Supporting Information

A comprehensive analysis of RAS-effector interactions reveals interaction hotspots and new binding partners

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 Table S1. Human proteins containing RAS association (RA) domain

No.	Entry	Protein name
1	Q9NS23	RASSF1, NORE2, PDA32
2	P50749	RASSF2, CENP34, RASFADIN
3	Q86WH2	RASSF3
4	Q9H2L5	RASSF4
5	Q8WWW0	RASSF5, RAPL, NORE1
6	Q6ZTQ3	RASSF6
7	Q02833	RASSF7, HRC1
8	Q8NHQ8	RASSF8, HOJ1
9	O75901	RASSF9, PCIP1, PAMCI
10	A6NK89	RASSF10
11	P55196	AF6, AFDN, MLLT4
12	Q12967	RALGDS, RALGEF, RGF, RGDS
13	O15211	RALGDSL2, RAB2L
14	Q9NZL6	RGL1
15	Q9BSI0	RGL2
16	Q3MIN7	RGL3
17	Q9Y4G8	PDZGEF1, RAPGEF2, RAGEF1
18	Q8TEU7	PDZGEF2, RAPGEF6, RAGEF2
19	Q9P212	PLCε1, PPLC, NPHS3
20	Q13671	RIN1, JC99
21	Q8WYP3	RIN2, JC265
22	Q8TB24	RIN3
23	Q5U651	RAIN, RASIP1
24	Q7Z5R6	RIAM, APBB1IP, PREL1, RARP1
25	Q96JH8	RADIL, RASIP2
26	Q14451	GRB7, B47
27	Q13322	GRB10, GRB-IR, Meg1, RSS
28	Q14449	GRB14
29	Q15036	SNX17
30	Q96L92	SNX27
31	Q70E73	RAPH1, PREL2
32	P52824	DGKQ
33	Q96P48	ARAP1
34	Q8WZ64	ARAP2
35	Q8WWN8	ARAP3
36	B2RTY4	MYO9A
37	Q13459	MYO9B
38	Q9HD67	MYO10
39	Q9P2F6	ARHGAP20

Table S	2. Human protein	s containing RAS binding (RB) domain
No.	Entry	Protein name
1	P10398	ARAF, RAFA1, PKS
2	P15056	BRAF, NS7, p94
3	P04049	CRAF, CMD1NN, NS5
4	P42336	PI3Kα, p110α, CLAPO, CLOVE
5	P42338	ΡΙ3Κβ, p110β
6	P48736	ΡΙ3Κγ, ß110γ, ΡΙΚ3
7	O00329	ΡΙ3Κδ, p110δ
8	O00443	PI3KC2A, PI3KC2 α
9	O00750	ΡΙ3ΚC2Β, ΡΙ3ΚC2β
10	O75747	PI3KC2G, PI3KC2 _γ
11	O14924	RGS12
12	O43566	RGS14
13	Q13009	TIAM1
14	Q8IVF5	TIAM2, STEF

Table S2. Human proteins containing RAS binding (RB) domain

No.	Entry	Protein name	Reference
1	Q8IZJ4	RGL4	[1]
2	O95398	RAPGEF3, Epac1	[1, 2]
3	Q8WZA2	RAPGEF4, Epac2	[1, 3]
4	Q92565	RAPGEF5, Repac	[1]
5	O00522	KRIT1, Krit	[1, 4]
6	P19367	HK1	[5]
7	Q9BPZ7	SIN1, MAPKAP1	[6]
8	Q9BYB0	SHANK3	[7, 8]
9	Q9UPX8	SHANK2	[8]
10	Q8N9S9	SNX31	[9]
11	Q75LH2	FLJ10324	[10]

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No.	Entry	Protein name
1	P01112-1	HRAS1, p21HRAS
2	P01112-2	HRAS2, p19HRAS
3	P01111	NRAS
4	P01116-1	KRAS4A
5	P01116-2	KRAS4B, RASK2
6	Q7Z444	ERAS, KRAS2, HRASP
7	P11233	RALA
8	P11234	RALB
9	P10301	RRAS, RRAS1
10	P62070	RRAS2 TC21
11	O14807	RRAS3, MRAS
12	Q92963	RIT1, RIT, RIBB, ROC1
13	Q99578	RIT2, RIN, ROC2
14	P62834	RAP1A, KREV1
15	P61224	RAP1B
16	P61225	RAP2B
17	P10114	RAP2A
18	Q9Y3L5	RAP2C
19	Q15382	RHEB1
20	Q8TAI7	RHEB2
21	Q9Y272	RASD1, AGS1, DEXRAS1
22	Q96D21	RASD2, RHES, TEM2
23	O95057	DIRAS1, RIG, GBTS1
24	Q96HU8	DIRAS2
25	O95661	DIRAS3, ARHI, NOEY2, RHOI

