2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Table 1. Standard chemicals and reagents are listed in the alphabetical order.

Name	Company
Acetic anhydride	Sigma
Acrylamide mix [Rotiphorese® Gel 40 (29:1)]	Roth
L-Adenine hemisulfate salt	Sigma
Agar	Difco
Agarose	Invitrogen
Ammmonium chloride	Merck
Ammonium persulfate (APS)	Invitrogen
Ampicillin	Sigma
L-Arginine HCl	Sigma
Bacto peptone	Difco
Bacto yeast extract	Difco
Blocking reagent	Boehringer
Bovine serum albumin (BSA)	Sigma
Bradford reagent	Sigma
Brefeldin A	Sigma
Bromophenol blue	Fluka
Calcium chloride	Merck
Chloroform	Merck
Citric acid	Merck
Complete mini protease inhibitor cocktail tablets	Roche
4'6-diamino-2-phenyl-indole (DAPI)	Serva
Dextran blue	Fluka
Dextran sulfate	Sigma
Diethylpyrocarbonate (DEPC)	Sigma
Dimethyl sulfoxide (DMSO)	Merck
Disodiumhydrogen phosphate	Merck
Dithiothreitol (DTT)	Sigma
dNTPs	MBI Fermentas
DPBS (PBS for cell culture)	BioWhittacker
Dulbecco's Modified Eagle's Medium (DMEM) with 4,5 g/l glucose	BioWhittacker
Ethanol	Merck
Ethidium bromide	Serva
Ethylenediaminetetraacetic acid (EDTA)	Merck
Fetal Bovine Serum (FBS)	Biochrom AG
Fluoromount-G	Science Services
Formamide	Merck
Formaldehyde	Roth
GeneRuler 1 kb and 100 bp DNA ladders	MBI Fermentas
Glacial acetic acid	Merck
Glucose	Merck
L-Glutamine solution for cell culture	BioWhittacker

Glutaraldehyde	Sigma
Glycerol	Merck
Glycine	Sigma
Heat inactivated sheep serum (HISS)	Gibco
Heparin sodium salt	Sigma
Herring sperm DNA	Roche
Hydrogen peroxide	Merck
L-Histidine HCl monohydrate	Sigma
Hybridime human placental DNA	HT
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium (HEPES)	Sigma
L-Isoleucine	Sigma
Isopropanol	Merck
Kanamycin	Invitrogen
L-Leucine	Sigma
Lithium acetate	Sigma
Lithium chloride	Merck
L-Lysine HCl	Sigma
Magnesium chloride	Merck
Magnesium sulfate	Merck
Maleic acid	Merck
Manganese chloride	Sigma
®-Mercaptoethanol	Merck
Methanol	Merck
L-Methionine	Sigma
3-[N-Morpholino]propanesuflonic acid (MOPS)	Sigma
N,N,N ,N –Tetramethylethylenediamine (TEMED)	Invitrogen
Nocodazole	Sigma
Nonidet P40 (NP40)	Fluka
OPTIMEM with GLUTAMAX	Gibco
Paraformaldehyde (PFA)	Sigma
Pd(N)6 random hexamers	Pharmacia
Penicillin/streptomycin antibiotic solution for cell culture	Invitrogen
Phenol	Roth
Phenol red	Fluka
L-Phenylalanine	Sigma
Polyethylene glycol 3350 (PEG 3350)	Sigma
Polyethylene glycol 6000 (PEG 6000)	Merck
Polyfectamine transfection reagent	Qiagen
Potassium acetate	Merck
Potassium chloride	Merck
Potassium dihydrogen phosphate	Merck
Precision Plus Protein Kaleidoscope Standards	Bio-Rad
L-Proline	Sigma
Protector RNase Inhibitor	Roche
Ribonucleic acid from Baker’s yeast type III (tRNA)	Sigma
Rubidium chloride	Sigma
Salmon sperm DNA	Sigma
Sephadex G-50	Pharmacia
Sodium acetate	Merck
Sodium azide	Sigma
Sodium chloride	Merck

Sodium citrate	Merck
Sodium deoxycholate	Sigma
Sodium dihydrogen phosphate	Merck
Sodium dodecyl sulfate (SDS)	Serva
Sodium hydroxide	Merck
Tetramisole hydrochloride (levamisole)	Sigma
Thiamine hydrochloride	Sigma
L-Threonine	Sigma
Tris (hydroxymethyl)-aminomethane	Merck
Triton X-100	Roth
TRIzol reagent	Invitrogen
L-Tryptophane	Sigma
Trypton	Difco
Tween 20	Sigma
L-Tyrosine	Sigma
L-Valine	Sigma

2.1.2 Buffers and solutions

Aqueous solutions were prepared using autoclaved Millipore water. For sterilization, solutions were autoclaved or passed through a 0.45 μm filter (Millipore). Solutions required for SDS polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis and immunofluorescence are listed in Table 2. Solutions used for whole mount and section *in situ* hybridization are listed in Table 3.

Table 2. Solutions used for SDS-PAGE, Western blot analysis and immunofluorescence.

