Materials and Methods

Embryo culture and injections

Embryos of *Xenopus laevis* were obtained by artificial fertilisation. They were maintained in 10% normal amphibian medium (NAM). *Xenopus* embryos were injected at the one cell stage with antisense morpholino oligonucleotides (MOs) (dissolved in water) obtained from GeneTools, LLC or with sense RNA obtained by *in vitro* transcription.

Morpholino oligonucleotides are listed below:

Drf1MO	5'-GCAGAACAGAGATCACACTGGCCAT-3'
TreslinMO	5'-TGTGACGGTAATGGCCCATACGATT-3'
Cut5MO	5'-GGCTCGTTTTCACTCGAAGCCATTT-3'
RecQ4MO	5'-CCTTAACCTCATTATAGCGCTCCAT-3'
Cdc6A	5'-CTGGTGCTTGGCATGGCTGCTTGTC-3'
Cdc6B	5'-AATTCAGTCAGAAATAACCAGGCTC-3'

The control morpholino (coMO) is the standard control morpholino from Gene Tools.

cDNA preparation and qRT PCR

Total RNA was isolated from *Xenopus* embryos using the TriPure reagent (Invitrogen) according to the manufacturer's instructions, followed by a LiCl precipitation. mRNA expression was validated by quantitative RT-PCR using the LightCycler 480 (Roche). Reverse transcription was carried out using the Transcriptor First Strand cDNA kit (Roche) followed by quantitative real-time PCR using a LightCycler 480 SYBR Green I master kit (Roche) following the manufacturer's instructions. Primer sequences are listed below:

CUT5FW	5'-AAGACAGTCCAGGCAGGCTA-3'
CUT5REV	5'-CGAATTCACAGGCTTCCAAT-3'
DRF1FW	5'-TAAAATGCCTCCTCCAATCG-3'
DRF1REV	5'-GCACCGTAGGTCAGGAATGT-3'
RECQ4FW	5'-CCCGGTGCATCAGTTTAGTT-3'
RECQ4REV	5'-TTGCCCTGTTTCACCTTTTC-3'
TRESLINFW	5'-TCTGGGAAATGTGTGTTCCA-3'
TRESLINREV	5'-TCATTGAAGGGGCTTTCATC-3'
CDT1FW	5'-AATCCACAGCAACGACTTCC-3'
CDT1REV	5'-CGCAGGATTCTAGCCAGTTC-3'
ORC2FW	5'-TTATCAGCAATGTCGCGAAG-3'
ORC2REV	5'-CACTCCATCCATGCCTTTTT-3'
ORC3FW	5'-TGAGTTGCTCCGACAAGATG-3'
ORC3REV	5'-GTGGGGCTGCATTAAGATGT-3'
MCM2FW	5'-GGATGAGATGTTGGCAAGGT-3'
MCM2REV	5'-ACAGTAATTGGGATGCTGCC-3'
MCM3FW	5'-GAGCACAGATGTTGCAAGGA-3'
MCM3REV	5'-GCATACTGCACAAGCTCCAA-3'

MCM4FW	5'-CCTCTCCATTGCTAAGGCTG-3'
MCM4REV	5'-GGTTTTCTTGGGGTTCCATT-3'
MCM5FW	5'-TACTGCAGAGCGAAATGTGG-3'
MCM5REV	5'-CGGTCTCTGTAGCAAAAGGC-3'
MCM6FW	5'-GATCAAGGCGAGGATGAAAA-3'
MCM6REV	5'-AATCCAGCATTTGAGGATGG-3'
PSF1FW	5'-GGTCTTTGGGTGGTGAAGAA-3'
PSF1REV	5'-CTTGTCGGATCAGTTGCTCA-3'
PSF2FW	5'-ATGTCGAATTGTCCCTCCAG-3'
PSF2REV	5'-GAGCCGCAGTTTTGCTATTC-3'
PSF3FW	5'-CACTATGGTTGGCGAAAGGT-3'
PSF3REV	5'-AGTGAGGTCCCATCTTGTGC-3'
SLD5FW	5'-ACCTGTCCCCTGAGGAGTTT-3'
SLD5REV	5'-CTCTGGTTCCACCAGGATGT-3'
CDC45FW	5'-CGTTCTTCTCCTGGTTGCTC-3'
CDC45REV	5"-TTTGCACCGCAGTTTATGAG-3"

Cloning and *in vitro* transcription

cDNA was prepared from *Xenopus* eggs as described above. The coding sequence of *Xenopus treslin* was amplified by PCR and cloned into the BamHI, StuI site of a PCS2 vector containing an in frame C-terminal Flag tag, cloned in its StuI, XhoI site. The coding sequence and 6 base pairs from the 5' UTR of *Xenopus recq4* (against which the MO was designed), were amplified by PCR, using a forward primer containing an XhoI restriction site and subcloned blunt end into the Zero Blunt TOPO PCR Cloning vector from Invitrogen. The insert was cut out with XhoI and cloned into the XhoI site of the PCS2 vector. The *Xenopus drf1* ORF cloned into the Nde1 BglII site of the pCITE4a vector from NOVAGEN was a gift from Tatsuro Takahashi (Osaka University, Japan). The *Xenopus cut5* ORF cloned in PCS2 was a gift from Haruhiko Takisawa (Osaka University, Japan).

To obtain sense RNA from these constructs for injection, the *treslin, cut5, drf1* and *recq4* containing plasmids were digested respectively with NOT1, Asp718, SpeI and Asp718. SP6 RNA polymerase was used for *in vitro* transcription of *treslin, cut5* and *recq4* and T7 RNA polymerase for *drf1*. The ORF of *Xenopus cdc45* was amplified from cDNA by PCR and cloned into the ClaI/XhoI site of PCS2.

Antibodies

Polyclonal rabbit antibodies against Cut5, Drf1, RecQ4 and Treslin were kind gifts from Haruhiko Takisawa, Tatsuro Takahashi (Osaka University, Japan) and John Diffley respectively (CRUK Clare Hall, UK). The polyclonal rabbit anti-Cdc45 antibody and the polyclonal rabbit anti-CyclinE antibody were a gift from Dr Vincenzo Costanzo (CRUK Clare Hall, UK). Anti-Flag M2 antibody was from Sigma. Anti-Phospho-Chk1 (Ser345) (133D3) rabbit monoclonal antibody was from Cell Signaling and recognises *Xenopus* Ser 342. Mouse monoclonal Anti-Chk1 antibody (DCS-310) was from Medical and Biological Laboratories Co., LTD. (provided by Caltag Medsystems). Polyclonal rabbit

anti-Cdk1 pTyr15 was from Acris (by 2B Scientific). Polyclonal rabbit anti-Cdc6 was a gift from Ron Laskey. Polyclonal rabbit anti-Mcm2 (ab4461), Mcm4 (ab4459), Mcm5 (ab17967), HistoneH3 (ab18521) and monoclonal mouse anti-Mcm7 (ab2360) were from Abcam. Monoclonal mouse anti-Orc1 (NB100-2738) and polyclonal rabbit anti-Pcna (NBP1-78105) were from Novus Biologicals.

Western blotting

Anti-Cut5, anti-Drf1, anti RecQ4 and anti-Flag were used at concentrations of 1/5000, 1/10000, 1/4000 and 1/1000 respectively in PBS with 0.1% Tween and incubated overnight at 4°C. Anti-Treslin was used at a concentration of 1/200 in PBS with 0.1% Tween and incubated overnight at 4°C. Anti-Phospho-Chk-1 (Ser345) was used at a concentration of 1/1000 in TBS+0.1%Tween and 5%BSA O.N. at 4°C. Anti-Chk1, anti-Cdk1 pTyr15, anti-CyclinE, anti-Cdc6, anti-Mcm2, anti-Mcm4, anti-Mcm5, anti-HistoneH3, anti-Orc1 and anti-Pcna were used at a concentration of 1/1000 in PBS+0.1%Tween and incubated 1 hr at RT. Anti-Cdc45 was used at a concentration of 1/3000 in PBS+0.1%Tween and incubated 1 hr at RT. Goat anti-rabbit and goat anti-mouse HRP coupled antibodies from Pierce were used as secondary antibodies.

