CHAPTER 4

Materials and Methods

4.1 Constructs

Constructs for the different experiments were generated according to standard cloning procedures[235]. Fragments or entire coding regions of genes were amplified by polymerase chain reaction (PCR) using Taq (Fermentas, Burlington, Ontario, Canada), Vent (New England Biolabs (NEB), Ipswich, Massachusetts, USA) or KOD (Novagen, Merck Bioscience, Darmstadt, Germany) polymerase and the cycler program given in Table 4.1. Primers were designed to incorporate required restriction sites, tags or thrombin cleavage sites into the PCR products. Amplified products were purified with the PCR Purification Kit (QIAGEN, Hilden, Germany) and subject to cleavage by restriction endonucleases. After agarose gel electrophoresis, cleaved fragments were cut out of the gel and purified with the Gel Purification Kit (QIAGEN). The target vectors were prepared similarly, but in most cases, prior to gel purification, their 5'-ends were dephosphorylated by calf intestine alkaline phosphatase (Fermentas). Fragments and vectors were ligated with T4 ligase (NEB) overnight at 16 °C. Ligation products were transferred into *E. coli* (see Chapter 4.5) by electroporation[292,293] or heat shock[294]. Sequences of all constructs were confirmed by DNA sequencing (Invitek GmbH, Berlin, Germany and SEQLAB Sequence Laboratories Göttingen GmbH, Göttingen, Germany). Primers used for the different PCRs are listed in Table 4.3. For details about templates, fragment borders (numbering according to full-length proteins), restriction sites or target vectors of the created constructs, refer to Table 4.2.

Table 4.1: Standard cycler program for PCRs
Annealing temperatures and elongation times are optimized for individual PCR reactions.

Step	Temperature	Time	Number of cycles
initial denaturation	94 °C	2 min	1
denaturation	94 °C	30 sec	30
annealing	optimized (50–65 °C)	30 sec	
elongation	72 °C	20–60 sec/kbp	
final elongation	72 °C	2 min	1

Table 4.2: Details about constructs used in this work

Construct Name	Template	Fragment Boarders	Target Vector	Restriction Sites	Primers	Protein ID (Swiss-Prot)
[1]GYF	–	280–342	pTFT74	–	–	O95400
GST-GYF	IRALp962D081Q2	280–342	pGEX4T-1	BamHI-XhoI	1/2	
GYF bait		256–342	pGBKT7	EcoRI-BamHI	3/4	
GYF prey		256–342	pGADT7	EcoRI-BamHI	3/4	
CD2BP2 bait		full-length	pGBKT7	NcoI-BamHI	5/6	
CD2BP2 prey		full-length	pGADT7	NcoI-BamHI	5/6	
K4 bait		1–190	pGBKT7	EcoRI-BamHI	7/8	
K6 bait		1–255	pGBKT7	EcoRI-BamHI	7/9	
EGFP-CD2BP2		full-length	pEGFP-C3	XhoI-BamHI	10/11	
PERQ2 GST-GYF	[2]cDNA clone HJ03496	531–596	pGEX4T-1	BamHI-XhoI	12/13	O75137
PERQ2 prey			pGADT7	NcoI-BamHI	14/15	
PERQ2 bait			pGBKT7	NcoI-BamHI	14/15	
PERQ2-GYF			[1]pTFT74	NcoI-HindIII	16/17	
SMY2 GST-GYF	[3]genomic DNA of yeast strain S288C	193–290	pGEX4T-1	BamHI-XhoI	18/19/20/21	P32909
SMY2-GYF		193–290	pET28-adaptor	BamHI-XhoI	18/19/20/21	
SYH1 GST-GYF		150–226	pGEX4T-1	BamHI-XhoI	22/23	Q02875
PR-SYH1 GST-GYF		141–226	pGEX4T-1	BamHI-XhoI	24/23	
LIN1 GST-GYF	[3]genomic DNA of yeast strain S288C	282–340	pGEX4T-1	BamHI-XhoI	25/26	P38852
		262–340			27/28	
		264–340			29/28	
		253–340			30/28	
		264–335			29/31	
		253–335			30/31	
		232–340			32/28	
Q9VKV5 GST-GYF	clone GH13760	260–319	pGEX4T-1	BamHI-XhoI	33/34	Q9VKV5
GYN4 GST-GYF	[4]genomic P1 clone MBD2	546–604	pGEX4T-1	BamHI-NotI	35/36	Q9FMM3
GYN4-PR GST-GYF		546–619	pGEX4T-1	BamHI-NotI	35/37	

