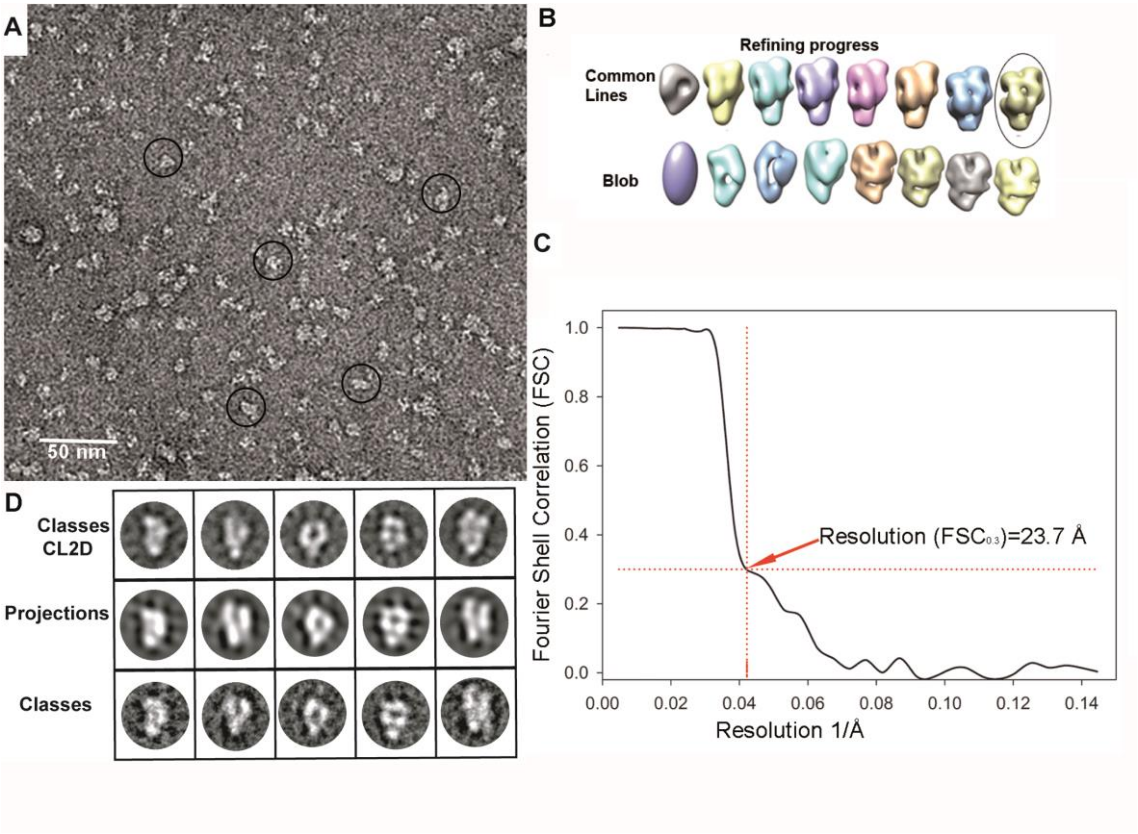
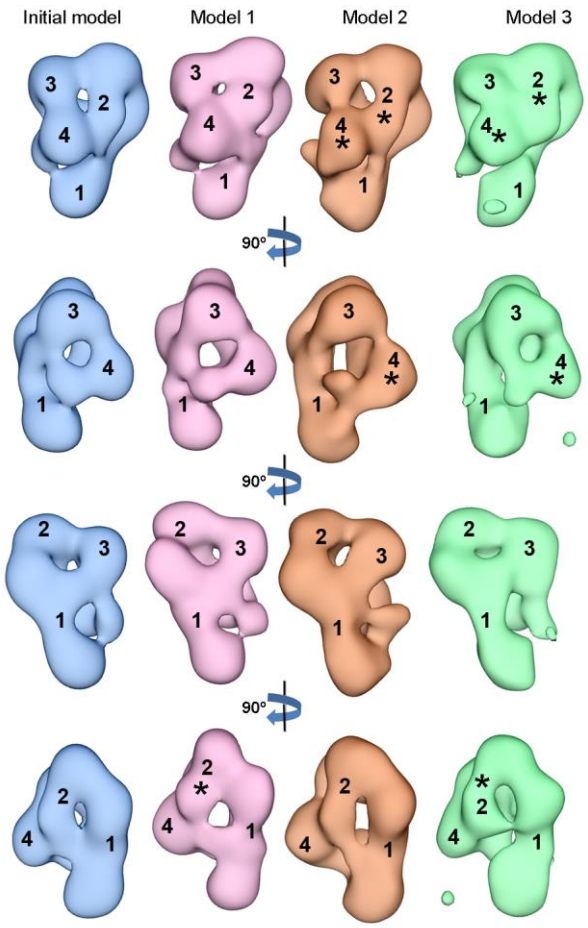
Supplementary Figures

