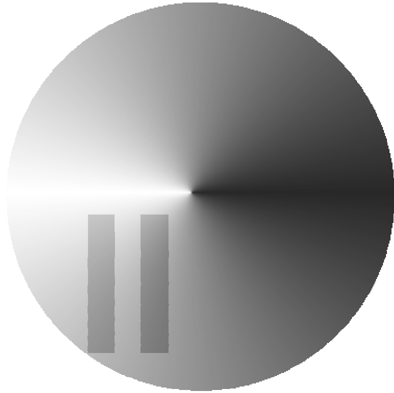
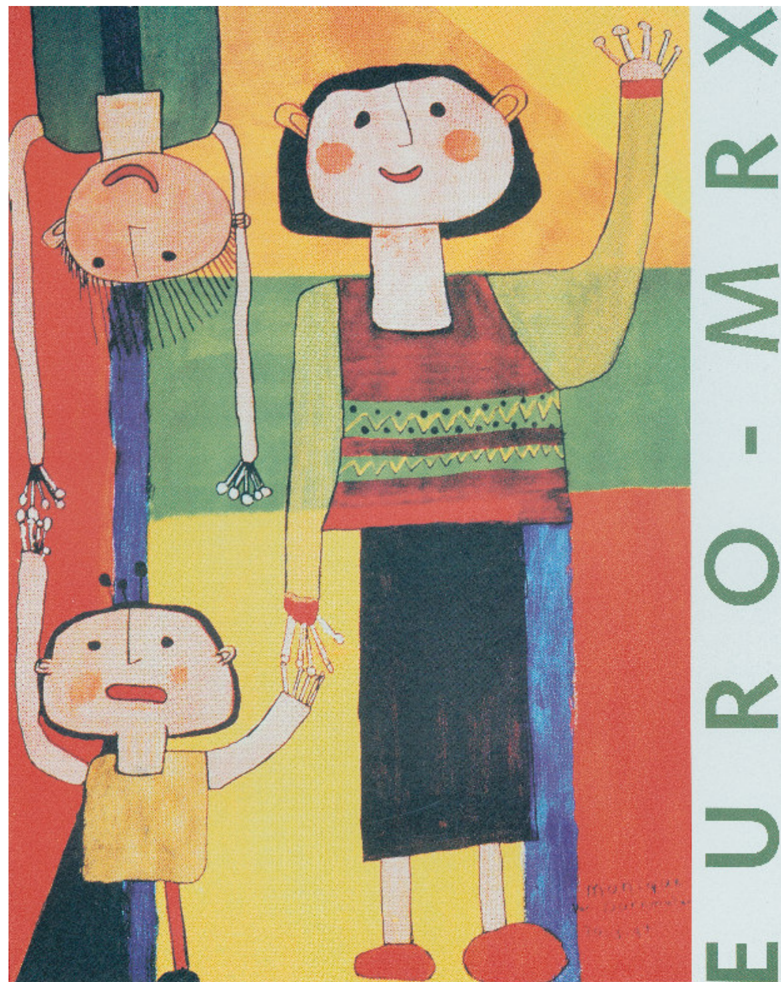


Materials & Methods



'Euro-MRX Logo' – The Netherlands, 1997, Monique van Dooremalen.

The Euro-MRX Consortium is a collaborative effort from several human genetics departments to collect patient material from families with presumed X-linked mental retardation. Their logo was created by a patient with Down's syndrome in creative studio 'D.C. De Ronde Hoep'. The German magazine 'Ohrenkuss', exclusively written by patients with Down's syndrome, is another initiative that challenges society's views on mental 'disability'.

IIA. Materials

A. Chemicals and chemical compounds

Table IIA-1 lists all chemicals, other than those exclusively employed for cell culturing, relevant to this study. The CAS registry number for each chemical is included as a unique identifier.

Table IIA-1 | **Chemicals and chemical compounds**

Chemical	CAS registry number	Supplier
Acetic acid	64-19-7	Merck
Acetic anhydride	108-24-7	Sigma
Acetonitrile	75-05-8	Merck
$^{32}\alpha$[P]dCTP	NA[§]	Amersham Biosciences
$^{33}\alpha$[P]UTP	NA[§]	Amersham Biosciences
$^{35}\alpha$[S]UTP	NA[§]	Amersham Biosciences
Ammonium acetate	631-61-8	Merck
APS	7727-54-0	Biorad
ATP	51963-61-2	Roche
Benzil	134-81-6	Sigma
Betaine	107-43-7	Sigma
β-mercaptoethanol	60-24-2	Merck
Biotin-16-dUTP	None	Roche
Boric acid	10043-35-3	Merck
Brij 58	9004-95-9	Serva
Bromphenol blue	115-39-9	Serva
Calcium chloride	10043-52-4	Merck
CBB R 250	6104-59-2	Serva
Cesium chloride	7647-17-8	Gibco BRL
Chelex 100	68954-42-7	BioRad
Chloroform	67-66-3	Merck
Citric acid	77-92-9	Merck
CTP	652154-13-7	Roche
DAPI	28718-90-3	Serva

Table IIA-1 | **Chemicals and chemical compounds**		
Chemical	**CAS registry number**	**Supplier**
dATP	1927-31-7	Fermentas & Roth
dCTP	102783-51-7	Fermentas & Roth
DEPC	1609-47-8	Sigma
Dextran blue	87915-38-6	Fluka
Dextran sulfate	9011-18-1	Pharmacia
dGTP	93919-41-6	Fermentas & Roth
1,4-diazobicyclo-2,2,2-octane	280-57-9	Serva
DIG-11-dUTP	123625-64-9	Roche
Disodium hydrogen phosphate	7558-79-4	Merck
DMSO	67-68-5	Sigma
DTT	3483-12-3	Invitrogen
dTTP	93939-78-7	Fermentas & Roth
dUTP	102814-08-4	Roche
EDTA	60-00-4	Merck
EGTA	67-42-5	Sigma
EtBr	1239-45-8	Serva
Ethanol	64-17-5	Merck
Ficoll 400	26873-85-8	Pharmacia
Formaldehyde – 37.0% (v/v)	50-00-0	Fluka Biochemika
Formamide	75-12-7	Fluka Biochemika
GA	111-30-8	Sigma
Glycerol	56-81-5	Merck
Glycine	56-40-6	Merck
Glycogen	9005-79-2	Roche
GTP	56001-37-7	Roche
HEPES	7365-45-9	Calbiochem
Hydrochloric acid – 32.0% (v/v)	7647-01-0	Merck
Imidazole	288-32-4	Fluka
IPTG	367-93-1	Appligene Oncor
Isopropanol	67-63-0	Merck
Latrunculin B	76343-94-7	Calbiochem
Lead citrate	512-26-5	Fluka
Lithium chloride	7447-41-8	Merck
LLnL	110044-82-1	Sigma
Magnesium chloride	7786-30-3	Merck
Methanol	67-56-1	Merck
3-methyl-1-butanol	123-51-3	Sigma
NP 40 Substitute	9063-89-2	Fluka

Table IIA-1 | **Chemicals and chemical compounds**

Chemical	CAS registry number	Supplier
PEG 6000	25322-68-3	Merck
PFA	30525-89-4	Merck
Phenol	108-95-2	Roth
Phenol red	143-74-8	Sigma
PIPES	5625-37-6	Sigma
PMSF	329-98-6	Sigma
Poly-vinylpyrrolidone	9003-39-8	Sigma
Potassium chloride	7447-40-7	Merck
Potassium dihydrogen phosphate	7778-77-0	Merck
SDS	151-21-3	Roth
Sephadex G-50	9048-71-9	Amersham Biosciences
Silver nitrate	7761-88-8	Merck
Sodium acetate	127-09-3	Calbiochem
Sodium azide	26628-22-8	Serva
Sodium borohydride	16940-66-2	Merck
Sodium cacodylate	6131-99-3	Merck
Sodium carbonate	497-19-8	Merck
Sodium chloride	7647-14-5	Roth
Sodium citrate	6132-04-3	Merck
Sodium deoxycholate	302-95-4	Merck
Sodium dihydrogen phosphate	7558-80-7	Merck
Sodium fluoride	7681-49-4	Sigma
Sodium hydroxide	1310-73-2	Merck
Sodium perchlorate	7791-07-3	Merck
Sodium thiosulfate	10102-17-7	Merck
Sucrose	57-50-1	Gibco BRL
TEAA	67533-12-4	Transgenomic
TEMED	110-18-9	Gibco BRL
Toluidine blue O	92-31-9	Sigma
Triethanolamine	102-71-6	Merck
Triethylamine	121-44-8	Fluka Biochemika
Tris	77-86-1	Merck
Tris·HCl	1185-53-1	Merck
TRITC-phalloidin	219920-04-4	Molecular Probes
Triton X-100	9002-93-1	Roth
Tween-20	9005-64-5	Sigma
Uranyl acetate	6159-44-0	Fluka
Urea	57-13-6	Merck

Table IIA-1 | **Chemicals and chemical compounds**		
Chemical	**CAS registry number**	**Supplier**
UTP	19817-92-6	Roche
Water	7732-18-5	Baxter Deutschland
X-gal	7240-90-6	Appligene Oncor
Xylene	1330-20-7	Roth
Xylene cyanol	2650-17-1	Sigma

§ Even after repeated enquiries, Amersham Biosciences was not able to supply this information.

B. Reagents

B.1. Biological reagents

B.1.1. General

Table IIA-2 lists all general biological reagents, other than those exclusively employed for cell culturing, relevant to this study.

Table IIA-2 | **Biological reagents – General**	
Reagent	**Supplier**
Agarose	Invitrogen
Milk powder	Protifar
NuSieve agarose	FMC Bioproducts

B.1.2. RNA/DNA

Table IIA-3 lists all nucleic acid-related reagents relevant to this study. PCR primers are listed in Appendix E.

Table IIA-3 | **Biological reagents – RNA/DNA**	
RNA/DNA	**Supplier**
Cot-1 DNA	Gibco BRL
E. coli total RNA	Courtesy of D. Meunier, MPI-MG, Berlin, Germany
GeneRuler 100 bp DNA Ladder Plus	Fermentas
GeneRuler 1 kb DNA Ladder	Fermentas
Herring sperm DNA	Sigma
Hybridime	HT Biotechnology
$pd(N)_6$	Amersham Pharmacia
Yeast total RNA	Courtesy of Dr. E. Minina, MPI-MG, Berlin, Germany

B.1.3. Protein

B.1.3.1. General

Table IIA-4 lists all general protein-related reagents relevant to this study.

Table IIA-4 | **Protein-related reagents – General**

Protein	MW (kDa)	Supplier
BSA, cryst. lyophil.	67	Serva
BSA-C	~67	Aurion
hKIAA1202 peptide	3	Biosyntan
Protein A-agarose	NA	Roche
Protein G-agarose	NA	Roche
Rainbow coloured protein MW marker	14.3 – 220	Amersham Biosciences
RNAguard RNase Inhibitor	NA	Amersham Pharmacia Biotech

B.1.3.2. Antibodies and immunoglobulins

Table IIA-5 lists all primary and secondary antibodies, and immunoglobulins relevant to this study.

Table IIA-5 | **Protein-related reagents – Antibodies and immunoglobulins**

Antigen	Clonality	Isotype	Conjugated with	Dilution§			Supplier
				WB	IF & EM	Co-IP (µg)	
				Primaries			
11 C-terminal Actin amino acids	Polyclonal	Rabbit IgG	-	1:500 in PBST$^{M\text{-}5.00\%}$	NA	1.00	Sigma
C-terminus of Actin	Polyclonal	Goat IgG	-	1:1000 in PBST$^{M\text{-}5.00\%}$	1:100 in 1stAbD	1.00 – 2.50	Santa Cruz Biotech-nology
Avidin D	Polyclonal	Goat IgG	FITC	NA	1:200 in FISH – AbD	NA	Vector Laboratories
Cdc34†	Polyclonal	Rabbit IgG	-	1:330 in PBST$^{BSA\text{-}1.00\%}$	NA	7.50	Abcam
11 C-terminal hCul1 amino acids	Polyclonal	Rabbit IgG	-	1:500 in PBST$^{BSA\text{-}1.00\%}$	NA	5.00	Zymed Laboratories
DIG	Monoclonal	Mouse IgG	Cy3	NA	1:200 in FISH – AbD	NA	Dianova
hGolgin-97	Monoclonal	Mouse IgG$_{1,\kappa}$	-	NA	1:500 in 1stAbD	NA	Molecular Probes
HA epitope	Monoclonal	Mouse IgG$_1$	-	1:1000 in PBST$^{M\text{-}5.00\%}$	NA	1.00 – 1.50	Covance
hKIAA1202 peptide	Polyclonal	Rabbit IgG	-	1:300 in PBST$^{BSA\text{-}1.00\%}$	1:10 in 1stAbD & 1:3 – 1:10 in EM – Buffer B	5.00	BioGenes
rProhibitin	Polyclonal	Rabbit IgG	-	NA	1:100 in 1stAbD	NA	Abcam
hRoc1 AA 97 – 108	Polyclonal	Rabbit IgG	-	1:250 in PBST$^{BSA\text{-}1.00\%}$	NA	10.0	Abcam

Table IIA-5 | **Protein-related reagents – Antibodies and immunoglobulins**

Antigen	Clonality	Isotype	Conjugated with	Dilution[§]			Supplier
				WB	IF & EM	Co-IP (µg)	
hSkp1 (p19)	Monoclonal	Mouse IgG_1	-	1:250 in $PBST^{BSA\text{-}1.00\%}$	NA	7.50	Zymed Laboratories
bUbiquitin	Monoclonal	Mouse IgG_1	-	1:200 in $PBST^{BSA\text{-}2.00\%}$	NA	5.00	Santa Cruz Biotech-nology
V5 epitope	Monoclonal	Mouse IgG_{2a}	-	1:3000 in $PBST^{M\text{-}5.00\%}$	1:500 in 1[st]AbD & 1:50 – 1:100 in EM – Buffer B	1.00 – 2.00	Invitrogen
V5 epitope	Polyclonal	Rabbit IgG	-	-	1:20 – 1:300 in EM – Buffer B	-	Abcam
hVimentin	Polyclonal	Neat goat serum	-	1:1000 in $PBST^{M\text{-}5.00\%}$	1:100 in 1[st]AbD	5.00	Chemicon
Secondaries							
Mouse-IgG	Polyclonal	Donkey IgG	Cy3	NA	1:1000 in 2[nd]AbD	NA	Dianova
Goat-IgG	Polyclonal	Donkey IgG	Cy3	NA	1:200 in 2[nd]AbD	NA	Dianova
Rabbit-IgG	Polyclonal	Goat IgG	Cy3	NA	1:200 in 2[nd]AbD	NA	Dianova
Mouse-IgG	Polyclonal	Goat IgG	FITC	NA	1:1000 in 2[nd]AbD	NA	Dianova
Goat-IgG	Polyclonal	Donkey IgG	FITC	NA	1:100 in 2[nd]AbD	NA	Dianova
Rabbit-IgG	Polyclonal	Donkey IgG	FITC	NA	1:250 in 2[nd]AbD	NA	Dianova
Mouse-IgG	Polyclonal	Goat IgG	Gold – 10 nm	NA	1:20 – 1:50 in EM – Buffer B	NA	BB International
Rabbit-IgG	Polyclonal	Goat IgG	Gold – 10 nm	NA	1:10 – 1:80 in EM – Buffer B	NA	BB International
Mouse-IgG	Polyclonal	Goat IgG	Gold – 15 nm	NA	1:20 – 1:50 in EM – Buffer B	NA	BB International
Mouse-IgG	Polyclonal	Goat IgG	HRP	1:2000 in PBST	NA	NA	Dianova
Goat-IgG	Polyclonal	Donkey IgG	HRP	1:30000 in $PBST^{M\text{-}5.00\%}$	NA	NA	Dianova
Rabbit-IgG	Polyclonal	Donkey IgG	HRP	1:2000 in PBST	NA	NA	Amersham Biosci-ences

Table IIA-5 | **Protein-related reagents – Antibodies and immunoglobulins**

Antigen	Clonality	Isotype	Conjugated with	Dilution§			Supplier
				WB	IF & EM	Co-IP (µg)	
Control immunoglobulines							
NA	Polyclonal	Mouse IgG	NA	NA	NA	1.00 – 2.00	Santa Cruz Biotech-nology
NA	Polyclonal	Goat IgG	NA	NA	NA	1.00 – 5.00	Santa Cruz Biotech-nology
NA	Polyclonal	Rabbit IgG	NA	NA	NA	1.00 – 5.00	Santa Cruz Biotech-nology

§ The composition of each diluent is specified in Table IIA-11.
† The exact antigen is proprietary information from Abcam.

B.1.3.3. Enzymes

B.1.3.3.1. General

Table IIA-6 lists all general enzymes relevant to this study. The EC number for each enzyme is included as an additional identifier. Most enzymes are recombinant.

