Methods

Culturing primary human fibroblasts

IMR90, ATCC# CCL-186, a diploid primary human fibroblast adherent cell line derived from fetal lung tissue, was obtained from LGC Promochem (Wesel, Germany) at population doubling (PDL) 20. All culture passages and population doubling were recorded. All experiments were performed with IMR90 cells under a PDL of 50.

Cells were cultured at 37˚C with 5% $C0_2$ in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mg/ml glucose, 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 50 µg/ml penicillin and 50 µg/ml streptomycin. Cultures at ~80% confluence were routinely split 1:3 in 10 cm culture dishes as follows. The cells were washed 2x in prewarmed PBS. 1 ml PBS containing 0.25% (w/v) Trypsin-0.03% EDTA was added to the dishes and placed at 37^0C for 5-10 minutes. After the cells detached from the dishes, 1 ml prewarmed culture medium was added and the cells transferred to a 50 ml falcon tube. Culture medium was added to 30 ml and the cells replated at 10 ml per 10 cm dish.

Plating of IMR90 cells for transfections were carried out essentially as described above with the following exceptions. After the cells were incubated in 1 ml PBS containing 0.25% (w/v) Trypsin-0.03% EDTA at 37^0C for 5-10 minutes, 1 ml culture medium was added and the cells were transferred into a 15 ml falcon tube. An aliquot of 10 µl cell suspension was removed and mixed with trypan blue and counted in a Neubauer Haemocytometer. The cell concentration was adjusted accordingly by the addition of complete media. Cells were plated 15 hours prior to transfection at the following concentrations: 2 x 10^3 cells in 80 µl

per well (ø 6.5 mm) of a 96 well dish, 2.25 x 10^5 cells in 4.5 ml per 6 cm dish and 5.0 x 10^5 cells in 8 ml per 10 cm dish.

Freezing and thawing of IMR90 was performed as follows. Cells were grown to 80% confluence, washed, trypsinized and pelleted at 1200 rpm (~200 x g) in an Eppendorf 5804R refrigerated centrifuge at 4^0C for 4 minutes. The cell pellet was resuspended in 1 ml pre-warmed growth medium, a small aliquot removed for trypan blue staining and counted with a haemocytometer. The cells were resuspended to 1 x 10^6 cells/ml in culture medium + 10% Dimethyl sulfoxide (DMSO), aliquoted into liquid nitrogen storage tubes and placed in a Styrofoam box at –80^0C. The cells were transferred to liquid nitrogen storage tanks (-180^0C) 48 hours later. Cells were thawed by gentle agitation in a 37^0C water bath and the contents transferred to a 75 cm ventilated flask containing prewarmed growth medium.

Serum starvation of primary human fibroblasts

Cells were plated as above in 6 or 10 cm dishes. IMR90 cells routinely attached to the culture dish within 10 hours at which point they were washed 3x with prewarmed PBS to remove all traces of FCS. The cells were cultured in growth medium without FCS for 3 days prior to transfection. After 3 days, transfections were performed with antisense oligonucleotides as described below. Serum (10% final) was added directly to the culture medium 24 hours post transfection.

Preparation of antisense oligonucleotides and transfection lipids

Phosphorothioate antisense oligonucleotides or Geneblocs (GBs) were synthesized according to modifications described by Thompson et al., see refrences in [97] . GBs were obtained through atugen AG (Berlin, Germany) from BioSpring (Frankfurt am Main, Germany). The cationic transfection lipid NC147 was sythesized by and obtained from atugen AG (Berlin, Germany). Lyophilized GBs were resuspended in 1x sterile PBS to yield a concentration of 200 µM and

stored in RNase-free Eppendorf tubes at –80˚C. GBs were thawed on ice prior to use. Lyophilized NC147 was resuspended in ddH_20 to 1 mg/ml, stored at 4^0C in glass vials, and used at a final concentration of 1.6 µg/ml for all experiments.