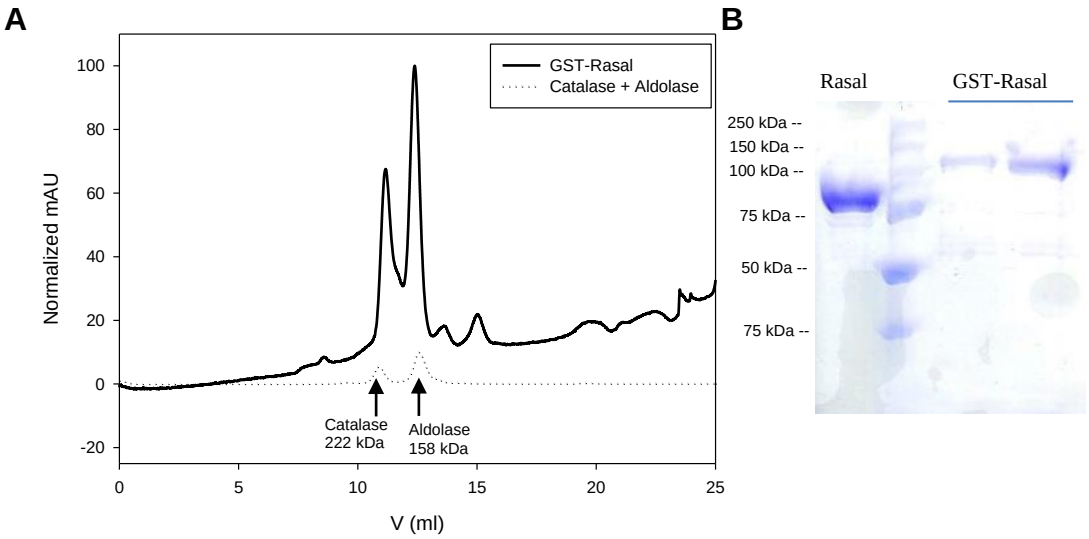


Supplementary Figure S1. Negative staining EM analysis of Rasal

(A) Example of a negative staining EM image of Rasal. Selected individual particles are circled. Bar, 50 nm. (B) Representation of the iterative angular refinement for 3D reconstruction of Rasal. The refinement was performed simultaneously using EMAN, starting from two initial models, blob and common lines. The final model used for subsequent refinement by Projection Matching is circled. (C) Computed FSC curve for the Rasal Projection Matching final reconstruction. Resolution was estimated to be 23 Å based on the 0.3 criterion. (D) The 3D reconstruction quality can be assessed by comparison of the reference-free 2D classes (CL2D, top row) with the 3D map projections and the generated class averages from EMAN (center and bottom, respectively), based on the 3D reconstruction of Rasal.