DNA combing on dissociated animal cap cells

Embryos were injected at the one cell stage into the animal pole with 300 pg of *cut5*, *drf1, recq4* and *treslin* mRNA. At 6 hrs 30 mins post fertilisation, the vitelline membrane of the embryos was removed, 30 animal caps per sample were cut and the animal cap cells were dissociated for 10' in Calcium- and Magnesium- free medium (75 mM Tris pH 7.5, 880 mM NaCl, 10 mM Kcl, 24 mM $NaHCO_3$). Pigmented cells were removed. The dissociated cells were incubated for 10' in 5-Iodo-2'-deoxyuridine (IdU) (SIGMA) at a concentration of 50 μM. At 7 hrs 10 mins post fertilisation, cells were fixed in 100% EtOH for 10', spun for 6' at 4000 rpm at RT, washed in PBS and spun for 6' at 4000 rpm at RT. The cell pellet was dissolved in 50μl PBS and cells were embedded in an agarose plug by adding 50μl of 1% Low Melting Point agarose in PBS, kept at 65°C. The plug was allowed to solidify on ice and then incubated twice in 0.43 M EDTA with 0.85% *N*-Lauroylsarkosine sodium salt (SIGMA) and 1 mg/ml Proteinase K (Roche) at 55°C for 5h and 24 h respectively to digest all proteins. The supernatant was removed and the plug was washed briefly 3x in TE ph 6.5. To remove proteinase K, the plug was incubated 2x 30' at 55°C in TE ph6.5 with 0.1 mM PMSF. Afterwards, the plug was equilibrated 3x30' at RT in 50 mM MES monohydrate (FLUKA) pH 6.5 in H2O. The supernatant was removed and the plug was melted in 200 μl (= two plug volumes) MES (50 mM pH 6.4) for 20' at 68°C and immediately equilibrated at 42°C. The agarose was digested by overnight digestion with 4 Units of β agarase (New England Biolabs). 40 μl sample was stretched onto a 22mm Square Silanized Glass Cover Slide. DNA was fixed onto the slide by a 5' incubation in Methanol-Acetic acid 3/1 and denatured in 2.5 M HCl for 1 hr. Slides were then incubated for 1' in 70%, 90% and 100% EtOH, dried, washed 4x5' in PBS and blocked for 1 hr in 3% BSA + 0.1% Tween 20 at room temperature. IdU was detected with a mouse anti-BrdU antibody (BD) used at a 1/20 dilution for 1 hr at 37°C. This signal was visualized with a secondary rabbit anti-mouse and a tertiary donkey-anti

rabbit, both Alexa Fluor 488 coupled (2mg/ml, Invitrogen). In between all antibody incubations, slides were washed 3x5' in PBS + 0.1% Tween 20. Antibody incubations were done in PBS with 1%BSA and 0.1%Tween 20. Secondary and tertiary antibodies were diluted 1/50, secondary antibodies were incubated for 1 hr at 37°C and tertiary antibodies were incubated for 1/2 hr at 37°C. DNA was stained with a mouse anti-ssDNA antibody (Millipore), diluted 1/300 in PBS with 3%BSA and 0.1%Tween 20 and incubated ON at 4°C. The signal was visualized with a secondary rabbit anti-mouse and a tertiary donkey anti-rabbit antibody, both AF594 coupled (2mg/ml, Invitrogen). Strands were analyzed with ImageJ and the data were compiled using excel.

DNA replication in egg extract

Cut5, RecQ4, Drf1, Treslin and Cdc45 proteins were translated with the TNT T7/SP6 reticulocyte lysate system from PROMEGA according to the manufacturers protocol.

In DNA replication reactions diluted with reticulocyte lysate, a total volume of 155 µl was used. A mixture of 77.5 µl egg extract; 50 µl reticulocyte lysate; 7.75 µl energy mix (150 mM Creatine Phosphate (Roche), 20 mM ATP (Roche), 2 mM EGTA; 20 mM $MgCl_2$); *Xenopus* sperm chromatin (amount of nuclei as indicated); 3.8 µl Creatine Kinase (Roche) (4 mg/ml in 50% glycerol) and H_2O (to obtain a total volume of 152 µl) was brought to room temperature for 10' after which 3 µl α^{32}PdATP (0.74 Mbq/µl) was added to the reaction. Addition of the radioactive label is indicated as time 0. For undiluted DNA replication reactions, the respective volumes were 88 µl egg extract, 5 µl energy mix, *Xenopus* sperm chromatin (amount of nuclei as indicated), 2.5 µl Creatine Kinase and H_2O (to obtain a total volume of 97µl) and 3 µl α^{32}PdATP. DNA replication was stopped at the indicated timepoints by adding an equal volume of 2x STOP buffer (2%SDS; 80 mM EDTA, 600 mM NaCl). Samples were treated with RNAse A for 1 hr at 37°C (final conc of 0.1 mg/ml) and then overnight with proteinase K at 37°C (final conc 0.5 mg/ml). After phenol/chloroform extraction and ethanol precipitation of the sample, DNA pellets were dissolved in H_2O and loaded onto a 1% agarose gel. The dried gel was exposed for autoradiography.

Chromatin precipitation of replicating sperm nuclei in egg extract

Xenopus sperm chromatin was replicated in undiluted egg extract as described above. Replication of the sperm chromatin was stopped after 15 min at 23°C by diluting the reaction 20 fold in ice cold NIB buffer (50 mM KCl, 50 mM Hepes, 5 mM $MgCl_2$, 2mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 0.5% triton) + protease inhibitor cocktail (Roche). 20 µl of the reaction was removed for the input. The remaining reaction volume was pipetted onto an equal volume of NIB+30% sucrose and spun for 5' at 4°C at 4200 g. 20 µl of the supernatant was saved for loading onto the final gel. The remaining supernatant was removed and discarded without disturbing the chromatin/supernatant interface. This interface was washed twice by carefully adding and removing 200 µl NIB buffer. Thereafter, the reaction was spun for 15 min at 4°C at 6000 g.

The supernatant was discarded and the pellet dissolved in 20 µl EB buffer (Qiagen). The samples were sonicated and analysed by SDS PAGE and Western blot.

Movies

Movies were made with a Leica MZ FL III microscope at 19°C unless otherwise stated.

Preparation of libraries for transcriptome sequencing

Total RNA was prepared from *Xenopus* embryos at the indicated timepoints using the Tripure reagent (Invitrogen) according to the manufacturer's instructions, followed by LiCl precipitation. mRNA-Seq libraries were prepared with the TruSeq kit (Illumina) and sequenced on an Illumina HiSeq 2000 instrument in Paired End mode at 100 base length.

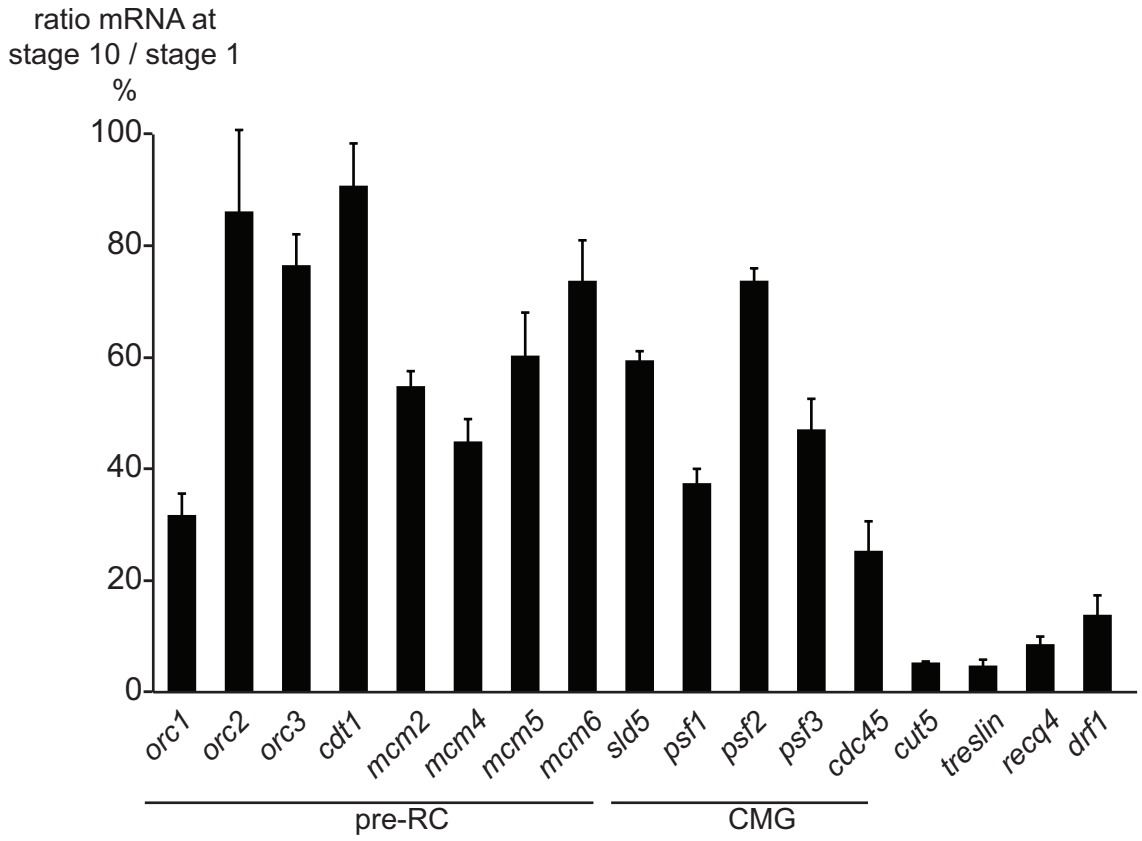
Bioinformatics

The resulting fastq files from the HiSeq were filtered for low quality reads (<Q20), low quality bases were trimmed from the ends of the reads (<Q20) and adapters were removed using cutadapt (http://code.google.com/p/cutadapt/). Fragments where the reads overlapped were joined using FLASH (http://ccb.jhu.edu/software/FLASH/). The resulting reads were mapped against the *Xenopus laevis* 6.1 genome using TopHat (http://tophat.cbcb.umd.edu/).

