3 METHODS

3.1 Cell culture

3.1.1 Culture conditions

The human Burkitt's lymphoma cell line BL60-2 was cultured in RPMI-1640 supplemented with 10% heat-inactivated **f**etal **c**alf **s**erum (FCS), 25 μg/ml gentamicin, 1mM sodium pyruvate, 2 mM Glutamax, 10 mM Hepes pH 7.5, 20 nM bathocuproine disulfonic acid and 50 μM □-thioglycerol at 37°C and 5% CO_2. H9 cells were cultured in RPMI-1640 with 10% heat-inactivated FCS and gentamicin. HeLa and murine embryonal fibroblasts (MEF) were cultured in Dulbecco's MEM with 10% heat-inactivated FCS and gentamicin.

3.1.2 Apoptosis

3.1.2.1 Induction of apoptosis

Apoptosis was induced in BL60-2 cells by incubation with 1.3 μg/ml goat anti-human IgM $F(ab)_2$ antibody, 250 μM **ca**lcium **i**onophore A 23187 (Ca-I) or irradiation with 120 mJ/cm^2 UV light, respectively. About 80% confluent HeLa cell layers were irradiated with 120 mJ/cm^2 UV light or treated with 1μM **st**auro**s**porine (STS) to induce apoptosis. To trigger apoptosis in the T cell line H9, cells were pre-incubated with 1 μg/ml anti-CD95 antibody for 30 min, before the crosslinking $F(ab)_2$ goat anti-mouse IgG antibody was added for additional 3,5 h. B and T cells were harvested by centrifugation. For HeLa cells, the culture medium containing detached apoptotic cells was collected, still adherent cells were incubated with trypsin/EDTA solution for about 5 min in the incubator, and then combined with the former culture medium and centrifuged. Cells were washed twice with PBS and cell pellets were frozen in liquid nitrogen and stored at -80°C for further analysis.

3.1.2.2 Inhibition of caspase function by specific peptides

For the inhibition of caspase activity, cells were incubated with different irreversible cell permeable peptide inhibitors at a concentration of 100 μM:

- z-DEVD-fmk (for inhibition of caspase-3 like caspases)
- z-VAD-fmk (for inhibition of a broad spectrum of caspases)

The inhibitors were dissolved in DMSO (stock 50□mM) and were added to the culture medium 1□h prior to induction of apoptosis. DMSO alone was added for a control. The final DMSO concentration did not exceed 0.5%.

3.1.2.3 Quantification of apoptosis rate

After 24 h of treatment, the percentage of apoptotic cells was evaluated by staining the cells with 5 µg/ml acridine orange in PBS and counting of cells with fragmented apoptotic versus normal nuclei as observed by fluorescence microscopy.

3.1.3 Proliferation assay

10,000 BL60-2 cells in 200 µl medium/well were transferred into a 96-well-plate. Cells were left untreated or stimulated with anti-IgM antibodies as described above. After 24 h, 1 µCi/well [6-^{3}H]-thymidine (10 Ci/mmol, 1 mCi/ml) was added. The cells were incubated for an additional 20 h. The plate was then frozen and thawed at 37°C and the contents were transferred to a unifilter plate using a vacuum pump. After drying, 25 µl Microscint 20 were added to each well. The plate was then sealed and counts per minute (cpm) were measured in a Topcount scintillation counter. Triplicates were used for the calculation of proliferation.

3.1.4 Transient transfection of cell lines

The evening before transfection, 3*10^{5} cells per well were transferred into a 6-well plate. The following day, equimolar amounts of plasmid (about 1 µg) were mixed with 6 µl Metafectene and 200 µl medium without supplements and incubated for 30 min at room temperature (RT). The mixture was then added to the cells and the 6-well plates were stored again in the incubator. 4 h past transfection, the medium was exchanged twice. Transfection efficiency was controlled after 24 h by FACS (**f**luorescence **a**ctivated **c**ell **s**orting) analysis of cells transfected with pEGFP-N3 in parallel.

