2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All standard chemicals and reagents were purchased from Sigma and Merck unless indicated otherwise. The manufacturer of materials and equipment used is indicated only if it is different from standard lab materials and equipment.

[α-^{32}P]dCTP	Amersham™
Agar	Difco™
Agarose	Invitrogen™
Ampicillin	Sigma™
BSA	Sigma™
Complete™, Mini Protease Inhibitor Cocktail Tablets	Roche™
Cresol red	Aldrich™
DAPI	Serva™
Dextran blue	Fluka™
Diethylpyrocarbonate (DEPC)	Aldrich™
dNTPs	Roth™
Ethidium bromide	Serva™
GeneRuler™ 1 kb and 100 bp DNA ladders	MBI Fermentas™
Herring sperm DNA	Roche™
Hybridime™ human placental DNA	HT Biotechnology™
IPTG	Serva™
Kanamycin	Invitrogen™
Magnesium chloride	Perkin Elmer™
pd(N)$_6$ random hexamers	Pharmacia™
PEG $_{6000}$	Merck™
QickHyb®	Stratagene™
Rainbow™ Protein Molecular Weight Marker	Amersham™
Sephadex™ G-50	Pharmacia™

SDS	Serva™
TEMED	Invitrogen™
TRIzol®Reagent	Invitrogen™
Trypton	Difco™
Tween™ 20	Sigma™
Vectashield™ Mounting Medium	Vector Laboratories™
X-gal	Invitrogen™
Yeast extract	Difco™

2.1.2 Solutions and media

Aqueous solutions were prepared using autoclaved Millipore™ water. For sterilisation, if necessary, solutions and media were autoclaved or passed through a 0.45µm filter (Millipore™).

DEPC-H_2O	0.1% (v/v) DEPC, double autoclaved
Ethidium bromide (EtBr)	10 mg/mL EtBr
Cresol red	1 mM Cresol red
Herring sperm DNA	10 mg/mL
PEG hybridisation solution	250 mM NaCl 125 mM di-sodium hydrogen phosphate 1 mM EDTA 7% (w/v) SDS 10% (w/v) PEG_{6000}
LB (Luria Bertani) medium	10 g/L trypton 5 g/L yeast extract 10 g/L NaCl
LB agar	15 g agar per 1 L LB medium

5X OLB (-dCTP)	0.1 mM dATP, dGTP, dTTP 1 M HEPES 0.425 mM $pd(N)_6$ 25 mM $MgCl_2$ 250 mM Tris 0.36% (v/v) ß-Mercaptoethanol
1X PBS	137 mM NaCl 2.7 mM KCl 10.1 mM Na_2HPO_4 1.8 mM KH_2PO_4 pH 7.3
Lysis Buffer	10 mM Tris/HCl pH 7.5 25 mM EDTA 75 mM NaCl
20X SSC	300 mM sodium citrate 3 mM NaCl pH 7.0
1X TE	10 mM Tris 1 mM EDTA pH 7.5
50X TAE	2 M Tris 5.71 % acetic acid (v/v) 50 mM EDTA pH 8.0
10X MOPS	200 mM MOPS 10 mM EDTA 85 mM sodium acetate pH 7.0
Sucrose HEPES Buffer	0.32 M sucrose 4 mM HEPES pH 7.2
Yeast Lysis Buffer	2% Triton X100 100 mM NaCl 10 mM Tris pH 8.0 1 mM EDTA

10X Laemmli Buffer	0.25 M Tris 1.92 M Glycine 1 % SDS
10X PCR Buffer	100 mM Tris-HCl pH 8.3 500 mM KCl 0.01% (w/v) gelatin

2.1.3 Enzymes

All restriction endonucleases for cloning, sequencing and Southern blotting were purchased from New England Biolabs™, Invitrogen™ or MBI Fermentas™ GmbH. Reactions were performed in supplied reaction buffers according to the protocol provided by the manufacturer. Additional enzymes used are listed in Table 2 below.

Table 2: Additional enzymes

Enzyme	Concentration	Company
AmpliTaq™	5 U/µl	Perkin Elmer™
Taq DNA Polymerase	5 U/µl	Qiagen™
Cloned *Pfu* DNA Polymerase	2.5 U/µl	Stratagene™
Klenow fragment	2 U/µl	Roche™
SuperScript™ II RNase H- reverse transcriptase	200 U/µl	Invitrogen™
RNAguard™ RNAse Inhibitor	32.6 U/µl	Amersham™
Proteinase K	10 mg/ml	Invitrogen™
RNase A	50 µg/ml	Roche™
T4 Polynucleotide Kinase	10 U/µl	Fermentas™
T4 DNA Ligase	400 U/µl	Promega™

2.1.4 Kits

All kits, listed in Table 3, were used for purification as instructed by the manufacturer.