Solution	**Components**
10×Protease inhibitor solution	1 tablet of complete mini protease inhibitor cocktail per 1 mL H_2O
Cell lysis buffer	150 mM NaCl 50 mM Tris-HCl (pH 7.5) 1% NP40
2×Sample buffer	125 mM Tris-HCl (pH 6.8) 4% SDS 10% Glycerol 0.006% Bromophenol blue 2% β-Mercaptoethanol
Protein loading buffer	350 mM Tris-HCl (pH 6.8) 10% SDS 30% Glycerol 9,3% DTT 0.012% Bromophenol

1×Electrophoresis buffer	25 mM Tris-HCl 250 mM Glycine 0.1% SDS
Western blot transferring buffer	192 mM Glycine 25 mM Tris-HCl 20% Methanol
1×PBS (pH 7.3)	137 mM NaCl 2.7 mM KCl 10.1 mM Na_2HPO_4 1.8 mM KH_2PO_4
PBST	0.1% Tween 20 in 1×PBS
Blocking buffer for Western blot	5% (w/v) nonfat dry milk in 1×PBST
Blocking solution for immunocytochemistry	1×PBS 10% FBS or 3% BSA 0.1% Triton X-100

Table 3. Solutions used for whole mount and section *in situ* hybridization

Solution	**Components**
DEPC- H_2O	0.1% (v/v) DEPC in H_2O
DEPC-PBS	0.1% (v/v) DEPC in 1×PBS
DEPC-PBST	0.1% (v/v) Tween 20 and 0.1% DEPC in 1×PBS
PBST/Glycine	0.2% (w/v) Glycine in DEPC-PBST
PBST/Tetramisole	0.05 % (w/v) Tetramisole in DEPC-PBST
RIPA buffer	0.05% SDS 150 mM NaCl 1% (v/v) NP40 0.5% (w/v) Sodium deoxycholate 1 mM EDTA 50 mM Tris-HCl in DEPC- H_2O
PFA/Glutaraldehyde	4% PFA/PBS (pH 7.4) 0.2% Glutaraldehyde
20×DEPC-SSC (pH 7.0)	300 mM Sodium citrate 3 M NaCl 0.1 % DEPC

Heparin	100 mg/mL Heparin in 4 ×DEPC-SSC
Hybe buffer	50 % Formamide 0.1 % Tween 20 50 µg/mL Heparin 5×DEPC-SSC (pH 4.5, adjusted with citric acid) diluted in DEPC- H_2O
SSC/FA/T	2×SSC 50% Formamide 0.1 % Tween 20
5×MABT	0.5 M Maleic acid (pH 7.5) 0.75 M NaCl 5 % Tween 20
RNase solution	0.5 M NaCl 10 mM Tris-HCl (pH 7.5) 0.1 % Tween 20
Alkaline phosphatase buffer	100 mM NaCl 50 mM Mg Cl_2 100 mM Tris-HCl (pH 9.5) 0.05 % (w/v) Tetramisole 0.1 % Tween 20
10×Wash buffer	4 M NaCl 1 M Tris-HCl (pH 7.5) 0.5 M EDTA
TNE	10 mM Tris-HCl (pH 7.5) 500 mM NaCl 1 mM EDTA
NTMT (pH 9.5)	100 mM NaCl 100 mM Tris-HCl (pH 9.5) 50 mM Mg Cl_2 0.1% Tween 20
Hybridization solution (for paraffin sections)	10 mM Tris-HCl (pH 7.5) 600 mM NaCl 1 mM EDTA 0.25% SDS 10% Dextran sulfate 1× Denhardt solution 200 µg/mL Ribonucleic acid from Baker's yeast type III 50% Formamide

2.1.3 Antibodies and kits

Table 4. List of antibodies

Antibody	Source
Anti-HA antibody Rabbit	Sigma
Monoclonal anti-Flag antibody Rabbit	Sigma
Polyclonal anti-Flag antibody Mouse	Sigma
Alexa Fluor® 546 goat anti-rabbit IgG	Molecular Probes
Alexa Fluor® 488 goat anti-mouse IgG	Molecular Probes
Anti-Rabbit IgG (Goat), peroxidase conjugate	Oncogene
Anti-Mouse IgG (Goat), peroxidase conjugate	Oncogene
Anti-digoxigenin-AP	Roche
Anti-Actin Rabbit	Sigma
Anti- γ-Adaptin Mouse	Bd Biosciences
Anti-AKAP450 Mouse	Bd Biosciences
Anti-Elastin #RA75 Goat	EPC
Anti-ERK1 Mouse	BD Biosciences
Anti-ERK2 Mouse	BD Biosciences
Anti-Fibulin-4 Mouse	Dr. Lihua Y. Marmorstein
Anti-Fibulin-5 #4075 Rabbit	Dr. Elaine Caroline Davis
Anti-GM130 Sheep	Dr. Francis A. Barr
Anti-GMAP210 Mouse	BD Biosciences
Anti-PDI Mouse	Stressgen
Anti-SP-B Goat	Santa Cruz
Anti-SP-C Goat	Santa Cruz
Anti-TGN46 Sheep	Serotec
Anti-β-Tubulin Mouse	Sigma
Anti-Vimentin Mouse	Sigma

Table 5. List of commercial kits

Kit	Company
BigDye terminator cycle sequencing ready reaction kit	PE Biosystems
10×DIG RNA labelling mix	Roche
BM Purple AP Substrate	Roche
DAB Kit	Vector
Lectin Blocking Kit	Vector
Lectin Kits	Vector
QIAprep spin miniprep kit	Qiagen
QIAgen plasmid midi and maxi kits	Qiagen
QIAquick gel extraction kit	Qiagen
Revert Aid kit	Fermentas
Roti™-Lumin Chemiluminescence substrate	Roth
SYBR green	Applied Biosystems
VECTASTAIN® ABC Kits	Vector

