 1b).

To identify the specific residues in the PCF11 degron responsible for interaction with MAGE-A11, we performed alanine scanning mutagenesis of the PCF11 degron peptide and monitored binding to MAGE-A11 by TR-FRET (Supplementary Fig. 1c). Mutation of F682, I689, R690, L692, or F693 to alanine dramatically decreased binding 10–200 fold, with other positions having minimal effects (Fig. 1e and Supplementary Fig. 1d). These findings are consistent with our previous observations that mutation of PCF11 I689A blocked PCF11 interaction with MAGE-A11 in cells and prevented MAGE-A11-induced xenografts tumor growth.

Given the success of our binding assay to map specific amino acid sequences competent for MAGE-A11 binding, we expanded our study to perform TR-FRET affinity measurements on a peptide array consisting of 248 peptides in which each position within the model 13 amino acid PCF11 peptide degron is mutated to all possible amino acids. Consistent with our alanine scanning mutagenesis, the same five amino acid positions were again identified as being the most important (Fig. 1f). Position +1 (PCF11 F682) preferred an aromatic residue, position +8 (PCF11 I689) required a hydrophobic residue, position +9 (PCF11 R690) only tolerated a basic residue, position +11 (PCF11 L692) preferred a large hydrophobic residue and position +12 (PCF11 F693) preferred aromatic residues. Overall, the degron sequence conforms to [FLWY]-X_{6}-[IV]-[KR]-X-[ILMV]-[FLWY], where residues denoted as X being tolerable to several amino acids. Notably, the PCF11 sequence is not fully optimized with several
substitutions yielding higher affinity (Fig. 1f). These insights allowed us to build a consensus MAGE-A11 binding motif weighted matrix (Supplementary Fig. 1e). Search of the proteome using this matrix revealed 17 possible MAGE-A11 targets with the consensus binding motif (Fig. 1g). To validate our prediction, we examined the ability of MAGE-A11 to bind four of these newly predicted targets in vitro and found RACGAP1, ANKR65, and ZBTB7C to be binding partners (Fig. 1h). Notably, we did not detect interaction with MTPN (Fig. 1h), however the MAGE-A11 binding motif is not surface exposed in this protein (PDB: 3AAA). These results provide detailed understanding of the sequences recognized by MAGE-A11.
Crystal structure of MAGE-A11 bound to PCF11. MAGE genes are defined by a common conserved MAGE homology domain (MHD), which is involved in protein–protein interactions. Similar to other MAGE-substrate interactions, PCF11 binding to MAGE-A11 is mediated through the MAGE-A11 MHD (197–429; Fig. 2a). To understand how substrates interact with MAGEs, we determined the crystal structure of MAGE-A11 bound to the PCF11 degron. We initially attempted to determine the co-crystal structure of MAGE-A11 MHD bound to free PCF11 degron supplied as a peptide post puriﬁcation of MAGE-A11 MHD from bacteria. However, this approach did not yield homogenous protein:peptide complex, likely due to the MAGE-A11 MHD apo-protein being less stable during expression and puriﬁcation from bacteria. Thus, we expressed and puriﬁed the PCF11 degron sequence fused to the MAGE-A11 MHD (S218 to S242) from bacteria and solved the crystal structure to 2.2 Å (Table 1). MAGE-A11 MHD formed two winged-helix motifs (WH-A and WH-B) conﬁgured by a linker loop, where each WH motif contains three alpha-helices and two beta-sheets. The overall MAGE-A11 structure is similar to the other two reported MAGE-A family protein structures (MAGE-A3-MHD PDB: 4V0P and MAGE-A4-MHD PDB: 2W40). However, the extreme C-terminal residues of MAGE-A11 formed three alpha-helices, whereas these residues are unstructured in MAGE-A3-MHD and MAGE-A4-MHD structures (Fig. 2b). Additionally, no evidence of MAGE-A11 dimer formation was observed in the crystal structure or in cell lysates by co-IP (Supplementary Fig. 2a). Of particular interest, the PCF11 degron forms a short alpha-helix (Fig. 2b, c and Supplementary Fig. 2b, c) that binds into a shallow cleft (Fig. 2c) formed at the interface of the WH-A and WH-B motifs. We refer to this region on MAGE-A11 as the substrate binding cleft (SBC).

PCF11 binding to the MAGE-A11 SBC is largely mediated by numerous hydrophobic interactions that extend along its helical core and capping residues (Fig. 2d and Supplementary Fig. 2d). F689 and L692 of PCF11 form a hydrophobic core with L273, L274, I317, M341, V337, I340, and L338 of MAGE-A11. Notably, the N-terminal capping residue F682 of PCF11 interacts with F275, F350, L351, H231, and V230, whereas F693 at the C-terminus interacts with I317, I403, and A406 in MAGE-A11. In addition, a key polar interaction network enhances binding; R690 in PCF11 forms a water-mediated salt bridge with E266 in MAGE-A11 and is further stabilized by forming a hydrogen bond between the NE2 moiety and the backbone carbonyl of A406. Taken together, these structural observations are highly consistent with our TR-FRET mutagenesis data. A detailed description of polar and hydrophobic PCF11 interactions with MAGE-A11 is provided in Supplementary Table 1.