GYN4 bait	[4]genomic P1 clone MBD2	546–604	pGBKT7	NcoI-NotI	35/36	Q9FMM3
GYN4-PR bait		546–619	pGBKT7	NcoI-NotI	35/37	
Q9FZJ2-GYF	[5]genomic clone F17L21	474–534	[1]pTFT74	NcoI-HindIII	38/39	Q9FZJ2
AKNA bait	DKFZp667H017Q2	795–894	pGBKT7	NcoI-NotI	40/41	Q96RR9
CD2 bait	[1]CD2 construct	245–351	pGBKT7	EcoRI-BamHI	42/43	P06729
CD2 prey		245–351	pGBKT7	EcoRI-BamHI	42/43	
[6]CD2	–	full-length	pCDM8	–	–	
[6]CD2taa236	–	1-235	pCDM8	–	–	
MAGD1 bait	IRALp962H0828Q2	357–514	pGBKT7	NcoI-NotI	44/45	Q9Y5V3
NEDD4 bait	IRAK-p961C1479Q2	267–328	pGBKT7	NcoI-NotI	46/47	P46934
NPWBP bait	IRALp962P0114Q2	388–551	pGBKT7	NcoI-NotI	48/49	Q9Y2W2
PI31 bait	IRAKp961G1751Q2	214–271	pGBKT7	NcoI-NotI	50/51	Q92530
SmB bait	IMAGp998D118415Q3	148–231	pGBKT7	NcoI-NotI	52/53	P14678-2
EYFP-SmB		full-length	pEYFP-C1	HindIII-SalI	54/55	
SWAN bait	IRAL-p962K1725Q2	700–869	pGBKT7	NcoI-NotI	56/57	Q9NTZ6
WWP2 bait	IRAK-p961B0119Q2	293–491	pGBKT7	NcoI-NotI	58/59	O00308
[7]PC89-X_9	–	–	–	–	–	–
PC89-X_2PPPX_3	–	–	[7]PC89	EcoRI-BamHI	60/61	–
pET28-adaptor	–	–	pET28	NcoI-HindIII	62/63	–
[8]Yep352-5'+3xHA/EAP1	–	full-length	Yep352-5'	–	–	P36041
[9]GAL-MSL5-HA(3)	–	full-length	–	–	–	Q12186

Indicated template DNA, vectors, and complete constructs were kind gifts of:
[1]Christian Freund, Protein Engineering Group and [3]Anette Diehl, NMR-unterstützte Strukturforschung, Leibniz-Institut für Molekulare Pharmakologie (FMP) and Freie Universität, Berlin, Germany; [2]Takahiro Nagase and [4]Satoshi Tabata, Kazusa DNA Research Institute, Chiba, Japan; [5]Arabidopsis Biological Resource Center – Stock donors: Teresa Mozo, Institut für Genbiologische Forschung Berlin GmbH, Berlin and Thomas Altmann, Max-Planck-Institut für Molekulare Pflanzenphysiologie, Department Willmitzer, Golm, Germany; [6]Ellis Reinherz, Department of Medicine, Dana-Farber Cancer Institute, Boston, USA; [7]Gianni Cesareni, Department of Biology, University of Rome Tor Vergata, Rome, Italy; [8]Nahum Sonenberg, Department of Biochemistry and McGill Cancer Center, McGill University, Montreal, Canada; [9]Michael Rosbash, Howard Hughes Medical Institute, Department of Biology, Brandeis University, Waltham, USA. Clone GH13760 was purchased from ResGen/Invitrogen (Invitrogen Corporation, Carlsbad, California, USA). All other template DNAs were obtained from Deutsches Ressourcenzentrum für Genomforschung, Berlin. Sequences of primers are listed in Table 4.3.

Table 4.3: List of primers used in this work

Sequences are given in 5'-3' direction. N- or C-terminal borders of the protein fragments, encoded by the resulting PCR products, are indicated by numbers in brackets, preceding or succeeding a string, respectively. For internal primers, the number is replaced by the letter i. The asterisk marks the presence of a stop codon. Sequencing primers appear in a separate part of the table. For each indicated vector, the first primer is for forward sequencing, the second for reverse sequencing. Constructs of standard vectors were sequenced with primes according to the manufacturer's protocol and are not listed here. Primers were obtained from BioTeZ, Berlin-Buch GmbH, Berlin, Germany.

