Results

Testing of amplicons for Real-time rtPCR

Total RNA was prepared using the Invisorb total RNA isolation kits (InVitek GmbH, Berlin, Germany). A stock of total RNA was made from asynchronously proliferating primary human fibroblasts, IMR90 cells and used as a template for subsequent Taqman reactions. The amplicons for the mcm genes were designed using the Primer Express Oligo Design program (ABI, Foster City, CA) using the manufacturer's suggested parameters. A list of the amplicons is found in the materials section. Figure 3 shows the expected sizes of the amplicons for the mcm genes as designed. Real-time rtPCR on IMR90 total RNA using these amplicons listed was performed as described in the methods section. The products were mixed with an equal volume of 2x DNA loading buffer and separated through a 15% agarose gel. All of the amplicons produced robust PCR products in accordance with their predicted size (figure 3). Secondary, non-specific bands were negligible.

A

Amplicon Sizes for Replication Genes

	Base Pairs		Base Pairs
Orc 1	88	Mcm 2	95
Orc 2	145	Mcm 3	93
Orc 3	81	Mcm 4	69
Orc 4	127	Mcm 5	146
Orc 5	93	Mcm 6	119
Orc 6	119	Mcm 7	82

B

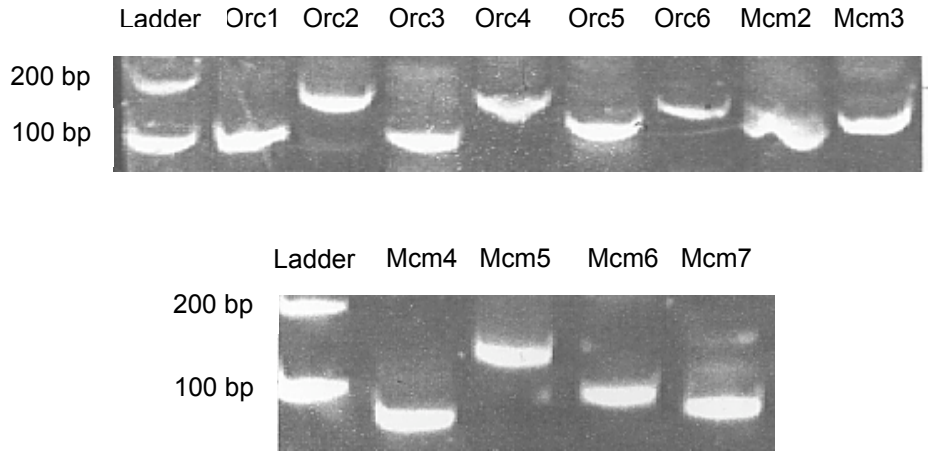


Figure 3 Amplicons for Real-time rtPCR against mcm and orc replication gene products. (A) Expected sizes of Real-time rtPCR products of replication genes. **(B)** Gel electrophoresis of Real-time rtPCR products confirms amplicon functionality. 2.5 ng total RNA from asynchronously proliferating IMR90 cells was added to each 25µL reaction mixture. After the Real-time rtPCR was completed 9µl of each reaction was mixed with 1µl 10x DNA loading buffer and seperated through a 15% denaturing agarose gel and stained with ethidium bromide.

Design and production of mcm4 Geneblocs

The design and production of GeneBlocs (GBs) was a service provided through collaboration with atugen AG in Berlin, Germany. GBs are hybrid DNA:RNA antisense oligonucleotides synthesized according to modifications described by Thompson et.al (see refrence in [97]) and are designed to contain minimal internal repetitive sequences which may form secondary structures at physiological ionic conditions. GBs are 23 nucleotides in length and have the following structure: cap-nnnnnnnNNNNNNNNNnnnnnnn-cap. The cap is an inverted deoxy abazic modification which does not interfere with mRNA target binding, but increases the resistance to endonucleolytic cleavage within the cell [97, 98]. The DNA sequences (N) are phosphorothioate-linked deoxyribonucleotides (A,C,G,T). The RNA sequences (n) are 2'-O-methyl ribonucleotides (A,C,G,U). (a review concerning the design of antisense oligonucleotides is found in [98])

The Mcm4 full-length cDNA sequence (figure 2, GenBank accession number NM182746) was used to design a panel of eight GBs which were screened by transfection of IMR90 cells and quantifying Mcm4 mRNA levels after 24 hours by Real-time rtPCR. Control transfections were performed with either of two oligos. GeneBloc control, GBC, are scrambled sequence oligos representing all possible combinations of 23 nucleotides and were used only for the initial screening. The mismatch control, MM, was designed after a GeneBloc was found that efficiently downregulates its target and contains 6 mismatched nucleotides.

Screening of MCM4 Geneblocs in IMR90 cells

Mcm4 GeneBlocs 22173 through 22180 were transfected into IMR90 cells in 96 well dishes at 15, 30 and 60 nM final concentrations as described. Total RNA was isolated after 24 hours and Mcm4 mRNA levels analyzed by Real-time rtPCR. The results are shown in figure 4 and table 1. The levels of Mcm4 mRNA

in IMR90 cells transfected with GBs 22175 through 22180 were altered to ~50% below (GB22175 at 60 nM) or ~30% above (GB 22178 at 60 nM) those found in untransfected cells. Transfection of GB 22173 resulted in a Mcm4 mRNA down-regulation of 80% at 60 nM, 85% at 30 nM and 75% at 15 nM when compared to levels found in untransfected cells. Transfection of GB 22174 resulted in a Mcm4 mRNA down-regulation of 82% at 60 nM, 89% at 30 nM and 92% at 15 nM when compared to levels found in untransfected cells. Tranfection of the control GeneBloc GBC resulted in a Mcm4 mRNA up-regulation of 3% at 60 nM, 40% at 30 nM and 60% at 15 nM when compared to levels found in untransfected cells. The results show an inverse dose response for several GBs such that higher concentrations of GB were less effective.

IMR90 cells were retransfected with GBs 22173, 22174 and the GBC at 100, 50, 25, 12.5, 6.25 and 3.125 nM concentrations. The results are presented in figure 5 and table 2. Transfection of GB 22173 resulted in a Mcm4 mRNA down-regulation of 69% at 100 nM, 64% at 50 nM, 79% at 25 nM, 70% at 12.5 nM, 70% at 6,25 nM and 77% at 3.125 nM when compared to levels found in untransfected cells. Transfection of GB 22174 resulted in a Mcm4 mRNA down-regulation of 83% at 100 nM, 87% at 50 nM, 90% at 25 nM, 83% at 12.5 nM, 68% at 6,25 nM and 86% at 3.125 nM when compared to levels found in untransfected cells. Thus, GBs 22173 and 22174 do not show a dose dependent increase in downregulation.

GeneBloc 22174 was resynthesized at a larger and higher purity scale and has the following sequence: 5´- ccuagagACTGCTCACuugccac - 3´. Utilizing the GeneBloc 22174 sequence, a mismatch control was designed and synthesized with following sequence: 5´- ccua**c**agA**G**T**C**C**A**CTCuugc**g**ac - 3´. Small cap sequences are 2´ O-methyl modified ribonucleotides. Large cap sequences are DNA. Bold letters represent mismatched bases. See Figure 2 for position of GeneBloc 22174 within the mcm4 cDNA sequence.

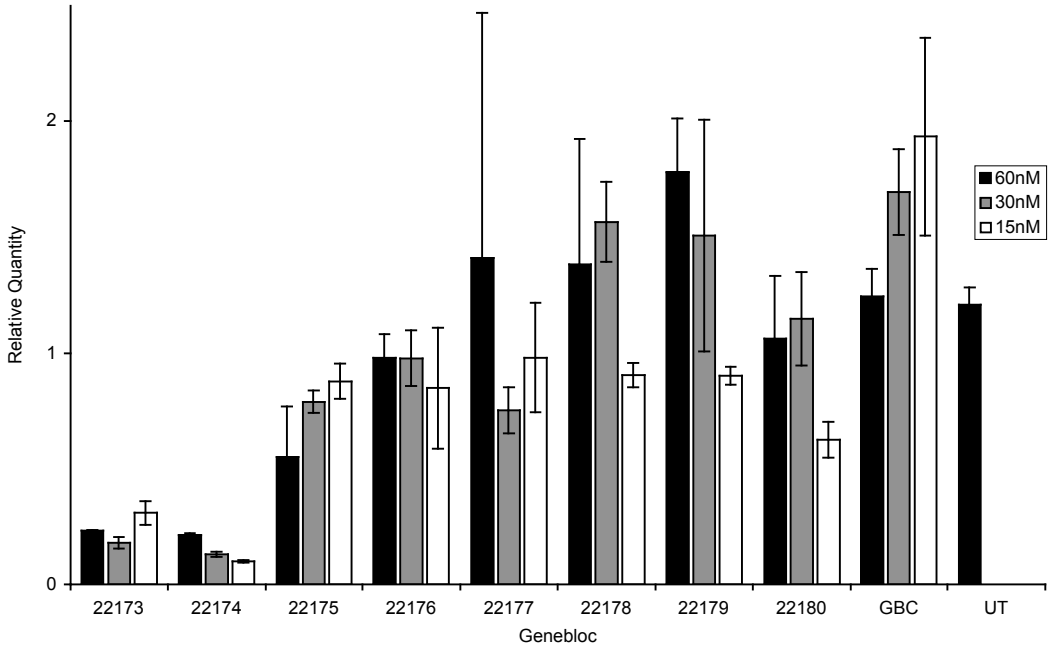


Figure 4 Real-time rtPCR (Taqman) analysis of Mcm4 mRNA levels in IMR90 cells transfected with mcm4 GeneBlocs(GBs) 22173 through 22180. IMR90 cells were transfected in 96 well plates with several concentrations of Mcm4 GeneBlocs in triplicates as described in methods. Twenty four hours post transfection, total RNA was isolated and subject to duplex Taqman analysis with amplicons against Mcm4 mRNA and the internal loading control of β-actin. Reactions were performed in triplicate and normalized to the level of β-actin in the same reaction. mRNA levels are expressed as a relative quantity to a standard curve generated from Mcm4 mRNA from untransfected cells. The experiment indentifies two GBs which strongly downregulate Mcm4 mRNA levels 24 hours after transfection.