Table 3: Kits

Kit Name	Company
QIAprep™ Spin Miniprep Kit	Qiagen™
QIAgen™ Plasmid Midi and Maxi Kits	Qiagen™
QIAquick™ Gel Extraction Kit	Qiagen™
MinElute™ Gel extraction Kit	Qiagen™
MicroSpin™ G-50 Columns	Amersham Biosciences™

Kit Name	Company
Advantage™ 2 PCR Kit	Clontech™
Expand™ Long Template PCR System	Roche™
Enhanced Chemiluminescence HRP Detection Kit	Amersham Biosciences™

2.1.5 Vectors

All vectors used for expression studies as well as those used for intermediate cloning steps are listed in Table 4.

Table 4: Cloning vectors

Name	Size (bp)	Resistance gene	Company
pGEM™-T Easy	3018	Ampicillin	Promega™
pCMV-Tag™2B	4324	Kanamycin/Neomycin	Stratagene™
pEGFP-N3	4729	Kanamycin/Neomycin	Clontech™
pEGFP-C1	4731	Kanamycin/Neomycin	Clontech™
pcDNA™3.1	5428	Ampicillin/Neomycin	Invitrogen™

2.1.6 Cell culture reagents

All cell culture reagents, listed in Table 5, were purchased sterile and free of mycoplasma.

Table 5: Cell culture reagents

Reagent	Company
Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1.5g/L glucose	Biowhittaker™
DMEM/F12	Biowhittaker™
Non-essential amino acids	Invitrogen™
Penicillin/streptomycin antibiotic solution	Invitrogen™
Fetal Bovine Serum (FBS)	Biowhittaker™
Lipofectamine™ 2000 Transfection Reagent	Invitrogen™

2.1.7 Antibodies

All primary and secondary antibodies used in this study are listed in Table 6.

Table 6: Antibodies

Name	Description	Company
anti-FLAG™	Mouse monoclonal	Stratagene™
anti-SAPK1b/JNK3	Rabbit polyclonal	Upstate Biotechnology™
anti-rabbit-HPRT	Donkey anti-rabbit IgG	Amersham Biosciences™
anti-mouse-Cy3	Donkey anti-mouse IgG	Dianova™
anti-mouse-FITC	Goat anti-mouse IgG	Dianova™

2.1.8 Human genomic clones

All human cosmid libraries, cosmids, BACs and IMAGE cDNA clones were obtained from the Resource Centre of the German Human Genome Project (RZPD). Cosmid libraries used are listed in Table 7.

Table 7: Cosmid libraries

Library Description	Library Name
Human Chromosome 2-specific	Livermore LLNL library 128 (RZPD)
Human Chromosome 4-specific	LANL library 503 (RZPD)
Human Chromosome X-specific	Los Alamos LANL library 153 (RZPD)

2.1.9 Bacterial material

For all recombinant DNA techniques competent *E. coli* DH5α cells were used.

2.1.10 Mammalian cell lines

Transient transfection experiments were carried out in either HeLa (human cervical cancer) or Neuro2A (mouse neuroblastoma) cells.

2.1.11 Translocation patients, XLMR families, and controls

All translocation patients in this study had been diagnosed with some degree of mental retardation (mild, moderate, or severe), and carried chromosome translocations, presumably balanced, observed by Giemsa banding in diagnostic cytogenetics laboratories. For each patient, either a lymphoblastoid or fibroblast cell line was available. DNA from 200 families collected by the European XLMR Consortium, including 14 families for which linkage data implicates the Xp11 region, was available for mutation screening. DNA from an additional 10 Australian MRX families for which linkage data implicated the Xp11 region

was also included in the panel for screening. The control panel consisted of 275 normal males and 65 normal females. All samples were obtained with informed consent.

2.1.12 Online resources for clone mapping and alignments

In silico sequence analysis was performed using a combination of databases and alignment tools available online. They are listed in alphabetical order (with URLs) in Table 8.

Table 8: Online resources for mapping and alignments

Institute and/or alignment tool	URL
BLAST at NCBI	http://www.ncbi.nlm.nih.gov/BLAST/
Celera Discovery System™	http://www.celeradiscoverysystem.com/
CEPH (Centre d'Etude du Polymorphisme Humain)/Généthon	http://www.cephb.fr/bio/ceph-genethon-map.html
GenBank	http://www.ncbi.nlm.nih.gov/Genbank/
Integrated X Chromosome database	http://www.ixdb.molgen.mpg.de/
National Center for Health Statistics	http://www.cdc.gov
NIX analysis	http://www.hgmp.mrc.ac.uk/
Online Mendelian Inheritance in Man (OMIM)	http://www.ncbi.nlm.nih.gov/Omim
PipMaker	http://bio.cse.psu.edu/pipmaker/
RZPD (Resource Zentrum Primary Database)	http://www.rzpd.de/
Sanger Institute	http://www.sanger.ac.uk/
UCSC Human Genome Working Draft	http://genome.ucsc.edu/
Whitehead Institute STS-Based Map of the Human Genome	http://www.broad.mit.edu/cgi-bin/contig/phys_map
XLMR Update (October 2003 Petro Chiurazzi)	http://www.xlmr.interfree.it/home.htm