Table IIA-6 | **Enzymes – General**

Enzyme	**EC**	**Application**	**$T_{max. activity}$ (°C)**	**Specific activity**	**Supplier**
AmpliTaq DNA polymerase	2.7.7.7	Conventional PCR	72	5 U/µl	Perkin Elmer
DNA polymerase I, Klenow fragment	2.7.7.7	RA labelling of DNA probes	37	5 U/µl	USB
DNA polymerase I, NT grade	2.7.7.7	Non-radioactive labelling of DNA probes	37	5 U/µl	Roche
DNase-free RNase A	3.1.27.5	Non-specific RNA digestion	37	50 U/mg	Roche
Expand DNA polymerase	2.7.7.7	Long-range PCR	68	5 U/µl	Roche
Lysozyme	3.2.1.17	Bacteriolysis	25 – 30	135 U/µg	Roche
Pepsin A (porcine)	3.4.23.1	Non-specific protein digestion	37	~15 milliAnson-U/mg[698]	Serva
Pronase B	3.4.21.81	Non-specific protein digestion	40 – 60	7 U/mg	Roche
Proteinase K	3.4.21.64	Non-specific protein digestion	65	30 U/mg	Fermentas
RNase-free DNase I	3.1.21.1	Non-specific DNA digestion	37	10 U/µl	Roche
SP6 RNA polymerase	2.7.7.6	*In vitro* transcription	37	≥ 20 U/µl	Roche
Superscript II RNase H^- reverse transcriptase	2.7.7.49	RT	42	200 U/µl	Invitrogen
T4 DNA ligase	6.5.1.2	DNA ligation	4 – 16	3 U/µl	Promega
T7 RNA polymerase	2.7.7.6	*In vitro* transcription	37	≥ 20 U/µl	Roche
TdT	2.7.7.31	Homo-polymeric cDNA tailing	37	15 U/µl	Invitrogen

B.1.3.3.2. Restriction endonucleases

Table IIA-7 lists all restriction endonucleases relevant to this study.

Table IIA-7 | **Enzymes – Restriction endonucleases**

Restriction Endonuclease	**$T_{max.\ activity}$ (°C)**	**Buffer**	**Thermal inactivation**	**Supplier**
*Aci*I	37	NEBuffer 3	65°C – 20'	New England BioLabs
*Age*I	37	Buffer O^+	65°C – 20'	Fermentas
*Alu*I	37	Buffer Tango	65°C – 20'	Fermentas
*Bam*HI	37	REact 3	NA	Gibco BRL
*Bgl*II	37	REact 3	NA	Gibco BRL
*Cla*I	37	REact 1	NA	Invitrogen
*Dra*I	37	Buffer B^+	65°C – 20'	Fermentas
*Eco*RI	37	REact 3	NA	Gibco BRL
*Hinc*II	37	REact 4	65°C – 10'	Gibco BRL
*Hind*III	37	REact 2	65°C – 20'	New England BioLabs
*Kpn*I	37	NEBuffer 1 + 0.01% (w/v) BSA	NA	New England BioLabs
*Nhe*I	37	REact 4	NA	Invitrogen
*Not*I	37	Buffer O^+	65°C – 20'	Fermentas
*Pvu*II	37	REact 6	NA	Gibco BRL
*Sac*II	37	REact 2	NA	Gibco BRL
*Sal*I	37	REact 10	NA	Gibco BRL
*Tai*I	65	Buffer R^+	NA	Fermentas
*Xba*I	37	REact 2	NA	Gibco BRL
*Xho*I	37	REact 2	NA	Gibco BRL

B.2. Non-biological reagents

Table IIA-8 lists all non-biological reagents relevant to this study.

Table IIA-8 | **Non-biological reagents**

Reagent	**Application**	**Supplier**
Acrylamide:bis-acrylamide	PAGE (e.g. SSCP, Western blotting)	Sigma
Aqueous phenol, pH 4.5 – 5.0	RNA extraction	Roth
Auto-radiographic emulsion NTB2	Photographic emulsion for use in mouse ISH using $^{33}\alpha$[P]UTP	Kodak
Bradford Reagent	Colourimetric measurement of protein concentration	Sigma

Table IIA-8 | **Non-biological reagents**

Reagent	**Application**	**Supplier**
Complete Mini	Protease inhibitor cocktail tablets	Roche
D-19 Developer	Photograpic developer for use in mouse ISH	Kodak
DPX Mountant for histology	Mounting medium for dark field micros-copy	Fluka
G153 Developer	Developer for the Curix 60	Agfa
G354 Fixer	Fixer for the Curix 60	Agfa
Gentle Buffer	Eluent for antibody affinity purification	Pierce Biotechnology
Immersol 518F	Immersion oil for magnifications ≥ 400×	Zeiss
K5 Nuclear Emulsion	Photographic emulsion for use in mouse ISH using $^{35}\alpha$[S]UTP	Ilford
Kodak Fixer	Photograpic fixer for use in mouse ISH	Kodak
Lipofectamine 2000	Transfection reagent	Invitrogen
6 × Loading Dye Solution	DNA loading dye	Fermentas
LR Gold	Acrylic embedding resin for use in EM	LR Company
LR White	Acrylic embedding resin for use in EM	LR Company
Mayer's Hemalum Solution	Hematoxylin-containing histological dye	Merck
Nail varnish	Sealing off of coverslips on microscope slides	Jade
Paraffin	Mounting of tissues for mouse ISH	Sherwood Medical Company
Ponceau S Staining Solution	Reversible protein staining	Sigma
Ready Value Cocktail	Scintillation liquid for the LS 6000TA scintillation counter	Beckman Coulter
Restore Western Blot Stripping Buffer	Removal of primary antibodies from PVDF membranes	Pierce Biotechnology
Ribonucleoside vanadyl com-plexes	RNase inhibitor	New England Bio-Labs
Roti-Histokitt	Mounting medium for use in histology	Roth
Sulfolink Matrix	Antibody affinity purification	Pierce Biotechnology
Trizol	RNA isolation	Gibco BRL
Vectashield mounting medium	Mounting medium for fluorescence mi-croscopy	Vector Laboratories
Western Lightning Chemilumines-cence Reagent Plus	Chemiluminscence-based detection sys-tem for western blotting	Perkin Elmer

C. Vectors & Plasmids

C.1. Vectors

Table IIA-9 lists all vectors relevant to this study.

Table IIA-9 | **Vectors**

Name	Type	Supplier
pBluescript II SK(+)	Ampicillin-resistant cloning vector, 2958 bp	Stratagene
pBTM116-D9	As pBTM117c, but adapted for site-specific recombination cloning	RZPD
pBTM117c	Ampicillin-resistant yeast expression vector with N-terminal LEX A DNA BD, 9350 bp	Courtesy of Dr. E. Wanker, MDC Berlin, Germany
pcDNA4/cMyc-V5-HisB	Ampicillin/Zeocin-resistant mammalian expression vector with N-terminal cMyc and C-terminal V5 and 6× His tag, 5122 bp	Courtesy of J. Ruschmann, MPI-MG, Berlin, Germany
pcDNA4/FLAG-V5-HisB	Ampicillin/Zeocin-resistant mammalian expression vector with N-terminal FLAG and C-terminal V5 and 6× His tag, 5128 bp	Courtesy of J. Ruschmann, MPI-MG, Berlin, Germany
pcDNA4/V5-HisB	Ampicillin/Zeocin-resistant mammalian expression vector with C-terminal V5 and 6× His tag, 5091 bp	Invitrogen
pDONR201	Kanamycin/Chloramphenicol-resistant cloning vector suitable for site-specific recombination cloning, 4470 bp	Invitrogen
pEGFP-C1	Kanamycin-resistant mammalian expression vector with N-terminal EGFP tag, 4731 bp	Clontech
pEGFP-N3	Kanamycin-resistant mammalian expression vector with C-terminal EGFP tag, 4729 bp	Clontech
pGAD426	Ampicillin-resistant yeast expression vector with N-terminal GAL4 AD, 7870 bp	Courtesy of Dr. E. Wanker, MDC Berlin, Germany
pGAD426-D3	As pGAD426, but adapted for site-specific recombination cloning	RZPD
pGEM-T Easy	Ampicillin-resistant TA-cloning vector, 3015 bp	Promega
pTL1-HA3	Ampicillin-resistant mammalian expression vector with N-terminal HA tag, ~4100 bp	Courtesy of Dr. E. Wanker, MDC Berlin, Germany

C.2. Plasmids

Table IIA-10 lists all plasmids constructed in the course of this study.

Table IIA-10 | **Plasmids**

Name	**Vector**	**Insert**	**Experimental use**
hFBXO25-ORF1/2-EGFP	pEGFP-N3	*Xho*I/*Sac*II hFBXO25 ORF1/2	Subcellular localisation
EGFP-hFBXO25-ORF1/2	pEGFP-C1	*Xho*I/*Sac*II hFBXO25 ORF1/2	Subcellular localisation
hFBXO25-ORF1/2-V5	pcDNA4/V5-HisB	*Nhe*I/*Bam*HI hFBXO25 ORF1/2	Subcellular localisation, co-IP
hFBXO25-ORF1ΔF-V5	pcDNA4/V5-HisB	*Xho*I/*Xba*I hFBXO25 ORF1ΔF	Co-IP
hFBXO25-S244L-V5	pcDNA4/V5-HisB	hFBXO25 ORF1-S244L	Co-IP
hFBXO4-V5	pcDNA4/V5-HisB	*Bam*HI/*Not*I hFBXO4	Co-IP, positive control
hFBXO7-V5	pcDNA4/V5-HisB	*Eco*RI/*Not*I hFBXO7	Co-IP, positive control
pGEM-mFbxo25	pGEM-T Easy	*mFbxo25* exons 6 – 10	*mFbxo25* ISH
hKIAA1202-Blue	pBluescript II SK(+)	*Eco*RI/*Cla*I hKIAA1202 ORF I	Starting point for subcloning
hKIAA1202-EGFP	pEGFP-N3	*Eco*RI/*Sal*I hKIAA1202 ORF I	Subcellular localisation
EGFP-hKIAA1202	pEGFP-C1	*Eco*RI/*Sal*I hKIAA1202 ORF I	Subcellular localisation
hKIAA1202-V5	pcDNA4/V5-HisB	*Hin*dIII/*Kpn*I hKIAA1202 ORF I	Subcellular localisation, co-IP
hKIAA1202-HA	pcDNA4/HA-HisB	*Hin*dIII/*Kpn*I hKIAA1202 ORF I	Co-IP
HA-hKIAA1202	pTL1-HA3	*Hin*dIII/*Kpn*I hKIAA1202 ORF I	Co-IP
cMyc-hKIAA1202-V5	pcDNA4/cMyc-V5-HisB	*Hin*dIII/*Kpn*I hKIAA1202 ORF I	Co-IP
FLAG-hKIAA1202-V5	pcDNA4/FLAG-V5-HisB	*Hin*dIII/*Kpn*I hKIAA1202 ORF I	Co-IP
MOM-hKIAA1202-V5	pcDNA4/MOM-V5-HisB	*Hin*dIII/*Kpn*I hKIAA1202 ORF I	MOM recruitment assay
GAL4AD-hKIAA1202-Y2H-1/2/3/4/5/6	pGAD426	*Sal*I/*Not*I hKIAA1202 Y2H-1/2/3/4/5/6	Y2H screen, 'prey'
LexA-DBD-hKIAA1202-Y2H-1/2/3/4/5/6	pBTM117c	*Sal*I/*Not*I hKIAA1202 Y2H-1/2/3/4/5/6	Y2H screen, 'bait'
GAL4AD-hKIAA1202-Y2H-4 – 6	pGAD426	*Sal*I/*Not*I hKIAA1202 Y2H-4 – 6	Y2H screen, 'prey'
LexA-DBD-hKIAA1202-Y2H-4 – 6	pBTM117c	*Sal*I/*Not*I hKIAA1202 Y2H-4 – 6	Y2H screen, 'bait'

Table IIA-10 | **Plasmids**			
Name	**Vector**	**Insert**	**Experimental use**
hKIAA1202-Y2H-4-V5	pcDNA4/V5-HisB	*Hin*dIII/*Apa*I hKIAA1202 Y2H-4	GA oligomerisation assay
pGEM-mKiaa1202-1	pGEM-T Easy	*mKiaa1202* exons 4 – 6	*mKiaa1202* ISH, probe 1
pGEM-mKiaa1202-2	pGEM-T Easy	*mKiaa1202* exons 7 – 9	*mKiaa1202* ISH, probe 2

D. Buffers and solutions

Table IIA-11 lists all buffers and solutions relevant to this study. Please note that buffers and solutions are tabulated at stock concentrations but were used at diluted working concentrations, unless otherwise mentioned.

Table IIA-11 | **Buffers and solutions**	
Buffer	**Composition**
Blood Cell Lysis Buffer	400 mM Tris·HCl, pH 8.0; 60.0 mM EDTA, pH 8.0; 150 mM NaCl; 34.7 mM SDS *ad volumina* ddH_2O
Brij-Mix	0.800% (w/v) Brij 58; 7.70 mM sodium deoxycholate; 40.0 mM Tris, pH 8.0; 50.0 mM EDTA, pH 8.0 *ad volumina* ddH_2O
Buffer C	50.0 mM Tris·HCl, pH 7.5; 300 mM NaCl; 5.00 mM EDTA, pH 8.0; 3.10 mM NaN_3 *ad volumina* ddH_2O
CBB R 250 Destaining Solution	1.75 M glacial acetic acid; 9.86 M methanol *ad volumina* ddH_2O
CBB R 250 Staining Solution	1.21 mM CBB R 250; 1.75 M glacial acetic acid; 9.86 M methanol *ad volumina* ddH_2O
Cross-linking – Buffer A	34.2 mM Na_2HPO_4; 15.8 mM NaH_2PO_4; 250 mM NaCl *ad volumina* ddH_2O, pH 7.2
Cross-linking – Buffer B	13.7 mM Na_2HPO_4; 6.30 mM NaH_2PO_4; 150 mM NaCl; 1.00 mM DTT; 5.00 mM GA; 680 mM glycerol; 0.500‰ (v/v) NP 40 Substitute *ad volumina* ddH_2O, pH 7.2
50× Denhardt's	1.00% (w/v) Ficoll 400; 1.00% (w/v) poly-vinylpyrrolidone; 1.00% (w/v) BSA *ad volumina* ddH_2O
DEPC-treated ddH_2O	6.80 mM DEPC *ad volumina* ddH_2O

Table IIA-11 | **Buffers and solutions**

Buffer	**Composition**
DHPLC – Buffer A	100 mM TEAA; 19.2 mM acetonitrile *ad volumina* ddH_2O
DHPLC – Buffer B	100 mM TEAA; 4.79 M acetonitrile *ad volumina* ddH_2O
EM – Buffer A	20.0 mM Tris·HCl, pH 7.4; 150 mM NaCl *ad volumina* ddH_2O
EM – Buffer B	0.300% (w/v) BSA-C; 20.0 mM Tris·HCl, pH 7.4; 150 mM NaCl *ad volumina* ddH_2O
Erylysis Buffer	10.0 mM Tris·HCl, pH 8.0; 320 mM sucrose; 5.00 mM $MgCl_2$; 1.00% (v/v) Triton X-100 *ad volumina* ddH_2O
FISH – AbD	1.00% (w/v) BSA; 0.100% (v/v) Tween-20 *ad volumina* 4× SSC
FISH – Blocking Buffer	5.00% (w/v) BSA; 0.100% (v/v) Tween-20 *ad volumina* 4× SSC
FISH – Mounting Medium	12.3 M glycerol; 100 mM Tris·HCl, pH 8.0; 205 mM 1,4-diazobicyclo-2,2,2-octane *ad volumina* ddH_2O
FISH – Wash Buffer	0.100% (v/v) Tween-20 *ad volumina* 4× SSC
IF – 1^{st}AbD	0.200% (v/v) Tween-20; 4.00% (w/v) BSA *ad volumina* PBS
IF – 2^{nd}AbD	3.00% (v/v) HeLa total cell lysate in Pagano Lysis Buffer; 0.200% (v/v) Tween-20; 4.00% (w/v) BSA *ad volumina* PBS
IF – Fixing Solution	1.20 M PFA; 10.0 mM GA; 12.0% (v/v) 10× PEM *ad volumina* ddH_2O
10× Lämmli Buffer	250 mM Tris; 1.90 M glycin; 6.94 mM SDS *ad volumina* ddH_2O
2× Magic Mix	8.00 M urea; 15.0 mM Tris·HCl, pH 7.5; 1.20 M glycerol; 34.7 mM SDS; 59.7 μM bromphenol blue; 143 mM β-mercaptoethanol *ad volumina* ddH_2O When used as lysis buffer, add 1 Complete Mini proteinase inhibitor tablet per 10 ml shortly before use.
Mouse ISH – Hybridisation Buffer ($^{33}\alpha$[P]UTP)	12.5 M formamide; 3.00 M NaCl; 20.0 mM Tris·HCl, pH 7.5; 10.0 mM NaH_2PO_4, pH 8.0; 5.00 mM EDTA, pH 8.0; 10.0% (w/v) dextran sulfate; 1:50 50× Denhardt's; 0.500‰ (w/v) yeast total RNA *ad volumina* DEPC-treated ddH_2O
Mouse ISH – Hybridisation Buffer ($^{35}\alpha$[S]UTP)	13.6% (v/v) 20× SSC; 0.450‰ (w/v) *E. coli* total RNA; 0.910‰ (w/v) herring sperm DNA; 0.182% (w/v) BSA; 9.10 mM ribonucleoside vanadyl complexes; 11.4 M formamide; 9.10% (w/v) dextran sulfate; 273 mM DTT *ad volumina* DEPC-treated ddH_2O