Primer Number	Construct / Primer Name	Primer Sequence
1	GST-GYF (280-)	CTACTGGATCCGATGTGATGTGGGAATATAAG
2	GST-GYF (-342*)	AGTAGCTCGAGTTAGGTGTAGAGGTCAAAGTCAAT
3	GYF bait and prey (256-)	GCGCTGAATTCGCGGAGGAGGAACTGGAGACC
4	GYF bait and prey (-342*)	AGCGCGGATCCTTAGGTGTAGAGGTCAAAGTC
5	CD2BP2 bait and prey (1-)	CTACTCCATGGAGCCAAAGAGGAAAGTGACCTTC
6	CD2BP2 bait and prey (-342*)	AGTAGGGATCCTTAGGTGTAGAGGTCAAAGTCAAT
7	CD2BP2 K4/K6 bait (1-)	GCGCTGAATTCCCAAAGAGGAAAGTGACCTTC
8	CD2BP2 K4 bait (-190*)	AGCGCGGATCCTTAAGGCCCCTTTCTCCCTTTGCC
9	CD2BP2 K6 bait (-255*)	AGCGCGGATCCTTACAACTCCTCAGCGAACATGTC
10	EGFP-CD2BP2 (1-)	GCATCACTCGAGATGCCAAAGAGGAAAGTGACC
11	EGFP-CD2BP2 (-342*)	AGCTGGATCCCGGGTGTAGAGGTCAAAGTCAAT
12	PERQ2 GST-GYF (531-)	ATCTATGGATCCGAAGCAATGCAGAAGTGGTATTAC
13	PERQ2 GST-GYF (-596*)	CTATCTCGAGTTATGGACCTGGAGAAAAGGGAAC
14	PERQ2 bait and prey (531-)	ATCTATCCATGGAAGCAATGCAGAAGTGGTATTAC
15	PERQ2 bait and prey (-596*)	CTATGGGATCCTATGGACCTGGAGAAAAGGGAAC
16	PERQ2-GYF (531-)	ATCTATCCATGGCGCATCATCATCATCATCATGAAGCAATG-CAGAAGTGG
17	PERQ2-GYF (-596*)	CTATGAAGCTTCATGGACCTGGAGAAAAGGGAAC
18	SMY2 GST-GYF (193-)	ATCTATGGATCCATGGGTAACGGAATGTCACAACTCCCAG
19	SMY2 GST-GYF (-i)	GTCCGTATCATACTTTTCGAGTTTAGTCATTAACTCACC
20	SMY2 GST-GYF (i-)	CTCGAAAAGTATGATACGGACCCATTCACTACTTTTGACAA-ACTTC
21	SMY2 GST-GYF (-290*)	CTATGCTCGAGAGAATCAGAGCTGGTTGTTTGAAC
22	SYH1 GST-GYF (150-)	ATCTATGGATCCATGGAATCTCAGTGGAAATACATTGACTC
23	(PR-) SYH1 GST-GYF (-226)	CTATGCTCGAGAACAATGAAATCAAAGGCGACAAAAG
24	PR-SYH1 GST-GYF (141-)	ATCTATGGATCCATGGGTAATCAACCCCCACCACC
25	LIN1 GST-GYF (282-)	ATCTATGGATCCACAAAGTTATGGGGCTTCAAGTGG
26	LIN1 GST-GYF (-340*)	CTATGCTCGAGTTACATGAAACTTAAACACGATACATG
27	LIN1 GST-GYF (262-)	ATCTATGGATCCATGGTTCAGGATGCTATAGAAGAGG
28	LIN1 GST-GYF (-340)	CTATGCTCGAGCATGAAACTTAAACACGATACATG
29	LIN1 GST-GYF (264-)	ATCTATGGATCCGATGCTATAGAAGAGGAAATTTTC
30	LIN1 GST-GYF (253-)	ATCTATGGATCCGAAGTATATGAATATAACCGC
31	LIN1 GST-GYF (-335)	CTATACTCGAGTTACGATACATGTATCCAGTTTTC
32	LIN1 GST-GYF (232-)	ATCTATGGATCCGTTATTCACGGAATCGAGC
33	Q9VKV5-GYF (260-)	ATCTATGGATCCATGGAGGTGACCTGGGAGTTTAAATG
34	Q9VKV5-GYF (-316*)	CTATGCTCGAGCAAGTACAAATCGAAATCTATGCG
35	GYN4/-PR (546-)	ATGTACCATGGGATCCGAACTTTCACTCTATTATAAAGATC
36	GYN4 (-604*)	CTATGCGGCCGCTACCGTAAATGTGGCATTACATCAC
37	GYN4-PR (-619*)	CTATGCGGCCGCTAATTTTGCTTGGCACCAGTAAAACC

38	Q9FZJ2-GYF (474-)	ATCTATCCATGGCGCATCATCATCATCATCATGAGTTCTTA -TTCTTGTATATTGATCC
39	Q9FZJ2-GYF (-534*)	CTATGAAGCTTCATTCTGCCTTTATATATGACATGACC
40	AKNA bait (795-)	ATGTACCATGGGATCCCTGGAAGTTGATGGG
41	AKNA bait (-894*)	CTATGCGGCCGCTAGCCGCTTCCCTCCAGGC
42	CD2 bait and prey (245-)	GCGCTGAATTCAATGATGAGGAGCTGGAGACA
43	CD2 bait and prey (-351*)	AGCGCGGATCCTTAATTAGAGGAAGGGGACAA
44	MAGD1 bait (357-)	ATGTACCATGGCCTGGCAGAACCCAGTC
45	MAGD1 bait (-514*)	CTATGCGGCCGCTACTGTGAGGCACGCGAGTTG
46	NEDD4 bait (267-)	ATGTACCATGGGATCCGAATTAGAGCCTGGCTGGG
47	NEDD4 bait (-328*)	CTATGGAATTCGCGGCCGCTATAGGTTGTCCTGAGGGG
48	NPWBP bait (388-)	ATGTACCATGGGATCCCAGCAGCAGGCTCCGCC
49	NPWBP bait (-551*)	CTATGCGGCCGCTATGCACTTGTATCATCCGCC
50	PI31 bait (214-)	ATGTACCATGGGATCCAGATCTGGCTTCCCAAGAGC
51	PI31 bait (-271*)	CTATGCGGCCGCTACAGGTACATGTCATCGTAGC
52	SmB bait (148-)	ATGTACCATGGGTACTGTTGCAGCCGC
53	SmB bait (-231*)	CTATGCGGCCGCTAAAGAAGGCCTCGCATCCC
54	EYFP-SmB (1-)	CAGATAAGCTTCTATGACGGTGGGCAAGAGCAG
55	EYFP-SmB (-231*)	AGTTAGTCGACTTAAAGAAGGCCTCGCATCCCAG
56	SWAN bait (700-)	ATGTACCATGGGATCCGCAGGAGGTGAAGAGCATG
57	SWAN bait (-869*)	CTATGCGGCCGCTAAATAGACACAGTAAAGGGCATG
58	WWP2 bait (298-)	ATGTACCATGGAATTCCCAGCGGCTGCCCAG
59	WWP2 bait (-491*)	CTATGCGGCCGCTAATAAGCACCAGGGGAACC
60	focused library (1-)	CGGTGAATTCCGAAAACGAAGTCATNNKNNKCCGCCTCCTN -NKNNKNNKGTTCAGAGGGATCCCGC
61	focused library (-15)	GCGGGATCCCTCTGAAC
62	pET28 adaptor (1-)	ATCTATCCATGGCGCATCATCATCATCATCATCTTGTCCCA -AGAGGATCCATCTTTCTATAAGCTTATCTAT
63	pET28 adaptor (-14)	ATAGATAAGCTTATAGAAAGATGGATCCTCTTGGGACAAGA -TGATGATGATGATGATGCGCCATGGATAGAT