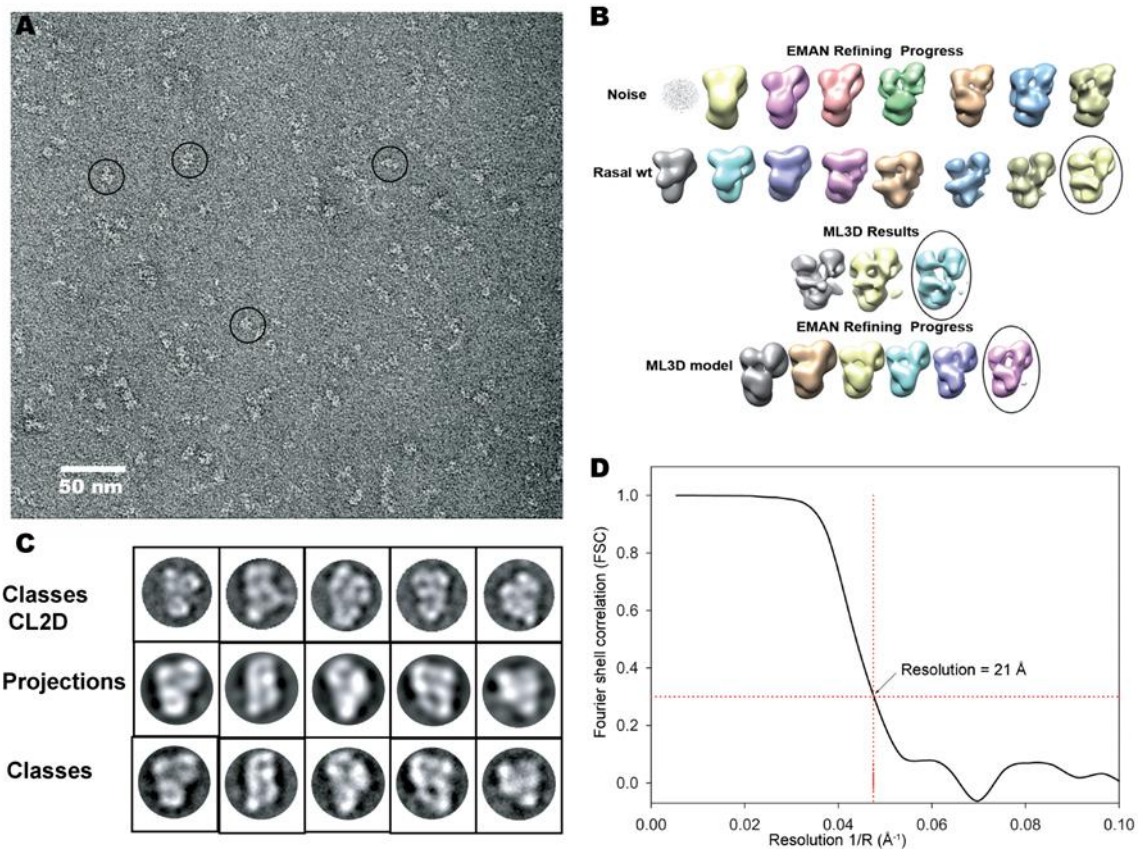


Supplementary Figure S2. Rasal flexibility. Four orthogonal views (vertical) of the Rasal 3D reconstruction obtained with EMAN (initial model), and the three volumes obtained from the 3D classification procedure (ML3D). From the whole set of particles, 40% are assigned to Model 1, 25% to model 2 and 35% to model 3. Asterisks indicate the regions with the highest conformational flexibility.



