4 RESULTS

4.1 DNA replication is repressed in apoptotic cells

4.1.1 IgM-crosslinking causes apoptosis in over 70% of BL60-2 cells after 24 hours and leads to a complete inhibition of DNA replication

IgM-induced apoptosis of B cells, which is mediated by (self)antigens *in vivo*, can be mimicked in cell culture by crosslinking the surface IgM molecules with anti-IgM antibodies. To study B cell receptor triggered apoptosis I used the Burkitt's lymphoma cell line BL60-2, which is highly sensitive towards treatment with crosslinking anti-IgM antibodies (136). 24 hours after addition of anti-IgM $F(ab)_2$ fragments to the cell culture, over 70% of the cells displayed fragmented nuclei after acridine orange staining, which is a reliable marker for apoptosis (data not shown).

In addition, proliferation (reflected by [^{3}H]-thymidine incorporaion) of BL60-2 cells was reduced to less then 7% in cells treated with anti-IgM $F(ab)_2$ fragments for 24 hours compared to untreated cells (Figure 7). This result indicates that virtually all cells respond to the apoptotic stimulus, which is important for the subsequent results.

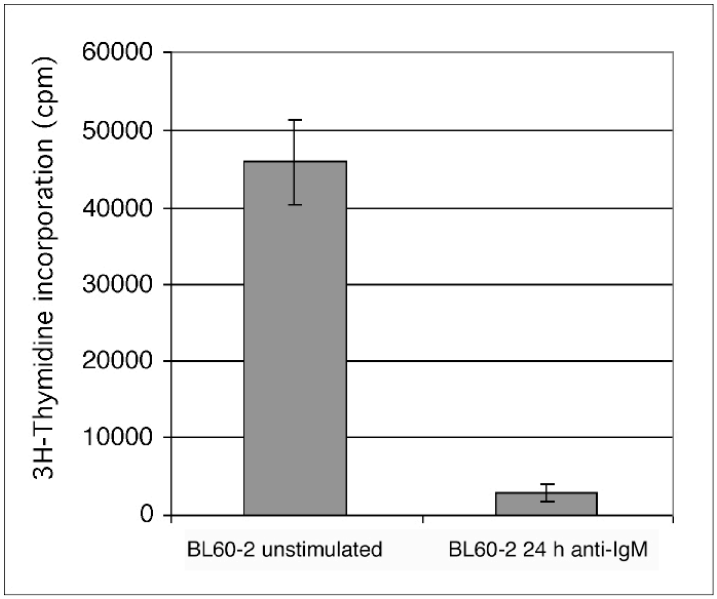


Figure 7. Proliferation is reduced to less then 7% after 24 hours of IgM-crosslinking. BL60-2 cells were left unstimulated or incubated with anti-IgM antibody, respectively. After 24 hours [6-^{3}H]-thymidine was added and cells were incubated for additional 20 hours. Counts per minute (cpm) were measured in a scintillation counter.

4.1.2 mRNA of Mcm2-7 and Cdc6 declines in apoptotic BL60-2 cells

As proliferation is substantially reduced in apoptotic BL60-2 cells, the mRNA levels of genes involved in the initiation of DNA replication in unstimulated and apoptotic cells were analyzed.

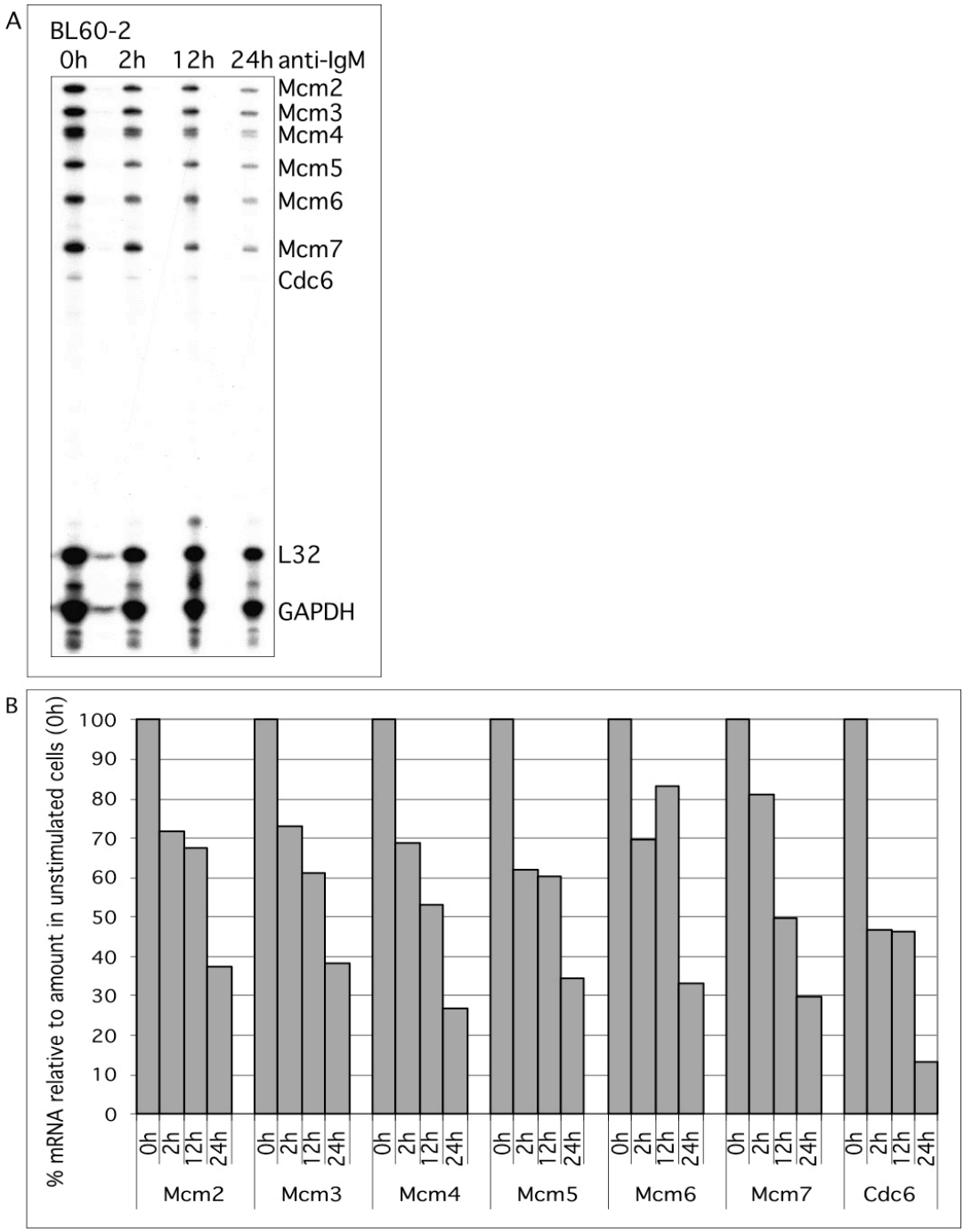


Figure 8. mRNA levels of Mcm2-7 and Cdc6 are reduced early in apoptotic BL60-2 cells. (A) 10 μg total RNA from unstimulated BL60-2 cells and cells treated with anti-IgM antibodies for 2, 12 and 24 hours were analyzed by RNase protection assay for the respective mRNA levels of Mcm2-7 and Cdc6. (B) Band intensities were quantified with the intensities in unstimulated cells set to 100% and normalized relative to the corresponding average values of the housekeeping genes L32 and GAPDH.

Total RNA was prepared from unstimulated cells and cells treated with anti-IgM antibodies for 2, 12 and 24 hours and analyzed by **R**Nase **p**rotection **a**ssay (RPA) (Figure 8A). Although the total mRNA level is reduced in apoptotic cells, evaluation of the band intensities and comparison with the corresponding average values of the housekeeping genes L32 and GAPDH revealed a significantly higher reduction in mRNA level already 2 hours after induction of apoptosis for all tested Mcms (62 – 81% when compared to controls) and most profoundly for Cdc6 (47% when compared to controls) (Figure 8B). This represents an early stage in apoptotic BL60-2 cells, where effector caspases are not jet activated (52).

4.1.3 Mcm3 and Cdc6 are selectively cleaved in response to diverse apoptotic stimuli

Another mechanism to hinder pre-RC formation in apoptotic cells is the destruction of the remaining pre-RC proteins: caspase-3 was shown to selectively cleave Mcm3 in response to various apoptotic stimuli (137). To examine whether this cleavage does also occur in IgM-induced B cell apoptosis and whether additional proteins that participate in the pre-RC complex are also cleaved, whole cell protein extracts from untreated BL60-2 cells and cells incubated with crosslinking anti-IgM antibodies for 24 hours were analyzed by SDS-PAGE. Staining of the corresponding immunoblots with antibodies revealed an apoptotic cleavage of Mcm3 and Cdc6, while Mcm2 and Mcm4-7 remained unchanged (Figure 9A).
The apoptotic Mcm3 fragment has an apparent size of ~90 kDa and corresponds to the fragment described by Schwab et al. (137). Its first visible appearance was narrowed down to 8-12 hours past induction of apoptosis by anti-IgM antibodies (Figure 9B). The main cleavage product of Cdc6 with an apparent size of ~45 kDa was first visible after 4 hours with a second fragment of ~35 kDa appearing after 16 hours (Figure 9C).
In addition, I analyzed Mcm3 and Cdc6 in BL60-2 cells, HeLa cells and the T cell line H9 in response to other apoptotic stimuli i.e. calcium ionophores, UV irradiation, staurosporine and CD95-antibodies. Calcium ionophores insert into the plasma membrane, where they function as selective pores leading to an influx of calcium ions into the cells. The elevated calcium level mimics the release of calcium from the endoplasmatic reticulum, which is part of the apoptotic signaling cascade, and thereby leads to programmed cell death. UV irradiation induces DNA damage. If this damage is not revertible by DNA repair mechanisms, the cell dies by apoptosis. Staurosporine is a broad-spectrum kinase inhibitor. CD95-antibodies lead to crosslinking of the death-domain

containing CD95-receptor in the T cell plasma membrane, thereby inducing a classical death receptor-mediated apoptosis.