Vector	Sequencing Primer	Primer Sequence
pTFT74	T7	TAATACGACTCACTATAGG
	pTFT74-3'	TCAGCGGTGGCAGCAGCCA
pGAD-GL	human lung Y2H-5'	AATACCACTACAATGGATG
	T7	TAATACGACTCACTATAGG
pEYFP-C1 pEGFP-C3	E(G/Y)FP-5'	ATGGTCCTGCTGGAGTTCGTG
	E(G/Y)FP-3'	GGTATGGCTGATTATGATCAGT
PC89	PC89-5'	TACCCTCGTTCCGATGCTG
	PC89-3'	GCTGAGGCTTGCAGGGAG

4.2 GYF Domain Constructs

The domain borders for human CD2BP2-GYF were derived from the structure. For the other GYF domains, the estimated borders were obtained from sequence alignment (see Fig. 1.3 and Table 4.2). In the case of GYN4 and SYH1, additional constructs were designed to include an

internal PRS (GYN4 residues 546–619; termed GYN4-PR and SYH1 residues 141–226; termed PR-SYH1).

4.2.1 Constructs for *In Vitro* Experiments

For expression of the GYF domains as glutathione S-transferase (GST) fusion proteins, the corresponding PCR products were cloned into pGEX4T-1 (GE Healthcare, Little Chalfont, United Kingdom). Expression constructs of untagged CD2BP2- and His_6-tagged PERQ2-GYF were obtained by cloning the respective PCR fragments into pTFT74[295]. Cloning of the SMY2-GYF encoding PCR product into a modified pET28 vector (pET28 adaptor) set the domain in frame with an N-terminal His_6-tag, separated by a thrombin cleavage site.

4.2.2 Bait and Prey Constructs

For yeast two-hybrid analysis, PCR products coding for full-length CD2BP2, a fragment comprising its GYF domain or PERQ2-GYF were ligated into pGADT7 (Clontech, Mountain View, California, USA). PCR products, comprising the coding region of GYN4-GYF with (GYN4-PR bait) or without internal PRS (GYN4 bait) or comprising the coding region of PERQ2-GYF were introduced into the bait vector pGBKT7 (Clontech) for yeast two-hybrid screens.

4.2.3 Fluorescence Protein Fusion Constructs

DNA coding sequences for full-length CD2BP2 and SmB (from Smith antigen nomenclature) were cloned into the pEGFP-C3 and pEYFP-C1 (Clontech) vector, respectively, for colocalization experiments[296].

4.3 Constructs of Potential GYF Domain Interaction Partners

PCR products which encode the proline-rich regions of the proteins AKNA (AT-hook protein), MAGD1 (melanoma-associated antigen D1), NEDD4 (neural precursor cell expressed developmentally down-regulated protein 4), NPWBP (Npw38-binding protein), PI31 (proteasome inhibitor PI31 subunit), SmB, SWAN (SH3/WW domain anchor protein in the nucleus), and WWP2 (WW domain-containing protein 2), and a DNA fragment, coding for the complete cytoplasmic tail of CD2 (cell differentiation antigen 2), were inserted into the pGBKT7 vector.

4.4 Library Construction

Construction of the focused library RKRSHXXPPPXXXVQR in the phagmid vector PC89 was similar to the procedure described elsewhere[297]. For synthesis of the oligonucleotide insert, containing degenerate codons at defined positions flanked by an EcoRI and BamHI restriction site, 2 nmol of each focused library primer were used in a PCR reaction (total volume: 500 µl) with 0.4 mM deoxyribonucleotide triphosphate, 2.5 mM $MgCl_2$, and 0.1 units (U)/µl Taq polymerase (Fermentas). The annealing temperature was set to 50 °C, the elongation time, at 72 °C, to 30 sec. The double-stranded 66-mer was purified by gel-filtration using sephadex G50, equilibrated in H_2O. After EcoRI - BamHI digestion of the PCR product for 3.5 h at 37 °C, the restriction enzymes were heat inactivated at 75 °C for 25 min. The cleaved 53-mer fragment was purified by native Tris-borate EDTA buffered polyacrylamide gel-electrophoresis (PAGE) on a 20 % (w/v) polyacrylamide gel with 3.6 % bisacrylamide. The band of correct size was excised and the DNA was eluted with elution buffer (10 mM Mg-acetate, 500 mM NH_4-acetate) by repeated snap-freezing, followed by a 4 h incubation at 37 °C and overnight incubation at 4 °C. After Na-acetate precipitation[235] and dissolution in H_2O, the DNA fragment was ligated to 1 µg EcoRI - BamHI cleaved, dephosphorylated PC89 for 18 h at 15 °C, using an insert : vector ratio of 2.5 in a total volume of 40 µl. The ligation mix was heated at 75 °C for 20 min and purified using StrataClean™ resin (Stratagene, La Jolla, California, USA). 40 µl ElectroTen-Blue® electroporation-competent cells (Stratagene; see Table 4.4) were transformed by electroporation with 4–5 µl ligation mix and incubated for 90 min at 37 °C in 1.5 ml rich medium (SOC or 2xYT medium[235], see Table 4.5). A total of 320 µl competent cells were transformed and spread on 46 Pedri dishes (15 cm diameter) containing 2xYT agar plus 100 µg/ml ampicillin. Colonies (5.5×10^6 in total, which correspond to a 1.7 fold coverage of the library) were resuspended in medium with 20 % glycerol and stored at -80 °C. PC89 and the PC89–nonapeptide-library (X_9) were a gift from Gianni Cesareni (Dipartimento di Biologia, Universita di Roma).

