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Titration of Four Replication Factors is essential for the *Xenopus laevis* Mid-Blastula Transition

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Abstract

The rapid, reductive early divisions of many metazoan embryos are followed by the mid-blastula transition (MBT), during which the cell cycle elongates and zygotic transcription begins. It has been proposed that the increasing nuclear to cytoplasmic (N/C) ratio is critical for controlling the events of the mid-blastula transition (MBT). We show that four DNA replication factors, Cut5, RecQ4, Treslin, and Drf1, are limiting for replication initiation at increasing N/C ratios *in vitro* and *in vivo* in *Xenopus laevis* . The levels of these factors regulate multiple events of the MBT including the slowing of the cell cycle, the onset of zygotic transcription, and the developmental activation of the kinase Chk1. This work provides a mechanism for how the N/C ratio controls the MBT and shows that the regulation of replication initiation is fundamental for normal embryogenesis.

The early cell divisions in many metazoa are rapid and maintained by maternally supplied products. Bulk zygotic transcription begins at the mid-blastula transition (MBT), also referred to as the start of the maternal-zygotic transition (MZT) (1), and is accompanied by the onset of cell movement, the activation of cell cycle checkpoints and the elongation and asynchrony of cell divisions (2-6). This elongation of the cell cycle is coincident with a reduction in the density and synchrony of DNA replication initiation events (7-9), leading to the possibility that replication factors themselves might be important regulators of cell cycle duration during development. To test this hypothesis we measured the abundance of replication initiation factors in *Xenopus laevis* embryos. Components of the first step of replication initiation, a process called ‘licensing’ or pre-replicative complex (pre-RC) assembly, remained constant or increased in abundance during embryogenesis (Fig. 1A - Orc1 and Mcm2, 4, 5 and 7).

After pre-RC formation, two kinases are required to assemble active replisomes, Cyclin-Dependent Kinase (CDK) and Dbf4-Dependent kinase (DDK). The abundance of the non-catalytic subunit of DDK, which in *Xenopus* embryos is Drf1 (Dbf4-related factor 1), becomes greatly reduced by the time of gastrulation (10, 11). Both the mRNA and protein levels of three other replication factors, *cut5*, *treslin* and *recq4* also decreased during embryogenesis (Fig. 1A and Fig. S1). Treslin and RecQ4 are orthologues of Sld3 and Sld2,

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The supporting online material consists of 10 figures, 1 movie and methods.

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the Cyclin-Dependent Kinase (CDK) targets required for replication initiation in yeast, whereas Cut5 is the orthologue of their interaction partner Dpb11 (12-14). Reduction in the levels of these replication factors during early cell divisions suggested that their abundance might be a determinant of embryonic replication initiation rates. To test this, we added *in vitro* translated (IVT) RecQ4, Treslin, Cut5, and Drf1 to *Xenopus* egg extracts and analyzed the replication of sperm nuclei *in vitro*. Increased expression of these proteins in extracts resulted in a corresponding increase in DNA synthesis when these factors were overexpressed in combination (Fig. S2A-E). Furthermore, increasing the nuclei:extract ratio, to mimic the increasing N/C ratio of the early embryo, caused an exponential decline in DNA synthesis, which was suppressed by overexpression of RecQ4, Treslin, Cut5, and Drf1 (Fig. 1B and Fig. S2C). In addition, whereas neither the pre-RC component Orc2 nor histone H3 were titrated out from extracts or on chromatin at increasing nuclei:extract ratios (Fig. 1C) (15), RecQ4, Cut5, Drf1 and Treslin were significantly depleted from extracts (unbound) and less abundant on chromatin at nuclei:extract ratios that resulted in reduced replication activity (Fig. 1C and Fig. S2F). We conclude that these four factors are limiting for DNA synthesis rates in *Xenopus* egg extracts in a N/C ratio-dependent manner.

