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ONLINE METHODS

Y2H interaction and modified Y2H. Since native CED-9 is a transmembrane protein, for all Y2H and pull-down experiments we used a protein lacking the C-terminal transmembrane segment (residues 249 to 280), CED-9ΔTM. DB-CED-9ΔTM (wild-type, K207E or W214R) was transformed into yeast cells (MaV203)[6] by standard PEG/LiAc heat shock transformation and checked for auto-activation. AD-CED-4, AD-SPD-5 or F25F8.1 were transformed in yeast cells containing DB-CED-9ΔTM. EGL-1 or CED-4 was expressed as a fusion to the SV40 nuclear localization sequence (NLS) on pMAD16 (this manuscript) a vector that carries a geneticin resistance gene for

transformant selection. Co-transformants were then tested for the expression of *lacZ* and *URA3* reporter genes[32].

Quantitative β-galactosidase assay. MaV203[6] yeast cells cotransformed with the different sets of vectors were grown overnight in selective media (SC-Leu-Trp-Ura + 100 μg/ml geneticin), then diluted in fresh media to an OD_{600nm} of 0.1. Once cultures reached an OD_{600nm} of 0.4-0.5, yeast cells were harvested by centrifugation, then washed in 1ml of ice-cold β-galactosidase assay buffer and lysed by adding 20 μl of chloroform and 30 μl of SDS 10%. β-galactosidase reactions were started by adding 0.8 mg of ONPG (Sigma), then stopped (once a color change was observed) by adding 0.5 ml 1M Na_2CO_3. Reaction times were recorded. Lysates were cleared by centrifugation, then OD_{420nm} was measured and recorded. β-galactosidase units are calculated as $1/OD_{600nm}$ * 1000/time (minutes) * OD_{420nm}. Units were normalized to the wild-type interaction. The Student's *t*-test was used to evaluate the significance of the difference of EGL-1 effect on CED-9(wild-type)/CED-4, CED-9(G169E)/CED-4, CED-9(wild-type)/SPD-5 and CED-9(wild-type)/F25F8.1 comparing the β-galactosidase activity ratio in absence/presence of EGL-1.

Generation of *ced-9* alleles. The *ced-9*ΔTM ORF was mutagenized over 30 cycles of PCR using Platinum *Taq* HiFi DNA polymerase (Invitrogen). PCR products were cloned by Gateway reaction into pDONR-Express[8]. Full-length *ced-9*ΔTM clones were selected by plating *E. coli* on selective media containing kanamycin and IPTG, generating about

5 x 10^5 transformants. All clones were scraped from plates, plasmids were isolated, and their products transferred by Gateway reaction into pDEST-DB vector.

Isolation of *ced-9* edgetic alleles insensitive to EGL-1. Ten micrograms of the CED-9ΔTM mutant library were transformed by standard PEG/LiAc heat shock transformation into yeast cells (MaV203)[6] containing AD-CED-4 and EGL-1 fused to the SV40 nuclear localization sequence (NLS). Co-transformants were selected on selective media (SC-Leu-Trp-Ura + 100 μg/ml geneticin), then tested for the expression of *lacZ* and *URA3* reporter genes[32]. *ced-9* ORFs were PCR-amplified directly from yeast colonies, sequenced, then re-introduced by gap repair in fresh yeast cells containing AD-CED-4 and EGL-1, then retested. DB-CED-9 proteins recovered from the screen were also tested for auto-activation.

Yeast two-hybrid screening. DB-CED-9ΔTM was used as bait in Y2H screens against two libraries: a mixed stage cDNA library (AD-cDNA library)[33], and a normalized ORFeome library (AD-ORFeome library)[34]. Co-transformants were plated on selective media (SC-Leu-Trp-His +20mM 3AT). AD-Y interactors were PCR-amplified directly from yeast colonies, then sequenced. From the cDNA library screen, a fragment of SPD-5 interacted with CED-9 (370 C-terminal amino acids). This fragment was used in all Y2H experiments.

Reverse Y2H selections. To carry out R-Y2H, MaV203 yeast cells were transformed with one of the AD fusion proteins and the DB-CED-9 library, and then plated on

selective media (SC-Leu-Trp + 0.2% 5-FOA). An average of 3-5 million yeast transformants were obtained for each R-Y2H screen. 5-FOA resistant colonies were picked and streaked onto selective media (SC-Leu-Trp-His + 20mM 3AT) and for β-galactosidase assays. R-Y2H alleles were PCR-amplified directly from yeast cells and PCR products were sequenced. Forward and reverse traces were aligned with wild-type *ced-9* ORF using Seqman (DNA Star package). Only single non-synonymous missense mutants were kept for further analysis. All other changes were discarded. In parallel, interactions were retested against all partners (CED-4, SPD-5 and F25F8.1)[32] and attributed a score from 0 (-) to 3 (+++), from loss-of-interaction to wild-type interaction.