Table IIA-11 | **Buffers and solutions**

Buffer	**Composition**
Mouse ISH – Proteinase K Digestion Buffer ($^{33}\alpha$[P]UTP)	50.0 mM Tris·HCl, pH 7.5; 5.00 mM EDTA, pH 8.0; 600 mU/ml proteinase K *ad volumina* DEPC-treated ddH_2O
Mouse ISH – Proteinase K Digestion Buffer ($^{35}\alpha$[S]UTP)	200 mM Tris; 2.00 mM $CaCl_2$; 60.0 mU/ml proteinase K *ad volumina* DEPC-treated ddH_2O
Mouse ISH – RNase A Buffer ($^{33}\alpha$[P]UTP)	100 mM Tris·HCl, pH 7.5; 50.0 mM EDTA, pH 8.0; 4.00 M NaCl; 1.00 U/ml RNase A *ad volumina* ddH_2O
Mouse ISH – RNase A Buffer ($^{35}\alpha$[S]UTP)	1.00 U/ml RNase A *ad volumina* 2× SSC, pH 7.0
Mouse ISH – Wash Buffer ($^{35}\alpha$[S]UTP)	10.0% (v/v) 20× SSC; 12.5 M deionised formamide; 10.0 mM DTT *ad volumina* ddH_2O
Ni-NTA Elution Buffer	50.0 mM NaH_2PO_4; 300 mM NaCl; 250 mM imidazole; 0.500‰ (v/v) Tween-20 *ad volumina* ddH_2O, pH 8.0 Add 1 Complete Mini proteinase inhibitor tablet per 10 ml shortly before use. Keep at 4℃ at all times.
Ni-NTA Lysis Buffer	As Ni-NTA Elution Buffer, but with 10.0 mM instead of 250 mM imidazole
Ni-NTA Wash Buffer	As Ni-NTA Elution Buffer, but with 20.0 mM instead of 250 mM imidazole
NT-Buffer	500 mM Tris·HCl, pH 7.9; 50.0 mM $MgCl_2$; 0.500‰ (w/v) BSA *ad volumina* ddH_2O
Nucleus Buffer	10.0 mM Tris·HCl, pH 7.5; 25.0 mM EDTA, pH 8.0; 75.0 mM NaCl *ad volumina* ddH_2O
5× OLB	236 µM Tris·HCl, pH 8.0; 23.3 mM $MgCl_2$; 1.00 M HEPES, pH 6.6; 48.6 mM β-mercaptoethanol; 0.190 mM dATP; 0.190 mM dGTP; 0.190 mM dTTP 50 A_{260} units $pd(N)_6$ per 25 ml *ad volumina* TE, pH 7.5
Pagano Lysis Buffer	50.0 mM Tris·HCl, pH 7.5; 250 mM NaCl; 5.00 mM EDTA, pH 8.0; 0.100% (v/v) Triton X-100; 50.0 mM NaF; 0.100 mM Na_3VO_4; 0.100 mM PMSF *ad volumina* ddH_2O Add 1 Complete Mini proteinase inhibitor tablet per 10 ml shortly before use. Keep at 4℃ at all times.
10× PBS	1.37 M NaCl; 27.0 mM KCl; 100 mM Na_2HPO_4; 20.0 mM KH_2PO_4 *ad volumina* ddH_2O, pH 7.4
PBST	0.100% (v/v) Tween-20 *ad volumina* PBS
$PBST^{BSA\text{-}0.500/1.00/2.00\%}$	0.500/1.00/2.00% (w/v) BSA *ad volumina* PBST
$PBST^{M\text{-}5.00\%}$	5.00% (w/v) milk powder *ad volumina* PBST

Table IIA-11 | **Buffers and solutions**

Buffer	**Composition**
PEG Buffer	85.5 mM Na_2HPO_4; 39.5 mM NaH_2PO_4; 250 mM NaCl; 243 mM SDS; 1.00 mM EDTA, pH 8.0; 10.0% (w/v) PEG 6000 *ad volumina* ddH_2O, pH 7.2
10× PEM	1.00 M PIPES; 50.0 mM EGTA; 20.0 mM $MgCl_2$ *ad volumina* ddH_2O, pH 7.0
5× SDS-PAGE Loading Buffer	2.15 M β-mercaptoethanol; 520 mM SDS; 22.4 mM bromphenol blue; 6.84 M glycerol; 138 mM Tris·HCl, pH 6.8 *ad volumina* ddH_2O
Serra's	10.3 M ethanol; 1.75 M acetic acid *ad volumina* formaldehyde – 37% (v/v)
Southern hybridisation – Denaturation Buffer	1.50 M NaCl; 500 mM NaOH *ad volumina* ddH_2O
Southern hybridisation – Neutralisation Buffer	1.50 M NaCl; 500 mM Tris·HCl, pH7.5 *ad volumina* ddH_2O
20× SSC	3.00 M NaCl; 300 mM Na-citrate *ad volumina* ddH_2O, pH 7.0
SSCP – 2× Sample Buffer	24.6 M deionised formamide; 10.0 mM EDTA, pH 8.0; 1.50 mM bromphenol blue; 1.90 mM xylene cyanol *ad volumina* ddH_2O
SSCP – Developer Solution	375 mM NaOH; 2.60 mM $NaBH_4$; 1.50‰ (v/v) formaldehyde *ad volumina* ddH_2O
SSCP – DNA Fixation Solution	1.70 M ethanol; 87.4 mM acetic acid *ad volumina* ddH_2O
Stop Solution for RA Labelling	10.0 ml TES; 5 spatula tips dextran blue; 1 spatula tip phenol red
50× TAE	2.00 M Tris; 1.00 M acetic acid; 50.0 mM EDTA, pH 8.0 *ad volumina* ddH_2O
10× TBE	900 mM Tris; 900 mM boric acid; 20.0 mM EDTA, pH 8.0 *ad volumina* ddH_2O
TE Buffer – pH 7.5/8.0	10.0 mM Tris·HCl; pH 7.5/8.0; 1.00 mM EDTA, pH 8.0 *ad volumina* ddH_2O
TES	10.0 mM Tris·HCl, pH 7.5; 5.00 mM EDTA, pH 8.0; 6.94 mM SDS *ad volumina* ddH_2O
Tris·HCl pH 7.0/7.5/8.0	1.00 M Tris·HCl *ad volumina* ddH_2O, pH 7.0/7.5/8.0
Western hybridisation – 5× Blotting Buffer	240 mM Tris; 195 mM glycine; 6.94 mM SDS *ad volumina* ddH_2O
Western hybridisation – Stripping Buffer	150 mM glycine, pH 2.5; 13.9 mM SDS *ad volumina* ddH_2O

E. Cell culture

E.1. General reagents

Table IIA-12 lists all cell culture reagents, other than media and cells, relevant to this study. Where applicable, the CAS registry number is included.

Table IIA-12 | **Cell culture reagents – General**

Reagent	CAS registry number	Supplier
Agar	NA	Difco
Ampicillin	69-53-4	Sigma
Chloramphenicol	56-75-7	Sigma
Colcemid	477-30-5	Gibco BRL
Collagen	9007-34-5	Roche
Dextrose	14431-43-7	Sigma
DPBS	NA	Cambrex
FBS	NA	Biochrom
Glucose	14431-43-7	Merck
Herbimycin A	70563-58-5	Alexis Biochemicals
Kanamycin	70560-51-9	Gibco BRL
L-Glu	56-85-9	Cambrex
NEAA	NA	Cambrex
Penicillin/Streptomycin	113-98-4/3810-74-0	Cambrex
Peptone	NA	Difco
Retinoic acid	302-79-4	Sigma
Sodium orthovanadate	13721-39-6	Sigma
Sodium pyruvate	113-24-6	Biochrom
Tryptone	NA	Difco
Yeast extract	NA	Difco
Yeast nitrogen base w/o amino acids	NA	Difco
Zeocin	11006-33-0	Invitrogen

E.2. Media

Table IIA-13 lists all cell culture media relevant to this study.

Table IIA-13 | **Cell culture reagents – Media**

Medium	Formulation	Supplier
DMEM	Cambrex' DMEM is formulated as described by Dulbecco and Freeman[699].	Cambrex

Table IIA-13 | **Cell culture reagents – Media**

Medium	**Formulation**	**Supplier**
EMEM	Cambrex' EMEM is formulated as described by Eagle[700] with the following adaptations: omission of 2.00 mM L-Glu and 5.00 – 10.0% (v/v) FBS; addition of 0.800 mM $MgSO_4 \cdot 7H_2O$ and 28.2 µM phenol red; 26.2 instead of 23.8 mM $NaHCO_3$ and 0.200 mM L-Cys instead of 0.100 mM L-Cystine.	Cambrex
LB – Liquid Culture	1.00% (w/v) tryptone; 0.500% (w/v) yeast extract; 170 mM NaCl *ad volumina* ddH_2O, pH 7.0	NA
LB – Plate	LB – Liquid Culture medium supplemented with 1.50% (w/v) agar	NA
Neuro-2A – Differentiation Medium	Essentially formulated as described by Riboni *et al.*[701]. 2.00% (v/v) FBS; 100 U/ml penicillin; 68.6 µM streptomycin; 2.00 mM L-Glu; 1.00% (v/v) NEAA; 20.0 µM retinoic acid *ad volumina* DMEM	NA
Opti-MEM with GlutaMAX I	Contains GlutaMAX I, 28.6 mM sodium bicarbonate, HEPES, sodium pyruvate, hypoxanthine, thymidine, trace elements, growth factors, 0.280 µM phenol red. The exact formulation is proprietary information from Invitrogen.	Invitrogen
RPMI-1640	Cambrex' RPMI-1640 is formulated as described by Moore *et al.*[702].	Cambrex
SD – Liquid Culture	0.67% (w/v) yeast nitrogen base w/o amino acids; 100 mM dextrose Supplemented as described in Adams *et al.*[703], but omitting relevant ingredients for drop-out media *ad volumina* ddH_2O	NA
SD – Plate	SD – Liquid Culture medium supplemented with 2.00% (w/v) agar	NA
SH-SY5Y – Differentiation Medium	A combination of differentiation media described by Preis *et al.*[704] and Rogers *et al.*[705]. 15.0% (v/v) FBS; 100 U/ml penicillin; 68.6 µM streptomycin; 2.00 mM L-Glu; 10.0 µM retinoic acid; 236 nM herbimycin A; 10.0 µM activated sodium orthovanadate *ad volumina* DMEM	NA
SOC	2.00% (w/v) tryptone; 0.500% (w/v) yeast extract; 8.50 mM NaCl; 10.0 mM $MgCl_2$; 2.5 mM KCl; 20.0 mM glucose *ad volumina* ddH_2O, pH 7.0	NA
YPD – Liquid Culture	1.00% (w/v) yeast extract; 2.00% (w/v) peptone; 100 mM dextrose *ad volumina* ddH_2O	NA
YPD – Plate	YPD – Liquid Culture medium supplemented with 2.00% (w/v) agar	NA
2× TY	1.60% (w/v) tryptone; 1.00% (w/v) yeast extract; 85.5 mM NaCl *ad volumina* ddH_2O, pH 7.0	NA

F. Cells

F.1. Mammalian cell lines

Table IIA-14 lists all mammalian cell lines relevant to this study. For those cell lines obtained from a cell culture collection, the ID number is included.

Table IIA-14 | **Mammalian cell lines**

Cell line	Origin	ID number
Control lymphoblastoids	EBV-transformed 46,XX B-lymphocytes	NA
COS-7	African green monkey kidney	DSMZ ACC 60
HeLa	Human cervical carcinoma	ECACC 93021013
Neuro-2A	Mouse neuroblastoma	DSMZ ACC 148
Patient lymphoblastoids	EBV-transformed 46,X,t(X;8)(p11.2;p22.3) B-lymphocytes	NA
Primary fibroblasts	Human 46,XX skin	NA
SH-SY5Y	Human neuroblastoma	DSMZ ACC 209
U373 MG	Human glioblastoma astrocytoma	ECACC 89081403

F.2. Bacterial and yeast strains

Table IIA-15 lists all bacterial and yeast strains relevant to this study. Apart from genes on the F' episome, which are WT, genes listed under 'Relevant genotype' signify mutant alleles.

Table IIA-15 | **Bacterial and yeast strains**

Strain	Species	Relevant genotype	Use
DH5α	*E. coli*	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 Δ*lac*U169 (φ80 *lacZ* ΔM15)	Conventional plasmid amplification
JM110	*E. coli*	*rpsL* (str^r) *thr leu thi*-1 *lacY galK galT ara tonA tsx dam dcm supE*44 Δ(*lac-proAB*) F´[*traD*36 $proAB^+$ $lacI^q$ *lacZ* ΔM15]	Plasmid amplification without adenine methylation of GATC in cloned DNA
L40ccua	*S. cerevisiae*	*MATa his3Δ200 trp1-901 leu2-3,112 LYS2::*$(lexAop)_4$*-HIS3 ura3::*$(lexAop)_8$*-lacZ ADE2::*$(lexAop)_8$*-URA3 GAL4 gal80 can1 cyh2*	Y2H
L40ccα	*S. cerevisiae*	*MATα his3Δ200 trp1-910 leu2-3,112 ade2 LYS2::*$(lexAop)_4$*-HIS3 URA3::*$(lexAop)_8$*-lacZ GAL4 gal80 can1 cyh2*	Y2H

Table IIA-15 | **Bacterial and yeast strains**

Strain	Species	Relevant genotype	Use
SURE 2	*E. coli*	e14$^-$(McrA$^-$) Δ(*mcrCB-hsdSMR-mrr*)171 *endA*1 *supE*44 *thi*-1 *gyrA*96 *relA*1 *lac*$^-$ *recB recJ sbcC umuC*::Tn*5* (*kan*^{r}) *uvrC* F´[*proAB*$^+$ *lacI*^{q} *lacZ* ΔM15 Tn*10* (*tet*^{r}) Amy *cam*^{r}]	Plasmid amplification with reduction of rearrangements and deletions in cloned DNA
XL1-Blue	*E. coli*	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac*$^-$ F´[*proAB*$^+$ *lacI*^{q} *lacZ* ΔM15 Tn*10* (*tet*^{r})]	Conventional plasmid amplification

G. Molecular biology kits

Table IIA-16 lists all molecular biology kits relevant to this study.

Table IIA-16 | **Molecular biology kits**

Name	Application	Supplier
BigDye Terminator Cycle Sequencing Kit	DNA sequencing chemistry	Applied Biosystems
Dynabeads Oligo(dT)$_{25}$	mRNA isolation	Dynal Biotech
Microcon YM-30	Protein concentrator with a MW cut-off of 30 kDa	Millipore
MinElute Gel Extraction Kit	Concentrated DNA recovery after agarose gel elctrophoresis	Qiagen
QIA Expressionist	Affinity purification of 6× His-tagged proteins with Ni-NTA agarose	Qiagen
QIAfilter Midi Cartridge	Filter unit for the QIAprep Midiprep Kit	Qiagen
QIAprep Midiprep Kit	Plasmid isolation from up to 25 ml of high-copy plasmid culture	Qiagen
QIAprep Miniprep Kit	Plasmid isolation from up to 5 ml of high-copy plasmid culture	Qiagen
QIAquick Gel Extraction Kit	DNA recovery after agarose gel electrophoresis	Qiagen
QIAquick PCR Purification Kit	Purification of first-strand cDNA preparations	Qiagen
QIAshredder	Shearing of genomic DNA and cellular membranes	Qiagen
QuantumRNA 18S Internal Standards	Semi-quantitative RT-PCR	Ambion
QuickChange Site-Directed Mutagenesis Kit	*In vitro* site-directed mutagenesis	Stratagene

H. Materials, plastic- & glassware and disposables

Table IIA-17 lists all materials, plasticware and disposables relevant to this study. All DURAN glassware is from Schott.