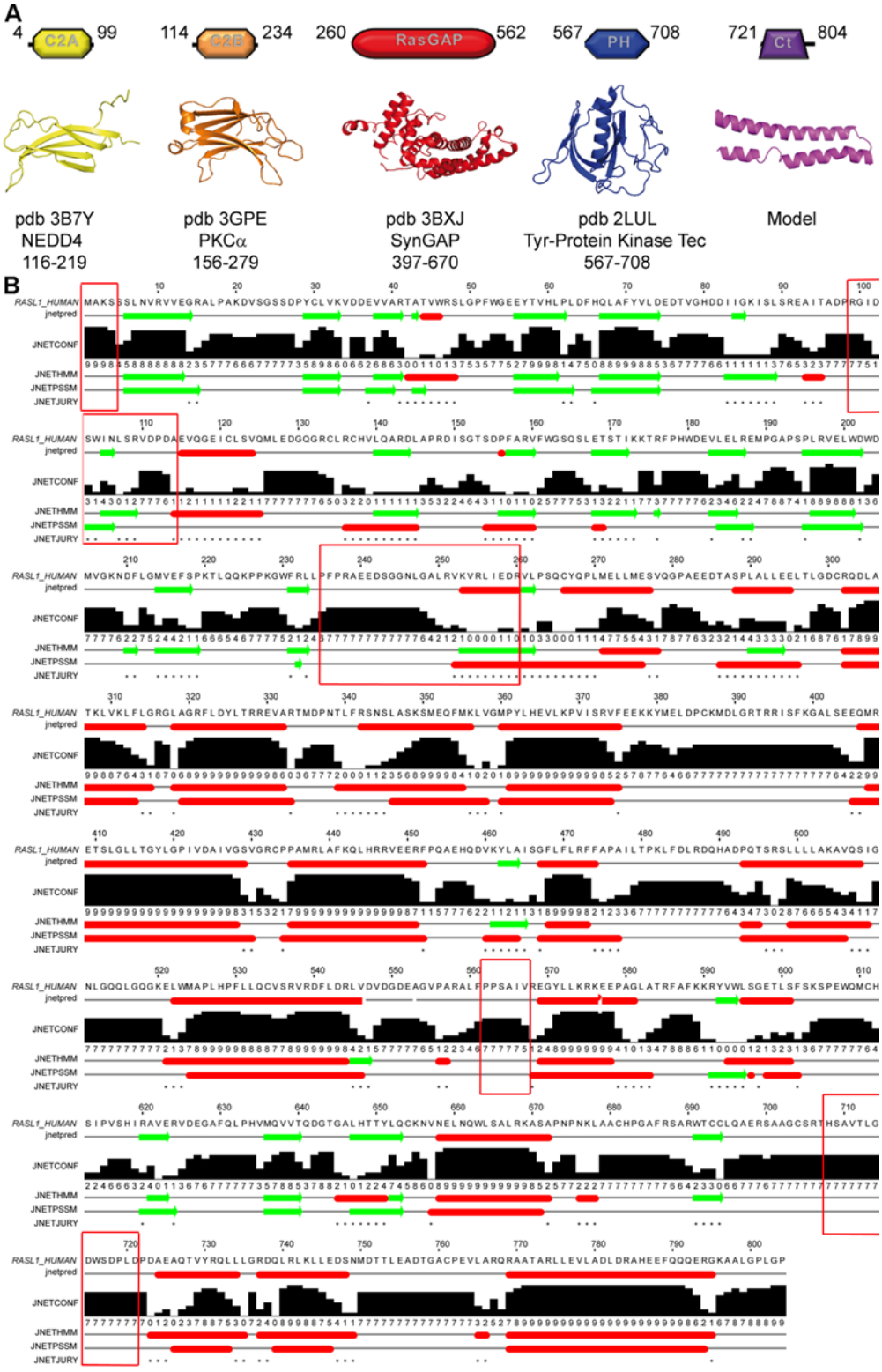
Supplementary Figure S3. GST-Rasal characterization

(A) Size exclusion chromatography, using Superdex-200 10/300 GL, of the GST-Rasal sample. Catalase and aldolase were used as molecular weight standards. GST-Rasal eluted in two peaks, corresponding to dimer (240 kDa) and monomer (120 kDa). (B) Two fractions of eluted GST-Rasal were resolved by SDS-PAGE. Rasal and molecular weight markers are shown.



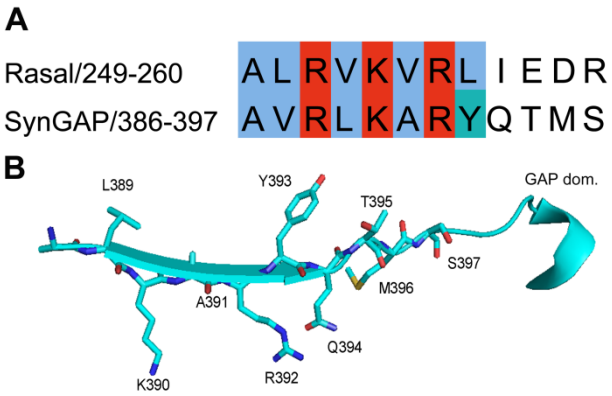
Supplementary Figure S4. Negative staining EM analysis of GST-Rasal

(A) Example of a negative staining EM image of GST-Rasal. Selected individual particles are circled. Bar 50 nm. (B) Representation of the iterative angular refinement for 3D reconstruction of GST-Rasal. The refinement was performed simultaneously using EMAN, starting from two initial models, noise and low resolution-filtered Rasal. The final model using Rasal as initial model (circled) was used as an initial model for ML3D classification. The most consistent volume obtained in ML3D (circled) was filtered and refined using EMAN. The final model is circled. (C) The 3D reconstruction quality can be assessed by comparison of the reference-free 2D classes (CL2D, top row) with the 3D map projections and the generated class averages from EMAN (center and bottom, respectively), based on the 3D reconstruction of GST-Rasal. (D) Computed FSC curve for the GST-Rasal final volume. Resolution was estimated to be 21 Å based on the 0.3 criterion.