2.2 Methods

2.2.1 DNA Isolation

DNA was extracted from lymphoblastoid and fibroblast cell lines according to standard techniques (Miller et al. 1988). Essentially, cells were resuspended in lysis buffer (see section 2.1.2), and subject to overnight proteinase K digestion, followed by phenol-chloroform extraction, extraction with chloroform alone, and ethanol precipitation. Approximately 500 µg DNA was obtained from $1x10^8$ lymphoblastoid cells; approximately 100 µg DNA was obtained from $2x10^7$ fibroblast cells. Plasmid and cosmid DNAs were isolated using QIAprep™ plasmid miniprep and midiprep kits according to supplied protocols.

2.2.2 RNA Isolation

Total RNA from fibroblast or lymphoblastoid cell lines was isolated using TRIzol™ Reagent (Invitrogen™) according to the manufacturer's protocol, which is based on the acid guanidine thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987). To prevent RNase contamination, all steps were performed using RNase-free solutions, pipette tips and reaction tubes. Approximately 500 µg RNA was obtained from $1x10^8$ cells lymphoblastoid cells; approximately 100 µg RNA was obtained from $2x10^7$ fibroblast cells.

2.2.3 Fluorescence *in situ* hybridisation (FISH)

Chromosome analysis was performed using standard high-resolution techniques. For breakpoint mapping, CEPH and ICRFy900 YAC probes selected from the Whitehead Institute and the Integrated X Chromosome database contigs, and BACs and PACs from the Sanger Institute and UCSC "Golden Path" were used. Cosmid clones were selected from the chromosome-specific cosmid libraries available from RZPD. Clones were prepared by standard techniques, labelled with appropriately coupled dUTPs by nick translation or directly labelled by DOP-PCR, and used as probes in FISH as described previously (Wirth et al. 1999).

2.2.4 Preparation of DNA probes for hybridisation

All of the following PCR reactions were done with AmpliTaq™ (Perkin Elmer™), using the supplied buffer (1.5mM $MgCl_2$ final concentration). PCR conditions included a 2-5 minute denaturation at 94°C, 30-40 cycles of 94°C for 1 minute, annealing for 1 minute, and 72°C for 1-2 minutes (depending on product size), followed by 7 minutes at 72°C. Annealing temperature (T_A) for each set of primers, and all relevant primer sequences, together with descriptive names (for primers, based on corresponding clone sequence; for probes, based on corresponding patient study, relevant chromosome, or appropriate gene name), are indicated in the tables below. Amplification of product was confirmed in 0.8 to 2 % agarose gels, and fragments were purified using either the QIAquick™ Gel Extraction Kit or the MinElute™ Gel extraction Kit (Qiagen™), according to the manufacturer's protocol. All primers, shown in 5' to 3' orientation, were synthesised at MWG Biotech™. Relevant X chromosome genomic probes are listed in Table 9, probes used for localisation of autosome breakpoints are listed in Table 10, and probes for northern blots are listed in Table 11.

Table 9: Primers for amplification of relevant X chromosome probes

Probe	Primer Name	Primer Sequence	T_A
Patient 1 - BP (1584 bp)	596C15-41642fw	TTTTCTACTGAGAGGTCTTCTGG	59°C
	596C15-43225rv	TTTAGAGTCTCACACTATCACCC	
Patient 2-1 (1020 bp)	bwxD27-79678fw	ATTCATCTCTCAGGGATTGTAG	57°C
	bwxD27-80697rv	CATTTAGTGGCACTATAACAGG	
Patient 2-2 (1011 bp)	bwxD27-49071fw	TAGATTTCTGTATAGTTTCTCTAC	55°C
	bwxD27-50081rv	ATCATTAAAAAGTCAAGAAACAAC	
Patient 2-3 (731 bp)	bwxD27-14606fw	ATTCTCATTTGCCTGGAATGG	57°C
	bwxD27-15336rv	GTATACCATAGCCACATTAGC	
Patient 3-1 (966 bp)	83I24-18500fw	GCCAGTCTCTAAGTTCCAAAG	59°C
	83I24-19465rv	ACCCTCATTCATTTTACACCAG	
Patient 3-2 (986 bp)	83I24-61390fw	TGCACAGTGTATTTGGCACTG	59°C
	83I24-62375rv	GGTCCAGTGACATTACTTTGC	
Patient 3-3 (940 bp)	83I24-129191fw	TGATAGTGAGGCAAGTGTGTG	59°C
	83I24-130130rv	ATGCCATCTCTCTACCTTGTC	
Patient 3-BP* (1026 bp)	83I24-9087fw	ATGCACTACAGAGTTTATGTGAG	59°C
	83I24-10112rv	TTCTCTTGGGCTTTACTCTATTG	

*This probe was used for breakpoint (BP) identification by Southern blot.