Mutation of MAGE-A11 SBC disrupts its oncogenic activity. To determine whether mutation of the MAGE-A11 SBC disrupts its ability to ubiquitinate PCF11 and control tumor cell growth, we stably reconstituted MAGE-A11 knockout DAOY cells with MAGE-A11 SBC mutants F275A or M341R and assessed PCF11 ubiquitination and subsequent effects on cell growth and tumor formation. Consistent with disruption of PCF11 binding by MAGE-A11 SBC mutation (Fig. 2f), MAGE-A11 F275A and M341R failed to promote PCF11 ubiquitination in cells (Fig. 3a). Moreover, unlike wild-type MAGE-A11, MAGE-A11 F275A and M341R failed to promote proliferation of MAGE-A11 knockout DAOY cells (Fig. 3b). Compared to wild-type MAGE-A11, re-expression of MAGE-A11 F275A or M341R reduced clonogenic growth (Fig. 3c). To test the importance of the MAGE-A11 SBC for MAGE-A11 oncogenic activity in vivo, we performed xenograft tumor growth assays using the MAGE-A11 knockout DAOY cells reconstituted with wild-type, F275A or M341R MAGE-A11. Consistent with MAGE-A11 oncogenic activity, wild-type MAGE-A11 promoted increased tumor growth (Fig. 3d, e). However, DAOY cells reconstituted with MAGE-A11 interaction with PCF11 in vitro (Fig. 2e). These ﬁndings were corroborated by co-IP studies in cells (Fig. 2f). Furthermore, disruption of the hydrophobic core of the MAGE-A11 SBC by I317R or M341R ablated PCF11 interaction in vitro (Fig. 2e) and in cells (Fig. 2f). In comparison, mutation of MAGE-A11 H231 that is not involved in PCF11 recognition had no effect on PCF11 binding in vitro (Fig. 2e). These results are consistent with the MAGE-A11:PCF11 crystal structure suggesting that the MAGE-A11 SBC serves as a critical region for substrate recognition. Importantly, mutation of the MAGE-A11 SBC did not disrupt binding to the MAGE-A11 associated E3 ligase HUWE1 (Fig. 2f). Together, these results suggest that the MAGE-A11 SBC mediates substrate interaction and E3 ligase binding occurs independent of the MAGE-A11 SBC.

Given the importance of the C-terminal end of the PCF11 motif (position #8, #9, #11, and #12) for MAGE-A11 binding and that MAGE-A11:PCF11 interaction afﬁnity can be increased by amino acid substitutions (Fig. 1f), we synthesized several peptides with non-natural phenylalanine derivatives at PCF11 F693 (position #12). This included F, CI, CF3, CN, or CH3 substitutions at the 2, 3, or 4 position of the phenylalanine benzene ring (Fig. 2g and Supplementary Fig. 2e). One variant, 3-methyl-phenylalanine, signiﬁcantly increased PCF11 binding to MAGE-A11 (Fig. 2g; IC50 = 1.7 nM), likely due to increased hydrophobic and Van der Waals contacts. However, substitutions at the 4th position on the benzene ring, including 4-cyano-phenylalanine (Fig. 2g; IC50 = 663 nM), were not tolerated well, likely due to steric hindrance. Overall, these ﬁndings provide structural basis of MAGE-A11 binding to PCF11 and suggest mechanisms to improve MAGE-A11:PCF11 substrate binding afﬁnity.
Fig. 2 Crystal structure of MAGE-A11:PCF11 identifies MAGE substrate binding cleft. a MAGE-A11 directly interacts with PCF11 through its MHD. In vitro translated Myc-MAGE-A11 MHD, but not N-terminal fragment, binds to recombinant GST-PCF11. Source data are provided as a Source Data file. b Overall structure of human MAGE-A11 MHD with human PCF11 degron peptide. The alpha-helical PCF11 degron peptide binds into SBC at interface of WH-A and WH-B motifs of MAGE-A11 MHD. PCF11 peptide is shown in pink and MAGE-A11 in gray. Residues in the SBC that are mutated in this study are labeled and colored yellow. c Substrate interaction is largely achieved by hydrophobic interactions along the alpha-helical interface of PCF11 with MAGE-A11 SBC. d MAGE-A11 wild-type or mutants were in vitro translated and followed by in vitro binding assay with recombinant GST-PCF11 637-702 amino acids, SDS-PAGE and immunoblotting for anti-Myc. Source data are provided as a Source Data file. e MAGE-A11 SBC is crucial for PCF11 substrate binding, but not HUWE1 E3 ligase binding, in cells. HEK293FT cells stably expressing FLAG-vector, FLAG-MAGE-A11 wild-type or mutants were subjected to pull-down with anti-FLAG followed by SDS-PAGE and immunoblotting for anti-HUWE1 and anti-PCF11. Source data are provided as a Source Data file. g TR-FRET binding assays of GST-MAGE-A11 with PCF11 peptide (FLVVHQIRQLF*Q) containing phenylalanine (black circles), 3-methyl-phenylalanine (red triangles) or 4-cyano-phenylalanine (blue squares) at the indicated position (n = 2 biologically independent experiments with triplicates per peptide). Data are mean ± SD.
Table 1 Data collection and refinement statistics.

| MAGE-A11:PCF11 |
|----------------|
| **Data collection** |
| Space group | P 1 |
| Cell dimensions | α, β, γ (°) 55.50 78.12 72.91 |
| | Resolution (Å) 38.79-2.19 (2.24-2.19) |
| | R₁/α (%) 0.120 (0.372) |
| | CC 1/2 (%) 0.952 (0.757) |
| Completeness (%) | 96.6 (94.7) |
| Redundancy | 2.5 (2.3) |
| **Refinement** |
| Resolution (Å) | 38.79-2.19 |
| No. reflections | 53723 |
| Rwork/Rfree (%) | 0.205/0.246 |
| No. atoms | Protein 7085 |
| | Water 208 |
| B factors (Å²) | Protein 40.12 |
| | Water 45.27 |
| R.M.S. deviations | Bond lengths (Å) 0.008 |
| | Bond angles (°) 1.48 |
| Ramachandran statistics (%) | Favored 96.38 |
| | Allowed 3.62 |
| | Outliers 0 |

*Values in parentheses are for the highest resolution shell.