Table IIA-17 | **Materials, plasticware and disposables**

Item	Application	Supplier
Adhesive PCR film	Disposable cover for PCR plates	ABgene
Biomax MS X-ray film 18 × 24 cm & 30 × 40 cm	Sensitive X-ray film	Kodak
Cell culture dish Ø 90 mm	Culturing and isolating of mammalian cell foci	Biochrom
Cell culture flask 75 & 150 cm^2	Culturing of mammalian adherent cells	Biochrom
Cell culture flask 250 ml	Culturing of mammalian suspension cells	Nunc
Cell culture plate 6-well, Ø 35 mm/well	Culturing of mammalian adherent cells for fluorescence and confocal microscopy	Biochrom
Cell culture plate 24-well, Ø 15 mm/well	Expanding of stably transfected mammalian cell-lines	Biochrom
Cell scraper 30 & 38 cm	Scraping of mammalian adherent cells	Biochrom
Chromatography paper	Blotting paper for protein transfer after SDS-PAGE onto PVDF Western Blotting Membrane	Whatman
Disposable reaction tube 14 ml	Growing of up to 5 ml of bacterial liquid o/n culture	Greiner Bio-One
Disposable reaction tube 30 ml	Preparing of mammalian genomic DNA and total RNA	Sarstedt
Disposible syringe 1 ml	Mechanical disruption of mammalian cells	Becton Dickinson
Economy Mini-Spin Column	Disposable poly-propylene column for Ni-NTA affinity purification of his-tagged proteins	Pierce Biotechnology
Eppendorf tube 1.5 & 2.0 ml	Disposable reaction vessel	Roth & Eppendorf
Falcon tube 15 & 50 ml	Disposable reaction vessel	Greiner Bio-One
Glass bead Ø 2.85 – 3.30 mm	Stopper in Chelex 100/Sephadex G-50 columns	Roth

Table IIA-17 | **Materials, plasticware and disposables**

Item	Application	Supplier
Glass coverslip 20 × 20 mm	Coverslip for fluorescence and confocal microscopy	Menzel-Gläser
Human Brain Blot IV	Northern blot containing adult human amygdala, caudate nucleus, corpus callosum, hippocampus, whole brain, substantia nigra and thalamus poly-A$^+$ RNA	Clontech
Human Brain Blot V	Northern blot containing adult human amygdala, caudate nucleus, corpus callosum, hippocampus, whole brain and thalamus poly-A$^+$ RNA	Clontech
Human foetal brain cDNA library no. 564, ~36864 clones	cDNA library of human foetal brain, enriched for full-length cDNAs; screened with probes encompassing the *hKIAA1202* 5' end	RZPD
Human Fetal MTN Blot II	Northern blot containing foetal human brain, lung, liver and kidney poly-A$^+$ RNA	Clontech
Human large cDNA collection I no. 451, ~7680 clones	cDNA library of human adult skeletal muscle and spinal cord with an average insert size of ~5.4 kb – ~6.0 kb; screened with probes encompassing the *hKIAA1202* 5' end	RZPD
Human large cDNA collection II no. 313, ~14208 clones	cDNA library of human adult bone marrow, adipose tissues and foetal skin with an average insert size of > 2 kb; screened with probes encompassing the *hKIAA1202* 5' end	RZPD
Human MTN Blot	Northern blot containing adult human heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas poly-A$^+$ RNA	Clontech
Hybond ECL Membrane	Nitrocellulose membrane for peptide dot blot	Amersham Pharmacia Biotech
Hypercassette 18 × 24 cm & 30 × 40 cm	Cassette for exposing X-ray film	Amersham Pharmacia Biotech
Inoculation loop 1 & 10 µl	Inoculation of bacterial liquid cultures	Nunc
Microtitre plate 384-well	Plates for liquid culture handling in automated Y2H	Nunc
Mouse MTN Blot	Northern blot containing adult mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis poly-A$^+$ RNA	Clontech
Needle 27G¾	Mechanical disruption of mammalian cells	Becton Dickinson
Nickel grid 200 mesh Ø 3.05 mm	Support for EM sections	EM Sciences
OmniTray	Multi-use tray for colony gridding in Y2H screening	Nunc

Table IIA-17 | **Materials, plasticware and disposables**

Item	**Application**	**Supplier**
Parafilm	Temporary cover for reaction vessels	Pechiney Plastic Packaging
Pasteur pipette	Glass pipette for Chelex 100/Sephadex G-50 columns	Roth
PCR plate 96-well	Set of 8 × 12 reaction tubes for PCR	ABgene
PCR tube 8-strip	Set of 1 × 8 reaction tubes for PCR	Perkin Elmer
Petri dish Ø 85 mm	Agar plates for bacterial culture	Greiner Bio-One
Pipette tip 0.1 – 10 µl, 20 – 200 µl & 200 – 1000 µl	Disposable tips for pipetting	Roth
PVDF Western Blotting Membrane	Membrane for western blotting	Roche
Quick-Seal centrifuge tube 5, 39 & 100 ml	Poly-allomer centrifuge tubes for the VTi 65, VTi 50 and 45 Ti rotors	Beckman Coulter
Roti-Nylon plus	Cationic nylon membrane for Southern blotting and Y2H screening	Roth
Scalpel	Disposable scalpel for cutting DNA from agarose gels	Aesculap
Serological pipette 2, 5, 10 & 25 ml	Disposable pipette for cell culture	Corning
Super RX X-ray film 18 × 24 cm	Conventional X-ray film	Fuji
Superfrost Plus glass slide 25 × 75 × 1 mm	Microscope slide for dark field, fluorescence and confocal microscopy	Menzel-Gläser
Thermanox coverslip Ø 25 mm	Coverslips for EM	Nunc
UVette	Cuvet for UV spectroscopy	Eppendorf

I. Laboratory equipment and instruments

Table IIA-18 lists all equipment and instruments relevant to this study

Table IIA-18 | **Laboratory equipment and instruments**

Instrument	Application	Brand
ABI 3730 capillary sequencer	Automated DNA sequencing	Applied Biosystems
AF30-WS Self Contained Ice Machine	Ice machine for producing ice flakes	Scotsman
AMD Athlon XP 1600+	Personal computer for data analysis & management, knowledge acquisition and supportive administrative purposes	Zero One
Axiocam digital camera	Fluorescence microscopic imaging	Zeiss
Axioplan 2 microscope	Fluorescence, dark field and bright field microscopy	Zeiss
B 5050 E incubator	37°C incubator for growing bacterial colonies on agar plates	Heraeus
Biomek 2000	Automated single-step pippeting for Y2H screening	Beckman Coulter
Biomek FX	Automated pipetting of 384-well plates for Y2H screening	Beckman Coulter
BP 2100 S Balance d = 0.01 g	Precision balance	Sartorius
BP 61 Balance d = 0.1 mg	Analytical balance	Sartorius
BTR10 roller	Tube roller mixer	Ratek
CA/REV 6 Cleanbench	Laminar flow hood for culturing mammalian cells	Clean Air
Casy 1	Cell counter for automated counting of mammalian cells	Schärfe Systems
Chemical Hood	Laminar flow hood for general laboratory purposes	Thurm Labortechnik
Controlled Environment Incubator Shaker	37°C incubator with shaking platform for growing liquid bacterial cultures	New Brunswick Scientific
Curix 60	Developing machine for developing X-ray film	Agfa
DCS-S75 digital camera	Phase contrast microscopic imaging	Sony
Deep freezer	Incubation and storage of samples at -20 – -25°C	Bosch

Table IIA-18 | **Laboratory equipment and instruments**		
Instrument	**Application**	**Brand**
DMIRE2 inverted microscope	Phase contrast microscopy of differentiating mammalian cells	Leica
DXC-9100P Progressive 3 CCD digital camera	Dark field microscopic imaging	Sony
E.A.S.Y. 440K Gel Documentation System	Imaging of EtBr-stained agarose gels	Herolab
EBA 12 R cooled microcentrifuge	Cooled table-top centrifuge for Eppendorf tubes	Hettich
Eclipse TS100	Inverted light microscope for studying mammalian cell cultures	Nikon
Expression 1680 Pro 1600 × 3200 dpi, 48 bit	Colour flatbed scanner with transparency unit for digitalising documents and developed X-ray films	Epson
F25 water bath	Cooled water bath for SSCP with the MultiphorII flatbed system	Julabo
Fastscan-F114FX 1k CCD digital camera	EM imaging	TVIPS
Fridge	Incubation and storage of samples at 4 °C	Bosch
GeneAmp PCR System 9700	Thermocycler for PCR amplification	Applied Biosystems
Herasafe HS18 Cleanbench	Laminar flow hood for microbiological purposes	Heraeus
Horizon 11.14 Horizontal Gel Electrophoresis System	Chamber for separating nucleic acids by agarose gel electrophoresis	Gibco BRL
Hydra-96 Microdispenser	Automated pipetting of 96-well plates for PCR amplification	Robbins Scientific
IP-2717 Regulated Power Supply	Power supply for electrophoresis	Heathkit
K2 High Throughput Picker	Spotting and gridding robot (384 format) for Y2H screening	KBiosystems
L8-70M Ultracentrifuge	Ultracentrifuge for CsCl-DNA preparations	Beckman Coulter
LS 6000TA scintillation counter	Scintillation counter	Beckman Coulter
LSM 510 confocal microscope	Confocal microscopy	Zeiss
5415C microcentrifuge	Table-top centrifuge for Eppendorf tubes	Eppendorf
2020 Microplate Absorbance Reader	Spectrophotometer for measuring protein concentrations	Anthos
Mini Trans-blot Cell	Chamber for separating protein samples by SDS-PAGE	BioRad
Mono-staltic pump 2-6301	Pump for pouring gradient PAGE gels	Buchler Instruments

Table IIA-18 | **Laboratory equipment and instruments**

Instrument	**Application**	**Brand**
Multiphor II	Flatbed system for SSCP	Amersham Pharmacia Biotech
PCR7250T WAVE Nucleic Acid Fragment Analysis System	DHPLC-based high-throughput mutation screening	Transgenomic
Philips CM100	Transmission EM	FEI Company
Pipet·lite 2.0 – 20.0 µl & 20.0 – 200.0 µl	Multichannel pipette for handling up to 12 × 2.0 – 200.0 µl	Rainin
Pipetman 0.10 – 2.00 µl, 2.0 – 20.0 µl, 20.0 – 200.0 µl & 200 – 1000 µl	Pipettes for handling volumes between 0.10 – 1000 µl	Gilson
Polytron PT 3100 homogeniser with PT-DA 3012/2T knife	Homogenising of mouse tissues to isolate total RNA	Kinematica
Powerpette Plus	Pipetting aid for up to ~30 ml	Jencons
Promax 2020 shaker	Reciprocating mixer platform	Heidolph
REAX 2000 vortexer	Vortex mixer	Heidolph
RM2135 microtome	Sectioning of paraffin-embedded mouse tissues	Leica
Rotanta 46 R cooled swing-out centrifuge	Pelleting of mammalian cells	Hettich
Rotina 48 R swing-out centrifuge	Pelleting of bacterial cells	Hettich
Series 900 mini-monitor	Handheld Geiger-Müller counter	Dosimeter, Artisan Electronics Corp.
Sorvall GS-3 rotor max. 13700 *g*	Rotor for pelleting bacterial cultures > 50 ml in the Sorvall RC-5B centrifuge	Kendro
Sorvall RC-5B Refrigirated Superspeed Centrifuge	Cooled centrifuge for pelleting of mammalian genomic DNA, total RNA and > 50 ml of bacterial cultures	DuPont Instruments
Sorvall SS-34 rotor max. 50000 *g*	Rotor for pelleting mammalian genomic DNA and total RNA in the Sorvall RC-5B centrifuge	Kendro
SPD 111V Speed Vac	Heatable vacuum system for concentrating biological samples	Savant
Steri-cycle CO_2 incubator 371	37 °C, 5% CO_2 incubator for growing mammalian cell cultures	Thermo Electron Corp.
45 Ti, VTi 50 and VTi 65 rotors max. 235000, 242000 & 404000 *g* respectively	Rotors for the L8-70M Ultracentrifuge	Beckman Coulter

Table IIA-18 | **Laboratory equipment and instruments**		
Instrument	**Application**	**Brand**
ThermoForma 758 Ultra-freezer	Storage of samples at -80 – -86°C	Thermo Electron Corp.
Thermomixer 5436	Heat block for incubations between RT – 95°C	Eppendorf
Trans Blot SD	Semi-dry transfer cell for protein transfer after SDS-PAGE onto PVDF Western Blotting Membrane	BioRad
Ultracut E microtome with diamond knife	Sectioning of LR White/Gold-embedded mammalian cells	Leica
Ultrospec 3100 *Pro*	Spectrophotometer for measuring DNA and RNA concentrations	Biochrom
WB10 waterbath	Waterbath for incubations between RT – 95°C	Memmert

J. Software, algorithms and databases

J.1. Software packages & programs

Table IIA-19 lists all software packages & programs relevant to this study. In case software is available free of charge, the URL from which it can be retrieved is mentioned under 'Developer'. Software reported in the literature, is also referenced under 'Developer'.

Table IIA-19 | **Software packages & programs**			
Software	**Version**	**Application**	**Developer**
Adobe reader	7.0.5	Management of PDF files	Adobe http://www.adobe.com/products/acrobat/readermain.html
AxioVision LE	4.3.0.101	Recording, analysing and processing of fluorescence images	Zeiss
BioEdit	5.0.9	Analysis and investigation of DNA sequence chromatograms	706 http://www.mbio.ncsu.edu/BioEdit/bioedit.html
CorelXara	1.2	Object-oriented vector graphics package	Xara
Deep View Swiss-PdbViewer	3.7	Calculation of molecular models and visualisation of PDB files	707 http://www.expasy.org/spdbv/
E.A.S.Y. Win32	3.99.199	Capturing of agarose gel pictures	Herolab

Table IIA-19 | **Software packages & programs**

Software	**Version**	**Application**	**Developer**
EMBOSS	2.10	*In silico* manipulation and investigation of DNA and protein sequences	708 http://www.hgmp.mrc.ac.uk/Software/EMBOSS/
EndNote	7.0.0	Tool for managing bibliographic data	Thomson ISI Researchsoft
Gap4	4.10	Analysis of DNA sequence chromatograms	709 http://staden.sourceforge.net/manual/gap4_unix_toc.html
GCG	10.3	*In silico* manipulation and investigation of DNA and protein sequences	Accelrys
GeneDoc	2.6.002	Multiple sequence alignment editor and shading utility	710 http://www.psc.edu/biomed/genedoc/
ImageQuant	5.2	Densitometric analysis	Molecular Dynamics
LSM Image Browser	3.2.0.104	Acquiring, analysing and processing of confocal images	Zeiss
NCBI ORF Finder	NA	Identification of ORFs in deduced amino acid sequences	Tatusov and Tatusov, unpublished http://www.ncbi.nlm.nih.gov/gorf/gorf.html
NJPlot	NA	Plotting of phylogenetic trees	711 http://pbil.univ-lyon1.fr/software/njplot.html
Office 2000	9.0.3821 SR-1	Package aiding in data analysis and supporting administrative work	Microsoft
Paint Shop Pro	8.00	Raster graphics package	Jasc Software
Photoshop Elements	2.0	Aquiring images from the Expression 1680 Pro flatbed scanner	Adobe
PyMOL	0.98	Generation of ray-traced molecular models and visualisation of PDB files	DeLano Scientific LLC http://pymol.sourceforge.net/
Seqman II	5.00	Analysis of DNA sequence chromatograms	DNAStar
Staden	1.5.3	*In silico* manipulation and investigation of DNA and protein sequences	712 http://staden.sourceforge.net/
TreeView	1.6.6	Displaying of phylogenetic trees	713 http://taxonomy.zoology.gla.ac.uk/rod/treeview.html
VisualGrid	3.4.1	Array analysis package used in automated Y2H	GPC Biotech

Table IIA-19 | **Software packages & programs**			
Software	**Version**	**Application**	**Developer**
Wavemaker	4.1	Calculation of melting temperatures and operation of the PCR7250T WAVE Nucleic Acid Fragment Analysis System	Transgenomic
Windows 2000 Professional	5.00.2195	Operating system	Microsoft
X-Win32	5.0.3	Unix client for Windows	StarNet Communications Corp.

J.2. Algorithms

Table IIA-20 lists all algorithms relevant to this study. URLs for implementations of these algorithms are mentioned under 'Reference & URL'. Algorithms reported in the literature, are referenced under 'Reference & URL'.