Table 10: Primers for amplification of relevant autosome probes

Probe Name	Primer Name	Primer Sequence	T_A
Chr 2-1 (1034 bp)	421J10-18035fw	AGAAGGACAGATTTTCTTACTG	56°C
	421J10-19069rv	CAAATCTAGTATTTCACCAGAG	
Chr 2-2 (990 bp)	421J10-88322fw	AGCATTGCCTACTTCATCTCC	59°C
	421J10-89311rv	GAGAACTGTAGCCTTCTTGAG	
Chr 14-BP* (1260 bp)	966I7-101623fw	TAAAGGCTTGTGCTTGAGAAGC	59°C
	966I7-102882rv	GGTAACTTCATCATGCCAATGG	
Chr 4-1 (1038 bp)	†MAPKCel-14463fw	GGGAACATCTTCCCTAAAGG	59°C
	†MAPKCel-15500rv	ATCATTTCCACTGGTCAGGG	
Chr 4-2 (1051 bp)	†MAPKCel-49930fw	GAGAACAAGATTTGCCTTCAG	57°C
	†MAPKCel-50981rv	AAGTGTACAATTCCCAGAAGC	
Chr 4-3 (1101 bp)	†MAPKCel-74952fw	GAGGAAACATTTTCCAGGATAG	57°C
	†MAPKCel-76052rv	AATAATGCAGAGATGTAGGCTC	
Chr 4-4 (1095 bp)	†MAPKCel-107399fw	TGTATACCTACCTCTTCTTTGC	57°C
	†MAPKCel-108493rv	CCAGCCAAATTGGTTGAATTAG	
Chr 4-5 (874 bp)	†MAPKCel-122858fw	AAGATTCCTGACCAAGGCAC	57°C
	†MAPKCel-123731rv	AAGCAAGTCTTGTCATTTGCC	
Chr 4-BP* (1298 bp)	†MAPKCel-77714fw	GGGTATGCTTATCACCTTCAAAG	60°C
	†MAPKCel-79011rv	ATACCACTGGCTGCTAAGAAGG	

†MAPCel refers to sequence from Celera Discovery System™ corresponding to JNK3 (also referred to as MAPK10)
*Theses probes were used for breakpoint (BP) identification by Southern blot.

Table 11: Primers used for amplification of cDNA northern blot probes

Probe	Primer Name	Primer Sequence	T_A
NXT2 (397 bp)	DC9exon2fw	TTATGTAGATCAGGCATGTAG	55°C
	DC9exon2rev	ACTAGACCAATCTTGAAAACG	
ABCB7 (591 bp)	ABC7(62-82)fw	GCTCAAGATGGCGCTGCTCG	61°C
	ABC7(631-652)rv	CCAATCAGAACTGCTGTTGCC	
ZNF41 (479 bp)	ZNF41fw621	AGTCAGAGGCTGCCTTCAAG	60°C
	ZNF41rv1099	AGTAGAGGAAGGGCTATGGG	

2.2.5 RT-PCR experiments

Five µg of RNA was used for reverse transcription with Superscript™ II (Invitrogen™), essentially according to the manufacturer's protocol, but in the presence of RNAguard™ (Amersham™ Pharmacia™), using random hexamers for priming. Amplification of *HPRT* with intron-spanning primers (and in some cases also *G3PDH* with non-intron-spanning primers) served as a cDNA loading control. For studies on *ZNF41,*

breakpoint-spanning primers, and primers 3' and 5' to the breakpoint were used to amplify translocation patient and control *ZNF41* transcripts. Splice variants in XLMR patient and controls were amplified with one of the two forward primers F1 or F2 and the reverse primer RX (5'-CTG TTC AAG GCA GCA TGG TC-3' T_A 60°C) that is specific for the known *ZNF41* variants 41.3 and 41.6. All PCR reactions were done essentially as described for probe amplification. Cycle number and extension times were adjusted as necessary; primers and annealing temperatures not indicated above are listed in Table 12 below.