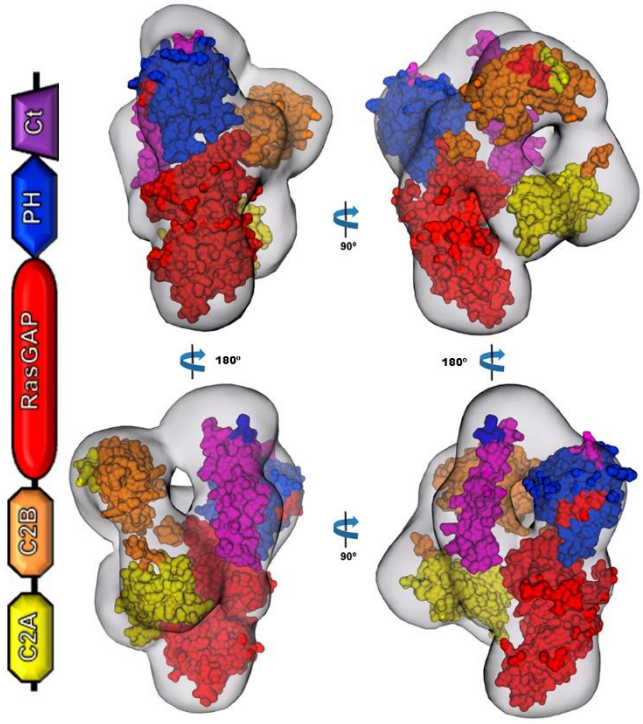
Supplementary Figure S5. Rasal sequence homology and structure prediction

(A) 3D structures of homologous domains used for docking into the Rasal and GST-Rasal 3D reconstructions. (B) JNET secondary structure prediction for Rasal. Links between domains, not present in 3D structures, are labeled with red rectangles.



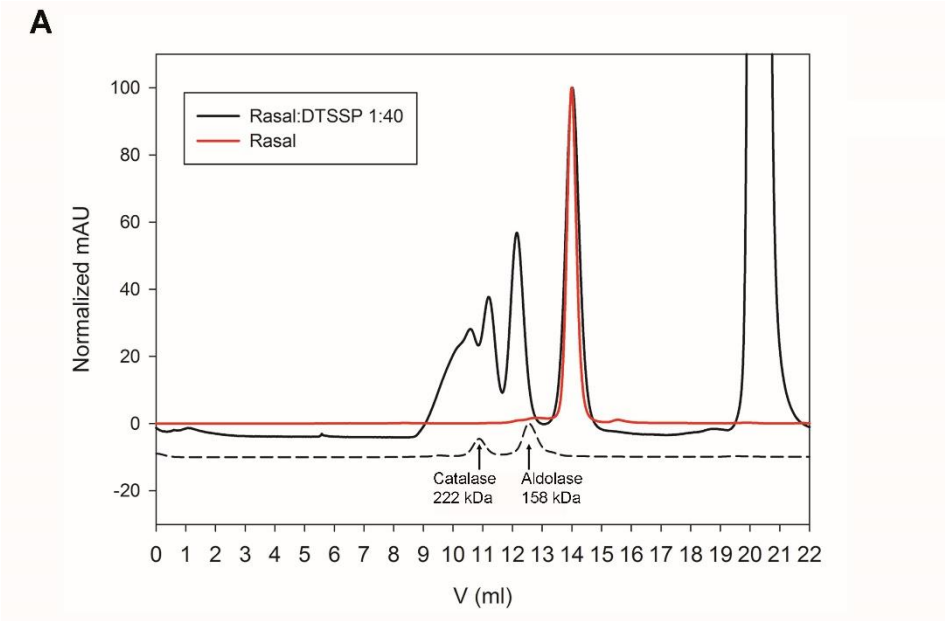
Supplementary Figure S6. Rasal and SynGAP sequence alignment

(A) Sequence alignment of Rasal and SynGAP residues preceding the GAP domain, using the Clustal X color scheme. (B) SynGAP x-ray structure of this region. The residues with high homology correspond to a β-sheet preceding the GAP domain.



Supplementary Figure S7. Rasal domain topology

Four views of homologous 3D structures of the distinct domains and the Ct 3D model, represented as surface models, docked into the Rasal 3D reconstruction.