4.5 Bacterial Strains and Media

For cloning, the *E. coli* bacterial strain DH5α (GIBCO, Invitrogen Corporation, Carlsbad, California, USA) or XL1-Blue (Stratagene) were routinely utilized (Table 4.4). Constructs, based on the vector pCDM8 (CD2 and CD2taa236) were amplified in MC1061/P3 (Invitrogen). Protein expression took place in *E. coli* BL21 (DE3) and BL21 (DE3) pLysS (Novagen, Merck Bioscience, Darmstadt, Germany). Phages were produced in *E. coli* XL1-Blue. For library construction, ElectroTen-Blue® electroporation-competent cells (a variant of the XL1-Blue strain, Stratagene) were used to insure high transformation efficiencies.

Table 4.4: *E. coli* strains used in this work

***E. coli* strain**	**Genotype**	**Source**
BL21 (DE3)	F^-, *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
BL21 (DE3) pLysS	F^-, *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysS [Cam^R]	Novagen
DH5α	F^- Φ80*lac*ZΔ*M15* Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ	GIBCO
MC1061/P3	F^- *hsd*R (r_k^-, m_k^+) *ara*D139 Δ(*ara*ABC-*leu*)7679 *gal*U *gal*K Δ*lac*X74 *rps*L *thi mcr*B [P3: Kan^R Amp^R (am) Tet^R (am)]	Invitrogen
ElectroTen-Blue® electroporation-competent cells	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr)173 end*A1 *sup*E44 *thi-1 rec*A1 *gyr*A96 *rel*A1 *lac* Kan^R [F´ *proAB lacI*^{q}*Z.M15* Tn*10* (Tet^R)]	Stratagene
XL1-Blue	*rec*A1 *end*A1 *gyr*A96 *thi-1 hsd*R17 *sup*E44 *rel*A1 *lac* [F´ *proAB lacI*^{q}*Z.M15* Tn*10* (Tet^R)]	Stratagene

Table 4.5: Media used for bacterial growth
Composition of different media for *E. coli* cultures. For preparation of solid media, 15 g/l agar were added. If required, anitibiotics at the following concentrations were added to the media: 100 µg/ml ampicillin, 34 µg/ml kanamycin (Roth, Karlsruhe, Germany), 34 µg/ml chloramphenicol (Eurobio; Les Ulis Cedex, France). Asterisks indicate media for which the pH was adjusted to 7.0 with NaOH.

LB*	**Minimal Medium A**	**SOC***	**2xYT***
10 g/l tryptone	10.5 g/l K_2HPO_4	20 g/l tryptone	6 g/l tryptone
5 g/l yeast	4.5 g/l KH_2PO_4	5 g/l yeast extract	10 g/l yeast extract
extract	0.5 g/l Na-citrate	0.5 g/l NaCl	5 g/l NaCl
5 g/l NaCl	0.12 g/l $MgSO_4$	0.186 g/l KCl	
	1 g/l NH_4Cl or 0.75 g/l $^{15}NH_4Cl$	0.95 g/l $MgCl_2$	
	8 g/l glucose or 2 g/l [^{13}C] glucose	3.6 g/l glucose	

4.6 Protein Preparation

Proteins were expressed in *E. coli* BL21 (DE3) and BL21 (DE3) pLysS and purified from the soluble fraction after sonication. GST-fusion proteins and His_6-tagged GYF domains were purified by affinity chromatography using glutathione-sepharose and Ni^{2+}-NTA agarose according to the manufacturer's manual (GE Healthcare), respectively, and dialyzed against phosphate buffered saline (PBS). Untagged CD2BP2-GYF domain was purified by ion exchange chromatography (Mono Q® HR 10/10, GE Healthcare) and subsequent gel filtration (Superdex® 75, GE Healthcare) in 50 mM Na-phosphate buffer, pH 6.3. The His_6-tag of SMY2-GYF and the GST-tag of SYH1-GYF were cleaved off by incubation with thrombin (Calbiochem, Merck Bioscience; 10 U/mg protein, at 4 °C and 16 °C overnight in PBS, respectively) and were removed by gel filtration (Superdex® 75) in PBS. To obtain NMR samples of SMY2-, PERQ2-, and CD2BP2-GYF for titration experiments, cells expressing the tagged or untagged GYF domain constructs were grown on defined medium (minimal medium A, see Table 4.5) supplemented with $^{15}NH_4Cl$. For backbone resonance assignments of SMY2-GYF, defined medium was used, supplemented with [^{13}C] glucose and $^{15}NH_4Cl$.

4.7 SPOT Analysis

Membrane SPOT synthesis was kindly performed by Angelika Ehrlich. Peptides were synthesized on Whatman 50 cellulose membranes first by semi-automated SPOT synthesis[233,298] (Abimed; Software LISA, Jerini AG, Germany) and later on with an Auto-Spot Robot (ASP 222: Invartis AG, Köln, Germany). The synthesis using N-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry on β-alanine functionalized cellulose membranes was performed according to standard protocols[298,299]. Membranes were probed with GST-GYF fusion proteins as described elsewhere[300]. Briefly, membranes were incubated with GST-GYF fusion proteins (40 µg/ml) overnight at 4 °C. After washing, bound GST-GYF fusion protein was detected with rabbit polyclonal anti-GST antibodies (Z-5, Santa Cruz, California, USA) and horseradish peroxidase (HRP) coupled anti-rabbit IgG antibodies (Rockland, Gilbertsville, Pennsylvania, USA). An enhanced chemiluminescence substrate (SuperSignal West Pico, Pierce Illinois, USA) was used for detection by a LumiImager™ (Diagnostics, Mannheim, Germany).