Table 12: Primers for RT-PCR analyses in Patient 3

Product	Primer Name	Primer Sequence	T_A
ZNF41 (5') (304 bp)	ZNF41fw41 (F1)	CTTCGGAGCTGACACTAAGC	60°C
	ZNF41rv344 (R1)	AAGCTGAGCTGGCATCTGTG	
ZNF41 (BP) (296 bp)	ZNF41fw407 (F2)	ATGGCAGCTAATGGGGACTC	60°C
	ZNF41rv702 (R2)	CCTGAACAGCTCTGATGTGG	
ZNF41 (3') (479 bp)	ZNF41fw621 (F3)	AGTCAGAGGCTGCCTTCAAG	60°C
	ZNF41rv1099 (R3)	AGTAGAGGAAGGGCTATGGG	
HPRT (504 bp)	Hprtfw	TGGCGTCGTGATTAGTGATG	60°C
	Hprtrv	TATCCAACACTTCGTGGGGT	
G3PDH (688 bp)	G3PDH 137for	ACCCCTTCATTGACCTCAACTAC	61°C
	G3PDH 824rev	TGCTTCACCACCTTCTTGATGTC	

2.2.6 Isotope-labelling of probes for hybridisations

For hybridisations, 20-100 ng of gel purified PCR-amplified probe DNA was labelled by random priming in oligo labelling buffer (OLB) in the presence of [α-^{32}P]dCTP and Klenow fragment polymerase. To remove excess non-incorporated dNTPs and random hexamers, labelled probe was mixed with dextran blue/phenol red dye, separated on a Sephadex G-50 column and eluted with TES buffer, and denatured for 5-10 minutes at 95°C. Oligo probes were labelled with polynucleotide kinase in the supplied buffer solution (MBI Fermentas^{TM}) in the presence of [γ-^{32}P]ATP, and purified by MicroSpin^{TM} G-50 Columns (Amersham Biosciences^{TM}) according to the manufacturer's protocol.

2.2.7 Library screening

Cosmid libraries were prehybridised at 65°C for 1-4 hours in PEG hybridisation buffer supplemented with 0.1 mg/mL denatured herring sperm DNA as blocking reagent.

Labelled DNA probes were denatured for 5-10 min at 95°C, chilled on ice, pre-incubated with human placental DNA (Hybridime™, HT Biotechnology™) for 1-2 hours in PEG hybridisation buffer to block repetitive sequences, and added to the prehybridisation buffer. Membranes were incubated overnight at 65°C. Washing with 0.2X SSC/0.1%SDS was repeated as necessary at temperatures from 55°C – 65°C. Autoradiographs were exposed at -80°C from 12 hours to three days, as necessary for clarity of observed signals.

2.2.8 Southern blot hybridisations

Genomic DNAs were digested with appropriate restriction enzymes, separated in 1% agarose gels with a 1kb DNA ladder (GeneRuler™, MBI Fermentas™) molecular weight marker for reference, and transferred to Roti®-Nylon plus membranes (Roth™) by overnight alkaline transfer in 10X SSC. Membranes were rinsed in 2X SSC, and DNA was fixed by UV crosslinking. Hybridisation and washing were as for library screening. Autoradiographs were exposed at -80°C for up to 1 week.

2.2.9 Northern blot hybridisations

For northern blot hybridisations, 20 to 25 μg of total RNA from patient and control lymphoblastoid cell lines was denatured in formamide loading buffer (50% deionised formamide and 6% formaldehyde in 1X MOPS) and separated in a 1% agarose gel with 3.7% formaldehyde in 1X MOPS buffer. RNA was transferred to Roti®-Nylon plus membranes (Roth) by overnight alkaline transfer in 20X SSC. Membranes were rinsed in 2X SSC, and RNA was fixed by UV crosslinking. Visualisation of ribosomal RNAs by ethidium bromide staining served as an approximate size standard. Human multiple tissue northern blots were purchased (Clontech™). Blots were probed with labelled oligo or cDNA probes (described in sections 2.2.6) in QuikHyb™ (Stratagene™), essentially according to manufacturer's protocol (68°C for cDNA probes; 42°C for oligo probes). To control for RNA loading, blots were hybridised either to the *β-actin* cDNA probe provided, or to the *G3PDH* fragment described in section 2.2.5. Following washing as needed in 0.2X SSC/0.1%SDS, blots were exposed to radiographic film for up to 2 weeks. Each commercial blot contained 2 μg poly(A)$^+$ RNA from the following tissues: #7760-1 (human adult RNAs): heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas; #7756-1 (human foetal RNAs):

brain, lung, liver and kidney; #7769-1 (human brain RNAs): amygdala, caudate nucleus, corpus callosum, hippocampus, brain (whole), subsantia nigra, thalamus.