3.1.5 Immunofluorescence

PBS:	0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 14 mM NaCl
PBS-T:	PBS + 0.05% Tween 20
Mowiol solution:	4.2 ml 99.5% glycerine, 2.4 g Mowiol 4-88, 12 ml 0.2 M Tris-HCl pH8.5

Cells were harvested by trypsinization 4 h past transfection and 5000 cells/well were grown on 10-well slides. 24 h past transfection, cells were fixed with 5% paraformaldehyd in PBS for 20 min at RT, washed three times with PBS, permeabilized with 0.1% triton X-100 in PBS for 10 min at RT, washed again and blocked with 5% non-fat dry milk (for anti-FLAG antibody) or 1% bovine albumine, fraction V (for anti-V5 antibody) in PBS-T for 30 min at RT. Slides were incubated with the first antibody diluted 1:200 in 3% milk in PBS-T or 1% bovine albumine in PBS-T, respectively, over night at 4°C. Following three washing steps with PBS-T, the secondary biotinylated antibody was diluted 1:200 in 3% milk / 1% bovine albumine and applied for 1 h at RT. Slides were washed again with PBS-T and finally incubated with streptavidine alexa fluor 568 conjugate diluted 1:200 plus 2.5 µg/ml DAPI (4.6-**d**i**a**midino-2-**p**heny**l**indole) in 3% milk / 1% bovine albumine for 1 h at RT. After three more washing steps with PBS-T, the cells were covered with mowiol solution and cover slips and analyzed with a Leica DLMB microscope. Digital pictures were taken with a Leica DC500 camera using the IM1000 1.20 software.

3.1.6 Staining of apoptotic cells with CaspACE FITC-VAD-fmk

24 h past transfection, cells were harvested by trypsinization, $1*10^5$ cells were resuspended in 200 µl medium with 0.4 µl CaspACE FITC-VAD-fmk and placed in the incubator for 30 min. Cells were washed once with PBS, resuspended in PBS and analyzed using a FACSCalibur flow cytometer and the CellQuest 3.3 software.

3.2 DNA techniques

3.2.1 Conventional DNA preparation protocols

TBE: 0.1 M Tris-base, 0.1 M boric acid, 2.5 mM EDTA

All standard methods like plasmid-preparation, cloning methods, electrophoresis on agarose gels in TBE etc. were done according to Sambrook et al. (134) or according to the manufacturer's instructions. Modifications of any of these protocols are indicated where necessary.

3.2.2 PCR

For the cloning of short DNA fragments for TNT assay (3.4.4) and RPA (3.3.6) Taq polymerase and buffer were used, while the Advantage-HF 2 PCR kit was applied for the

cloning of longer fragments or full length Mcm3 and Cdc6. In both cases, PCR reactions were carried out as follows:

PCR samples:		PCR program:	
	1 ng template DNA		94°C 5 min
	5 µl 10 x buffer		94°C 1 min
	2 µl dNTP mix (10 mM each)		60°C 1 min (25 times)
	20 pmol primer 1		72°C 1 min
	20 pmol primer 2		72°C 5 min
	1 µl polymerase		
	H_2O ad 50 µl		

For sewing-PCRs, 2 or 3 overlapping fragments were generated in individual PCR reactions. The products were purified on agarose gels and combined in a second PCR step, in which they were amplified with the two outer oligonucleotides.

3.2.3 Sequencing

For sequencing reactions 6 pmol primer, 2 µl Big Dye terminator ready reaction mix and H_2O ad 10 µl were added to 1 µg plasmid DNA. The following program was run in a DNA thermal cycler:

Sequencing program:		
	96°C 30 sec	
	55°C 15 sec	25 times
	60°C 4 min	

Samples were precipitated, resuspended in 25 µl template suppression reagent and boiled for 5 min at 95°C. Sequencing was carried out by an ABI PRISM 310 Genetic Analyzer. The acquired sequences were analyzed and aligned with Factura 2.2.2 and GeneWorks 2.3.1 software.