Xenopus laevis genome build 6.1 from Xenbase (http://www.xenbase.org) was used as a reference genome. Transcript sequences obtained from NCBI RefSeq (November 2012) were assigned to the genome using BLAT. The resulting mappings were filtered by a mismatch threshold as well as requiring 90% of the transcript to match the genome and all exons to match a single scaffold. To prevent spurious matching, the genome was filtered to only include scaffolds with length >10kb. This resulted in 11,876 transcripts mapping to the genome. This mapping was used as a junction file for Tophat 2 (http://tophat.cbcb.umd.edu/) which was used to map the RNA-Seq reads to the genome. Read counts were then generated for each of the transcripts.

Read counts for each transcript were normalised by the total read count for the sample and transcript length to give RPKM expression values. Relative expression between treated and control was calculated as a log fold change for each transcript at each time point. Transcripts were selected for mean expression above 2 RPKM over all time points in either control or treated, leaving 8037 genes. This set was further filtered by stipulating a 2 fold difference between maximum and minimum values over the time course in either control or treated samples, which resulted in a final selection of 1206 genes. The log fold change at each time point was then plotted for the remaining transcripts. A mean and confidence interval was also calculated and plotted for these values at each time point. Finally, raw expression and log fold changes were plotted for selected genes and compared to qPCR data.

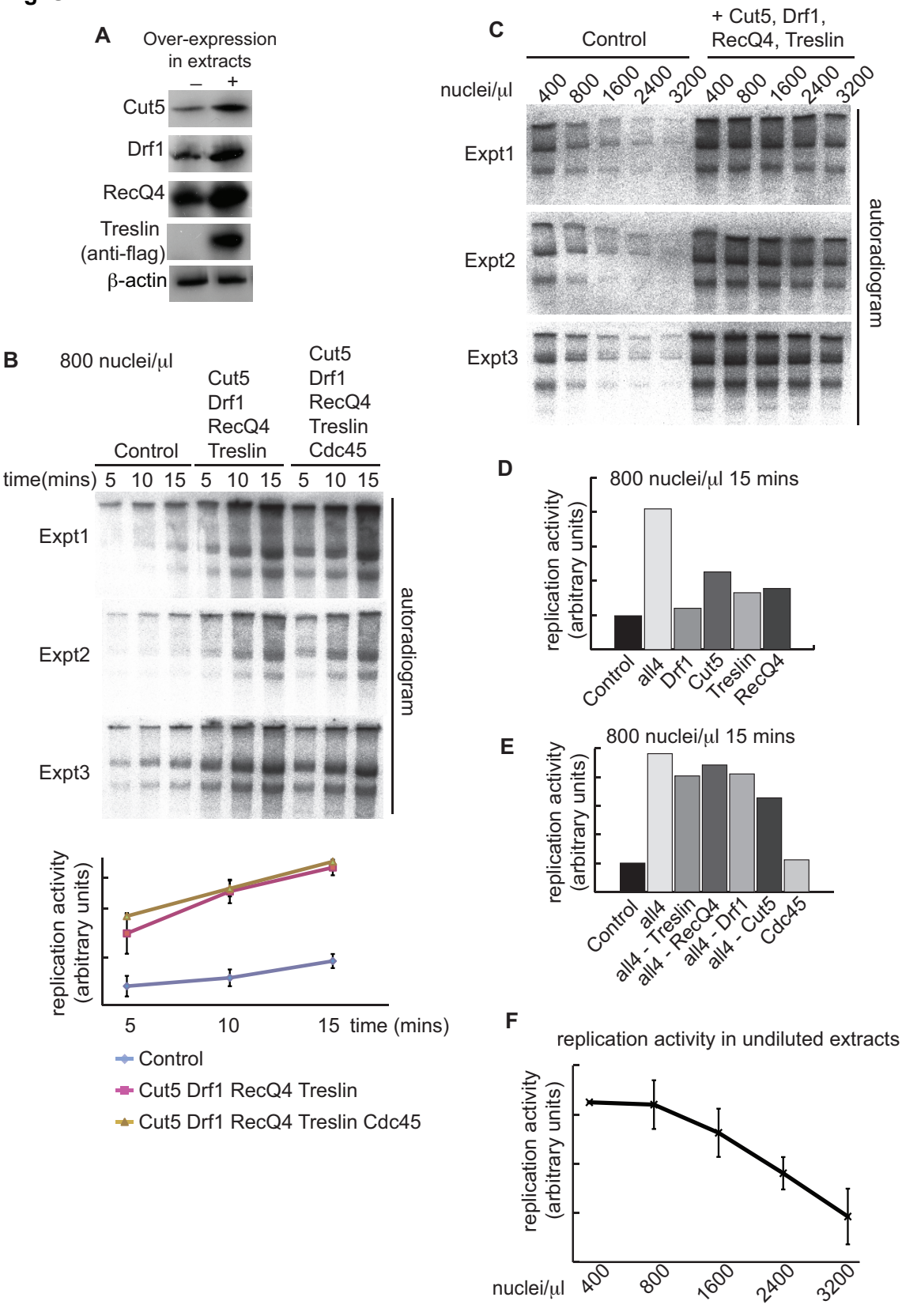
Fig. S1.



mRNA expression levels of genes involved in DNA replication at stage 10 compared to stage 1

Graph of mRNA expression levels of replication initiation genes at stage 10 (gastrula) versus stage 1 (fertilised embryo). mRNA levels were measured by qRT PCR (in triplicate) and values were normalised to *ODC* (Ornithine decarboxylase 1). The mRNA expression levels of *ODC* do not change from stage 1 to stage 10. The Pre-RC (pre-replicative complex) includes proteins involved in replication licensing, while the CMG complex (Cdc45-MCM-GINS) is the active form of the eukaryotic replicative helicase.

Fig. S2


A
Over-expression in extracts
– +
Cut5
Drf1
RecQ4
Treslin (anti-flag)
β-actin
B
800 nuclei/μl
Control
Cut5 Drf1 RecQ4 Treslin
Cut5 Drf1 RecQ4 Treslin Cdc45
time(mins) 5 10 15 5 10 15 5 10 15
Expt1
Expt2
Expt3
autoradiogram
replication activity (arbitrary units)
5 10 15 time (mins)
Control
Cut5 Drf1 RecQ4 Treslin
Cut5 Drf1 RecQ4 Treslin Cdc45
C
Control
+ Cut5, Drf1, RecQ4, Treslin
nuclei/μl 400 800 1600 2400 3200 400 800 1600 2400 3200
Expt1
Expt2
Expt3
autoradiogram
D
800 nuclei/μl 15 mins
replication activity (arbitrary units)
Control all4 Drf1 Cut5 Treslin RecQ4
E
800 nuclei/μl 15 mins
replication activity (arbitrary units)
Control all4 all4 - Treslin all4 - RecQ4 all4 - Drf1 all4 - Cut5 Cdc45
F
replication activity in undiluted extracts
replication activity (arbitrary units)
nuclei/μl 400 800 1600 2400 3200

Overexpression of Treslin, Cut5, RecQ4 and Drf1 increases the efficiency of DNA replication in egg extracts.

A. Western blot of *Xenopus* egg extracts after the addition of IVT Cut5, Drf1, RecQ4 and Treslin in rabbit reticulocyte lysate. Control (-) is the egg extract + reticulocyte lysate with empty plasmid. Treslin is Flag tagged and detected with an anti-Flag antibody.

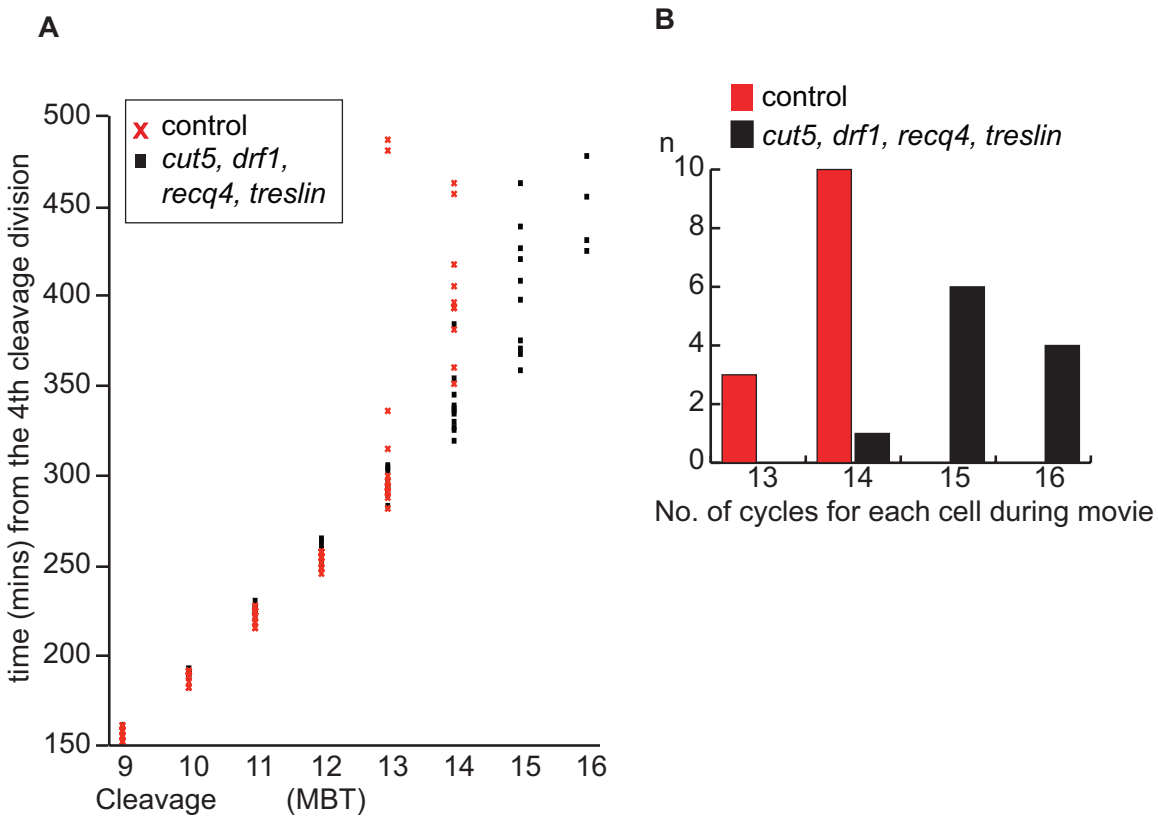
B. *Xenopus* sperm chromatin was added at 800 nuclei/μl of egg extract either supplemented with rabbit reticulocyte lysate + empty vector (control) or rabbit reticulocyte lysate + vectors encoding *cut5, drf1, recQ4* and *treslin* or rabbit reticulocyte lysate + vectors encoding *cut5, drf1, recQ4* and *treslin* and *cdc45* in the presence of α–^{32}P-dATP. Reactions were stopped at the indicated times, loaded onto an agarose gel and visualized by autoradiography. Triplicate experiments are shown. (Below) Intensities of the bands were analysed by ImageJ and plotted. Error bars show standard deviation of the triplicate experiments above. This experiment shows that another replication initiation factor, Cdc45 is not limiting in these conditions in *Xenopus* egg extracts.