2.2.10 Breakpoint cloning (Patient 3)

Breakpoint cloning was performed essentially as described previously (Siebert et al. 1995). Patient DNA was digested overnight with *Eco*RI restriction endonuclease, and ethanol precipitated. Approximately 1 µg of total digested DNA was ligated to preannealed adaptor oligos (for sequence, see table below) using T4 DNA ligase (Promega), overnight at 14°C in a 50 µL reaction. Following ligase denaturation (10 minutes at 70°C), and 1:10 dilution of ligation reaction, 5 µL were used as template for 50 µL PCR reactions with AP1 and sequence-specific Primer 1. After 1 minute 30 seconds at 94°C cycles included 30 seconds at 94°C, 30 seconds annealing (at T_A), and 3 minutes extension at 72°C, and were followed by 7 minutes at 72°C. The PCR reaction was diluted and used as a template for a nested PCR with AP2 and Primer 2, with the same conditions except for annealing temperature modification. For both reactions, touchdown PCR was employed: for AP1 and Primer 1, 2 cycles for each T_A 67°C, 65°C, and 63°C preceded 30 cycles for T_A 61°C; for AP2 and Primer 2, 2 cycles for each of T_A 65°C, 63°C, 61°C and 59°C preceded 30 cycles for T_A 58°C. A fragment of approximately 700 base pairs was obtained, gel purified, and reamplified with AP2 and Primer 2 before cloning into pGEM®-T Easy™ vector. The inserted breakpoint fragment was sequenced using the BigDye™ Terminator Chemistry (PE Biosystems™) and separated on an ABI 377 DNA sequencer. Primers and adaptor oligo sequences are listed in Table 13.

Table 13: Primer/oligo sequences for Patient 3 breakpoint cloning.

Name	Function	Sequence
Adaptor(long)	Adaptor	CTAATACGACTCACTATAGGGCTCGAGCGG CCGCCCGGGCAGGT
AdaptorEcoRIPhos*		AATTACCTGCCCGG
Primer 1	Initial PCR	CACACAGATAGAGACGATACA
AP1		GGATCCTAATACGACTCACTATAGGGC
Primer 2	Nested PCR	TCTTACAGACCATCTCTAAACC
AP2		TATAGGGCTCGAGCGGC

* 5’ phosphate modification

2.2.11 Mutation screening of *ZNF41* by denaturing high performance liquid chromatography (DHPLC)

PCR products ranging from 100-300 nucleotides in length and corresponding to the complete known human *ZNF41* coding sequence plus up to 40 nucleotides of neighbouring intronic sequences were amplified from genomic DNA and analysed by DHPLC. The coding sequence screened corresponds to *ZNF41* mRNA transcript variant 1 (also referred to as *ZNF41.1*, GB Accession # NM_007130), plus 3' exon 2 extension indicated by the sequence *ZNF41.6* (GB Accession # AJ010021) and 5'exon 3 extension indicated by the sequences *ZNF41.6* and *ZNF41.3* (GB Accession # AJ010018). Exon 5 was amplified in 8 overlapping fragments (exons 5a-h). PCR reactions were carried out in 50 µl reaction volumes containing 100 ng of genomic DNA, 300 pmol of each primer, 0.4 mM dNTPs, 1U AmpliTaq™ DNA polymerase (Perkin Elmer™), and 2-4 mM $MgCl_2$, with the exception of exon 5d, which was amplified using 1.5U Taq DNA polymerase and Q-Solution™ (Qiagen™). An initial 5 minute denaturation at 95°C was followed by 35 cycles of 1 minute at 95°C, 1 minute of annealing, and 1.5 minutes extension at 72°C, and then a final extension step of 10 minutes at 72°C. Sequences of primer pairs (in 5' to 3' orientation) and specific amplification conditions (T_A and $MgCl_2$ concentration) are indicated in Table 14. Products were checked by 1.5 % agarose gel electrophoresis before analysis by WAVE nucleic acid fragment analysis system (Transgenomic™). Paired products were denatured at 95°C for 5 minutes and slowly brought to room temperature. Re-annealed DNA duplexes were injected and eluted with a linear acetonitrile gradient at a flow rate of 0.9 mL/min, with a mobile phase consisting of a mixture of buffer A (0.1M triethylammonium acetate, or TEAA, with 0.1% acetonitrile) and buffer B (0.1M TEAA with 25% acetonitrile). Melting profiles and resolution temperatures for each fragment were predicted by the Transgenomic™ WAVEMAKER™ software version 4.1. For each exon, runs were repeated with 2-4 temperatures (listed in Table 15), which spanned the predicted optimal temperature range. For each pair of patient or control samples exhibiting exceptional elution profiles, DNA was re-amplified, gel purified using the QIAquick™ gel extraction kit (Qiagen™), sequenced using the BigDye™ Terminator Chemistry (PE Biosystems™) and separated on an ABI 377 DNA sequencer.