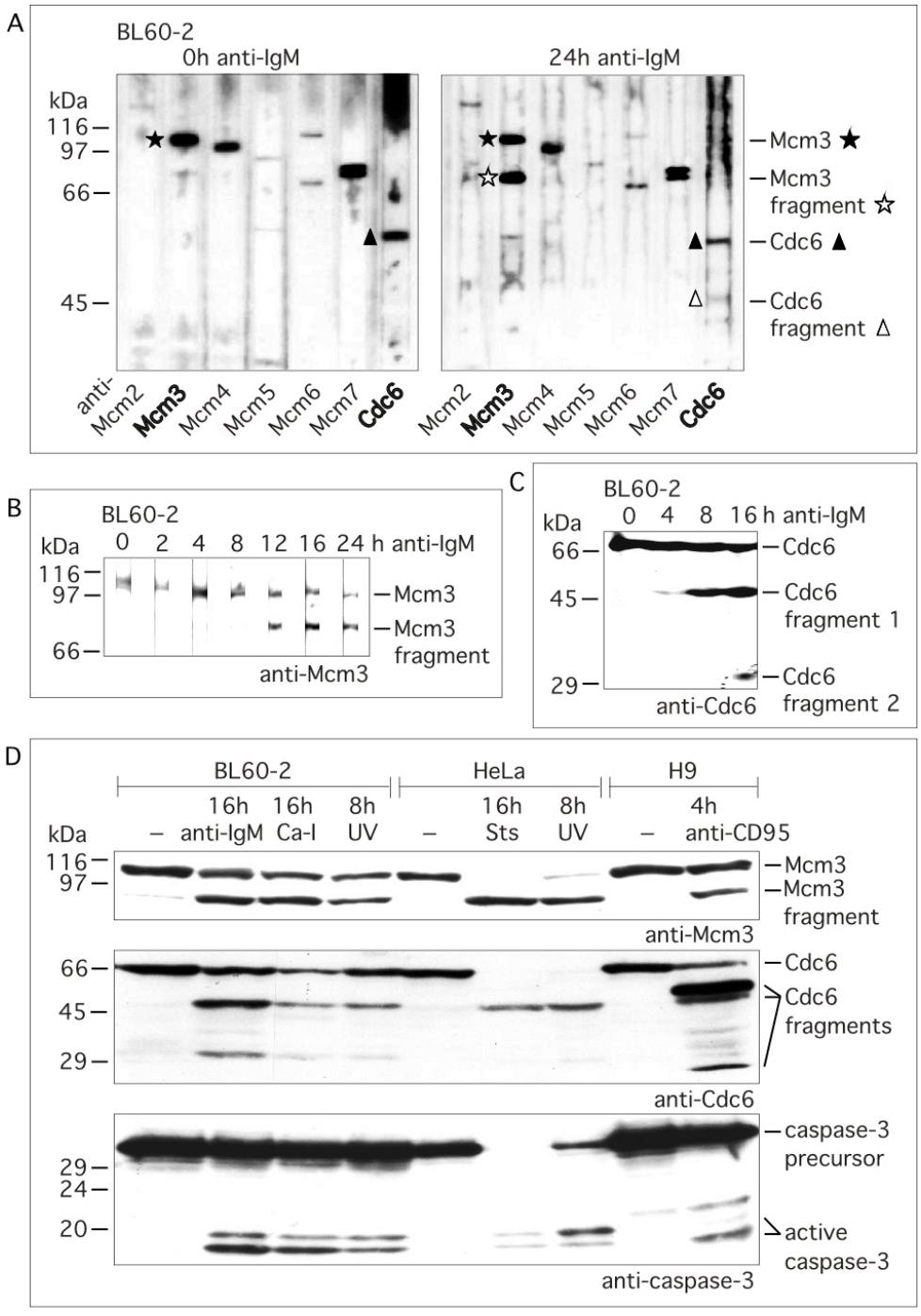


Figure 9. Mcm3 and Cdc6 are cleaved in response to various apoptotic stimuli. (A) Immunoblots of whole cell protein extracts from unstimulated and apoptotic BL60-2 cells 24 hours after induction of apoptosis with anti-IgM antibodies were stained with the indicated antibodies against pre-RC components. (B) and (C) To study the kinetics of Mcm3 and Cdc6 cleavage, protein was prepared at different points in time after induction of apoptosis and analyzed on immunoblots with anti-Mcm3 or anti-Cdc6 antibody, respectively. (D) Whole cell protein extracts were prepared from BL60-2 cells, HeLa cells and H9 cells before and after induction of apoptosis by the indicated stimuli and screened for cleavage of Mcm3, Cdc6 and caspase-3 in immunoblots.

As caspases are activated by cleavage, I screened in parallel for the active caspase-3 fragment. I found cleavage of both Mcm3 and Cdc6 in all three cell lines and in response to all tested apoptotic stimuli. The cleaved proteins were always accompanied by activated caspase-3. The cleavage pattern was the same for all tested stimuli, except for Cdc6 in anti-CD95 induced apoptosis of H9 cells, where fragments of deviating sizes were generated (Figure 9D).

4.1.4 Caspase-3 and -7 cleave recombinant Mcm3 and Cdc6 *in vitro*

Because cleavage of Mcm3 and Cdc6 was accompanied by the appearance of activated caspase-3 and prevented when cells were pre-incubated with the broad spectrum caspase inhibitor z-VAD-fmk or the caspase-3 family inhibitor z-DEVD-fmk (Figure 10A), a caspase-dependent cleavage of both proteins in apoptotic cells was assumed.

To verify cleavage by caspases *in vitro*, recombinant His-tagged proteins were generated. Recombinant Mcm3 protein was expressed in *E. coli* and recombinant Cdc6 protein in the baculovirus system. The proteins were purified and their cleavage by recombinant effector caspases was analyzed. Recombinant Mcm3 was cleaved by caspase-3 and caspase-7, which recognize the same optimal cleavage site DEVD, but not by caspase-6 (optimal sequence: VEHD) (Figure 10B) (47). The produced fragment corresponds in size to the Mcm3 fragment generated in apoptotic cells (~90 kDa). The cleavage was prevented by the addition of the caspase inhibitor z-DEVD-fmk. The same results were obtained for the cleavage of Mcm3 in HeLa nuclear extract incubated with recombinant caspases (data not shown).

Incubation of recombinant Cdc6 with recombinant caspase-3 led to generation of two protein fragments corresponding to the ones seen in apoptotic cells. However, caspase-7 selectively generated the larger fragment. A very limited cleavage occurred upon incubation with caspase-6 (Figure 10B). As described above, a deviating pattern of Cdc6 fragments was observed in apoptotic H9 cells after crosslinking of CD95 (Figure 9D). The apoptotic pathway started by ligation of death-domain containing receptors involves the activation of caspase-8 or caspase-10 in humans (Figure 1). Therefore, I analyzed whether one of these caspases could be responsible for the generation of the deviating Cdc6 fragments. This seems not to be the case, as recombinant caspase-8 and caspase-10 did not cleave recombinant Cdc6 (Figure 10B), even though both enzymes were enzymatically active, as demonstrated by their cleavage of caspase-3 contained in BL60-2 extracts (data not shown). In addition, I found that a combined incubation of recombinant Cdc6 with caspase-3 and caspase-8 or caspase-3 and caspase-10 generated the same

fragments as incubation with caspase-3 alone (data not shown). Again the same results were obtained by incubation of HeLa nuclear extract with the recombinant caspases (data not shown).