Analysis of CED-9 mutants by western blots. Glutathione-S-transferase (GST) tagged CED-9ΔTM mutants were transiently transfected into HEK-293T cells. Lysates were separated on Nu-PAGE acrylamide gels (Invitrogen). Following transfer to PVDF membranes, GST-tagged proteins were detected with a rabbit anti-GST polyclonal antibody (Sigma). Protein sample loading was controlled by probing membranes with a mouse anti-α-tubulin antibody (Sigma).

Validating interactions by co-affinity purification (co-AP). Glutathione-S-transferase (GST) tagged CED-9ΔTM and Myc-tagged full-length partners[35] were transiently transfected into human HEK-293T cells. Cleared lysates were incubated with glutathione-sepharose beads (Amersham Biosciences). Purified complexes and control lysate samples were separated on Nu-PAGE acrylamide gels (Invitrogen). Following transfer to PVDF membranes, Myc and GST-tagged proteins were detected with a

mouse anti-Myc monoclonal antibody (clone 9E10) and a rabbit anti-GST polyclonal antibody (Sigma). Co-AP results were scored from 0 (-) to 3 (+++), from complete loss-of-interaction to wild-type.

CED-9 structures. Three CED-9 X-ray crystallographic structures have been solved to date: CED-9 alone (PDB ID code 1OHU)[19], CED-9 in complex with the EGL-1 BH3 peptide (PDB ID code 1TY4)[18], and CED-9 in complex with a CED-4 dimer (PDB ID code 2A5Y)[16]. Since these structures differ in the amino acids missing at the N- and C-termini, amino acids that are not present in all three structures were discarded to allow comparison between these structures. The common region used extends from E75 to R237. The structures were fitted to the wild-type CED-9 sequence. For the CED-9 and the CED-9/EGL-1 structures, selenomethionine were replaced by methionine. For the CED-9 and the CED-9/CED-4 structures, serine residues at positions 107, 135, and 164 were replaced by the original cysteine residues. The CED-9/EGL-1 structure contains a leucine to proline substitution at position 148. This proline residue was replaced by a leucine residue using Modeller[36]. Modeller was also used to add the missing T161 and D162 residues in the CED-9/CED-4 structure, and missing side chains. All tertiary structures were optimized with HyperChem™ (release 6.1 for Windows - Hypercube) by a conjugated gradient procedure using the AMBER96 force field until reaching an RMS gradient lower than 0.01 kcal/(Å mol). Figures of tertiary structures were generated with PyMol (http://www.pymol.org).

Analysis of surface exposure of edgetic/non-edgetic residues. The relative solvent-accessible surface areas (%ASAs) were calculated for the three CED-9 structures as before[37]. Residues were called accessible if their %ASA was above 10% in at least one of the three structures, or buried if below this threshold in all three structures. This criterion was chosen to take into account variations between the three available CED-9 structures. The analysis was then repeated using ASA cutoffs of 20 and 30%.

For the same reason, statistical tests were carried out for each residue using the maximal %ASA observed in the three CED-9 structures. The maximal %ASA values of the 19 partner-specific edgetic residues were averaged and compared to the value obtained with 1,000,000 sets of 19 residues picked at random in CED-9. Picking the same residue several times for the same set was prohibited because edgetic alleles isolated several times were only counted once. Similar tests were done with random sets of 16 and 23 residues for the edgetic alleles defective for two interactions, and for the non-edgetic alleles, respectively. The observed average maximal %ASA was then compared to the distribution of the average of the random sets to determine the statistical significance by empirical *P*-value. Randomizations done with the three structures independently or with the average %ASA of the three structures gave similar results.

W73 (SPD-5-specific edgetic allele) and E241 (non-edgetic allele) residues were not included in the ASA analysis because they are not present in all three structures. When alleles were mutated at the same position but with distinct substitutions having different interaction profiles (*e.g.*, CED-9(G82E) and CED-9(G82R), which are defective for SPD-

5 only and for both CED-4 and SPD-5, respectively), the corresponding residue was included in both sets (one or two-interaction defective edgetic alleles in our example).

Analysis of CED-9/CED-4 interactions in the co-crystal structure. The CED-9/CED-4 interface was analyzed on the co-crystal structure after the addition of hydrogen atoms with PyMol. The non-edgetic E241 residue was included since it is present in the tertiary structure. When alleles were mutated at the same position but with distinct substitutions having different interaction profiles (*e.g.* CED-9(P106Q) and CED-9(P106R), which are defective for CED-4 only and for both CED-4 and SPD-5, respectively), the corresponding residue was included in both sets (one or two-interaction defective edgetic alleles in our example).

CED-9 residues that are at the CED-9/CED-4 interface were detected using a distance cutoff of 4.0 Å. At this cutoff 41 of 175 CED-9 residues are in contact with CED-4. *P*-values were calculated by the hypergeometric test. We calculated the probability of having 6/6, at least 7/14, or maximum 1/24 residues at the interface, for the set of residues defective for CED-4 only, for CED-4 and another partner, and for all three partners, respectively.

For each residue of CED-9, the minimal distance to CED-4 was calculated as the distance between the two closest atoms. For each set of residues (residues defective for CED-4 only, for CED-4 and another partner, and for all three partners), the average distance to CED-4 was calculated and compared to the average obtained for 1,000,000 sets of 6, 14 or 24 residues, respectively, picked at random in CED-9. Picking the same residue several times for the same set was prohibited. Empirical *P*-values were

calculated via the comparison of the observed average to the distribution of the random sets.