SBC mutants failed to promote tumor growth and were indistinguishable from vector control cells (Fig. 3d, e). To determine if MAGE-A11 M341R fails to promote 3′-UTR shortening, tumors were isolated and transcriptome analysis was performed. Consistent with MAGE-A11 M341R failing to bind to and ubiquitinate PCF11, MAGE-A11 M341R tumors had significantly lengthened 3′-UTRs compared to wild-type MAGE-A11 (Fig. 3f, g). Together, these results suggest that the MAGE-A11 SBC is required for its ability to regulate PCF11, promote alternative polyadenylation and drive tumor growth.

MAGE SBC mediates substrate recognition and specificity. In humans there are at least 40 distinct MAGE genes that each contain at least one broadly conserved MHD, with some MHDs being >90% identical. However, not all MAGES are redundant and their MHDs have been shown to recognize distinct substrates, including MAGE-A11:PCF11, MAGE-A3:AMPK, and MAGE-F1:MSM19. Thus, specific sequence elements in the MHD likely dictate substrate specificity. Mapping sequence conservation of MAGES to the MAGE-A11:PCF11 structure revealed that the majority of residues that are highly conserved across MAGES are on the MHD backside away from PCF11 binding site (Fig. 4a). However, the most variable residues are concentrated around the MAGE SBC, suggesting that these are likely key positive and negative selection filters that dictate substrate specificity across the MAGE protein family. This is exemplified in comparing the SBC of MAGE-A11 with -A3 (Fig. 4b). Although the MHD structures are highly similar (RMSD 1.07; MAGE-A3 (108-293) to MAGE-A11 (221-406)), detailed examination of the SBCs revealed important differences. For example, PCF11 R690 forms a salt bridge with E266 in MAGE-A11 SBC (Fig. 4b). However, in the MAGE-A3 SBC this residue has undergone a charge swap to lysine (Fig. 4b and Supplementary Fig. 3). This along with other sequence variation in the SBC likely dictate substrate specificity for MAGE ligases. Consistent with these observations, the ability to bind PCF11 is highly specific to MAGE-A11 with none of the other ten MAGE-A proteins having detectable binding (Fig. 4c), despite overall sequence conservation between these MHDs being quite high (>80% similarity).

To determine whether other MAGEs utilize a similar SBC formed at the interface of WH-A and WH-B to mediate substrate binding, we mutated this region in MAGE-A3 SBC (F162A, I204R, L228R, or Y301A) and MAGE-F1 SBC (F129A, L196R, or W269A) that correspond to those mutated in the MAGE-A11 SBC (Fig. 4d, f and Supplementary Fig 3) disrupted their ability to bind AMPK and MSM19 substrates, respectively (Fig. 4e, g). Additionally and consistent with MAGE-A11, mutation of MAGE-A3 SBC or MAGE-F1 SBC did not impair interaction with their cognate E3 ligase binding partners, TRIM26 and NSE1, respectively (Fig. 4e, g). Consistent with this, di-leucine residues previously shown to contribute to ligase binding do not occupy the SBC (Fig. 2d).

Together, these results suggest that the mechanism for substrate recognition is conserved across MAGE proteins and is mediated the MHD SBC.

Discovery of small molecule inhibitors targeting MAGE-A11. MAGE-A11 expression is typically restricted to reproductive tissues, but is aberrantly expressed in many tumor types where its expression correlates with poor patient prognosis. This, the cancer-specific MAGE-A11 E3 ubiquitin ligase is an ideal therapeutic target. Our findings suggest that disruption of MAGE-A11 interaction with PCF11 may be a viable strategy to design cancer-specific therapeutics. Although disruption of protein–protein interactions is difficult, several examples have emerged, including a small molecule agonist that inhibits T-cell proliferation through the inhibition of IL-2 binding to its receptor IL2Ra, small molecule inhibitors of the anti-apoptotic proteins Bcl-2, Bcl-XL, and Bcl-w and small molecule antagonists block HD2M binding to p53.

To identify inhibitors of MAGE-A11, we performed a TR-FRET-based high-throughput screen of 31,407 compounds (summarized in Supplementary Fig. 4a) to identify small molecules that block MAGE-A11 binding to PCF11 in vitro. From this primary screen, 205 compounds (0.65% hit rate, Z′ = 0.82) that had >30% inhibition (Supplementary Fig. 4b, c) were cherry-picked for follow-up dose response screening. A Z′-factor value >0.5 indicated that the screen is robust. After hit clustering and structure-activity relationship (SAR) analysis from our primary and secondary screening results, we assayed an additional 500 analogs of nine scaffold groups obtained by substructure search of an in-house library collection (Fig. 5a and Supplementary Data 1). Of these scaffolds, the 145 quinoline analogs (Scaffold-4 in Supplementary Data 1) showed the most significant effects on MAGE-A11:PCF11 binding with reasonable SAR and range of activity (Fig. 5a). To expand SAR of the quinoline series, we extended diversity around the scaffold and conducted a single point TR-FRET screen of 797 additional quinoline analogs followed by dose response experiments for 59 compounds (>30% inhibition at 15 µM) (Supplementary Fig. 4d and Supplementary Data 2 and 3). From these primary and secondary screens, one of the 4-Aminoquinoline analogs, SJ521054, emerged as a promising hit with robust activity in
Indeed, SJ521054 stabilized PCF11 protein levels in MAGE-A11 expressing HEK293FT cells but had no effect on vector control cells (Fig. 5d). Strikingly, regardless of their proliferation rate, SJ521054 decreased the viability of MAGE-A11 expressing DAOY and HEK293FT cells to a much greater extent than vector control MAGE-A11-negative cells (Fig. 5e and Supplementary Fig. 4f, g).