Table S4. Human proteins containing RAS-related GTP-binding domain

	HRAS	RRAS1	RAP1B	RAP2A	RALA	RHEB	RIT1
RASSF1	52±10	33±4	26±2	22±2	18±3	37±3	136±26
RASSF2	147±26	122± 14	67±9	47±5	167±20	44±7	n.b.
RASSF3	500±164	435±61	116±26	100±18	139±15	64±9	n.b.
RASSF4	193±31	n.b.	101±21	88±10	191±46	47±7	58±9
RASSF5	1.0±0.1	56±4	4.0±1	2.0±0.2	49±10	46±7	n.b.
RASSF6	91±13	112±20	65±18	53±19	n.b.	56±13	98±27
RASSF7	140±67	30±5	72±13	68±9	101±30	76±16	34±3
RASSF8	n.b.	114 ±13	66±15	67±21	115±9	102±21	76±7
RASSF9	179±61	n.b.	74±18	66±15	n.b.	143±38	27±4
RASSF10	n.b.	99±10	73±6	67±16	n.b.	150±44	55±11
CRAF	0.3±0.1	3.3±1	30±7	n.b.	n.b.	35±9	139±40

 Table S5. Dissociation constants determined for the RAS-effector interactions.

Dissociation constants (K_d values \pm SE calculated by matrix inversion using GraFit program) were determined by evaluating the fluorescence polarization data (Figures S5 and S6) shown in Figure 3 as bar charts. No binding (n.b.) stands for K_d values higher than 500 μ M.

Table S6. Published structures of the RAS and Effector protein complexes.

Structures	PDB code	Res. (Å)	Ref. ^a
RB domains			
RAP1A-GTP-CRAF RB	1C1Y	2.2	[1]
RAP1A(E30D/K31E)-GppNHp-CRAF RB	1GUA	2.0	[2]
RAP1A(E30D/K31E)-GDP-CRAF RB(A85K/N71R)	3KUC	1.92	[3]
HRAS-GDP-CRAF-RB(A85K)	3KUD	2.15	[3]
HRAS-GppNHp-CRAF-RB	4G0N	2.45	[4]
HRAS(Q61L)-GppNHp-CRAF-RB	4G3X	3.25	[4]
KRAS-GppNHp-ARAF-RB	2MSE	NMR	[5]
HRAS(G12V)-GppNHp-PI3Kγ-RB(V223K/V326A)	1HE8	3.0	[6]
HRAS-GppNHp-Byr2-RB	1K8R	3.0	[7]
RA domains			
HRAS(D30E/E31K)-GppNHp-RASSF5-RA (L285M/K302D)	3DDC	1.8	[8]
HRAS(G12V)-GTP - GRAB14-RA/PH (K272A/E273A)	4K81	2.4	[9]
HRAS-GppNHp-RALGDS	1LFD	2.1	[10]
HRAS(G12V)-GTP-PLCε(Y2176L) RA2	2C5L	1.9	[11]
HRAS-GppNHp-Afadin RA1	6AMB	2.5	[12]
RAP1B-GppNHp-Rasip1 RA	5KHO	2.78	[13]

^a References are listed below.

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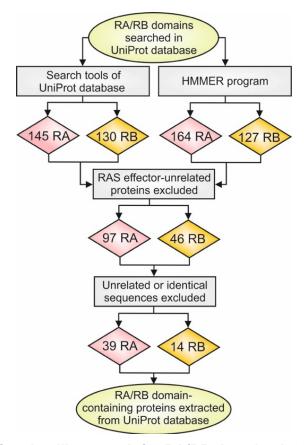


Figure S1. Flowchart of an *in-silico* search for RA/RB domains in data bases. RAS effector proteins in the human proteome were selected in a stepwise search. The initial search for input sequences, containing RA/RB domains, was performed in the UniProt database for proteins containing annotated RA/RB domains. Then HMMER was used to identify more sequence related domains. In the next step, the ClustalW sequence alignments of the output sequences were manually inspected and carefully processed to obtain all output sequences, such as 41 RA (in 39 different proteins) and 16 RB (in 14 different proteins) domains (Fig. S2 and S3), depicted in Tables S1 and S2.