Table 14: Primers and conditions for PCR amplification of *ZNF41* fragments for analysis by DHPLC

ZNF41 Exon	Forward primer	Reverse primer	T_A	[$MgCl_2$]
2	GTTGTCAGCAGGAG AGCCTG	TGTCTCCAGGGT GGCAGGTC	64°C	3 mM
3	GCATTAAAAACAAC TTGCTATC	ATGGAAATACCC TCATTAGCAG	56°C	2 mM
4	CCAATCAGCTGGAC CCAATC	GTGATACTGACT TCCATGTCC	62°C	4 mM
5a	CTGCTGATTGTTCTT TGTGTCG	ATTGTGTCACAG TTATGGAGTC	61°C	4 mM
5b	ATAATTCATGTGAC TACCAAGC	CCATTACACATT CAGTACACAC	61°C	4 mM
5c	CCACCCACCATCAG AAAATTC	TCAGATGTCTAA AGAGGTCTG	59°C	3 mM
5d	AGAAACCCTACAAA TGCAGTG	CTCCAGTATGAA TTCTCTGATG	59°C	2.25 mM
5e	GGCCTTCATCCAGA AATCAC	CCACAGCCATTG CACTTATAG	61°C	4 mM
5f	AAAGGCTTTTACTG ACCAGTC	TGCATTCATAAG GCTTCTCTC	59°C	4 mM
5g	AGAAATCGCACTTC ATTGCGC	TCTGGATGAAAG CTTTCCCAC	61°C	4 mM
5h	AGTCTCATACTGGA GAAAGAC	CTGCTATTAGAT ACCTGATGC	59°C	3 mM

Table 15: Products sizes and temperatures used for DHPLC runs

ZNF41 Exon	Product size	T_1	T_2	T_3
2	247 bp	61.9°C	62.5°C	64.0°C
3	254 bp	61.0°C	61.9°C	62.6°C
4	186 bp	59.8°C	61.8°C	63.0°C
5a	338 bp	54.9°C	55.5°C	56.8°C
5b	313 bp	55.4°C	56.7°C	57.7°C
5c	351 bp	57.2°C	58.4°C	58.9°C
5d	336 bp	56.9°C	57.9°C	58.9°C
5e	333 bp	57.0°C	58.0°C	Not applicable
5f	326 bp	57.0°C	58.3°C	59.5°C
5g	339 bp	56.3°C	58.0°C	59.0°C
5h	263 bp	56.0°C	57.0°C	57.8°C

2.2.12 Cell fractionation and western blotting

Cell fractionation was performed essentially as described previously (Gray and Whittaker 1962). Cells were suspended in sucrose HEPES (hydroxyethylpiperazine ethanesulfonate) buffer supplemented with β-mercaptoethanol (1%) and Complete™ Protease Inhibitor Cocktail (Roche), and lysed manually with a dounce homogeniser. Pellet P_1 (nuclei and cell debris) was obtained by centrifugation at 1000*g* for 10 minutes. This pellet was resuspended in sucrose buffer, and the centrifugation was repeated. The combined supernatants of this step (S_1) were centrifuged at 17000*g* to obtain pellet P_2 (cytoplasmic components including mitochondria). The remaining supernatant (S_2) was centrifuged at $1x10^5 g$ for 1 hour to obtain the microsomal fraction (P_3), and the remaining supernatant S_3. All pellets were resuspended in yeast lysis buffer, also supplemented with Complete Protease Inhibitor Cocktail (Roche). Protein fractions were denatured at 95°C for 5 minutes in 1X Laemmli buffer, and separated on a 10% polyacrylamide gel according to standard procedures. Following semi-dry electrotransfer to microporous polyvinylidene difluoride (PVDF) membrane, proteins were blocked in 5% milk in PBST (1X PBS supplemented with 0.1% Tween™ 20), for minimum 1 hour, and hybridised overnight at 4°C to anti-JNK3 (Upstate Biotechnology™) diluted 1:2000 in 1% BSA/PBST. Blots were washed 3x 5 minutes in PBST, and hybridised to anti-rabbit IgG conjugated to horseradish peroxidase. Following 3x 5 min washes in PBST, proteins were visualised by enhanced chemiluminescence (ECL) detection (Amersham Biosciences™) and exposure to radiographic film.