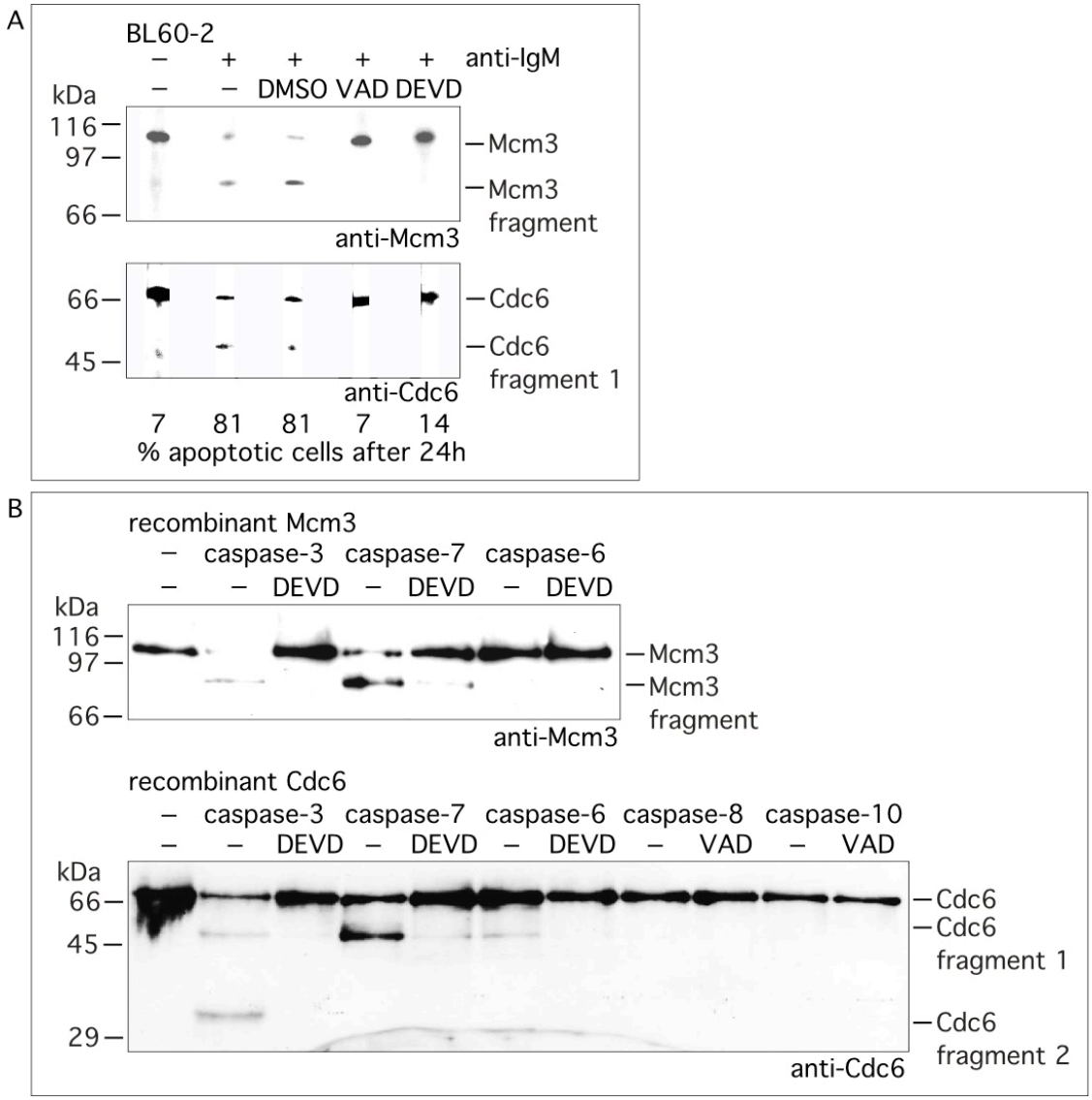


Figure 10. Recombinant Mcm3 and Cdc6 are cleaved by caspase-3 and caspase-7 *in vitro*. (A) BL60-2 cells were pre-incubated for 1 hour with 100 μM caspase-inhibitor z-VAD-fmk or z-DEVD-fmk, respectively, before induction of apoptosis with anti-IgM antibodies. Whole cell protein extracts were analyzed for inhibition of Mcm3 and Cdc6 cleavage on immunoblots. (B) Recombinant Mcm3 and Cdc6 proteins were incubated with the indicated recombinant caspases for three hours at 37°C. Caspase inhibitors z-DEVD-fmk or z-VAD-fmk were added as indicated. Cleavage of Mcm3 and Cdc6 was evaluated on immunoblots using the described antibodies.

4.1.5 Identification of caspase-3 cleavage sites in Mcm3 and Cdc6

As mentioned above, the optimal caspase-3 cleavage site is DEVD, but the two aspartic acids are clearly the most critical determinants of enzyme specificity (47). The Mcm3 amino acid sequence contains 13 putative caspase-3 cleavage sites of the sequence

DxxD. To identify the actual site used by the enzyme, I constructed five overlapping PCR products spanning the Mcm3 sequence, each of them containing several of the putative cleavage sites (Figure 11).

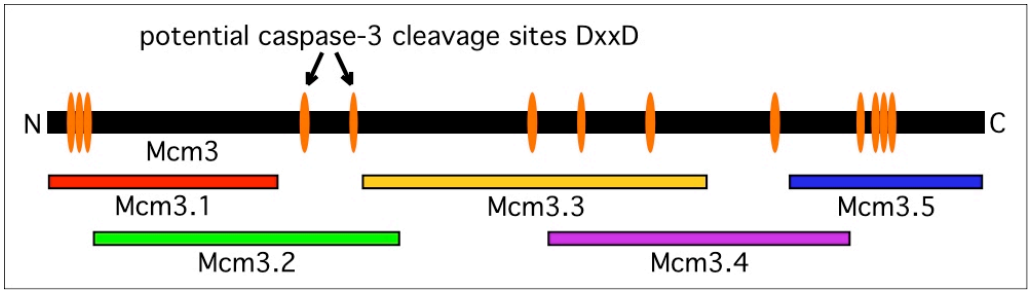


Figure 11. Potential caspase-3 cleavage sites in Mcm3 and Mcm3 fragments used in TNT assays. Mcm3 contains 13 ideal potential caspase-3 cleavage sites of the amino acid sequence DxxD. To identify the site used in apoptotic cells, the overlapping fragments Mcm3.1 – Mcm3.5 were generated and analyzed for cleavage by caspase-3 in TNT assays.

The corresponding radiolabeled polypeptides produced in an *in vitro* **t**ranscription a**n**d **t**ranslation assay (TNT) were analyzed for cleavage by caspase-3. A clear cleavage that was inhibited by z-DEVD-fmk occurred only in fragment Mcm3.5 (Figure 12). The four DxxD sites in this fragment (Figure 11) were then individually mutated. The mutation of two aspartic acids in fragment Mcm3.5A to alanine resulted in the prevention of cleavage by caspase-3, while mutations in fragments Mcm3.5B-D had no effect (Figure 12).

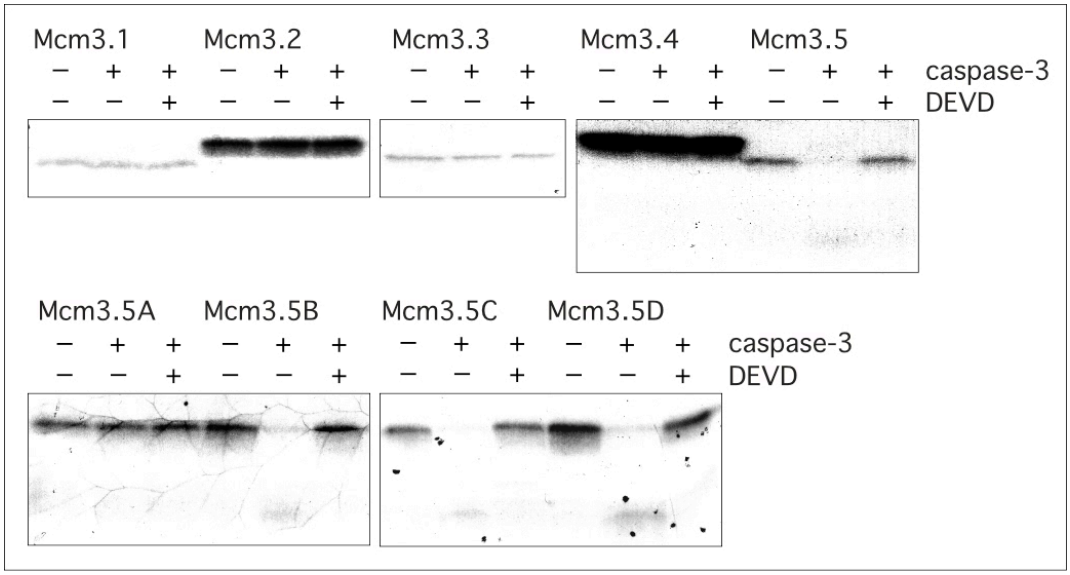


Figure 12. A single site in Mcm3 is selectively cleaved by caspase-3 in TNT assays. Polypeptides Mcm3.1 – Mcm3.5 (see Figure 11) were generated in TNT assays and analyzed for cleavage by caspase-3. Cleavage was identified solely for fragment Mcm3.5, which contains four ideal caspase cleavage sites. Aspartic acids of these sites were individually mutated to alanines. Cleavage of the fragment with a mutation in site A was prevented selectively, thereby identifying the intact site Mcm3.5A as caspase-3 target sequence.

A N-terminally FLAG-tagged full length Mcm3 containing the same mutation as the fragment Mcm3.5A (FLAG-Mcm3D701A) was generated by site-directed mutagenesis (Figure 14), overexpressed in HeLa cells and examined for cleavage in response to an apoptotic stimulus. 18 hours past transfection, apoptosis was induced by addition of 1 □M staurosporine. Another six hours later whole cell protein was prepared and analyzed by SDS-PAGE and immunoblot. At this time overexpressed FLAG-Mcm3wt was cleaved, yielding a fragment identical to the one generated from intrinsic Mcm3 protein, while the mutant protein FLAG-Mcm3D701A was not cleaved (Figure 13A). I therefore identified the caspase-3 site in Mcm3, used for cleavage in apoptotic cells, at DAKD[701].

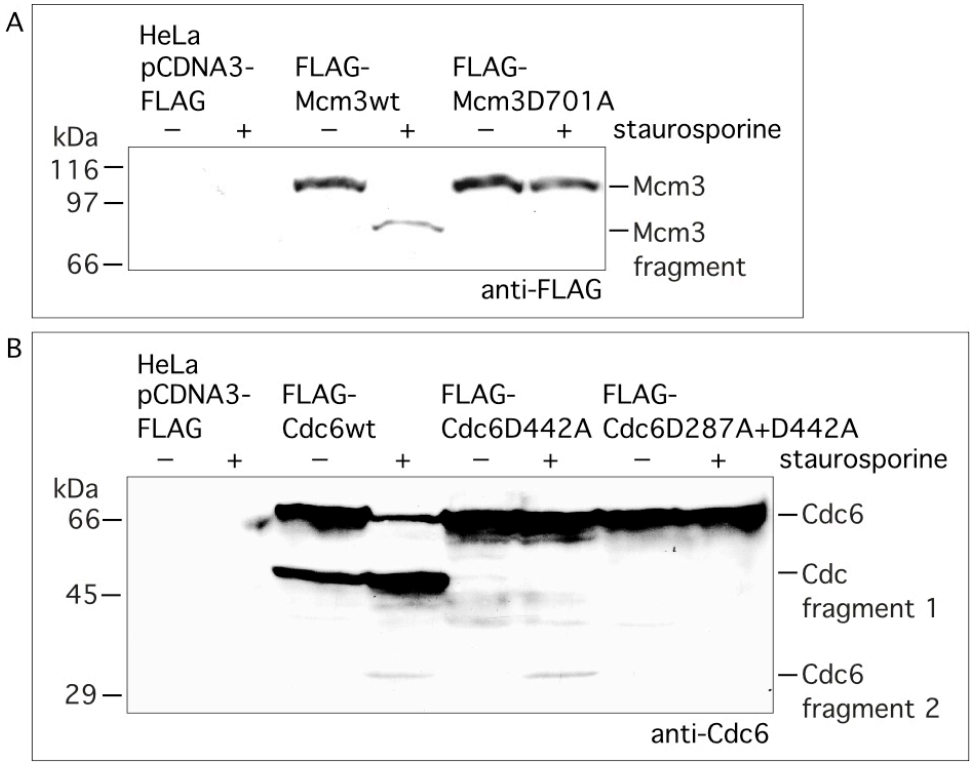


Figure 13. Overexpressed Mcm3 and Cdc6 with mutated caspase sites are not cleaved after induction of apoptosis. (A) FLAG-tagged Mcm3wt and the caspase-resistant mutant Mcm3D701A were overexpressed in HeLa cells. Apoptosis was induced 18 hours past transfection by addition of 1μM staurosporine. After additional six hours whole cell protein was prepared and the extracts were analyzed by immunoblot with anti-FLAG antibody. (B) FLAG-tagged Cdc6wt and Cdc6 variants with mutated caspase site(s) were overexpressed in HeLa cells. Apoptosis was induced as described above. Protein extracts were analyzed by immunoblot using the anti-Cdc6 antibody.