C. Same as B, but with increasing nuclear concentrations during incubation. After 15 minutes of *in vitro* replication, equal amounts of DNA for each reaction (relative to the input sperm DNA) were loaded on an agarose gel prior to autoradiography. This data therefore represents the amount of DNA replication/ng of input sperm DNA. These triplicate experiments are the data used for the graph in Figure 1B.

D, E. As in B, reactions were stopped after 15 minutes. all4 refers to extracts over-expressing Treslin, Cut5, RecQ4 and Drf1. all4-Treslin for example refers to over-expression of Cut5, RecQ4 and Drf1. Over-expression of these factors individually causes a minimal effect on replication activity in extracts (D), but over-expression of 3 out of 4 causes a significant increase in replication activity (E). This is similar to our results in budding yeast, where over-expression of any combination of 3 of the 4 limiting factors could cause a significant increase in late origin firing (20). Importantly Treslin, Drf1, RecQ4 and Cut5 are not incorporated into replisomes. As a result, they are free to recycle after initiation events. We hypothesise that the increase in replication activity with over-expression of any combination of 3 of the 4 factors is because under these conditions only the recycling of the 4th factor limits the rate at which replication initiation can occur. We do not know the mechanisms by which these factors are recycled after replication initiation. E also shows that over-expression of Cdc45 alone has very little effect on replication activity in extracts.

F. Replication activity of undiluted extracts at increasing nuclear:extract ratios. *Xenopus* sperm chromatin was added to undiluted egg extract in the presence of α–^{32}P-dATP. Reactions were stopped at 15 minutes, loaded onto an agarose gel prior to autoradiography. Triplicate repeats were quantified by ImageJ and represented here as an average, with the replication activity at 400 nuclei/μl normalised to 1. Error bars are standard deviation. The chromatin binding of the limiting factors in one of these triplicate repeats is presented as Figure 1C.

Fig. S3

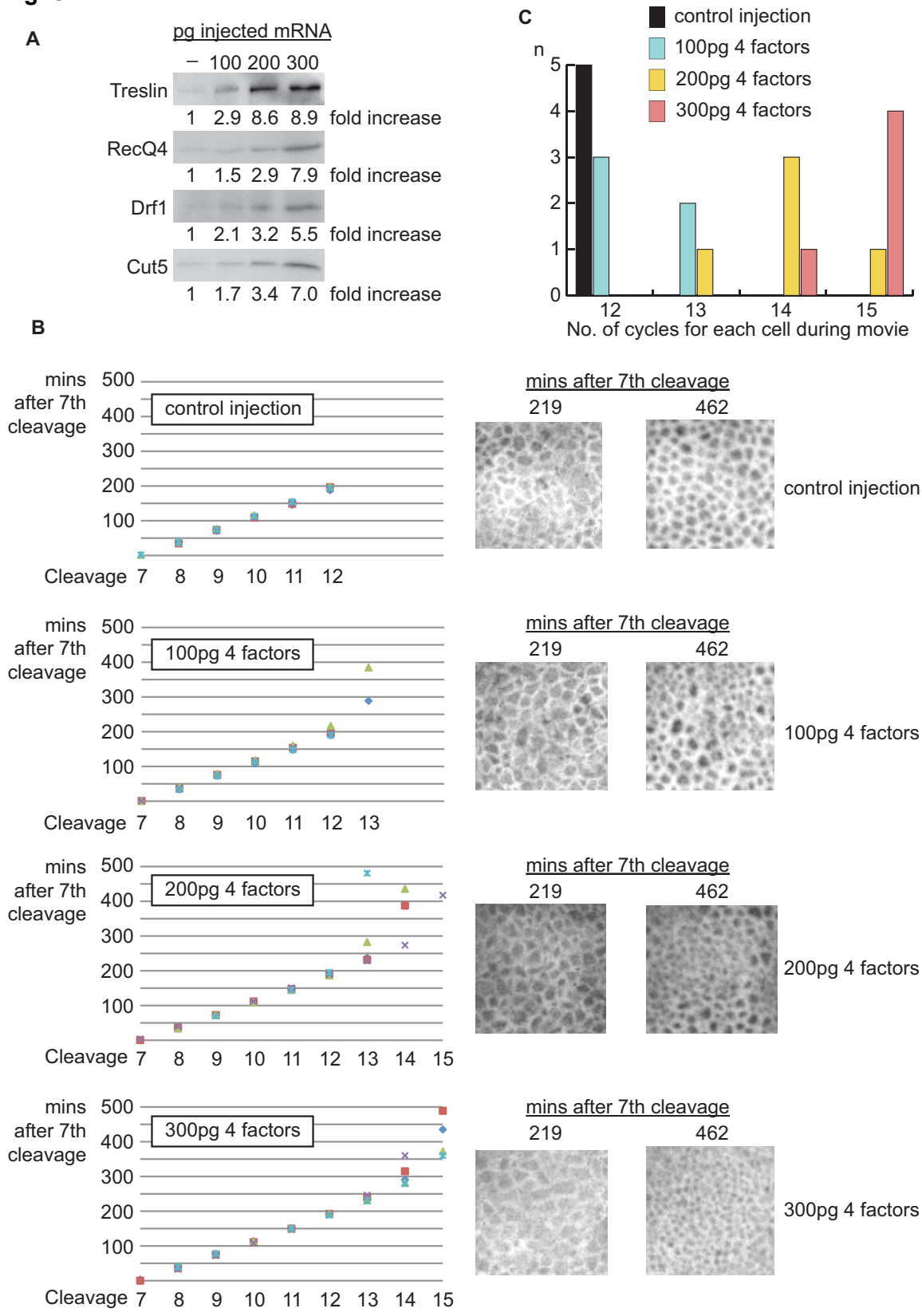


Overexpression of Cut5, Drf1, RecQ4 and Treslin causes extra synchronous divisions after the MBT.

A. 15 individual cells from a time lapse movie were followed through the early embryonic divisions. Note that this movie is a longer movie than Movie S1. Each time point corresponds to the cleavage of an individual cell from embryos that were injected with water (control - red cross) or 300pg mRNA each of *cut5, drf1, recQ4* and *treslin* (black square). Cleavages 1-8 are excluded for simplicity. Note that even after over-expression of RecQ4, Treslin, Drf1 and Cut5 the cell cycle elongated at cleavages 15/16. This elongation at cycle 15 could be due to the onset of other events, such as the introduction of gap phases, or could reflect the continued depletion of these factors by the additional cycles.

B. Representation of the number of cell cycles each cell in A undergoes until 500 mins after cleavage 4.

Fig. S4


A
pg injected mRNA
– 100 200 300
Treslin
1 2.9 8.6 8.9 fold increase
RecQ4
1 1.5 2.9 7.9 fold increase
Drf1
1 2.1 3.2 5.5 fold increase
Cut5
1 1.7 3.4 7.0 fold increase
C
control injection
100pg 4 factors
200pg 4 factors
300pg 4 factors
n
No. of cycles for each cell during movie
B
mins after 7th cleavage
control injection
100pg 4 factors
200pg 4 factors
300pg 4 factors
Cleavage
219
462

Cut5, Drf1, RecQ4 and Treslin show a dose dependent effect on extra synchronous divisions after the MBT.

