The eukaryotic translation initiation factor eIF4E, a potent onco-gene, is highly regulated. One class of eIF4E regulators, including eIF4G and the 4E-binding proteins (4E-BPs), interact with eIF4E using a conserved YXXXLΦ-binding site. The structural basis of this interaction and its regulation are well established. Really Interesting New Gene (RING) domain containing proteins, such as the promyelocytic leukemia protein PML and the arenaviral protein Z, represent a second class of eIF4E regulators that inhibit eIF4E function by decreasing eIF4E’s affinity for its m7G cap ligand. To elucidate the structural basis of this inhibition, we determined the structure of Z and studied the Z-eIF4E complex using NMR methods. We show that Z interacts with eIF4E via a novel binding site, which has no homology with that of eIF4G or the 4E-BPs, and is different from the RING recognition site used in the ubiquitin system. Z and eIF4G interact with distinct parts of eIF4E and differentially alter the conformation of the m7G cap-binding site. Our results provide a molecular basis for how PML and Z RINGS reduce the affinity of eIF4E for the m7G cap and thereby act as key inhibitors of eIF4E function. Furthermore, our findings provide unique insights into RING protein interactions.

The structural basis underlying the effects of PML and Z RINGS on eIF4E was unknown. Further, the features for recognition of RINGs with proteins other than ubiquitin conjugating enzymes (Ubc) had not been reported. To address these issues, we used NMR methods to study the structural aspects of the Z-eIF4E interaction as a prototypic example of RING-eIF4E interactions. We report the structure of an arenaviral protein, Z, and demonstrate that eIF4E recognition by the RING domain of Z differs substantially from the recognition of ubiquitin conjugating enzymes (Ubc) by RINGs. In particular, residues within the first zinc-binding site make key contacts with eIF4E. A structural comparison of Z, PML, and RINGs acting as ubiquitin ligases suggested molecular underpinnings for their different interactions. In addition, Z binds to regions of the dorsal surface of eIF4E that differ from those used by eIF4G and the 4E-BPs. This leads to differential effects on the conformation of the distal cap-binding site of eIF4E. Our findings provide the foundation for further studies aimed at elucidating the biological consequences of RING-eIF4E interactions, unique RING-protein interactions, and for interactions of arenavirus and host cell proteins.


The authors declare no conflict of interest.

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Data deposition: Coordinates of the Z structure were deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2KO5), and chemical shift data are in the BioMagResBank, www.bmrb.wisc.edu (accession no. 15660).

1To whom correspondence should be addressed. E-mail: Katherine.Borden@umontreal.ca.

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Results and Discussion

Solution Structure of the Z RING Protein. As a first step in understanding RING-eIF4E interactions, we solved the solution structure of the 99 residue Z protein from LASV. We chose LASV Z because of its better solution behavior than PML RING (19). This Z construct associates with eIF4E and reduces cap binding to the same extent as previously used constructs derived from LCMV Z (Fig. S1), making it an ideal candidate to investigate RING-eIF4E interactions by NMR.

The RING domain of Z (residues 30 to 70) forms a well-defined structure comprising a helix (residues 50–58), two short antiparallel β-sheets (β1: residues 42–44, 47–49; and β2: 63–64, 69–70) and two loop regions: 35–39 (loop 1) and 58–62 (loop 2). For residues 30 to 70, the rmsd with respect to the mean coordinate positions is 0.39 (±0.06) and 0.91 (±0.23) Å for backbone and heavy atoms, respectively (Fig. 1B and Table S1). These residues are relatively rigid on the ns/ps timescales, while residues in loop 1 have some degree of motion (Fig. 1C). In contrast, the N terminus (1–29) and C terminus (71–99) exhibit very low heteronuclear NOE values, consistent with these being disordered.

Two zinc atoms bind the RING in a typical cross-brace fashion (Fig. S2A). Interestingly, when Z was compared to the structurally diverse family of RINGs using the DALI program (20), no significant superimposition was obtained (DALI score <3.0) due to its unique conformation around site II. Specifically, its topology differs from other RINGs by the location of the first two zinc ligating residues within site II being arranged on either side of the first small β-strand (β1). Further, Z has a second small β-sheet (β2) also around site II (Fig. L4). A comparison of the structure of Z and PML RING domains revealed that these are quite homologous around site I, in which certain aromatic and charged side chains have nearly identical positions (Fig. S3).

The rmsd between PML (residues 9–16, 28–32) and Z (residues 31–38, 49–53) is 1.9 Å (Fig. S3F), which is striking given the low identity between these proteins (Fig. S2C). Loop 1 in PML and Z adopts a more open conformation than found in other RINGs such as BRCA1 or Cbl, neither of which bind eIF4E (16). This conformational difference is likely important for eIF4E recognition (see below).