Table IIA-20 | **Algorithms**	
Algorithm	**Reference & URL**
Analysis of genomic sequence, incl. exon and gene prediction	
Fex FGene	714 http://www.softberry.com/berry.phtml
FGenes	715 http://www.softberry.com/berry.phtml
GeneFinder	Wilson *et al.*, unpublished http://ftp.genome.washington.edu/cgi-bin/genefinder_req.pl
GeneMark	716 http://bioweb.pasteur.fr/seqanal/interfaces/genemark.html
Genscan	717 http://genes.mit.edu/GENSCAN.html
Grail	718 http://bioweb.pasteur.fr/seqanal/interfaces/grailclnt.html
Hexon	714 http://searchlauncher.bcm.tmc.edu/seq-search/gene-search.html
HMMgene	719 http://www.cbs.dtu.dk/services/HMMgene/
MZEF	720 http://rulai.cshl.org/tools/genefinder/
NIX	Williams, unpublished http://www.hgmp.mrc.ac.uk/Registered/Webapp/nix/
RepeatMasker	Smit, unpublished http://www.repeatmasker.org/

Table IIA-20 | **Algorithms**	
Algorithm	**Reference & URL**
RESCUE-ESE	721 http://genes.mit.edu/burgelab/rescue-ese/
tRNAscan	722 http://lowelab.ucsc.edu/tRNAscan-SE/
Analysis of protein sequence, incl. prediction of TM regions and secondary and tertiary structure	
Antigenic	Rice, unpublished http://bioweb.pasteur.fr/seqanal/interfaces/antigenic.html
Coils	723 http://www.ch.embnet.org/software/COILS_form.html
DAS	724 http://www.sbc.su.se/~miklos/DAS/
Digest	Bleasby, unpublished http://bioweb.pasteur.fr/seqanal/interfaces/digest.html
DSC	725 http://bioweb.pasteur.fr/seqanal/interfaces/dsc.html
HTH	Rice, unpublished http://bioweb.pasteur.fr/seqanal/interfaces/helixturnhelix.html
Kyte – Doolittle hydropathy	726 http://www.expasy.ch/tools/protscale.html
Moment	727 http://shannon.mbi.ucla.edu/DOE/Services/moment/
PHD	4 http://cubic.bioc.columbia.edu/
Phyre	Kelley; unpublished http://www.sbg.bio.ic.ac.uk/phyre/
PIX	Williams, unpublished http://www.hgmp.mrc.ac.uk/Registered/Webapp/pix/
PRED-TMR	728 http://o2.db.uoa.gr/PRED-TMR/
Predator	729 http://bioweb.pasteur.fr/seqanal/interfaces/predator.html
PSort	730 http://psort.nibb.ac.jp/
Seg	731 ftp://ncbi.nlm.nih.gov/pub/seg/seg
Sigcleave	Rice, unpublished http://bioweb.pasteur.fr/seqanal/interfaces/sigcleave.html
SignalP	732 http://www.cbs.dtu.dk/services/SignalP/
Simpa96	733 http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_simpa96.html

Table IIA-20 | **Algorithms**	
Algorithm	**Reference & URL**
Split35	734 http://split.pmfst.hr/split/
TMAP	735 http://www.mbb.ki.se/tmap/
TMHMM	736 http://www.cbs.dtu.dk/services/TMHMM/
TMpred	737 http://www.ch.embnet.org/software/TMPRED_form.html
Sequence alignment and phylogenetic analysis	
ClustalW	738 http://www.ebi.ac.uk/clustalw/index.html
Dialign	739 http://bibiserv.techfak.uni-bielefeld.de/dialign/
GeneNest	740 http://genenest.molgen.mpg.de/
Global sequence alignment	741 http://www.ebi.ac.uk/emboss/align/
Local sequence alignment	742 http://www.ebi.ac.uk/emboss/align/
MultiPipMaker	743 http://www.bx.psu.edu/miller_lab/
NCBI BLAST	744 http://www.ncbi.nlm.nih.gov/BLAST/
PipMaker	745 http://www.bx.psu.edu/miller_lab/
REPuter	746 http://bibiserv.techfak.uni-bielefeld.de/reputer/

J.3. Databases

Table IIA-21 lists all databases relevant to this study. The applications for these databases are explained under IIB.C.1.4.

Table IIA-21 | **Databases**	
Database	**URL**
Nucleic acids	
Ensembl Genome Browser	http://www.ensembl.org/
NCBI Entrez	http://www.ncbi.nlm.nih.gov/
UCSC Genome Bioinformatics	http://genome.ucsc.edu/

Table IIA-21 | **Databases**

Database	**URL**
	Protein
Pfam	http://www.sanger.ac.uk/Software/Pfam/
Prosite	http://www.expasy.org/prosite/
SBASE	http://hydra.icgeb.trieste.it/~kristian/SBASE
Swiss-Prot Protein knowledgebase	http://www.expasy.org/sprot/

IIB. Methods

A. Experimental procedures

A.1. Isolation

A.1.1. DNA

A.1.1.1. Isolation of DNA from Epstein-Barr virus-transformed lymphocytes

- Resuspend 0.5 – 1.0 $\times 10^8$ EBV-transformed lymphocytes in Nucleus Buffer.
- Add 1.40 U/ml pronase B and 17.4 mM SDS. Incubate o/n at 37°C.
- Extract with phenol, phenol/chloroform and chloroform.
- Precipitate the DNA with isopropanol in the presence of 100 mM NaCl.
- Wash twice in 70.0% (v/v) ethanol and air-dry.
- Dissolve in 1.00 ml TE buffer, pH 7.5 by o/n end-over-end incubation at 37°C.
- RNase-treat 60' with 2.50 U/ml RNase A at 37°C.
- Recover the DNA by phenol/chloroform and chloroform extractions.
- Precipitate with ethanol in the presence of 300 mM NaAc, pH 4.8.
- Wash in 70.0% (v/v) ethanol and air-dry.
- Dissolve in 1.00 ml TE buffer, pH 7.5 by o/n end-over-end incubation at 37°C.
- Measure the DNA concentration using conventional UV spectroscopy.

A.1.1.2. Isolation of DNA from blood

- Incubate 1.00 vol. of blood for 4.0' at RT with 4.00 vol. of Erylysis Buffer.
- Centrifuge 5.0' at 1300 *g*.
- Resuspend in 0.300 – 1.00 vol. Blood Cell Lysis Buffer.
- RNase-treat 30' with 7.50 U/ml RNase A at 37°C.
- Add 1.25 M $NaClO_4$ and gently mix end-over-end.
- Extract with chloroform and precipitate with ethanol.
- Wash twice with 70.0% (v/v) ethanol and air-dry.
- Dissolve in TE buffer, pH 8.0. by o/n end-over-end incubation at 37°C.

- Measure the DNA concentration using conventional UV spectroscopy.

A.1.1.3. Isolation of high-purity plasmid DNA from bacterial cells

- Pick a single colony from a plated transformation.
- Inoculate 5.00 or 25.0 ml of liquid, antibiotic-containing LB medium.
- Shake the culture o/n at 225 rpm and 37°C.
- Mix 500 µl of o/n culture with 4.50 M glycerol. Store glycerol stocks at -80°C.
- Harvest the plasmid from the remaining culture using Qiagen's Miniprep Kit (5 ml) or Qiagen's Midiprep Kit (25 ml) according to the manufacturer's instructions. Use the Midiprep Kit in conjunction with the QIAfilter Midi Cartridge.
- Measure the plasmid concentration using conventional UV spectroscopy.

A.1.1.4. Isolation of ultra pure plasmid DNA from bacterial cells

Courtesy of D. Vogt, MPI-MG, Berlin, Germany.

- Pick a single colony from a plated transformation.
- Inoculate 2.0 ml of antibiotic-containing 2× TY medium and shake at least 6.0 hrs at 225 rpm and 37°C. Use 50 – 200 µl to inoculate 200 ml of antibiotic containing 2× TY medium and shake o/n at 225 rpm and 37°C.
- Prepare and store glycerol stocks as described in the previous section.
- Centrifuge the culture 10.0' at 8300 *g*.
- Resuspend the pellet in 11.0 ml 25.0% (w/v) sucrose/50.0 mM Tris, pH 8.0.
- Add 98 U/µl lysozyme; 82.0 mM EDTA, pH8.0 and 15.0 ml Brij-Mix.
- Centrifuge 30' at 70400 *g* and 10°C.
- Add 6.10 mM CsCl and 760 µM EtBr to the supernatant.
- Centrifuge 18.0 hrs at 160000 *g* and 20°C.
- Collect the plasmid DNA and centrifuge 18.0 hrs at 187000 *g* and 20°C.
- Extract the EtBr with isopropanol. Add 3.00 vol. ddH_2O and 2.80 vol. isopropanol. Vortex.
- Flash-freeze in liquid nitrogen. Thaw and vortex. Centrifuge 30' at 38700 *g*.
- Wash twice with 70.0% (v/v) ethanol and air-dry. Dissolve in 1.00 vol. TE buffer, pH 7.5.
- Assay the DNA integrity on a 1.00% agarose gel.
- Measure the plasmid concentration using conventional UV spectroscopy.

A.1.2. RNA

A.1.2.1. Isolation of total RNA from cell lines

- Resuspend 0.5 – 1.0 $\times 10^8$ EBV-transformed lymphocytes or fibroblasts in 10.0 ml Trizol.
- Incubate 5.0' at RT, add 2.10 M chloroform and vortex vigorously.
- Incubate 3.0' at RT and centrifuge 15.0' at 12000 *g* and 4°C.
- Precipitate the RNA with isopropanol. Wash twice with 75.0% (v/v) ethanol, air-dry and dissolve in DEPC-treated ddH_2O.
- Precipitate with ethanol in the presence of 300 mM NaAc, pH 4.8 and air-dry.
- Dissolve in 500 (lymphoblastoid cells) or 250 µl (fibroblasts) DEPC-treated ddH_2O.
- Check the integrity of the RNA on a 1.00% agarose gel and measure its concentration using conventional UV spectroscopy.

A.1.2.2. Isolation of total RNA from mouse tissue

Collaboration with D. Meunier, MPI-MG, Berlin, Germany.

(C57BL/6 × C3H) x C57BL/6 mice were sacrificed by cranial dislocation prior to tissue collection. Tissue samples were homogenised in 1.00 ml Trizol per 75.0 mg of tissue. RNA was prepared from the homogenates as described in the previous section.

A.1.2.3. Isolation of poly-A^+ RNA from total RNA

Poly-A^+ RNA was isolated from total RNA using the Dynabeads Oligo$(dT)_{25}$ magnetic beads according to the manufacturer's recommendations.

A.1.3. Protein

A.1.3.1. Isolation of total protein from mammalian cells

Suspension cells:

- Centrifuge 10.0^6 cells/ml 10.0' at 160 *g* and 4°C. Aspirate the medium and keep cells on ice at all times.

Adherent cells:

- Seed 1.0 – 3.5 $\times 10^4$ cells/cm^2 24 hrs prior to protein extraction.
- Treat the cells 2.0 hrs with 25.0 µM LLnL prior to protein isolation in case blocking of the proteasome is desired.
- Aspirate the medium and wash with RT DPBS.

- Exchange the RT DPBS for 150 µl/cm^2 ice-cold PBS and keep cells on ice at all times.
- Scrape the cells and transfer them to a microfuge tube. Centrifuge 10.0' at 160 *g* and 4°C.

Suspension and adherent cells:

- Wash the pellet with 4°C PBS and centrifuge 10.0' at 160 *g* and 4°C.
- Resuspend in ~1.00 µl/cm^2 Pagano Lysis Buffer/Buffer C/2× Magic Mix.
- Lyse 60' by gentle end-over-end mixing at 4°C (Pagano Lysis Buffer and Buffer C) or 3'' at RT (2× Magic Mix).
- Pass the lysate several times through a 27G¾ syringe (Pagano Lysis Buffer and Buffer C) or through a QIAshredder column (2× Magic Mix), according to the manufacturer's recommendations.

The protein concentration was determined using Bradford's assay[747] by adding one vol. of Bradford Reagent to four vol. of 1:1000 – 1:4000 diluted sample. The shift in the adsorption maximum of the CBB G-250 dye, contained in the Bradford Reagent, was measured at 595 nm and compared to a BSA standard curve. All samples, the BSA standard and the blanks were measured in duplicate.

A.1.3.2. Isolation of his-tagged proteins from mammalian cells

- Prepare a cell pellet of cells expressing the his-tagged protein as described in the previous section.
- Resuspend the cell pellet in 3.30 µl/cm^2 Ni-NTA Lysis Buffer.
- Rotate 60' end-over-end at 4°C.
- Apply the lysate to a QiaShredder column and centrifuge 2.0' at 16100 *g* and 4°C.
- Remove the column and centrifuge the flow-through 10.0' at 1000 *g* and 4°C.
- Transfer the supernatant to a clean Eppendorf tube and add 0.670 µl/cm^2 Ni-NTA agarose slurry.
- Mix 60' end-over-end at 4°C.
- Equilibrate a poly-propylene Mini-Spin Column with 1.00 vol. of Ni-NTA Wash Buffer.
- Apply the mixture to the column and allow the fluid to drain by gravity flow and the Ni-NTA agarose beads to set on the column's frit.
- Wash the beads 5 times with 5.30 µl/cm^2 Ni-NTA Wash Buffer.

- Apply 8 times 0.330 µl/cm^2 Ni-NTA Elution Buffer and collect all 8 fractions.
- Estimate the protein concentration in each of the fractions by western hybridisation (see IIB.A.4.6.) and Bradford's assay (see previous section).

A.2. Separation

A.2.1. Agarose gel electrophoresis

DNA fragments ranging from ~100 bp to ~10.0 kb were size-separated on 0.800% – 3.00% (conventional agarose) or 3.00% – 4.00% (NuSieve agarose) gels at 1.60 – 17.9 V/cm in TAE. DNA was visualised by addition of 1.27 µM EtBr. After electrophoresis, the gel was inspected on a UV-light box and documented using the E.A.S.Y. 440K Gel Documentation System.

A.2.2. Poly-acrylamide gel electrophoresis

A.2.2.1. General poly-acrylamide gel electrophoresis

DNA fragments ranging from ~50 bp to ~500 bp were size-separated on 8.00% (49:1) – 11.0% (29:1) acrylamide:bis-acrylamide gels at 7.20 V/cm in TBE. DNA was visualised by silver staining (see next section).

Denatured and reduced protein samples were size-separated at 0.400 V/cm (o/n 5.00 – 15.0% gradient gel) – 23.1 V/cm (~1.0 hr conventional mini gel) in Lämmli Buffer. To visualise proteins, gels were incubated for 45' at RT in CBB R 250 Staining Solution, followed by destaining in CBB R 250 Destaining Solution till distinct bands appeared.

A.2.2.2. Single-strand conformation polymorphism analysis

Collaboration with U. Fischer and J. Ruschmann, MPI-MG, Berlin, Germany.

All PCR amplifications (see IIB.A.3.1) of *hKIAA1202* exons were run with 3.00 mM $MgCl_2$ on 100 ng of XLMR patient genomic DNA. To destabilise GC-rich stretches of sequence, 700 mM DMSO was added to reactions 'Exon 1/1' and 'Exon 1/2'. All reactions were run using Cycle B, except for 'Exon 8/2' which was run using Cycle C. Primers are listed in Appendix E, cycling conditions in Appendix F.

- Mix 1.00 vol. of a 200 bp – 350 bp amplicon with 1.00 vol. of 2× SSCP Sample Buffer.
- Denature the samples 10.0' at 95°C and chill them on ice.

- Run o/n, at 15 and 20°C, on native 29:1 and 49:1 acrylamide:bis-acrylamide gels containing 274 mM glycerol as described in the previous section.
- After electrophoresis, incubate the gels twice for 3.0' in DNA Fixation Solution, stain 10.0' in 6.00 mM $AgNO_3$ and rinse twice with ddH_2O.
- Immerse the gel up to 20.0' in Developer Solution and fix 5.0' in 700 mM Na_2CO_3.
- Assess shifts on a light box.

A.2.3. High-throughput denaturing high-performance liquid chromatography

Collaboration with B. Moser, MPI-MG, Berlin, Germany.

All reagents for PCR amplification (see next section) of *hKIAA1202* exons on XLMR patient genomic DNA were combined using the Hydra-96 Microdispenser. Amplicons were generated as described in the previous section, apart from amplicon 'Exon 6/1' which was divided into amplicons 'Exon 6/1a' and 'Exon 6/1b' and from amplicon 'Exon 8/1' which was split into amplicons 'Exon 8/1a' and 'Exon 8/1b'. These latter two amplicons were run using Cycle C. Primers are listed in Appendix E, cycling conditions in Appendix F, and DHPLC conditions in Appendix G.

- Assay the integrity and concentration of PCR products on 96-well agarose gels.
- Pool 2 times 20.0 µl of corresponding PCR products, denature 5.0' at 95°C and reanneal by gradual cooling down to RT.
- Using the PCR7250T WAVE Nucleic Acid Fragment Analysis System, allow the samples to heat to 2 – 4 different temperatures, as calculated by the Wavemaker software, encompassing the melting temperature of the corresponding WT helix.
- Allow DHPLC Buffers A and B to mix, as calculated by the Wavemaker software, establishing an acetonitrile step gradient, flowing at a rate of 0.9 ml/min.
- Assay DHPLC profiles and repeat the analysis for samples suggesting hetero-duplex formation, by newly pooling the patient DNAs with WT DNA.
- Confirm results by direct sequencing of newly amplified PCR products.

A.3. Amplification

A.3.1. Polymerase chain reaction

Unless otherwise noted, all PCR reactions were performed in a GeneAmp PCR System 9700 thermocycler in a total volume of 25.0 or 50.0 µl with 300 nM of each primer, 200 –

500 nM dNTPs, 1.50 – 1.75 mM $MgCl_2$ and 50.0 (AmpliTaq) – 75.0 (Expand) mU/µl of DNA polymerase. Except when indicated differently, amplification was achieved using conventional cycling conditions (Cycle A) as mentioned in Appendix F. Primers are listed in Appendix E.

A.3.2. Reverse transcription polymerase chain reaction

First-strand cDNA synthesis was performed on human fibroblast, human foetal brain and mouse adult brain total RNA and on human poly-A^+ fibroblast RNA. Subsequent PCR amplification was performed on 1/30th of purified cDNA preparation as a template, using conventional cycling conditions. Each reaction was carried out in parallel on RNA with and without the addition of reverse transcriptase in order to verify the absence of genomic DNA contamination. Primers are listed in Appendix E, cycling conditions in Appendix F.