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A MAIL K VW	YNHETS	AFESETKVRVNSI	EVIKQLLQKFKIENSPQD	FALHIIFA	TGEQ RRLKKTD I P L LQRL LQ CPSEKN	VARIFLMDK
<pre>EK EL KVW</pre>	3 <u>ASSF7</u> A A M E L K V W	V D G I Q R V V C G V S E Q T T C Q	EVVIALAQAIGQTG	RFVLVQRLR	- EKERQLLPQECP VGAQATCG QFASD'	DVQFVLRRT
BEKK IVW	3ASSF8 M E L K V W	VDGVQRIVCGVTEVTTCQ	EVVIALAQAIGRTG	RYTLIEKWR	- DTERHLAPHENP I ISLNKWG QYASD'	DVQLILRRT
D T C I KL SVW	3ASSF9 E E K E I V V W	V C Q E E K L V C G L T K R T T S A	DVIQALLEEHEATFGEKRFLLGKP	· SDYCIIEKWR	- GSERVLPPLTRI LKLWKAWG DEQPN	NMQFVLVKA
D CT R 1 S WE L GE CG S Y Y Y S ! L U T S P F V K Y F WE L GE CG S Y Y S ! L U T S P F V K Y F WE L GE CG S Y Y S ! L U T S P F V K Y F WE L GE CG S Y Y S ! L U T S P F V X Y F WE L GE CG S Y Y S ! L U T S P F V Y F WE L GE CG S Y Y S ! L U T S P F V Y F	<u>ASSF10</u> S E K K I S V W	I CQEEKLVSGLSRRTTCS	DVVRVLLEDGRRRRRRQRRLGSAGI	JPH GPELPEPP NE DDE DD DEAL	P Q GM L C G P F Q C Y C I V E K WRG F E R I L P N K T R I L R L W G E E Q E N	N V R F V L V R S
BARV IR NS BARV IR SS BARV IR	RGL1 DTCIIRISVE	D - NNGNMYKS IMLTSQDKTP.	AVIQRAMLKHNLDSDPAE	EYELVQVIS	- EDKELVIPDSANVFYAMNSQVN	NFDFILRKK
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K PL UVK VH - - - N N D - N N T KS M V KS T L N KY KY H P DOUV R VF F - - - N N D N T KS M V D S K P DOUV R VF F - - - - N N D N N D S K N N N D S K N N N S K N N D S K N N N D S K N N N S K N N N S K N N N S K N N N S K N N N S K N N N S K N N N S K N N N S K N N N S K N N N S K N N N S K N N N S K N N N S K N N N N S K N N N N S K N N N N N N N N N N N N N N N N N N N	RGL3 E A R V I R V S I D	N - DHGNLYRS LLTSQDKAP	SVVRRALQKHNVPQPWAC	DYQLFQVLP	- GDRV LL I PDNAN VFYA MSPVAPI	PRDFMLRRK
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F KR QV KVY F SR AL OV PS F KR QV KVY OBK AA GN FA TK GT RV PS S GGT R I Y OBK AA GN FA TK GT RV SS S GGT R I Y OBK AA GN FA TK GT RV SS KK L VI WH OBS LK PN IN VKT ILLST A GD F OT VY OBK AA GN FA TK GT RV SY A GD F OT VY OBK AA GN FA TK GY AY VS VR TT PI A GD F OT VY OBK LI E E KA FE FOH IK VP AA GD L L LE VY OBK LI E E KA FE FOH SI TI R TS PI A A H H I Y P OL L E E KA FE FOH SI TI R TS PI A A H H I Y P OL L E E KA FE FOH SI TI R TS PI A A H H I Y P OL L E E KA FE FOH SI TI R TS PI A A H H I Y P OL C T L KY SP A N H H I Y P OL C T L KY SP A N H H I Y P OL C T T Y SP A N H H I K P OL C T T Y SP A N H H I K P OL C T T Y SP A N H H I K P OL C T T Y SP A N H H I K P OL C T T Y SP	GRB10 A K Q D V K V F S	ED GTSKVVEILADMTAR	DLCQLLVYKSHCVDDN	SWT LVEHHP	- HLGLERCLEDHELVVQVES T MASE :	- SKFLFRKN