Table 1 mRNA levels in GeneBloc transfected IMR90 cells after 24 hours

GeneBloc #		Relative Quantity	Std.Dev.	% Downregulation
GB 22173	60 nM	0.23	0.00	**80.83**
	30 nM	0.18	0.02	**85.14**
	15 nM	0.31	0.05	**74.50**
GB 22174	60 nM	0.21	0.01	**82.38**
	30 nM	0.13	0.01	**89.27**
	15 nM	0.10	0.00	**91.85**
GB 22175	60 nM	0.55	0.22	**54.23**
	30 nM	0.79	0.05	**34.61**
	15 nM	0.88	0.08	**27.27**
GB 22176	60 nM	0.98	0.10	**18.80**
	30 nM	0.98	0.12	**19.04**
	15 nM	0.85	0.26	**29.79**
GB 22177	60 nM	1.41	1.06	**(16.74)**
	30 nM	0.75	0.10	**37.63**
	15 nM	0.98	0.24	**18.86**
GB 22178	60 nM	1.38	0.54	**(14.51)**
	30 nM	1.57	0.17	**(29.61)**
	15 nM	0.91	0.05	**25.11**
GB 22179	60 nM	1.78	0.23	**47.27**
	30 nM	1.51	0.50	**(24.67)**
	15 nM	0.90	0.04	**25.32**
GB 22180	60 nM	1.06	0.27	**11.92**
	30 nM	1.15	0.20	**4.97**
	15 nM	0.63	0.08	**48.09**
GBC	60 nM	1.24	0.12	**(2.97)**
	30 nM	1.69	0.19	**(40.22)**
	15 nM	1.94	0.43	**(60.13)**
Unt		1.21	0.08	

N.B.: Values in parenthesis indicate an upregulation above untransfected levels.
Percent downregulation = 100% - ((Relative Quantity$_{(exp)}$/Relative Quantity$_{(unt)}$) x 100)

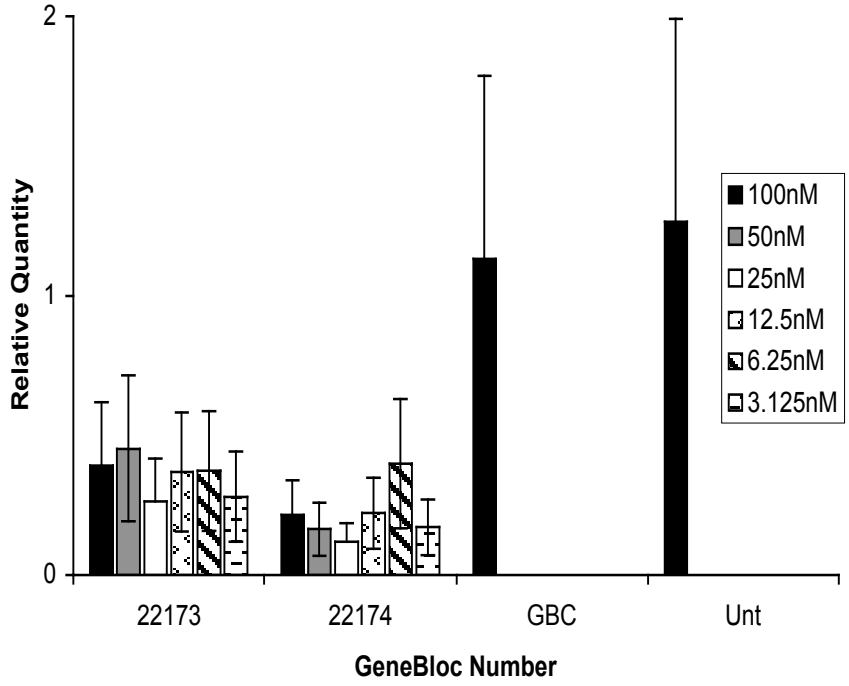


Figure 5 Taqman analysis of Mcm4 mRNA levels from IMR90 cells transfected with various concentrations of Mcm4 GeneBlocs 22173 and 22174. IMR90 cells were transfected in 96 well plates in triplicates as described in methods. Twenty four hours post transfection, total RNA was isolated and subject to duplex Taqman Analyis with amplicons against Mcm4 mRNA and the internal loading control of β-actin. Reactions were performed in triplicate and normalized to the level of β-actin in the same reaction. MRNA levels are expressed as a relative quantity to a standard curve generated from Mcm4 mRNA from untransfected cells .

Table 2 mRNA levels in IMR90 cells 24 hours after transfection at increasing concentrations of GB 22173 or GB 22174.

GeneBloc #		**Relative Quantity**	**Std.Dev.**	**% Downregulation**
GB 22173	100 nM	0.39	0.23	**69.09**
	50 nM	0.45	0.26	**64.20**
	25 nM	0.26	0.15	**79.12**
	12.5 nM	0.37	0.21	**70.89**
	6.25 nM	0.37	0.21	**70.62**
	3.125 nM	0.28	0.16	**77.85**
GB 22174	100 nM	0.21	0.12	**83.00**
	50 nM	0.16	0.10	**86.95**
	25 nM	0.12	0.07	**90.67**
	12.5 nM	0.22	0.13	**82.54**
	6.25 nM	0.40	0.23	**68.45**
	3.125 nM	0.17	0.10	**86.48**
GBC	100 nM	1.13	0.65	**10.23**
Unt		1.26	0.73	

N.B.: Percent downregulation = 100% - ((Relative Quantity$_{(exp)}$/Relative Quantity$_{(unt)}$) x 100)

Time course of Mcm4 depletion in IMR90 cells transfected with GB22174

A time course experiment was performed to determine the kinetics of Mcm4 mRNA downregulation. In addition, the levels of Mcm3 and 5 mRNA were analyzed. Asynchronously proliferating IMR90 cells were transfected as described with a final concentration of 60 nM GB 22174 or the mismatch control (MM) and split 1:4 into 3.5 cm dishes after 5 hours. Total RNA was isolated at the indicated time points and subjected to duplex Taqman analysis containing the β-actin and one of three specific Mcm amplicons. The data analyzed for relative expression levels are shown in figure 6 and table 3 .

The level of Mcm4 mRNA in GB 22174 transfected IMR90 cells is down-regulated in an apparent time-dependent manner to 75% of the level found in untransfected cells. The level of Mcm3 mRNA is reduced by 57% after 48 hours whereas the level of Mcm5 mRNA is reduced by 15% within the same time period. The levels of Mcm3 and 4 in cells tranfected with the mismatch control (MM) are approximately equal to those observed in untransfected cells. The one exception is the level of Mcm5 mRNA in mismatch control transfected cells, which is 38% higher than untransfected cells. The down-regulation kinetics of mRNA levels parallel the disappearence at the protein level of Mcm3 and 5 (see discussion). Within 15 hours post-transfection the levels of Mcm3 and 5 mRNA increase significantly (50 and 60 % respectively) above untransfected levels. Whether this increase is the result of a transcriptional upregulation cannot be addressed without analysis of the levels found in mismatch control transfected cells at the same time points.

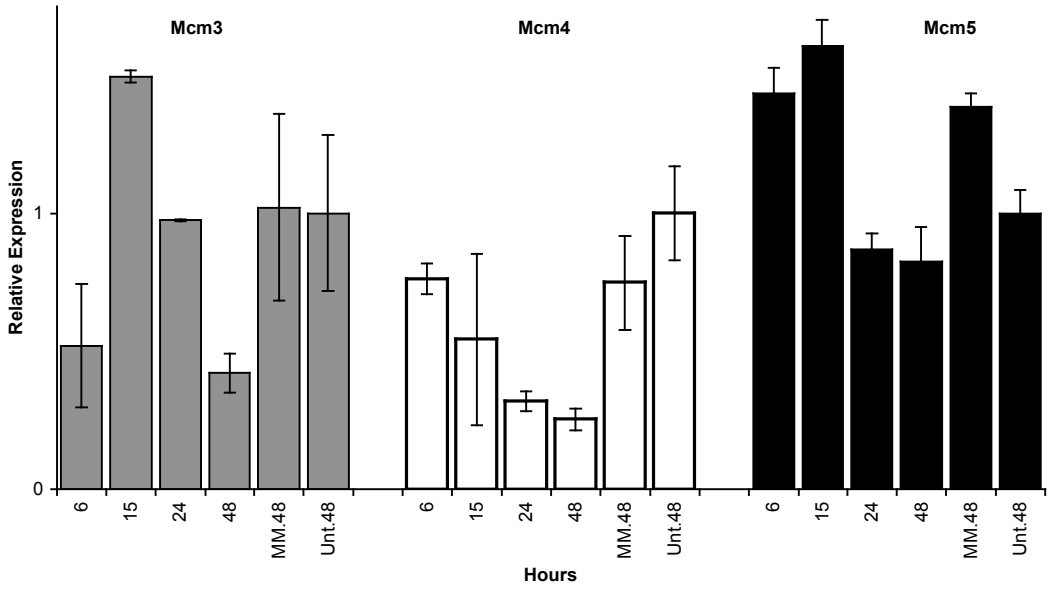


Figure 6 Real-time rtPCR (Taqman) Analysis of Mcm 3, 4 and 5 mRNA levels in IMR90 cells transfected with mcm4 GeneBloc (GB) 22174: time course. IMR90 cells were transfected in 6 cm plates as described in methods. Five hours post-transfection the cells were split 1:4 into a 6 well plate. At the time points indicated, total RNA was isolated and subject to duplex Taqman analysis containing the appropriate mcm amplicon and a β-actin internal control amplicon. mRNA expression levels were normalized to β-actin and are relative to the expression of untreated samples.

Table 3 mRNA levels in IMR90 cells transfected wit GB 22174 at 60 nM: time course

	Relative Expression	Std.Dev.	% Downregulation
Mcm3			
6 hr	0.52	0.22	**48.06**
15 hr	1.49	0.02	**(49.48)**
24 hr	0.98	0.00	**2.40**
48 hr	0.42	0.07	**57.96**
MM 48 hr	1.02	0.34	**(2.10)**
Unt	1.00	0.28	
Mcm 4			
6 hr	0.76	0.06	**23.69**
15 hr	0.54	0.31	**45.66**
24 hr	0.32	0.04	**68.14**
48 hr	0.25	0.04	**74.74**
MM 48 hr	0.75	0.17	**25.26**
Unt	1.00	0.17	
Mcm5			
6 hr	1.43	0.09	**(43.40)**
15 hr	1.61	0.10	**(60.77)**
24 hr	0.87	0.06	**12.94**
48 hr	0.83	0.12	**17.35**
MM 48 hr	1.39	0.05	**(38.51)**
Unt	1.00	0.08	

N.B.: Values in parenthesis indicate an upregulation above untransfected levels.
Percent downregulation = 100% - ((Relative Expression$_{(exp)}$/Relative Expression$_{(unt)}$) x 100)

MCM4 protein synthesis is blocked in IMR90 cells transfected with GB 22174

To determine the effects on MCM4 protein levels, asynchronously proliferating IMR90 cells were transfected in 6 cm dishes with 60 nM final concentrations of GBs 22173, 22174 or the mismatch control (MM) as described. IMR90 whole cell extracts were prepared 3 and 7 days after transfection and immunoblotted with human anti-MCM4 and β-actin. The results are presented in figure 7. MCM4 protein is undetectable in IMR90 cells transfected with GB 22174 after 3 days and remains below detectable levels up to 7 days post-transfection. Transfection with the MM control does not alter MCM4 protein levels when compared to untransfected cells. MCM4 levels in IMR90 cells transfected with GB 22173 remain detectable 7 days post transfection. Equal loading of protein was confirmed by probing for β-actin. Thus, GB 22174 provides an efficient and sustained down regulation of Mcm4 mRNA and consequently, blocks MCM4 protein synthesis. Transfection of mismatch control (MM) has no effect on the level of MCM4 protein. IMR90 cells transfected with GB 22173, even though producing a strong mRNA downregulation, did not abolish MCM4 protein even after 7 days in this experiment.

