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Tipin/Tim1/And1 protein complex promotes Polα chromatin binding and sister chromatid cohesion

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The Tipin/Tim1 complex plays an important role in the S-phase checkpoint and replication fork stability. However, the biochemical function of this complex is poorly understood. Using *Xenopus laevis* egg extract we show that Tipin is required for DNA replication in the presence of limiting amount of replication origins. Under these conditions the DNA replication defect correlates with decreased levels of DNA Polα on chromatin. We identified And1, a Polα chromatin-loading factor, as new Tipin-binding partner. We found that both Tipin and And1 promote stable binding of Polα to chromatin and that this is required for DNA replication under unchallenged conditions. Strikingly, extracts lacking Tipin and And1 also show reduced sister chromatids cohesion. These data indicate that Tipin/Tim1/And1 form a complex that links stabilization of replication fork and establishment of sister chromatid cohesion.

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Introduction

Chromosomes are duplicated with remarkable speed and accuracy, with no molecules of DNA left unreplicated or replicated more than once. To allow complete replication of large eukaryotic chromosomes, cells license thousands of replication origins distributed along the chromosome. Origin licensing consists of recruitment of the essential helicase activity, Mcm2–7, on the DNA following coordinated binding of origin-recognition complex (ORC), Cdc6 and Cdt1 (Gillespie *et al*, 2001). Once two adjacent origins fire, the two converging forks have to progress until they meet to ensure the complete replication of the DNA segment between the origins. Forks can stall if they encounter DNA damage. Under these conditions a replication-pausing complex stabilizes the stalled forks, while a checkpoint response is activated to halt cell-cycle progression and to allow fork restart following DNA repair (Branzei and Foiani, 2005). Tipin, together with Tim1 and Claspin, is a member of the replication-pausing complex and is important to mediate the intra-S-phase checkpoint (Ito *et al*, 2001; Mayer *et al*, 2004; Noguchi *et al*, 2004; Nedelcheva *et al*, 2005; Chou and Elledge, 2006; Krogan *et al*, 2006; Gotter *et al*, 2007; Unsal-Kacmaz *et al*, 2007; Yoshizawa-Sugata and Masai, 2007). Studies of Claspin and Tim1 orthologues in yeast, respectively Mrc1 and Tof1, showed that they are also important for control of normal progression of DNA replication (Hodgson *et al*, 2007). Furthermore, a reduction in the expression levels of mammalian Tim1 results in decreased rate of DNA synthesis (Chou and Elledge, 2006; Gotter *et al*, 2007; Unsal-Kacmaz *et al*, 2007; Yoshizawa-Sugata and Masai, 2007). All these data clearly indicate that Tipin and Tim1 are active components of the replication fork, and that beyond their well-established role in fork stabilization they may have a more direct role during DNA replication. An essential additional mechanism to ensure full replication under stress is achieved by licensing many more origins than the ones that will be actually used. These supplementary licensed origins remain 'dormant' during S-phase (Blow and Ge, 2008) and fire only under stress when replication forks are stalled or slowed ensuring complete genome replication (Woodward *et al*, 2006; Ge *et al*, 2007; Blow and Ge, 2008; Ibarra *et al*, 2008).

We have investigated the role of Tipin during DNA replication using *Xenopus laevis* egg extract. We found that Tipin is required for efficient DNA synthesis. Tipin's role becomes evident when dormant origins are suppressed. Under these conditions, absence of Tipin leads to drastic reduction in the level of Polα on the chromatin. We have also identified And1 (Ctf4) as a new binding partner of Tipin, and demonstrate that these two proteins collaborate in the loading and/or stabilization of Polα on DNA. In addition, the Tipin/And1-depleted extract show loosening of sister chromatid cohesion. This is in agreement with the cohesion defect already observed in the yeast orthologue mutants, *csm3* (Marston *et al*, 2004; Mayer *et al*, 2004; Xu *et al*, 2004) and *ctf4* (Hanna *et al*, 2001; Petronczki *et al*, 2004; Zhou and Wang, 2004; Xu *et al*, 2007). These data provide biochemical evidence that factors involved in DNA replication and checkpoint signalling also contribute to establishment and maintenance of chromosome cohesion (Suter *et al*, 2004; Warren *et al*, 2004).

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Results

Tipin is required for DNA replication under minimal licensing condition

We have previously shown that Tipin is associated with the replication fork and is required for fork stability and fork restart (Errico *et al*, 2007). However, its role during DNA replication is unclear. Reduction of Tipin/Tim1 levels in human cells by siRNA resulted in a delay in S-phase progression (Chou and Elledge, 2006; Unsal-Kacmaz *et al*, 2007; Yoshizawa-Sugata and Masai, 2007), whereas depletion of Tipin (and consequently Tim1) from *Xenopus* egg extract had no measurable impact on DNA replication (Errico *et al*, 2007).