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X MEV 1 RV H + +	ŝ		FAVAEA LEKYGLEKENPK	DYCIARVMLPPG AQHSD	DEKGAKEIILDDDECPLQIFREWPSDKGI	ILVFQLKRR
40 E / K 1 Y P E K K K A T X Y S Y Y T P A C / X Y Y Y Y Y A D A C / Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y			QV LDN LMDKSHCGYSL	DWS LVETVS	- ELQMERIFEDHENLVENLLNWT RDSQ I	- NKLIFMER
GDL 1 C Y Y L E E K K A F C M K Y P A GDL L E Y Y V E R K E P D S 1 R S Y GDL ME Y Y V E R K E P D S 1 R S Y A Y H H Y P 0 L S T E S D A S C Y T A T K A Y H H Y P 0 L S T E S D A S C Y T A T K N E H T R Y P 6 A S E G T Y C G P A R K N E H T R Y P K D G N C A Y S K T T WN K S I P L K I F A K D G N C A Y S K T T WN	DGKQ A QE V L K I Y P G	WLKVGVAYVSVRVTPKSTAR	SVVLEVLPLLGRQAESPE	SFQLVEVAMGC	: RHV QRTM LMDE QP L LDR L QD I R QMS V R QV S Q	QTRFYVAES
GDLL LEVY BERE POCS I I RISP GDL MEVY I E GOLP POCS I I RISP AAYHLH I YP OLS T E S Q AS CRVT AT KI MET LR I YP OLS T E S Q AS CRVT AT KI RDE LE AL I HRQEM S T V C F G G S S CK T I NISI KS I PLK I F A KD I G N C A Y S KT I T VM NI KS I PLK I F A	ARAP1 GDF ICTVY L	EEKKAETEQHIKVPASMTAE	ELTLEILDRRNVGIREKD	YWTCFEVNE	- REEAERPLHFAEKVLP-ILHGLGTI	TDSHLVVKK
ADLIMEVY		PDCSIIRSP	ELTNDILAIKNIIPTKGD	IWATFEVIE	- NEELERPLHYKENVLEQVLRWS SLAEPG:	GSAYLVVKR
A A Y TH TYP GLST I E SGASCKYLA TKI NEHT LR I YP GLAI SEGT I YCPI PARKI RDE I E AL I HRQEMT ST VYCHGGSSCK TT I NSI KSI P LK I F A KD I GNCAY SKT I TVMNS		EQQLPDNCVTLKVSPTLTAE	ELTNQV LEMRGTAAGM - D	LWVTFEIRE	- HGELERPLHPKEKVLEQALQWC QLPEPC	CSASLLLKK
NEHLERTYPG-ALSEGLIYCP PARK RDELEALIHRQEMTSTVYCHGGGSCKITINS KSIPLKIFAKDIGNCAYSKTITVMN		STTESQASCRVTATKDSTTS	DVIKDA ASLRLDGTKC	Y V L V E V K E	SGGEEWV LDANDS - PVHRV LLWPRRA QDEHPQEDG	GYYFLLQER
KDE E A L I H R Q E M T S T V Y C H G G S C K I T I N S I K S I P L K I F A K D I G N C A Y S K T I T V M N		A I SEGT I Y CP I PARKNSTAA	EVIESLINKLHLDKTKC	Y V L A E V K E	FGGEEWILNPTDC-PVQRMMLWPRMALENRLSGED	DYRFLLREK
	КУ	VYCHGGGSCK T NSHTTAG	EVEKLIRGLAMEDSRN	MFALFEYNGHVDKAIESRTV VOLWWNCOK	/ V A D V L A K F E K L A A T S E V GD L P W K F Y F K L Y C F L D T D N V P K D S V E	EFAFMFEQA
	¥	UIGNCAYSKIIIVMNSULAN	EVINMSLPMLGI GSERU	Х Q L W V N S G K	EEAPYPLIGHEYPYGIKMSHLKDSALLIPGSKDSI	TPFNLQEP