To validate the activity of SJ521054 in orthogonal assays, we examined (1) its ability to disrupt MAGE-A11-induced degradation of PCF11 and (2) the cytotoxicity towards MAGE-A11-positive cells compared to isogenic MAGE-A11 negative cells.

TR-FRET for disrupting MAGE-A11:PCF11 interaction (IC_{50} 0.40 μM; Fig. 5b, c).

Fig. 3 Mutation of MAGE-A11 SBC disrupts its oncogenic activity. a MAGE-A11-induced ubiquitination of PCF11 depends on its substrate recognition by MAGE-A11 SBC. Ubiquitinated proteins from FLAG-vector, FLAG-MAGE-A11 wild-type, or SBC mutants stably expressing HEK293FT cells were treated with 10 μM MG132 for 4 h and isolated with tandem ubiquitin binding entity-agarose followed by SDS-PAGE and immunoblotting for endogenous PCF11. Source data are provided as a Source Data file. b MAGE-A11 SBC mutants fail to promote proliferation of MAGE-A11 knockout DAOY cells. Wild-type, MAGE-A11-knockout DAOY cells, or those reconstituted with MAGE-A11 were counted for cell proliferation at the indicated time points (n=3 biologically independent experiments per group). Data are mean ± SEM. p-value by two-way ANOVA followed by Tukey’s multiple comparisons test is indicated (****P < 0.0001). c-e MAGE-A11 knockout DAOY cells reconstituted with MAGE-A11 SBC mutants reduced clonogenic growth and xenograft tumor growth of MAGE-A11-knockout DAOY cells. Stable expression of wild-type MAGE-A11 or SBC mutants in MAGE-A11-knockout DAOY cells were assayed for clonogenic growth (c) (n=3 biologically independent experiments per group, two-tailed unpaired Student’s t test, *P < 0.05; **P < 0.01). Data are mean ± SEM and xenograft tumor growth in mice (n=6 per group). Tumor burden (d) and tumor weight (e) are shown. Data are mean ± SD, p-value by two-way ANOVA followed by Tukey’s multiple comparisons test is indicated in d (****P < 0.0001), ordinary one-way ANOVA followed by Sidak’s multiple comparisons test is indicated in e (****P < 0.0001, ****P < 0.0001, ****P < 0.0001). Red circle, Vector; Blue square, MAGE-A11 WT; Green triangle, MAGE-A11 F275A; and Pink triangle, MAGE-A11 M341R. f MAGE-A11 M341R mutant fails to promote mRNA 3’UTR shortening. Transcriptome analysis of tumors (n=3 per group) from MAGE-A11 knockout DAOY cells stably expressing FLAG-MAGE-A11 wild-type or M341R mutant was determined and scatterplot of Percentage of Distal polyA site Usage Index (PDUI) is shown. False discovery rate ≤ 0.05 and ΔPDUIs ≥ 0.2 are colored. The shift towards distal polyadenylation site is significant (p = 5.9 × 10^{-16}, binomial test). g Representative RNA-seq density plots are shown.
To extend upon the discovery of SJ521054, nine additional derivatives were designed, synthesized, and evaluated by the TR-FRET assay for their ability to disrupt MAGE-A11:PCF11 interaction and their physicochemical properties were determined along with selected previous hits for comparison (Supplementary Data 4). From these data, we identified SJ1008066 as a lead compound that is cell permeable and has improved activity in the TR-FRET assay (IC$_{50}$ 0.13 µM) and solubility (Fig. 5f, Supplementary Fig. 5a, and Supplementary Data 4). To evaluate direct binding of SJ521054 and SJ1008066 to MAGE-A11 MHD, we performed Nano-differential scanning fluorimetry (NanoDSF) assay (Supplementary Fig. 5b, c). Both compounds stabilized MAGE-A11 in a six-point does response confirming on-target activity (Fig. 5g and Supplementary Fig. 5b, c). Furthermore,
Fig. 4 The SBC region mediates substrate recognition and specificity across the MAGE family. a Sequence conservation of MAGE-A genes mapped onto MAGE-A11:PCF11 structure. b Comparison of MAGE-A3 and -A11 SBC reveals key sequence differences in critical substrate binding residues. c In vitro GST-pulldown assays reveal that GST-PCF11 binds in vitro translated Myc-MAGE-A11, but no other Myc-MAGE-A protein. Source data are provided as a Source Data file. d-g Mutation of MAGE-A3 or -F1 SBC disrupts binding to their respective substrates AMPK and MMS19, not the E3 ligases TRIM28 and NSE1. Surface representations of MAGE-A3 (protomer model formed by PDB ID 4V0P for residues 1-296 and MAGE-A11 based homology model for C-terminal residues 297-311) (d) and MAGE-F1 (SWISS-MODEL based on PDB:4VOP) (f) show positioning of SBC residues mutated. Consistent with Fig. 2c, MAGE atoms within 5 Å of modeled PCF11 (based on superimposition of MAGE-A11:PCF11) are colored cyan. Residues in the SBC that are mutated and colored yellow. Co-IP of MAGEs with substrates and ligases were determined in HeLa cells (e) or HEK293FT cells (g) upon transfection of the indicated constructs for 48 h, IP with anti-Myc, SDS-PAGE, and immunoblotting for indicated proteins. Source data are provided as a Source Data file.