Previous work using different eukaryotes, including *X. laevis* and humans, demonstrated that Mcm2–7 complexes are loaded on the chromatin in a 20-fold excess over the number of chromatin-bound ORC molecules and over the number of active replication origins (Edwards *et al*, 2002; Oehlmann *et al*, 2004). Many hypotheses have been proposed about the possible function of this excess of MCM, termed as the 'MCM paradox' (Hyrien *et al*, 2003). Blow and co-workers showed that one of these functions is to license 'dormant' replication origins that are inactive during unperturbed S-phase, but can be used to allow complete DNA replication under conditions of replicative stress (Woodward *et al*, 2006; Ge *et al*, 2007; Ibarra *et al*, 2008).

We reasoned that in an embryonic cell system, such as *X. laevis* egg extract, in which there are more origins than in somatic cells, the presence of dormant origins could mask a replication defect phenotype in the Tipin-depleted extract. It has been shown that the addition of Geminin (a Cdt1 inhibitor that prevents MCM loading on the chromatin) to the extract shortly after addition of sperm nuclei reduces the number of MCM complexes loaded on the DNA to a minimum required to support efficient DNA replication without altering the inter-origin distance (Woodward *et al*, 2006). This condition is referred as 'minimum licensing'; the amount of MCM on the chromatin is reduced to 10% when compared with the normal 'excess' condition, which is also referred to as 'maximum licensing' (Woodward *et al*, 2006; Ge *et al*, 2007; Blow and Ge, 2008; Ibarra *et al*, 2008). To test our hypothesis and better dissect the role of Tipin at the replication fork, we have assessed the efficiency of DNA replication in the Tipin-depleted extract under 'minimum licensing' conditions (Figure 1A–C). Addition of Geminin to the mock or Tipin-depleted extract 3 min after addition of sperm nuclei reduced the amount of MCM on the DNA as expected (Supplementary Figure S1A). We have performed a time-course experiment (Figure 1B) and measured the efficiency of DNA replication at 120 min post nuclei addition (Figure 1C). We found that, under minimal licensing conditions the Tipin-depleted extract showed 70% reduction in the overall efficiency of DNA replication (Figure 1B and C, lane 2). The defect was specific, since it could be rescued by adding back Tipin/Tim1 recombinant protein (Figure 1B and C, lane 3) (since Tipin depletion concomitantly depletes most of Tim1; Errico *et al*, 2007) or mock-depleted extract (Figure

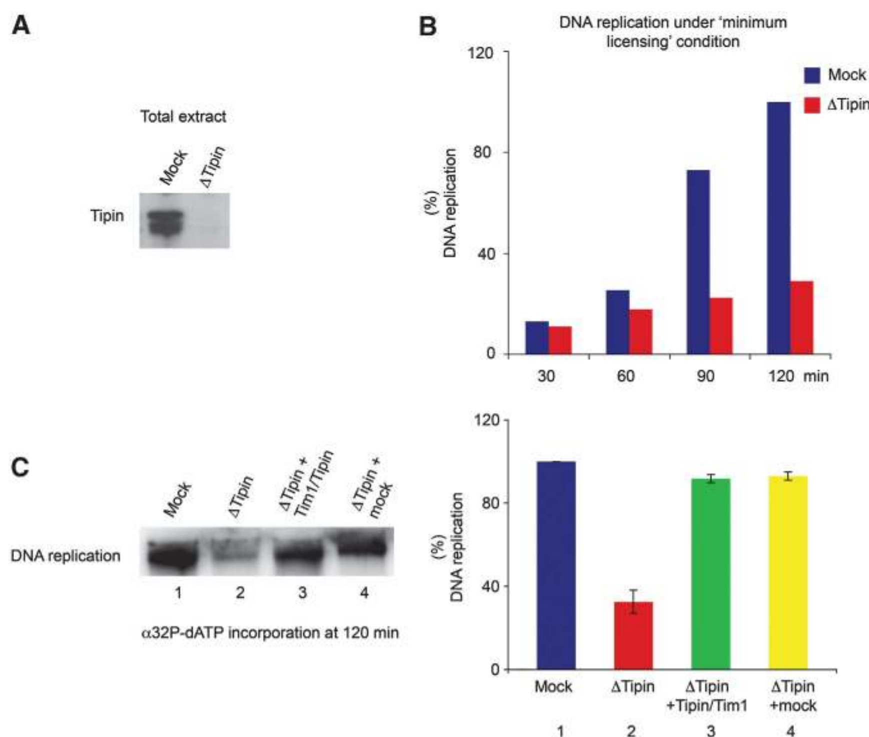

Figure 1 Tipin is required for efficient DNA replication under 'minimum licensing' conditions. (**A**) Immunoblot to assess Tipin depletion from egg extract. (**B**) Time course of DNA replication in 'minimum licensed', mock- (blue bar) and Tipin-depleted extract (red bar) (right panel). (**C**) The efficiency of DNA replication in 'minimum licensed', mock- (lane 1) and Tipin-depleted extract (lane 2) at 120 min post nuclei addition was tested. The defect in DNA replication observed in the Tipin-depleted extract (lane 2) was rescued by the addition of Tipin/Tim1 recombinant protein complex (lane 3) or mock extract (lane 4). Three independent experiments are averaged in the bar graphs. The error bars are standard deviation from the mean value.