Figure S2. Multiple sequence alignment of human RA domains. Amino acid sequences of 41 RA domains of 39 RA domain-containing proteins were aligned by using ClustalW and implemented in BioEdit with default multiple alignment parameters. Asterisks highlight RAS-binding amino acids of the respective effectors as indicated in red, green, magenta, blue, orange, and purple. Underlined proteins were biochemically investigated in this study.

CRAF * *	* * * *	* * *
CRAF NT I R V F L	PNKQRTV-VNVRNGMSLH	ORTV - VNVRNGMS LHDCLMKALK VRG-LOPECCAVFR I LHEHKGKKARLDWNTDAAS LI GEE LQVDF L-
BRAF P I V R V F L P N K		ORTV - VPARCGVT VRDS LKKALM MRG - LIPECCAVYR IQ DGEKKPIGWDTDISWLT GEELHVEV L
ARAF GT V K V Y L P N K		QTV - VTVRDGMS VYDSLDKALK VRG - LNQDCCVVYR LI KGRKTVTAWDTA I APLD GEELIVEV L
RGS12-RB1 KHCCIHL PDG	- PDGTSCV - VAVKAGFS IK	TSCV - VAVKAGFS IKDILSGLCE RHG - INGAAADLF IV GGDKPLVLHQDSSILE SRDLRLEKR-
RGS12-RB2 LF R LD L VP I		NRSVGLKAKPTKPVTEVLRPVVARYG-LDLSGLLVRLSGEKEPLDLGAPISSLDGRVVLEEK-
RGS14-RB1 K Y C C V Y L P D G		TASL-ALARPGLTIRDMLAGICEKRG-LSLPDIKVYLVGNEQALVLDQDCTVLADQEVRLENRI
RGS14-RB2 F E L E L T A I	- TALERVVRISAKPTKR LQ	ERVVRISAKPTKRLQEALQPILEKHG-LSPLEVVLHRPGEKQPLDLGKLVSSVAAQRLVLDTLP
TIAM1 TPSWFCL	- PNNQPAL - TVVRPGDT AR	AM1 TPSWFCL PNNQPAL-TVVRPGDT ARDTLELICK THQ-LDHSAHYLR LKFLIENKMQLYVPQ PEEDIYELL-
TIAM2 I QT Y V HF QD	QDNHGVTVGIKPEHR VE	NHGVTVGIKPEHRVEDILTLACKMRQ-LEPSHYGLQLRKLVDDNVEYCIPAPYEYMQQQVYDEI-
PI3KV NNCIFIVIHR	- STTSQTIKVSPDDTPG AILQ	PJ3Ky INNCIFIVIHRSTTSQTIKVSPDDTPGAILQSFFTKMAKKSLMDIPESQSEQDFVIRVCGRDEYLVGETPIKNFQWVRHCLKNGEEIHVVLDT
PI3Ka KGQIIVVIWVIVS	PNNDKQKYTLKINHDCVPEQVIA	EA I RKKTRSM LLSS EQLKLCVLEYQGKYIIK V CGCDEYFLEKYPLSQYKYIRSC I M LGRMPNLMLN
PI3KB GGK LI VAVHF	- ENCQDVFSFQVSPNMNPIKVNE	LA IQKRLT IHGKEDEVSPYDYV LQ VSGRVEYVFGDHPLIQFQYIRNCVMNRALPHFILV
PI3K5 NRALLVNVKF EGS	- EGSEESFTFQVSTKDVPLALMA	EESFTFQVSTKDVPLALMACALRKKAT VFRQPLVEQPEDYT IQ VNGRHEYLYGSYPLCQFQYICSCLHSGLTPHLTMVH
PI3KC2G KTKFNIHIFI DNS	- DNST QP LHFMPCANYL VK	T QPLHFMPCANY LVKDLIAEILHFCTNDQLLPKDHIISVCGSEEFLQNDHCLGSHKMFQKDKSVIQLHQK
PI3KC2A NASVKVSIDI EGF	- EGFQLPVTFTCDVSST VEI	QLPVTFTCDVSSTVEIIIMQALCWVHDDLNQVDVGSYVLKVCGQEEVLQNNHCLGSHEHIQNCRKWDTEIRLQLLT
PI3KC2B E V N L K V T V L C D R I		QEALTFTCNCSST VDLLIYQTLCY THDDLRNVDVGDFV LK PCGLEEFLQNKHALGSHEY IQYCRKFDIDIRLQLME

Figure S3. Multiple sequence alignment of human RB domains. Amino acid sequences of 16 RB domains of 14 RB domain-containing proteins were aligned by using ClustalW and implemented in BioEdit with default multiple alignment parameters. Asterisks highlight RAS-binding amino acids of the respective effectors as indicated in green and red. Underlined proteins were biochemically investigated in this study.