4.8 Phage Display

Phages displaying the nonapeptide (X_9) or the focused peptide library, fused to the major capsid protein, were produced by transforming *E. coli* XL-1 Blue cells with PC89 constructs, superinfecting transformed cells with the VCS-M13 helper phage (Stratagene) and overnight incubation in 2xYT medium in the presence of 100 μg/ml ampicillin and 34 μg/ml kanamycin (30 °C, 270 rpm shacking). Phage particles were purified by three successive polyethylene glycol (PEG)/NaCl precipitations[301], each comprising the following steps: (1) addition of 20 % (v/v) of a 20 % (w/v) PEG 6000 solution, 2.5 M NaCl, to the phage solution (culture supernatant in the first step) and incubated for 1 h on ice, (2) sedimentation of precipitated phage particles by centrifugation for 15 min at 4000 rpm, and (3) resuspension of phages in PBS followed by a centrifugation step (2 min at 14000 rpm) to remove remaining bacterial contaminations. Library screening was performed as follows: 30–50 μl of GST-GYF loaded glutathione-sepharose 4B beads (GE Healthcare) were incubated with 5 x 10^9–5 x 10^{11} infectious particles at 4 °C overnight in PBS on a rotor. After washing 3 times with PBS, bound phages were eluted with 800 μl of 100 mM glycine HCl, pH 2.2 and neutralized with 48 μl 2 M Tris. For phage amplification, *E. coli* XL-1 Blue cells (see Table 4.4) were infected with eluted phage followed by superinfection with helper phage and subsequent incubation as described above. After three or six rounds of panning, the inserts of selected phages were sequenced to identify their displayed peptide.

4.9 Preparation of Peptides

Peptides used in this work (Table 4.6) were synthesized by Annerose Klose, Dagmar Krause, and Dr. Michael Beyermann. Automated solid-phase synthesis (ABI 433A, Applied Biosystems Foster City, California, USA) using standard Fmoc chemistry in a batch-wise mode were performed as described previously for the synthesis of corticotropin releasing factor (CRF) analogs[302]. After final cleavage/deprotection using trifluoroacetic acid/H_2O (9/1), crude peptides were purified by preparative high pressure liquid chromatography (HPLC) to give final products of 95 % purity according to HPLC analysis. The peptides were characterized by Heidemarie Lerch and Dr. Eberhard Krause using mass spectrometry, which gave the expected masses.

Table 4.6: Peptides used in this work
The peptides CD2 and Pep-1 have an unmodified terminal carboxy group, all others comprise an amide group at the C-terminus.

Peptide	Sequence
AKNA	VSMKPPGFQAS
CD2, CD2S	SHRPPPPGHRV
CD2-G8W	SHRPPPPWHRV
CD2L	HPPPPPGHRSQAPSHRPPPPGHRVQH
GYN4 internal	AKSGPPPGFTGAKQN
MAGD1	AWQNPPGWQTPPGWQTPPGWQGPPDW
MSL5S1	SSIAPPPGLSG
MSL5L1	SIAPPPGLSGPPGFSN
MSL5L2	DINKPTPPGLQGPPGL
Pep-1	KETWWETWWTEWSQPKKKRKV
PD1	EFGPPPGWLGR
SmB-1	PMGRGAPPPGMMGPPPGMRPPM
SmB-2	GTPMGMPPPGMRPPPPGMRGLL
snRNP A	MPPPGMIPPPGLAPGQIPPGAM

4.10 NMR Titration Experiments

All NMR experiments were performed on either Bruker DRX600 or DMX750 instruments equipped with standard triple-resonance probes. Data processing and analysis were carried out with the XWINNMR (Bruker, Karlsruhe, Germany), Prosa/XEASY[303], and Sparky[304] software packages. The NMR samples of CD2BP2-GYF (untagged) were buffered with 50 mM Na-phosphate, pH 6.3, those of PERQ2- (His_6-tagged) and SMY2-GYF (His_6-tag removed) with PBS. In the NMR titration experiments, increasing amounts of peptides were added to ^{15}N-labeled samples of GYF domains. The gradual change of chemical shifts in ^{1}H-^{15}N-HSQC spectra allowed unambiguous assignment of the resonances of ligand-bound GYF domains. The sum of the chemical shift changes for ^{1}H and ^{15}N atoms of a resonance peak in a sample with peptide was determined as: $[(10*\Delta^{1}H)^2 + (2*\Delta^{15}N)^2]^{1/2}$, where $\Delta^{1}H$ and $\Delta^{15}N$ are the chemical shift changes given in ppm. Data were analyzed using the Microcal™ Origin™ program (Mircocal Software, Northampton, Massachusetts, USA) by assuming a simple two-state binding model. Titrations of the peptides SmB-1, SmB-2, and CD2 to 0.4 mM samples of CD2BP2-GYF were performed at 298 K (Fig. 6.4). Further titrations (4 and Fig. 9.7) to 0.2 mM samples of CD2BP2-GYF or SMY2-GYF took place at 297 K and a titrations with a 0.6 mM sample of PERQ2-GYF and AKNA peptide (Fig. 9.11) was performed at 296 K. Backbone assignment of the SMY2-GYF domain was based on CBCA(CO)NH[305], CBCANH[306], and HNCO[307] experiments with SMY2-GYF in PBS at 299 K. ^{1}H spectra were recorded at the following conditions: CD2BP2-GYF 0.1 mM at 298 K, PERQ2-GYF 0.2 mM at 297 K, and SMY2-GYF 1.2 mM at 299 K.