Other MCM proteins are degraded in IMR90 cells after MCM4 depletion

The kinetics of MCM protein down regulation was determined by whole cell extract immunoblotting. IMR90 cells were transfected in 10 cm dishes with 60 nM final concentrations of GB 22174 or the mismatch control (MM) as described. Whole cell extracts were prepared at 24, 48, 72 and 120 hours after transfection and immuno- blotted with human anti-MCM2, anti-MCM3, anti-MCM4, anti-MCM5 , anti-PCNA and anti-β-actin antibodies. The results are shown in figures 8 and 9.

MCM2 and MCM4 are not detected whereas MCM3 and MCM5 protein levels remain unchanged at 24 hours, Figure 8. At 48 hours, MCM3 has been abolished while MCM5 protein levels remain unchanged. At 72 hours, MCM2, MCM4 and MCM5 proteins are not detected. The proliferating cell nuclear antigen, PCNA, a proliferative marker with a chromatin bound half-life of 30 hours (ref) and a soluble half life of 275 hours, remains detectable at 72 hours. MCM proteins have been shown to have a half-life of up to 21 hours [80]. After 120 hours MCM2, MCM4, MCM5 and PCNA are undetectable, figure 9. MCM3 protein levels were not determined at 72 and 120 hours, but by extension from figure 8, are presumed to remain undetectable (see discussion). The levels of MCM2, 3, 4 and 5 in mismatch control (MM) transfected cells remain unchanged throughout the time course as compared to untransfected (Unt) cells.

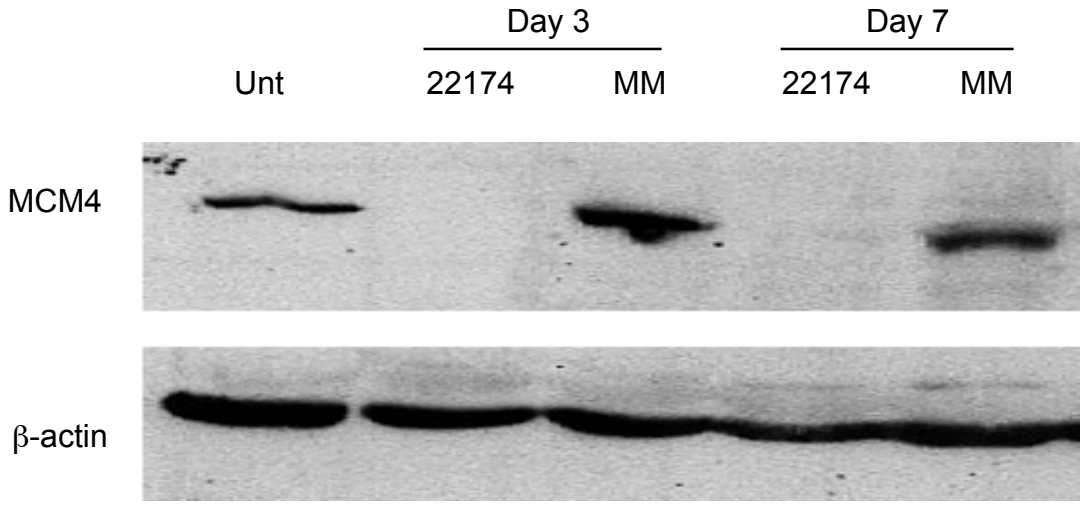


Figure 7 Mcm4 GeneBloc 22174 prevents the synthesis of MCM4 in IMR90 cells. Asynchronous IMR90 cells were plated in a 10 cm plate and transfected with 60 nM 22173 or 22174 mcm4 GeneBloc as described. At days 3 and 7, whole cell extracts were isolated and immunoblotted with human anti-MCM4. GeneBloc 22174 prevents MCM4 synthesis when compared to untreated (Unt) and control transfections (MM). β-actin serves as a loading control. Immunoblotting with anti-MCM4 was performed on whole cell extracts as described in the methods section.

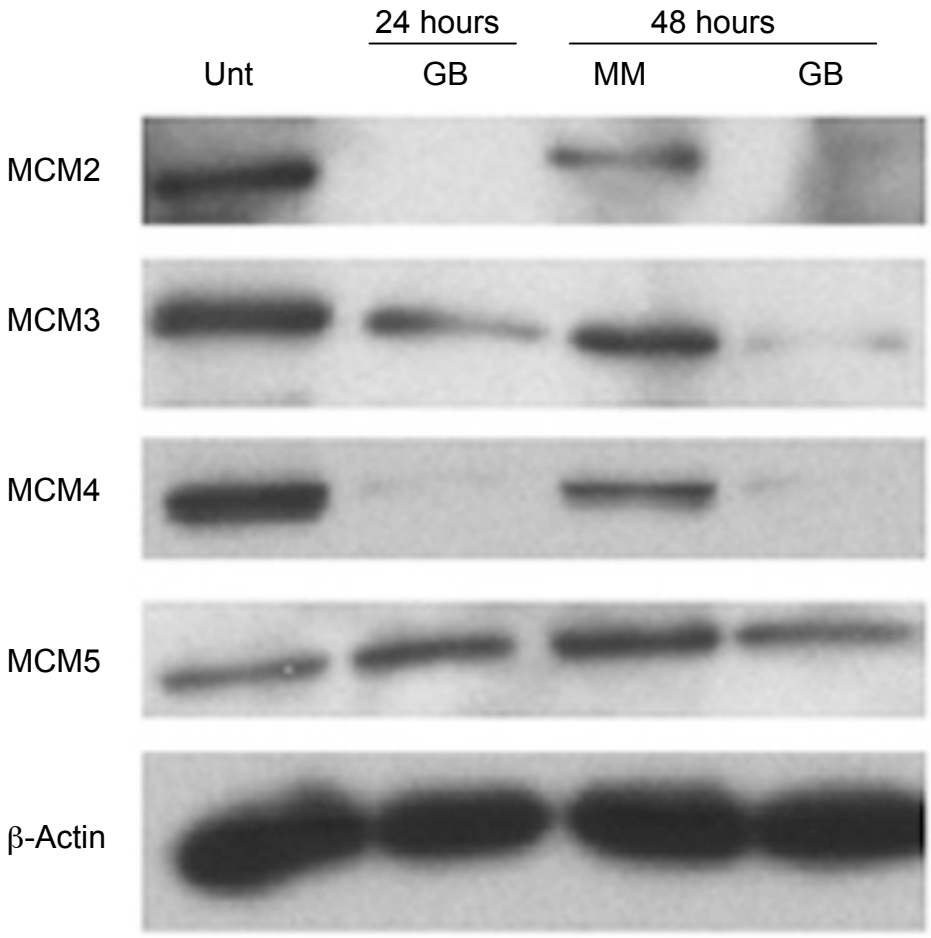


Figure 8 Effect of Mcm4 GB22174 on other MCM proteins in IMR90 cells. IMR90 cells were transfected with 60 nM 22174 mcm4 (GB) or control Genebloc (MM) in 10 cm plates as described in methods. Ablation of MCM4 also results in the rapid degradation of MCM2, a putative MCM2,4,6 sub-complex member after 24 hours. At 48 hours, MCM3 is degraded, were as MCM5 remains unchanged (see text for further discussion). Immunoblotting of whole cell extracts was performed as described in the methods section.

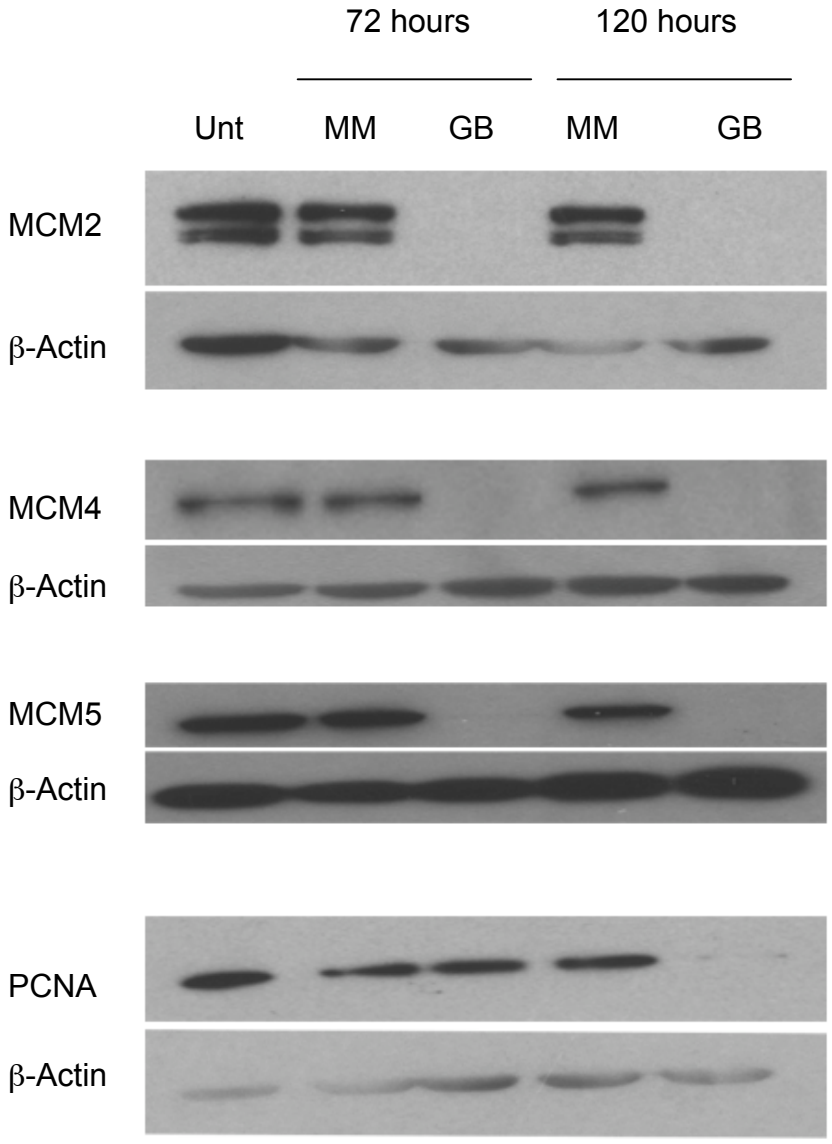


Figure 9 Effect of Mcm4 GB22174 on other MCM proteins after extended periods of GB treatment. IMR90 cells were transfected with 60 nM 22174 mcm4 (AS) or control Genebloc (MM)in 10 cm plates as described in methods. After 72 hours, ablation of MCM4 results in the complete degradation of the MCM complex although PCNA is still detectable. Only after 120 hours is PCNA below detection limits (see text for further discussion). Control transfection (MM) shows no alteration of MCM and PCNA levels when compared to untreated cells (Unt). Immunoblotting of whole cell extracts was performed as described in the methods section.

