Antibodies

Antibody	Isotype	Reference	Company
actin	mouse monoclonal	A-1804	Sigma-Aldrich (St Louis, MO, USA)
ASH2	rabbit polyclonal	A300-107A	Bethyl laboratories (Montgomery, TX, USA)
BCL-3	rabbit polyclonal	sc-185	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
FLAG	rabbit polyclonal	F7425	Sigma-Aldrich (St Louis, MO, USA)
FLAG (M5)	mouse monoclonal	F4042	Sigma-Aldrich (St Louis, MO, USA)
H3K4me3	rabbit polyclonal	pAb-MEHAHS	Diagenode (Liege, Belgium)
HA-probe	rabbit polyclonal	sc-805	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
HDAC1	rabbit polyclonal	sc-7872	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
HDAC3	rabbit polyclonal	sc-11417	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
HDAC4	rabbit polyclonal	sc-11418	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
HDAC6	rabbit polyclonal	#2162	Cell signaling Technology (Beverly, MA, USA)
histone H3	rabbit polyclonal	ab1791 (ChIP grade)	Abcam (Cambridge, UK)
IκBα	rabbit polyclonal	sc-371	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
MLL1	rabbit polyclonal	A300-086A	Bethyl laboratories (Montgomery, TX, USA)
NEMO	rabbit polyclonal		Gift from Dr. R. Weil (Institut Pasteur, Paris, France)
NF-κB p65	rabbit polyclonal	sc-109	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
NF-κB, p100/p52	mouse monoclonal	#05-361	Upstate/Millipore (Billerica, MA,USA)
NF-κB, p100/p52	rabbit polyclonal	ab1595	Gift from Dr. E. Dejardin (GIGA-R, Liege, Belgium)
NF-κB, p50	rabbit polyclonal	sc-114	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
RelB	rabbit polyclonal	sc-226	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
TRX2	rabbit polyclonal	A300-113A	Bethyl laboratories (Montgomery, TX, USA)

Cell culture

<table>
<tr><th>Cell line</th><th>Medium (BioWhitakker, Walkersville)</th></tr>
<tr><td>HEK293 (human embryonic kidney 293)
293FT, Phoenix ecotropic</td><td rowspan="5">DMEM
+ 10% fetal bovine serum
+ 2mM glutamine
+ antibiotics (100U/ml penicillin + 100µg/ml streptomycin)</td></tr>
<tr><td>HeLa (human cervical cancer)</td></tr>
<tr><td>NIH3T3 (mouse embryonic fibroblasts)</td></tr>
<tr><td>MEF (mouse embryonic fibroblasts) wild type</td></tr>
<tr><td>MEF NEMO$^{-/-}$ (a gift from Dr. M. Pasparakis, Institute for Genetics, Centre for Molecular Medicine (CMMC), University of Cologne, Germany)</td></tr>
<tr><td>HUT78 (human cutaneous T cell lymphoma)</td><td>RPMI 1640
+ 10% fetal bovine serum
+ 2mM glutamine
+ antibiotics (100U/ml penicillin + 100µg/ml streptomycin)</td></tr>
<tr><td>164T2, S19 (mouse T lymphoma cell lines, a gift from Dr. Y. St-Pierre, INRS-Institut Armand Frappier, University of Québec, Laval, Québec, Canada)</td><td>RPMI 1640
+ 10% fetal bovine serum
+ 2 mM glutamine
+ antibiotics (100U/ml penicillin + 100µg/ml streptomycin)
+ 10mM Hepes
+ 50 µM β-mercaptoethanol</td></tr>
</table>

Reagents

♦ TNFα was purchased from Roche Applied Sciences (Basel, Switzerland).
♦ The LTβR agonistic antibody was kindly provided by Dr. E. Dejardin (Dejardin *et al.*, 2002).

Plasmids

♦ **Hut78 pcDNA3.1-FLAG/ Hut78 pBabe puromycine**: Hut78 coding sequence (accession number U09609) was subcloned by PCR from a p100 pMT2T expression vector (Bours *et al.*, 1992) into the pcDNA3.1-FLAG expression vector for immunofluorescence and co-immunoprecipitation analysis

and into the pBabe puromycin retroviral vector for infection experiments. The 3' primer used for the PCR had the following sequence:

- **3'**: 5'-CCGGAATTCTCAGGAGGCTGAAAAGGTGCGAGCGTTCAC-3'

where the underlined nucleotides denote the sequence encoding the SAS amino acids (numbers 667 to 669) found at the C-terminal end of Hut78. As a result, the Hut78 expression construct codes for the first 666 amino acids of p100 (accession number NM_002502) followed by the SAS peptide and the stop codon.