Supplementary Figure S8. Size exclusion chromatography of Rasal and DTSSP-crosslinked Rasal (1:40 Rasal:DTSSP molar ratio). Elution profiles of catalase and aldolase are shown as molecular weight standards. Non-crosslinked Rasal eluted as a single peak at a size <158 kDa, in accordance with its monomeric structure. Crosslinked Rasal eluted in 3 peaks, a main peak that overlapped with non-crosslinked Rasal, and two high molecular weight peaks possibly due to non-specific DTSSP-driven oligomerization or stabilization of unstable oligomers. Fractions of the peak corresponding to the monomer were digested and analyzed by mass spectrometry.

Supplementary Table S1. Detailed information of crosslinked peptides identified at false discovery rate (FDR) = 0.0. Nineteen Inter-domain crosslinked peptides; twelve intra-domain crosslinked peptides. Five dimeric crosslinked peptides.

Score	m/z	M+H+	Peptide 1	From	To	Peptide2	From	To	Domains	Res1	Res2	FDR
199	487.75516	1947.99881	[SSSLNVR]	4	10	[LAFKQLHR]	439	446	C2A-GAP	4	442	0
185	479.58363	1436.73634	[VKVR]	252	255	[SSSLNVR]	4	10	C2A-GAP	253	4	0
198	908.45861	1815.90994	[SSSLNVR]	4	10	[GKAALGPLGP}	795	805	C2A-Ct	4	796	0
142	605.30368	1813.89649	[SSSLNVR]	4	10	[EGYLLKR]	569	575	C2A-PH	4	574	0
183	597.06425	2385.23517	[VKVR]	252	255	[VFWGSQSLETSTIKK]	160	174	C2B-GAP	253	173	0
167	540.79546	2160.16001	[VKVR]	252	255	[TLQQKPPKGWFR]	221	232	C2B-GAP	253	228	0
160	500.86548	2500.29829	[VKVR]	252	255	[KTRFPHWDEVLELR]	174	187	C2B-GAP	253	174	0
147	718.1457	3586.69939	[SNSLASKSMEQFMK]	343	356	[KTRFPHWDEVLELR]	174	187	C2B-GAP	348	174	0
196	565.04586	2257.16161	[LAFKQLHR]	439	446	[KEEPAGLATR]	576	585	GAP-PH	442	576	0
189	437.23119	1745.90293	[VKVR]	252	255	[KEEPAGLATR]	576	585	GAP-PH	253	576	0
148	400.9629	1600.82977	[VKVR]	252	255	[KASAPNPNK]	669	677	GAP-PH	253	669	0
145	639.94179	3195.67984	[KEEPAGLATR]	576	585	[LVGMPYLHEVLKPVISR]	357	373	GAP-PH	576	368	0
143	708.83436	2832.31561	[KEEPAGLATR]	576	585	[SNSLASKSMEQFMK]	343	356	GAP-PH	576	348	0
139	516.77974	2064.09713	[EGYLLKR]	569	575	[LAFKQLHR]	439	446	GAP-PH	574	442	0
184	518.95564	1554.85237	[VKVR]	252	255	[GKAALGPLGP}	795	805	GAP -Ct	253	796	0
167	881.08849	2641.25092	[GKAALGPLGP}	795	805	[SNSLASKSMEQFMK]	343	356	GAP -Ct	796	348	0
147	751.91196	3004.62601	[GKAALGPLGP}	795	805	[LVGMPYLHEVLKPVISR]	357	373	GAP -Ct	796	368	0
196	709.03227	2125.08226	[GKAALGPLGP}	795	805	[KEEPAGLATR]	576	585	PH-Ct	796	576	0
135	644.67505	1932.0106	[EGYLLKR]	569	575	[GKAALGPLGP}	795	805	PH-Ct	574	796	0
221	579.7899	2316.13777	[KEEPAGLATR]	576	585	[KEEPAGLATR]	576	585	Dimer PH-PH	576	576	0
201	837.8754	3348.47977	[SNSLASKSMEQFMK]	343	356	[SNSLASKSMEQFMK]	343	356	Dimer GAP-GAP	349	349	0
188	440.44456	2198.19369	[LAFKQLHR]	439	446	[LAFKQLHR]	439	446	Dimer GAP-GAP	442	442	0
151	967.51677	1934.02626	[GKAALGPLGP}	795	805	[GKAALGPLGP}	795	805	Dimer Ct-Ct	796	796	0
144	392.56374	1175.67667	[VKVR]	252	255	[VKVR]	252	255	Dimer GAP-GAP	253	253	0
200	723.21669	4334.26376	[LAFKQLHR]	439	446	[RVEERFPQAEHQDVKYLAISGFLFLR]	447	472	GAP-GAP	442	461	0
197	568.2943	2837.44239	[LAFKQLHR]	439	446	[RISFKGALSEEQMR]	395	408	GAP-GAP	442	399	0