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1B and C, lane 4). We have also measured the efficiency of DNA replication in the mock and Tipin-depleted extract in the presence of 10 000 nuclei per microlitre, a condition known to increase the inter-origin distance (Walter and Newport, 1997). Surprisingly, under these conditions we were not able to detect defects in DNA replication in the Tipin-depleted extract (Supplementary Figure S1B), suggesting that replisomes can travel longer distance even in the absence of Tipin.

Tipin interacts with Polα and is required for its association with the chromatin under 'minimum licensing' conditions

One of the proposed roles for Tipin and Tim1 is to couple the helicase with the replisome. This would minimize excessive unwinding of the DNA (Errico *et al*, 2007; Gotter *et al*, 2007). Consistent with this hypothesis, we have previously demonstrated an interaction between Tipin and Mcm7 (Errico *et al*, 2007) in *X. laevis*, while others have shown the association of Tipin/Tim1 complex with other member of the replisome such as Mcm6, Mcm7 (Chou and Elledge, 2006), Mcm2, RPA, Polδ and Polε (Gotter *et al*, 2007) in human cells. We next asked whether Tipin was also able to interact with DNA polymerases in *X. laevis* egg extract; indeed Tipin immunoprecipitates, both from extract (Figure 2A) and nuclei (Supplementary Figure S2), were found to contain Polα (p180). In order to determine the role of Tipin during DNA replication, we took advantage of the 'minimum licensing' conditions and asked whether the loading of replisome components on the chromatin was defective. Tipin was depleted from an interphase *Xenopus* eggs extract (Figure 2B) and chromatin was isolated under 'maximum or minimum' licensing conditions (Figure 2C). We found that under 'minimum licensing' conditions the amount of Polα (p180) on the chromatin was greatly reduced in the Tipin-depleted extract (Figure 2C), whereas the level of Orc1 remained unchanged (Figure 2C). In addition we monitored the binding of key replication fork proteins such as Cdc45 and Sld5 of the GINS complex. We found that under minimum licensing conditions the binding of these proteins was unaffected in the presence or absence or Tipin (Figure 2C). These data suggest that under these conditions replication fork structures are preserved, and that the Tipin/Tim1 complex is specifically required to promote stabile binding of Polα (p180) to replication forks.

Analysis of replication forks in the Tipin-depleted extract by molecular combing

We next examined origins distribution and fork progression in the mock or the Tipin-depleted extract, under 'maximum or minimum' licensing conditions, by molecular combing (Herrick *et al*, 2000; Marheineke and Hyrien, 2001, 2004). Sperm nuclei were incubated in egg extract (mock or Tipin-depleted) supplemented with digoxigenin–dUTP, in order to label the entire replicated DNA. Biotin–dUTP was added at different times (40 and 60 min) after the addition of digoxigenin and DNA replication was allowed to advance to completion (up to 120 min). For these experiments, replication initiation zones are defined as regions replicated prior to addition of biotin–dUTP and, therefore, will appear as digoxigenin-positive, but biotin-negative (gap), tracts (also defined as the replication eye) (Figure 3A). The inter-origin distance (eye-to-eye distance, ETED) is calculated as the distance between midpoints of adjacent eyes (Marheineke and Hyrien, 2004; Labit *et al*, 2008).

Overall, we found that both under 'maximum' or 'minimum' licensing conditions the peak of the distribution of inter-origin distance was approximately 10–20 kb, consistent with previous observations (Woodward *et al*, 2006), although