- Add 960 mU/µl RNase inhibitor and 125 mU/µl DNase I to 0.100% (w/v) of RNA and incubate 30' at 37°C.
- Add 1.80 mM EGTA, pH 8.0 and heat inactivate 10.0' at 65°C.
- Set up, in duplicate, reaction mixtures containing 0.250‰ (w/v) of DNase I-treated RNA, 500 nM dNTPs, 10.0 mM DTT, 12.5 ng/µl $pd(N)_6$ and 960 mU/µl RNase inhibitor.
- Denature 10.0' at 65°C, chill 5.0' on ice, anneal 10.0' at 25°C and 2.0' at 42°C.
- Add 9.50 U/µl Superscript II reverse transcriptase to each sample of the first set of reactions. Add the same volume of DEPC-treated ddH_2O to the corresponding samples of the second set of reactions.
- Incubate 1.5 hrs at 42°C and heat inactivate the reverse transcriptase 15.0' at 70°C.
- Purify each reaction on a QIAquick PCR Purification column according to the manufacturer's recommendation.
- Assay 1/30th of each reaction on a 1.00% agarose gel and check for genomic contamination by PCR amplification of *hG3PDH* on each cDNA.

A.3.3. Semi-quantitative reverse transcription polymerase chain reaction

Semi-quantitative RT-PCR was performed using the 'QuantumRNA 18S Internal Standards' kit according to the manufacturer's guidelines. Essentially, the linear range of amplification was established for each set of GSPs by densitometric analysis of size-separated, EtBr-

stained aliquots, which were taken from the reaction mixture at regular intervals (every other cycle, cycles 21 – 37). Using cycling conditions resulting in linear amplification, the ratio of *18S rRNA* primer:competimer was established in multiplex RT-PCR reactions on cDNA preparations from mouse adult brain total RNA. These reactions contained a set of GSPs and ratios of *18S rRNA* primer:competimer, ranging from 1:35 to 1:50. The ratio resulting in RT-PCR products of equal intensity, as monitored by densitometric analysis, was then used to establish expression levels of the gene of interest in mouse cDNAs derived from E12 (head and body), E14.5 and E18 (both stages, brain and body) embryos.

A.3.4. Rapid amplification of complementary DNA ends

Whereas cDNA preparations were homo-polymerically tailed for 5' RACE, such cDNA tailing was superfluous for 3' RACE due to the natural occurrence of a poly-A tail at the 3' end of mRNAs.

- Add 200 nM dATP to 0.300 vol. of purified first-strand cDNA (see IIB.A.3.2).
- Denature 3.0' at 94°C and briefly chill on ice.
- Add 600 mU/µl of TdT and incubate 15.0' at 37°C.
- Heat inactivate the TdT 10.0' at 65°C.

Second-strand synthesis and amplification of the tailed cDNA were performed using the following primers on $1/20^{th}$ of tailed cDNA (5' RACE) or $1/30^{th}$ of purified cDNA (3' RACE) as a template: RACE-T_{18}-AP1 (30.0 nM), RACE-AP1n and a GSP located in the first or the second exon for 5' RACE or in the penultimate or the ultimate exon for 3' RACE. All amplifications were run using Cycle D. A second round of amplification was performed using RACE-AP2n (nested in RACE-AP1n) and a nested GSP using conventional cycling conditions. Primer sequences are listed in Appendix E, cycling conditions in Appendix F.

A.3.5. Suppression polymerase chain reaction

- Pre-digest 60.0 – 70.0 µg of genomic patient and control DNA 2.0 hrs with 150 mU/µg of a 'six-cutter' endonuclease in 6.00 vol. at $T_{max.\ activity}$ and pipet up and down occasionally.
- Add an additional 450 mU/µg of endonuclease and incubate o/n at 37°C.
- Extract the DNA with phenol/chloroform and precipitate it with ethanol.
- Redissolve in 0.200 vol. of 10.0 mM Tris·HCl, pH7.0.

- Denature 31.0 μM of long adaptor (5'-CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC GGG CAG GT-3') and 31.0 μM of short adaptor (5'-TGG ACG GG-N_2H-3') 60" in 0.130 vol. ddH_2O at 65°C.
- Anneal the adaptors 15.0' at 20°C.
- Combine the annealed adaptors with the digested genomic DNA and ligate o/n at 4°C with 230 mU/μl of T4 DNA ligase in 0.400 vol.

One fifth of the ligation was than employed as template DNA in a PCR reaction with AP1-F and a reverse GSP using Cycle E. A second round of PCR was performed with AP2-Fn, nested in AP1-F, and a nested reverse GSP using conventional cycling conditions. Primers are listed in Appendix E, cycling conditions in Appendix F.

A.3.6. *In vitro* mutagenesis

In vitro mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit according to the manufacturer's guidelines. Introduction of a local mutation was based on a PCR approach, using Cycle F. Primers are listed in Appendix E, cycling conditions in Appendix F.

A.3.7. DNA sequencing

Courtesy of J. Ruschmann, S. A. Kunde and D. Gröger, MPI-MG, Berlin, Germany.

PCR products (20.0 pg/bp) or plasmid DNA (100 ng) were sequenced (Cycle G) in a total volume of 10.0 μl using the BigDye Terminator Cycle Sequencing Kit according to the manufacturer's recommendations. Primers are listed in Appendix E, cycling conditions in Appendix F.

- Precipitate sequenced DNA 45' with 2.50 vol. 100% (v/v) ethanol at 2700 *g* and 19°C.
- Wash twice for 15.0' with 10.0 vol. of 70.0% (v/v) ethanol at 2700 *g* and 19°C.
- Remove all traces of ethanol by spinning the samples briefly upside-down followed by an additional, 'upright' 1' centrifugation under vacuum.
- Resuspend the DNA pellets in 1.00 M betaine and shake 45' at RT before loading the samples on an ABI 3730 capillary sequencer.

A.4. Hybridisation

A.4.1. Fluorescent *in situ* hybridisation

Courtesy of C. Menzel and L. Van Zutven, MPI-MG, Berlin, Germany.

Preparation of metaphase chromosomes:

- Arrest cells in metaphase by adding 270 nM colcemid to EBV-transformed lymphoblastoid cultures or human peripheral blood lymphocytes 60' prior to cell harvest.
- Centrifuge 10.0' at 160 *g* and 4°C.
- Resuspend the cell pellet in 50.0 mM KCl and incubate 20.0' at 4°C.
- Fix the cells o/n at 4°C in 18.5 M methanol – 4.40 M acetic acid.
- Drop-splash the cells on microscopic slides.

Treatment of microscopic slides:

- Treat microscopic slides 60' with 5.00 U/ml RNase A in 2× SSC, pH 7.0 at 37°C.
- Treat an additional 10.0' with 1.50 milliAnson-U/ml Pepsin A in10.0 mM HCl at 37°C.
- Rinse thrice in 2× SSC, pH 7.0 and dehydrate in an ethanol series (70.0%, 80.0%, 100%; all v/v, 10.0' each).
- Denature chromosomes 60" at 75°C in 17.6 M formamide (in 2× SSC, pH 7.0) and dehydrate in an ethanol series.

Labelling of DNA probes (YAC DNA):

- Mix 0.0200‰ (w/v) YAC DNA with 10.0 mM β-mercaptoethanol, 50.0 µM each dATP, dCTP and dGTP, 37.5 µM dUTP, 12.5 µM DIG-11-dUTP or biotin-16-dUTP, 3.4×10^{-4} U/µl DNase I and 130 mU/µl DNA polymerase in NT-Buffer.
- Incubate 2.0 hrs at 15°C.
- Add 28.0 mM EDTA, pH 8.0 to stop the labelling reaction.
- Ethanol precipitate the labelled YAC DNA in the presence of Cot-1 and herring sperm DNA.
- Resuspend in equal volumes of deionised formamide and 20.0% (w/v) dextran sulfate (in 4× SSC, pH 7.0).

Labelling of DNA probes (BAC DNA):

- Whereas YAC DNA was DNase-treated and labelled simultaneously, 0.100‰ (w/v) BAC DNA was DNase-treated 45' with 2.00×10^{-4} U/µl DNase I at 37°C in NT-Buffer prior to labelling.
- Labelling was performed as described for YAC DNA.

Hybridisation of DNA probes on metaphase chromosomes:

- Mix 10.0% (w/v) dextran sulfate, 0.500‰ (w/v) herring sperm DNA, 0.100‰ (w/v) Cot-1 DNA and 0.0100‰ (w/v) DIG-11-dUTP-labelled DNA in 12.5 M formamide (in 2× SSC, pH 7.0).
- Pre-anneal repetitive sequences 20.0' with Cot-1 DNA at 37°C.
- Denature the hybridisation mixture 5.0' at 70°C.
- Apply 30.0 µl of the hybridisation mixture to each slide and seal under a coverslip.
- Hybridise o/n in a moist chamber at 37°C.
- Wash the slides twice for 5.0' in 12.5 M formamide (in 2× SSC, pH 7.0) at 42°C and twice for 5.0' in 0.1× SSC, pH 7.0 at 60°C.

Detection of labelled probes

- Incubate the slides at least 30' at 42°C in Blocking Buffer and rinse briefly in Wash Buffer.
- Apply FITC-conjugated α-avidin or Cy3-conjugated α-DIG antibodies to detect the labelled probes.
- Wash thrice for 5.0' at 42°C in Wash Buffer.
- Counterstain the chromosomes 3.0' with 2.72 µM DAPI in 2× SSC, pH 7.0.
- Mount the slides in Mounting Medium.
- Investigate the signals with an epifluorescence microscope and record them with a digital camera.

A.4.2. Mouse *in situ* hybridisation

A.4.2.1. Mouse *in situ* hybridisation – $^{33}\alpha$[P]UTP

Collaboration with Dr. E. Minina, MPI-MG, Berlin, Germany.

Tissue collection, fixation, embedding and sectioning:

- (C57BL/6 × C3H) x C57BL/6 mice were sacrificed by cranial dislocation prior to tissue collection.
- Fix the tissue samples o/n at 4°C in Serra’s.
- Exchange the Serra’s for ice-cold 70.0% (v/v) isopropanol and incubate 2.0 hrs at 4°C. Incubate another 3 – 4 times 2.0 hrs at 4°C in 70.0% (v/v) isopropanol. Incubate o/n at 4°C in 70.0% (v/v) isopropanol.

- Exchange the 70.0% (v/v) isopropanol for 100% (v/v) isopropanol and incubate 2 – 3 times 2.0 hrs at 4°C in 100% (v/v) isopropanol.
- Incubate the tissue samples 2 × 5.0' in xylene and infiltrate them 20.0' at 60°C (under vacuum) in 1:1 xylene:paraffin, 60' at 60°C (under vacuum) in paraffin and an additional 2.0 hrs at 60°C (under vacuum) in fresh paraffin.
- Arrange the tissue in a small container, embed in 60°C paraffin and allow the paraffin to solidify o/n at 4°C.
- Cut 7.0 µm sections from the embedded tissue using a microtome and mount by floating them in a 42°C waterbath and arranging them on microscopic slides.
- Dry sections 30' at 50°C and o/n at 40°C. Store slides at 4°C.

Labelling of RNA probes:

- PCR amplify a suitable probe from mouse brain cDNA as described under IIB.A.3.1.
- TA-clone the amplicon in pGEM-T Easy as described under IIB.A.5.2, IIB.A.5.3 and IIB.A.1.1.3.
- Linearise the cloned template DNA as described under IIB.A.5.1.
- Increase the volume two-fold with DEPC-treated ddH_2O.
- Extract the DNA twice with 1.00 vol. of phenol and once with 1.00 vol. of chloroform.
- Ethanol precipitate in the presence of 3.00 M NaAc, pH 5.2.
- Dissolve the pellet in 10.0 mM TE, pH7.5 to a final concentration of 0.0200 – 0.100% (w/v).
- Mix 0.0250‰ (w/v) linearised DNA with 500 nM of each NTP, 250 mU/µl RNase inhibitor, 2.00 U/µl T7 (anti-sense) or SP6 (sense) RNA polymerase and 4.00 µCi/µl $^{33}\alpha$[P]UTP in Transcription Buffer provided with the enzyme.
- Incubate 60' at 37°C.
- Add 500 mU/µl RNase-free DNase I and incubate 30' at 37°C.
- Increase the volume five-fold with DEPC-treated ddH_2O and add 2.00×10^{-5}‰ (w/v) glycogen, 500 mM LiCl and 2.50 vol. 100% (v/v) ethanol.
- Precipitate the RNA by a 30' incubation at -20°C and a subsequent 15.0' centrifugation at 21900 *g* and 4°C.
- Wash the RNA pellet twice with 80.0% (v/v) ice-cold ethanol and air-dry 5.0'.
- Resuspend in ~2.5 times the original transcription volume of DEPC-treated ddH_2O to obtain a final concentration of $\sim 1.8 \times 10^6$ cpm/µl.

- Just before use, denature the labelled probe by heating 5.0' at 95°C followed by brief chilling on ice and use at a 1:20 dilution.

Pre-hybridisation of embedded tissue sections:

- Deparaffinise tissue sections by incubating twice for 10.0' in xylene.
- Rehydrate by immersion in an ethanol series (twice 100%, 95.0%, 75.0%, 50.0%, 30.0%; all v/v, 10.0' each).
- Incubate 5.0' in 145 mM NaCl, followed by incubation in PBS for 5.0'.
- Fix 30' in 1.33 M PFA (in PBS).
- Wash 5.0' in PBS, treat 5.0' with 200 mM HCl and wash again 5.0' in PBS.
- Incubate 3.0' in Proteinase K Digestion Buffer ($^{33}\alpha$[P]UTP), wash 5.0' in PBS, fix 10.0' in 1.33 M PFA (in PBS) – 20.0 mM GA and wash 5.0' in PBS.
- Acetylate 10.0' in 130 mM triethanolamine – 26.5 mM acetic anhydrid, wash 5.0' in PBS and incubate 5.0' in 145 mM NaCl.
- Dehydrate the sections by immersion in an ethanol series (30.0%, 50.0%, 75.0%, 95.0% and twice 100%; all v/v, 2.0' each) and air-dry the slides 15.0' at RT.

Hybridisation of RNA probes on mouse tissue sections:

- Apply 60.0 µl of Hybridisation Buffer ($^{33}\alpha$[P]UTP) to each slide and cover with a coverslip.
- Hybridise o/n at 65°C under a 12.5 M formamide (in 5× SSC) atmosphere.
- Remove the coverslip and wash the slides 30' in 5× SSC at 55°C and 30' in 2× SSC at 55°C.
- RNase-treat 30' in RNase A Buffer ($^{33}\alpha$[P]UTP) at 37°C and incubate 30' in 12.5 M formamide (in 2× SSC) at 55°C.
- Wash twice for 30' in 2× SSC at 55°C.
- Dehydrate by immersion in an ethanol series (30.0%, 50.0%, 75.0%, 95.0% and twice 100%; all v/v, all in 300 mM NH_4Ac, 10.0' each) and air-dry the slides 15.0' at RT.
- Hybridise serial sections with sense and anti-sense probes.

Detection of labelled probes:

- Inspect the signal by exposing a BioMax X-ray film o/n.
- Dip the slides in NTB 2 auto-radiography emulsion and air-dry 1.5 hrs.
- Store 10.0 days at 4°C, develop 5.0' with D-19 Developer at 15°C, rinse 15.0' with tap water at RT and fix 15.0' with Kodak Fixer at 20°C.

- Remove the photographic emulsion from the back of each slide and counterstain the sections 3.0' in 6.50 mM toluidine blue O.
- Dehydrate by immersion in an ethanol series (30.0%, 50.0%, 75.0%, 95.0% and twice 100%; all v/v, 60'' each) and incubate 30' in xylene.
- Mount the sections in DPX Mountant and cover the slides with coverslips.
- Investigate sections using dark field microscopy and photograph them with a mounted digital camera.

A.4.2.2. Mouse *in situ* hybridisation – $^{35}\alpha$[S]UTP

Collaboration with D. Meunier, MPI-MG, Berlin, Germany.

Tissue collection, fixation, embedding and sectioning:

Collection, fixation, embedding and sectioning of tissues was performed identical to the procedures described in the previous section.