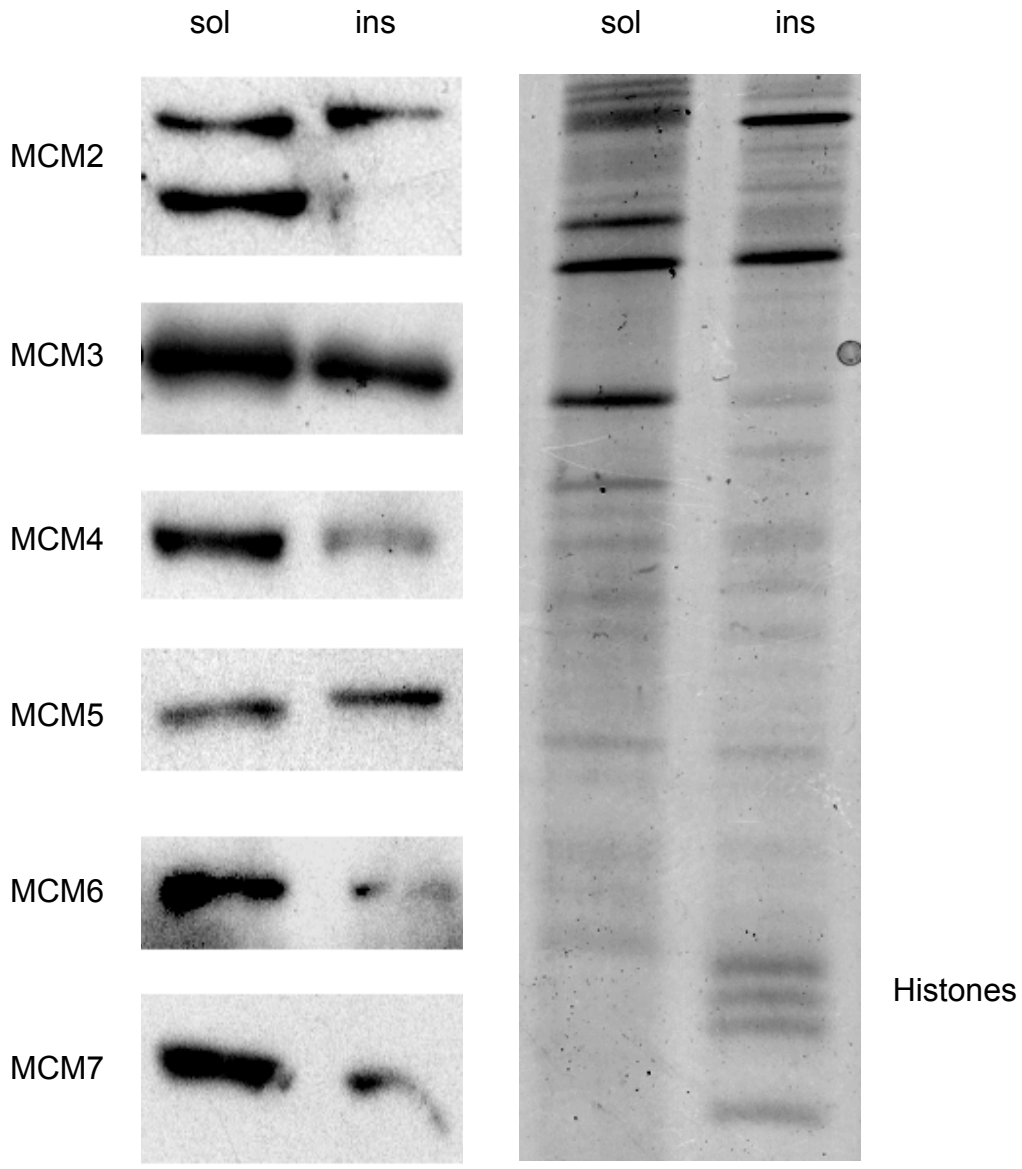


Figure 10 Biochemical analysis of soluble and insoluble MCM proteins in IMR90 cells. Fractionation and immunoblotting of cellular extracts were prepared from asynchronously IMR90 cells as described in methods. Fractionated cell extracts from approximately 2.5 x 10^4 cells was used for each immunoblot. Coommassie blue visualization of histone proteins separated through a 22% polyacrylamide gel as described in the methods section shows successful fractionation of soluble (sol) and insoluble (ins) proteins.

The subcellular localization of MCM proteins in IMR90

Fractionated cell extracts were prepared from asynchronously proliferating IMR90 cells as described in the methods section. Fractionation was performed in the presence of 100mM NaCl which preserves the status of chromatin bound proteins [48]. Proper fractionation of soluble and insoluble proteins separated through a 22% polyacrylamide gel is confirmed by the presence of histones in the insoluble fraction. Fractionated proteins were separated through an 8% polyacrylamide gel and immunoblotted with human anti-MCM antibodies 2 through 7 as described. The results are shown in figure 10.

MCM2 is present as two distinct phosphorylated forms. The slower migrating, hypophosphorylated species is found at approximately equal levels in both soluble and insoluble fractions whereas the faster moving hyperphosphorylated species is found exclusively in the soluble fraction [51]. MCM3 is found in both fractions with a slight increase in the soluble fraction. Although not immediately obvious, closer inspection reveals the presence of at least two distinct phosphorylated MCM4 species which are found in both fractions [101]. However, the majority of both phosphorylated MCM4 species is found in the soluble fraction. MCM5 is found at approximately equal levels in both fractions. The majority of MCM6 and MCM7, as with MCM4, is found in the soluble fraction.

The subcellular localization of MCM is altered in IMR90 cells depleted of MCM4

To determine the effects on MCM localization in response to down-regulation of MCM4, IMR90 cells were plated in 6 cm dishes, transfected with 60 nM final concentrations of GB 22174 or the mismatch control (MM) and split 1:2 into 3.5 cm dish 5 hours after transfection. Fractionated extracts were prepared at 15 and 30 hours post transfection, separated through an 8% polyacrylamide gel and immunoblotted with human anti-MCM2, anti-MCM3, anti-MCM4 and anti-MCM5. Proper fractionation of soluble and insoluble proteins separated through a 22%

polyacrylamide gel is confirmed by the presence of histones in the insoluble fraction. The results are presented in figure 11.

The localization of MCM2, MCM3, MCM4 and MCM5 in mismatch control (MM) transfected cells remains unchanged after 30 hours and agrees with the data presented in figure 10. Soluble MCM4 protein is virtually undetectable whereas insoluble chromatin bound MCM4 is still detectable 15 hours post transfection. MCM2 is present in both fractions at reduced levels and like MCM4 at 30 hours is released from the chromatin. After 30 hours, MCM4 seems to have been solubilized and is no longer found on the chromatin. As with MCM4, the levels of soluble and chromatin bound MCM2 are decreased after 15 hours. Interestingly, after 30 hours, only soluble MCM2 is detected.

Although previous transfections have consistantly resulted in a complete disappearence of MCM4 after 24 hours, this is not the case in the experiment presented in figure 11. This may be due to a sub-optimal (in this case, too high cellular density which would decrease transfection efficiency) cellular concentration prior to transfection or perhaps a residual effect of the trypsinization and replating of transfected cells. The efficiency of MCM4 down regulation in cells split 5 hours after transfection was tested by immunoblotting (data not shown). The kinetics of down regulation was indeed delayed such that after 48 hours, MCM4 levels were drastically reduced but still detectable. After 72 hours, MCM4 protein was undetectable. Thus, ablation of MCM4 results in an initial destruction of soluble sub-complexes followed by a solubilization of chromatin bound MCM2 and MCM4 subunits. MCM3 and MCM5 subunits are found at approximately equal levels in both fractions at 30 hours which agrees with figure 10 and the kinetics of subunit degradation found in whole cell extracts as presented in figure 8 (see also discussion).

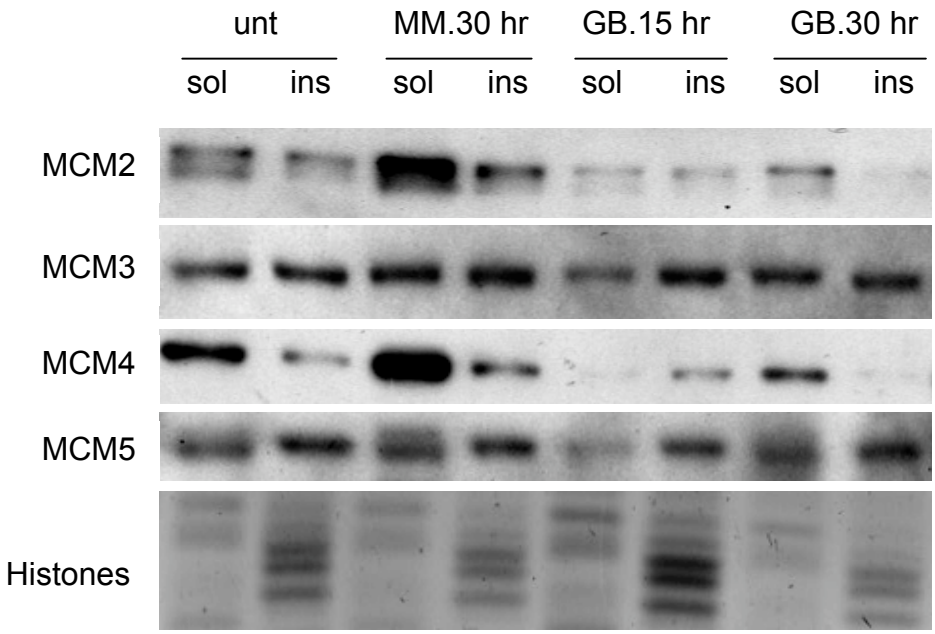


Figure 11 Chromatin bound MCM proteins are solubilized after treatment with Mcm4 GB22174. Asynchronously proliferating IMR90 cells were plated 6 cm dishes, transfected with 60 nM GB 22174 or control GB (MM) and split 5 hours after transfection 1:2 into 3.5 cm dishes. At 15 and 30 hours, fractionated proteins were isolated and immunoblotted as described. Histone proteins visualization by coomassie staining confirmed proper fractionation. Fifteen hours post-transfection, soluble MCM4 is degraded rapidly followed by its translocation from the chromatin bound fraction at 30 hours. MCM2 levels are decreased but detectable at 15 hours. MCM2 is translocated from the chromatin bound fraction at 30 hours. MCM3 and MCM5 soluble fractions are slightly after 30 hours, while the chromatin bound levels remain unchanged.