Identification of a Unique Mode of RING Interaction. To map the Z binding surface for eIF4E we employed 2D 1H-15N heteronuclear single quantum correlation (HSQC) NMR chemical shift mapping and transferred cross saturation (TCS) experiments (21) (Fig. 2). Note that throughout this study we used cap-free eIF4E. Upon addition of eIF4E, 15N Z undergoes relatively large chemical shift changes and/or line broadening (in red and green, respectively, in Fig. 2A–C) for residues around site I and on the underside of the RING. In particular, residues in loop 1 of site I were most affected, suggesting these are at the interacting surface with eIF4E (Fig. S4 and Fig. 2A–C). Interestingly, in the free form, loop 1 was somewhat less ordered than the rest of the Z RING (Fig. 1B and C), which may be important for facilitating local rearrangement upon eIF4E binding. Also, the N and C termini of Z are not perturbed by eIF4E addition consistent with previous studies where deletion of these regions did not affect the Z-eIF4E interaction (16).

We performed TCS experiments (21) to distinguish chemical shift perturbations arising due to ligand binding from conformational changes distal to the interaction surface. The TCS experiment showed large reductions in signal intensity for residues around site I of Z, particularly loop 1 (F30, K32, S33, W35, and the H6 side chain of N38), confirming chemical shift mapping that these residues are at the complex interface (Fig. 2D). Mutations W35A, F36A, N38A, or K39A within loop 1 of Z also abrogated its association with eIF4E (Fig. 2E) further underlining the importance of these residues. Consistent with previous data, mutation of site II did not reduce association with eIF4E (Fig. 2E) (16). In contrast, mutation of site I or addition of EDTA, both of which unfold Z, abrogated its association with eIF4E (16), Fig. 2E). This is consistent with mutational and biophysical studies indicating that the integrity of site I, but not site II, is required for folding of PML and Z RINGs (16). Studies with BRCA1 and PML RINGs suggested that zinc binding is anticooperative with site I being the higher affinity site (22). This previous study suggested that the pliability in site II may enable RINGs to bind different partners as a function of zinc levels.

Altogether, our studies indicate that the region comprising residues 30 to 39 interacts with eIF4E. This region is highly conserved among known Z proteins (Fig. S2B). Significantly, the Z recognition site for eIF4E does not utilize a YXXXXL α-helix motif as used by eIF4G and the 4E-BPs. Thus, this represents a previously undescribed type of eIF4E binding domain.

BRCA1 and Cbl RINGs bind Ubcs using a shallow mainly hydrophobic groove formed by the central helix and zinc sites (Fig. S5A) (23). In the comparative region of Z and PML, residues K32, H47, R63, and K68 form a positively charged groove (Fig. 2 and Fig. S5B) and association with eIF4E is mediated via a surface formed by the residues around loop 1, which is distinct to the region used by other RINGs to interact with Ubcs. The conformation of site I is conserved between PML and Z, and differs from that observed in BRCA1 and Cbl. These analyses reveal that the molecular underpinnings of RING-Ubc and RING-eIF4E interactions are markedly different (see Fig. S5 for details).

Mapping of the Interaction Site on eIF4E. To characterize the binding site of eIF4E for Z, we monitored perturbations in the NMR signal of 15N-eIF4E as a function of Z addition (Fig. S6) and performed TCS studies of the 2H, 15N-labeled eIF4E/unlabeled Z complex. The largest chemical shift perturbations were found for residues in helices 1 and 2 of eIF4E, together with residues preceding the initial β-strand (Fig. 3A). These were accompanied by significant line broadening, some of which were broadened beyond detection (green in Fig. 3A and B), e.g., the indole NH of W73. The TCS experiments confirmed the chemical shift...
perturbation mapping with residues H37, V69, E70, W73, A74, and W130 exhibiting the largest intensity changes (Fig. 3C).

Interestingly, no ordering of the N-terminal eIF4E arm was observed upon Z addition, in contrast to the eIF4G-eIF4E-cap ternary complex in yeast (24). These results indicate that Z binds to a region of eIF4E that only partially overlaps with the YXXXXLΦ class of regulators (see below).

**Modeling the eIF4E:Z Complex.** Attempts to use residual dipolar couplings to obtain the relative orientation of Z to eIF4E were unsuccessful given the complete insolubility of the complex in multiple alignment media. Further, the intermediate NMR exchange regime observed for the eIF4E-Z complex meant that it was not possible to obtain intermolecular NOEs due to loss of the resonances specifically at the interaction surface. Therefore, we generated a model of the eIF4E:Z complex with the restraint-driven docking program Haddock (25), using chemical shift perturbation, TCS, and mutagenesis data (Figs. 2, 3, and 4C). The Z-eIF4E complex was a good candidate for this strategy as neither protein undergoes large-scale conformational changes upon complex formation as seen by the limited extent of chemical shift perturbation, consistent with previous circular dichroism
A shift perturbations (Fig. 5) lie the differential effects on cap binding by analyzing chemical
tinct partners.