To test whether these factors are limiting for replication initiation *in vivo*, we increased the levels of all four factors in embryos by injection of mRNA into the fertilized egg (Fig. 2A). To analyze replication rates directly we dissected the animal caps of embryos at stage 8.5 (MBT), dissociated the cells, and pulse-labelled replication forks with IdU (Fig. 2B top). Anti-IdU immuno-fluorescence of labeled DNA stretched onto slides (Fig. 2B bottom) revealed that overexpression of RecQ4, Treslin, Drf1, and Cut5 resulted in an almost twofold increase in total replication (Fig. 2C i). The length of the IdU incorporation tracks remained constant after overexpression of the 4 factors (Fig. 2C ii), suggesting that replication elongation was not affected. By contrast, the gap between labels was reduced nearly twofold by overexpression of these factors (Fig. 2C iii), which is indicative of an increase in origin firing. From this analysis we conclude that RecQ4, Treslin, Drf1, and Cut5 are limiting for the frequency of initiation at the MBT in *Xenopus* embryos.

A major transition at the MBT is the lengthening of the cell cycle (4, 16). To test the importance of these limiting replication factors for cell cycle control at the MBT, we analyzed time-lapse movies of injected *Xenopus laevis* embryos (Movie S1). In control embryos, rapid synchronous divisions occurred until the MBT at cycle 12, after which cell divisions slowed and became asynchronous (Fig. 3A-C and Fig. S3), as previously described (17). In embryos that overexpress RecQ4, Treslin, Drf1, and Cut5, the rapid, synchronous cell divisions continued beyond cycle 12 (Fig. 3A-C and Fig. S3) and as a result these embryos had a considerably greater number of cells (Fig. 3A) and a higher DNA content after the MBT (Fig. 3D).

In accordance with a direct role for the N/C ratio in the titration of these factors in embryos, we observed a dose-dependency between the levels of overexpression of these factors and the number of rapid cell cycles that were induced after the MBT (Fig. S4). Furthermore, partial depletion of RecQ4, Treslin, Drf1, and Cut5 had the opposite effect to the overexpression and resulted in longer cell cycles at the MBT (Fig. S5C). We conclude that the titration of at least 4 replication factors, RecQ4, Treslin, Drf1, and Cut5 controls cell cycle length at the MBT in *Xenopus laevis*.

Shortening of the cell cycle at the MBT by overexpression of these limiting factors is unlikely due to indirect effects on the cell-autonomous developmental clock (18, 19) because the timing of Cyclin E degradation was unaffected in injected embryos (Fig. S6A). We also tested whether the limiting factors affected the checkpoint kinase Chk1, which becomes transiently activated at the time of the MBT (2, 3, 6). The combined

overexpression of RecQ4, Treslin, Drf1 and Cut5 together caused much earlier activation of Chk1 in *Xenopus* embryos (Fig. S6B,E).

We have previously shown that increasing the rates of replication initiation in budding yeast leads to checkpoint activation by the transient depletion of nucleotides (20). Since it has been shown that deoxyribonucleotide levels are reduced at the onset of the MBT in *Xenopus laevis* (21), we wondered whether the early activation of Chk1 upon overexpression of RecQ4, Treslin, Drf1, and Cut5 is due to the premature depletion of nucleotides. To test this, we co-injected a fourfold excess (200pmols) of dNTPs into the fertilized egg (22). Whereas injection of dNTPs alone had no effect on the developmental activation of Chk1 (Fig. S6C), co-injection of dNTPs together with overexpression of the 4 factors greatly reduced the activation of Chk1, particularly at the time of the MBT (Fig. S6D). This suggests that the developmental activation of Chk1 is, at least in part, caused both by the depletion of initiation factors and by limiting dNTP levels at the MBT. Co-injection of dNTPs together with the 4 factors, while partially suppressing the developmental Chk1 activation, did not affect the continuation of rapid cell divisions in these embryos (Fig. S7). This indicates that overexpression of RecQ4, Treslin, Drf1 and Cut5 sustains rapid cell divisions at the MBT independently of Chk1 activation levels.

Previous studies have suggested that elongation of the cell cycle is a requirement for the onset of zygotic transcription (23). To address directly the importance of coordinating replication with the onset of transcription, we analyzed the *Xenopus laevis* transcriptome by high-throughput sequencing of staged embryos. Overexpression of limiting replication factors delayed the expression of a large number of genes at the MBT (Fig. S8). Therefore the regulation of replication initiation densities and cell cycle length at the MBT is a primary event that controls the timely transcription of a subset of genes in *Xenopus* embryos. As is the case in flies (24), the dynamics of expression of several genes remained unaffected by the extra rapid cell cycles (Fig. S8A), underlining that several mechanisms regulate mRNA abundance at the MBT (1).