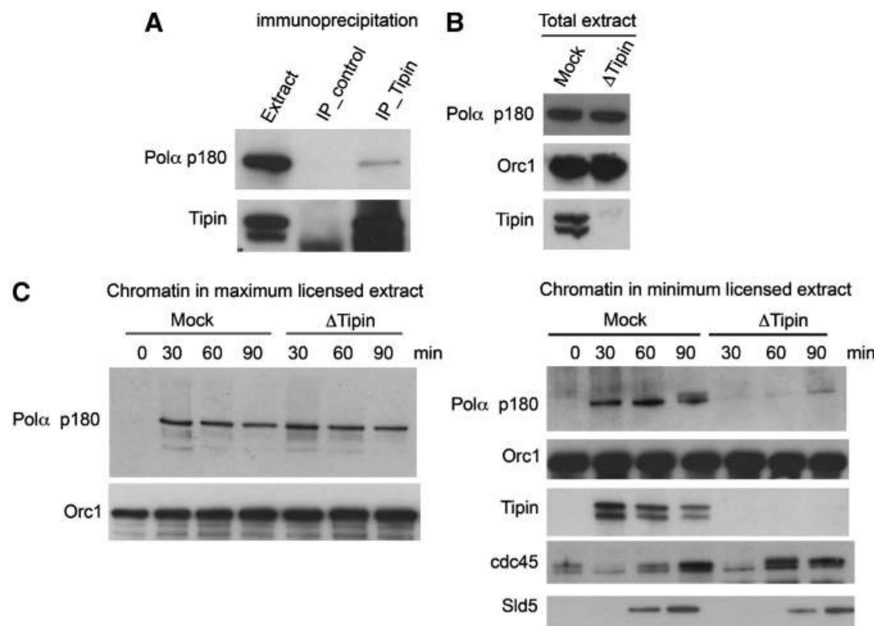

Figure 2 Tipin is required for Polα loading on the chromatin in 'minimum licensing' condition. (**A**) *Xenopus* extract was immunoprecipitated with either anti-Tipin or preimmune serum. Samples were probed with anti-Polα and anti-Tipin antibodies. (**B**) Immunoblot analysis to detect the level of Polα, Orc1 and Tipin in total extract (mock or Tipin depleted). (**C**) Immunoblot to detect the level of Polα, Orc1, cdc45, Sld5 and Tipin on the chromatin, at different time points (30, 60, 90 min), under 'maximum' (right panel) or 'minimum' licensing (left panel) condition.

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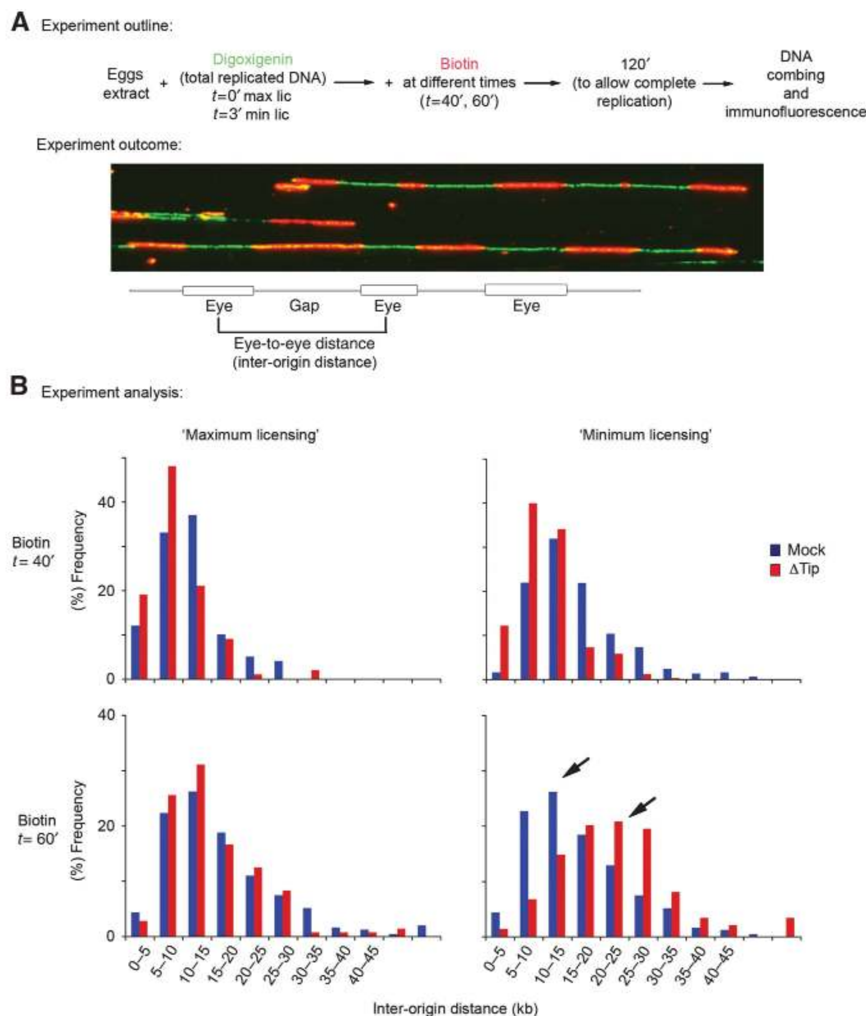

Figure 3 Analysis of inter-origin distance in the Tipin-depleted extract by molecular combing. (**A**) Scheme of the molecular combing experiment and visualization by immunofluorescence of DNA fibres. Green tracts represent origins of DNA replication (replication eye). (**B**) Distribution of inter-origins distance under 'maximum' and 'minimum' licensing condition in samples where biotin–dUTP was added at $t = 40$ min or at $t = 60$ min. The mock extract is represented by the red bar and the Tipin-depleted extract by the blue bar. Black arrows indicate a shift of the peak of distribution of inter-origins distances towards higher value in the 'minimum licensed', Tipin-depleted extract at $t = 60$ min. The difference between the distribution of inter-origin distance in the mock- and Tipin-depleted extract observed at 60 min in minimum licensing condition is statistically significant (t-test $P < 0.0001$).