Labelling of RNA probes:

- Generation, linearisation and precipitation of plasmid DNA were performed as described in the previous section.
- Mix 0.0500‰ (w/v) linearised DNA with 500 nM ATP, 500 nM CTP, 500 nM GTP, 20.0 mM DTT, 900 mU/µl RNase inhibitor, 2.0 U/µl T7 (anti-sense) or SP6 (sense) RNA polymerase and 2.0 µCi/µl $^{35}\alpha$[S]UTP in Transcription Buffer provided with the enzyme.
- Incubate 2.0 hrs at 37°C. Add 500 mU/µl RNase-free DNase I and incubate 15.0' at 37°C.
- Add 0.250% (w/v) *E. coli* total RNA, 6.90 mM DTT and 50.0% (v/v) aqueous phenol, pH 4.5 – 5.0 increasing the volume ten-fold. Vortex gently.
- Centrifuge 2.0' at 21900 *g* and collect the aqueous phase.
- Add 0.330 vol. Stop Solution for RA Labelling and apply to a Sephadex G-50 – Chelex 100 column.
- Collect the labelled probe and discard the unincorporated $^{35}\alpha$[S]UTP.
- Dilute 1.00 µl of probe 1:5000 in scintillation liquid and measure the RA decay.
- Add 0.100 vol. of 3.00 M NaAc, pH 5.2 and 2.50 vol. ethanol. Mix gently and incubate o/n at 20°C.
- Centrifuge 10.0' at 21900 *g* and 4°C. Wash the pellet with 70.0% (v/v) ethanol.

- Air-dry the pellet 5.0' at 37°C and dissolve in TE, pH7.5 – 3.50 mM SDS to a final concentration of ~5.0 $\times 10^5$ cpm/µl.
- Just before use, denature the probe by heating 10.0' at 80°C followed by brief chilling on ice and use at a 1:5 dilution.

Pre-hybridisation of embedded tissue sections:

- Deparaffinise tissue sections by incubating twice for 3.0' in xylene.
- Rehydrate by immersion in an ethanol series (100%, 95.0%, 90.0%, 70.0%; all v/v, 3.0' each).
- Fix 5.0' in 1.33 M PFA (in PBS, pH 7.5). Treat 10.0' with 200 mM HCl and wash twice for 10.0' in 2× SSC.
- Incubate 5.0' in Proteinase K Digestion Buffer ($^{35}\alpha$[S]UTP).
- Fix 5.0' in 1.33 M PFA (in PBS, pH 7.5) and rinse in PBS.
- Acetylate 10.0' in 110 mM triethanolamine – 26.5 mM acetic anhydrid and wash 5.0' in 2× SSC.
- Incubate the slides 3.0' in 70.0% (v/v) ethanol and store them at -20°C.

Hybridisation of RNA probes on mouse tissue sections:

- Apply 25.0 µl of Hybridisation Buffer ($^{35}\alpha$[S]UTP) and cover with parafilm.
- Hybridise o/n at 54°C under a 12.5 M formamide (in 4× SSC) atmosphere.
- Remove the parafilm and wash the slides twice for 20.0' in Wash Buffer ($^{35}\alpha$[S]UTP) at 54°C and 4 times 10.0' in 2× SSC at RT.
- RNase-treat 15.0' in RNase A Buffer ($^{35}\alpha$[S]UTP) at 37°C and incubate 15.0' in Wash Buffer ($^{35}\alpha$[S]UTP) at 54°C.
- Wash twice for 5.0' in 2× SSC at RT.
- Dehydrate by immersion in an ethanol series (70.0%, 90.0%, 95.0% and 100%; all v/v, 3.0' each) and air-dry the slides 15.0' at RT.
- Hybridise serial sections with sense and anti-sense probes.

Detection of labelled probes:

- Inspect the signal by exposing a BioMax X-ray film o/n.
- Dip the slides in K5 Nuclear Emulsion, prepared according to the manufacturer's recommendations and air-dry 60'.
- Store 3 – 4 weeks at 4°C, develop 5.0' with D-19 Developer at 14°C, rinse 30'' with tap water at RT and fix 15.0' with 1.20 M sodium thiosulfate.

- Rinse twice for 10'' with tap water and counterstain the sections 4.0' in Mayer's Hemalum Solution.
- Rinse twice with tap water and dip 10'' in 950 mM HCl [in 70.0% (v/v) ethanol].
- Wash 5 times 25.0' with tap water and dehydrate by immersion in an ethanol series (70.0%, 90.0%, 95.0% and 100%; all v/v, 2.0' each).
- Air-dry at least 30' and mount the sections using Roti-Histokitt.
- Investigate sections using dark field microscopy and photograph them with a mounted digital camera.

A.4.3. Southern hybridisation

Labelling of DNA probes:

Probes were typically 300 – 1200 bp gel-purified, PCR-amplified DNA fragments, labelled with α^{32}[P]dCTP to a specific activity of ~1.6×10^5 cpm/µl.

- Denature $8.7 \times 10^{-4} – 6.5 \times 10^{-2}$‰ (w/v) amplified DNA 5.0' in 5× OLB at 95°C in a volume of 23.0 µl and chill on ice.
- Add 800 nCi/µl α^{32}[P]dCTP and 200 mU/µl Klenow polymerase and incubate 1.5 hrs at 37°C.
- Add 2.00 vol. Stop Solution for RA Labelling and apply to a Sephadex G-50 – Chelex 100 column.
- Collect the labelled probe on 100 µg Hybridime and discard the unincorporated α^{32}[P]dCTP.
- Block repetitive sequences by denaturing 5.0' at 95°C and subsequent chilling on ice.
- Incubate the labelled probe 2.0 hrs at 65°C in 1.00 vol. of PEG Buffer at a final specific activity of ~3.2×10^6 cpm/ml.

Digestion, size-separation and blotting of genomic DNA on nylon membranes:

Ten µg of genomic DNA, extracted from patient and control lymphoblastoid cell lines (see IIB.A.1.1.1) were digested o/n with an appropriate endonuclease (see IIB.A.5.1).

- Load the digested DNA on a 0.800% agarose gel and size-separate o/n at 1.60 V/cm.
- Denature the DNA twice for 30' by gently shaking the gel in Denaturation Buffer.
- Incubate the gel twice for 20.0' in Neutralisation Buffer and rinse it with ddH_2O.
- Blot the DNA o/n with 10× SSC at 20°C onto a cationic nylon membrane by upward capillary transfer.

- Cross-link the DNA 2.0' to the membrane by UV irradiation at 302 nm.

Pre-hybridisation, hybridisation and signal detection

- Denature 2.00 mg of herring sperm DNA 5.0' at 95°C and cool down rapidly on ice.
- Pre-hybrididse the membrane 2.0 hrs at 65°C in 4.00 vol. of PEG Buffer containing 0.100‰ (w/v) denatured herring sperm DNA.
- Add the labelled probe and hybridise o/n at 65°C.
- Wash the membrane twice for 5.0' at 65°C in 2× SSC – 3.50 mM SDS and 15.0' at 65°C in 0.1× SSC – 3.50 mM SDS.
- Visualise RA signals by exposing X-ray film 8.0 – 100 hrs at -80°C.

A.4.4. Northern hybridisation

A Human Fetal MTN Blot II, a Human MTN Blot, a Human Brain Blot V and a Mouse MTN Blot were hybridised with 600 – 850 bp probes exactly as described in the previous section. Blots were reprobed with a *β-actin* or a *mG3pdh* control probe to check the loading and integrity of the blots.

A.4.5. Complementary DNA library screening

In order to screen RZPD's cDNA libraries 451 (human large cDNA collection), 313 (human large cDNA collection II) and 564 (human foetal brain cDNA library), RA labelling of probes, blocking of repetitive sequences, hybridisation and washing of membranes and signal detection were performed exactly as described under IIB.A.4.3.

A.4.6. Western hybridisation

- Briefly soak a PVDF membrane of the appropriate size in methanol, incubate it 5.0' in ddH_2O and 5.0' in Blotting Buffer with (proteins < 150 kDa) or without (proteins > 150 kDa) 5.00 M methanol.
- Soak 6 pieces of chromatography paper per membrane in Blotting Buffer.
- Place an SDS-PAGE gel containing size-separated proteins (see IIB.A.2.2.1) on top of the membrane and sandwich both together between two stacks of three soaked pieces of chromatography paper.
- Transfer the proteins 30' (proteins < 150 kDa) or 60' (proteins > 150 kDa) from the gel to the membrane by semi-dry electroblotting at 15.0 V.

- Block the membrane 60' at RT or o/n at 4°C in $PBST^{BSA-2.00\%}$ (α-Ubiquitin antibody) or in $PBST^{M-5.00\%}$ (all other antibodies).
- Apply primary antibodies and incubate the membrane 60' at RT (antibodies raised against a tag and α-Actin raised in rabbit) or o/n at 4°C (all antibodies).
- Wash the membrane thrice for 5.0' in PBST (α-mouse/rabbit IgG antibodies) or 5 times 5.0' in $PBST^{M-5.00\%}$ (α-goat IgG antibodies).
- Apply HRP-conjugated secondary antibodies and incubate the membrane 30' at RT.
- Wash the membrane 4 times 5.0' in PBST (α-mouse/rabbit IgG antibodies) or 6 times 5.0' in $PBST^{M-5.00\%}$ (α-goat IgG antibodies).
- Apply chemiluminescence reagent to the membrane as recommended by the manufacturer and incubate 2.0' at RT.
- Briefly rinse the membrane in PBST and expose X-ray film to visualise the signal.

In case the efficiency of protein transfer needs to be verified prior to immunodetection, the membrane was stained with Ponceau S Staining Solution according to the following protocol:

- Incubate the membrane 2.0' in Ponceau S Staining Solution.
- Remove the staining solution and wash the membrane with ddH_2O till bands become visible.
- Scan the membrane to document results.
- Destain the membrane for 10.0' – 15.0' in PBST.
- Block the membrane as described above and continue with immunodetection.

While primary antibodies were removed from the membrane using Restore Western Blot Stripping Buffer at 37°C according to the manufacturer's recommendations, secondary antibodies were removed at RT using the following protocol:

- Rinse the membrane briefly in PBST.
- Incubate the membrane twice for 10.0' in Stripping Buffer.
- Equilibrate briefly in 1.00 M Tris·HCl, pH 7.0 and rinse thrice with PBST.
- Apply the next secondary antibody.

A.4.7. Serum test on peptide dot blot

- Spot 2.00 µg of peptide on 1.0 cm^2 pieces of Hybond ECL Membrane.
- Air-dry 10.0' and block 30' at RT in $PBST^{BSA\text{-}0.500\%}$.
- Incubate o/n at 4°C in serial primary antibody dilutions or in serial dilutions of pre- and post-immunesera.
- Wash the membranes thrice for 5.0' in PBST and apply HRP-conjugated secondary antibodies.
- Incubate 30' at RT and wash thrice for 5.0' in PBST.
- Detect the signal as described in the previous section.

A.5. Molecular biology

A.5.1. DNA restriction

Endonuclease digestions of genomic DNA were carried out in two steps: a 3.0 hrs 'pre-digestion' incubation with 2.00 U/µg DNA was followed by an o/n incubation with an additional 3.00 U/µg DNA. Plasmid DNA and PCR products were digested o/n with 1.00 U/µg DNA.

Incubation temperatures and buffer conditions were as stated by the supplier. The amount of endonuclease never exceeded 5.00% (v/v) of the total reaction volume. When possible, the enzyme was heat-inactivated as recommended by the supplier. Endonucleases are listed in Table IIA-7.

A.5.2. DNA Ligation

Using 200 mU/µl of T4 DNA ligase, blunt-end and TA-ligation reactions were carried out o/n at 4°C; sticky end ligations o/n at 16°C.

A.5.3. Transformation

- Gently thaw DH5α, XL1-Blue, JM110 or SURE 2 competent cells from -80°C.
- Add 0.0200 – 1.00‰ (w/v) plasmid DNA to an aliquot of cells and incubate 30' on ice.
- Heatshock the cells 45' at 42°C in a water bath and cool them down 2.0' on ice.
- Add 1 ml of 37°C SOC medium and shake 60' at 225 rpm and 37°C.

- Plate 50.0, 100 and 500 µl transformed cells on LB-plates containing the appropriate antibiotic and incubate o/n at 37°C.

A.5.4. Immunoprecipitation

- Wash 5.00 vol. protein A- or protein G-coupled agarose slurry thrice for 10.0' at 4°C in Buffer C (α-Vimentin IP) or Pagano Lysis Buffer (all other IPs).
- Add 2.00 vol. of washed protein A/G-coupled agarose beads to 38.0 vol. 0.500 – 0.850‰ (w/v) protein lysate and mix 40' (over-expressed proteins) or 60' (endogenous proteins) end-over-end at 4°C.
- Centrifuge 2.0' at 1000 *g* and 4°C, and add 2.00 – 10.0 $\times 10^{-3}$‰ (w/v) primary antibody to the supernatant.
- Mix 60' (over-expressed proteins) or o/n (endogenous proteins) end-over-end at 4 °C.
- Add 3.00 vol. of washed protein A/G-coupled agarose beads and mix 60' (over-expressed proteins) or 2.0 hrs (endogenous proteins) end-over-end at 4 °C.
- Centrifuge 2.0' at 1000 *g* and wash the beads thrice for 10.0' at 4°C with 40.0 vol. Buffer C (α-Vimentin IP) or Pagano Lysis Buffer (all other IPs).
- Resuspend the beads in 1.00 vol. SDS-PAGE Loading Buffer or Magic Mix and denature 5.0' at 95°C.
- Continue with size-separation (see IIB.A.2.2.1) of the samples and western hybridisation (IIB.A.4.6).

A.5.5. Chemical cross-linking of proteins

- Affinity purify his-tagged proteins from mammalian cells as described under IIB.A.1.3.2.
- Use a Microcon protein concentrator according to the manufacturer's recommendations to:
  - ✓ Concentrate the protein thirty-fold.
  - ✓ Remove low MW contaminants by buffer exchange (~99.99%) using Buffer A.
  - Incubate 0.0150 – 0.0250‰ (w/v) of concentrate 30' at 25°C in Buffer A/Buffer B containing 0.00, 0.100, 0.500, 1.00, 2.50, 4.50, 6.50, 8.50, 10.0, 25.0, 50.0, 100 or 150 mM GA.

- *Alternatively*, incubate 0.0150 – 0.0250‰ (w/v) of concentrate 0.0, 5.0, 10.0, 15.0, 30, 60, 90 or 120 min. at 22°C in buffer A/Buffer B containing 5.00 mM GA.
- Add 330 mM Tris·HCl, pH 7.0 and incubate 10.0' at 25°C.
- Size-separate the samples on an SDS-PAGE gel and stain it with CBB R 250 (see IIB.A.2.2.1) or perform western hybridisation (see IIB.A.4.6).

A.5.6. Raising an α-hKIAA1202 antibody

The 28 AA peptide CLENPALDLSSYRAISSLDLLGDFKHAL which was selected as an antigen for the production of an α-hKIAA1202 antibody was synthesised commercially according to earlier published methods[748,749]. The peptide was HPLC-purified to > 80% and the synthesis was verified by MALDI-TOF[750]. The peptide was conjugated to LPH with 4-(N-maleimidomethyl)cyclohexanecarboxylic acid N-hydroxysuccinimide ester and used to immunise two rabbits (BioGenes). After eight booster injections at increasing intervals ranging from one week to one month, sera were collected.

The peptide-specific antibody was affinity-purified from the sera using a SulfoLink column, based on iodoacetyl – sulfhydryl chemistry. The peptide's sulfhydryl groups must be present in the reduced state before they can be coupled to the column's matrix. The state of the sulfhydryl groups was investigated by SDS-PAGE:

- Incubate 4.00 µg peptide 10.0' at 95°C in Magic Mix in the presence or absence of 14.3 mM β-mercaptoethanol.
- Chill on ice, size-separate on a 15.0% SDS-PAGE gel and stain the gel with CBB R 250 as described under IIB.A.2.2.1.

The CBB R 250 staining indicated that the sulfhydryl groups were present in the reduced form. The peptide was then bound to the column and the antisera were applied as recommended by the manufacturer. Next, the antibody was eluted at 4°C with:

- 5 times 2.00 ml Gentle Buffer
- 5 times 2.00 ml 50.0 mM PIPES, pH 5.8
- 5 times 2.00 ml 100 mM citric acid, pH 5.0
- 5 times 2.00 ml 50.0 mM glycine, pH 2.5
- 5 times 2.00 ml 100 mM triethylamine, pH 11.5

All elution fractions were assayed for hKIAA1202 affinity by peptide dot blot as described under IIB.A.4.7.

A.5.7. Automated yeast two-hybrid screening

Collaboration with Dr. U. Stelzl, MDC, Berlin, Germany.

To determine the identities and reading frames of 9665 cDNAs from the hEx1 human foetal brain library[751], cDNAs were sequenced from the 5' end. BLAST analyses against the sequence databases revealed a non-redundant set of 4275 cDNAs for subcloning. Clones yielding multiple fragments upon *Sal*I or *Not*I endonuclease digest were discarded. All other *Sal*I/*Not*I inserts were ligated into pGAD426 and pBTM117c to generate in-frame fusions to the GAL4 activation ('preys') and the LexA DNA binding ('baits') domains, respectively. An additional 2136 potential full-length ORFs were amplified from PCR source clones of the RZPD repository. PCR products were cloned in pDONR201 by site-specific recombination. Clones were sequenced and annotated using BLAST searches. Next, cDNA fragments were shuttled o/n into pGAD426-D3 ('preys') and pBTM116-D9 ('baits'). Prior to yeast transformation, the integrity of all constructs was assessed in triplicate using conventional restriction analysis and subsequent agarose gel electrophoresis. The redundancy of the generated clones was < 4.0%. While 48.0% of plasmids encoded full-length ORFs, 52.0% coded for C-terminal fragments[752].