2.1.4 Primers

Table 6. Probe primers

Name of Primer	Sequence
MSP01_for	5'-GCTGGACAGGGTCTATGGAA-3'
MSP01_rev	5'-TTGACCAGAGCACAGTGAGG-3'
MSP02_for	5'-TGTCTCAGCCAGTGCGATAC-3'
MSP02_rev	5'-TGGCCATTGCTATTTGTGAA-3'
MSP03_for	5'-TTGTCTACTGGCTGCACTGG-3'
MSP03_rev	5'-CCCTGAAAAGATGCCGATAA-3'
MSP04_for	5'-CAGAATGGTGTTCCCGAAGT-3'
MSP04_rev	5'-CCTGCTTGAACTTGCTTTCC-3'
MSP05_for	5'-AGCTAAGCAGGTGGACCAGA-3'
MSP05_rev	5'-GAAAGGGTGGCTCGTTTACA-3'
MSP06_for	5'-CAGGAATTGTGCTGCTGAAA-3'
MSP06_rev	5'-GTAGTGATAGCGGGGTGCAT-3'
MSP07_for	5'-GAACTGAAGCTTGGCTCTCG-3'
MSP07_rev	5'-GTAGTCATGGCCGTTTCGAT-3'
MSP08_for	5'-GGTAAAGCCAACACCAGGAA-3'
MSP08_rev	5'-ATGCCTGAGCTAGTGCCTGT-3'
MSP09_for	5'-CAGTGAGCTGGAGCATTGAA-3'
MSP09_rev	5'-CGCTGAAGTTGCAGTAGCAG-3'
MSP10_for	5'-CCTACTGGGCAGTGGTCATT-3'
MSP10_rev	5'-AGCTGGGAAAGTTCGAGTGA-3'
MSP11_for	5'-TGTATGCGGCTGACAGAGAC-3'
MSP11_rev	5'-GGAAAAAGCGTTCATTGGAA-3'
MSP12_for	5'-AAGAGAGGACCAGACCAGCA-3'
MSP12_rev	5'-GCTGCTGGAGTTGAAGTTCC-3'
MSP13_for	5'-GCTCTGCATCAGTGACGGTA-3'
MSP13_rev	5'-AAACCTGGGGAGCCACTACT-3'
MSP14_for	5'-CAGGCCTTGGATGGTACACT-3'
MSP14_rev	5'-TGATGTTGCCATCTGGGTAA-3'
MSP15_for	5'-GTGGGTGGAAAGGAGAACAA-3'
MSP15_rev	5'-TCTCTCAAGCCTTCCCTTCA-3'
MSP16_for	5'-GAGGCAGTCCTGAACGAGTC-3'
MSP16_rev	5'-GCACTGGGATACAAGGGAGA-3'
MSP17_for	5'-ACAGTCCCATTGCCTGTCTC-3'
MSP17_rev	5'-GATGTTGGCCACCGTAGAGT-3'
MSP18_for	5'-ATCCCCTTTTTGGGTTCATC-3'
MSP18_rev	5'-AATGGTGGTCCTGCATTAGC-3'
MSP19_for	5'-CAGTGGAGTGGACTTGCTGA-3'
MSP19_rev	5'-TGGTCGGTCCTGTGATTGTA-3'
MSP20_for	5'-AAGATGCCCTGAAAGAAGCA-3'
MSP20_rev	5'-GCACTTTGGGGCTATCATGT-3'
MSP21_for	5'-TGGCCACTCTTGCTCTAGGT-3'
MSP21_rev	5'-GCCTTCAGCTTGCCATAGTC-3'
MSP22_for	5'-TGGAAAACAAGGACCAAAGG-3'
MSP22_rev	5'-GAAGAGGCCATTCTGCTCTG-3'
MSP23_for	5'-CTCCTCTTGGTGGCTGACTC-3'
MSP23_rev	5'-AGATGCCCAATTTCATGAGC-3'