Tranfection of primary human fibroblasts with GeneBlocs

The initial screening of Geneblocs (GBs) was carried out by transfecting asynchronously proliferating IMR90 cells in 96 well plates. The cells were plated as described 15 hours prior to transfection. One hour prior to transfection, the growth medium was replaced. The transfection solution was prepared by mixing the GBs and the appropriate lipid at a 5x concentration in serum-free DMEM in 10 ml polycarbonate tubes. After a 45 minute incubation at 37˚C with 5% C02, 20 µl of the 5x GB-lipid was added to each well containing 80 µl growth medium giving a final 1x solution of both GB and lipid. The initial screening of GBs in 96 well plates were performed in triplicates at final concentrations of 15, 30 and 60 nM. GB dose response transfections were performed in triplicates at final concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 nM.

All transfections of IMR90 cells in 6 cm culture dishes were performed with a final GB concentration of 50 nM. IMR90 cells were plated 15 hours prior to transfection as described. One hour prior to transfection the growth medium was replaced. The transfection solution was prepared by mixing 1 µl of a 200 µM GB stock with 6 µl NC147 lipid in 0.5 ml serum-free DMEM in a 10 ml polycarbonate tube. After a 45 minute incubation at 37˚C with 5% C02 for 45 minutes, the mix was added to the culture dish (5 ml final volume).

Splitting of transfected IMR90 cultures in 6 cm culture dishes was performed as follows. Five hours after transfection, the GeneBloc containing medium was removed and saved. The cells were washed 1x with 5 ml pre-warmed PBS and 1 ml pre-warmed PBS containing 0.25% (w/v) Trypsin-0.03% EDTA was added to the dish and placed at 37˚C for 5-10 minutes. After the cells detached from the

dish the original GeneBloc containing medium was reapplied. The cells were gently dispersed by pipetting and split 1:2 or 1:4 into 3.5 cm culture dishes.

Transfections of IMR90 cells in 10 cm culture dishes were performed with a final GB concentration of 60 nM. IMR90 cells were plated 15 hours prior to transfection as described. One hour prior to transfection the growth medium was replaced. The transfection solution was prepared by mixing 3 µl of a 200 µM GB stock with 16 µl NC147 lipid in 2.0 ml serum-free DMEM in a 10 ml polycarbonate tube. After a 45 minute incubation at 37˚C with 5% $C0_2$, the mix was added to the culture dish (10 ml final volume).

Analysis of mRNA levels by Real-time rtPCR

Total RNA was isolated from IMR90 cells utilizing the Invisorb total RNA isolation kit according to the manufacturers instructions (InVitek GmbH, Berlin, Germany). Total RNA was eluted in 100 µl of RNase-free ddH_20. Real-time rtPCR (Taqman analysis) reactions were carried out in 50 µl containing 10 µl total RNA plus 40 µl premade Taqman master mix containing an internal control β-actin amplicon plus one of three target amplicons, mcm3, mcm4 or mcm5 (see materials). The reactions were mixed in RNase-free optical 96-well PCR plates, sealed with optical tape and placed in a Applied Biosystems Sequence Detector 7700 (ABI systems, Foster City , CA). The Taqman amplicons were designed with the Primer Express Program version 0.1 according to the manufacturers suggestions and are listed in the appendix (ABI, Foster City, Ca). The amplicons were synthesized by Metabion GmbH (Martensried, Germany), resuspended at 100 µM in RNase-free ddH_20 and stored at -80^0C. The cycle profile had the following conditions: 48^0C for 30 minutes, 95^0C for 10 minutes, followed by 40 cycles of 15 seconds at 95^0C and 1 minute at 60^0C. The data was analyzed for relative quantity or relative expression as noted in the text according to Applied Biosystems User Bulletin #2, December 11, 1997 and as described Heid et al., [99].