SJ521054 and SJ1008066 inhibited MAGE-A11 binding to PCF11 in cell lysates as determined by co-IP, with much less potent compounds having minimal effects (Fig. 5h and Supplementary Fig. 4e). Interestingly, both compounds did not disrupt interaction of MAGE-A3 with AMPKα1 or MAGE-F1 with MMS19 (Fig. 5i). Taken together, these results identify tool compounds that provide proof-of-principle that specific disruption of MAGE-A11:substrate interaction may be a means to discover cancer-specific therapeutics.

Discussion

MAGE genes were discovered almost three decades ago as reproductive tissue restricted genes that when aberrantly expressed in cancer elicit a cytotoxic immune response due to their antigenicity. This discovery set off a flurry of research on the potential of MAGEs to prime the immune systems of cancer patients to target tumor cells. However, the underlying function and biochemical activity of MAGE proteins were largely understudied. More recently, greater understanding into the normal physiological and disease functions of MAGEs has been elucidated—importantly, the MAGE family of proteins were biochemically defined as scaffolding proteins that diversify the function of E3 ubiquitin ligases through the recruitment of novel substrates for ubiquitination. However, many questions remained unanswered that have hindered our understanding of how MAGEs work and therapeutically targeting the many MAGE cancer-testis antigens. Chiefl y, the molecular and structural basis of how MAGEs identify substrates was unknown. The specific degenerate sequences recognized by any given MAGE was unclear and the sequence and structural features on MAGEs that mediate recognition were unknown. These questions have important implications into MAGE biology, including understanding how relatively similar MAGE proteins can have distinct targets that impinge on a variety of cellular pathways. Answering these questions will facilitate the design of MAGE chemical inhibitors. Here, we provide the first evidence of how MAGEs recognize substrates and identify chemical inhibitors of the cancer selective, oncogenic MAGE-A11.

We have determined the crystal structure of MAGE-A11 bound to its substrate PCF11. Strikingly, a small peptide of PCF11 forms short alpha-helix that binds into a hydrophobic cleft formed at the interface of WH-A and WH-B motifs within the MAGE-A11 MHD. Mutational analysis of this interface across three distinct MAGEs (MAGE-A3, -A11, and -F1) established that this region defines the substrate binding site on MAGEs. Thus, we refer to this region as the substrate binding cleft (SBC).

Notably, in the previously solved structures of MAGE-A3 and -A4, the SBC was occupied by unstructured intermolecular MAGE protein or tag residues, respectively. Specifically, MAGE-A3 was observed as a domain-swapped dimer where the C-terminus was observed as a random coil threaded through the SBC of the crystallographic symmetry related protomer. However, in the MAGE-A11:PCF11 structure, these residues form the terminal helix of a helical bundle structure at the C-terminus that partly contributes to substrate binding. In MAGE-A4, the N-terminal purification tag threads through the SBC as part of a domain-swapped symmetry related dimer. Our results suggest that interaction of the unstructured C-termini of MAGE-A3 and N-terminal tag of MAGE-A4 with the SBC may be induced during crystallization due to the absence of a natural substrate. This could lead to the hydrophobic cleft being filled unnaturally to allow protein stabilization and crystallization. In either case, the SBC appears to be an important binding site for protein–protein interaction, including substrates.

The MAGE family is evolutionarily conserved in all eukaryotes and underwent dynamic expansion from a single gene in protozoa to a large multigene family in eutherian mammals. Evolutionarily, type II MAGEs appeared earlier than type I MAGEs, but the MHD is a common feature to both type I and II MAGEs. MHDs are highly conserved (46% identical) in human, especially, the MHDs of MAGE-A subfamily share >80% protein sequence identity. However, distinct MHDs often recognize non-overlapping substrates. We found that this substrate selectivity is likely imparted by sequence diversity around the SBC that may provide key positive and negative filtering. In addition to binding substrates, the MHD also mediates binding to RING or HECT ligases. For example, MAGE-C2 MHD bound the coiled-coil region on TRIM28, MAGE-B18 bound a basic region of LNX1 and MAGE-G1 bound the WH-A motif of NSE1. Although the MHD mediates ligase binding, we found that mutation of MAGE-A3, -A11, or -F1 SBC did not disrupt binding to their cognate E3 ligases. This suggests that (1) SBC mutations do not cause global structural changes to the MHD and (2) ligase and substrate binding to the MHD occur independently on non-overlapping surfaces. Consistent with this observation, our previous co-crystal structure of MAGE-G1:NSE1 showed a distinct binding surface for NSE1 not restricted to the MHD SBC. Notably, the relative orientation of WH-A and WH-B of MAGE-G1 MHD in the MAGE-G1:NSE1 structure is quite distinct compared to the structures of MAGE-A3, -A4, and -A11:PCF11. Therefore, E3 binding may induce structural dynamics within the MHD and the relative positioning of the WH motifs may contribute to selective ligase binding between MAGEs. Additional MHD structures with both ligase and substrate bound will provide important answers to these questions.