To create an interaction-matrix, preys were transformed in the L40ccα yeast strain and baits in the L40ccua strain. Baits interacting with the activation domain alone (19.6%), were excluded from the automated Y2H analysis[752]. Interaction mating was performed by mixing replicated L40ccα strains with pooled (8 baits/pool) L40ccua strains and subsequent cultivation of the mixtures on YPD agar plates for 36 hrs at 30°C. After mating, clones were automatically picked and transferred to liquid SDII medium (-Leu -Trp). For selection of protein – protein interactions, diploid yeast cells were spotted on SDIV agar plates (-Leu -Trp -His -Ura) with and without nylon membranes. After incubating 120 – 144 hrs at 30°C, digitised images of the plates and membranes were assessed for growth and β-galactosidase activity. Putative interactions were confirmed by mating the positive preys identified in the initial screen with each of the 8 baits followed by SDII/SDIV selection as described.

A.6. Cell biological methods

A.6.1. Mammalian cell culture

Collaboration with S. Freier and H. Madle, MPI-MG, Berlin, Germany.

All mammalian cell lines were cultured under a 5.0% CO_2 atmosphere at 37°C. Adherent cells were seeded at a density of 5.2 – 35.0 $\times 10^3$ cells/cm^2. Suspension cells were maintained at a density of 10.0^6 cells/ml. Table IIB-1 lists the culturing conditions for those cell lines relevant to this study.

Table IIB-1 | **Culturing conditions for mammalian cells**

Cell line	**Medium**	**Passage**
COS-7	10.0% (v/v) FBS; 100 U/ml penicillin; 68.6 µM streptomycin *ad volumina* DMEM	Every 2 – 3 days, 1:4
HeLa	10.0% (v/v) FBS; 100 U/ml penicillin; 68.6 µM streptomycin; 2.00 mM L-Glu *ad volumina* DMEM	Every 3 – 4 days, 1:4
Lymphoblastoid cell lines	10.0% (v/v) FBS; 100 U/ml penicillin; 68.6 µM streptomycin; 2.00 mM L-Glu *ad volumina* RPMI-1640	Every other day, 1:2
Neuro-2A	10.0% (v/v) FBS; 100 U/ml penicillin; 68.6 µM streptomycin; 2.00 mM L-Glu; 1% (v/v) NEAA *ad volumina* DMEM	Every 7 days, 1:4
Primary fibro-blasts	As for HeLa	Every 3 – 4 days, 1:2
SH-SY5Y	15.0% (v/v) FBS; 100 U/ml penicillin; 68.6 µM streptomycin; 2.00 mM L-Glu *ad volumina* DMEM	Every 7 days, 1:4
U373 MG	10.0% (v/v) FBS; 100 U/ml penicillin; 68.6 µM streptomycin; 2.00 mM L-Glu; 1.00% (v/v) NEAA; 1.00 mM sodium pyruvate *ad volumina* EMEM	Every 3 – 4 days, 1:4

A.6.2. Transfection

Cells were either transfected in cell culture flasks (protein isolation) or in 6-well plates fitted with coverslips (IF).

- Seed 1.0 – 3.5 $\times 10^4$ cells/cm^2 24 hrs prior to transfection.
- Incubate 70.0 – 160 ng/cm^2 plasmid DNA and 2.50 µl Lipofectamine 2000 per µg DNA 30'' in 300 µl Opti-MEM with GlutaMAX I each.
- Mix the DNA and the Lipofectamine 2000 solutions and incubate 20.0' at RT.
- In the meantime, aspirate the medium from the cells and wash them once with DPBS.

- Apply antibiotics-free cell culture medium and add the DNA – Lipofectamine 2000 mixture.
- Incubate the cells 24 hrs under standard conditions prior to protein extraction or IF.

A.6.3. Fluorescence & immunofluorescence

A.6.3.1. Proteins tagged with enhanced green fluorescent protein

Transfection of vector DNA was used as a negative control.

- Aspirate the medium 24 hrs after transfection with EGFP-tagged constructs.

Live imaging:

- Rinse the coverslips briefly with PBS, mount them and seal them with nail varnish.
- Investigate the EGFP distribution with conventional epifluorescence microscopy and record the signals with a mounted digital camera within 1.5 hrs.

Imaging of fixed cells:

- Rinse the cells once in 1.2× PEM Buffer.
- Fix the cells 10.0' in Fixing Solution at RT and rinse thrice with PBS.
- Mount in Vectashield mounting medium supplemented with 2.72 µM DAPI and seal with nail varnish.
- Investigate the EGFP distribution with conventional epifluorescence (see IIB.A.6.6.1)/confocal (see IIB.A.6.6.2) microscopy and record the signals with a mounted digital camera.
- Store preparations at 4°C in the dark for up to one month.

A.6.3.2. V5-tagged and endogenous proteins

The following controls were performed for each experiment:

- ✓ Vector control (over-expression studies)
- ✓ Labelling with secondary antibody alone (single and double labelling)
- ✓ Labelling with both secondary antibodies alone (double labelling)
- ✓ Labelling of each structure separately (double labelling)

- Treat the cells 45' with 5.00 µM latrunculin B prior to IF in case disruption of the F-actin cytoskeleton is desired.
- Aspirate the medium 24 hrs after seeding 1.0 – 3.5 $\times 10^4$ cells/cm^2 or after transfection with V5-tagged constructs.
- Rinse once with 1.2× PEM (twice when latrunculin B has been applied).

- Fix the cells 15.0' in ice-cold methanol at -20°C (α-Prohibitin IF) or 10.0' in Fixing Solution at RT (all other IFs).
- Rinse thrice with PBS.
- Incubate the cells 5.0' in 0.200% (v/v) Triton X-100 (in PBS) and wash thrice for 5.0' with PBS.
- Block 60' in 1^{st}AbD at RT or o/n at 4°C.
- Incubate the cells o/n in the dark with the first antibody/antibodies (in 1^{st}AbD) at 4°C.
- Wash thrice for 15.0' with PBS.
- In the meantime, pre-associate the secondary antibody/antibodies 30' in 2^{nd}AbD at 37°C.
- Incubate the cells 45' in the dark with the pre-associated secondary antibody/antibodies at RT and wash thrice for 15.0' with PBS.

  TRITC-phalloidin staining:
  - ✓ Wash 15.0' (instead of thrice for 15.0') with PBS.
  - ✓ Stain the cells 30' in the dark with 12.8 nM TRITC-phalloidin at RT.
  - ✓ Wash 4 times 5.0' with PBS.
  - ✓ Mount a test slide and assess whether additional washing is desirable.

- Mount in Vectashield mounting medium supplemented with 2.72 µM DAPI and seal with nail varnish.
- Investigate the fluorescent signals with conventional epifluorescence (see IIB.A.6.6.1)/confocal (see IIB.A.6.6.2) microscopy and record them with a mounted digital camera.
- Store preparations at 4°C in the dark for up to one month.

A.6.4. Cell differentiation

- Seed 5.2 $\times 10^3$/cm^2 cells in 6-well plates fitted with coverslips (SH-SY5Y) or collagen-coated coverslips (Neuro-2A).
- Incubate 24 hrs under standard conditions.
- Aspirate the medium, wash briefly with DPBS and apply Differentiation Medium.
- Follow differentiation for 8.0 days by inverted phase contrast microscopy and record progress daily with a mounted digital camera (see IIB.A.6.6.1).

A.6.5. Stable transfection of U373 MG cells

To generate U373 MG cells stably expressing tagged proteins, 10.0^5 cells were transfected with linearised plasmid DNA. Two days after transfection, ~600, ~800 and ~1000 cells were seeded in culture dishes and cultured in 650 µM zeocin-containing medium. The optimal zeocin concentration had been established beforehand by culturing non-transfected U373 MG in the presence of increasing zeocin concentrations. After 48 hrs, foci were transferred to 24-well culture plates and expanded.

A.6.6. Microscopy

A.6.6.1. Bright field, dark field, phase contrast and epifluorescence microscopy

Conventional bright field, dark field, phase contrast and epifluorescence microscopy was executed according to standard procedures. Microscopes were fitted with 2.5×, 5×, 10×, 40× (all Zeiss Plan-Neofluar), 63× and 100× (both Zeiss Plan-Apochromat) objective lenses and 10× ocular lenses. Whereas oil immersion objectives were used for magnifications ≥ 400×, conventional dry optics were utilised for smaller magnifications.

The epifluorescence microscope was equipped with a Zeiss Filter set 25, consisting of 3 bandpass filters characterised by maximal transmissions at wavelengths of 400, 495 and 570 nm (excitation) and 460, 530 and 610 nm (emission). Prior to recording, each specimen was observed through a barrier filter completely eliminating cross-channel bleeding of Cy3/TRITC and EGFP/FITC fluorescence, circumventing documentation of artifactual doubly labelled structures. Settings 10.0% below the cut-off at which the appropriate negative controls did not yield a signal, were regarded as the maximum level at which to record IF experiments.

A.6.6.2. Confocal microscopy

Confocal microscopy was executed according to standard procedures. The microscope was equipped with a Zeiss Filter set 15, consisting of a bandpass filter with a maximal transmission at a wavelength of 546 nm (excitation) and a long pass filter with maximal transmission at ≥ 590 nm (emission). Lenses, barrier filter and exposure settings on the confocal microscope were as described in the previous section. The argon (488 nm, 15.0 mW) and helium – neon (543 nm, 0.5 mW) lasers were used at 80.0 – 95.0% of their max. power. 146.2 μm^2 areas were scanned at 3.20 µs/pixel (2048 × 2048 dpi, 12 bit). Unless otherwise noticed, the distance between optical sections was 500 nm. DAPI staining was recorded with an epifluorescence microscope and computationally merged with the corresponding confocal image.

A.6.6.3. Electron microscopy

Collaboration with Dr. R. Lurz and G. Lüder, MPI-MG, Berlin, Germany.

- Seed and transfect mammalian cells on Thermanox coverslips as described under IIB.A.6.2.
- Wash cells briefly in 50.0 mM sodium cacodylate, pH 7.4 and fix 1.5 hrs at RT in 1.33 M PFA – 20.0 mM GA (in 50.0 mM sodium cacodylate, pH 7.4).
- Dehydrate by immersion in an ethanol series (30.0%, 50.0%, 70.0%, 90.0%, 95.0%, 6 times 100%; all v/v, 10.0' each).
- Infiltrate 60' in 50.0% (v/v) LR White/Gold (in ethanol) at RT and o/n in 66.0% (v/v) LR White/Gold (in ethanol).
- Infiltrate twice for 12.0 hrs in 100% (v/v) LR White/Gold at RT and twice for 12.0 hrs in 100% (v/v) LR White/Gold containing 4.75 mM benzil at RT.
- Polymerise 48 hrs in 100% (v/v) LR White/Gold containing 4.75 mM benzil at RT.
- Cut 70.0 – 80.0 nm thick sections, transfer them to nickel grids and stabilise the sections by carbon coating using vacuum evaporation.
- Incubate 10.0' in Buffer A and 10.0' in Buffer B.
- Incubate 2.0 hrs at RT in Buffer B containing the primary antibody.
- Wash 4 times 10.0' in Buffer B.
- Incubate 2.0 hrs at RT in Buffer B containing the secondary antibody.
- Wash 4 times 10.0' in Buffer A.
- Post-stain the sections with uranyl acetate and lead citrate as described by Reynolds[753].
- Visualise samples with a transmission EM and record signals with a mounted digital camera.

B. Clinical procedures

B.1. 46,X,t(X;8)(p11.2;p22.3) patient characterisation

The patient was examined at the University Hospital Ulm, Germany. Karyotyping was performed at the Department of Medical Genetics, University of Ulm, Germany by Dr. G. Barbi.

B.2. X-linked mental retardation patient cohort, control panels and *hKIAA1202* mutation screening

DNA from 196 XLMR families of the Euro-MRX Consortium (J. Chelly *et al.*, Paris, France; J.-P. Frijns *et al.*, Leuven, Belgium; B. Hamel *et al.*, Nijmegen, The Netherlands; C. Moraine *et al.*, Tours, France; J. Gècz *et al.*, Adelaide, Australia and H.-H. Ropers *et al.*, Berlin, Germany) was available for mutation screening of *hKIAA1202*. Mutations in thirthy-one of these families were linked to Xp11. Controls A consisted of 73 healthy females and 22 healthy males, Controls B consisted of 46 healthy males.

All families and Controls A were screened for mutations by SSCP as described under IIB.A.2.2.2. Twenty-five Xp11-linked families and Controls B were screened for mutations by DHPLC as described under IIB.A.2.3. Twenty-one Xp11-linked families and Controls B were screened for variable repeats and for genomic rearrangements (2 male controls) employing PCR technology (see IIB.A.3.1) and Southern hybridisation (see IIB.A.4.3), respectively. In 24 Xp11-linked families and 29 male controls, an imperfect repeat in exon 6 was directly sequenced as described under IIB.A.3.7.

C. Computational procedures

C.1. Bio-informatics

C.1.1. Molecular biological software

Three (open source) software packages (Staden, GCG & EMBOSS) and two free applications (BioEdit & NCBI ORF Finder) were employed for the conceptual translation of nucleic acid sequences, the *in silico* manipulation of DNA and protein sequences and the study of DNA and protein properties. Gap4 and SeqManII were employed in the analysis of DNA sequence chromatograms generated after DNA sequencing. Details on all software are listed in Table IIA-19.

C.1.2. Prediction algorithms

C.1.2.1. Exon and gene prediction algorithms

Several nucleic acid sequence analysis programs, including a multitude of exon and gene prediction algorithms, were run and their output was visualised using the NIX WWW tool at the HGMP server. Programs included in NIX analysis were Genscan, Grail, Fex, Hexon,

MZEF, GeneMark, GeneFinder, Fgene, FGenes, HMMgene, RepeatMasker and tRNAscan. Putative exonic splice enhancers were predicted with the RESCUE-ESE algorithm. Details on each of these algorithms are listed in Table IIA-20.

C.1.2.2. General protein sequence analysis

Several peptide analysis programs, including a multitude of prediction algorithms, were run and their output was visualised using the PIX WWW tool at the HGMP server. Programs included in PIX analysis were PSort, DSC, Predator, Simpa96, PHD, Seg, Coils, TMpred, TMAP, DAS, HTH, SignalP, Sigcleave, Antigenic and Digest. Details on each of these algorithms are listed in Table IIA-20.

C.1.2.3. Transmembrane prediction

Table IIA-20 details the TM prediction algorithms that were applied. For Kyte-Doolittle hydropathy calculations[726], the window size was set to 19 and the TM threshold to 1.6.

C.1.2.4. Automated structural homology modelling

Protein sequences were submitted to the Phyre server. The returned models were manually refined on their PDB templates using Swiss-PdbViewer. Graphics were generated with PyMOL software.

C.1.3. Sequence alignment and phylogenetic analysis

C.1.3.1. Pairwise alignment

DNA and protein sequences were aligned against several databases using the BLAST tool. A pair of sequences was locally aligned using the Smith-Waterman algorithm. Global alignments were performed using the Needleman-Wunsch algorithm. Large genomic sequences were aligned with PipMaker. REPuter was used to screen genomic regions for unique repeats. Clustering of ESTs, based on sequence alignments, were visualised with GeneNest. Details on each of these algorithms are listed in Table IIA-20.

C.1.3.2. Multiple alignment

Local and global multiple alignments were performed with Dialign and ClustalW, respectively. Such alignments were manually refined. The phylogenetic data generated by ClustalW were visualised as phylogenetic trees using NJPlot or TreeView. Multiple alignments of sets of large genomic sequences were executed using MultiPipMaker. Shading of multiple protein alignments, performed with GeneDoc, were based on BLOSUM 62[754]. Species were phy-

logenetically ranked using the taxonomy browser at the NCBI. Details on each of the algorithms and programs are listed in Table IIA-20.

C.1.4. Databases and nomenclature

The nucleic acid databases (Table IIA-21) were consulted to subtract sequence information from genomic clones, ESTs, mRNAs, cDNAs and conceptually translated proteins and to compile BAC minimal tiling paths covering the inserts of FISH-mapped YAC clones. The protein databases (Table IIA-21) were consulted to subtract peptide sequences and protein domain information.

Numbering of genomic sequences starts with the first nucleotide. In case of coding sequence, nucleotide numbering starts from the first ATG with the corresponding methionine residue being the first amino acid in the protein. FBP nomenclature is according to Jin *et al.*[645]. Mutations are reported as recommended by den Dunnen and Antonarakis[755]. A list of gene/protein symbols, including their full names and database identifiers, can be found in Appendix H.

C.2. Image acquisition, analysis and processing software

Agarose gel pictures were captured with E.A.S.Y. Win32. Densitometric analyses were performed with ImageQuant software. Whereas epifluorescence images were recorded and analysed using AxioVision LE, confocal images were acquired and analysed with the LSM Image Browser program. Developed X-ray films were digitalised on an Expression 1680 Pro flatbed scanner. All images were processed using CorelXara and Paint Shop Pro.