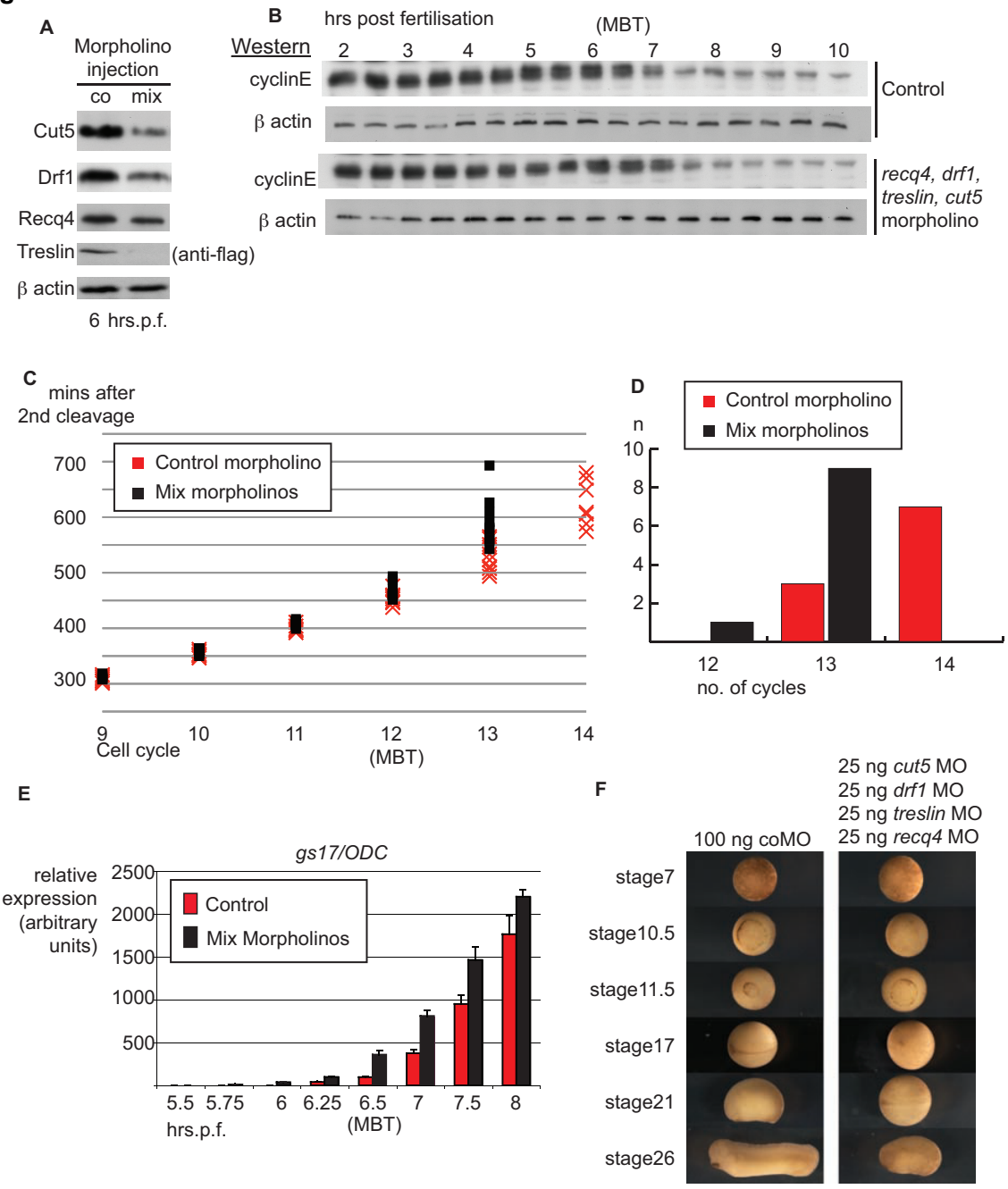
A. Western blot from embryos immmediately prior to the MBT (6 hrs.p.f) showing the different levels of over-expression of *cut5, drf1, recQ4* and *treslin*, by injection of 100-300pg of mRNA of each factor at the 1 cell stage. Western blots were quantified with ImageJ and the increase in expression relative to control-injected embryos is shown below each blot.

B. Time-lapse movies of injected embryos were performed at 19^{o}C until 500 mins after cleavage 7. Five individual cells in each embryo were followed through the early embryonic divisions. Cleavages 1-6 are excluded for simplicity.

Control embryos went through 12 divisions in this experiment, while embryos injected with the 4 factors underwent increasing numbers of divisions, with increasing synchrony dependent on the amount of mRNA injected. (Right) Still images from the time-lapse movies show that cell size and number are very similar for all embryos at 219 mins (MBT), while towards the end of the movie cell numbers are greater and cell sizes are smaller in correlation with the amount of over-expression of the 4 factors.

C. Representation of the number of cell cycles each cell in B undergoes until 500 mins after cleavage 7. Note that the number of cell divisions increased with the increasing dose of the 4 factors.

Fig. S5

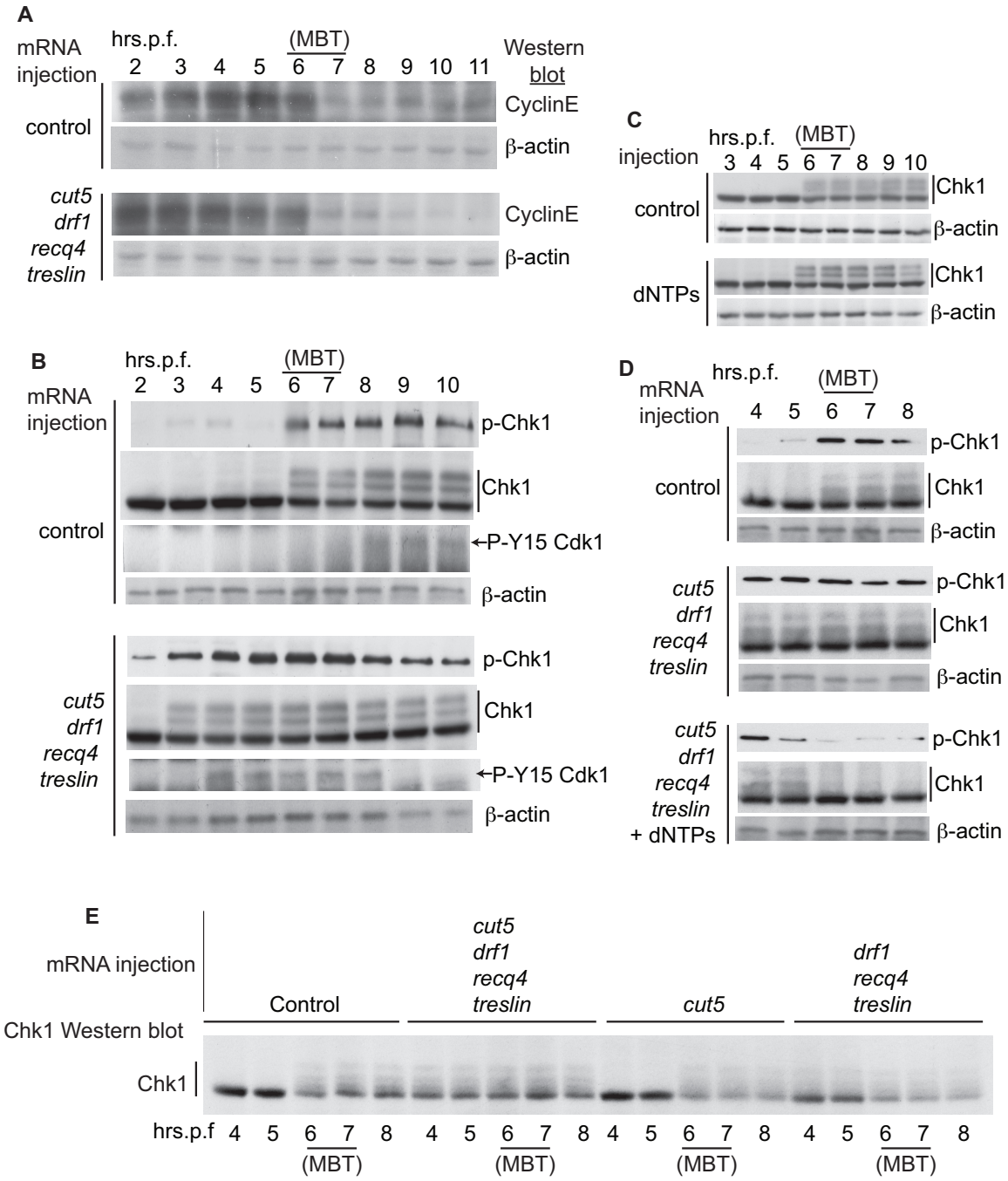


Partial knock down of Cut5, Drf1, RecQ4 and Treslin by morpholino injection elongates the cell cycle at the MBT.