To understand the physiological importance of the regulation of DNA replication during development we analyzed the phenotype of embryos overexpressing RecQ4, Treslin, Drf1, and Cut5. Embryos that overexpress these 4 factors failed to complete gastrulation and blastopore closure at stage 10.5 (Fig. 4) and underwent cell death by stage 17 (neurula), showing that titration of these factors is critical for embryogenesis.

If this developmental defect upon overexpression of RecQ4, Treslin, Drf1 and Cut5 is indeed due to increased rates of initiation, we hypothesized that a partial reduction in origin licensing might suppress this phenotype. Injection of embryos with morpholinos against the pre-RC component *cdc6* resulted in partial depletion of the Cdc6 protein (Fig. 4, top) and although this caused a slight delay in blastopore closure, 81% of these morpholino-injected embryos reached the neurula stage (Fig. 4). However, co-injection of these *cdc6* morpholinos together with overexpression of RecQ4, Treslin, Drf1 and Cut5 partially rescued the ability of these embryos to undergo gastrulation, and 38% of embryos survived until stage 17. This indicates that the developmental deficiency caused by overexpression of RecQ4, Treslin, Drf1 and Cut5 is at least in part due to the resulting increase in replication initiation. The *cdc6* morpholinos in combination with the overexpression of limiting initiation factors not only partially rescued the embryonic defect, but also restored normal cell cycle elongation and Chk1 activation at the MBT (Fig. S9). Together these experiments demonstrate that the regulation of replication initiation rates is necessary for several of the critical events of the MBT and for normal development in *Xenopus laevis*.

This study provides a mechanistic basis for the hypothesis put forward thirty years ago that passive depletion of limiting factors by the N/C ratio is the primary mechanism controlling events at the MBT (4). We speculate that the interplay between the limiting replication factors RecQ4, Treslin, Drf1, and Cut5, together with Chk1 activation and CDK inactivation forms a feedback loop (Fig. S10), which in accordance with work in flies (3, 6, 25, 26), underlies how S-phase length is regulated during development across eukaryotes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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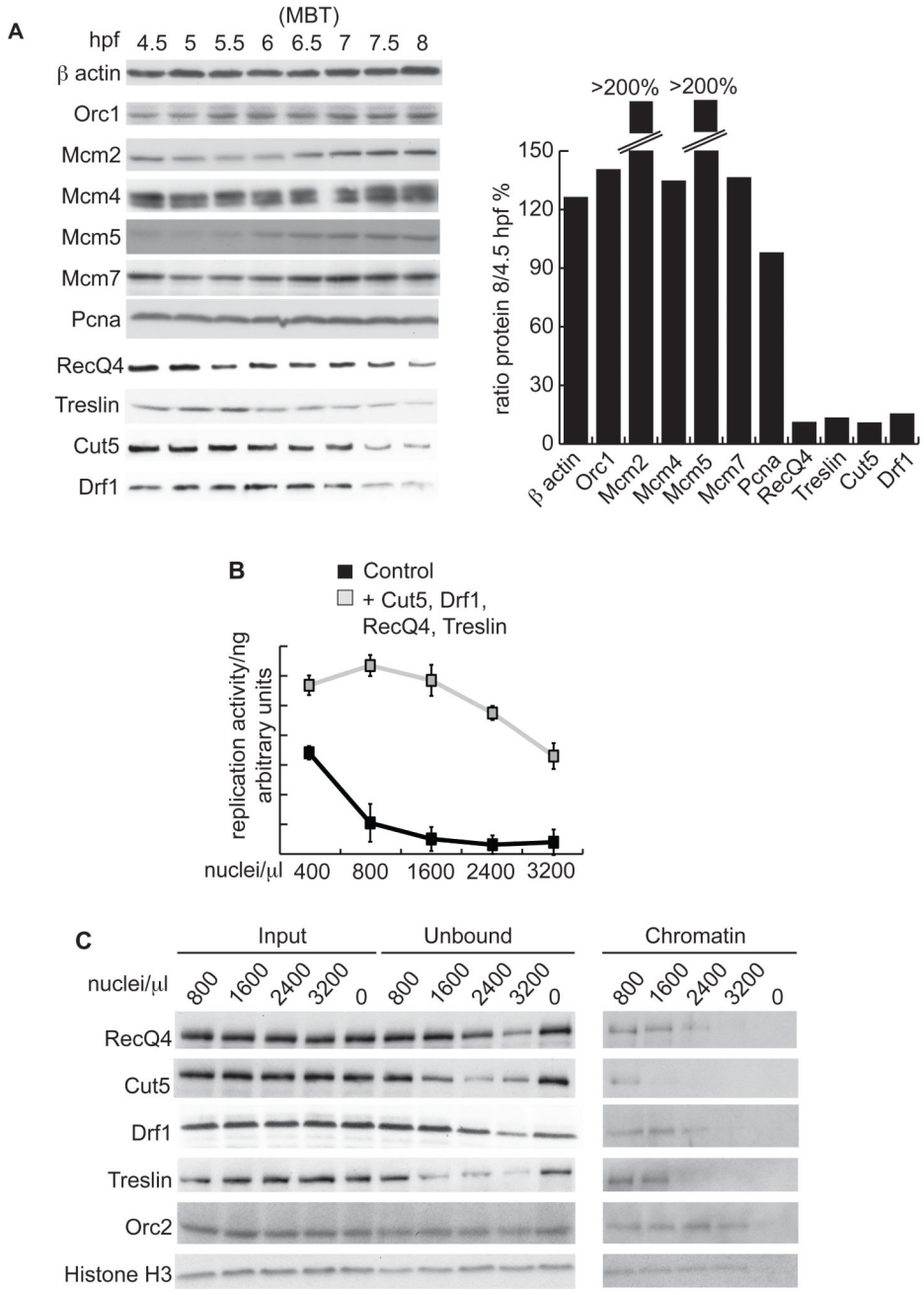