Finally, MAGEs and other cancer-testis antigens have been extensively proposed as cancer-specific therapeutic targets. Thus far there has been limited success. In this study we focus on targeting oncogenic MAGEs through blocking binding to substrates. We propose this approach will be of broad utility against the many cancer-specific MAGEs. As proof-of-principle we target MAGE-A11 and discover sub-micromolar 4-Aminoquinolines inhibitors of MAGE-A11 binding to PCF11. These tool compounds will help facilitate research into MAGE-A11 biology and give precedence for the development of future MAGE-directed therapeutics.
Methods

Cell culture and stable cell lines. HEK293FT, HeLa and DAOY cells were grown in DMEM (Gibco) supplemented with 10% (v/v) FBS (Hyclone), 2 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.25 mg/ml amphotericin B (Invitrogen). HEK293FT and DAOY cells were transfected with either HA-FLAG-vector, FLAG-MAGE-A11 wild-type or SBC mutants using Effectene (QIAGEN) according to the manufacturer’s protocol in 6 cm² plates. After 48 h, cells were selected with 1 µg/ml puromycin (Sigma) over 2 weeks.

Immunoprecipitation. HEK293FT cells were plated in 6 cm² plates and transfected 24 h later with Effectene (QIAGEN) according to the manufacturer’s protocol. After 48 h, cells were washed and scraped in cold PBS, spun down, and resuspended in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM DTT, 0.1% (v/v) Triton X-100, 10 mM N-Ethylmaleimide, and 1X protease inhibitor cocktail (Sigma)). Cell lysates were incubated with appropriate antibodies overnight at 4 °C and then with protein A beads for 2 h at 4 °C. Beads were then washed with lysis buffer three times and eluted with 2X SDS sample buffer.
Western blotting and antibodies. For western blotting, samples in SDS sample buffer were resolved on SDS-PAGE gels and then transferred to nitrocellulose membranes prior to blocking in TBS with 5% (w/v) milk powder or 3% (w/v) buffer. Membranes prior to blocking in TBST with 5% (w/v) milk powder or 3% (w/v)

Peptide synthesis. N,N-dimethylformamide (DMF), N-methylpyrrolidone (NMP), trifluoroacetic acid (TFA), 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), and N-methylmorpholine (NMM), were purchased from Millipore-Sigma (St. Louis, MO). O-(7-Azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HATU), 1-Hydroxy-7-azabenzotriazole (HOAT), and substituted Fmoc-phenylalanine analogs were purchased from ChemImpex (Wood Dale, IL). Fmoc-amino acids and preloaded Wang resins were purchased from Millipore-Sigma (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA). Black and gold volume plates and 384-well polypropylene compound plates were purchased from Corning Incorporated (Tewksbury, MA). The St. Jude FDA drug and Bioactive library (11,293 chemicals), Drug-like library (10,073 chemicals), and Lead-like library (10,041 chemicals) were custom assembled and purchased from commercial sources including Sigma, Microsource (Gaylordville, CT), selleckchem (Houston, TX), ChemDiv (San Diego, CA), Chembridge (San Diego, CA), Enamine (Monmouth Jct., NJ), and Life Chemicals (Niagara-on-the-Lake, ON, Canada). They were prepared and controlled with the help of a robotic peptide synthesizer (Overture, Millipore-Sigma, Billerica, MA). The St. Jude Bioactive library includes a broad range of small molecules including those that are known to interact with human proteins. The library is designed to be used in high-throughput screening experiments to identify new drug targets and to validate existing targets. The library is sorted by structure and function, and contains a variety of non-native compounds that can be used to probe the activity of small molecules in a wide range of biological systems.

Peptide purification. Peptides were purified using precolumns (PrepC18; Waters, Milford, MA) and prepacked reverse-phase columns (C18; Phenomenex, Torrance, CA) using gradient elution with acetonitrile and water containing 0.1% formic acid. Peptides of interest were then desalted using a C18 reversed-phase column (Waters, Milford, MA) and concentrated using a vacuum concentrator (Eppendorf AG, Hamburg, Germany). The final purity of the peptides was assessed by LC-MS/MS analysis and was typically greater than 95%.

Peptide binding to GST-fusion proteins. GST-fusion proteins were expressed in E. coli and purified using glutathione agarose affinity chromatography. The purified GST-fusion proteins were dialyzed against buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, and 0.1% Triton X-100 and then incubated with the peptides at a 1:10 ratio (protein:peptide) in a final volume of 50 µL. After incubation, the GST-fusion proteins were pelleted by centrifugation and the supernatant was analyzed by Western blotting using anti-GST antibodies.

Peptide binding to GST-fusion proteins was determined by Western blotting using anti-GST antibodies. The intensity of the bands was quantified using ImageJ software, and the binding affinity was calculated using a Scatchard plot. The dissociation constant (Kd) was calculated using the equation Kd = (slope × Vtotal) / (intercept - Vtotal), where Vtotal is the volume of the binding reaction and the slope and intercept were determined from a linear regression analysis of the data. The Kd values were expressed as the mean ± SEM of at least three independent experiments.

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and 200 µm integration time. The TR-FRET fluorescence emission ratio (TR-FRET signal) for each well was presented as 10,000 × 520 nm/490 nm, which was used directly for curve fitting, or further converted to %Inhibition based on the respective positive and negative controls. The graphic software GraphPad Prism 4.81 (GraphPad Software; La Jolla, CA, USA) was used to generate curves and derive IC50 values of tested peptides or compounds, if applicable.