A. Western blot from embryos immmediately prior to the MBT (6 hrs.p.f) showing the knock down of Cut5, Drf1, RecQ4 and Treslin after injection of 25ng each of morpholinos against the four factors at the 1 cell stage. The Western blots for RecQ4, Cut5, Drf1 and actin show the endogenous levels of protein from embryos injected with the mix of all 4 morpholinos (mix) relative to control morpholino (co) injected embryos. For Treslin, to check the efficacy of the morpholino, mRNA corresponding to Flag-

Treslin was injected into embryos, with or without the mix of morpholinos. The efficacy of the Treslin morpholino is evident from the reduction in Flag-Treslin protein.
B. Western blot of Cyclin E from control morpholino injected embryos or embryos injected with the mixture of 25ng each of the morpholinos against *cut5, drf1, recq4* and *treslin.* The degradation of Cyclin E was slightly delayed in the morpholino injected embryos at around the time of the MBT. This may reflect the delay in cell cycle progression by mix morpholino injection (see C and D below) at the MBT.
C. Time lapse movies of control injected embryos or embryos injected with the mixture of 25ng each of the morpholinos against *cut5, drf1, recq4* and *treslin* (mix) were made at 16^{o}C. The cleavages of 10 individual cells from 2 embryos for each condition were followed until 700mins after cleavage 2. Notably the cell divisions until cycle 12 are similar in both control and mix morpholino injected embryos. This likely reflects the fact that the timings of the early cleavage divisions are independent of DNA replication (17). At the MBT, cleavage 12 and in particular cleavage 13 are slowed by the partial reduction in the levels of Cut5, Drf1, RecQ4 and Treslin.
D. Representation of the number of cell cycles each cell in C undergoes until 700 mins after cleavage 2. Note that the number of cell divisions during this time course is reduced in embryos injected with the mix morpholinos against *cut5, drf1, recq4* and *treslin*.
E. qPCR analysis of the timing of expression of the *gs17* gene **(**Gastrula-specific protein 17) relative to *ODC* in staged embryos. Embryos were taken every 15 mins from 5hrs 30 minutes (5.5) until the MBT at 6hrs 30 minutes and then every 30 mins. In accordance with previous studies that show that inhibition of DNA replication leads to the early onset of zygotic transcription (23), the morpholinos against *cut5, drf1, recq4* and *treslin* (mix), that cause delayed cell cycle progression at the MBT (C and D above), cause the earlier transcription of the *gs17* gene.
F. Analysis of early embryogenesis in control morpholino injected embryos (coMO) or embryos injected with the mixture of 25ng each of the morpholinos against *cut5, drf1, recq4* and *treslin.* Stage 7 = pre-MBT, 10.5 = gastrula, 11.5 = mid-gastrula, 17 = neurula, 21 = tailbud, 26 = tadpole. Note that the mix morpholino injected embryos show a delay in embryogenesis from stage 10.5 onwards.

Fig. S6

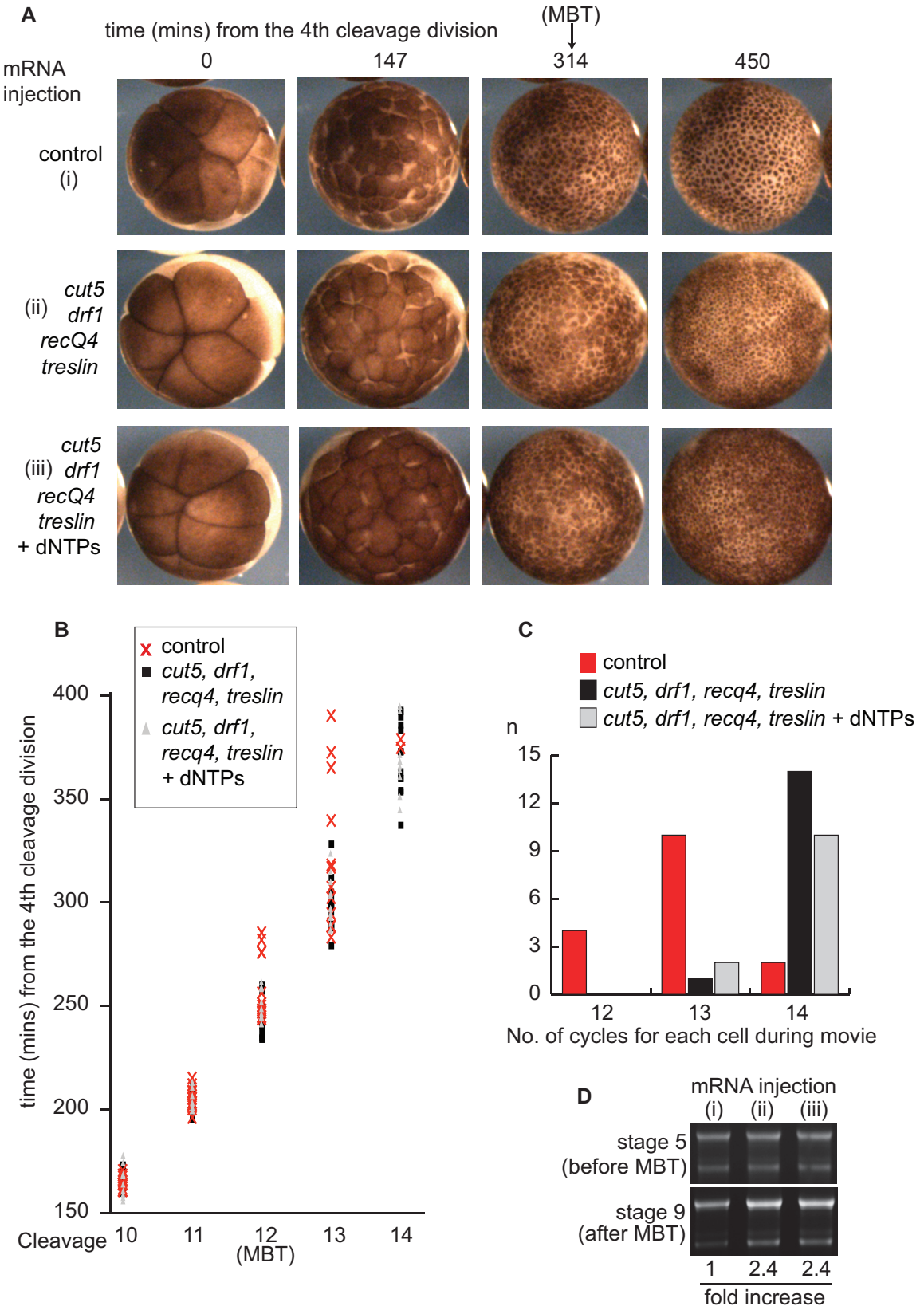


Limiting replication factors and dNTP levels control the developmental activation of Chk1.

A. Western blot showing the degradation of Cyclin E at the MBT from embryos either injected at the 1 cell stage with water (control) or with 300 pg mRNA each of *treslin, cut5, recq4* and *drf1*. This shows that the cell autonomous developmental clock is not

affected by over-expression of limiting replication factors. hrs.p.f stands for hours post fertilisation.
B. Western blot with antibodies recognizing phospho-Chk1 (anti phospho serine 342), total Chk1 and the tyrosine 15 phosphorylated form of Cdk1 (P-Y15 Cdk1). Note that in control embryos, Chk1 is activated at the MBT, resulting in increased Y15 phosphorylation of Cdk1, likely through degradation of Cdc25 (2). In embryos over-expressing Cut5, Drf1, RecQ4 and Treslin, Chk1 activation and phospho-Y15 Cdk1 occurs earlier.
C, D. As in B. Where indicated, 200pmol dNTPs were injected at the 1 cell stage. Note that coinjection of dNTPs with mRNA for *cut5, drf1, recQ4* and *treslin* leads to considerable suppression of the appearance of the active form of Chk1, particularly at the MBT.
E. As in B. While over-expression of Cut5, Drf1, RecQ4 and Treslin is sufficient for the premature activation of Chk1, increasing the levels of Cut5 alone resulted in Chk1 activation at the same time as control embryos. Over-expression of Drf1, RecQ4 and Treslin causes some early activation of Chk1, in accordance with a partial effect of over-expression of 3 of the 4 factors on replication rates *in vitro* (Fig. S2E).

Fig. S7


A
time (mins) from the 4th cleavage division
(MBT)
0
147
314
450
mRNA injection
control (i)
(ii) cut5 drf1 recQ4 treslin
(iii) cut5 drf1 recQ4 treslin + dNTPs
B
control
cut5, drf1, recq4, treslin
cut5, drf1, recq4, treslin + dNTPs
time (mins) from the 4th cleavage division
400
350
300
250
200
150
Cleavage
10
11
12 (MBT)
13
14
C
control
cut5, drf1, recq4, treslin
cut5, drf1, recq4, treslin + dNTPs
n
15
12
9
6
3
0
12
13
14
No. of cycles for each cell during movie
D
mRNA injection
(i) (ii) (iii)
stage 5 (before MBT)
stage 9 (after MBT)
1 2.4 2.4
fold increase

Coinjection of dNTPs with overexpression of Cut5, Drf1, RecQ4 and Treslin also causes extra rapid cell divisions after the MBT.

A. Images taken from a time-lapse movie (Movie S1) of injected embryos at the indicated times at 19^{o}C. Cleavage 4 at the 16-cell stage was set to time zero. The MBT occurs at around 314 mins after cleavage 4. Five separate movies were taken of 13 embryos for each injection condition (i-iii). Each movie shows qualitatively the same result. (i) = injected at the 1 cell stage with water (control), (ii) injected with 300 pg mRNA each of *treslin, cut5, recq4* and *drf1* and (iii) as (ii) + 200pmol dNTPs. The images for conditions i) and ii) are the same as those in Figure 3A. Note that embryos co-injected with dNTPs (iii) are very similar to those not injected with dNTPs (ii).