The caspase-3 cleavage sites in Cdc6 were identified in a similar manner. Cdc6 has three DxxD sites. Only one of them, DEMD[287], was used by caspase-3 in the TNT assay (data not shown). An additional site with the less typical sequence SEVD[442] was also identified in the TNT assay system (data not shown). Overexpression of FLAG-tagged Cdc6wt in HeLa cells and induction of apoptosis led to fragments identical to those derived from intrinsic protein. Overexpressed FLAG-Cdc6D442A (Figure 18) was only cleaved once

(the main fragment was missing, while the smaller fragment was still generated), while the double mutant FLAG-Cdc6D287A+D442A (Figure 18) was not cleaved at all (Figure 13B). I therefore identified the caspase-3 cleavage sites in Cdc6 at DEMD^{287} and SEVD^{442}.

4.1.6 The apoptotic Mcm3 fragment augments apoptosis in HeLa cells

In addition to plasmids containing N-terminally FLAG-tagged Mcm3wt and Mcm3 with mutated caspase site (FLAG-Mcm3D701A), plasmids expressing Mcm3 fragments corresponding to the ones produced by caspase cleavage in apoptotic cells, were generated (Figure 14): FLAG-Mcm3N consists of amino acids 1-700, while FLAG-Mcm3C includes amino acids 702-808. I could not detect FLAG-Mcm3C protein expression.

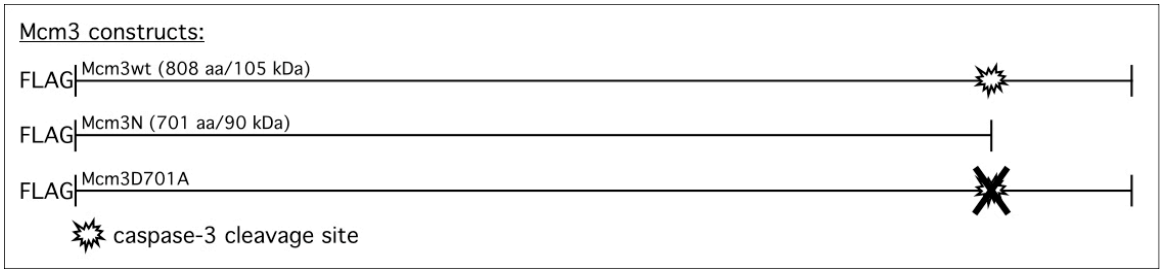


Figure 14. Mcm3 constructs. Constructs generated for overexpression in HeLa cells are depicted.

The intracellular localization of the proteins was examined by immunofluorescence with anti-FLAG monoclonal antibody. I detected overexpressed FLAG-Mcm3N and FLAG-Mcm3D701A (red) in the DAPI-stained (blue) nucleus like FLAG-Mcm3wt (Figure 15).

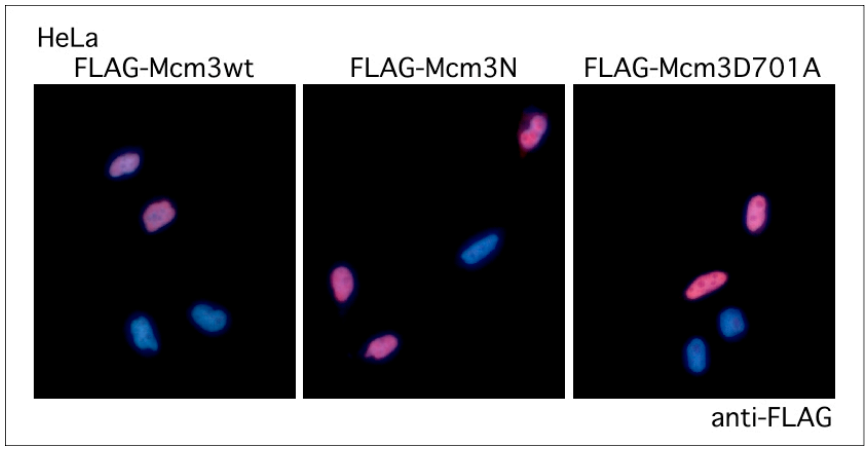


Figure 15. Localization of overexpressed Mcm3 variants in HeLa cells. FLAG-tagged Mcm3wt, Mcm3N and the caspase-resistant mutant Mcm3D701A were overexpressed in HeLa cells growing on 10-well-slides. 24 hours past transfection cells were fixed, permeabilized and stained with anti-FLAG M5 antibody (red fluorescence). Nuclei were visualized by the DNA-dye DAPI (blue). Intracellular localization of the proteins was observed by fluorescence microscopy.

I also analyzed whether the overexpressed proteins were able to co-precipitate intrinsic Mcm4 as an indicator for their ability to participate in MCM complex formation. Chromatin-bound proteins were excluded from the immunoprecipitation experiment, to avoid indirect co-immunoprecipitation of FLAG-Mcm3 proteins and Mcm4 through independent binding to chromatin. Although in a lower amount as FLAG-Mcm3wt, both FLAG-Mcm3N and FLAG-Mcm3D701A coprecipitated intrinsic Mcm4 (Figure 16).

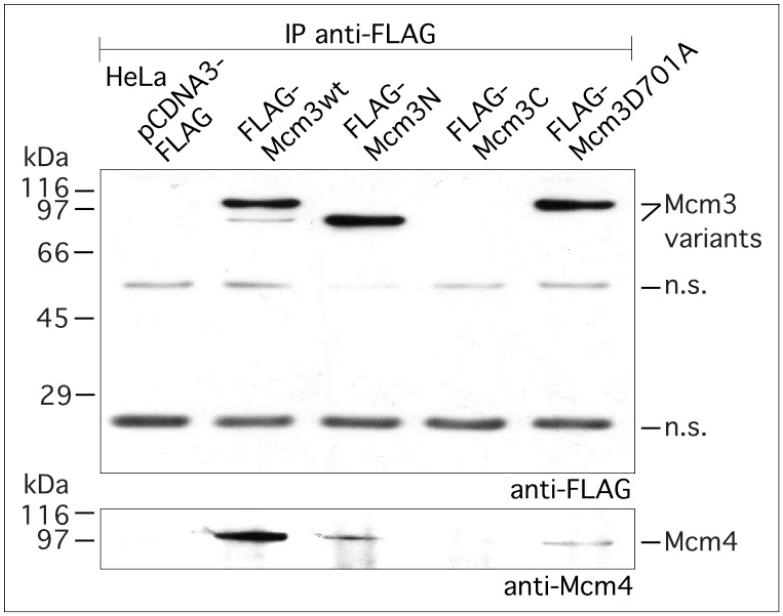


Figure 16. Binding of overexpressed Mcm3 variants to intrinsic Mcm4. FLAG-tagged Mcm3wt, Mcm3N and Mcm3D701A were overexpressed in HeLa cells for 24 hours. Cells were harvested and cellular proteins were prepared excluding chromatin-bound proteins. Extracts were incubated with biotinylated anti-FLAG antibody and subsequently with streptavidine-coated magnetic beads. Eluted fractions were analyzed by immunoblots for the overexpressed Mcm3 variants and coprecipitated intrinsic Mcm4.

Next, I analyzed whether overexpression of the proteins had a pro-apoptotic effect on the transfected cells. HeLa cells were stained with CaspACE FITC-VAD-fmk for active caspases (FL1) 24 hours past transfection. The FACS analysis repeatedly displayed in six independent experiments a significantly higher amount of cells with activated caspases after transfection with FLAG-Mcm3N (here 47% CaspACE-positive cells compared to 5% for the empty plasmid, which is a 9.4 fold induction), while FLAG-Mcm3wt (19%, 3.8 fold induction) and the caspase-resistant mutant FLAG-Mcm3D701A (14%, 2.8 fold induction) had a less pronounced effect on cell survival (Figure 17A).

The transfection efficiency in this experiment was 72% (evaluation of pEGFP control after 24 hours) and protein expression levels analyzed by immunoblot were comparable (Figure 17B). The above results indicate a pro-apoptotic function for FLAG-Mcm3N, which resembles the fragment generated from Mcm3 by caspase cleavage in apoptotic cells.

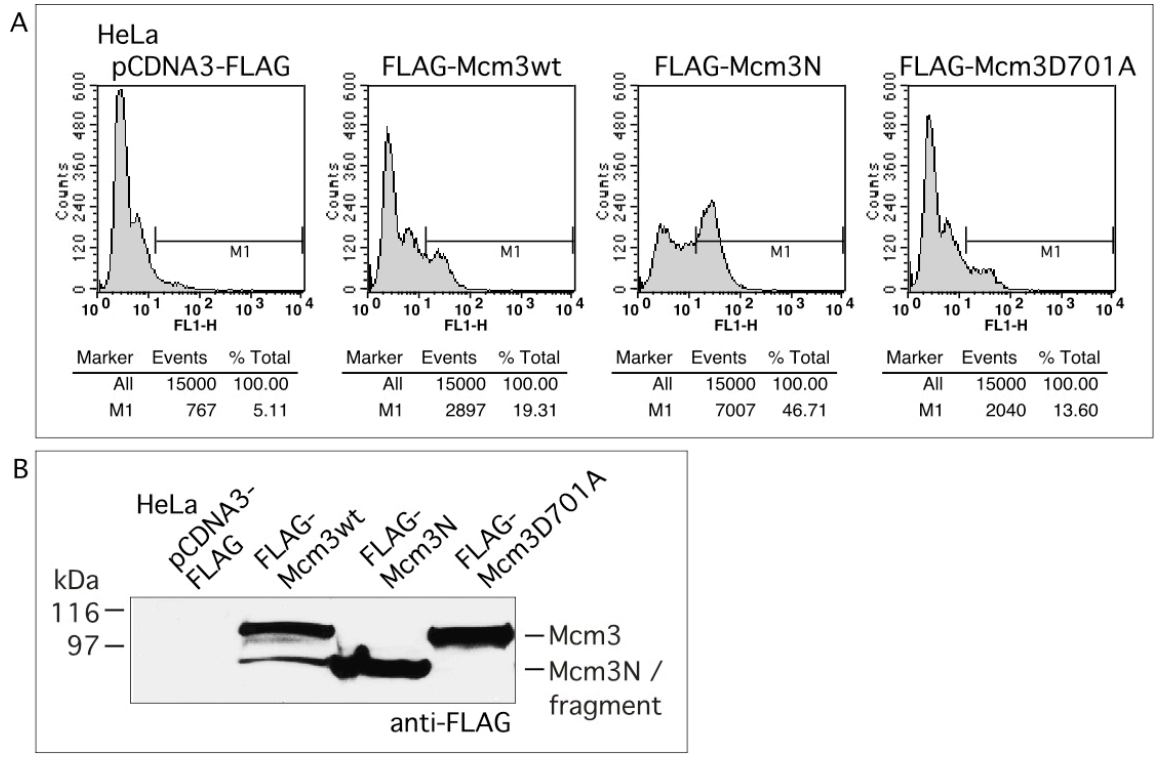


Figure 17. Overexpressed N-terminal Mcm3 fragment exhibits a pro-apoptotic effect on HeLa cells. FLAG-tagged Mcm3wt, Mcm3N, and Mcm3D701A were overexpressed in HeLa cells for 24 hours. (A) Cells were harvested, incubated in medium with CaspACE-FITC-VAD-fmk for 30 min at 37°C to stain for activated caspases and analyzed by flow cytometry. (B) In parallel, whole cell protein extracts were prepared and analyzed by immunoblot.