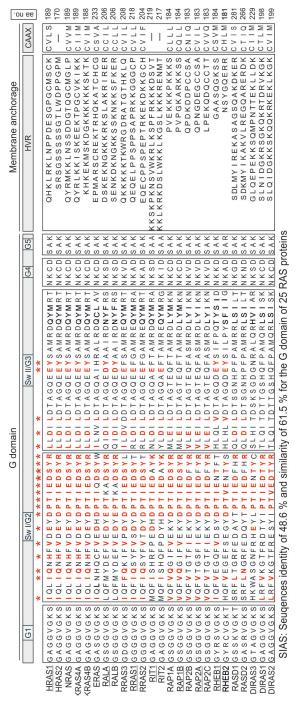


Figure S4. Multiple sequence alignment of human RAS protein family. Amino acid sequences of 25 RAS family proteins were aligned using ClustalW implemented in BioEdit with default multiple alignment parameters. Asterisks highlight effector-binding amino acids as indicated in red. Conserved signatures of the RAS proteins critical for GDP/GTP binding, GTP hydrolysis and proteins interactions are represented as G1 (or P loop for phosphate binding and magnesium ion coordination), G2 (or switch I for magnesium ion coordination and γ -phosphate binding, G3 (or switch II for γ -phosphate binding containing the catalytic glutamine), G4 (the major determinant of guanine base binding specificity) and G5 box (for guanine base binding). HVR (hypervariable region) and CAAX (C is cysteine, A is any aliphatic amino acid, and X is any amino acid) are critical motifs for association with the cell membrane. Underlined proteins were biochemically investigated in this study.

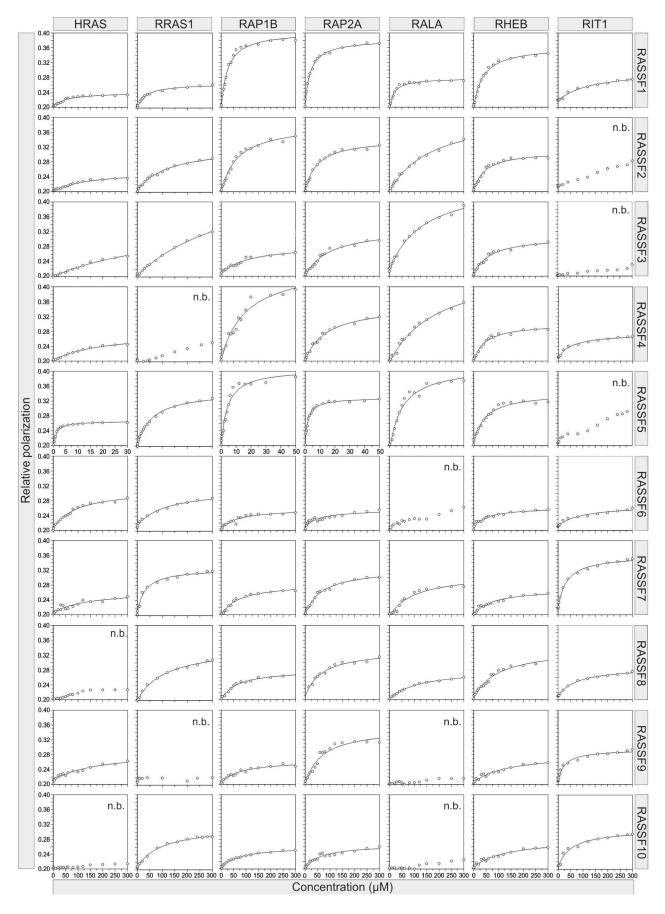


Figure S5. Fluorescence polarization measurements of RAS interactions with RASSF RA domains. Fluorescence polarization experiments were conducted to determine the dissociation constants (K_d) by titrating the active, mGppNHp-bound form of RAS proteins (1 µM) with increasing concentrations of the respective effector domains. The X-axis represents the concentration of the effector domain as MBP fusion proteins in µM and Y-axis represents fluorescence polarization. The lines through the data points indicate that equilibrium K_d values have been determined for the respective measurements. The K_d values are summarized in Figure 3 and Table S5.

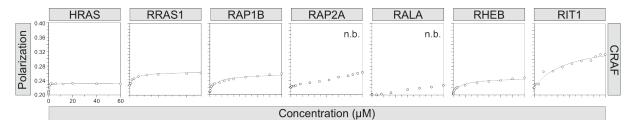


Figure S6. Fluorescence polarization measurements of RAS interactions with CRAF RB domain. Fluorescence polarization experiments were conducted to determine the dissociation constants (K_d) by titrating the active, mGppNHp-bound form of RAS proteins (1 µM) with increasing concentrations of CRAF RB domain. The X-axis represents the concentration of the effector domain as MBP fusion proteins in µM and Y-axis represents fluorescence polarization. The lines through the data points indicate that equilibrium K_d values have been determined for the respective measurements. The K_d values are summarized in Figure 3 and Table S5.