A (Fig. 4) increases the affinity of eIF4E for the cap (27). Binding footprints
finity of eIF4E for the cap (26). In contrast to Z, eIF4G binding
partners were the first class observed to reduce the af-
partners were added (Fig. S8). The resulting model showed a globally consistent
studies (15, 16). The resulting model showed a globally consistent orientation of Z with eIF4E (Fig. S7). Here, W35 and F36 of Z
interact with the hydrophobic cavity between helices 1 and 2 of eIF4E. Also, residues N38 and K39 on Z interact with H37 and E70 on eIF4E, consistent with mutational data (Figs. 2C and 4C).

How Does Z Decrease Affinity of eIF4E for the Cap? The RING class of eIF4E partners were the first class observed to reduce the affinity of eIF4E for the cap (26). In contrast to Z, eIF4G binding increases the affinity of eIF4E for the cap (27). Binding footprints of Z and the eIF4G peptide (eIF4Gp) from our NMR data indicate that they bind overlapping but distinct surfaces on eIF4E (Fig. 4A and B). Z binding is more centralized to helix 1, whereas eIF4Gp binding is more toward the top of the dorsal surface. To confirm these observations, we carried out pulldown experiments using a series of mutations in eIF4E to preferentially modulate eIF4Gp vs. Z binding (Fig. 4C and D). Note, all mutants studied were structured as observed by NMR and/or CD (Fig. S8A and C). We observed that mutations in the shallow groove between helices 1 and 2 (Y76A or W130F) substantially reduce Z binding but not binding to eIF4Gp. Conversely, mutation of residues toward the top of the dorsal surface (Q40A, K65A, V69G, D144A, and, to a lesser extent, D147A) reduced binding to eIF4Gp whereas these mutations did not substantially affect the binding of eIF4E to Z, although there was a slight reduction for Q40A. E70A and W73A mutants reduce both Z and eIF4Gp binding. Importantly, equal amounts of bait proteins were immobilized on the beads (Figs. 2E and 4C), and the same amount of soluble partners were added (Fig. S8B). In summary, eIF4E uses specific features on the dorsal surface to interact with structurally dis-
tinct partners.

We investigated whether the distinct binding footprints under-
lie the differential effects on cap binding by analyzing chemical shift perturbations (Fig. 5A and B and Fig. S9) and line broad-
ening (Fig. S10) in eIF4E for the two complexes. Interestingly, the chemical shift for the indole nitrogen of W130 was altered and substantially broadened in the Z complex, but not in the eIF4Gp complex (Fig. 5B). Further, W130 is at the Z interaction site according to TCS data. W130 is adjacent to β and β5 strands, which back onto the cap-binding site, and thus its perturbation could modulate cap binding (Fig. 5B and Fig. S10D). Y76, a res-
due adjacent to W130, underwent substantial line broadening in the wild-type eIF4E-Z complex, and its mutation abrogated bind-
ing for Z. Interestingly, many residues in the cap-binding site were perturbed by either addition of Z or mutations of Y76 or W130 (Fig. 3 and Figs. S10 and S11). Notably, mutation of Y76 or W130 did not abrogate eIF4Gp binding. Thus, altering the Y76-W130 interaction could be the initial step in a chain of events communicating Z binding to the cap-binding site. Inter-
estingly, W130 was proposed to be part of an allosteric track important for communication between the dorsal surface and the cap-binding site (28). Given the differing binding surfaces for Z and eIF4Gp, communication to the cap-binding site may go through different allosteric routes and thereby yield different outcomes.