minor differences attributable to kinetic variability and increased stressful condition due to the depletion procedure could be seen in mock-depleted extracts at 40 min. During early S-phase, the Tipin-depleted extract exhibited a higher frequency of shorter inter-origin distance when compared with the mock sample (Figure 3B), both in the presence or the absence of dormant origins ('maximum' or 'minimum' licensing). This phenomenon may be due to a defect in the intra-S-phase checkpoint (Errico *et al*, 2007) that normally suppresses origin firing (Shechter *et al*, 2004). Later in S-phase, there is no further substantial difference in the distribution of the inter-origin distance between the mock and depleted extract under 'maximum licensing' conditions. The peak of this distribution was instead shifted towards higher values in the Tipin-depleted extract under 'minimum licensing' (Figure 3B). To exclude the possibility that the shift towards higher inter-origin distance was due to merging of replication forks occurring at 60 min, due to eventual modifications of the fork rate, we calculated the average 'eye length' and the fork speed for each sample. Importantly, we found that there was no substantial difference in the various samples (mock or depleted, minimum or maximum licensing, comparing the same time points) (Supplementary Table S1). We have also plotted the average inter-origin distance (ETED) per fibre over the average eye-length (EL) per fibre for each sample (Supplementary Figure S3) and confirmed that only in the 'minimum licensed' ΔTipin sample (at 60 min) there is significant increase in fibres having large ETED (>25 kb) and small EL (<10 kb) values. These distances cannot be the result of merged forks, which would have instead caused an increase in the number of fibres with large ETED and high EL. Therefore, the observed shift of the peak of inter-origins distance in minimum licensed Tipin-depleted extract at 60 min possibly reflects the reduced efficiency of DNA replication due to a reduced number of active origins. This could be explained by the requirement for Tipin in promoting Polα binding to chromatin. We cannot also exclude sporadic collapse of a

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higher number of replication forks in Tipin-depleted extract, consistent with the role of Tipin in maintaining fork stability (Chou and Elledge, 2006; Errico *et al*, 2007). In any case, the biochemical data showing the presence of replisome components such as GINS and Cdc45 under minimal licensing condition without Tipin (Figure 2D) exclude the occurrence of massive fork collapse.

A new Tipin-interacting partner: And1

In order to understand the molecular basis of the interaction between Tipin and Polα, we performed immunoprecipitation experiments using egg extract using anti Tipin antibodies (Errico *et al*, 2007). Co-immunoprecipitated proteins were then fractionated by SDS–PAGE and analysed by mass spectrometry. Together with the Mcm2–7 complex, we identified And1 (data not shown) as a new Tipin-interacting protein. And1 orthologues in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively Ctf4 and Mcl1, have been shown to interact with DNA Polα (Miles and Formosa, 1992; Formosa and Nittis, 1999; Zhou and Wang, 2004; Tsutsui *et al*, 2005). Recently, Dutta and co-workers have shown that mammalian And1 interacts with both Mcm10 and DNA Polα. They also showed that in *Xenopus* egg extract Mcm10 is required for loading of And1 on chromatin and both proteins are required for recruitment of DNA Polα at the replication fork (Zhu *et al*, 2007). From this perspective the interaction between Tipin and And1 is intriguing and may contribute to explaining why under 'minimum' licensing condition Tipin-depleted extracts are defective in the association of DNA Polα with the chromatin.

To explore this possibility, we examined whether the Tipin–And1 interaction was occurring in egg extract. We found that Tipin immunoprecipitates from extract specifically contained And1 (Figure 4A) and that also And1 was able to co-immunoprecipitate Tipin (Figure 4A). The interaction was also confirmed by immunoprecipitation from nuclei (Supplementary Figure S2). This indicates that Tipin interacts with And1 *in vivo*. To address whether the interaction between Tipin and And1 was direct, we performed pull-down experiments using recombinant proteins. We found that differently from GST alone, GST–And1 is directly interacting with Tipin–6His (Figure 4B). Since both Tipin and And1 were able to co-immunoprecipitate Polα, we asked whether the interactions between Polα and these proteins were direct. We have performed pull-down experiments using GST, GST–And1 or GST–Tipin recombinant proteins and *in vitro* transcription translated ^{35}S-labelled Polα–p180 subunit. We showed that both GST–Tipin and GST–And1, but not GST alone, were able to pull down Polα–p180 (Figure 4C), suggesting that both Tipin and And1 are directly interacting with Polα.

Tipin and And1 are necessary for DNA replication

To understand the relevance of a Tipin/And1 interaction, we performed double depletion experiments using egg extract. Both antibodies, anti-Tipin and anti-And1, efficiently depleted the respective proteins from the extract (Supplementary Figures S4 and S5). We prepared Tipin- or And1-depleted extracts, as well as Tipin/And1 double-depleted extracts, and tested them for their ability to support DNA replication. As previously shown, Tipin depletion did not affect DNA replication (Errico *et al*, 2007), while And1