TR-FRET inhibitory activity characterization of a panel of PCF11 peptides against the MAGE-A11 MHD and FAM-PCF11_4 was performed as follows. In a 20 µl/well assay volume, dilutions of PCF11_1 to PCF11_8 peptides (concentrations ranged from 3.1 nM to 100 µM for peptides PCF11_1, PCF11_4, PCF11_5, PCF11_6 and PCF11_8 in a 1 to 2 dilution pattern for 16 concentration levels, and ranged from 18.9 nM to 100 µM for peptides of PCF11_1, PCF11_2, PCF11_3, and PCF11_7 in a 1 to 2 dilution pattern for 12 concentration levels), 200 nM FAM-PCF11_4, 5 nM Tb-anti-GST and 5 nM GST-MAGE-A11 were mixed together and incubated for 90 min before the TR-FRET signal for each well was measured. The final DMSO concentration was 1.1% (v/v). In each group, a well of groups with 1.1% (v/v) DMSO along with 200 nM FAM-PCF11_4, 5 nM Tb-anti-GST and 5 nM GST-MAGE-A11 was included as the negative control (SignalDMSO without GST-MAGE-A11, 100% inhibition). The signal of each peptide-treated well was measured. The levels (mean ± standard deviation), 200 nM FAM-PCF11_4, 5 nM Tb-anti-GST and 5 nM GST-MAGE-A11 group) was also included as the positive control (SignalGST-MAGE-A11 without GST-MAGE-A11, 100% inhibition). The signal of each peptide-treated well was normalized to the positive and negative controls to derive %inhibition of the peptide at a specific concentration with Eq. (1). Where applicable, the %Inhibition data were fit into a Sigmoidal dose response equation to derive IC50 values.

\[
\text{IC50} = \frac{\left[\text{Signal}_{\text{peptide}} - \text{Signal}_{\text{DMSO without GST-MAGE-A11}}\right]}{\left[\text{Signal}_{\text{DMSO without GST-MAGE-A11}} - \text{Signal}_{\text{GST-MAGE-A11 without GST-MAGE-A11}}\right]} \times 100\% \tag{1}
\]

%Inhibition = 100% − \frac{\text{Signal}_{\text{peptide}} - \text{Signal}_{\text{DMSO without GST-MAGE-A11}}}{\text{Signal}_{\text{DMSO without GST-MAGE-A11}} - \text{Signal}_{\text{GST-MAGE-A11 without GST-MAGE-A11}}} \times 100\% \tag{1}

where σ is the standard deviation of the negative control group (DMSO group), σ− is the standard deviation of the positive control group (DMSO without GST-MAGE-A11 group), mean+ is the mean of the negative control group (DMSO group), and mean− is the mean of the positive control group (DMSO without GST-MAGE-A11 group).

For TR-FRET dose-response test, a protocol similar to the primary screening was followed except that the final DMSO concentration was 0.8% (v/v) with 0.1% (v/v) from the FAM-PCF11_4 DMSO stock solution and 0.7% (v/v) from the compound-DMSO solution or the DMSO for the control wells (Pin Tool was used to transfer 140 nl of compound-DMSO solutions or DMSO). The inhibitors were arrayed directly into the wells (200 µl/well) and ranged from columns 21–24 of 384-well compound plates. The final tested compound concentrations ranged from 3.6 nM to 70 µM in a 1 to 3 dilution pattern for 10 concentration levels or ranged from 0.13 nM to 70 µM in a 1 to 2 dilution pattern for 20 concentration levels. The %Inhibitions of each tested inhibitor at individual concentrations were calculated by using Eq. (1) and were fit into a sigmoidal dose response equation to derive IC50 values with the GraphPad Prism 4.81 software, if applicable.

Thermal stability measurements. The thermal stability of the MAGE-A11 in the presence of ligand was measured using differential scanning fluorimetry. In all, 200 µl of ligand PCF11_6, diluted to achieve the desired final concentration in DMSO, was treated as an input to an Applied Biosystems Microfluidic Optical reaction plate using acoustic transfer. To each well, 20 µl thermal stability assay solution (10 µM His-SUMO-MAGE-A11 and 5 µM SyproOrange in 50 mM HEPES, 100 mM NaCl, 50 mM MgCl2, pH 7.5) was added, and the plate was sealed with Microamp optical adhesive film. The plate was exposed to a range of temperatures (25–99 °C, ramp 6 °C/min) while measuring the fluorescence of SyproOrange in an Applied Biosystems Mx3005p real-time PCR instrument. Each compound was analyzed twice as a positive control validating the assay. A temperature gradient of 1 °C min−1 from 25 to 95 °C was applied and the intrinsin protein fluorescence at 330 and 350 nm was recorded. The 350 nm to 330 nm ratios of fluorescence measurements were used to calculate the Tm values of each sample using the Boltzmann Equation. All experiments were performed in triplicate.

Nano-differential scanning fluorimetry measurements. For NanoDSF measurements, a Prometheus T48 instrument (NanoTemper Technologies) was used to determine the melting temperatures. Triplicate samples were prepared in a standard 384-well PCR plate with His-SUMO-MAGE-A11 at 10 µM and ligand concentrations as noted. After incubating the protein and ligands for at least 30 min, capillaries were filled with 10 µl of sample and placed on the sample holder. DMSO was used as a negative control while PCF11_6 peptide was used as a positive control validating the assay. A temperature gradient of 1 °C min−1 from 25 to 95 °C was applied and the intrinsic protein fluorescence at 330 and 350 nm was recorded. The 350 nm to 330 nm ratios of fluorescence measurements were used to calculate the Tm values of each sample using the Nanotemper PR-ThermControl software. Data were further analyzed and plotted using GraphPad Prism (v 8.3.1).

Protein expression and purification. GST-PCF11 (full-length human PCF11 or amino acids 637–702), GST-MAGE-A11 MHD (human MAGE-A11 MHD amino acids 218–429), or GST tag alone were included in BL21(DE3) cells at 16 °C with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). GST-tagged proteins were purified from bacterial lysates in lysis buffer (50 mM Tris pH 7.5, 150 mM KCl, 0.1 mM EDTA, 50 µM lysozyme, 100 mM X: 1, 100 µM X: 2, 100 µM X: 3, 100 µM X: 4, 100 µM X: 5, 100 µM X: 6, 100 µM X: 7) followed by Resource Q ion exchange chromatography (GE Amersham) and was concentrated to 15 mg/ml in 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM DTT and 5% (v/v) glycerol.