MSP24_for	5'-AAGGTGCATGGTTCTTGGTC-3'
MSP24_rev	5'-CCTCACAAAGCATTGGGTCT-3'
MSP25_for	5'-CGGGAAGGAGACACAGAGAG-3'
MSP25_rev	5'-GCGATACTCCTCCACAGAGC-3'
pid01_for	5'-CAGGCCTTCGATAAGCTGTC-3'
pid01_rev	5'-AGGCCAGGAGTAGAGGAAGC-3'
pid02_for	5'-TGGGGAACTGAGAAATGAGG-3'
pid02_rev	5'-CGGGTCTCAAGGAACACATT-3'
pid03_for	5'-TGGCTAAAGACCCGAAAATG-3'
pid03_rev	5'-GTTTTGGGGGAACACAAATG-3'
pid04_for	5'-CCTCTAGTCCCAAGGGAAGG-3'
pid04_rev	5'-GTCCTGGAGTCCTGGTGTGT-3'
pid05_for	5'-CGAGCCTTCTCCTACACCTG-3'
pid05_rev	5'-GTTTGATGTCCGCATCTCCT-3'
pid06_for	5'-TCACCTGAGAACTGGCCTCT-3'
pid06_rev	5'-GACTTGGCATGGTGTACGTG-3'
pid08_for	5'-ACCCGTGCTGTGTCTTATCC-3'
pid08_rev	5'-CTCTGCAGGTTCACACCAGA-3'
pid09_for	5'-AACCAACTCTGGGAGGTGTG-3'
pid09_rev	5'-AGTCCAGTGGGCTCTCAGAA-3'
pid10_for	5'-AAGTGAACCAATCCCCTGTG-3'
pid10_rev	5'-TCCTCCACCTACCTTCATGG-3'
pid11_for	5'-AGGAGCTGAGTCCTGATGGA-3'
pid11_rev	5'-GCCATGGTTGCTTGGTACTT-3'
pid12_for	5'-ACCTTCCTGGATTGATGCTG-3'
pid12_rev	5'-TGACAGCTTGCTTGGTTGAC-3'
pid13_for	5'-AAGGCATGTCCGTTTACCTG-3'
pid13_rev	5'-CCCTCAGTCCTCACTTCGAG-3'
pid14_for	5'-CTCCGAACCAGAGAAGTTCG-3'
pid14_rev	5'-TGGCATTGAAGATCAAGCTG-3'
pid15_for	5'-TGGACCTGTACCTGGCCTAC-3'
pid15_rev	5'-TGCTACTCGTGGGTGAGTTG-3'
pid16_for	5'-CTGGGAGCAGCTAATTCTGG-3'
pid16_rev	5'-GCTGCATTGTTCTCCTCACA-3'
pid17_for	5'-CCTCTGTTTCTGGAGCCTTG-3'
pid17_rev	5'-TGTCCCAGAATCACCTCTCC-3'
pid18_for	5'-CTCAGACACTGGCCTCCTTC-3'
pid18_rev	5'-CTCCCTGAGAACCAGCAAAG-3'
pid19_for	5'-AGGACGGTGCATGGAGTAAC-3'
pid19_rev	5'-TTTACATGCAGGTGCTGCTC-3'
pid20_for	5'-CCGAGACCTGAACTTTGAGC-3'
pid20_rev	5'-CCAGTGCCCTGATAACACCT-3'
pid21_for	5'-CAGGACCCACACAACTGATG-3'
pid21_rev	5'-ACCAGCATATCAGGGGACAG-3'
pid22_for	5'-GTGTTGGGGATAGGGGAGTT-3'
pid22_rev	5'-GGAAGCAGCAAGGATAGCAC-3'
pid23_for	5'-GTGCCTGTGAGATTCCTGGT-3'
pid23_rev	5'-GGGAACAAGAAATGGGGTTT-3'
pid24_for	5'-CAGAGTCAGAGACCCCTTGC-3'
pid24_rev	5'-ATTGTGCTGGGGACTGTAGG-3'
pid25_for	5'-AGCCCAGAGTCTCCTGACAA-3'

pid25_rev	5'-CCTAATGGCCCCCAGTATTT-3'
pid26_for	5'-AGACCACACAGTCCCCAGAC-3'
pid26_rev	5'-ACCTGAGGCAGTAGGCAGAA-3'
pid27_for	5'-GCATCCAGCCACCTTATGAT-3'
pid27_rev	5'-TGACGTTCCAGATGTCCTTG-3'
pid28_for	5'-GTGGTGCTGTGACAATGACC-3'
pid28_rev	5'-ACCTCCACCTGGGTTCTCTT-3'
pid29_for	5'-AAAACCCTGAGGAACGAGGT-3'
pid29_rev	5'-CTCTCCAGTTTTGCCTTTGC-3'
pid30_for	5'-CAGCTTCATCCTCGTCTTCC-3'
pid30_rev	5'-CGGGACTCAGTGTAGGGGTA-3'
pid31_for	5'-ATCCGCGGTACACTATGAGG-3'
pid31_rev	5'-AACCCATCTGGTCTGAGGTG-3'
pid32_for	5'-TCCTTGCTACAGGCAGGAGT-3'
pid32_rev	5'-CCAGCATGAGACCTCACAGA-3'
pid33_for	5'-CAAGCCGATCAGGAGTTTTC-3'
pid33_rev	5'-CTCCAAGATTCCCCACAGAA-3'
pid34_for	5'-CCCCAAACCCCATATCTCTT-3'
pid34_rev	5'-CAGCCTGACTGCCCATAAAT-3'
pid35_for	5'-TGGCAGAATGAGTGTTCAGC-3'
pid35_rev	5'-TGCTGTTCTTGCCAGTTTTG-3'
olfl3_for	5'-AGAGGCTGCGTTTGTCATCT-3'
olfl3_rev	5'-AGGGGCTTGAGAATTTGGTT-3'
miap4_for	5'-CGGCTCAGTGAGTTTCTTCC-3'
miap4_rev	5'-ATTGTGGGTAAAGCGTGGAG-3'
Tem8_for	5'-TCAATGAGAAGCCCTTTGCT-3'
Tem8_rev	5'-TTGACTCGTGCATTCTTTGC-3'
FKBP9_for	5'-GCGAGAAGCGTATCATCACA-3'
FKBP9_rev	5'-TGGAGGGTTGTAGTGGGAAG-3'
Efemp2_for	5'-CATAGCTCGCGAAAGGATT-3'
Efemp2_rev	5'-AGAGCGAGGCAGACACAAAT-3'
scotin_for	5'-ACCCTATTGCTGCTGCTGTT-3'
scotin_rev	5'-AGTTGTGGTGTTGGTCACGA-3'
Crtap_for	5'-CTACAGCTTCCGCAACTTCC-3'
Crtap_rev	5'-CTCGTCATCTGGATGCTTCA-3'
Fstl_for	5'-CAGACAGAAGCCACAGGACA-3'
Fstl_rev	5'-CTCTTCCTGGGCAGAGTGAC-3'