Preparation of whole cell extracts for immunoblotting

Cells were harvested by trypsinization and washed 2x with PBS to remove all traces of serum. The cell pellet was resuspended in a small volume of PBS and counted in a Neubauer Haemocytometer. The cells where lysed in 1x SDS loading buffer (0.25 M Tris pH 6.8, 5% Glycerol, 1% SDS, 1.25% 2-Mercaptoethanol, 1 mM PMSF) at 5×10^3 cells/µl, sonicated for 5 minutes at 60% amplitude in Bandelin Sonopulse sonicator, boiled for 5 minutes at 100^0C and placed on ice or stored at -20^0C until analysis.

Preparation of fractionated cell extracts for immunoblotting

Fractionation of cellular proteins was performed using the protocol of Dimitrova and Gilbert [100]. Cells were harvested by trypsinization, washed 2x with PBS to remove all traces of serum, resuspended in 75 µl ice cold CSK buffer (10 mM Pipes pH 6.8, 100 mM NaCl, 300 mM Sucrose, 3 mM MgCl2, 1 mM EGTA, 1 mM DTT, 50 mM sodium fluoride, 0.1 mM sodium vanadate, 0.5% Triton, 1 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml chymostatin, 1 µg/ml leupeptin, 1 µg/ml aprotinin) and incubated on ice for 3 minutes. The insoluble or chromatin bound proteins were pelleted at 7600 rpm in an Eppendorf 5457R refrigerated centrifuge for 3 minutes at 4^0C. The soluble fraction was decanted and saved. The pellet containing chromatin bound proteins was washed 1x and resuspended in 75 µl ice cold CSK buffer. To both fractions, 15 µl 4x SDS loading buffer was added. The insoluble fraction was sonicated for 3 minutes at 30% amplitude in a Bandelin Sonopulse sonicator. Proper fractionation was controlled for by visualizing histone proteins in the insoluble fraction by separation through a 20% polyacrylamide gel (see below).

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide mini-gels were assembled and poured into Hoefer mini VE Basic gel frames with a single aluminum coated dish separated from a clear glass dish by 1 mm teflon spacers. The 1 mm thick gel combs where made from Teflon. Mini-gels were 1 mm thick, 8 cm wide and 9.2 cm long. Proteins were separated through 8% polyacrylamide minigels for immunoblotting or 20% polyacrylamide minigels for separation of histones. Protein samples (2.5 x 10^4 cells/well) in 1x loading buffer were loaded into 15 well combs and run at 150 volts direct current (constant voltage) for 30 minutes and through the separation gel at 200 volts direct current (constant voltage) for at least 2 hours. The electrophoresis tank and upper chamber of the gel container were filled with 1x SDS running buffer (25mM Tris-HCl, 192mM glycin, 0.1% SDS). Power was supplied from a BioRad PowerPac 400 (Pharmacia).

Transfer of proteins to PVDF membranes

PVDF membranes (immobilon P, millipore) were treated as follows. Membranes were placed in 100% methanol for 1 minute, transfered into 60% ethanol for 1 minute and rinsed in ddH20 for 10 minutes. The membranes were soaked in 1x transfer buffer (25 mM Tris, 192 mM Glycin) for at least 5 minutes. Polyacrylamide gels containing separated proteins were placed on PVDF membranes sandwiched between 10 mm sponges, 2 mm Whatmann paper presoaked in 1x transfer buffer and placed in a wet transfer chamber (Pharmacia mini VE blotter) containing 1x transfer buffer. The proteins were transferred at 0.85A direct current (constant current) for 45 minutes. Power was supplied from a BioRad Power Pac 200 (Pharmacia).

Immunoblotting

PVDF membranes containing bound bound proteins were blocked 10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween 20, 0.5% NP40, 0.5% BSA, Fraction V, 2.5% non-fat dried dried milk (block milk) for 15 minutes at RT or overnight at 4^0C. The membranes were incubated with primary antibodies diluted in block milk for 1 hour at RT or overnight at 4^0C. The membranes were washed 1x in 1x TBST for 5 minutes and incubated for 45 minutes at RT in block milk containing a 1:5000 dilution of horseradish peroxidase conjugated secondary antibody. The membranes were washed 3x for 10 minutes in 1x TBST and bound antibodies visualized by chemiluminescence. (Primary human antibody dilutions: rabbit polyclonal MCM2, MCM3, MCM4, mouse monoclonal MCM5 and PCNA-1:1000. mouse monoclonal MCM6 and MCM7-1:200. mouse monoclonal β-actin-1:100,000.)