For crystallization, PCF11 peptide (human PCF11 amino acid 677–701) was fused at the N-terminus of MAGE-A11 MHD (218–429) with linker sequence GSSGR. The fusion protein was expressed in BL21(DE3) E. coli using the pSUMO3 vector. The protein complex was purified using a HisTrap Hp 5 ml column (GE Amersham) followed by Resource Q ion exchange chromatography (GE Amersham). The His-SUMO tag was subsequently cleaved off by addition of SENP protease digested by second HisTrap Hp 5 ml column (GE Amersham). The resulting fusion protein complex was concentrated to 15 mg/ml in 50 mM Tris, pH 7.4, 200 mM NaCl, and 5 mM DTT.

Crystallization, data collection, structure determination, and model quality. Crystals were grown by the sitting-drop vapor diffusion method at 20 °C. The well solution contained 0.2 M Na acetate, 0.1 M Bis Tris propane pH 7.5 and 20% (v/v) PEG 3500. The drop contained a 1:1 volume ratio of well solution to protein (15 mg/ml in 50 mM Tris, pH 7.4, 200 mM NaCl and 5 mM DTT). Crystals appeared overnight and matured in ~1 week. For cryo-preservation, the crystals were incubated in reservoir solution with 10% (v/v) glycerol prior to flash-cooling in liquid nitrogen. Data were collected at the Southeast Regional Collaborative Access Team (SER-CAT) Sector 22-BM beamline at 1.1Å. Data were integrated and scaled using HKL2000 v1.6.3.2 to 2.2 Å. The structure was solved by molecular replacement with Phaser v2.23 using MAGE-A3 (PDB ID 4V0P) as the search model. Iterative rounds of model building and refinement were performed with COOT v0.8.931 and Refmac v5.8.0.237, respectively. Processing and refinement statistics are summarized in Table 1.

The final model is of high quality, with four fusion protein protomers in the P1 cell. Although PCF11 peptide residues were readily identifiable in all protomers, the MAGE-A11 linker was disordered. All protomers are remarkably similar, with a Cα RMSD of <0.4 Å relative to protomer A (chain B 0.141, chain C 0.351, and chain D 0.349). Detailed descriptions and structural features have been deposited in the Protein Data Bank with accession number 6WJH. Figures were made using PyMOL35.

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glutathione Sepharose beads (GE, Amersham) for 1 h in binding buffer (25 mM Tris pH 8.0, 2.7 mM KCl, 137 mM NaCl, 0.05% (v/v) Tween-20, and 10 mM 2-mercaptoethanol). Bound beads were blocked for 1 h in binding buffer containing 5% (w/v) milk powder. In vitro translated proteins (5 μl; Promega TNT rabbit reticulocyte lysate quick SP6 system) were then incubated with the bound beads for 1 h in binding buffer containing 5% (w/v) milk powder. After four washes in binding buffer, the proteins were eluted in SDS-sample buffer, boiled, subjected to SDS-PAGE, and blotted with anti-Myc.

Tandem ubiquitin binding entity ubiquitination assay. SDS-PAGE, and blotted with anti-Myc.

Cell viability assay. To assess cell viability after SJ251054 treatment, a 1 x 10^4 cells/ml were treated with SJ251054 (0–40 μM) and incubated for 24 h prior to changing the media and adding alkaline Blue (Thermo Fisher Scientific) and incubating for 4 h at 37 °C. Plates were read by measuring the fluorescence with excitation wavelength at 540 nm and emission wavelength at 590 nm on an Enspire plate reader.

Xenograft tumor growth assays. In all, 3 x 10^6 MAGE-A11-knockout DAOY cells reconstituted with wild-type MAGE-A11 or SBC mutants were mixed with matrigel (Corning) before injection into the flank of NOD scid gamma mice (Jackson Lab; n = 6 for each group). Tumor size was measured 2–3 times a week during the duration of the experiment. All animal experiments were approved by the St. Jude’s Children’s Research Hospital’s IACUC.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. Structural data that support the findings of this study have been deposited in PDB with the accession code 6WHJ. Sequencing data that support the findings of this study have been deposited in the GEO with the accession code GSE148458. Source data are provided with this paper.

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Author contributions

P.R.P., S.W.Y. and X.H. conceptualized the study and designed experiments. X.H. and S.W.Y. performed protein based experiments with D.J.M., W.L., J.M., P.R., E.C.G. and C.T.G.X.H. and D.J.M. designed and executed crystallographic studies. W.L. performed data analysis and wrote the manuscript. We also thank the St. Jude X-Ray Center, Hartwell Center and Department of Chemical Biology and Therapeutics at St. Jude. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38. Data were collected at Southeast Regional Collaborative Access Team (SER-CAT) 22-BM beamline at the Advanced Photon Source, Argonne National Laboratory. SER-CAT is supported by its member institutions (see www.ser-cat.org/members.html), and equipment grants (S10_RR25528 and S10_RR028976) from the National Institutes of Health. This work was partially supported by a sponsored research agreement from Amgen (P.R.P.) and NIH/NCI R01CA193466 (W.Li).

Competing interests

P.R.P. is a paid consultant of Levo Therapeutics, Inc. and Amgen Inc. All other authors declare no competing interests.

Additional information

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