♦ **NLS mutants:** the FLAG-Hut78 mutants harboring one or multiple point mutations within the NLS sequence were generated by using FLAG-Hut78 as template and the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

♦ **ΔGRR mutants:** the Hut78ΔGRR construct that lacks amino acids 347 to 377 was generated by first cloning a PCR-generated fragment encompassing the region from the initial ATG to amino acid 346, using the following primers:

- **5'**: 5'-CCGGGATCCATGGAGAGTTGCTACAA-3'
- **3'**: 5'-CCGGAATTCGAAGGTGGGCAAGGCCTTC-3'

The second fragment encompassing a region from amino acid 378 to the stop codon was generated by PCR, using the following primers and subsequently inserted in frame:

- **5'**: 5'-CCGGAATTCTTCTTCCCCTCCTCCCTGGC-3'
- **3'**: 5'-CCGGAATTCTCAGGAGGCTGAAAAGGTGCGAGCGTTCAC-3'

The p52ΔGRR mutant was generated by PCR using Hut78ΔGRR as template and the following 3'primer:

- **3'**: 5'-CCGGTCGACTCACGTGGCGGCCATCTG-3'

♦ **Ankyrin mutants**: the FLAG-Ankyrin1 to FLAG-Ankyrin5 mutants were generated by introducing a stop codon after each ankyrin repeat by site-directed mutagenesis (Quick Change site-directed mutagenesis kit, Stratagene, La Jolla, CA, USA).

♦ **RHD mutants**: the FLAG-Hut78 mutants lacking N-terminal residues (RHD1 and RHD2) were generated by PCR using FLAG-Hut78 as template and the following 5'primers:

- **RHD1**: 5'-CCGGGATCCATCGAGGTGGACCTGGTA-3'
- **RHD2**: 5'-CCGGGATCCCAGCCCATCCATGATAGC-3'
- **RHD3**: 5'-CCGGGATCCGGGACGTGTCTGATTCCA-3'

♦ **K75A mutant:** this mutant was generated by using FLAG-Hut78 as template and the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

♦ **LB40 pBabe**: was obtained by PCR using p100 pMT2T as template and the following 3'primer:

• **3'**: 5'-CCGGAATTCTTAGAGGGGCTCCTCGTTTTC-3'

♦ **p100HB pBabe**: was obtained by PCR using p100HB pcDNA3.1 as template, a gift from Dr. Marie Körner (Institut André Lwoff, Villejuif, France, and the following 3'primer:

• **3'**: 5'-CCGGAATTCCTACAGAGCTGTATCACC-3'

♦ **BCL-3 pcDNA3.1-FLAG** was generated by PCR, using BCL-3 pMT2T as template (Viatour *et al*., 2004).

♦ **p52 pMT2T** was generated by Dr. V. Bours (Bours *et al*., 1992).

♦ **HDAC3, FLAG-HDAC1, -3, -4 and -6** were gifts from Dr. C. van Lint (Laboratory of Molecular Virology, IBMM, Free University of Brussels, Gosselies, Belgium).

♦ **pcDNA3.1-FLAG-WDR5-HA, -RbBP5-HA and -hASH2L-HA** expression constructs were generous gifts from J.-H Lee and D. G. Skalnik (Wells Center for Pediatric Research, Departments of Pediatrics and Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana, USA) and were previously described (Lee *et al*., 2007).

♦ **MISSION™ shRNA lentiviral construct targeting p100/p52** (NM_002502.2) with the following sequence 5'-CCGGCCCTATCACAAGA TGAAGATTCTCGAGAATCTTCATCTTGTGATAGGGTTTTT-3' (TRCN 0000006514) and control "non target" shRNA lentiviral constructs were purchased from Sigma-Aldrich (St Louis, MO, USA).

Co-Immunoprecipitations

For immunoprecipitations of overexpressed proteins, 293 cells ($3x10^6$) were transfected with the indicated expression vectors using FuGENE 6 (Roche Applied Science, Basel, Switzerland) and lysis was performed 24h after transfection. The following immunoprecipitation protocols were similar for endogenous or transfected proteins. Cell lysates were incubated overnight at 4°C with the indicated antibodies and subsequently incubated for 2h with protein A-agarose conjugate. Anti-HA immunoprecipitations were performed in parallel as negative controls. The resulting immunoprecipitates were washed five times with the lysis buffer and subjected to SDS-PAGE for Western blot analysis.