Residues W102 and W56 are important for binding cap. Stud-
ies of apo eIF4E and the eIF4E-cap complex showed that W102 rotates into the cap-binding site, whereas W56 and its adjacent loop moves as a hinge and together these tryptophans sandwich the m7G cap (27). Neither Z nor eIF4Gp perturbed W56 or W102 indole resonances. In our previous studies of apo-eIF4E, the backbone resonances corresponding to W56 and W102 were masked by residues in the flexible N-terminal arm of eIF4E. To overcome this, we designed a shorter eIF4E (s-eIF4E) lacking residues 4 to 26. In the s-eIF4E/Z complex, small chemical shift perturbations are observed for the backbone resonances of the W56-containing loop, while the W102-containing loop, is unaffected. Additionally, Z binding leads to substantial line broadening of residues in the phosphate-binding region (relative to other residues in the complex, e.g., W166 or E105) suggesting that this region is undergoing conformational exchange. These include R157, A158, and C89 (Fig. 5). In the eIF4E/eIF4Gp complex, residues in the phosphate-binding region (D90, R157, K162) also undergo substantial conformational changes, and residues W102, E105, and W166 backbone resonances (but not Trp side-chain resonances) exhibit small shifts suggesting a small conformational change in this region (Fig. 5A). In general, residues exhibit sharper linewidths in the eIF4Gp complex (e.g., L81, Y91) than in the Z complex or for apo-eIF4E (Fig. 5, Fig. S10B–D). Thus, Z binding appears to induce conformational exchange in the cap-binding site leading to reduced cap affinity, whereas eIF4Gp binding reduces mobility and prestructures eIF4E into a higher affinity state (27).

Conclusions
No structures of any Arenaviruses proteins were reported prior to these studies. Z folds as a RING domain with a unique topology around site II, but exhibits structural similarity with PML RING around site I. Our studies show that this site forms the binding surface for eIF4E, specifically around loop 1, and represents a unique eIF4E binding site with no homology to that used by eIF4G and the 4EBPs. The structural similarity of Z and PML at this site combined with previous mutational data for PML (16) suggests that PML likely uses a similar strategy to bind and regulate eIF4E. Our studies also provide unique insights into RING recognition (Fig. S5C and D).

Compared to the eIF4G-eIF4E complex, Z binds eIF4E on a distinct region of the dorsal surface centered on helix I. Further, Z and eIF4G differentially alter residues at the distal cap-binding site, providing a possible mechanism for the different effects of Z and eIF4G on cap affinity. Our results suggest that perturbing the interaction between W130 and Y76 leads to conformational
changes in the cap-binding site including increased conformational exchange (Fig. 6). The increased conformational exchange of eIF4E upon addition of Z, taken together with the substantial line broadening observed, suggests that dynamically driven allosteric could be an important contributor to the effect of Z on the cap affinity of eIF4E. Note that it is also possible that multiple allosteric routes could be used simultaneously.

Z is important to many viral processes including regulation of virus gene expression, virion assembly, and budding. Z is likely a pleiotropic mediator of arenavirus-host interactions given it interacts not only with other viral proteins but also with a variety of host proteins other than eIF4E, including PML and PRH (11, 29, 30). The mechanisms by which pathogenic arenaviruses overcome the host innate defense response remain to be determined, but appear to involve a complex network of cellular interactions including plasmocytoid dendritic cells (pDC) (31). Interferon regulatory factor 7 (IRF7) contributes to the control of type I interferon expression by pDCs, which plays an important role in the host antiviral response (32). Translation of IRF7 mRNA is influenced by the 4E-BP/eIF4E ratio (32), raising the intriguing possibility that the Z-eIF4E interaction contributes to altered IFN production in arenavirus-infected pDCs. The generation of recombinant LCM viruses with Z mutations will facilitate studies aimed at determining the importance of Z/eIF4E interactions in the context of the natural course of virus infection and reveal undiscovered aspects of arenavirus biology.

**Materials and Methods**

**Expression and Purification.** Z and eIF4E were expressed and purified as described (27, 33) and confirmed by mass spectrometry (see Fig. S12). Site-directed mutants for Z and eIF4E were prepared using the Quick-Change mutagenesis kit (Stratagene). The N-terminal truncated eIF4E (Δ4-26 eIF4E, or s-eIF4E) was generated by a PCR cloning method. All constructs were verified by sequencing.

**Fig. 5.** (A) An overlay of 15N-1H HSQC spectra of eIF4E showing residues in the core and the cap-binding site without (cyan), with Z (purple), or with eIF4Gp (red). (B) Chemical shift differences for the eight Trp indoles between the apo-eIF4E and either eIF4E/eIF4Gp (red) or eIF4E/Z (purple) complexes.

**Fig. 6.** Communication from the dorsal surface to the cap-binding site of eIF4E. Key amino acid side chains are displayed in stick mode (backbone amides displayed in Fig. 5A are in green). The backbone is color-coded according to chemical shift changes in the eIF4E/Z complex.
NMR Spectroscopy and Structure Calculations. NMR samples typically contained 0.2 mM protein in 93% H$_2$O/7% D$_2$O containing 20 mM phosphate buffer (pH 7.2), 200 mM NaCl, 10 mM tris(carboxyethyl)phosphine, 50 µM ZnSO$_4$, and 0.02% sodium azide. NMR experiments were acquired at 600 MHz on a Varian INOVA spectrometer equipped with an HCN cold probe at 17 K. The final assignment of resonances were completed (33). Three-dimensional $^{15}$N-edited and $^{13}$C-edited NOE spectra (100 ms mixing time) were acquired at 600 and 800 MHz. Heteronuclear $^{1}$H–$^{15}$N NOE spectra were recorded in an interleaved manner (34). Data were processed with NMRPipe (35) and analyzed with Sparky (36).