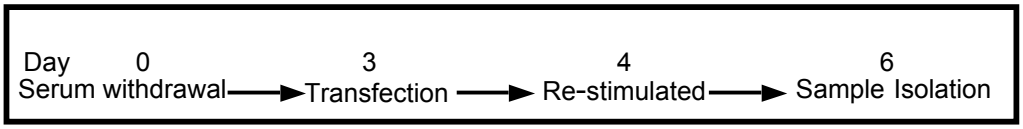
Transfection of serum starved IMR90 cells

Serum withdrawal of human primary fibroblasts results in the disappearance of MCM proteins after 48 hours with a concurrent cessation of proliferation [79, 81]. Within 20 hours after restimulation, MCM proteins are resynthesized and the cells begin to re-enter the cell cycle. To determine if subunits of the MCM complex are resynthesized in the absence of MCM4, IMR90 cells were starved of serum for 4 days and restimulated in the presence of Mcm4 GeneBloc. The schematic of this experiment is presented in Figure 12. Asynchronously proliferating IMR90 cells were plated in 6 cm dishes as described. After the cells had attached, they were thoroughly washed with PBS to remove all traces of FCS and cultured for 4 days in the absence of serum. Three days after serum withdrawal, the cells were transfected as described with 60 nM GB22174 or the mismatch control (MM). Twenty four hours after transfection, serum (10% final) was added directly to the GB containing growth medium. Whole cell extracts were prepared at 20 and 40 hours after restimulation and immunoblotted with human anti-MCM2 and MCM4 as described.

MCM2 and MCM4 proteins are barely detectable in serum starved cells after four days (figure 12). DNA synthesis was monitored by BrdU incorporation in serum starved IMR90 cells by flow cytometry and shows a low level of DNA synthesis (see section results:cell cycle analysis). Thus, a small population of cells are uneffected by serum withdrawal and continue to synthesize or down regulate MCM proteins slower than the majority of cells in the culture. MCM2 and MCM4 protein levels return to those found in asynchronously proliferating cells 40 hours after serum restimulation. MCM2 and MCM4 proteins in serum restimulated cells transfected with the control mismatch (MM) are resynthesized to presumably untransfected, asynchronous levels after 40 hours. Whole cell extracts from the MM treated cells were underloaded by about 50% as determined by comparison of β-actin levels with untransfected cells, thus by inference, the MCM2 and 4 protein levels are about equal with untransfected levels. Cells restimulated in the

presence of GB 22174, fail to resynthesize MCM2 and MCM4 proteins after 40 hours. Thus, MCM2 protein is not resynthesized in the absence of MCM4 protein.

A



B

	GO/G1	S	G2/M	BrdU
Asynchronous	81.4	9.3	11.1	+++
Serum Starved	90.8	2.7	6.5	+
Re-stimulated	69.0	11.5	19.7	+++
Re-stimulated + MM	65.9	9.8	24.5	+++
Re-stimulated + AS	83.0	6.0	11.1	+

C

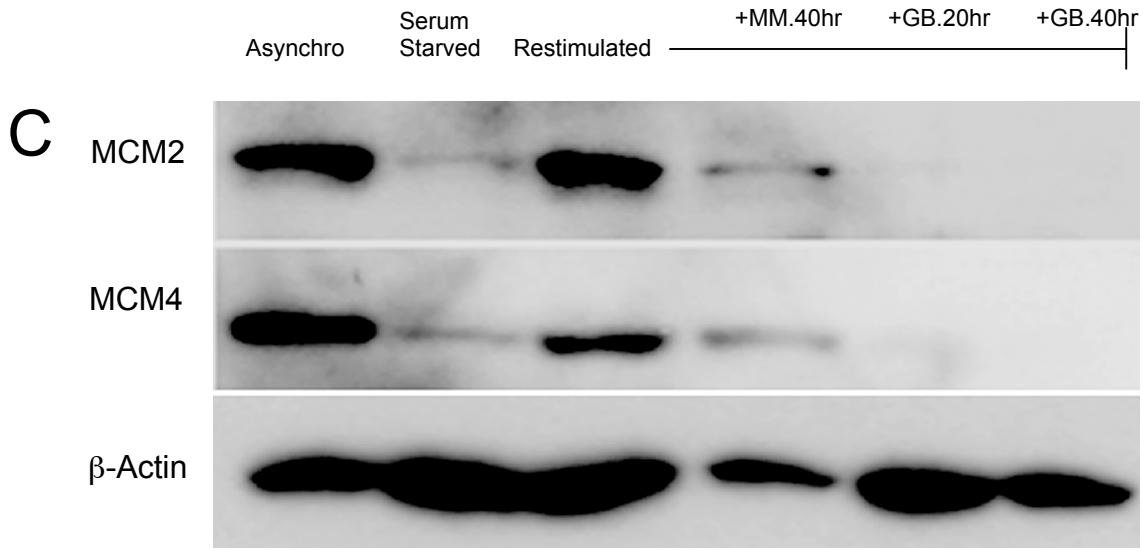


Figure 12 Serum starved IMR90 cells transfected with mcm4 GeneBloc 22174 (GB) fail to synthesize DNA upon restimulation. (A) Schematic diagram of starvation experiment. 3 days after serum starvation, IMR90 cell were transfected with either mcm4 (GB) or control (MM) GeneBlocs as previously described (see methods). 24 hours after transfection, the cell were restimulated by the addition of serum to the cell culture medium. **(B)** Table showing the percentage of cells in G0/G1, S and M phases and BrdU incorporation as determined by flow cytometry of cells from the experiment presented in (A). **(C)** Immunoblot of whole cell extracts from cells treated with Geneblocs as described in (A) shows a failure to synthesize MCM after restimulation. Immunochemistry was performed as described previously (see methods).

Cellular proliferation of IMR90 cells is drastically reduced in the absence of MCM

The proliferative capacity of IMR90 cells transfected with GB 22174 was determined by counting the number of population doublings over the course of 96 hours. Asynchronously proliferating IMR90 cells were transfected as described with a final concentration of 60 nM GB 22174 or the mismatch control (MM) and split 1:4 into 3.5 cm dishes after 5 hours. At the indicated time points, the cells were trypsinized, stained with trypan blue to determine the number of viable cells and counted in a Neubauer haemocytometer. The results are presented in figure 13.

Over the course of 96 hours, untransfected (unt) and mismatch (MM) transfected IMR90 cells reach a density of approximately 1.75×10^5 from a starting density of approximately 5.0×10^4 cells which is slightly less than 3 population doublings. After 72 hours, the cells approached 100% confluency and proliferation slowed through contact inhibition. In stark contrast, IMR90 cells transfected with GB 22174 were unable to complete more than a single population doubling within 96 hours. Light microscopy at 10x magnification acquired prior to counting revealed the near confluent growth of untransfected and mismatch control transfected IMR90 cells at 72 hours, figure 14. In contrast, the density of IMR90 cells transfected with GB 22174 remained low paralleling the numbers obtained by counting. The decrease in proliferation of GB 22174 transfected IMR90 cells as determined by cell number was not a result of significant cellular death, as the percentage of blue stained cells for samples at everytime point was never above 10% of the total number of cells counted (data not shown).

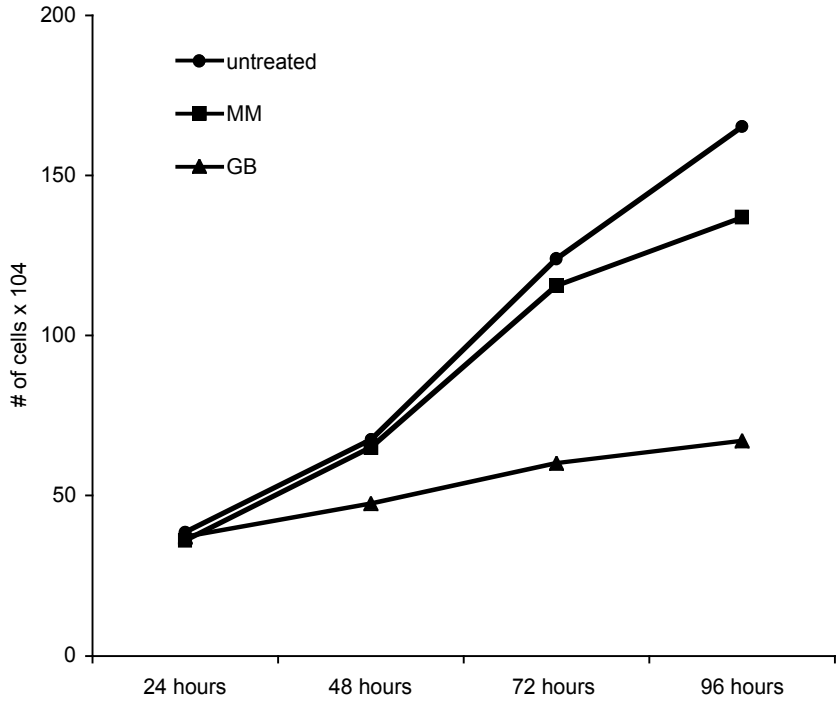


Figure 13 IMR90 cells transfected with mcm4 GeneBloc 22174 exhibit drastically reduced proliferation. IMR90 cells were transfected 6 cm plates as described in methods. Five hours post-transfection the cells were split 1:4 into a 3.5 cm dishes. At the time points indicated, the cells were harvested, stained with trypan blue and counted on a Neubauer Haemocytometer. The number of blue stained cells was subtracted from total cells and plotted. In contrast to untransfected (Unt) and control transfected (MM) cells which grow to conlfuence after approximately three population doublings, IMR90 cells transfected with mcm4 GeneBloc 22174 (GB) are only able to complete one population doubling within the same time. In no time point did the number of dead or blue stained cells equal more than 10% of the total cells counted (blue, dead cells plus white, viable cells)