4.1.7 Cdc6wt and the caspase-resistant mutant augment apoptosis in HeLa cells

In analogy to the Mcm3 plasmids, I generated plasmids containing N-terminally FLAG-tagged Cdc6wt, FLAG-Cdc6D287A+D442A, as well as the fragments FLAG-Cdc6N (amino acids 1-441) and FLAG-Cdc6C (amino acids 443-560), respectively (Figure 18).

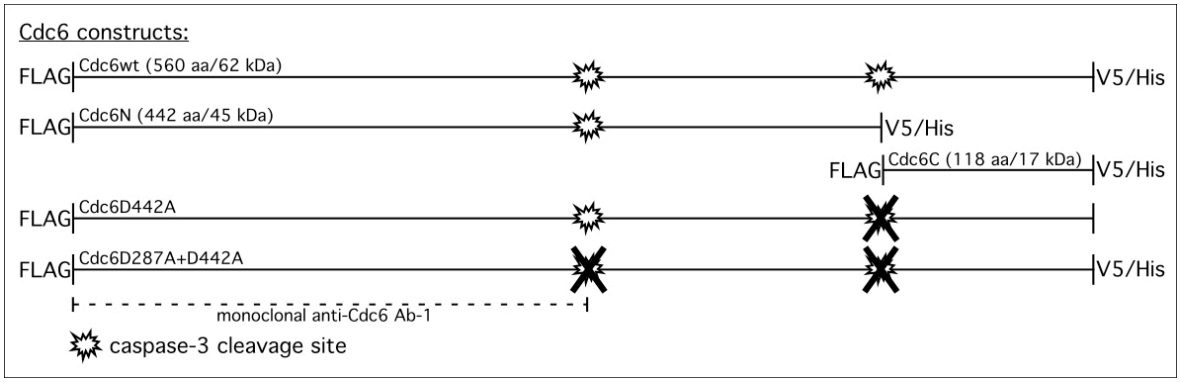


Figure 18. Cdc6 constructs. Constructs generated for overexpression in HeLa cells are depicted.

In immunofluorescence experiments, the intracellular localization of FLAG-Cdc6N and FLAG-Cdc6D287A+D442A (red) was either nuclear (colocalization with DAPI) or cytoplasmic, which resembles the localization of FLAG-Cdc6wt and corresponds to the cell cycle dependent differential subcellular localization of intrinsic Cdc6 (122). In contrast, the localization of FLAG-Cdc6C was exclusively cytoplasmic (Figure 19A). I further examined the localization of the apoptotic fragments generated from overexpressed wildtype Cdc6. Cdc6wt with the N-terminal FLAG-tag and an additional C-terminal V5/His-tag was overexpressed in HeLa cells. 24 hours past transfection apoptosis was induced with staurosporine and cells were fixed and stained with antibodies four hours thereafter. Interestingly, both the N-terminal FLAG-tagged fragment (red) and the C-terminal V5/His-tagged fragment (red) of Cdc6 were recognized by the corresponding antibodies exclusively in the cytoplasm of apoptotic cells with fragmented nuclei (blue) (Figure 19B).

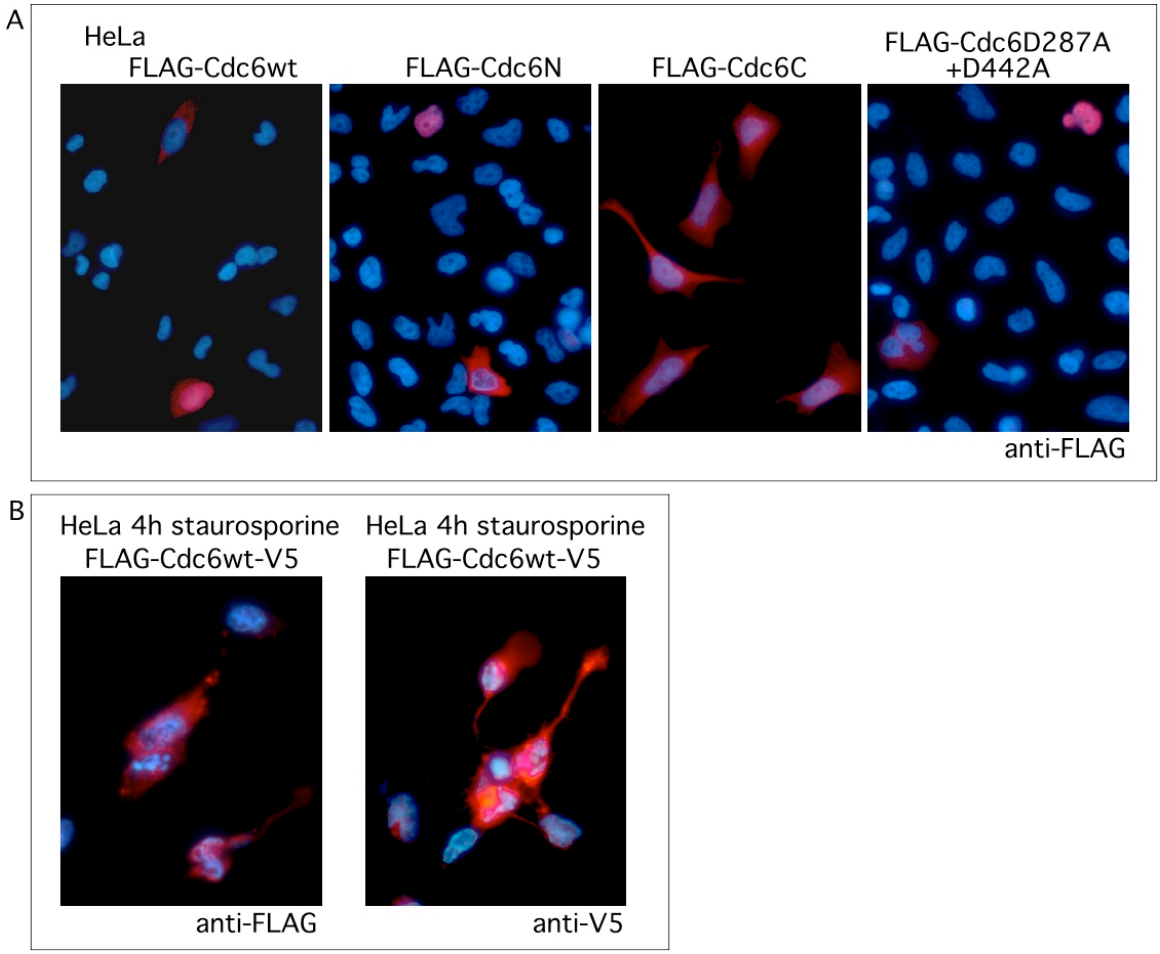


Figure 19. Localization of overexpressed Cdc6 variants in HeLa cells. (A) FLAG-tagged Cdc6 variants were overexpressed in HeLa cells and immunofluorescence was performed as described for Mcm3. (B) In addition, Cdc6wt with both N-terminal FLAG-tag (red fluorescence) and C-terminal V5-tag (red) was overexpressed in HeLa cells. 24 hours after transfection apoptosis was induced by 1 μM staurosporine. Cells were fixed 4 hours later and analyzed for intracellular localization of both tags (red) separately in cells with fragmented nuclei (blue).

To determine their functionality, I analyzed the capability of FLAG-Cdc6N, FLAG-Cdc6C and FLAG-Cdc6D287A+D442A to bind chromatin in comparison to FLAG-Cdc6wt, when overexpressed in starved murine embryonal fibroblasts (MEF) with downregulated intrinsic Cdc6 (115). Chromatin-binding assays displayed binding of FLAG-Cdc6N and FLAG-Cdc6D287A+D442A, which appeared even stronger than the binding of FLAG-Cdc6wt (Figure 20). FLAG-Cdc6C was not bound to chromatin in this assay.