Chemiluminescence

Immunoreacted proteins were detected using the SuperSignal Pico West (Pierce). Equal volumes of the Luminol/Enhancer and Stable Peroxide solutions were mixed, poured onto the immunoblotted PVDF membranes and incubated for 5 minutes at RT. Excess solution was drained away, the blot placed in clear plastic folder and exposed to Biomax MR film (Kodak). The film was developed through an AGFA Curix 60 (AGFA, Germany).

Coommassie staining and destaining of PVDF membranes

Equal loading of proteins for immunoblotting was performed by coommassie staining of proteins separated through 8% minigels as follows. After separation, the gels were placed in Coommassie staining solution for 0.5 hours on a rocking platform. The gels were destained in Coommassie destaining solution 1 (40% Methanol, 10% Glacial Acetic Acid) for 15 minutes followed by 20 to 30 minutes in Coommassie destaining solution II (7% Glacial Acetic Acid, 5% Methanol) with

2 to 3 solution changes until the the protein bands were clearly visible. The destained gel was air dried between cellophane foil (pre-soaked in 40% methanol, 2% glycerol) mounted and secured in a plexiglass frame and air-dried overnight. The same protocol was used when visualizing histone proteins separated through 20% polyacrylamide gels to control for proper fractionation of soluble and insoluble proteins. After immunoblotting and antibody signal detection, PVDF membranes were counterstained to control for efficient transfer and equal loading of protein samples. The membranes were placed in Coommassie staining solution for 0.5 hours on a rocking platform. Destaining was achieved after 10 minutes in Coommassie destaining solution I. Membranes were allowed to air dry.

Preparation of primary human fibroblasts for flow cytometry

Cells were trypsinized, washed 2x with PBS and pelleted at 1000 rpm (~200 x g) in an Eppendorf 5417R swinging bucket centrifuge for 4 minutes. The cells were resuspended in 0.5 ml ice cold PBS, injected into 4 ml ice cold 70% ethanol while vortexing and stored at 4˚ C for at least 24 hours before analysis. Prior to analysis the cells were washed 2x in PBS, resuspended in PBS containing propidium iodine (PI) (20 µg/ml) and DNase-free RNase A (50 µg/ml), incubated at 37˚C for 15 minutes in the dark and analyzed by flow cytometry. At least 2 x 10^5 cells were prepared for flow cytomteric analysis.

Flow cytometry

Fixed, PI stained cells were injected into 5 ml polystyrene round-bottom tubes through a cell-strainer cap to break up any clumped cells (Falcon tube 35-2235, Becton Dickinson, NJ). The cells were analyzed through a FACS Calibur flow cytometry cell scanner with an argon laser tuned to 488 nm (Becton Dickinson, NJ). Usage of the FACS Caliber was according to the suggested manufacturers protocols and those found in Chapter 7 of the Current Protocols in Cytometry (John Wiley and Sons Inc). The acquisition flow rate was kept below 300 events