***C. elegans* strains.** Methods for culturing *C. elegans* were described by Brenner[38]. All strains were grown at 20°C, maintained on NGM media on op50 bacteria, HB101 bacteria for growth in liquid culture or on HT115 bacteria for RNAi experiments. The following worm strains were used: Bristol strain N2, MT5523 [*unc-69(e587) ced-9(n1950n2161)*/qCi *dpy-19(e1259) glp-1(q339)*III]; MT1522 [*ced-3(n717)*IV]; DP38 [*unc-119(ed3)*III]; wild-type transgene, CED-9(K207E), CED-9(W214R).

Generation of transgenic *C. elegans* strains. A 4 kb genomic DNA fragment containing the *ced-9* operon (consisting of *cyt-1* and *ced-9*) and 400 bp of upstream and 600 bp of downstream flanking sequences was cloned from N2 genomic DNA, then cloned by Gateway reaction into pDONR223. Mutations were introduced in the 4 kb genomic DNA fragment with the Gene Tailor site-directed mutagenesis (SDM) kit (Invitrogen). Mismatched primers were designed according to the manufacturer's protocol. The full-length *ced-9* gene was sequenced from the SDM products to ensure that only the intended mutation was present and that the sequence was otherwise wild-type. The correct SDM product was cloned by Gateway reaction into pID2.02. Transgenic lines were generated by microparticle bombardment[39]. Homozygous transgenic worms were crossed into MT5523. Homozygous transgenic animals carrying the transgene were confirmed by PCR-amplification of *ced-9* and flanking vector specific sequences. The presence of the closely linked *unc-69*(*e587*) marker was used

to assess the presence of *ced-9*(*n1950n2161*). Genotypes of all crosses were confirmed by sequencing.

MT5523 [*unc-69(e587) ced-9(n1950n2161)*] was used as a negative control to compare the magnitude of the survival rate in transgenic worm strains. Number of embryos laid and survival rate were measured as before[40]. P0 animals that died within the first two days of the experiment were not analyzed. To evaluate the significance of both average number of embryos laid and the corresponding survival rate, we calculated the significance of differences compared to *ced-9* null allele worms ([*ced-9(n1950n2161)*] expressing wild-type *ced-9*, using a Student *t*-test for the average number of embryos laid and a binomial distribution test for the corresponding survival rate.

RNAi clones of *ced-4* and *cpb*-3 came from the *C. elegans* ORFeome-RNAi v1.1 library[41]. Since a *ced-9* RNAi clone was unavailable, this clone was transferred from an Entry clone (*C. elegans* ORFeome v1.0 library)[34] into pL4440-DEST-RNAi. RNAi on plates was carried out as described[42]. RNAi in liquid media was essentially performed as described[43]. Apoptotic germ cell corpses were identified and quantified based on their characteristic morphology under differential interference contrast (DIC) microscopy, or using SYTO-12 (Molecular Probes), as described[44].

Supplementary File	Title
Supplementary Figure 1	Summary of *ced-9* alleles described in the literature
Supplementary Figure 2	Co-affinity purification of CED-9 with its interaction-partners
Supplementary Figure 3	Interaction profiling of CED-4-defective alleles
Supplementary Figure 4	Interaction profiling of SPD-5-defective alleles
Supplementary Figure 5	Identification of F25F8.1-defective alleles
Supplementary Figure 6	Confirmation of edgetic alleles interaction-profile by Co-AP

Supplementary Figure 7	Solvent-accessibility of edgetic and non-edgetic residues versus random sets
Supplementary Figure 8	Western blots of CED-9 edgetic and non-edgetic mutants
Supplementary Figure 9	Y2H phenotypes of interactions between CED-9/SPD-5 and CED-9/F25F8.1 in absence or presence of EGL-1
Supplementary Figure 10	Y2H phenotypes of interaction between CED-9/SPD-5 in absence or presence of CED-4
Supplementary Table 1	Mutations identified in a random set of 100 clones from the CED-9ΔTM library
Supplementary Table 2	Alleles identified in the R-Y2H selection screen against CED-4
Supplementary Table 3	Alleles identified in the R-Y2H selection screen against SPD-5
Supplementary Table 4	Alleles identified in the Y2H screen against F25F8.1
Supplementary Tables 2-4	Excel worksheet with all alleles in Supplementary Tables 2-4
Supplementary Table 5	List of single amino acid change alleles
Supplementary Data 1	Isolation of *ced-9* edgetic alleles insensitive to EGL-1
Supplementary Data 2	Isolation of additional *ced-9* edgetic alleles
Supplementary Data 3	Structural analysis of edgetic and non-edgetic residues
Supplementary Data 4	SPD-5 and F25F8.1 binding sites on the CED-9 structure

Editorial Summaries:

AOP: A combination of forward and reverse two hybrid screening allows systematic identification of ‘edgetic’ or edge-specific alleles, which encode proteins that have lost a single physical interaction but for which other interactions remain unperturbed.

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