3.2.4 Site-directed mutagenesis

The QuikChange site-directed mutagenesis kit was used to insert mutations into long DNA stretches. Complementary oligonucleotides for both DNA strands, each including the desired mutation, were annealed to the denatured target plasmid and extended to nicked circular strands by the nonstrand-displacing DNA polymerase contained in the kit. The parental strands were digested by restriction enzyme DpnI, which selectively cleaves methylated DNA. The remaining newly synthesized DNA strands were transformed into competent *E. coli XLI-blue*, where the DNA nicks are repaired. Plasmids were prepared from bacterial clones and the mutation was controlled by sequencing (3.2.3).

3.2.5 DNA fragmentation

To induce apoptosis, BL60-2 cells were treated for 6 h with anti-IgM antibodies as described above (3.1.2.1) or left unstimulated. $1*10^7$ cells were then washed once with PBS. 0.5 ml lysis buffer contained in the InViSorb Apoptosis Detection Kit II were added to the pellets. After 5 min the suspension was applied to a DNA-binding column. The column was washed and the DNA finally recovered with 100 µl elution buffer at 70°C. 25 µl of each DNA sample were analyzed on a 1% agarose gel.

3.3 RNA techniques

3.3.1 Preparation of RNA

Cells were washed once with PBS and total RNA was prepared from cell pellets using the RNeasy Mini Kit. RNA quality was controlled on a 1.2% agarose / 1.1% formaldehyde gel (3.3.2.1).

3.3.2 Northern-blot analysis

Northern-blot analysis was performed essentially as described by Kroczek et al. (135). In brief, total RNA was isolated (3.3.1) and 8 µg RNA was electrophoretically separated on an agarose gel (3.3.2.1), transferred onto a Hybond-N membrane (3.3.2.2), cross-linked by UV-fixation and hybridized with a specific radioactively labeled DNA probe (3.3.2.3). Hybridization was detected by autoradiography.

3.3.2.1 RNA agarose gel electrophoresis

RNA-Gel:	1.2% Agarose 1.1% Formaldehyde 1 x MOPS	Premix:	1.3 x MOPS 3 M Formaldehyde 4% Formamide

Loading-buffer:
1 mM EDTA, pH 8.0
0.25% saturated Bromphenol Blue
0.25% saturated Xylene Cyanol
50% Glycerol

RNA was separated by denaturing agarose gelelectrophoresis in order to eliminate secondary structures as follows: 8 µg RNA (in 5.5 µl H_2O) were mixed with 19.5 µl of denaturing premix, 5 µl loading-buffer and 1 µl ethidium bromide (0.5 mg/ml). Samples

were denatured at 55°C for 15 min and kept on ice until loaded onto the gel. Electrophoresis was performed in 1x MOPS-buffer at 80 V under constant buffer circulation for about 4 h. Separation of the RNA was visualized on a UV-screen by detection of the 28S (4.7 kb), 18S (1.87 kb) and 5S (0.16 kb) rRNA bands.

3.3.2.2 RNA transfer by capillary forces

20 x SSC: 3 M NaCl, 0.3 M Tri-sodium citrate

The RNA-gel was equilibrated in 20x SSC for 10 min and transferred onto a Hybond-N membrane overnight by capillary forces. Fixation of the RNA to the membrane was performed by crosslinking with 120 mJ/cm^2 UV light. Transfer was controlled on a UV-screen by detection of rRNA bands.

3.3.2.3 Northern-blot hybridization

DNA-probes were cut from plasmids with restriction enzymes, yielding fragments sized between 300 and 500 bp. After boiling at 95°C for 5min, 50 ng of the fragments were used for the generation of radioactively labeled probes. Incorporation of [α-^{32}P]dCTP into complementary strands generated by Klenow polymerase was carried out with the HexaLabel DNA labeling kit according to the manufacturer's instructions. Unincorporated ^{32}P-labeled nucleotides were removed by separation on a Quick spin column.