Table 7. Other primers

Name of Primer	**Sequence**
pBTM117c_For	5'-TCTTCGTCAGCAGAGCTTCA-3'
pBTM117c_Rev	5'-CAGGTGGGACAGGTGAACTT-3'
pBTM-HsPost_4For	5'-GAGTCGACAGCCGATGAAGAGACTTTG-3'
pBTM-HsPost_3For	5'-GGGTCGACACAACCACAGGGCATTGAAAG-3'
pAC1b-HsPost_2Re	5'-TCGCGGCCGCTTCTACATCTAGTTGTTGC-3'
pBTM-HsPost_2Rev	5'-CTGCGGCCGCGCTTCTACATCTAGTTGTTGCA-3'
pAC1b-HsPost_1Re	5'-CAGCGGCCGCTGTGGCCAAAGCAGCTGAAA-3'
pBTM-HsPost_1Rev	5'-AGGCGGCCGCGTGGCCAAAGCAGCTGAAAT-3'
pBTM-HsPost_1For	5'-ATGTCGACGGCGCAAGGTTGGGCAGG-3'
mFLJ11752RCAS_Fo	5'-ACCACCATGGCTCAGGATTGGGC-3'

GT-vec-650-R	5'-CTTCGCTAGAAGCGGAAGAG-3'
pACT4-1b_Rev	5'-TTGCGGGGTTTTTCAGTATC-3'
pACT4-1b_For	5'-CACAACCAATTGCCTCCTCT-3'
hPOSTnonsenseMut	5'-TTGTTGTCGGCTTTTCTTCGCGGGGA-3'
hPOSTnonsenseMut	5'-GAAGAAAAGCCGACAACAACTTCAGCGA-3'
mMSP05-pET-30bFo	5'-ACCCATGGCGCCCCCCACCTGC-3'
mMSP05-pET-30bRe	5'-GCCTCGAGAATTGGGTCTTCTA-3'
Flag_BamH_Rev	5'-AGGATCCTTATTTGTCATCATCGTCCTTATA-3'
MSP05_CHICK_For	5'-ACCACCATGCTGCTGAGCCT-3'
MSP05_CHICK_Rev	5'-TTAAGGCATGCGGTTCATTT-3'
chMSP05-flag_REV2	5'-CCCTCGAGGCTGTCAATGTC-3'
MSP05ORF_for	5'-GCCACCATGTCACCAAAGACAC-3'
MSP05ORF_rev	5'-GGTCCACCTGCTTAGCTCTG-3'
M13-seq_For	5'-GTAAAACGACGGCCAGT-3'
M13-seq_Rev	5'-CAGGAAACAGCTATGAC-3'
ORFmsp04_for	5'-GCCACCATGGTGTTCCCGAAGTTGA-3'
ORFmsp04_rev	5'-TTAGCCTGTCTTCTGTGCCCCA-3'
ORFmsp08_for	5'-GCCACCATGGTGCCGCCACAGCT-3'
ORFmsp08_rev	5'-TCATCCTTGTTTCCTGGGCTGT-3'
ORFmsp13_for	5'-GCCGCCATGGACGCCAAGGTCGT-3'
ORFmsp13_rev	5'-TCACATCTTGAGCCTCTTGTTT-3'
ORFmsp18_for	5'-GCCACCATGGATGTTACTAAAGATG-3'
ORFmsp18_rev	5'-CTATGTAGCTACTGATCTCTGG-3'
ORFmsp18_rev2	5'-CATTATTTGTGCAGGCAGGTAA-3'
ORFmsp19_for	5'-GCCGCCATGGCCTATCCCCTGC-3'
ORFmsp19_rev	5'-TCAGTAGGCAAAGATGACATGG-3'
ORFmsp24_for	5'-GCCACCATGGAGCTAGACAGATGG-3'
ORFmsp24_rev	5'-TCATTTCATGTTTCTCTCAATC-3'
ORFmsp24_rev2	5'-ATGGTGGGCAAAATTACACTTC-3'
ORFpic01_for	5'-GCCGCCATGGTGCAAGAATCTGGG-3'
ORFpic01_rev	5'-TCACTCCATGTCCTCATTTACT-3'
rcas-5_for	5'-ACGCTTTTGTCTGTGTGCTGC-3'
Dmf04_rev	5'-ATCTCTGCAATGCGGAATTCAGTG-3'
msp17ORF_mouse_for	5'-GCCACCATGGTCTGCAAGGTGCT-3'
msp17ORF_mouse_rev	5'-TGCGTTCCTTTCTCCTCACT-3'
Posti1f_d_p350F	5'-TGGGATCTGATGTTCCCTTC-3'
Xg183GenoType-wt_R	5'-CGTCGAAAACGTCACTGAGA-3'
GT-xg183mut-R	5'-CCTGGCCTCCAGACAAGTAG-3'

2.2 Methods

2.2.1 RNA isolation

Total RNAs from cell lines and tissues were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol.

2.2.2 RT-PCR analysis

Reverse transcription (RT) were performed according to the manufacturer's instruction using the Superscript reverse transcriptase (Invitrogen) or water (for RT negative control). RT-PCR reactions were carried out in 20 μL reaction volume containing 1×reaction buffer (1.5 mM $MgCl_2$ final concentration), 0.2 μM of each primer pair, 0.2 mM dNTPs, 1 U *Taq* DNA polymerase and 2 μL of proper cDNA as template. Cycling conditions included an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at the specific annealing temperature, 1 minute at 72°C, and a final extension step at 72°C for 7 minutes. PCR products were analysed on 1.5 % agarose gels.

2.2.3 Cell culture and DNA transfection

All cells used in this study were cultured in the appropriate medium at 37°C in 5% CO_2. For DNA transfection experiment, cells were grown on glass coverslips in 6-well plates. Transient transfection was performed using Polyfect transfection reagent (jetPEI™) according to the manufacturer's protocol.