Immunofluorescence

HeLa cells were seeded on coverslips in six-well plates and subsequently transfected with the indicated expression plasmids using FuGENE 6 (Roche Applied Science, Basel, Switzerland). Twenty four hours later, cells were washed with PBS, fixed with 4% paraformaldehyde and permeabilized with ethanol for 6 min at -20°C. Fixed cells were incubated with the indicated antibodies for 45min at 37°C followed by 45min incubation at 37°C with a FITC-conjugated antibody IgG (Dako, Glostrup, Denmark) or an antibody conjugated to sulforhodamine acid chloride (Texas Red). Coverslips were then mounted on slides glasses with Prolong Gold Antifade (Invitrogen, Carlsbad, CA) and cells were observed by fluorescent microscopy (Nikon).

Retroviral infections of NIH3T3 cells

Retroviral infections of NIH3T3 cells were performed according to instructions from http://www.stanford.edu/group/nolan/retroviral_systems. Briefly, $2x10^6$ ecotropic phoenix cells were transfected using calcium phosphate with 10 µg of retroviral plasmids (pBabe-puromycin), either empty or encoding p52 or Hut78 and mutants. Forty-eight hours after transfection, supernatants were collected, filtered (0.2µm) and added with polybrene (8µg/ml) to $2x10^5$ NIH3T3 cells. Forty-eight hours post infection, NIH3T3 cells were treated for five days with puromycine (2µg/ml).

Lentiviral infections of HUT78 cells

For lentiviral infections of HUT78 cells, 293FT cells were transfected with 12µg of the “non target” lentiviral shRNA plasmid (used as negative control) or the shRNA construct that targets p100/p52 (NM_002502.2) and with 12 and 5µg of R8.91 and VSVG plasmids respectively, using the Mirus Bio’sTransIT®-LT1 reagent (Pittsburg, PA, USA). The supernatants from those infected cells were collected 48 hours post-transfection, added with polybrene to $5x10^5$ HUT78 cells and subjected to centrifugation for 10 min at 1200 rpm. This later step was repeated the next day and HUT78 cells were treated with puromycine (1µg/ml) 48 hours after the last infection.

Growth Curves

$20x10^3$ infected NIH3T3 cells were seeded in 6-well plates and counted every 2 days, starting 24h after plating. Medium was changed every 2 days. Each condition was plated and counted in duplicate.

Foci formation

For foci assays, $1.5x10^6$ infected NIH3T3 cells were seeded in a 10 cm plate and media was changed every 3 days. The cell cultures were followed for 3 weeks, and colonies were visualized with Giemsa staining.

Nude mice injection

For subcutaneous injection in nude mice, 10^6 infected NIH3T3 cells were resuspended in 200µl of PBS and injected in the flank of 5 weeks-old nude mice (Swiss strain, Wilmington, MA, USA). Evaluation of tumor growth was performed twice weekly.

Zymography

Conditioned media of cells incubated in serum-free medium for 24 or 48h were collected and centrifuged. Eighteen microliters of supernatant mixed with 6µl of loading buffer were applied onto a 10% polyacrylamide gel copolymerized with gelatin (1mg/ml). After electrophoresis, gels were renatured in 2% Triton X-100 for 1h and incubated overnight at 37°C in a buffer consisting of 50mM Tris-HCl (pH7,6) and 10mM $CaCl_2$. The gels were then stained with Coomassie brilliant blue in 40% methanol and 10% acetic acid and destained in 20% methanol and 10% acetic acid. Gelatinase activities were detected as transparent bands on the blue background.

Matrigel invasion assay

The assay was performed using a 24 well polycarbonate transwell permeable support with 8µm pores (Corning costar, Lowell, MA,USA), coated with 6.21µg of Matrigel (BD biosciences, Bedford, MA). HUT78 cells infected with distinct shRNA constructs were suspended in serum-free RPMI at a concentration of $5x10^5$cells/ml and 100µl were loaded into top chambers. The

lower chambers were filled with 0,6ml RPMI containing 10% FBS. After 72h of incubation at 37°C in a humidified 5% CO_2 incubator, cells that migrated through the Matrigel coated filters were recovered from the lower compartment and counted using a Thoma hemocytometer.