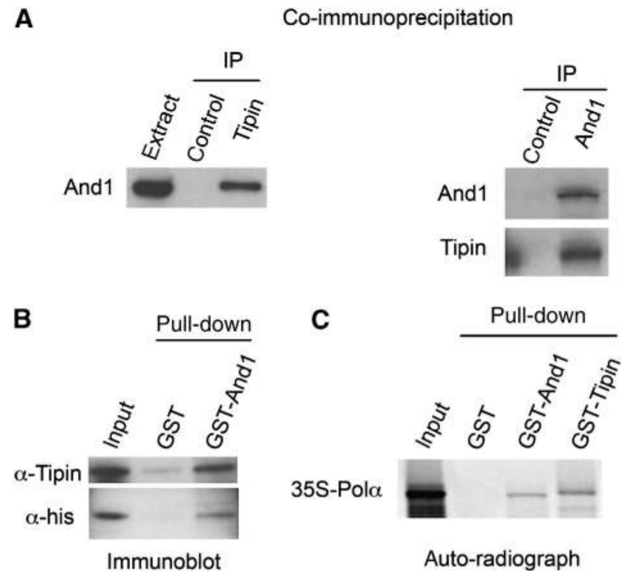

Figure 4 Tipin, And1 and Polα directly interact. (**A**) Equal amounts of extract were immunoprecipitated with either anti-Tipin, anti-And1 antibodies or pre-immune serum. Purified proteins were immunoblotted with the indicated antibodies to detect associated proteins. (**B**) Pull-down assay using GST or GST–And1 glutathione–Sepharose beads and recombinant Tipin–6His. The presence of Tipin–6His was detected both with anti-Tipin or anti-6His antibody. (**C**) Pull-down assay using GST, GST–And1 or GST–Tipin and *in vitro* translated ^{35}S-labelled Polα p180. Polα was detected by autoradiograph.

depletion induced moderate reduction in the efficiency of DNA replication (Zhu *et al*, 2007; Yoshizawa-Sugata and Masai, 2009). Interestingly, Tipin and And1 double depletion caused severe impairment of DNA synthesis, with a reduction of 70% in the overall efficiency of replication (Figure 5A). To understand the basis for such a defect in DNA replication, we analysed the loading on the chromatin of essential replication factors. We found that And1 and Tipin independently bind to chromatin with similar kinetics as the depletion of one protein does not significantly affect the binding of the other protein (Figure 5B). Moreover, Figure 5B shows that association of DNA Polα with chromatin is greatly reduced in Tipin/And1-depleted extract. This is also true, although to a lesser extent in And1 single depletion. Since previous data have also indicated the relevance of Mcm10 in such a process, we also checked Mcm10 level on the chromatin. We found no difference in the level of chromatin-bound Mcm10 (Figure 5C), consistent with its association with chromatin occurring before and independently of And1 (Zhu *et al*, 2007). Importantly, the binding of other replication proteins such as TopBP1 and Cdc45 was not significantly affected by the double Tipin–And1 depletion (Figure 5D). Taken together, these results clearly suggest that And1 and Tipin are specifically required for loading and/or stability of DNA Polα on the chromatin.

Tipin/And1-depleted extracts have a defect in sister chromatid cohesion

The two DNA molecules that arise from the replication fork must be held together until their separation in anaphase, a task performed mainly by a multi-protein complex named cohesin (Hirano, 2000).

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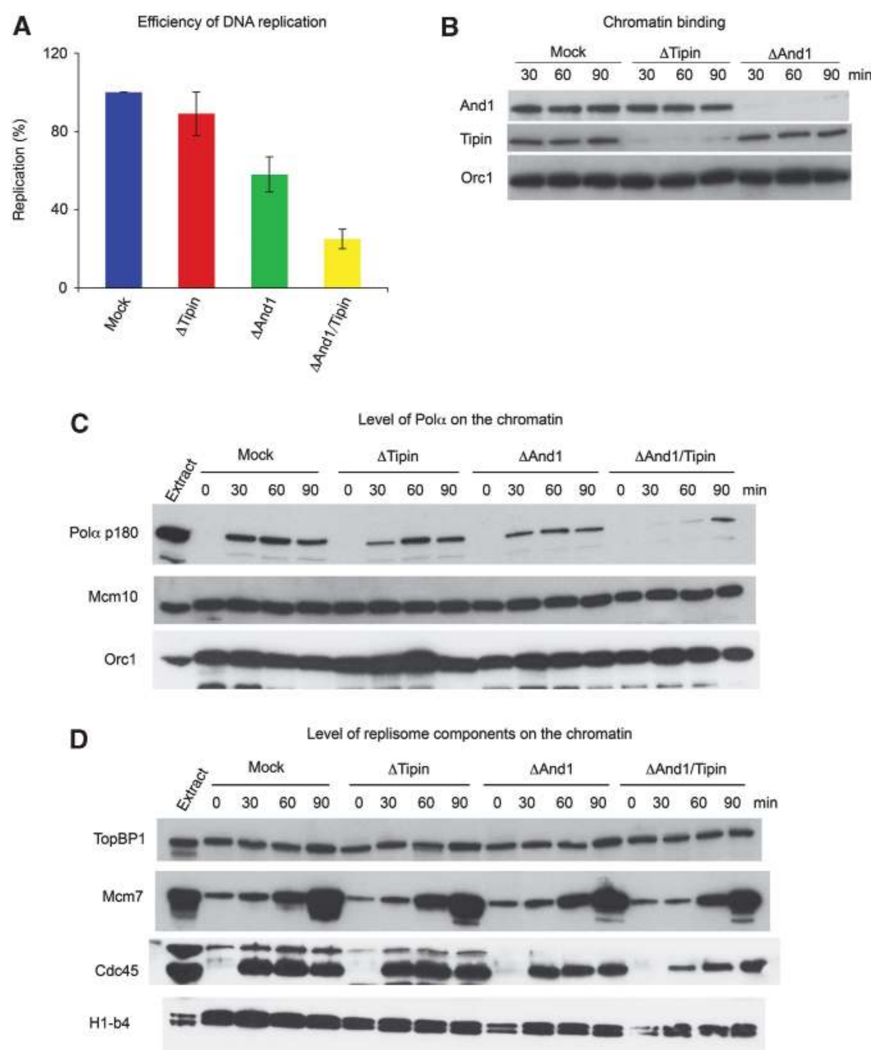