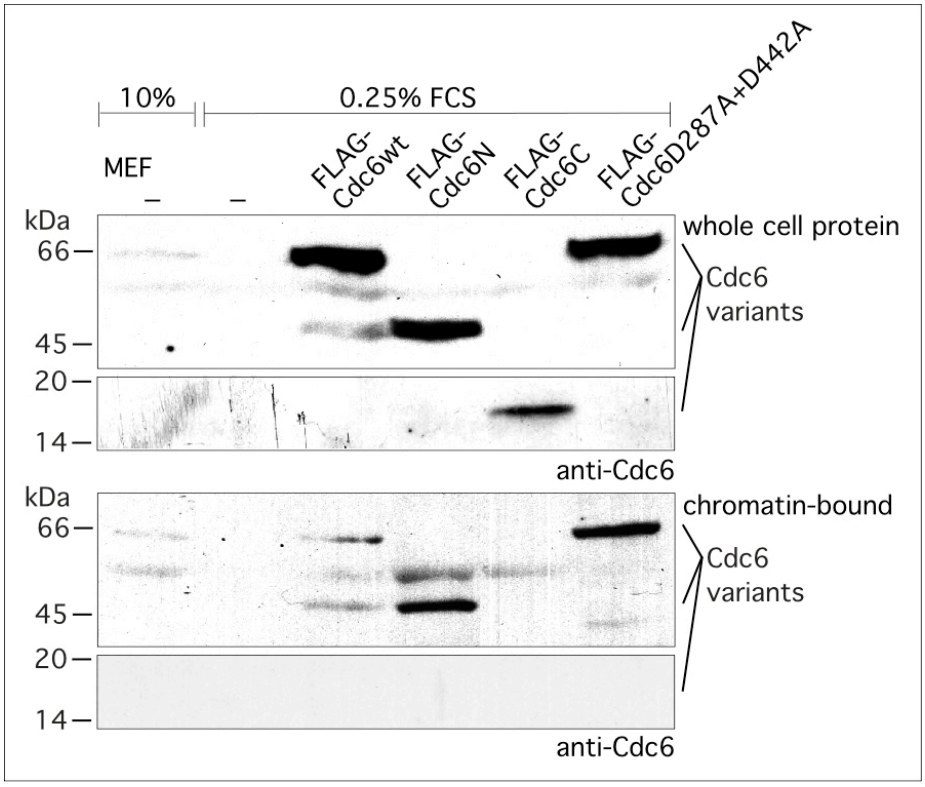


Figure 20. Chromatin binding of overexpressed Cdc6 variants. FLAG-tagged Cdc6 variants were overexpressed in starved MEF cells, which downregulate intrinsic Cdc6 protein. 28 hours past transfection cells were harvested and whole cell proteins or chromatin-bound proteins were prepared. Proteins of both preparations were analyzed and compared using immunoblots.

I also screened for pro-apoptotic properties of the four Cdc6 proteins using the same assay as described for Mcm3. I repeatedly found in six independent experiments that overexpressed FLAG-Cdc6wt (here 46% CaspACE-positive cells compared to 13% for the empty plasmid, which is a 3.5 fold induction) and the caspase-resistant FLAG-Cdc6D287A+D442A (46%, 3.5 fold induction) are considerably pro-apoptotic, while FLAG-Cdc6N (29%, 2.2 fold induction) and FLAG-Cdc6C (30%, 2.3 fold induction) displayed only minor effects on cell survival (Figure 21). The transfection efficiency in this experiment was 60% and protein expression levels analyzed by immunoblot were comparable (data not shown).

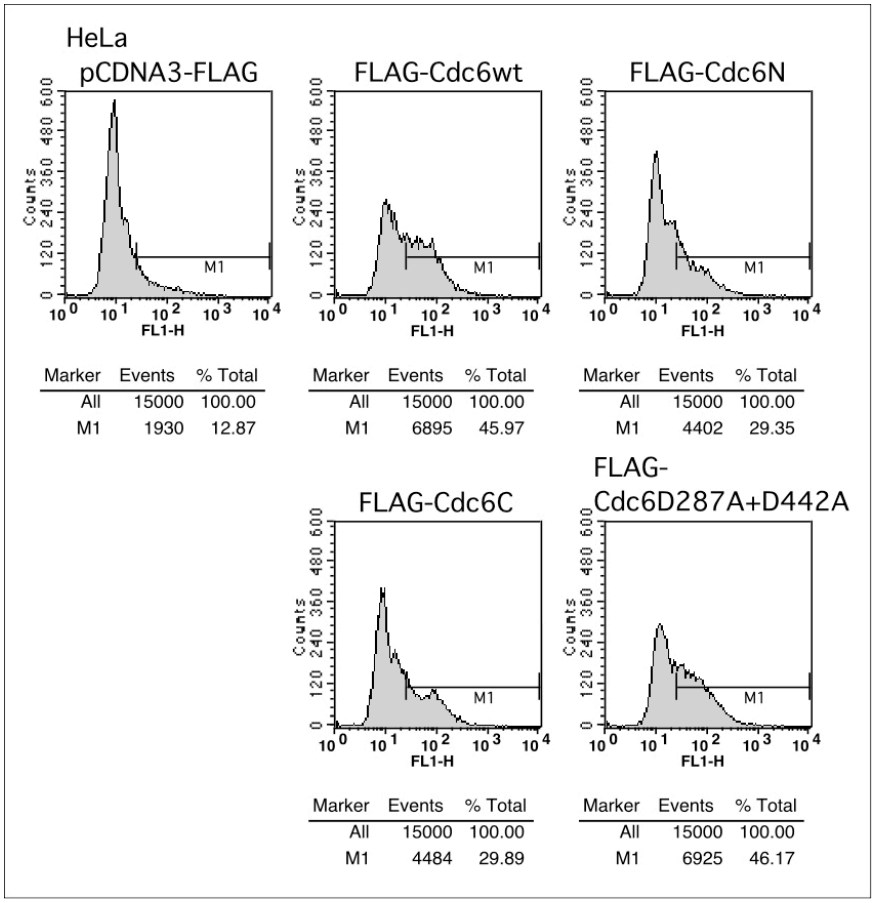


Figure 21. Overexpressed wildtype and caspase-resistant Cdc6 display a pro-apoptotic effect on HeLa cells. FLAG-tagged Cdc6 variants were overexpressed in HeLa cells. 24 hours past transfection cells were stained with CaspACE-FITC-VAD-fmk for activated caspases.

4.1.8 Search for a third caspase-3 cleavage site in Cdc6

An additional caspase-3 cleavage site in the Cdc6 protein was identified by Pelizon et al. at LVFD[99] while this work was in progress (138). I therefore tried to visualize the corresponding apoptotic Cdc6 fragment of ~52 kDa described by this group. The fragment was not detectable in apoptotic cell extracts with our anti-Cdc6 antibody, which binds in the N-terminal half of the protein. It was also not visible after generation of radiolabeled full length Cdc6 in the TNT assay system and cleavage of the protein with recombinant caspase-3 (no antibody is involved in the detection as proteins are visualized by autoradiography). Only one large fragment was generated upon incubation of Cdc6wt with the caspase and mutation of the two caspase-3 cleavage sites SEVD[442] and DEMD[287] prevented the cleavage (Figure 22A). In addition, I overexpressed Cdc6wt and the caspase-resistant Cdc6D287A+D442A with a N-terminal FLAG-tag and a C-terminal V5/His-tag in HeLa cells. Subsequently apoptosis was induced by staurosporine and whole cell protein extracts were prepared at different points in time past induction. The extracts were analyzed by immunoblots stained with anti-FLAG and anti-His antibodies.

Due to the attached tags, overexpressed Cdc6wt and Cdc6D287A+D442A as well as the apoptotic fragments appear slightly larger than the intrinsic proteins. Induction of apoptosis in cells expressing Cdc6wt led to cleavage at SEVD[442] and production of the main apoptotic fragments described above. The N-terminal fragment of ~45 kDa is recognized by the anti-FLAG antibody, while the C-terminal fragment of ~17 kDa is recognized by the anti-His antibody. Neither antibody detected the ~52 kDa fragment described by Pelizon et al. (Figure 22B). Furthermore, induction of apoptosis in cells expressing the double mutant Cdc6D287A+D442A, in which cleavage at the sites SEVD[442] and DEMD[287] is prevented, did not generate the additional fragment predicted, even though immunoblot analysis was done with both the N-terminal anti-FLAG and the C-terminal anti-His antibody (Figure 22B).

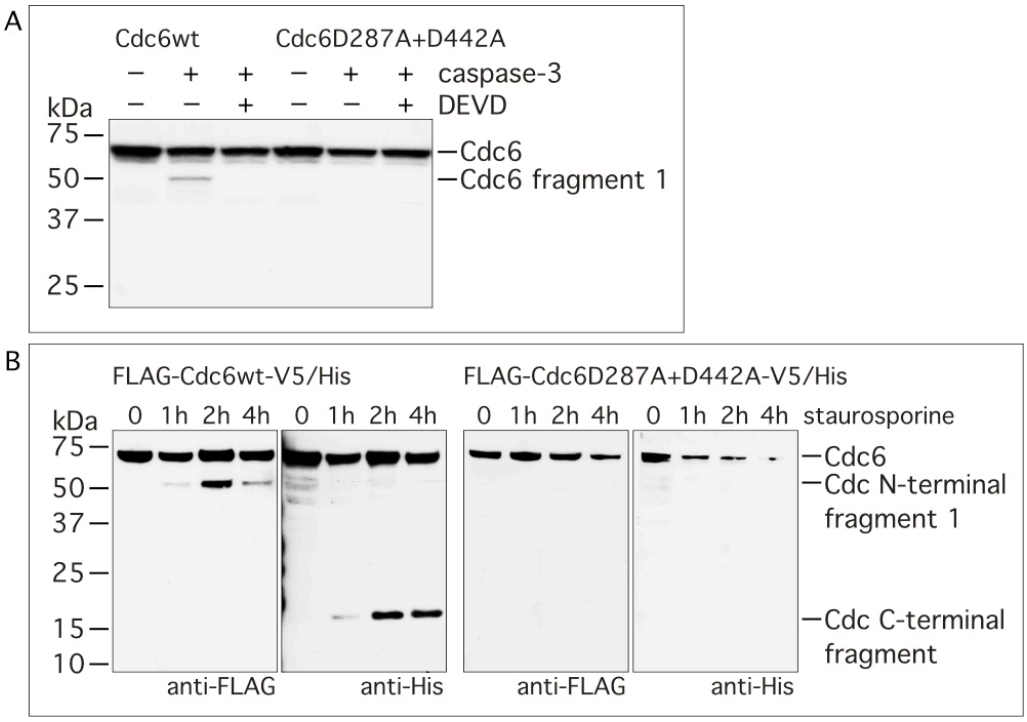


Figure 22. Cleavage of Cdc6 generates only one large fragment of about 45 kDa. (A) Full length Cdc6wt and Cdc6D287A+D442A were generated in TNT assays and incubated with caspase-3 with or without inhibitor. Samples were subjected to SDS-PAGE and bands were visualized by autoradiography. (B) Cdc6wt and Cdc6D287A+D442A with a N-terminal FLAG-tag and a C-terminal V5/His-tag were overexpressed in HeLa cells. 24 hours past transfection apoptosis was induced by staurosporine. Whole cell protein extracts were prepared at different points in time after induction of apoptosis and analyzed for Cdc6 fragments on immunoblots. Due to the attached tags, overexpressed Cdc6wt and Cdc6D287A+D442A as well as the apoptotic fragments appear slightly larger than the intrinsic proteins on immunoblots.