TCS experiments (21) were performed using a molar ratio of 10:1 for [100%-$^{15}$N]$^{1}$H$^{18}$O to unlabeled $^{1}$H$^{18}$O, and 4:1 for [100%-$^{15}$N]$^{1}$H$^{18}$O to unlabeled $^{1}$H$^{18}$O. Proton spectra were saturated at 0.9 ppm using a 1.5 s train of EOB (Edward-Obert) pulses of 20 ms separated by 1 ms and a relaxation delay of 1.5 s. The saturation frequency was shifted 25,000 Hz upfield for the off-resonance experiments. To evaluate the effect of the residual aliphatic protons within the labeled protein, and possible effects of spin diffusion from the high amide content, the TCS experiments were also carried out under identical conditions but without the unlabeled protein.

Distance restraints were obtained from 3D $^{15}$N-edited and $^{13}$C-edited NOE spectra. A total of 483 manually assigned distance restraints were classified according to peak intensities. Also, 30 experimental ϕ dihedral angles were obtained from values of J$_{HN-H}$ coupling constants derived from an HHNHA experiment (37). Hydrogen bonds were determined from a lyophilized amide proton from the second HSQC. For each hydrogen bond, two distance restraints were recorded at 10-min intervals at 20 °C. Amide protons were considered resistant to exchange and thus involved in hydrogen bonding, if the TCS experiments were also carried out under identical conditions but without the unlabeled protein.

NMR Titrations. All titrations were conducted with protein concentrations at or below 0.2 mM. The molar ratios were calculated to be 1:0.3, 1:0.6, 1:1, and 1:2 for both [100%-$^{15}$N]-eIF4E and [100%-$^{15}$N]-eIF4E$_{t}$ titrations. Two-dimensional $^{1}$H–$^{15}$N-HSQC NMR spectra were acquired for each titration point. Chemical shift perturbations for each resonance were calculated using the equation $\Delta \delta_{NN} = |\Delta \delta H_{NN} + \Delta \delta N_{NN}| / 2^{1/2}$ (41).

Pulldown Assays. Purified eIF4E was incubated with the fusion protein bound to either glutathione sepharose (GST-2 pulldown) or to Ni-$^{57}$-agarose beads (Ni$^{2+}$-agarose pulldown). 1 ml of binding buffer [PBS supplemented with 250 mM KCl and 0.5% (wt/vol) NP-40] for 1 h at room temperature while tumbling. Equivalent loading of the fusion proteins or eIF4E inputs (wild type and mutants) were verified by Coomassie blue staining. After incubation, beads were washed three times with 1 ml of washing buffer [PBS supplemented with 500 mM KCl and 1% (wt/vol) NP-40]. The presence of eIF4E was determined by Western blotting. In the His-eIF4E pulldown, the carrier protein Mdh with a six-histidine tag was used for the control (42).

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Supporting Information

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Supplemental Methods.

Structure Calculation. Structures were calculated using the CNS protocol (6) starting with an extended molecule. Initial structures were calculated with no zinc ion restraints, in order to identify these residues in an unbiased manner. After the Zn ligands were unequivocally identified, distance and angle constraints were added to maintain the tetrahedral bonding geometry of the two sites as described (7). In the next round, the resulting models were used to identify hydrogen bonds. Eleven were consistently observed. After another round, the structure having the lowest energy and least violations was used as input for the TALOS program (8). TALOS generated 9 additional ϕ dihedral and 30 unique ψ dihedral angle restraints that were used in a final refinement using XPLOR.