2.2.4 Immunofluorescence analysis

Cells were fixed in 3-4% (wt/vol) paraformaldehyde, quenched for 10min with 50nM ammonium chloride and permeabilized with 0.1% (vol/vol) Triton X-100 or Saponin for 5-10min. Antibody labelling was performed for 1h or over night. Secondary antibodies were conjugated to Alexa 488, Alexa 555 or Cy3 and DNA was stained with DAPI. Coverslips were mounted with Fluoromount.

2.2.5 Chicken bone marrow system

Chicken bone marrow cells harvested from inside of the femurs of E18 chicken embryos were cultured in 10% FCS/α-MEM till 80% confluency. Subdivided cells were infected by different RCAS viruses harboring different targets and maintained in the same medium till

100% confluency. Replace the growth medium with stimulation medium (containing 10mM beta-Glycerophosphate and 50µg/mL Ascorbic acid) and monitor the mineralization process by ALP activity and Alizarin red stain every two days by standard lab protocol.

2.2.6 Expression analysis in mouse embryos

E12.5 mouse embryos were fixed over night with 4% paraformaldehyde. E14.5 mouse embryos were embedded in Tissue Tec (Leica), 10 µm cryosections were cut and fixed with 4% paraformaldehyde. Embryos and sections were incubated in X-Gal staining solution (1mg/mL X-Gal, 5mM K3[Fe(CN)6], 5mM K4[Fe(CN)6], 2mM $MgCl_2$, 0,2% NP-40) for 24h at 37°C. Counterstaining of sections was done using nuclear fast red.

2.2.7 Yeast two-hybrid analysis and GST pull-down

These experiments were performed by Johannes Egerer in the lab of Dr. Francis A. Barr (MPI for Biochemistry, Martinsried). The yeast reporter strain PJ69-4A was transformed with pAct2-Scyl1bp1 constructs (pAct2: Clontech, Heidelberg, Germany) as prey and the respective Rab-/Arf-/Arl-GTPases constructs in the pFBT9 bait vector (pGBT9 [Clontech] modified to carry kanamycin resistance) following the Clontech laboratories yeast protocol handbook. Yeast was plated onto synthetic medium (-LW) and grown for 3d at 30°C. Colonies were picked and restreaked onto synthetic QDO medium and grown for another 2-3d. Growth indicated interaction of bait and prey proteins.

For pulldown assays, 500mg recombinant GST-Rab6 wild type, Rab6/T27N GDP-locked mutant or Rab1 wild type protein were coupled to 50 mL of packed glutathione-Sepharose beads (GE Healthcare) for 1h at 4°C in a total volume of 500 mL PBS. Beads were washed three times in 500 mL NE100 buffer (20mM Hepes/NaOH pH 7.5, 100mM NaCl, 10mM EDTA, 0.1% Triton X-100) and then mixed with 1.5 mL HeLa-L extract (1.5mg protein) in NL100 buffer (20mM Hepes/NaOH pH 7.5, 100mM NaCl, 5mM $MgCl_2$, 0.1% Triton X-100, protease inhibitor cocktail (Roche Diagnostics) and supplemented with a final concentration of 100mM GDP or GTP as described by Christoforidis et al. (Christoforidis and Zerial, 2000). Beads were incubated at 4°C for 2h and washed three times with 500 mL NL100 buffer that included 100mM GDP or GTP. Bound protein was subsequently eluted by rotating beads for 10min in 500 mL NE200 buffer (20mM Hepes/NaOH pH 7.5, 200mM NaCl, 20mM EDTA, 0.1% Triton X-100) at 4°C. After pelleting the beads by centrifugation

for 3min at 400×g and 4°C, the supernatant was incubated once with 50 mL glutathione-Sepharose for 10min at 4°C in order to remove resident recombinant Rab protein. The beads were again pelleted by centrifugation at 400×g 4°C for 3min and the supernatant subjected to protein precipitation using trichloric acetic acid and analysed by Western blotting analysis.

2.2.8 RNA interference

HeLa cells were cultured at 37°C and 5% CO_2 in DMEM containing 10% FCS. Cells were plated on coverslips at a density of 20,000 cells/well in a six-well plate and used 24h later. Cells were transfected with siRNA oligos designed against Rab6a (13nM), Rab6b (13nM), or Scyl1BP1 (10nM) and grown for 72h.

2.2.9 SDS-PAGE and Western blot analysis

Polyacrylamide gels were prepared according to standard protocols. Protein samples were heated at 95°C in protein loading buffer for 5 min and separated by 10-15% SDS-PAGE. For Western blot analysis, proteins were transferred from gels to microporous polyvinylidene difluoride (PVDF) membrane Immobilon-P (Millipore) using a mini tank transfer unit TE22 (Amersham Bioscience) according to the manufacturer's instructions. After transfer, blots were incubated with Western blot blocking buffer for 1 hour at room temperature, and then incubated overnight at 4°C with primary antibodies diluted in blocking buffer. Blots were washed three times in PBST, and incubated for 1 hour at room temperature with peroxidase-conjugated anti- rabbit IgG or anti-mouse IgG. Following 3 times washes in PBST, blots were incubated with Roti-Lumin chemiluminescence substrate (Roth) according to the manufacturer's protocol and exposed to Fuji X-ray film.