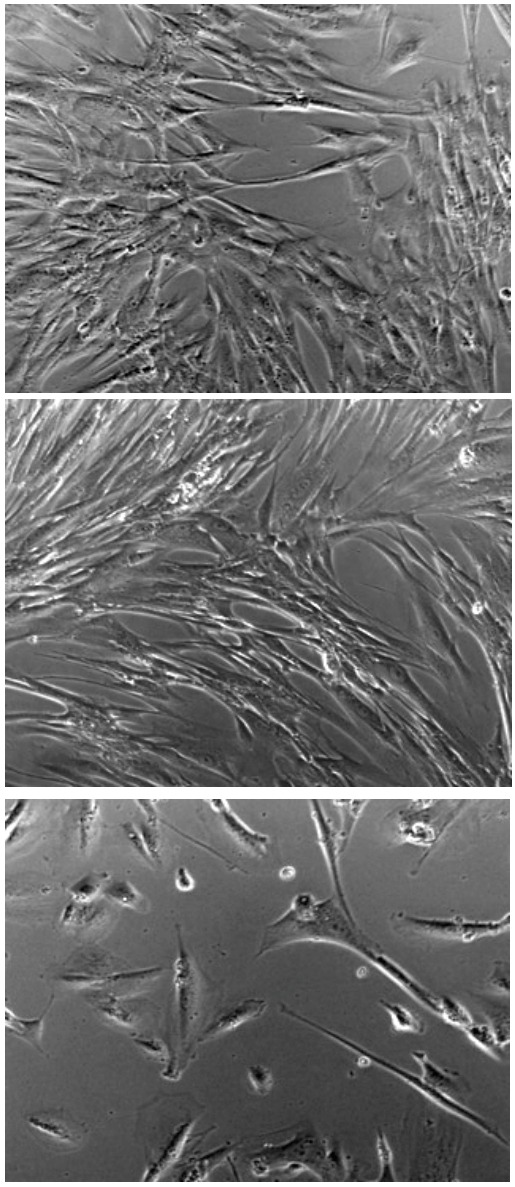


Figure 14 Light microscopy reveals the inability of Mcm4 GB 22174 transfected IMR90 cells to proliferate. IMR90 cells in 6 cm dishes were transfected as described in method. Five hours post-transfection the cells were split 1:4 into a 6 well plate. At the 72 hours, the cells were photographed with 10x objective prior to trypan blue staining and counting for the proliferation curve (see figure 13). In comparison to untransfected (Unt) and control (MM) mismatch transfected cells, AS treated cells were unable to achieve confluence and exhibit a flattened morphology.

The cell cycle distribution of MCM4 depleted IMR90 cells is drastically altered

The cell cycle distribution of GB 22174 transfected IMR90 cells was determined by flow cytometry of propidium iodide (PI) stained cells to measure DNA content. Cells in G0/G1 have a 2C DNA content which increases to 4C at the end of S-phase. PI intercalates into double stranded DNA and fluorescent emission increases in proportion to the amount of DNA. Asynchronously proliferating IMR90 cells were transfected as described in 6 cm dishes with a final concentration of 60 nM GB 22174 or the mismatch control (MM) and split 1:4 into 3.5 cm dishes after 5 hours. At the indicated time points, the cell were trypsinized, washed and fixed in 70% ethanol as described. Prior to analysis the cells were washed 2x in PBS, resuspended in PBS containing propidium iodide (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. Histogram graphs of Counts (number of cells, y-axis) versus FL2-A (DNA content, x-axis) were generated and are shown in figures 15 and 16.

The cell cycle profile of mismatch (MM) control transfected IMR90 cells with the exception of a slight increase in the number of cells in the G1/S peak, remains unaltered in comparison to untranfected cells at 72 and 120 hours post transfection. In contrast, GB 22174 transfected IMR90 cells accumulate at G1/S-phase boundary and G2/M phase peak after 72 hours. After 120 hours, there is an increase in the number of cells in S and G2/M phases which is either mitochondrial replication or a further traversal into the respective stages. While the number of cells in the G2/M phase peak have decreased in number, it cannot be determined if this represents a traversal out of M phase or again mitochondrial replication (see next sections and discussion). A total of 20000 ungated events were acquired for each sample and the number of cells shown under each histogram graph represents at least 75% of all acquired events.

The absence of massive cell death in transfected IRM90 cells confirms the non-toxicity of both the GB (22174 and the MM) and the lipid NC147 after 120 hours continuous exposure. It is further confirmed that a single transfection is sufficient to produce a sustained downregulation of MCM4 protein synthesis resulting in a drastic alteration of proliferation and the cell cycle distribution.

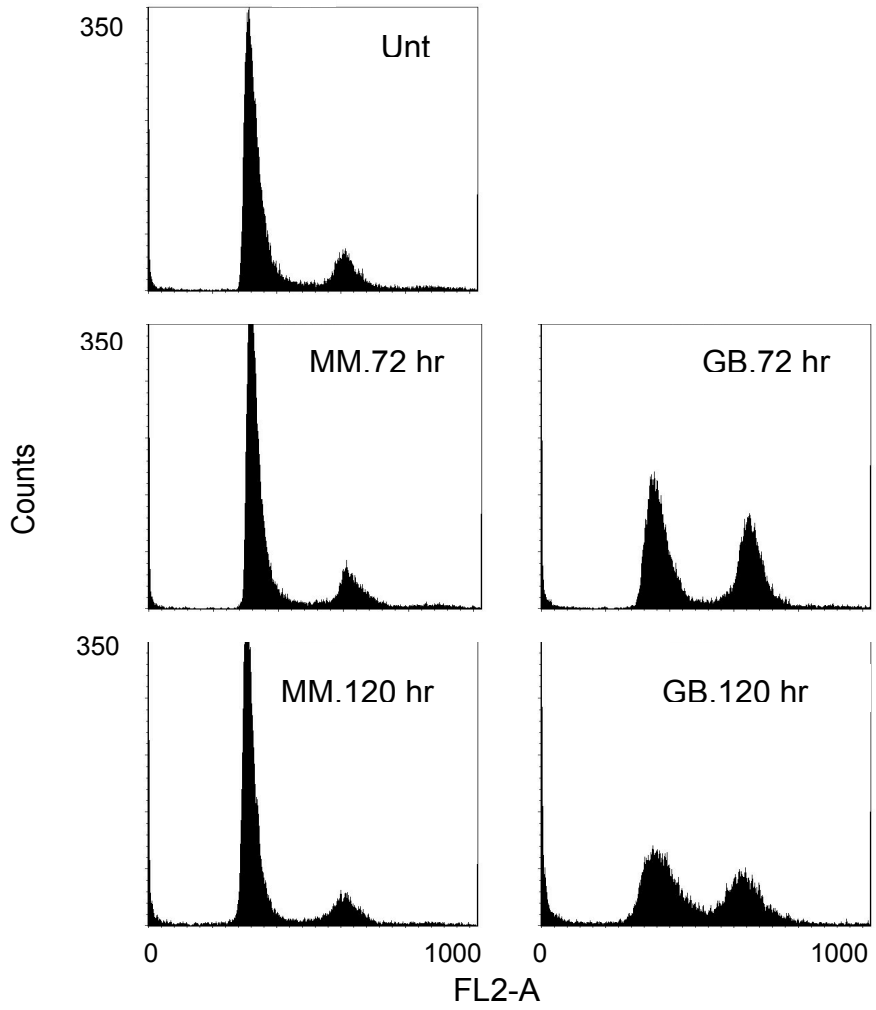


Figure 15 IMR90 cells treated with mcm 4 Genebloc 22174 (GB) exhibit drastically altered cell cycle profiles. IMR90 cells were transfected with 60nM mcm4 GeneBloc 22174 (GB) or control (MM), in 6 cm plates as described in methods. After 72 hours, the cells have accumulated at the G1/S phase boundary indicating a failure to traverse S phase. The cells also accumulate in the G2/M peak and taken together with figure 16, are stalled in G2. After 120 hours, the cells appear to have traversed into, but do not complete S phase. Preparation of cells for flow cytometery is described in the methods section. Prior to analysis, the cells were washed twice in PBS, resuspended in PI/RNase solution analyzed with a Becton Dickinson FACS calibur (see methods). Histograms of numbers of cells,(counts,linear) versus FL2-A (PI, DNA content, Linear), of 20,000 ungated events were acquired and the graphs above represent at least 18,500 cells for each sample indicating that cell death is not responsable for the cell cycle profile alterations.

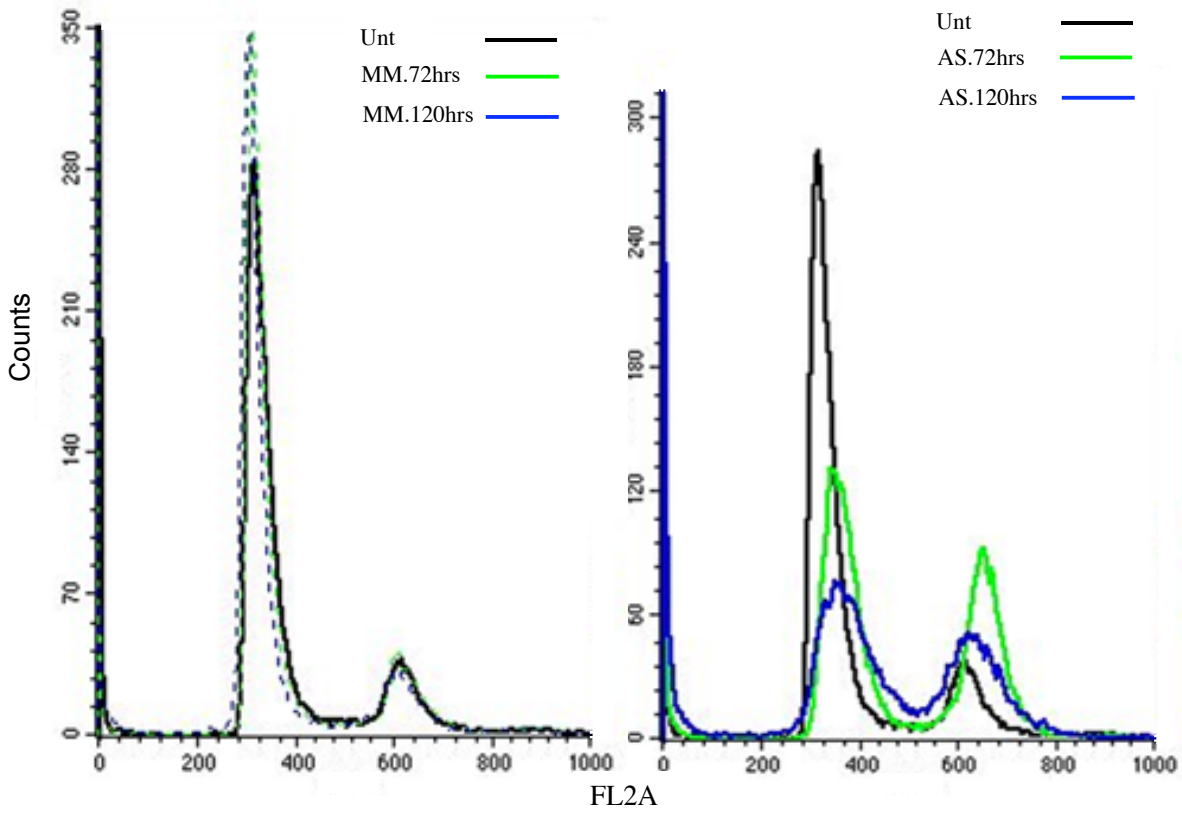


Figure 16 IMR90 cells treated with mcm 4 Genebloc 22174 (AS) exhibit drastically altered cell cycle profiles:overlay. see legend for figure 15.