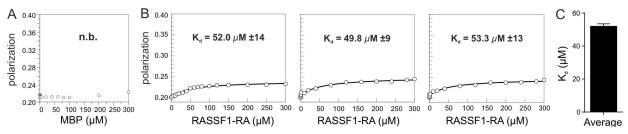


Figure S7. Control fluorescence polarization measurements. (A) Titration of mGppNHp-bound HRAS with purified MBP as a control experiment showed that MBP itself does not bind HRAS. (B) Three independent measurement for the interaction between HRAS•mGppNHp with RASSF1-RA resulted in very similar changes in fluorescence polarization. (C) The K_d values, determined for the three measurements in C, revealed a standard error of 1.021, which is far below the estimated standard error for the initial measurement in the manuscript (\pm 10). SE was calculated using GraphPad Prism software. This Result allow the assumption that the standard error for that measurement in this study is the maximum probable error predicted for that measurement, which would be a lower number in triplicate measurements.

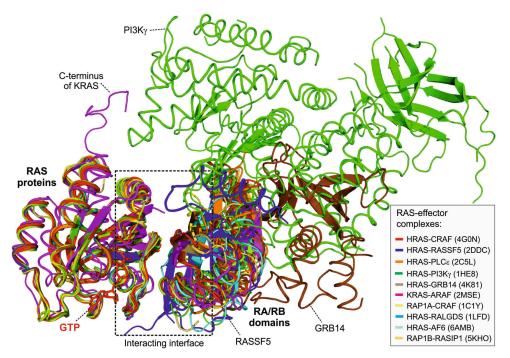


Figure S8. Superposition of all available RAS–effector complex structures. Ten structures of RAS-RA/RB domain complexes (see the inset and more details Table S6) were overlaid in ribbon representation. The interface residues (boxed), which were used for the generation of the matrix in Figure 3, are highlighted in Figure S9. Additional properties outside the interaction interface (box) are indicated.

	4 4 4 6 C C C C	[4] [4] [4]	[2]	[9]	B3 ♦	[8]	[10]
L52 D54 L56 Εq3 Υ64 β3 α2	A04 A15 L>100 E 61 L>100 A14 L86 K87 V88 R89 G90 A14 L86 K87 V88 R89 G90 A14 L86 K87 V88 R89 G90	D46 K47 L49 K50 V51 R52 G53	A>50 A,50 A,6.4 A,555 S,257 L258 M259 D260 β3 A	D307 K308 F309 M310 V311 V312 D313 N314	K2154 C2170 K2171 A2172 K2173 Y2174 S2175 L2176 S2177	A3 A100 D834 K835 H836 N837 L838 D839 E840 D841 α1 = β3 = β33	A0.043 A1.04 K78 F79 R\$0 P\$1 D\$2 M\$3 R\$4 M\$5 α1
H27 V29 E31 D33 P34 T35 I36 E37 D38 S39 Y40 R41 β2	A 6.0 A 2.8 A 2.8 A 0.15 A 0.15 A 0.15 A 0.15 A 0.13 A 0.15 A 0.1	P26 N27 K28 Q29 <mark>R3</mark> 0 T31 V32 V33 T34 D4	A ³² R226 S227 T228 T229 S230 Q231 T232 I233 K234 K254 K β2	P283 L284 D285 A286 1287 K288 Q289 M290 H291	K 1.0;E 24.5 L 184 P2 146 Ε2147 Ω2148 P2149 R2150 T2151 V2152 I2153 K2	K3.83 K0.41 K0.41 A18.2 A11 A5.6 F29 A100 N810 G811 N812 M813 Y814 K815 S816 1817 L818	Gç3 N54 F55 A56 T57 K58 C59 I60 R61 β2
A 12/1 12/4 025 HRAS G α1	B RAP14/wt 1.2 (mM) R n.b. TC21/wt 0.37 (mM) R n.b. RRAS/wt 1.3 (mM) R n.b. HRAS/wt 12 R 650 HRAS/wt 12 A 0.4 HRAS/wt 1.2 A 1.2 HRAS/wt 0.03 HRAS/wt 0.13 T57 R59 CRAF RB 11	RAP1A/wt 15 (mM) R n.b. TC21/wt 20 (mM) R n.b. RRAS/wt 70 (mM) R n.b. HRAS/wt 700 T20 K22 ARAF RB 81	HRAS/wt 2:9 5 50 F221 K223 PI3Kg RB 61	HRAS/wt 0.08 A 0.55 A 0.41 F239 K241 RASSF5 RA	HRAS/wt 1.4 F2138 Q2140 PLCe RA2 91	HRAS/wt 1.94 A 13.2 HRAS/wt 3.5 I801 R803 RALGDS RA 91	RAPTAW 0.04 A 0.2 HRAS/Wt 0.1 V42 A 8.6 AF6 RA1 91

Figure S9. Secondary structures, the corresponding interacting residues, and published dissociation constants (K_d) for respective interactions are illustrated for HRAS G domain (A) and various RA/RB domains (B). The secondary structures and the corresponding interacting residues (above the secondary structures) were extracted from the structures deposited in the PDB (Table 6). Color codes of the interacting residues (blue, red, green, black; bold) correspond to those in Figures 3 and S2-S4. Determined K_d values for the Interaction of defined RA/RB domains with different RAS family proteins and the variants of the interacting residues are represented above the interacting residues, respectively. The numbers on the right side refer to the original studies (see references below).