Fig. S1. (A) Z directly interacts with eIF4E. GST-pulldown assays were conducted on bacterially purified proteins as described in Materials and Methods and verified by SDS-PAGE and as described (1–4). Soluble eIF4E was incubated with sepharose beads loaded with GST or GST fused to LFV-Z in 0.5 mL of binding buffer [phosphate-buffered saline (PBS) supplemented with 0.25 M KCl and 0.5% (wt/vol) NP-40] for 1 h at room temperature while tumbling. After incubation, beads were washed three times with 1 mL of washing buffer [PBS supplemented with 0.5 M KCl and 1% (wt/vol) NP-40], and the presence of eIF4E was determined by Western blotting using a monoclonal antibody to eIF4E. The eluted fraction (E), the three washes (W1, W2, and W3), the beads (B) and input (I) are shown. I represents 1% of the total input protein. (B) Cap-binding assays. In order to show that Lassa Z derived from PreScission protease constructs (Zpp) was as active as Lassa Z derived from Factor Xa (ZXa) or LCMV Z derived from thrombin cleavage (Zt), we monitored the ability of each of these to abrogate the binding of eIF4E to a m7G cap column. One microgram of eIF4E with or without the different Z proteins was incubated with 30 microliters of m7GDP-sepharose beads in a total volume of 500 microliters of buffer (PBS supplemented with 1 mM DTT and 2 mM MgCl2). After the mixture was incubated on a shaker for 2 h at 4°C, the resin was washed three times with 1 mL of buffer, and aliquots of beads were analyzed on SDS gel followed by Western blotting.
Fig. S2. (A) Z amino acid sequence from LASV (left) and schematic presentation of the cross-braced zinc-binding pattern in Z (right). The β-strands and α-helix are shown as arrows and cylinder, respectively; Cys and His residues as circles and square, respectively. (B) Sequence alignment of Z in different arenaviruses. Zn$^{2+}$-coordinating residues are boxed in light blue and other highly conserved amino acids are shown in yellow. The secondary structure of the protein Z is shown above the aligned sequence (arrows indicate β-strands and cylinder indicate α-helix). The residue numbering of Z is given at the top. Residues undergoing large chemical shift perturbation and/or line broadening are marked with an asterisk below the conservation information (see main text for more details). Abbreviations: Ippy, Ipyp virus; Mop., Mopeia; Mob., Mobala; Pir., Pirital; W.A., Whitewater Arroyo; B.C.; Bear Canyon; All., Allpahuayo; Pich., Pichinde; Oli., Oliveros; Tam., Tamiami; Lat., Latino; Gua., Guanarito; Ama., Amapari; Cup., Cupixi; Sab., Sabia; Jun., Junin; Tac, Tacaribe; Mach., Machupo. (C) Sequence alignment of different RING domains discussed in the main text.
Fig. S3. Schematic view of site I for Z (A, 2KOS), PML (B, 1BOR), Cbl (C, 1FBV), and BRCA1 (D, 1JM7). (E) Sequence alignment around site I of the proteins shown in panels A to D. Residues shown on the different structures were highlighted in blue (positive), red (negative), green (aromatic), and yellow (aliphatic). (F) Superposition of the backbone atoms of Z (red) and PML (yellow). (G,H) Potential map of the surface of Z (G) and PML (H) around site I calculated with Molmol. The electrostatic potential is scaled from −0.5 kcal mol$^{-1}$ (red) to 0.5 kcal mol$^{-1}$ (blue). Z site II has a unique conformation with an extra β-sheet. This appears characteristic of the variation of site II structures in the RING family and also consistent with the fact that site II is not required for association with eIF4E because only site I is required for the ability of either PML or Z to interact with eIF4E. A structural comparison of site I revealed that PML and Z RINGs have a unique conformation relative to other RINGs. Their site I, and especially the small loop delineated by the first pair of cysteines (A,B,F), is oriented differently relative to the helix (or helical turn in PML) than Ubc binding RING domains such as Cbl or BRCA1 (C,D). Interestingly, recognition of Cbl, for example, with the Ubc involves a hydrophobic residue (Ile383) located in this loop. Instead, Z and PML present polar residues at that position (Ser33 and Gln59, respectively). The position of this loop in Ubiquitin ligase RING domains likely allows for the presentation of this hydrophobic residue (Ile). In addition, residues in Z that are in the binding site region are conserved in PML. Particularly, the aromatic residue F30, the positively charged residues K32 and K39, and the negatively charged residues E37 (A,G) are in equivalent positions to residues F54, R56, K65, and E63 in PML RING (B,H). Importantly, the binding interface on eIF4E also contains many charged (K36, H37, E70, D71, H78, E132, E140, D143), aliphatic (I35, L39, V69, Y76, L131, L135, L138), or aromatic residues (W73, Y76) that could interact with these residues on PML or Z. Cross-peaks of most of these residues broadened and/or shifted upon Z addition. Even though sequence alignment of residues in loop 1 for both Z and PML with the same region in Cbl or BRCA1 RINGs revealed similar residues (E), their position in the structure is very different (see F378, K382, E386, and K389 of Cbl in C vs. F30, K32, E37, and K39, respectively, in A) highlighting the different conformations of these two groups of RINGs. In summary, the two eIF4E inhibitors (PML and Z) are highly homologous around site I including the positioning of important side chains, whereas in other RING domains, the conformation of site I and the positions of these side chains differ significantly.