2.2.10 Synthesis of RNA probes for *in situ* hybridization

The part of the coding sequence corresponding to each candidate gene was cloned into the pTA1 vector. By using M13 primers, linearized DNA products were amplified by PCR. Starting from this step, all solutions were treated with DEPC. The reaction was performed in a total volume of 20 µL containing 1 µg of template DNA, 1× DIG labelling mix, 40 units of RNase inhibitor, 1× transcription buffer (Roche), and 40 units of T7 or SP6 RNA polymerase. After incubation for 2 hours at 37°C, 20 units of RNase-free DNase I were added and maintained at 37°C for 15 minutes in order to remove the DNA templates. The reaction was terminated by adding 2 µL of 0.2 M EDTA, and synthesized RNA was

precipitated using 1/10 volume of 4 M LiCl and 2.5 volumes of ethanol, and diluted in 100 µL DEPC-H_2O. The quality of the RNA probes was inspected on 1% agarose gel.

2.2.11 Whole mount *in situ* hybridization

Mouse embryos were isolated and then fixed in 4% PFA/PBS overnight at 4°C. Washing embryos twice with DEPC-PBST at room temperature, twice with 50% methanol/DEPC-PBST, and finally once with 100% methanol. Embryos were then put in fresh 100% methanol and stored at –20°C. Hybridization with DIG-labelled probes was performed briefly described as below. Embryos were rehydrated at 4°C in 75%, 50%, 25% methanol/DEPC-PBST (10 minutes for each) and washed twice in ice-cold DEPC-PBST. Afterwards, embryos were bleached with 6% hydrogen peroxide for 1 hour at 4°C, followed by 3 times washes in DEPC-PBST (10 minutes each). Younger embryos (E10.5 and E11.5) were digested for 3 minutes with 10 µg/µL proteinase K in proteinase K buffer at room temperature. For older embryos (E12.5), proteinase K concentration was up to 20 µg/µL and the reaction time was up to 5 minutes. After digestion, embryos were washed twice in PBST/Glycine, twice in DEPC-PBST, 3 times in RIPA buffer, 3 times in DEPC-PBST and fixed for 20 minutes in PFA/Glutaraldehyde. Subsequently, embryos were washed 3 times in DEPC-PBST and incubated in hybe buffer:DEPC-PBST (1:1 dilution) for 10 minutes, followed by a single washing step in hybe buffer at room temperature. Prehybridization was performed in hybe buffer at 65°C. Prior to hybridization, DIG-labelled RNA probes were diluted 1:100 and denatured for 5 minutes at 80°C, and added together with tRNA (100 µg/mL) to the prehybridization solution. Hybridization was performed at 65°C overnight. In order to remove unbound probe, embryos were washed twice for 30 minutes in hybe buffer at 65°C, followed by a single washing step in 50% RNase solution/50% hybe buffer at room temperature and by digestion with RNase A (100 µg/mL in RNase solution) for 1 hour at 37°C. Subsequently, embryos were incubated for 5 minutes in 50% RNase solution/50% SSC/FA/T at room temperature, and washed with SSC/FA/T at 65°C (twice for 5 minutes, 3 times for 10 minutes and 6 times for 30 minutes). After cooling down to room temperature embryos were washed for 10 minutes with (1:1) SSC/FA/T/1× MABT and subsequently twice for 10 minutes with 1× MABT. Prior to the incubation with antibodies, embryos were blocked with 10% Blocking reagent (Boehringer) diluted in 1×MABT for 1 hour at the room temperature. At the same time 1×MABT solution containing 1% Blocking reagent and alkaline phosphatase-conjugated anti-DIG antibodies (diluted 1:5000) was prepared and incubated for 1 hour at 4°C. After the blocking step, embryos were incubated overnight at

4°C in anti-DIG-antibody-containing solution and subsequently transferred to room temperature and washed 8 times for 1 hour in fresh PBST/Tetramisole solution in order to remove unbound antibodies. Staining was based on the enzymatic reaction performed by alkaline phosphatase (AP) in AP buffer using the BM Purple AP Substrate (Roche) according to the manufacturer's protocol. After the reaction, embryos were washed in alkaline phosphatase buffer, fixed in PFA/Glutaraldehyde and stored at 4°C. Images of the stained embryos were captured using the Leica MZ 12.5 stereomicroscope (Leica) coupled to the AxioCam HRc camera and the AxioVision 4.2 image analysis software.

2.2.12 Section *in situ* hybridization

The fixed embryos (overnight in 4% PFA/PBS) were washed twice in fresh DEPC-PBS, followed by 1 hour incubation in 70% ethanol. After embedding embryos in paraffin, 7 µm thick sections were cut and were attached to glass slides, baked for 1 hour at 60°C, dewaxed in xylene and rehydrated using 100%, 75%, 50% and 25% ethanol concentrations. Then slides were washed twice in DEPC-PBS, followed by a fixation step in 4% PFA/PBS for 10 minutes at room temperature. After 2× washes in fresh DEPC-PBST, mouse sections were digested with proteinase K (1.5 µg/mL) for 10 minutes and washed again 2 times in DEPC-PBST, followed by a second fixation step in 4% PFA/PBS for 5 minutes. Afterwards, sections were acetylated for 10 minutes with 0.25% acetic anhydride, washed twice in DEPC-PBST and prehybridised for 1-4 hours in hybridization solution (for paraffin sections) in a humified slide box. 2 µL of the specific probe was denatured in 100 µL of hybridization solution (for paraffin sections) and hybridized to the slides at 65°C overnight. On the next day slides were rinsed with 5×SSC, washed with 1×SSC/50% formamide for 30 minutes at 65°C and in TNE for 10 minutes at 37°C, followed by RNase digestion (20 µg/mL diluted in wash buffer). Later, slides were washed in TNE for 10 minutes at 37°C, followed by a single wash in 2×SSC for 20 minutes at 65°C and 2× washes in 0.2×SSC in the same conditions. For detection of DIG-labelled probes, slides were washed twice with 1×MABT at room temperature and blocked in 1×MABT containing 20% heat inactivated sheep serum (HISS). 1:2500 diluted alkaline phosphatase-conjugated anti-digoxygenin antibodies were preincubated for 2 hours at 4°C in 5% HISS/1×MABT and pipetted onto the slides. After overnight incubation at 4°C, slides were washed 3 times in 1×MABT, incubated for 10 minutes in NTMT and developed with BM Purple AP Substrate (Roche). After the reaction, slides were rinsed with NTMT, washed twice for 5 minutes in PBS.