2.2.13 Recombinant DNA techniques

Purified and digested plasmids and PCR products were ligated into appropriate vectors with T4 DNA ligase (Promega™) and heat-shock transformed or electroporated into competent *E. coli* DH5α cells according to standard protocols. Complete coding sequences for all constructs used in expression studies were verified by sequence analysis. Constructs with N-terminal JNK3 and C-terminal EGFP were created by PCR as follows: JNK3α2 was amplified with cloning primers 1 and 2 (see table below), digested with *Bgl*II and *Bam*HI and ligated into pEGFP-N3 (Clontech™) digested with the same enzymes. Prior to ligation, digested vector was treated for 1 hour with calf intestinal phosphatase, to reduce the background due to vector self-ligation. The mutant was created by the same technique, using

primers 1 and 3. Construction of reciprocal constructs, with C-terminal JNK3, involved restriction endonuclease digest of the constructs described above with the same enzymes, and cloning into the appropriately digested pEGFP-C1 construct (Clontech™), which harbours an N-terminal EGFP protein tag. For FLAG-tagged constructs, similar techniques were employed. Two partial *JNK3* clones available from RZPD (IMAGp998G1411658Q2 and IMAGp998I2410772Q2) were used in a combination of PCR amplification reactions and restriction digests that enabled us to create a clone containing the complete coding sequence of *JNK3α2*, fused in frame with the FLAG motif present in the pCMVTag-2B vector (Stratagene™). Using this clone as a template, primers 4 and 5 were used to amplify the FLAG-JNK3 wild type coding sequence, and primers 4 and 6 were used to amplify the FLAG-JNK3 truncation coding sequence. Following restriction digest, both were cloned into the pcDNA3.1 vector to create the expression constructs used for the immunofluorescence experiments shown. All relevant primer sequences are shown in Table 16.

Table 16: Primers used for construction of JNK3 expression constructs

Primer	Descriptive Name	Sequence
1	jnk3GFPN3bgliifw	ATTAGATCTGTGGTATTTATGAGCCTCCATTTC
2	newjnk3wtGFPbam	ATTGGATCCTTTCCTGCAACAACCCAGGGG
3	jnk3mutGFPN3bamhirv	CTTGGATCCGTCCCTTCCTGGAAAGAG
4	pcdnakpnFLAGjnkfw	ATAGGTACCGTGGTATTTATGGATTACAAGGA TGAC
5	pcdnaXbaJNKwtSTOP	CTAGTCTAGATCACCTGCAACAACCCAG
6	JNK3aa267stopXhoIrv	ATTACTCGAGTTAGTCCCTTCCTGG

2.2.14 Cell culture conditions and transfection experiments

Both HeLa and Neuro2A cells were cultured according to standard conditions. HeLa cells were maintained in DMEM/F12 medium with 1.5g/L glucose and 10% FBS, supplemented with L-glutamine, and penicillin/streptomycin. Neuro2A cells were maintained in DMEM with 1.5g/L glucose and 10% FBS, supplemented with L-glutamine, non-essential amino acids, and penicillin/streptomycin. All transient transfection experiments were done with Lipofectamine 2000 (Invitrogen), essentially according to the manufacturer's recommendations, except that antibiotics were removed. Cells were seeded at a density of 1.5 – 2.5 x10^4 per cm^2 and transfected at approximately 80-90% confluency, with 2-4 µg plasmid DNA for 10 cm^2 of adherent cells. As recommended by the manufacturer, Lipofectamine

volume was optimised with respect to DNA and cell density, and was typically approximately 2.5 µL/µg transfected plasmid DNA. Following transfection, cells were maintained in medium free of antibiotics.

2.2.15 Immunofluorescence

For immunofluorescence 24 and 48 hours post-transfection, cells were washed once in PBS and fixed to glass coverslips in 4% paraformaldehyde/PBS for 10 minutes. Experiments were conducted essentially according to standard techniques: after fixation, cells were washed once in PBS and permeabilised for 10 minutes in 0.2% Triton™ X 100/PBS, then washed 3X in PBS (including one wash with 0.5% glycine/PBS to remove excess paraformaldehyde). Cells were blocked for 30 minutes in block/wash buffer (0.5% BSA/PBS with 0.05% Tween™ 20), then incubated for 1.5 hours at room temperature with primary antibody (anti-FLAG) in block/wash buffer (1:300 dilution). Cells were washed 3x 5 minutes in block/wash buffer, and incubated for 30 minutes at room temperature with secondary antibody (anti-Cy3), diluted in block/wash buffer (1:1000). After washing 3x 5 minutes in block/wash buffer, cells were mounted onto slides in Vectashield™ Mounting Medium (Vector Laboratories) containing DAPI (Serva™) at 1 µg/mL and visualised by fluorescence microscopy with a Zeiss Axioskop epifluorescence microscope (Carl Zeiss) equipped with single band pass filters for excitation of green, red, blue and infrared fluorescence (Chroma Technologies™), and 63x and 100x plan-neofluoar lenses. Digital black-and-white images were recorded with a cooled CCD camera (Hamamatsu Photonics™) and merged to RGB-images by the ISIS fluorescence image analysis system (MetaSystems™). Images were further processed using Adobe Photoshop™ to match the fluorescence intensity seen in the microscope.