Fig. S4. Z perturbation during titration with eIF4E. Superposition of heteronuclear single quantum correlation (HSQC) NMR spectra collected for Z/eIF4E ratios 1:0 (green), 1:1 (blue), 1:3 (orange), and 1:5 (red). Some of the peaks that undergo larger shifts or broadening upon complex formation are identified.
Fig. S5. Potential map of the surface of Cbl (A, 1FBV) and Z (B) calculated with Molmol (left). For both structures, the surface maps the electrostatic potential on scale from −0.5 kcal mol⁻¹ (red) to 0.5 kcal mol⁻¹ (blue). In A, the black arrow indicates the shallow hydrophobic groove into which the two loops of the Ubc bind (see C). The side chains of residues that were found to contact the Ubc domain are displayed in yellow. In B, the white arrow indicates the position of this groove in Z. Note that this groove is more basic on Z and is not used for binding with eIF4E. Rather, eIF4E binds a different region on Z (displayed as a red arrow). (C) Complex interface between UbcH7 (yellow) and the RING domain of Cbl (brown) (PDB ID code 1FBV). (D) Complex interface between eIF4E (yellow) and Z (cyan) (Haddock-derived model, same as Fig. S7C). In both complexes, the side chains of residues that coordinate the zinc atoms in the ring domains (spheres labeled “I” and “II” for zinc site I and zinc site II, respectively), and the residues at the interface are shown. Note that residues that are represented in the eIF4E/Z complex (D) were mutated and shown to be important to complex formation. The orientation of the two RING domains in C and D are identical and represents a 35° rotation along the vertical axis compared with the structure of Z in Fig. 1A. To visualize the region on Cbl where Ubc binds and to compare with Z, both RING domains in A and B were rotated by 120° compared with C and D.
Fig. S6. eIF4E perturbation during titration with Z. Superposition of HSQC NMR spectra collected for eIF4E/Z ratios 1:0 (blue), 1:1 (red), 1:3 (green), and 1:5 (yellow). Some peaks that undergo larger shifts or broadening upon $^1$H – $^{15}$N complex formation are identified (side chains are boxed).
Docking of the eIF4E/Z complex was performed using the software HADDOCK 5 in combination with CNS based on a combination of NMR titration, transferred cross saturation, and mutagenesis data for both proteins. The starting structures for the docking were the 10 structures deposited in the PDB for the apo-eIF4E (PDB ID code 2GPQ) and for Z (PDB ID code 2K05). A 2.0 Å distance was used to define the ambiguous interaction restraints. Residues 1–40, 67–70, and 138–147 of eIF4E and 1–30, 35–40, and 71–99 of Z were defined as flexible. During the rigid body energy minimization, 200 structures were calculated. The 50 best solutions based on the intermolecular energy were used for the semiflexible simulated annealing followed by a refinement in explicit water. Finally, the solutions were clustered using a 2.5 Å rmsd based on the pairwise backbone rmsd matrix after superposition on the backbone of eIF4E. (A) Active and passive residues are shown on the eIF4E (left) and Z (right) structures. (B) Superposition of the 10 best eIF4E-Z complex models from the lowest-energy Haddock cluster. The rmsd with respect to the mean coordinate positions for backbone atoms of residues 40–217 (eIF4E) plus 30–70 (Z) is 2.0 Å. (C) Representative structure of the lowest-energy structural model of the eIF4E-Z complex. Expansion of the binding interface is shown on the right. In addition to residues W56 and W102 that are important for cap binding, residues that appear to play a role in the complex formation are represented.
Fig. S8.  (A) The far-UV CD spectra of wild-type eIF4E and the different eIF4E mutants used in this study. (B) Loading of the eIF4E wild-type and mutants that were used in the GST and His pulldown assays (see Fig. 4). (C) $^1$H-$^{15}$N HSQC spectra of the Z wild-type and different mutants of Z. Residues mutated are located in loop 1 (see main text for more details).
Fig. S9. Chemical shift differences between apo-eIF4E and the binary eIF4E/4Gpeptide (KKQYDREFLDFQFMPA, from human eIF4GII) or eIF4E/Z complexes. The right panels represent the chemical shift differences for the eight Trp indoles HN. Regions located on the cap (pink) and the dorsal (yellow) surface on eIF4E are shown.
Fig. S10. (A) The line broadening at elf4E/Z ratio of 1:5 is shown on the elf4E backbone (left, cap-binding site; right, dorsal surface) from low (thin) to substantial (wide) broadening. Chemical shift perturbations are also shown as in Fig. 3B, in addition to peaks that exhibit very low intensity (green) and that overlap (purple). An overlay of a region of $^{15}$N-$^1$H HSQC spectra of elf4E showing residues L81 (B) and Y91 (C) without (green) and with (blue) the elf4G peptide (containing the elf4E binding site). In contrast to elf4E/Z complex for which signals of L81 and Y91 broaden, the signal of these cross-peaks become sharper upon addition of the elf4G peptide to elf4E; while many residues, including L62, are not altered significantly. (D) The apo-elf4E structure showing the cap-binding site (left, W56 and W102) and the protein binding site (right, W73). Two residues, L81 and Y91 from the core of the protein, are also shown.
Fig. S11. (Left) Per-residue plot of chemical shift perturbations between eIF4E wild-type and different eIF4E mutants. Backbone and tryptophan side chains are shown. (Right) Chemical shift perturbations of backbone $^{1}H$ and $^{15}N$ resonances are mapped onto the three-dimensional structure of eIF4E (apo form). The protein residues are color-coded according to the extent of chemical shift perturbation from white (no variation) to red (large changes). The mutated residue is shown in green. If Z binding to W130 and/or Y76 is important for communicating its effects to the cap-binding site, one would expect that mutation of these residues might lead to chemical shift perturbation of residues on this side of eIF4E. Analysis of chemical shifts between mutant and wild-type proteins indicate that this is the case. For instance, the Y76A and W130F mutant proteins show perturbation of residues G88, Y91, and N155 relative to wild-type eIF4E. These residues were also preferentially line broadened in the Z bound form of wild-type eIF4E. In addition, chemical shift perturbations are also observed on the W56 loop. Thus, mutation of these residues key to Z binding also lead to perturbation of the cap-binding site. Importantly, we do not observe identical chemical shift perturbations for either mutation or the Z-eIF4E complex. This is expected as mutation of eIF4E and binding of Z to eIF4E are very distinct events. Nonetheless, the effects of these mutations are consistent with a model whereby Z binding on the dorsal surface of eIF4E initiates propagation of a conformational response through eIF4E to the phosphate binding part of the cap-binding site (Fig. 6). The RING of PML or Z can then reduce the affinity of eIF4E for the cap and impair its biological functions.
Fig. S12. Mass spectrum of the protein Z. (A) ES-MS spectra plotting ion abundance as a function of the mass/charge ratio. (B) Hypermass reconstruction of the spectrum. Purified Z protein was extensively dialyzed against 20 mM NH₄OAc buffer (pH 6.5) and then lyophilized. An aliquot of the protein was solubilized in a MS buffer (1:1 acetonitrile/water solution with 0.1% trifluoroacetic acid) to a final concentration of 40 μM. The sample was electrosprayed directly into a PE Sciex API-III triple quadrupole mass spectrometer. Experimental mass was calculated by Hypermass and Hypermass Reconstruct programs within Biomultiview 1.0 (supplied by the manufacturer).
Table S1. Structure statistics of the protein Z