Affymetrix microarray analysis

Total RNAs were extracted from NIH3T3 cells stably infected with empty virus or virus expressing p52 or Hut78 and cultured in triplicates, using the Nucleospin® RNA II kit (Macherey-Nagel, Düren, Germany). The integrity of the RNAs from those 9 distinct experimental conditions was checked with the Agilent Bioanalyzer using the RNA 6000 Nano kit (Agilent, Santa Clara, CA, USA). Double-stranded cDNAs were generated using the Superscript II RT kit (Invitrogen, Carlsbad, CA, USA) and biotin-labelled cRNA was generated using the GeneChip One-cycle Target Labeling kit (Affymetrix, Santa Clara, CA, USA). cRNAs were hybridized with the GeneChip® Mouse Genome 430 2.0 Array. Briefly, double-stranded cDNA was synthesized routinely from five micrograms of total RNA primed with a poly-(dT) –T7 oligonucleotide. The cDNA was used in an *in vitro* transcription reaction (IVT) in the presence of T7 RNA polymerase and biotin-labelled modified nucleotides during 16 hours at 37°C. Biotinylated cRNA was purified and then fragmented (35-200 nucleotides), together with hybridization controls and hybridized to the microarrays for 16 h at 45°C. The hybridized biotin-labeled cRNA was revealed using the Fluidics Station (Affymetrix, Santa Clara, CA, USA) by successive reactions with streptavidin R-phycoerythrin conjugate, biotinylated antistreptavidine antibody and streptavidin R-phycoerythrin conjugate. The arrays were finally scanned in an Affymetrix/Hewlett-Packard GeneChip Scanner 3000. Data were processed using the MAS 5.0 software (Affymetrix, Santa Clara, CA, USA).

Real-Time PCRs

Total RNAs were extracted using the EZNA Total RNA kit (Omega Bio-tek, Norcross, GA, USA) and cDNAs were synthesized using the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, Glen Burnie, MD, USA). Subsequent PCRs were carried out using the Power SYBR Green PCR master kit (Applied Biosystems, Foster City, CA, USA) on the LightCycler 480 (Roche Applied Sciences, Basel, Switzerland). Primers to amplify IκBα and MMP9

sequences from NIH3T3 and HUT78 cells as well as for the amplification of GAPDH and 18S sequences (for normalization purposes in NIH3T3 and HUT78 cells, respectively) are listed here:

Name	Forward Primer	Reverse Primer
GAPDH mouse	5'-TGTGTCCGTCGTGGATCTGA-3'	5'-CCTGCTTCACCACCTTCTTGA-3'
IκBα mouse	5'-CGGACAGCCCTCCACCTT-3'	5'-CACATTTCAACAAGAGCGAAACC-3'
MMP9 mouse	5'-TTCCCCAAAGACCTGAAAACC-3'	5'-GCCCGGGTGTAACCATAGC-3'
18S human	5'-AACTTTCGATGGTAGTCGCCG-3'	5'-CCTTGGATGTGGTAGCCGTTT-3'
MMP9 human	5'-GGACGATGCCTGCAACGT-3'	5'-CAAATACAGCTGGTTCCCAATCT-3'

ChIP assays

Cells were cross-linked with 1% paraformaldehyde and sonicated using the Bioruptor™ (Diagenode, Liege, Belgium). ChIP assays were performed using the OneDay ChIP kit (Diagenode, Liege, Belgium) using 1.5 to $2x10^6$ cells per experimental condition and according to the protocol described by the manufacturer. Subsequent PCRs were carried out using the Power SYBR Green PCR master kit (Applied Biosystems, Foster City, CA, USA) on the LightCycler 480 (Roche Applied Sciences, Basel, Switzerland). The sequences of the primers used are listed below. Input DNA was analysed simultaneously and used as normalization. For normalization of the ChIP assays in HUT78 cells, the signal obtained from a noncoding region (downstream of the albumin gene (Kouskouti & Talianidis, 2005)) was used to compensate for possible fluctuations arising during handling. For the histone-related ChIPs, H3K4me3 ChIP values were normalized according to the total H3 signal (as detected using the corresponding anti-H3 antibody).

Promoter	Primer Forward	Primer Reverse
IκBα mouse	5'-GCTGCAGGGAAGTACCTAGAG-3'	5'-CCCTGAGTGGCTGGAAAGTC-3'
mmp9 mouse	5'-CTTTAAACAGAAGAGGAAGGATAGTGC-3'	5'-CCTGATAGAGTCTTTGACTCAGCTTC-3'
mmp9 human	5'-TCCGCCCCCAGATGAAG-3'	5'-ACACTCCAGGCTCTGTCCTCTTT-3'