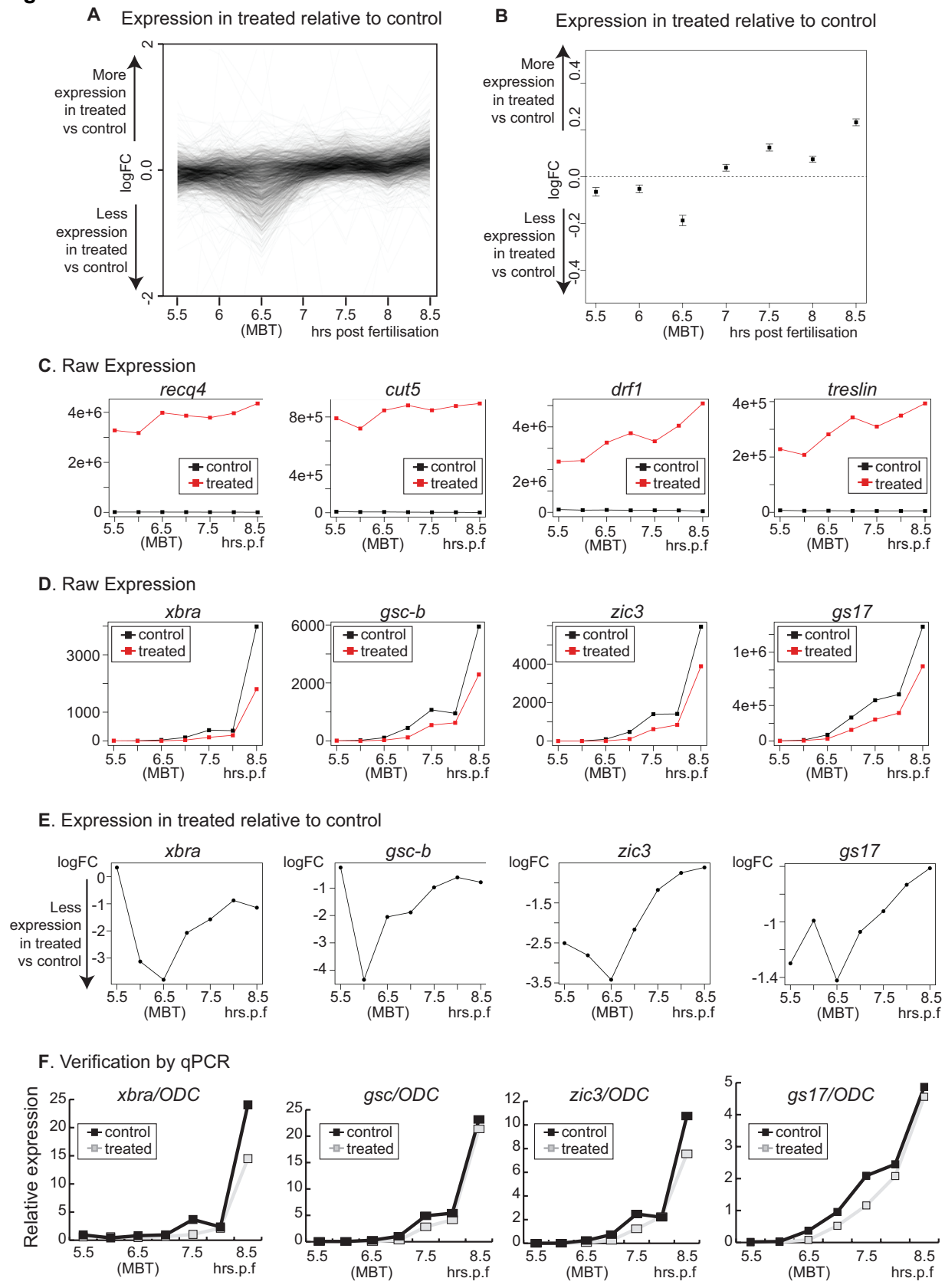
B. 15 individual cells from a time lapse movie were followed through the early embryonic divisions. Note that this movie is not the same as the Movie S1 and therefore this experiment represents a biological repeat of Figure 3B. Each time point corresponds to the cleavage of an individual cell from embryos that were injected with water (control - red cross), 300pg mRNA each of *cut5, drf1, recQ4* and *treslin* (black square) or 300pg mRNA each of *cut5, drf1, recQ4* and *treslin* + 200pmol dNTPs (grey triangle). Cleavages 1-9 are excluded for simplicity.

C. Representation of the number of cell cycles each cell in B undergoes until 400 minutes after cleavage 4.

D. Embryos were injected as in A (conditions i-iii) and half the DNA content of a single embryo at stages 5 and 9 was loaded onto an agarose gel and stained with Ethidium Bromide. This gel is representative of triplicate repeats. Below - DNA amount at stage 9 was quantified from the gel above using ImageJ and fold increase relative to the amount of DNA in control embryos (i) is represented. Note that a subset of this same image was used in Fig. 3D.

Together this figure indicates that co-injection of dNTPs does not affect the extra number of rapid cell cycles after the MBT by over-expression of Treslin, Cut5, RecQ4 and Drf1. Therefore this effect on the cell cycle is likely to be independent of Chk1 activation levels (Fig. S6D).

Fig. S8


A
Expression in treated relative to control
More expression in treated vs control
Less expression in treated vs control
logFC
hrs post fertilisation
(MBT)
B
Expression in treated relative to control
C. Raw Expression
recq4
cut5
drf1
treslin
control
treated
hrs.p.f
D. Raw Expression
xbra
gsc-b
zic3
gs17
E. Expression in treated relative to control
F. Verification by qPCR
xbra/ODC
gsc/ODC
zic3/ODC
gs17/ODC
Relative expression

Over-expression of Cut5, Drf1, RecQ4 and Treslin delays the onset of transcription.

A. RNA was isolated from staged embryos every 30 mins between 5hrs 30mins post fertilisation (5.5) and 8hrs 30mins (8.5), either injected with water (control) or injected with 300pg of mRNA each for *cut5, drf1, recQ4* and *treslin* (treated). mRNA was polyA selected, converted to cDNA libraries and sequenced. By this method we identified 8037 genes that were above the minimum detection threshold. The mRNA levels of the majority of these 8037 genes do not change during this time course, either in control or treated samples, and therefore for these genes, zygotic transcription or maternal mRNA degradation is insignificant (or they cancel each other out). Therefore to select for genes that might be transcribed during the MBT, we analysed in detail the 1206 genes that fluctuated at least 2 fold (either up or down) across any timepoints in either control or treated samples. For these 1206 genes we plotted the fold change (logFC) of the mRNA levels between treated samples relative to control (graph A - Each black line corresponds to the relative expression of each of the 1206 genes). Any mRNA that is similarly abundant in control and treated samples will have a value of 0. An mRNA that is more abundant in treated (over-expressing the 4 factors) versus control will have a positive value. An mRNA that is less abundant after over-expression of these 4 factors relative to control will have a negative value. This graph indicates that while a large number of genes do not show a difference between control and treated conditions, a significant proportion of the mRNAs are less abundant in the embryos over-expressing the 4 factors, specifically at the time of the MBT (6.5 hrs.p.f). This is consistent with a delay in the zygotic transcription of this subset of genes by the over-expression of these 4 factors.