<table>
<thead>
<tr>
<th>Restraints for final structure calculations</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total NOE restraints</td>
<td>483</td>
</tr>
<tr>
<td>Sequential ((i - j) = 1)</td>
<td>183</td>
</tr>
<tr>
<td>Medium range ((1 &lt;</td>
<td>i - j</td>
</tr>
<tr>
<td>Long range ((</td>
<td>i - j</td>
</tr>
<tr>
<td>Hydrogen bond restraints*</td>
<td>22</td>
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<tr>
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<td>39</td>
</tr>
<tr>
<td>(\psi) dihedral angles restraints</td>
<td>30</td>
</tr>
</tbody>
</table>
| Statistics for structure calculations (<SA>)
| RMSD from idealized covalent geometry     |     |
| Bonds (Å)                                 | 0.0036 ± 0.0002 |
| Angles (degrees)                          | 0.814 ± 0.042   |
| Improper (degrees)                        | 0.454 ± 0.024   |
| RMSD from experimental restraints: distances (Å)
| Ramachandran plot statistics (%)<sup>§</sup>|
| Residues in most favored regions          | 76.6 ± 2.6      |
| Residues in additional allowed regions    | 19.9 ± 2.0      |
| Residues in generously allowed regions    | 1.2 ± 0.8       |
| Residues in disallowed regions            | 2.3 ± 0.0       |

*The amide protons implicated in all these hydrogen bonds were found to slowly exchange with D₂O.

<sup>†</sup> <SA> refers to the ensemble of the 10 structures with the lowest energy from 50 calculated structures.

<sup>‡</sup> No distance restraint in any of the structures included in the ensemble was violated by more than 0.4 Å.

<sup>§</sup> Generated using PROCHECK on the ensemble of the 10 lowest-energy structures, residues 30–70. The residues located in the N and C-termini are more flexible.