Figure 5 Tipin and And1 are both required for DNA replication and for the efficient association of Polα to the chromatin. (**A**) The efficiency of DNA replication was measured in mock and depleted extracts (ΔTip, ΔAnd1, ΔAnd1/Tip). Three independent experiments are averaged in the bar graphs. The error bars are standard deviation from the mean value. (**B**) Sperm nuclei were added to *Xenopus* extract, mock or depleted (ΔTip, ΔAnd1), and chromatin was harvested at different times. Chromatin-bound proteins were analysed by SDS–PAGE and immunoblot analysis with the indicated antibodies. (**C**) Immunoblot analysis to detect the level of Polα, Orc1 and Mcm10 on the chromatin, at different time points, in mock and depleted extracts (ΔTip, ΔAnd1, ΔAnd1/Tip). Orc1 staining was used for normalization. (**D**) Immunoblot analysis to detect the level of Mcm7, TopBP1, Cdc45 on the chromatin, at different time points, in mock and depleted extracts (ΔTip, ΔAnd1, ΔAnd1/Tip). Histone H1-b4 staining was used for normalization.

Several data suggest a role of replication-fork components in the establishment of sister chromatid cohesion. Many replication-associated proteins from budding yeast are known to support efficient sister chromatid cohesion (Hanna *et al*, 2001; Mayer *et al*, 2001, 2004; Naiki *et al*, 2001; Skibbens, 2004; Warren *et al*, 2004; Xu *et al*, 2004, 2007). Recently a genetic analysis has placed Ctf4 (And1), Csm3 (Tipin) and Tof1 (Tim1) in the same pathway leading to sister chromatid cohesion (Xu *et al*, 2007). Moreover, the same analysis led to the conclusion that Csm3 and Tof1 are required for establishment of cohesion in S-phase, but not for maintenance of cohesion during G_2/M (Xu *et al*, 2007). Thus, we next asked whether the role of these factors (Ctf4, Csm3 and Tof1) in establishment of cohesion was conserved in higher eukaryotes. To analyse cohesion in our experimental system, sperm nuclei were added to interphase extract, mock or depleted (ΔTip, ΔAnd1, ΔTip/And1), together with biotin–dUTP to monitor replication. Once replication was completed, the reaction mixtures were driven into mitosis by addition of 1 volume of CSF-arrested extract (mock), which promoted chromosome condensation. Chromosomes were then fixed, spun over coverslips and analysed by immunofluorescence with an antibody recognizing the condensin XCAP-E, which labels the axis of each chromatid (Figure 6A). Biotin incorporation was detected with an Alexa 594–streptavidin conjugate. The majority of chromosomes that had completed replication, as judged by incorporation of biotin–dUTP, had paired sister chromatids. However, there was a general loosening of the pairing between the sister chromatids in the chromosomes from the depleted extract (Figure 6A and B). The average distance between chromatids was larger for chromosomes assembled in Tipin- (0.60 ± 0.15), And1- (0.60 ± 0.16) and Tipin/And1 (0.75 ± 0.19)-depleted extracts as compared to those