After RNA-transfer the Hybond-N membrane was incubated in 10 ml ExpressHyb solution for 1 h at 68°C. The radioactively labeled denatured DNA-probe was added at a concentration of 1-3*10^6 cpm/ml ExpressHyb solution and incubated over night at 68°C. Subsequently, the membrane was washed according to step 1-3 and then exposed to X-omat LS films using intensifying screens at –80°C for autoradiography.

Washing steps:
step 1: 2 x SSC, 0.1% SDS, 30 min at RT
step 2: 2 x SSC; 0.1% SDS, 30 min at 68°C
step 3: 0.1 x SSC; 0.1% SDS, 30 min at 68°C

3.3.3 Atlas cDNA expression array

15 μg total RNA (3.3.1) was incubated with RNase-free DNase I, purified, precipitated and recovered in H_2O as described in the Atlas cDNA expression array's manual. Thereafter, integrity of the RNA was again checked on an agarose / formaldehyde gel as described above (3.3.2.1). 3.2 μg of the DNA-free RNA from unstimulated or apoptotic BL60-2 cells and gene specific primers were used to produce radioactively labeled first-strand cDNA

with incorporated [□-^{33}P]dATP (3000 Ci/mmol, 10 mCi/ml). Probes were purified by column chromatography. 1.1*10^7 cpms of each labeled probe were used for hybridization of Atlas human cDNA expression array I membranes at 68°C overnight as described in the protocol. After washing, the membranes were exposed to a phosphoimaging screen overnight. Relative intensities of the spots were evaluated using the TINA 2.08e software. Spots on different membranes (unstimulated / 12 h anti-IgM) were normalized with the help of background and housekeeping gene signals.

3.3.4 High density oligonucleotide array expression analysis (Affymetrix GeneChip arrays)

Using 10 µg of total RNA (3.3.1), cDNA synthesis was performed using the SuperScript 2 RT-PCR Kit with a T7-(dT)24 primer containing a T7 RNA polymerase promoter sequence. The resulting cDNA was subjected to *in vitro* transcription in the presence of biotinylated UTP and CTP. After fragmentation, 10 µg of target cRNA were hybridized to HuGeneFL Arrays. Hybridization, washing and staining were performed according to the standard Affymetrix protocol. Fluorescence intensities were measured with a confocal laser scanner. Absolute and comparison analysis of primary data files was performed with the Microarray Suite 4.0 software.

3.3.5 RNase protection assay I (Pharmingen)

RNase **p**rotection **a**ssays (RPAs) were performed using the RiboQuant multi-probe RNase protection assay system with the template set hOrc or custom made RPA probe sets, respectively. [□-^{32}P]UTP-labeled (3000 Ci/mmol, 10 mCi/ml) antisense RNA was synthesized by T7 polymerase from the templates according to the manufacturer's instructions and hybridized overnight to 10 □g total RNA prepared from BL60-2 cells as described above (3.3.1). Unhybridized antisense (probe) and sense (cellular) RNA was then digested by RNase, leaving behind only hybridized double-stranded RNA. These RNA fragments were subjected to electrophoresis on a denaturing 6% acrylamide gel. The gel was dried under vacuum and exposed to X-omat LS films using intensifying screens at –80°C. Relative band intensities were evaluated using the TINA 2.08e software.

3.3.6 RNase protection assay II (Ambion)

Templates were generated using gene specific oligonucleotides and performing individual PCRs (3.2.2) leading to fragments sized between 92 bp and 385 bp. The PCR products were ligated to the T7 promoter adapter contained in the Lig'nscribe Kit. In a second PCR step the templates for antisense transcription were created. Following the MAXIscript protocol (Ambion) [α-^{32}P]UTP-labeled antisense RNA was produced from 1 µg of the prepared DNA template. After the removal of the template DNA by DNase treatment, the entire RNA samples were loaded onto a denaturing 6% acrylamide gel. After electrophoresis, the gel was exposed to a X-omat LS film for 1-3 min. Film and gel were aligned and the RNA fragments (visible on the film) were cut from the corresponding area of the gel. The labeled antisense RNA was then eluted from the gel piece by submerging the excised fragment in elution buffer supplied in the RPA III kit and Cerenkov counts were determined in a scintillation counter. Depending on the length of the fragment, approximately 1*10^5 cpms were used for hybridization to 1 µg of total cellular RNA (3.3.1). Hybridization and single-stranded RNA digestion were carried out as described in the manual. Samples were analyzed on denaturing 6% acrylamide gels, which were vacuum-dried and exposed to X-omat LS films.