IMR90 cells transfected with GB22174 fail to synthesize DNA

Asynchronously proliferating IMR90 cells were plated in 6 cm dishes and transfected with either GB 22174 or the mismatch control (MM) as described. To monitor DNA synthesis, bromodeoxyuridine (BrdU, 10μM final) was added directly to the dishes and incubated for 3 hours prior to harvesting at 48 hours post-transfection. Untransfected IMR90 cells without BrdU incorporation were incubated with the FITC conjugate anti-BrdU antibody as a control. The cells were fixed, incubated with FITC conjugate anti-BrdU, resuspended in PBS containing propidium iodide and DNase-free RNase and analyzed by dual color flow cytometer as described. Dot-blot graphs of DNA content (FL2-A, x-axis, linear amplification) versus BrdU reactivity (FL1-H, y-axis, logorithmic amplification) were generated and are shown in figure 17.

Untransfected IMR90 cells, not incubated with BrdU, but probed with FITC conjugated anti-BrdU exhibit no FITC reactivity as shown by a lack of cells above 10^2 along the y-axis. Untransfected cells, incubated with BrdU for 3 hours show a significant population of BrdU positive, S-phase cells. In contrast, MCM4 GeneBloc transfected cells show a drastic reduction in the number of BrdU positive, S-Phase cells. The small population of BrdU positive transfected cells may represent a population of untransfected cells or in corrolation with the finding in *Xenopus*, S-phase continues at a reduced speed with reduced MCM complex levels [102, 103]. Thus, DNA synthesis is drastically reduced in human primary fibroblasts when mcm gene expression is blocked. BrdU incorporation in mismatch transfected cells is indistinguishable from untransfected cells (data not shown and see figure 12).

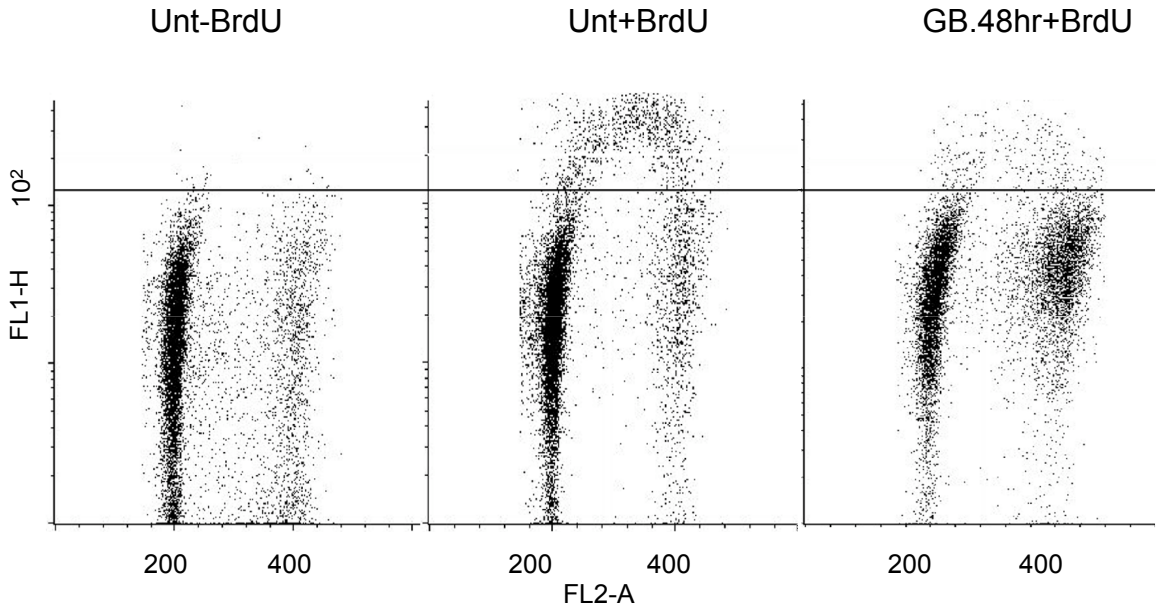


Figure 17 IMR90 cells treated with mcm 4 Genebloc 22174 (GB) show a substantial reduction of BrdU incorporation after 48 hours. IMR90 cells transfected in 6 cm plates as described in methods. 10μM BrdU was added to the growth medium and allowed to incorporate for 3 hours prior to harvesting at 48 hours post-transfection. Prior to flourescent flow cytometry, fixed cells were immunoreacted with FITC conjugated anti-BrdU and treated with PI/RNase solution as described in the methods section. Dot blot graphs of FL1-H (BrdU reactivity, logrithmic) versus FL2-A (DNA content, linear) were acquired by flourescent flow cytometry with a Becton-Dickinson FACS Calibur as described. Unt-BrdU, control untransfected cells without BrdU incubation. Unt+BrdU, untransfected cells incubated with BrdU. GB.48hr+BrdU, GB transfected cells with BrdU incubation. BrdU positive cells are above 10^2.

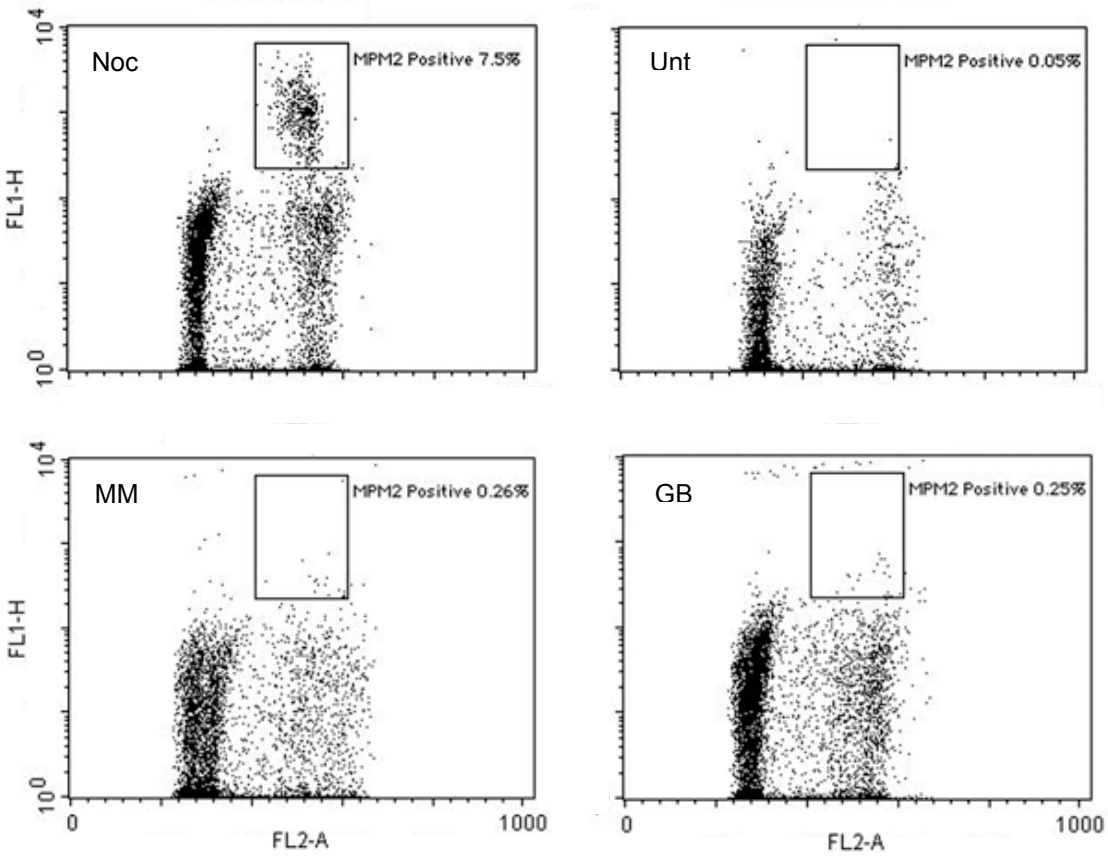


Figure 18 Mcm4 GeneBloc 22174 (GB) IMR90 transfected cells do not accumulate in mitosis as determined by MPM2 immunoreactivity. IMR90 cells were transfected in 6 cm plates as described in methods. 10μM Nocadozal was added to untransfected cells 18 hours prior to harvesting. 48 hours after transfection, the cells were harvested by trypsinization, washed in PBS, fixed in 70% ethanol and stored. Prior to analysis, the cells were immunoreacted with MPM2 antibody as described and resuspended in PI/RNase solution analyzed flourescent flow cytometry through a Becton Dickinson FACS calibur (see methods). Histograms were obtained by plotting FL1-H (FITC, MPM2 positive, logorithmic) versus FL2-A (PI, DNA content, linear). The percentage of MPM2 positve cells are shown within the boxes of each histogram. Unt, untreated cells, Noc, 10mM Nocadozole treated cells, MM, mismatch control transfected cells, GB, mcm4 22174 antisense transfected cells.

IMR90 cells depleted of MCM4 accumulate in G2

To determine if the accumulation of mcm4 GeneBloc transfected IMR90 cells at the G2/M phase peak represent an accumulation in mitosis, MPM2 reactivity was investigated. MPM2 antibody specifically recognizes a phosphorylated epitope found in phosphoproteins such as MAP2, HSP70, cdc25 and DNA topoisomerase IIα, most of which are phosporylated at the onset of mitosis [104]. MPM2 has been used successfully to identify mitotic cells by fluorescent activated flow cytometry . Asynchronously proliferating IMR90 cells were plated in 6 cm dishes and transfected with 60 nM concentrations of GB 22174 or the mismatch control (MM). A positive control for MPM2 of untransfected IMR90 cells treated with 10 µM Nocodazole for 18 hours was included. Nocodazole specifically inhibits the formation of spindle fibers arresting the cells in mitosis. The cells were fixed, incubated with monoclonal anti-MPM2, incubated with FITC conjugated goat anti-mouse, resuspended in a solution containing propidium iodide and DNase-free RNase and analysed on dual color flow cytometer as described. Dot-blot graphs of DNA content (FL2-A, x-axis, linear amplification) versus MPM2 reactivity (FL1-H, y-axis, logorithmic amplification) were generated and are shown in figures 15 and 16.