For cryo-sections, the embryos were placed into a chamber filled with Tissue Tec (Leica), and frozen in dry ice/ethanol. The frozen blocks were stored at –20°C and were sectioned at 10 µm with the HM 560 Cryo-Star cryostat (MICROM International GmbH). Hybridization with DIG-labelled probes and signal detection was performed with the use of the Genesis RSP 150 automation system supplied with the Gemini pipetting software (Tecan Group Ltd.). Images were captured using the Leica DMR light microscope (Leica) coupled to the AxioCam HRc camera and the AxioVision 4.2 image analysis software.

2.2.13 Quantitative PCR

RNA was isolated from organs of P5 mice using the Trizol (Invitrogen) method. 1µg of RNA was reverse transcribed using the Revert Aid kit (Fermentas) and random hexamers. Quantitative PCR was performed with SYBR green (Applied Biosystems) and the following primers: Scyl1bp1-f TCGAGCTCCAAAGCCAGATT, Scyl1bp1-r TCTTTCCGCAATGGCTTGAG, Gapdh-f GGGAAGCCCATCACCATCTT, Gapdh-r CGGCCTCACCCCATTTG.

2.2.14 Immunohistochemistry

Deparaffinize sections in 2 changes of xylene, 15 minutes each. Hydrate in 2 changes of 100% ethanol for 3 minutes each, 95% and 70% ethanol for 3 minute each. Then rinse in distilled water followed by 0.3% H_2O_2 treatment for 20 min. Put sections in Citrate Buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) and cook in microwave (Rank 3, 3min; RT 5min; Rank 2, 3min; RT, 30min). Rinse sections in PBS and block sections with for 30 minutes with 5% BSA (or suitable serum). Incubate sections with primary antibody at appropriate dilution in primary antibody dilution buffer for 1 hour at room temperature or overnight at 4 °C. Rinse sections with PBS/Tween 20 for 2×2 min. Incubate sections with secondary antibody at appropriate dilution for 1 hour at room temperature. Rinse sections with PBS followed by VECTASTAIN ABC KIT according to the manufacturer's protocol.

2.2.15 Lectin blot and lectin histochemistry

Tissues from E18.5 mouse embryos were homogenized individually in 400 µL of lysis buffer lysis buffer (50mM HEPES, 50mM NaCl, 10mM EDTA, 10% Glycerol, 1% Triton X-100) plus Proteinase Inhibitor Mixture using an ultrathurrax followed by an over night incubation at 4°C. The homogenate was centrifuged at 10,000 × g for 15 min at 4°C. After

removing the supernatant, resulting precipitate (ECM) was dissolved in double strength laemmli buffer. After determining protein concentration, this fraction was subjected to SDS/PAGE, and the proteins were transferred to a poly(vinylidene difluoride) (PVDF) filter. The filter was blocked with 5% BSA-PBS including 20 μg/mL avidin for 20 h at 4°C. After washing three times with PBS including 0.05% Tween-20 (PBST), the filter was reacted with 2.5 μg/mL biotinylated lectin (Vector Laboratories, Burlingame, CA) in 5% BSA-PBS including 10 μM biotin for 1 h at room temperature. The filter was washed three times with PBST and reacted with horseradish peroxidase (HRP)-avidin for 30 min. The filter was washed three times with PBST, and bound signals were visualized by using a SuperSignal West Pico Kit (Pierce Biotech). For lectin histochemistry, thin- sections from a paraffin-embedded E15 embryo were subjected to the lectin analysis described above, except diaminobenzidine (DAB) was used for signal detection.

2.2.16 Mass spectrometric analysis of N- and O-linked glycans

These experiments were performed by Willy Morelle (CNRS/USTL, France). The total N- and O-linked glycans from tissue proteins were released as described by Faid et al (Faid et al., 2007). MALDI-TOF MS experiments were carried out on Voyager Elite DE-STR Pro instrument (PersSeptive Biosystem, Framingham, MA, USA) equipped with a pulsed nitrogen laser (337 nm) and a gridless delayed extraction ion source. The spectrometer was operated in positive reflectron mode by delayed extraction with an accelerating voltage of 20 kV and a pulse delay time of 200 nsec and a grid voltage of 66%. All spectra shown represent accumulated spectra obtained by 400-500 laser shots. Sample was prepared by mixing a 1 μL aliquot (5 to 10 picomoles) with 1μL of matrix solution, on the MALDI sample plate. The matrix solution was prepared by saturating a methanol-water (1:1) with DHB (10 mg/mL). The reference samples were anonymous plasma samples from patients without CDG or a lysosomal storage disease.

2.2.17 Databases and bioinformatic anaylsis

NCBI and Ensembl databases were used for the reference sequence of a gene and its related analysis. Prediction of the secreted proteins was based on the bioinformatic analysis of the mouse protein sequence in the following link (http://www.cbs.dtu.dk/services/SignalP/).