Figure 1. Initiation factors are limiting for replication *in vitro* in *Xenopus laevis*

A. (Left) Western blot of DNA replication factors from *Xenopus* embryos at the indicated hours post fertilization (hpf) (. The MBT occurs at 6 hours 30 mins (6.5). (Right) Quantification of western blots using Image J. Protein levels are represented as the ratio between 8/4.5 hpf. Pcna is required for DNA replication elongation and did not change in abundance during early embryogenesis.

B. Replication of *Xenopus* sperm chromatin nuclei in IVT diluted egg extract. Sperm chromatin was added at the indicated concentrations either supplemented with rabbit reticulocyte lysate + empty vector (control) or + vectors encoding *cut5, drf1, recq4* and

treslin (grey). The reactions were quantified relative to input DNA to represent replication activity per ng of sperm DNA. n=3, error bars show standard deviation.
C. Western blot of chromatin binding of replication factors at increasing sperm nuclei to extract ratios. *Xenopus* sperm nuclei of the indicated ratios were replicated for 15 mins in undiluted egg extract. Input and chromatin unbound fractions were equally loaded by volume, while the chromatin fractions were normalized to the same DNA concentration.

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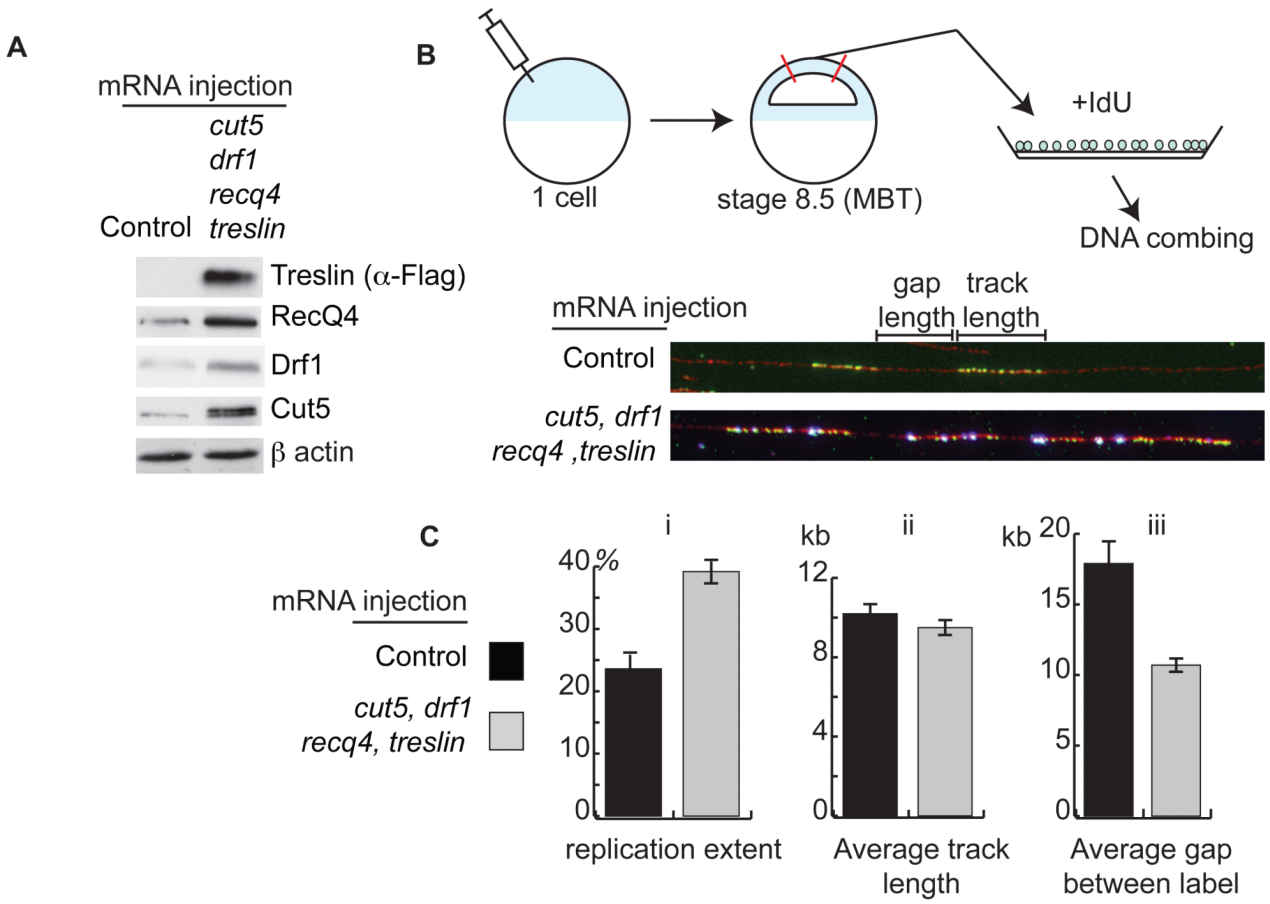

Figure 2. Cut5, Drf1, RecQ4 and Treslin are limiting for replication *in vivo* in *Xenopus laevis*

A. Western blot from stage 9 (post MBT) embryos either injected at the 1 cell stage with water (control) or with 300 pg mRNA each of *treslin*, *cut5*, *recq4*, and *drf1*.

B. (Top) sSchematic representation of experimental design. *Xenopus* embryos were injected at the one cell stage as in A in the animal pole. At 6.5 hpf (stage 8.5) animal caps were cut and their cells dissociated. The cells were given a pulse with IdU and incorporation was visualized by anti IdU immunofluorescence after stretching DNA onto slides (bottom, DNA-red, IdU-green).

C. Analysis from B of (i) replication extent (%), (ii) average IdU track length (kb) and (iii) average gap between label (kb). n = 96 and 93 fibers for control and overexpressing embryos, respectively.