per second to ensure proper resolution and when needed PBS was added to the tube to adjust the concentration of cells. At least 2 x 10^4 ungated events were acquired with a FSC threshold of 52 for each sample. Doublet discrimination (DMM) to distinguish between clumped and mitotic cells was set for FL2. Cell cycle distribution was determined by histograms of number of cells, counts, versus FL2-A (DNA content, flourescent intensity of PI, linear amplification, x-axis). Dual color flow cytometry was performed for FITC versus PI flourecent intensity. The data was analyzed by dot blot graphs of FL1-H (FITC intensity, logorithmic amplification, y-axis) versus FL2-A (DNA content, flourescent intensity of PI, linear amplification, x-axis). Propidium iodine (PI) is excited at 488 nm and has a peak emission of 617 nm. Fluorescein isothiocyanate FITC is excited at 488 nm and has a peak emission of 519 nm. FL1 compensation (FL2-%FL1) was set between 7,5 and 15%. FL2 compensation (FL1-%FL2) was set between 1 and 3%. Data acquisition and control of the flow cytometer was performed with the CellQuest Pro program (BD Biosciences, Heidelberg, Germany) on a Apple G4 computer (Apple Computers, Cupertino, Ca) according to the manufacturer´s sugestions.

BrdU incorporation and detection

DNA synthesis was monitored by the incorporation of bromodeoxyuridine (BrdU), a thymidine analog, as follows. BrdU (Sigma-Aldrich, St.Louis, Mo) was dissolved in ddH20 at 100 mM at 37^0C for 1 hour and sterilized through a 0.45 micron nitrocellulose filter (schleicher & schull). BrdU (10μM final) was added to growth medium 3 hours prior to harvesting. The cells were harvested and fixed for flow cytometry as described above. To detect incorporated BrdU, fixed IMR90 cells were pelleted and washed 1x in PBS, resuspended and incubated in 2M HCl for 30 minutes at 37^0C to denature the DNA. The cells were pelleted and resuspended in 1ml 0.1 M $Na_2B_4O_7$-10 H_20 (Borax) and incubated for 10 minutes at RT to neutralize the HCl. The cells were washed 1x in PBS, 1x in PBS-0.5% BSA, resuspended in 100 μl of PBS-0.5% BSA containing 1 μg FITC conjugated

anti-BrdU antibody (Catalog # 347583, Becton Dickson). The cells were incubated at RT for 30 minutes in the dark, washed in 1x PBS, resuspended in 500 µl PBS containing propidium iodine (20 µg/ml) and RNAase A (50 µg/ml) for 15 minutes at 37^0C and analyzed by dual color flow cytometry (FL1-H vs. FL2-A) as described above.

Detection of MPM2 reactivity by flow cytometry

Asynchronously proliferating IMR90 cells were plated in 6 cm dishes and transfected with either GeneBloc 22174 or the mismatch control as described. As a positive control for MPM2 reactivity, untransfected IMR90 cells were treated with 10µM Nocodozole for 18 hours. The cells were fixed for flow cytometry as described. The fixed cells were washed in 1x PBS containing 0.5% BSA and incubated at RT with 10 µg/ml monoclonal mouse monoclonal anti-MPM2 (Upstate) in 1x PBS containing 0.5% BSA for 1 hour . The cells were washed in 1x PBS-0.5% BSA and incubated at RT with 10 µg/ml FITC conjugated goat anti-mouse in 1x PBS-0.5% BSA for 1 hour . The cells were washed in 1x PBS-0.5% BSA, pelleted by centrifugation at 200x g for 5 minutes. The pellet was resuspended in PBS containing propidium iodine (20 µg/ml) and DNase-free RNase (50 µg/ml), incubated in the dark for 15 minutes at 37^0C and analyzed by dual color flow cytometry (FL1-H vs. FL2-A) as described above.

Hoechst staining of DNA for microscopy

IMR90 cells were plated and transfected in 6 cm dishes containing sterile round glass cover slips. The cells were fixed in 3.7% Paraformaldehyde (PFA) for 8.5 minutes and washed with PBS containing Ca^{2+}/Mg^{2+}. The cells were permeabilized by immersion in 0.1% Triton/PBS for 8.5 minutes, washed 1x with PBS, 1x with ddH_2O, stained with Hoechst 33342 at 1 µg/µl in ddH_20 for 5 minutes, and washed with ddH_20 for 5 minutes. Coverslips were mounted on glass microscope slides with a small drop of PVA/DABCO Mounting solution, dried overnight at 4^0C and visualized by flourescent microscopy.