187	422.48877	1686.93325	[VKVR]	252	255	[LAFKQLHR]	439	446	GAP-GAP	253	442	0
184	582.30363	2326.19269	[VKVR]	252	255	[RISFKGALSEEQMR]	395	408	GAP-GAP	253	399	0
181	587.72625	2934.60214	[LAFKQLHR]	439	446	[FFAPAILTPKLFDLR]	473	487	GAP-GAP	442	482	0
168	525.89605	2625.45114	[VKVR]	252	255	[LVGMPYLHEVLKPVISR]	357	373	GAP-GAP	253	368	0
164	523.62487	3136.71284	[LAFKQLHR]	439	446	[LVGMPYLHEVLKPVISR]	357	373	GAP-GAP	442	368	0
160	743.17626	3711.85219	[SNSLASKSMEQFMK]	343	356	[LVGMPYLHEVLKPVISR]	357	373	GAP-GAP	348	368	0
160	679.19007	4070.10404	[LAFKQLHR]	439	446	[FFAPAILTPKLFDLRDQHADPQTSR]	473	497	GAP-GAP	442	482	0
152	593.98086	3558.84878	[VKVR]	252	255	[FFAPAILTPKLFDLRDQHADPQTSR]	473	497	GAP-GAP	253	482	0
143	566.27547	2262.08005	[VKVR]	252	255	[SNSLASKSMEQFMK]	343	356	GAP-GAP	253	349	0
136	724.778	3619.86089	[ISFKGALSEEQMR]	396	408	[LVGMPYLHEVLKPVISR]	357	373	GAP-GAP	297	368	0

Supplementary Methods

DTSSP crosslinking assays

Rasal at 40 μM was incubated with 1.6 mM DTSSP (Thermo Scientific) for 30 min. Then, 50 mM Tris was added to stop the crosslinking reaction. The crosslinked Rasal was purified by SEC using Superdex 200 10/300 increase resin. Fractions of the monomer peak were pooled for the XL-MS experiments.

Trypsin digestion, LC-MS/MS analysis and peptide identification

Purified crosslinked Rasal was precipitated using the methanol/chloroform method and the protein pellet was dissolved in 10 μl 8 M urea, 25 mM ammonium bicarbonate. After vigorous vortexing, the urea concentration was reduced to 2 M by addition of 30 μl of 25 mM ammonium bicarbonate, followed by 100 ng trypsin (proteomics grade; Sigma-Aldrich); digestion was allowed to proceed at 37 °C for 5 h. The resulting peptide mixture was speed-vac dried and redissolved in 0.1% formic acid.

For LC-MS/MS analysis, we used a nano-LC Ultra HPLC (Eksigent) coupled online with a 5600 triple TOF mass spectrometer through a nanospray III ion source (both from AB Sciex) equipped with a fused silica PicoTip emitter (10 μm × 12 cm; New Objective). The HPLC setup included an Acclaim PepMap 100 trapping column (100 μm × 2 cm, 5 μm particle size; Thermo Scientific) and an Acquity UPLC BEH C18 column (75 μm × 150 mm, 1.7 μm particle size; Waters). Solvents A and B were 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. Peptides were fractionated at a 250 nl/min flow rate at 40 °C in gradient elution conditions consisting of 2% solvent B for 1 min, a linear increase to 30% B in 109 min, a linear increase to 40% B in 10 min, a linear increase to 90% B in 5 min and 90% B for

5 min. The ion source was operated in positive ionization mode at 150 °C with a potential difference of 2300 V. Each acquisition cycle included a survey scan (350–1250 m/z) of 250 ms and a maximum of 25 MS2 spectra (100–1500 m/z).

For peptide identification, PeakView 1.2 (AB Sciex) was used to convert raw MS/MS data to an mgf file that was searched against a custom-made database containing the sequence of human Rasal. The MS/MS ion search was performed using StavroX 3.5.1 with the following settings: trypsin as enzyme allowing 2 and 3 missed cleavages for Arg and Lys residues, respectively, oxidation of Met as variable modification, DSP/DTSSP as crosslinker, and MS and MS/MS tolerances of 20 ppm. Peptide identifications were filtered at a FDR=0, corresponding to a score ≥135.