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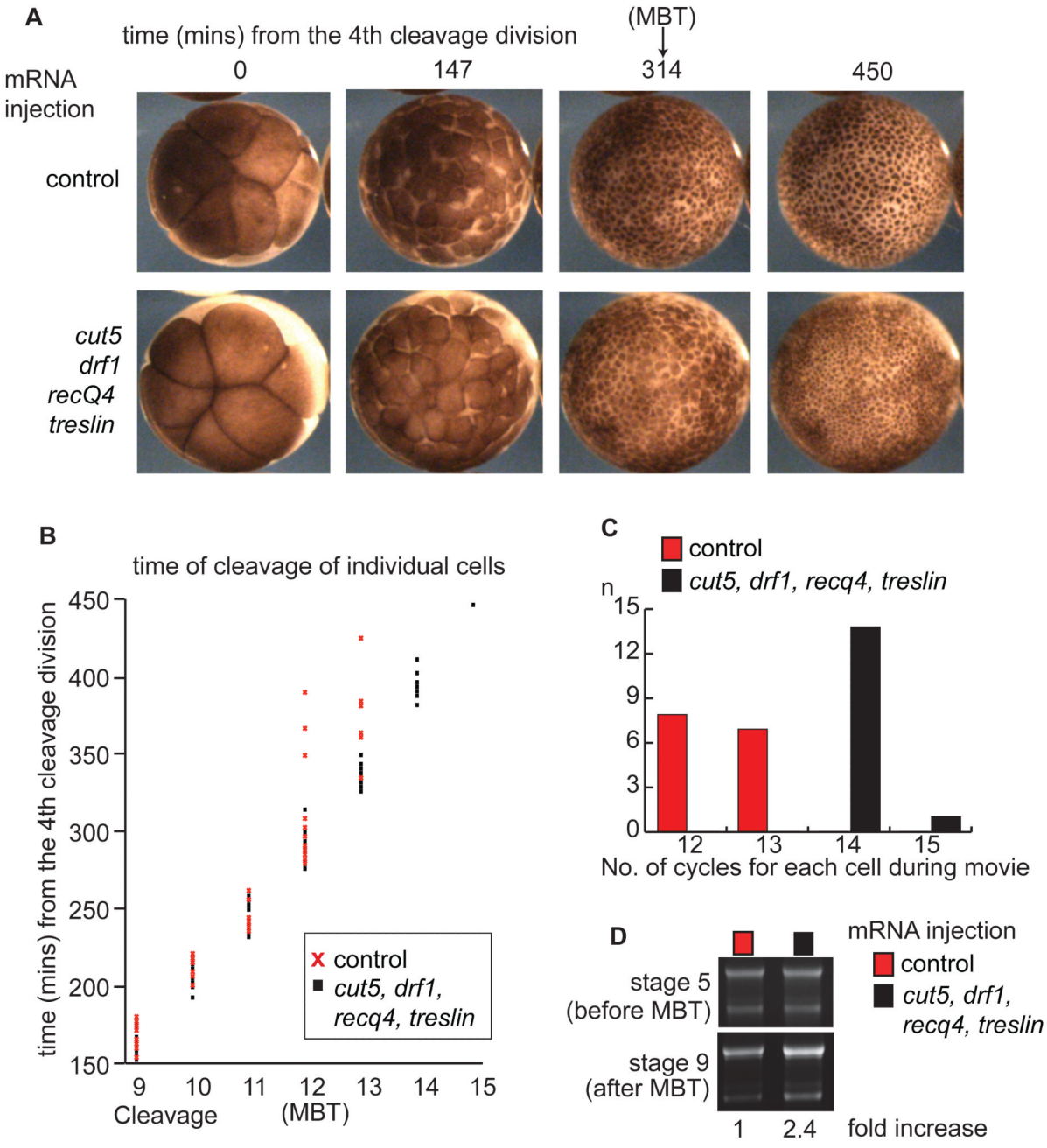

Figure 3. Overexpression of Cut5, Drf1, RecQ4 and Treslin causes extra rapid cell divisions after the MBT

A. Images taken from a time-lapse movie (Movie S1) of embryos injected at the 1 cell stage as in Fig.2A. Cleavage 4 at the 16-cell stage was set to time zero.

B. 15 individual cells from the embryos in Movie S1 were followed through early divisions until 450 minutes after cleavage 4. Each time point corresponds to the cleavage of an individual cell. Cleavages 1-8 are excluded for simplicity.

C. Representation of the number of cell cycles each cell in B undergoes until 450 minutes after cleavage 4.

D. Embryos were injected as in A 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 using ImageJ and fold increase relative to control is represented.