4.11 Fluorescence Titrations

Fluorescence titration experiments were performed with 3 µM samples of SMY2- and SYH1-GYF (tag removed), His_6-tagged PERQ2-GYF and GST-tagged GYN4 constructs in PBS at 25 °C. Fluorescence of GYF domains or fusion constructs was excited at 280 nm in the presence of increasing amounts of peptides (Table 4.6) on a Perkin LS-50B fluorimeter (Perkin Elmer, Wellesley, Massachusetts, USA) and the emission spectra were recorded between 300 and 400 nm. Centroid shifts were calculated using the software SpecWin (a kind gift of Sebastian Modersohn). Binding data were analyzed in a similar manner as data from NMR titration experiments.

4.12 Cell Culture and Lysis

4.12.1 Human Cell Lines

Jurkat J77 cells were maintained in supplemented RPMI 1640 medium (Biochrom AG, Berlin, Germany; Table 4.7) in a 37 °C humidified incubator with 5 % CO_2. Cells were washed 3 times with PBS and lysed for 30 min by gentle rocking in lysis buffer (25 mM Tris pH 7.4, 137 mM NaCl, 1 % (v/v) NP-40, 0.5 mM PMSF, 5 µg/ml of each aprotinin and leupeptin, 5 mM EDTA, 2 mM Na-orthovanadate). Cell lysates were centrifuged in order to remove insoluble cell debris and were either used directly or snap frozen for storage at -80 °C. HeLa S3 cells were maintained in supplemented Dulbecco's Modified Eagle's Medium (DMEM; BioWhittaker Molecular Applications, Walkersville, Maine, USA).

Table 4.7: Media used for cell cultures
Supplements of RPMI 1640 and DMEM media for Jurkat J77 and HeLa S3 cells, respectively, and composition of synthetic drop-out (SD) medium for yeast. [1] Biochrom AG, [2] Sigma-Aldrich Corporation, [3] BD Bioscience, San Jose, California, USA

RPMI 1640 and DMEM	Synthetic Drop-out
10 % (v/v) fetal bovine serum[1]	6.7 g/l yeast-nitrogen base (including ammonium sulfate)[2]
0.29 mg/ml glutamine[1]	drop-out mix[3] according to the manufactorer's protocol
50 µg/ml streptomycin sulphate[1]	20 g/l glucose or galactose
50 U/ml penicillin[1]	pH set to 5.8

4.12.2 Yeast Cells

Yeast strain BY4741 (a gift from Prof. Dr. Christine Lang) was transformed with GAL-MSL5-HA(3) (a gift from Prof. Dr. Michael Rosbash) or Yep352-5'+3xHA/EAP1 (a gift from Prof. Dr. Nahum Sonenberg) and cells were grown in synthetic drop-out (SD) medium, lacking uracil (SD-Ura), for plasmid maintainance. Protein expression was induced using galactose instead of glucose as the carbon source in the case of GAL-MSL5-HA(3). EAP1 is under the control of its native promoter in Yep352-5'+3xHA/EAP1[308] and therefore does not require induction of expression. Exponentially growing cells were harvested at an absorption at 600 nm (A_{600}) of 0.7–1.4, washed with 0.9 % NaCl, and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 1 % DMSO, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 tablet/100 ml Complete Mini EDTA-free protease inhibitor cocktail; Roche, Mannheim, Germany) supplemented with PMSF to a final concentration of 3.4 mM and Complete Mini EDTA-free protease inhibitor cocktail to ~ 2,8 tablets/10 ml. Cells were lysed by vortexing three times for 1 min with ~ 3 g glass beads (425–600 microns, acid washed; Sigma-Aldrich Corporation, St. Louis, Montana, USA) per gram of cell wet weight. Cell debris was removed by centrifugation (20 min at 16000 rpm) and lysates were snap frozen for storage.

4.13 Transfection and Fluorescence Microscopy

3 x 10^6 Jurkat J77 cells were transfected by nucleoporation (performed by Tobias Zech) using 2.0 µg of DNA, the Amaxa Nucleofection Kit V (Amaxa Biosystems, Cologne, Germany) and a Nucleofector device (program T-14). 24 h after transfection Jurkat J77 cells were fixed on poly-L-lysine coated slides (Menzel-Glaeser) with 4 % paraformaldehyde / PBS for 10 min at room temperature. HeLa S3 cells were grown on glass coverslips in 12 well plates, transiently transfected with Lipofectamine plus (GIBCO) and 0.4 µg DNA / well according to the manufacturers protocol and directly fixed on the coverslips 24–48 h after transfection, as described above. Nuclei were stain with Hoechst 33258 (5 µg/ml in H_2O) for 2 min, and cells were washed twice with PBS. For detection of CD2, cells were incubated 1 h at 37 °C with mouse $T11_1$ antibody and washed three times with PBS. After 30 min incubation with anti-mouse Cy3-coupled secondary antibody at 37 °C, the cells were washed accordingly and fixed, as described above. Cells were mounted in Flouromount G mounting medium for fluorescence microscopy (Southern Biotechnology, Birmingham, Alabama, USA). Live transfected HeLa S3 cells were also examined by confocal laser scanning microscopy in standard medium 24 h post transfection (by Tobias Zech). Conventional fluorescence analysis was performed using the Leica

DMLB (Deerfield, Illinois, USA) microscope. Images were recorded with a cooled Sensiocam CCD camera. For confocal laser scanning microscopy a LSM 510 (Carl Zeiss, Jena, Germany) with a 100 x / 1.3 objective and an argon laser (488 nm) was used. Images were processed by using Axiovision 3.0 (Carl Zeiss) and Adobe Photoshop (Adobe Systems, Mountain View, California, USA) programs.