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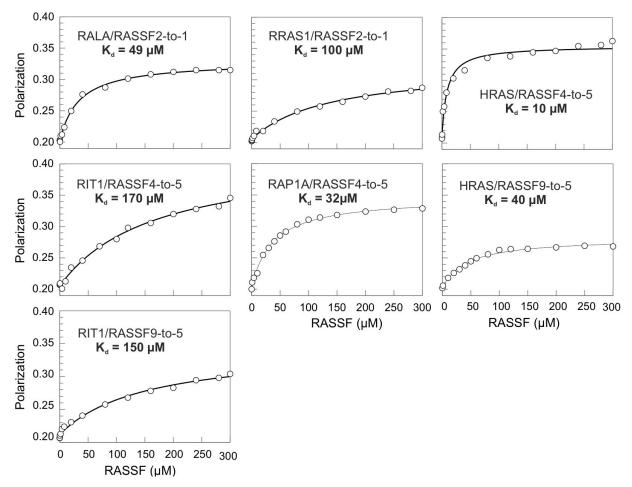


Figure S10. Fluorescence polarization measurements of the interaction of RASSF hotspot variants with various RAS family proteins. Fluorescence polarization experiments were conducted to determine the dissociation constants (K_d) by titrating the active, mGppNHp-bound form of RAS proteins (1 μ M) with increasing concentrations of the RA domains of RASSF hotspot variants (RASSF2-to-1 A186K/Y187D/V190K/T191H, RASSF4-to-5: Y185D/S187I/V188K/ N188L and RASSF9-to-5: V40D/G42I/L43K/K45L/R46H; see Figure 3, boxed residues). The X-axis represents the concentration of the effector domain as MBP fusion proteins in μ M and Y-axis represents fluorescence polarization. The lines through the data points indicate that equilibrium K_d values have been determined for the respective measurements. The K_d values are also summarized in Figure 4.

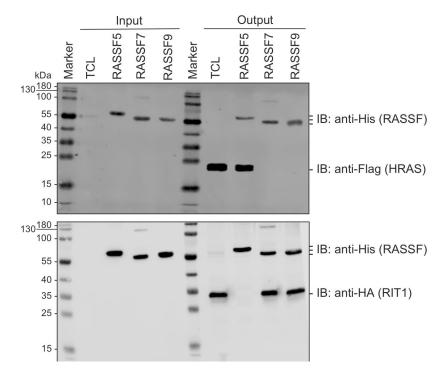


Figure S11. His-tag pull-down assay of RIT1 and HRAS binding by RASSF1-RA, RASSF7-RA and RASSF9-RA. HA-RIT1 and FLAG-HRAS, overexpressed in HEK 293T cells, were pulled down using His-tagged MBP-RA domains of RASSF5 (63 kDa), RASSF7 (54 kDa) and RASSF9 (55 kDa). Therefore, His-tagged RASSF-RA proteins (20 µg per protein and experiment) were first coupled to the Ni-NTA beads (100 µl per experiment) before mixing them with the cell lysates (200 µg per experiment). Note that His-pulldown assay did not work when cell lysate and the His-tagged proteins were added to the bead at the same time. Immunoblots of total cell lysates (TCL), evaluated using an Odyssey Fc Imaging System (LI-CORE Biosciences), were served as loading control to detect HA-RIT1 and FLAG-HRAS, respectively, analyzed by immunoblotting (IB) using anti-HA (SC-805, Santa Cruz) and anti-FLAG (F7425, Sigma) antibodies. Input samples represent the quantity of the RASSF proteins prior to being added to the beads and output samples indicate the quantity of the bound RASSF proteins to the beads after pull down. An anti-His (RM146, Thermo Fisher) antibody was used for detection of His-tagged RASSF5, RASSF7 and RASSF9 in input and output as loading control. Input and output samples showed that the RASSF proteins are comparably bound to the beads.