3.4 Biochemical methods for protein analysis

3.4.1 Preparation of whole cell protein extracts

SDS lysis buffer: 2 mM EDTA, 1% SDS, 10 mM Tris-HCl, pH 7.5

For the preparation of whole cell protein extracts, fresh or frozen cell pellets were resuspended in SDS lysis buffer and boiled for 15 min at 95°C. After centrifugation for 15 min at 12,000 x g at RT, total protein concentrations of the lysates were determined by the BCA assay. Samples were either used immediately for analysis or stored at -80°C.

3.4.2 Immunoblotting

2 x Laemmli buffer:	100 mM Tris-base, 20% glycerol, 8% SDS, 10% β-mercaptoethanol, 0.01% bromphenolblue, pH 6.8
Transfer buffer:	25 mM Tris-base, 192 mM glycine, 20% methanol, pH 8.3
TBS-T:	0.5 M NaCl, 20 mM Tris, 0.1% Tween-20
Coomassie solution:	0.25% coomassie brilliant blue R-250, 10% glacial acetic acid, 45% ethanol
Destaining solution:	7.5% glacial acetic acid, 25% ethanol

50 µg protein in Laemmli sample buffer were separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes. Membranes were blocked with 5% non-fat dry milk in TBS-T for 30 min at RT. Incubation with the first antibody was done in 3% milk in TBS-T for 1 h at RT. After washing the membranes four times with TBS-T for 10 min, they were incubated in 3% milk in TBS-T with HRP-conjugated secondary anti-mouse or anti-rabbit antibody for 1 h at RT, washed four times for 10 min with TBS-T and developed with enhanced chemiluminescence kit (ECL) as recommended by the manufacturer. Finally membranes were stained with coomassie solution for 1-3 min and destained to visualize the total protein as a loading control.

Primary antibodies were used as follows: rabbit anti-Mcm2 1:500, rabbit anti-Mcm3 1:2000, rabbit anti-Mcm4 1:2000, rabbit anti-Mcm5 1:1000, rabbit anti-Mcm6 1:1000, rabbit anti-Mcm7 1:1000, mouse anti-Cdc6 Ab-1 1:200, rabbit anti-Caspase-3 1:1000, mouse anti-FLAG M5 1:4000, rabbit anti-His tag ab9108 1:4000 and rabbit anti-DNA-PK p350 1:500.

As secondary antibodies, goat anti-rabbit IgG-HRP sc-2004 1:2500 and goat anti-mouse IgG-HRP 1:5000 were used, respectively.

3.4.3 Expression and cleavage of recombinant proteins

3.4.3.1 Expression in Escherichia coli

LB medium:	10 g bacto-tryptone, 5 g yeast extract, 5 g NaCl ad 1 l H_2O
Caspase buffer:	20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2

Mcm3 cloned into the pRSET B T7 expression vector was transformed into competent *E. coli BL21(DE3)pLysE*. Bacteria were grown in LB medium containing 34 µg/ml chloramphenicol and 50 µg/ml carbennicillin. Protein expression was induced at OD_{600} = 0.4 by addition of 1 mM IPTG to the culture. Bacteria were harvested 2 h after induction by centrifugation at 5000*g for 20 min at 4°C. His-tagged Mcm3 protein was purified using a Ni-NTA column under denaturing conditions according to the QIAexpressionist protocol. The eluted fractions were analyzed by SDS-PAGE, fractions of comparable protein concentrations were pooled and dialyzed in 0.5 l caspase buffer for 1 h at 4°C. The buffer was exchanged for fresh 0.5 l and dialysis was continued at 4°C over night.