4.14 GST Pulldown Assays

Glutathione-sepharose 4B beads (GE Healthcare), loaded with GST or GST-fusion proteins, were incubated with cell lysates at 4 °C in the absence or presence of 1 mM competing peptides. Beads were washed with PBS. Bound proteins were eluted in SDS sample buffer, separated by SDS-PAGE, and transferred onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes. Blots were probed with the appropriate primary and HRP conjugated secondary antibodies and developed as described in Chapter 4.7. For the pulldown of SmB/B' with CD2BP2-GYF (performed by Dr. Heuer), 100 µg GST or GST-fusion protein was incubated with 50 µl of glutathione-sepharose 4B beads for 2 h at 4 °C. Beads were washed three times with PBS. Cell lysates (~ 3×10^8 cells per experiment), pre-cleared with 50 µl of GST-loaded glutathione-sepharose 4B beads, were incubated with GST, GST-GYF (CD2BP2) or GST-hSH3C^{300} loaded beads for 2–4 h at 4 °C. Where required, a specific (SmB-2; Table 4.6) or non-specific peptide (Pep-1) was added to the lysates. Beads were washed 5 times with 10 ml of PBS prior to elution, SDS-PAGE, and transfer of bound proteins onto a PVDF membrane. For Western blot, an anti-SmB/B' primary (sc-5485; Santa Cruz) and anti-goat HRP coupled secondary antibody (Rockland) was used.

For the pulldown of HA-tagged MSL5 or EAP1 (eIF4E-associated protein 1) with the GYF domains of SYH1 and SMY2, 25 µl of glutathione-sepharose 4B beads, loaded with GST or GST-fusion proteins, were incubated with 100 µl yeast lysates overnight in the absence or presence of competing peptide MSL5L1 (Table 4.6). Beads were washed 3 times with PBS. For Western blotting, anti-HA (BD Bioscience, San Jose, California, USA) and anti-rabbit HRP coupled antibody (Rockland) was used.

4.15 Yeast Two-Hybrid Analysis

Yeast two-hybrid experiments were performed with the MATCHMAKER™ GAL4 two-hybrid system 3 according to the manufacturer's manual (Clontech). For library screens, the pGBKT7 bait construct encoding the GAL4 DNA-binding domain fused to the GYF domain of PERQ2 or GYN4, either with or without its PRS extension, was introduced into the yeast strain AH109, followed by transformation with the human lung cDNA library in the pGAD-GL vector (Clontech) or the Horwitz and Ma *Arabidopsis thaliana* two-hybrid library[309] from the Arabidopsis Biological Resource Center (ABRC), respectively. Plasmids of cotransformants growing on SD medium deficient for either His, Leu, and Trp (medium stringency) or adenine (Ade), His, Leu, and Trp (high stringency) were rescued from yeast according to a modified MATCHMAKER™ protocol. After incubation with lyticase, cells were lysed by SDS and freeze-thawing. Subsequently, lysates were mixed with N3 buffer of the QIAGEN plasmid isolation kit and plasmid preparation followed the QIAGEN protocol. Selected candidates were sequenced to identify the polypeptides interacting with the respective GYF domains. To confirm candidates obtained by the screens, the corresponding bait and prey vector combinations were introduced into yeast and cells were cultured on low (SD medium without Leu and Trp), medium or high stringency media. Binding of suggested interaction partners, derived from SPOT analysis, to either the GYF domain of PERQ2, CD2BP2 or to full-length CD2BP2, was tested accordingly: yeast strain AH109 was cotransformed with pGBKT7 bait constructs encoding the proline-rich regions of the candidates and the different pGADT7-GYF prey constructs. Cotransformants were cultured as described above.

4.16 Software

Vector NTI® (Invitrogen) was used for the design of cloning strategies and primers. Analysis of sequenced constructs and sequence alignments were performed with the Accelrys GCG package (San Diego, California, USA). Electropherograms were inspected with Chromas (Griffith University, Southport, Queensland, Australia). Fluoresence microscopy images were recorded with the help of the program Axiovision 3.0 (Carl Zeiss). Western blot and SPOT analysis images were recorded and quantified using the LumiAnalyst software (Roche Diagnostics, Mannheim, Germany). For NMR data processing and analysis, XWINNMR (Bruker), Prosa/XEASY[303], and Sparky[304] software were used. Graphical presentations of protein structures were obtained with Sybyl (Tripos, Inc., St. Louis, Montana, USA) and MolMol 2K.2[310]. Images from MolMol 2K.2 were rendered using POV-Ray (Persistence of Vision Raytracer Pty. Ltd., Victoria, Australia). Titration data were analyzed using SpecWin and Microcal™ Origin™ (Mircocal Software). The following web-based applications on the respective servers (given in brackets) were used in this work for sequence analysis and database searches: different types of BLAST search (National Center for Biotechnology Information[C]), DALI (EMBL-EBI[K]), Genscan (EnsEMBL[B]), NetPhos (NetPhos 2.0 Server[M]), PatMatch (SGD[N]), Pattern Search (Pôle Bioinformatique Lyonnais[O]), ProtPram (Expasy[P]), SCOP[I], SMART[D], and SUPERFAMILY[H]. Databases used for searches include DALI[Q], Interpro[G], PDB[J], SGD[N], Swiss-Prot/TrEMBL (Expasy[P]), TAIR[F], and Unigene[L].

[M] www.cbs.dtu.dk/services/NetPhos
[N] www.yeastgenome.org/
[O] pbil.univ-lyon1.fr/
[P] www.expasy.ch
[Q] ekhidna.biocenter.helsinki.fi/dali/start