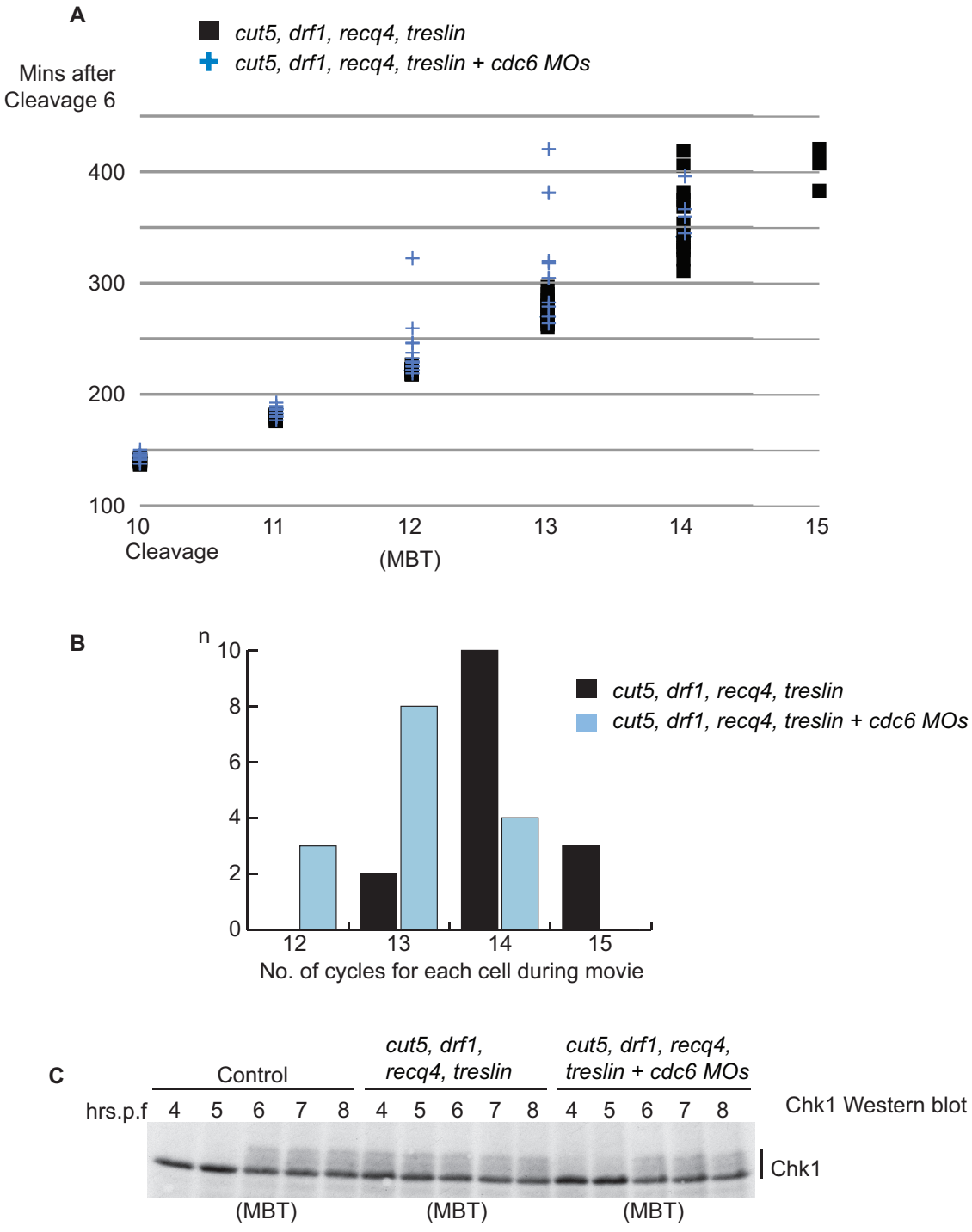
B. Representation of the average values for the fold change (logFC) in treated embryos (over-expressing the 4 factors) over control (water injected) from the graph in A. Error bars are standard deviation. Note that there is a significantly lower relative expression of genes in treated over control at the MBT. There also appears to be a slight increase in mRNA abundance in treated over control after the MBT (7.5-8.5 hrs.p.f). This may be due to the fact that this data is not normalised for DNA content and embryos over-expressing the four factors undergo more cell cycles and have a higher DNA content after the MBT (Fig. 3). This increased DNA content will tend to over-represent mRNA abundance in the treated embryos after the MBT and suggests that the delay in transcription caused by the over-expression of limiting factors will be even more significant per cell than reflected in A.

C. Although A and B are consistent with a delay in transcription at the MBT in embryos over-expressing the 4 factors, we wanted to check this by analysing the raw expression data for genes we expected to be transcribed at the MBT. As controls we first analysed the levels of the 4 factors that are over-expressed by mRNA injection at the 1 cell stage. Reassuringly the mRNA for *recq4, drf1, cut5* and *treslin* are much more abundant in the mRNA injected embryos (treated) versus the control.

D. Four genes we expected to be zygotically expressed around the time of the MBT; *xbra, gsc-b, zic3* and *gs17* were indeed transcribed at the time of the MBT in control samples. This transcription was clearly delayed for all 4 genes after over-expression of Cut5, Drf1, RecQ4 and Treslin as the raw expression profiles are lower from the MBT onwards relative to control.

E. Representation of the data in D as a relative fold change (logFC) of treated samples over control. All 4 genes show a trough of relative expression at the time of the MBT, consistent with a delay in transcription of these genes after over-expression of Cut5, Drf1, RecQ4 and Treslin.
F. To verify the raw expression data from the high-thoughput sequencing, we again isolated mRNA from staged embryos as described in A and performed qPCR on the genes represented in D. qPCR data was normalised to the ODC control. This qPCR data, in accordance with D above, also shows a delay in the accumulation of *xbra, gsc-b, zic3* and *gs17* mRNA in embryos over-expressing Cut5, Drf1, RecQ4 and Treslin relative to control embryos. Although it may seem that the delay in transcription in this data (approximately 30mins) is less than the number of additional rapid cycles induced by over-expression of Cut5, Drf1, RecQ4 and Treslin (for example see Fig. 3B), these transcriptome data is not normalised for DNA content of the embryo. Since over-expression of Cut5, Drf1, RecQ4 and Treslin causes extra cell divisions, the higher DNA content of these embryos will tend to over-represent mRNA abundance in treated embryos relative to control.

Fig. S9



Knock down of Cdc6 restores the elongation of the cell cycle and the timely activation of Chk1 after over-expression of Cut5, Drf1, RecQ4 and Treslin.

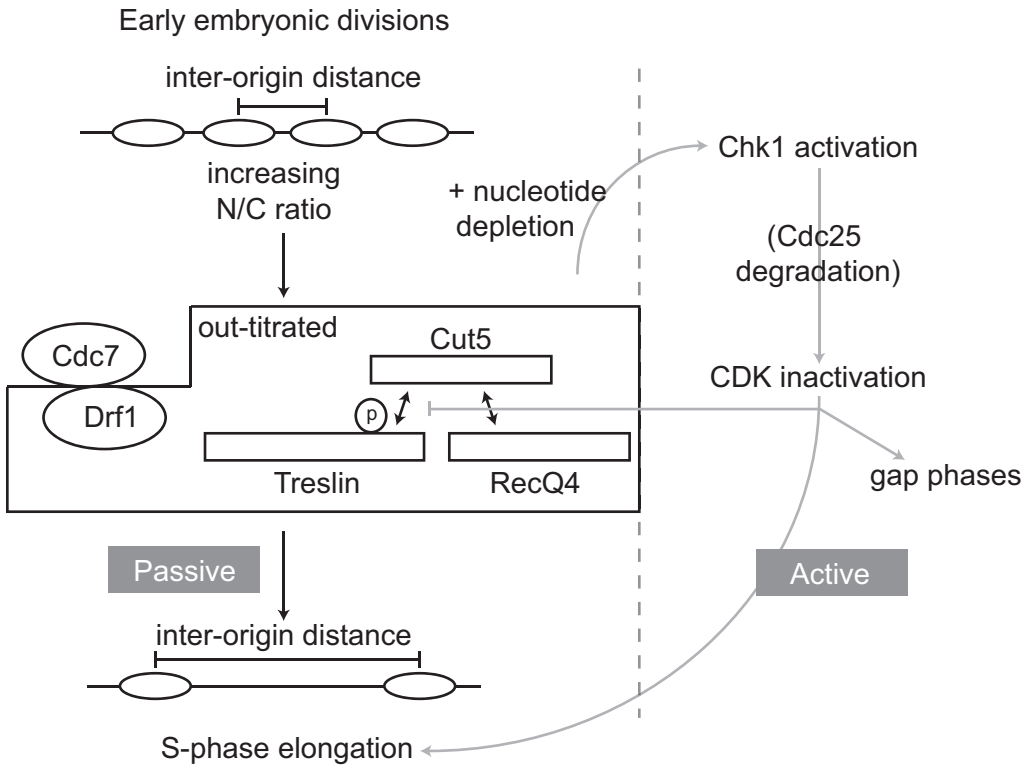
A. Time-lapse movies of embryos injected with 300pg each of mRNA *recq4, drf1, cut5* and *treslin* or 300pg each of mRNA *recq4, drf1, cut5* and *treslin* + *60pg cdc6*

morpholinos were performed at 19^{o}C until 450 mins after cleavage 6. Fifteen individual cells from these movies were followed through the early embryonic divisions. Cleavages 1-9 are excluded for simplicity. The *cdc6* morpholinos elongated the cell cycle at cycle 12 and 13 in spite of over-expression of Cut5, Drf1, RecQ4 and Treslin. This suggests that the number of active origins indeed determines the cell cycle length at the MBT in *Xenopus*.

B. Representation of the number of cell cycles each cell in A underwent until 450 mins after cleavage 6. Note that the number of cell divisions during this time course is reduced in embryos injected with the *cdc6* morpholinos together with mRNA for *cut5, drf1, recq4* and *treslin*.

C. Western blot of developmental Chk1 activation, as determined by the increase in the lower mobility, phosphorylated form of Chk1. Embryos injected with mRNA encoding *cut5, drf1, recq4* and *treslin* activated Chk1 early, while co-injection of *cdc6* morpholinos suppressed this early activation. This experiment indicates that the early activation of Chk1 in embryos over-expressing Cut5, Drf1, RecQ4 and Treslin is indeed due to the increased replication initiation rates in these embryos.

Fig. S10



Model proposing how S-phase elongation is controlled at the MBT.

The increasing nuclear to cytoplasmic (N/C) ratio during early divisions causes the out-titration of at least four replication initiation factors (Cut5, Treslin RecQ4, and Drf1) that become limiting for origin firing. This provides a passive mechanism for the lengthening of S-phase. Increasing inter origin distance together with nucleotide depletion leads to activation of Chk1 and subsequent CDK inactivation. This results in further cell cycle regulation by reducing the activity of the limiting factors directly (*12, 27*) and by introducing gap phases. Chk1 activation and CDK inactivation provide an active pathway to control cell cycle length during development.

Movie S1

Embryos injected at the animal pole at the 1 cell stage in triplicate as follows; control injected (bottom 3 embryos), injected with mRNA for *treslin, cut5, recq4* and *drf1* (middle 3 embryos) and injected with mRNA for *treslin, cut5, recq4* and *drf1* + 200pmol dNTPs (top 3 embryos). Movie was taken at 19^{o}C. Each frame is 3 minutes apart.