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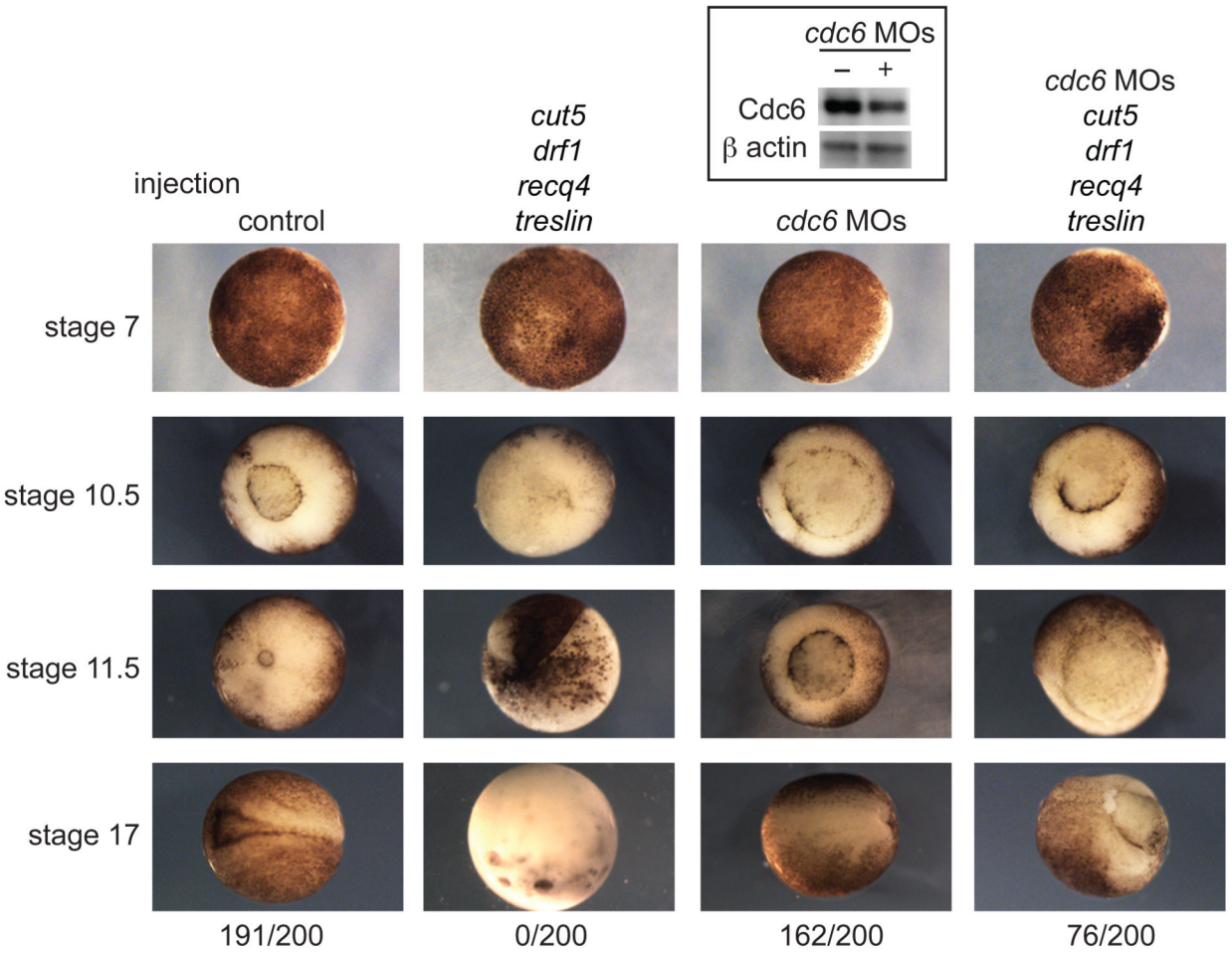

Figure 4. Increasing inter-origin distances at the MBT is critical for gastrulation and neurulation

Images of embryos injected with water (control - first column), *treslin*, *cut5*, *recq4*, and *drf1* mRNA (second column), *cdc6* morpholinos (MOs – third column) and embryos injected with a mixture of the *cdc6* morpholinos and mRNA for *treslin*, *cut5*, *recq4*, and *drf1* (fourth column). Stage 7 = pre-MBT, 10.5 = gastrulation, 11.5 = mid-gastrulation, 17 = neurula. The number of embryos out of 200 surviving at stage 17 is indicated at the bottom. (Top) Western blot of knockdown of Cdc6 after morpholino injection.