4.2 IgM-mediated signaling changes the mRNA transcription profile

To determine the differential mRNA expression pattern of genes in apoptotic versus non-apoptotic cells, total RNA was prepared from unstimulated cells and cells treated with anti-IgM antibodies for 4 and 12 hours, respectively. Cells harvested after 4 hours of stimulation represent an early stage of B cell apoptosis before the effector caspase-3 is activated (caspase-3 activation is first seen in immunoblots after 8 hours of treatment, data not shown). After 12 hours of stimulation BL60-2 cells are in a late stage of apoptosis reflected by the appearance of fragmented nuclei, phosphatidylserine exposure and active effector caspases.

The RNA samples were analyzed by high-density oligonucleotide microarrays (HuGeneFL GeneChip arrays, Affymetrix), cDNAs spotted on nylon membranes (Atlas arrays), and in RNase protection assays (RPA). The GeneChip arrays I used contain probe sets for 7070 human genes in total. I found 199 genes (2.8%) with enhanced mRNA levels 4 hours after induction of apoptosis (152 at 12 hours), while the expression levels of 75 genes (1.1%) were reduced (58 at 12 hours).

In addition to the oligonucleotide microarrays with a fluorescence-based detection, I investigated the differential transcription levels in apoptotic and non-apoptotic cells on Atlas membranes composed of cDNAs with a detection based on radioactive ^{32}P. 48 out of the 588 genes represented on the membranes (8.2%) were positively regulated in apoptotic cells (more than 2 PSL/mm^2 at least at one point in time, factor > 1.3). 58 genes (9.9%) showed reduced mRNA levels. I further analyzed 24 of the differentially regulated genes in RNase protection assays.

As shown in Table 1-5, I found a good correlation of the results obtained with the three independent methods. In a few cases the data differed with respect to the time (4 versus 12 hours), at which the enhanced or reduced transcription of a gene was first detected, but the tendency (positive, negative or no regulation) was identical for almost all genes analyzed.

4.2.1 DNA repair genes are repressed in apoptotic cells

DNA fragmentation is an important apoptotic mechanism leading to a complete and irreversible degradation of chromosomal DNA. A distinct DNA fragmentation is detectable in apoptotic BL60-2 cells after 6 hours of treatment with anti-IgM antibodies (Figure 23).

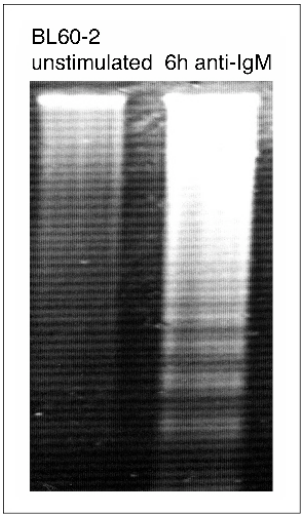


Figure 23. Fragmented DNA in BL60-2 cells 6 hours after induction of apoptosis. BL60-2 cells were cultured with 1.3 μg/ml anti-IgM antibody to induce apoptosis. After 6 hours of stimulation, DNA was isolated and analyzed on a 1% agarose gel.

Inopportune DNA repair in apoptotic cells would obstruct orderly DNA fragmentation and would therefore be counterproductive for the apoptotic degradation of the cell. I found decreased mRNA levels for the following six proteins involved in DNA repair.

The level of DNA ligase I mRNA decreased after stimulation with anti-IgM antibodies (Table 1). DNA ligase I catalyses base-excision-repair and has a function in DNA replication, where it connects the Okazaki-fragments of the lagging strand (139,140).

		0h anti-IgM	4h	12h
DNA ligase I	Atlas	-	-	-
	Chip	1032 P	944 P (-1.1)	946 A (-1.5)
DNA ligase III	Atlas	3.3	-	0.7 (-4.6)
	Chip	488 P	443 P (-1.1)	331 P (-1.2)
DNA ligase IV	Atlas	4.2	-	1.4 (-2.9)
	Chip	145 A	216 P (~1.4)	248 P (~1.3)
DNA-PKcs	Atlas	13.5	-	10.1 (-1.3)
	Chip	2228 P	2545 P (1.1)	1767 P (-1.3)
Ku70	Atlas	-	-	-
	Chip	10334 P	12012 P (1.2)	7201 P (-1.4)
Ku80	Atlas	-	-	-
	Chip	7660 P	6977 P (-1.1)	4725 P (-1.6)

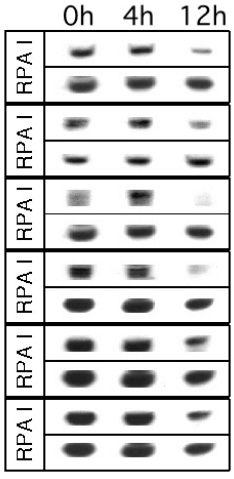


Table 1. mRNA levels of DNA repair proteins are diminished in apoptosis. Apoptosis was induced in BL60-2 cells with 1.3 μg/ml anti-IgM antibody. RNA was prepared at different points in time and subjected to analysis by Atlas array, HuGeneFL microarray or RNase protection assay (I = Pharmingen; II = Ambion), respectively. Evaluated counts for the different genes are shown with fold changes (4 hrs/0 h; 12 hrs/0 h) added in brackets. Values are given in PSL (photo-stimulated luminescence)/mm^2 for Atlas array. For GeneChip arrays average difference, absolute call (A = absent; P = present) and fold change are depicted. A tilde "~" character preceding the fold change value denotes that this value is an approximation due to the low average difference value for one of the data points compared. In the RPA section, the upper bands represent the tested mRNA while the lower bands show the corresponding GAPDH controls.

Less DNA ligase III mRNA was detectable in BL60-2 cells after IgM-crosslinking (Table 1). The mRNA of DNA ligase III exists in two splice variants. The larger variant III forms a complex with the DNA single-strand break repair protein XRCC1 (141). The smaller variant III is probably involved in recombination events during meiosis (142).

DNA ligase IV mRNA was diminished in both Atlas array and RPA (Table 1). When analyzed by GeneChip microarrays, an increase in mRNA was seen (it needs to be noted that the acquired values were in total very low and the significance of the increase is therefore questionable).

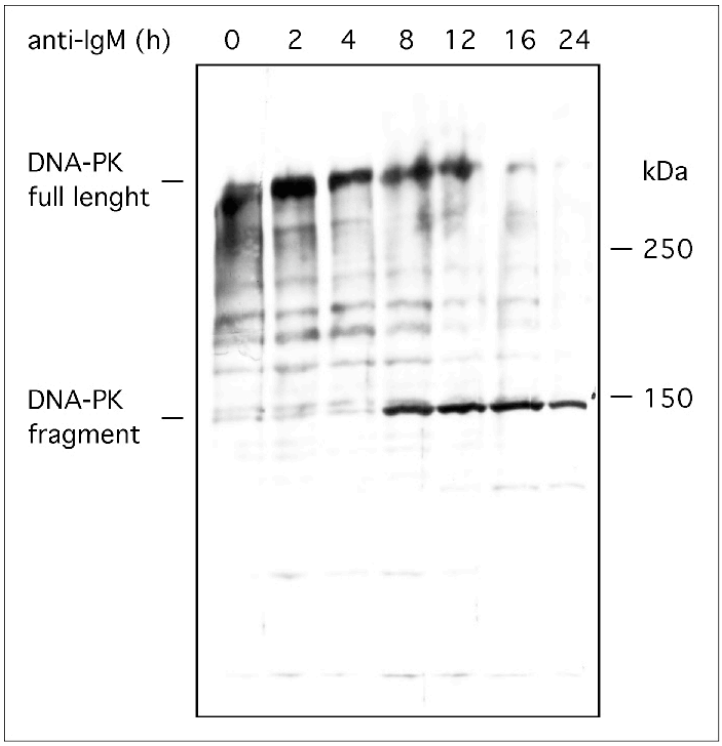


Figure 24. Cleavage of DNA-PK in BL60-2 cells starts 8 hours after induction of apoptosis. Apoptosis was induced in BL60-2 cells with 1.3 μg/ml anti-IgM antibody. Whole cell protein was prepared by SDS-lysis at different points in time and 100 μg protein / lane was separated by SDS-PAGE. The gel was blotted onto PVDF membrane and an immunoblot was performed using anti-DNA-PK antibody.

The level of DNA-PKcs (catalytic subunit of DNA-PK) mRNA decreased in BL60-2 cells after IgM-crosslinking (Table 1). DNA-PK (DNA-dependent protein kinase) is the only known kinase activated by double-stranded DNA ends. Its regulatory subunit is the Ku70/Ku80 heterodimer (143). DNA-PKcs protein is cleaved by caspases in apoptotic HL-60 cells (66). To check whether this additional mechanism to inactivate DNA-PK in apoptosis is present in BL60-2 cells also, I performed an immunoblot with total protein extracts prepared at different points in time after induction of apoptosis. Staining with anti-DNA-PK antibody revealed that cleavage of DNA-PK occured in apoptotic BL60-2 cells 8 hours after IgM-crosslinking (Figure 24). Cleavage was blocked by pre-incubation of the

cells with the caspase inhibitors z-DEVD-FMK (specific for Caspase-3 family) and z-VAD-FMK (broad spectrum caspase inhibitor), respectively (data not shown).

In addition, both components of the regulatory subunit of DNA-PK, Ku70 and Ku80, were downregulated on mRNA level in apoptotic BL60-2 cells (Table 1).

4.2.2 Transcription regulators show altered expression levels in apoptotic BL60-2 cells

I found changes in mRNA expression levels for the following transcription regulators in apoptotic versus non-apoptotic BL60-2 cells.

ID2 mRNA accumulated after IgM-crosslinking (Table 2). ID2 is a dominant-negative antagonist of basic helix-loop-helix transcription factors, which can be activated by oncoproteins of the myc family. It then interferes with the anti-proliferative effects of proteins belonging to the Rb family, thereby allowing cell cycle progression (144).