3.4.3.2 Expression in the baculovirus system

Cdc6 was expressed using the baculovirus system and was kindly provided by the group of Dr. Manfred Gossen at the MDC Berlin. Purification of the His-tagged protein and dialysis was performed as described above (3.4.3.1).

3.4.3.3 Caspase-mediated cleavage of recombinant proteins

Caspase buffer: 20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2

For the determination of caspases cleaving the protein of interest, 60 ng recombinant Mcm3 or 15 ng recombinant Cdc6 were incubated in caspase buffer for 3 h at 37°C with 60 ng recombinant human caspase-3, caspase-6, caspase–7, caspase-8 or caspase-10, respectively. As a control for specificity, 100 μM z-DEVD-fmk or 100 μM z-VAD-fmk were added. Samples were analyzed by immunoblot.

3.4.4 TNT coupled reticulocyte lysate system

Caspase buffer: 20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, pH 7.2
Destaining solution: 7.5% glacial acetic acid, 25% ethanol

Overlapping PCR fragments spanning the protein of interest were fused to a T7 promoter sequence by sewing-PCR (3.2.2). Mutations were inserted into the fragments by PCR-mediated mutagenesis. TNT (**t**ranscription a**n**d **t**ranslation) assays were performed using the TNT coupled reticulocyte lysate system according to the manufacturer's instructions with 250 ng of the DNA fragments and 10 μl L-[4,5-^{3}H]leucine (161 Ci/mmol). 10 μl of the reactions were incubated in caspase buffer containing 1 μg/ml aprotinin and 1mM PMSF with or without 100 ng recombinant human caspase-3 and 100 μM z-DEVD-fmk for 2 h at 37°C. The samples were analyzed on SDS-PAGE gels. Gels were fixed in Coomassie destaining solution for 30 min, soaked in Amplify solution for 30 min, dried under vacuum and exposed to X-omat LS films using intensifying screens at -80°C.

3.4.5 Immunoprecipitation

CSK buffer: 10 mM PIPES pH7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100, 1 mM ATP, 1 tablet/10 ml Complete protease inhibitor cocktail (115)

For immunoprecipitation cells were harvested by trypsinization 24 h after transfection. Pellets of $1*10^6$ cells were washed twice with PBS and resuspended in 1 ml CSK buffer. The tubes were incubated on ice for 20 min and centrifuged at 800*g for 5 min at 4°C. The supernatant, containing the soluble non-chromatin bound proteins, was incubated with 6.3 µg anti-FLAG biotinylated M5 monoclonal antibody under rotation for 1 h at 4°C. 80 µl magnetic Dynabeads M-280 streptavidine were added and rotation was continued for another hour. The beads were separated using a magnet and washed three times with 0.5 ml cold CSK buffer. Bound proteins were eluted by heating the beads for 10 min at 70°C in 40 µl NuPAGE LDS sample buffer and analyzed on 4-12% NuPAGE Bis-Tris gels according to the manufacturer's instructions.

3.4.6 Chromatin-binding assay

CSK buffer:	10 mM PIPES pH7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 0.5% Triton X-100, 1 mM ATP, 1 tablet/10 ml Complete protease inhibitor cocktail (115)

For chromatin-binding assays $2*10^6$ murine embryonal fibroblasts (MEF) were transfected with equimolar amounts of DNA (about 7.5 µg) and 80 µl Metafectene. After 4 h the medium was exchanged for DMEM containing only 0.25% FCS. Cells were starved for 24 h, harvested by trypsinization and split in half. After washing with PBS, one pellet was boiled in 30 µl SDS lysis buffer as described above (3.4.1) for the preparation of whole cell protein. The second pellet was resuspended in 1 ml CSK buffer. The suspension was left on ice for 20 min and centrifuged at 800*g for 5 min at 4°C. The pellet was resuspended in 0.5 ml CSK buffer, incubated on ice for 5 min and centrifuged again. The resulting pellet, containing chromatin and associated proteins, was finally boiled in 30 µl SDS lysis buffer. Both preparations were analyzed and compared by immunoblot.