An 18 hour treatment of untransfected IMR90 cells with 10 µM Nocodazole results in ~7.5% MPM2 positive cells. Untransfected IMR90 cells not treated with nocodazole exhibit little MPM2 reactivity (0,05% positive cells). Mismatch control and MCM4 Genebloc transfected both showed approximately the same percentage of MPM2 reactivity (0,25% positive cells), and may be an artifact of the transfection and not related to any decrease in MCM levels. Thus, IMR90 cells transfected with MCM4 GeneBlocs do not arrest in mitosis as determined by MPM2 reactivity. It must be concluded from these experiments that the accumulation of cells in the G2/M phase peak as determined by flow cytometry (figures 16 and 16) and the absence of MPM2 reactivity, is indicitive of a G2 arrest or representative of the incomplete firing of late origins.

Hoechst staining of IMR90 cells transfected with GB22174

Hoechst 33352 staining of IMR90 cell nuclei was performed to determine if IMR90 cells accumulate in mitosis after transfection with MCM4 GeneBlocs. Untransfected cells were grown on cover slips, stained with Hoechst Dye 33342 and photographs were made through a fluorescent light microscope as described. Figure 19 (A,B,C) shows three stages of mitosis in asynchronously proliferating IMR90 cells: A. Prometaphase is visualized as the nuclear membrane has broken down and the chromosomes are in a diffuse state, B. Alignment of chromatids along the metaphase dish during metaphase with visible microtubules, C. the separation of chromatids during anaphase/telophase with the appearance of a definite boarder around the chromosomes indicating the beginning of telophase. Figure 19 (D) shows cells in various interphase stages.

Hoechst 33342 staining was performed on IMR90 cells transfected with mcm4 GeneBloc 22174 in 6 cm dishes containing sterile glass coverslips. Cells were stained with Hoechst 33342 and photographed through a 10x objective. Figure 20 shows the results. There is little difference in nuclear morphology between untransfected and mismatch transfected IMR90 cells and no obvious mitotic events are present 72 hours post transfection. Both untreated and mismatch transfected cells had grown to a high density, in stark contrast to the low density of mcm4 GeneBloc transfected cells. There is no detectable accumulation of mcm4 GeneBloc transfected IMR90 cells in any mitotic stages. However, there seems to be increase in the proportion of larger, diffuse nuclei which could possibly indicate G2 phase. Taken together with a lack of MPM2 reactivity as presented in figure 18, it can be concluded that the accumulation of mcm4 GeneBloc transfected IMR90 cells in the G2/M phase peak as presented in figure 20 represents cells stalled in G2 (see discussion).

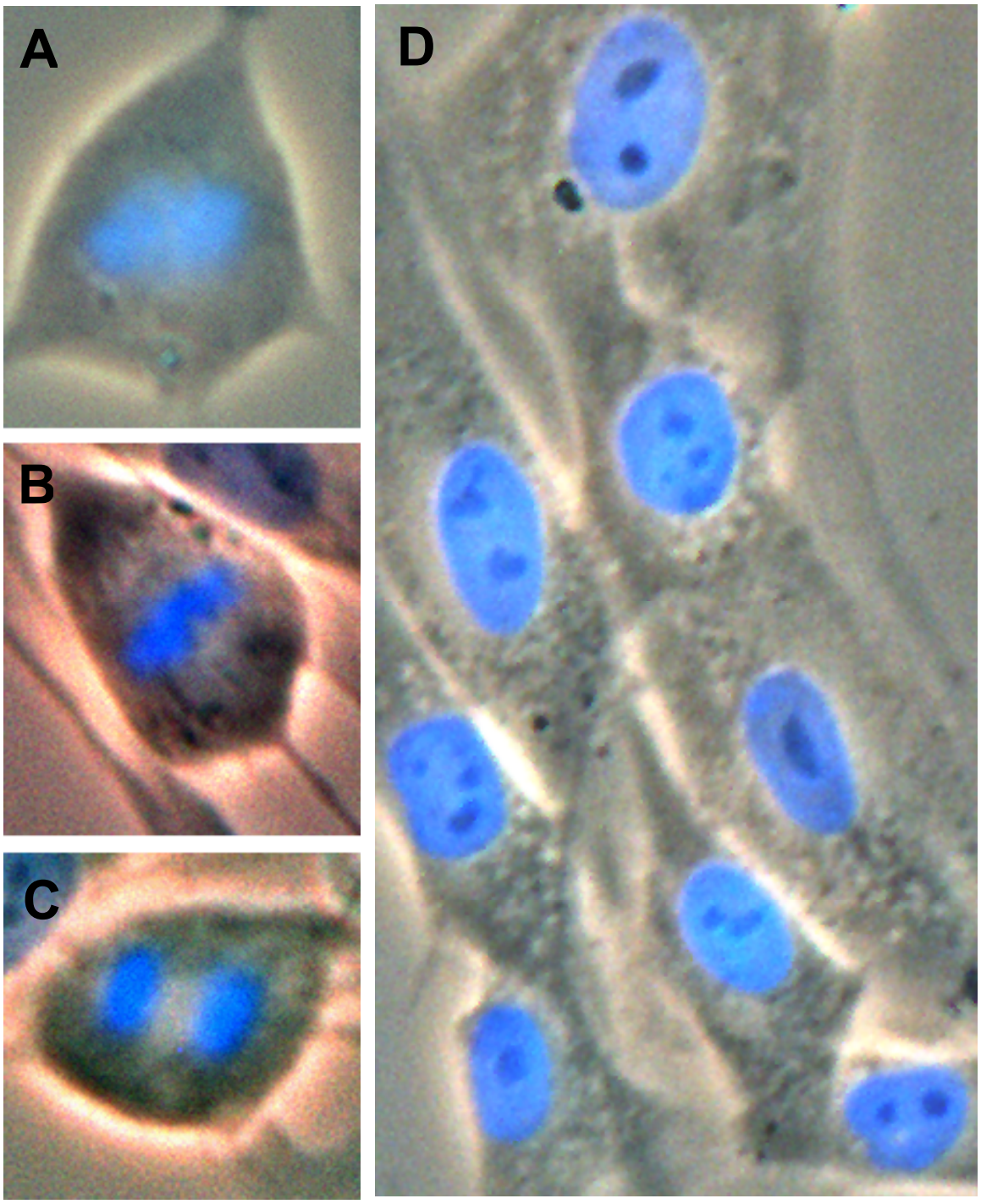


Figure 19 Hoechst staining of asynchronously proliferating IMR90 cells. IMR90 cells were grown on coverslips, fixed with 4% paraformaldehyde and stained with 1 µg/ml Hoechst dye and mounted on glass slides. The pictures were acquired through flourescent microscopy with a 10x objective. (A) prometaphase (B) Metaphase (C) Anaphase/Telophase (D) Interphase population

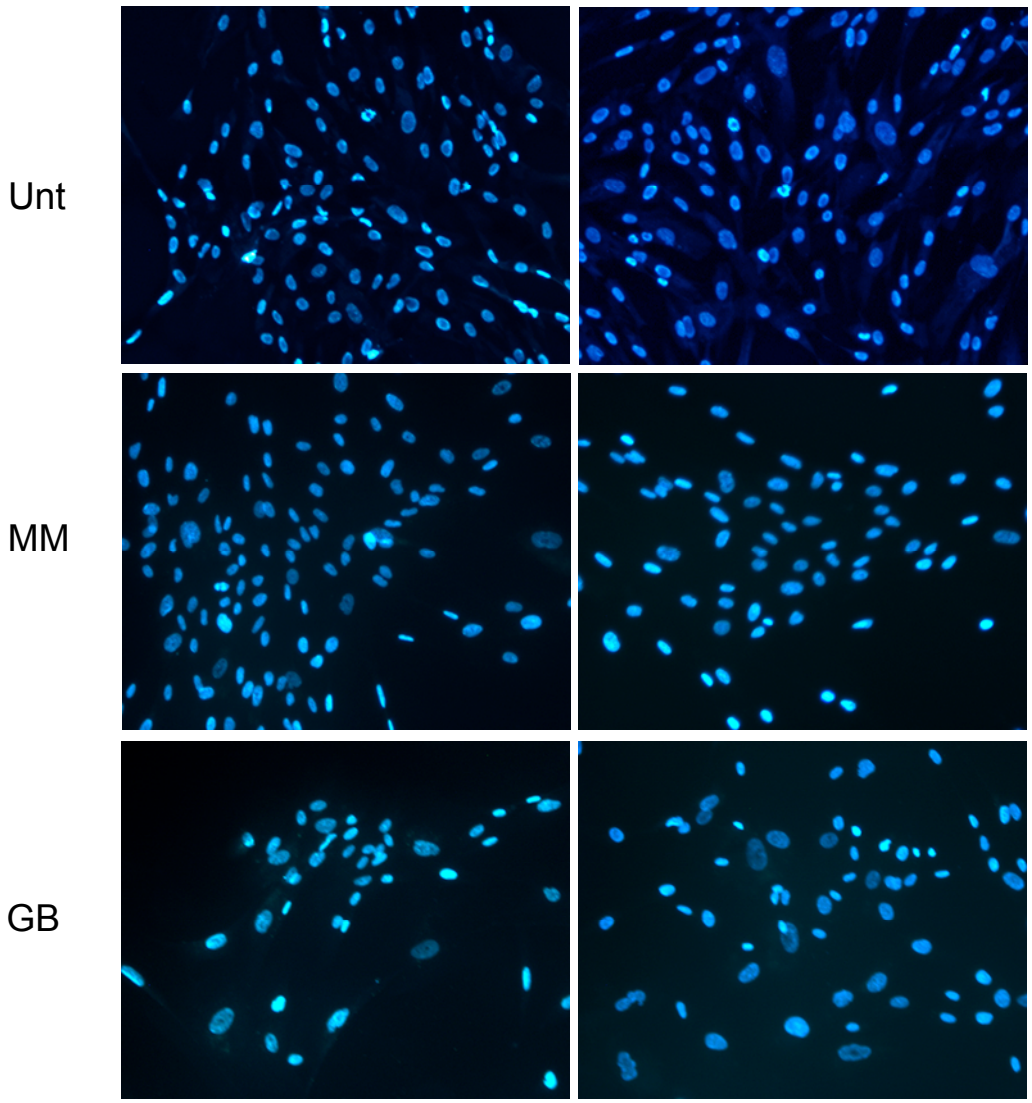


Figure 20 Hoechst staining of IMR90 cells transfected with mcm 4 GeneBlocs. IMR90 cells were transfected with 60 nM mcm4 GeneBloc 22174 (GB) in 6 cm plates containing glass coverslips as described in methods. The coverslips were removed and the cells fixed in 4% paraformaldehyde and stained with Hoechst dye 72 hours after transfection as described in the methods section. The coverslips were mounted on glass slides and photographed with a 4x objective a flourescent microscope. For each sample, two pictures where acquired. Unt, untreated MM, mismatch GB, GeneBloc.