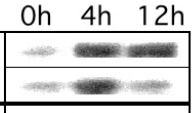
		0h anti-IgM	4h	12h		0h 4h 12h
ID2	Atlas	1.7	-	9.1 (5.4)	RPA II	
	Chip	712 A	799 P (1.6)	1109 P (2.2)		
NF-ATc	Atlas	5.7	-	15.3 (2.7)	RPA II	
	Chip	925 P	4114 P (4.7)	3096 P (3.6)		
EGR-1	Atlas	1.2	-	19.9 (17.3)	RPA II	
	Chip	150 P	1092 P (~6.6)	1343 P (~7.8)		
EGR-2	Atlas	-	-	-	RPA II	
	Chip	-6 A	1064 P (~8.1)	1763 P (~10.8)		
NAB2	Atlas	-	-	-		
	Chip	226 A	1060 P (5.1)	1129 P (5.5)		
TAFII30	Atlas	-	-	-	RPA II	
	Chip	36 A	3008 P (~17.3)	3729 P (~20.1)		
Topoiso-merase I	Atlas	21.0	-	7.5 (-2.9)		
	Chip	1793 P	679 P (-2.6)	829 P (-2.7)		
ETR101	Atlas	6.5	-	20.0 (3.1)	RPA I	
	Chip	4020 P	8147 P (2.0)	7920 P (2.2)		

Table 2. Transcription regulators differentially expressed after IgM-crosslinking.

NF-ATc, a cytoplasmic NF-AT (**n**uclear **f**actor of **a**ctivated **T** cells) protein, showed a strongly enhanced mRNA level in apoptotic BL60-2 cells after 4 hours of IgM-crosslinking, but the mRNA is diminished again after 12 hours of stimulation (Table 2). The NF-AT family of transcription factors takes part in the regulation of early immune response genes like cytokines in B and T lymphocytes (145).

Furthermore, the mRNA levels of two NF-AT target genes, transcription factors EGR-1 and EGR-2 (**e**arly **g**rowth **r**esponse), were highly elevated in apoptotic cells (Table 2). The immediate early gene EGR-1 is activated upon mitogenic stimuli, differentiation cues, tissue injury and B cell receptor (BCR) crosslinking (146). The EGR-2 (Krox-20) mRNA level is upregulated after stimulation of T cells with anti-CD3 and after IgM-crosslinking in the Burkitt's lymphoma line BL2 (147,148).

The mRNA level of NAB2 (Mader), another gene connected with the EGR proteins, was clearly enhanced after induction of apoptosis as detected by chip analysis (Table 2).

TAFII30, the 30kDa TATA-binding protein associated factor needed for RNA polymerase II mediated transcription of specific genes, showed an extremely augmented mRNA level in IgM stimulated BL60-2 cells (Table 2).

The mRNA of topoisomerase I was diminished in apoptotic BL60-2 cells (Table 2). Independent from its activity of relaxing supercoiled DNA, topoisomerase I exhibits an additional function in repressing basal transcription and enhancing transcription in response to an activator (149).

The mRNA of the immediate early gene ETR101 (pip92) accumulated moderately upon anti-IgM stimulation of BL60-2 cells (Table 2). ETR101 probably functions as a transcription activator in T cells (150). ETR101 is upregulated after IgM- or IgG-crosslinking in naive B cells, the immature B cell line WEHI-231 and the memory B cell line 2PK-3, respectively (151).

4.2.3 Two phosphatases are upregulated after induction of apoptosis

I found enhanced mRNA levels for two phosphatases in apoptotic BL60-2 cells.

The CD45 mRNA level was positively regulated in apoptotic BL60-2 cells (Table 3). The transmembrane phosphotyrosine phosphatase CD45 has been reported to play an important role in transducing the signal after IgM-crosslinking (152).

		0h anti-IgM	4h	12h
CD45	Atlas	-	-	-
	Chip	239 P	4589 P (19.3)	3922 P (16.4)
DUSP5	Atlas	-	-	-
	Chip	238 A	3344 P (13.0)	1938 P (7.0)

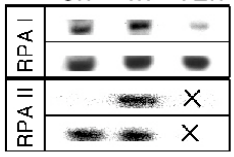


Table 3. Phosphatases with augmented mRNA levels in apoptotic BL60-2 cells.

The mRNA level of the **du**al-**s**pecificity **p**hosphatase 5 (DUSP5) increased extremely after stimulation of cells with anti-IgM antibodies. The mRNA amount peaked after 4 hours and

was again reduced after 12 hours of stimulation (Table 3). DUSP5 has the rare capability to hydrolyze phosphotyrosine, -serine and –threonine (153).

4.2.4 Genes belonging to various functional groups show altered mRNA levels after crosslinking of surface IgM molecules

I found enhanced mRNA levels for the following genes in apoptotic compared to normal cycling BL60-2 cells.

The immediate early gene TTP (**t**ris**t**etra**p**rolin, TIS11) showed a clearly enhanced mRNA level in apoptotic BL60-2 cells (Table 4). TTP belongs to a family of zinc finger proteins that is associated with RNA binding and seems to be part of a machinery that regulates the level of specific proteins by affecting the stability of the corresponding mRNA (154).

In addition, I analyzed the mRNA level of the TTP-related early response gene Berg36 (**B** cell **e**arly **r**esponse **g**ene 36; ERF-1) in unstimulated and apoptotic BL60-2 cells. This gene was not represented on the GeneChip arrays. The expression levels were therefore compared on Northern-blots. The mRNA amount of Berg36 was enhanced in BL60-2 cells as early as 10 minutes after induction of apoptosis by anti-IgM antibodies. The accumulation of mRNA reached its highest level after 30 minutes of stimulation with antibodies and was reduced again after 12 hours (Figure 25A). Whilst a similar increase occurred after treatment of BL60-2 cells with calcium ionophores, Berg36 mRNA was reduced after induction of apoptosis by UV irradiation (Figure 25B).

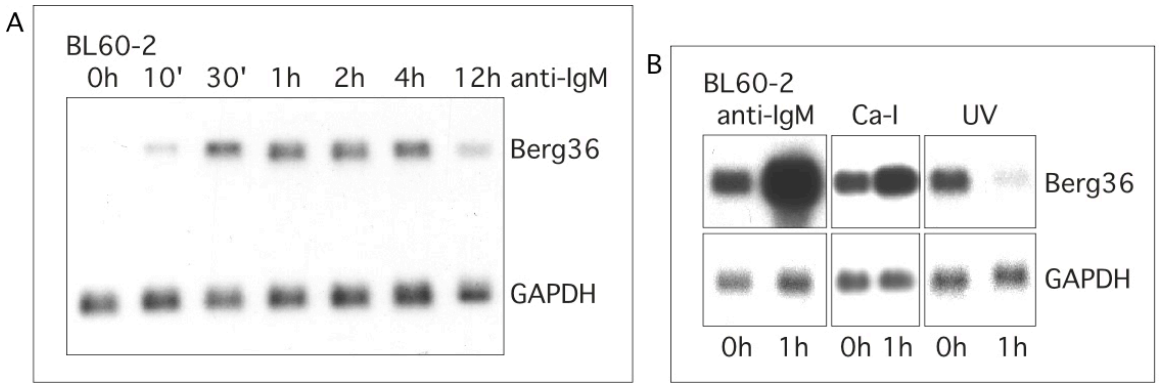


Figure 25. Berg36 mRNA is upregulated in response to IgM-crosslinking. (A) Northern-blot analysis revealed an increase in mRNA amount for the B cell early response gene Berg36 in BL60-2 cells as early as 10 minutes after induction of apoptosis with anti-IgM antibodies. (B) A similar upregulation occured in apoptosis induced by calcium ionophores, but not after irradiation with UV light.

RGS1 (BL34) mRNA level was upregulated in cells after anti-IgM stimulation (Table 4). RGS1 is a B cell specific inhibitor of G protein signaling (155).

NEFA (Nucleobindin 2) was another gene with moderately enhanced mRNA amount in response to the apoptotic stimulus (Table 4). NEFA protein is localized predominantly in the perinuclear region (ER, nuclear envelope, golgi). NEFA contains a leucine-zipper region and an EF-hand 2 motif, which permits protein-protein interactions and is a binding site for calcium ions (156).

		0h anti-IgM	4h	12h	0h 4h 12h
TTP	Atlas	1.1	-	6.5 (5.8)	RPA II
	Chip	-	-	-	
RGS1	Atlas	-	-	-	RPA II
	Chip	48 A	711 P (~5.7)	793 P (~5,9)	
NEFA	Atlas	-	-	-	RPA II
	Chip	407 P	1548 P (3.2)	2254 P (4.8)	
CD70	Atlas	0.4	-	2.3 (5.9)	RPA II
	Chip	509 P	1277 P (2.8)	1288 P (2.5)	

Table 4. Upregulation of various mRNAs in response to anti-IgM stimulation.

The CD70 mRNA level was augmented in apoptotic BL60-2 cells (Table 4). CD70 is a TNF-□ family member and the cellular ligand of CD27.

The expression of many additional genes was stimulated or repressed in apoptotic BL60-2 cells. Some genes with highly differential expression are listed in Table 5.

		0h anti-IgM	4h	12h	0h 4h 12h
1D-myo-inos. Triphosph. 3 kinase B	Atlas	-	-	-	RPA II
	Chip	363 P	2178 P (4.8)	2140 P (4.6)	
KIAA0053	Atlas	-	-	-	RPA II
	Chip	183 A	895 P (~5.9)	1162 P (6.3)	
Ninjurin 1	Atlas	-	-	-	
	Chip	823 P	-95.2 A (~-5.5)	211.9 A (-3.9)	
Scya2	Atlas	-	-	-	
	Chip	-37 A	650 P (~6.3)	618 P (~6.1)	
Scya4	Atlas	-	-	-	RPA II
	Chip	156 A	1709 P (~10.8)	1757 P (~10.7)	
c-fos	Atlas	-	-	-	RPA I
	Chip	61 A	853 P (~6.4)	1361 P (~8.4)	
EV12A	Atlas	-	-	-	RPA II
	Chip	362 P	1572 P (5.8)	1554 P (5.9)	
EMP3	Atlas	-	-	-	RPA II
	Chip	1179 P	4062 P (3.4)	6982 P (5.9)	

Table 5. Additional genes with differential mRNA levels in apoptotic BL60-2 cells.