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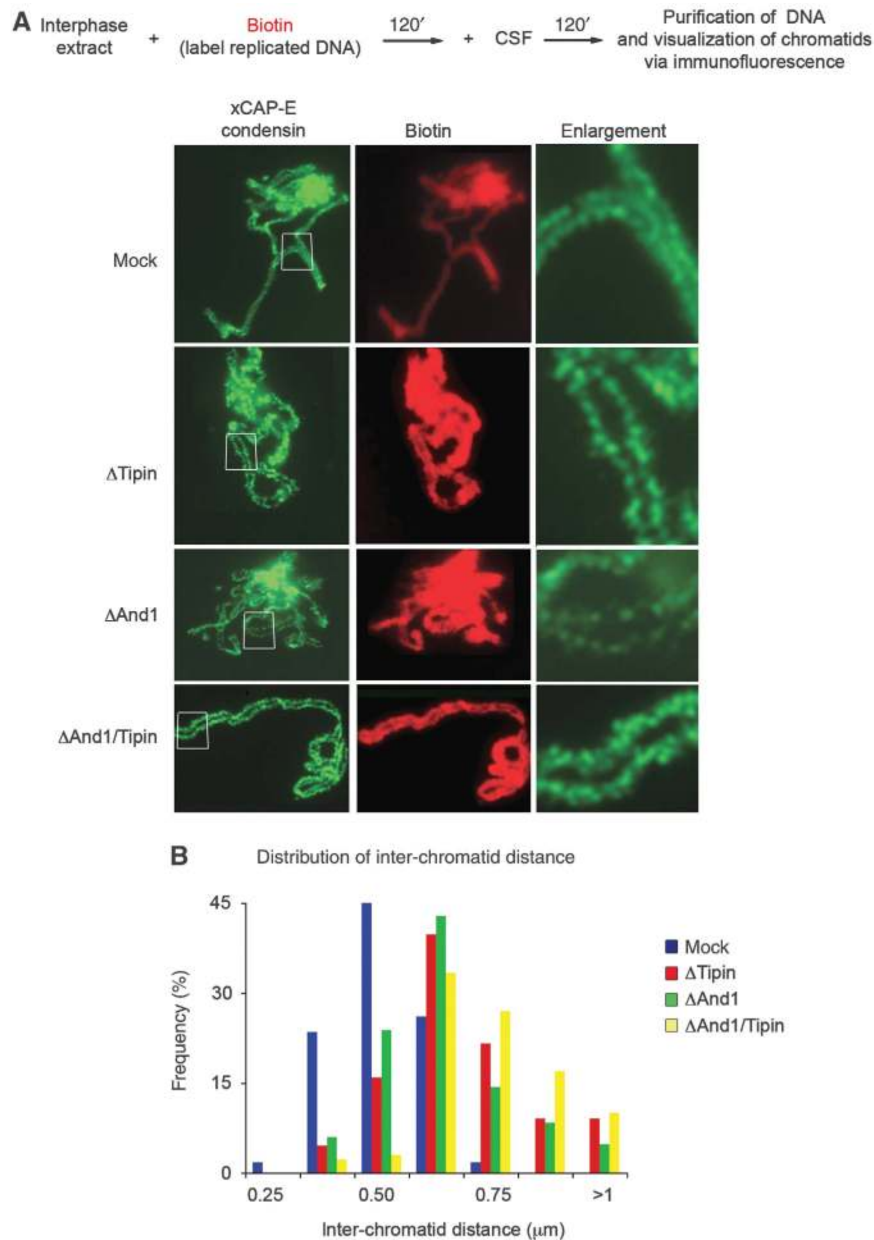

Figure 6 Tipin and And1 contribute to sister chromatid cohesion in S-phase. (**A**) Immunofluorescence analysis of mitotic chromosomes assembled in the indicated extract. Sister chromatids are stained with anti-condensin antibody, xCAPE (green). Biotin–dUTP was also added to the extracts to confirm that chromosome had replicated and detected with fluorescently labelled streptavidin (red). An enlarged section of the chromatids (boxed area) is also represented. (**B**) Distribution of the distance between sister chromatids in the different extracts.

assembled in the mock extract (0.43 ± 0.06). It is worth mentioning that the loosening of cohesion must have been generated in the depleted extract during DNA replication. Cohesion, indeed, could not be restored during chromosome compaction triggered by mitotic extract, although this extract was not depleted and, therefore, contained normal levels of And1 and Tipin. The cohesion defects observed were not due to impaired loading of cohesin on chromatin (Supplementary Figure S6). This suggests that inefficient establishment of sister chromatid cohesion results from a defect in the replication machinery rather than a structural problem with the cohesion apparatus, highlighting the intimate connection between DNA replication and sister chromatid cohesion.

Discussion

In this study, we report that, beyond its role in fork stability and checkpoint response, Tipin collaborates with And1 in the loading and/or stabilization of DNA Polα on chromatin. While And1's association with DNA Polα was already known (Zhu *et al*, 2007), we have demonstrated here that Tipin is able to directly interact with Polα. Tipin depletion does not significantly affect embryonic DNA synthesis under normal conditions (maximum licensing). However in the presence of fewer replication origins (minimum licensing), which mimic a condition closer to the somatic status, Tipin becomes an essential replication factor. During unchallenged

DNA replication many origins are initiated and the Tipin/Tim1 complex travels with the replication fork. In the event of the fork stalling, should enough forks stall a global checkpoint signal is activated that represses initiation at other sites throughout the genome and halts the cell cycle until replication can resume. However, in the presence of few stalled forks, if the checkpoint response is not strongly activated, nearby dormant origins could fire and rescue replication (Woodward *et al*, 2006). Under 'maximum licensing' conditions, we did not observe any difference in the efficiency of DNA replication between the mock- and Tipin-depleted extract. Our hypothesis is that under 'maximum licensing' conditions, when forks stall, the presence of nearby origins competent for initiation can rescue DNA replication even in the absence of Tipin/Tim1. When the number of competent origins is limited to the minimum required for efficient replication, the absence of Tipin/Tim1 becomes crucial. As shown by the molecular combing data, when in 'minimum licensed', Tipin-depleted extract forks stall, the extract reacts by firing more origins at early time points (early S-phase). However, later in S-phase, in the absence of dormant origins to maintain DNA replication, we detect a defect in the efficiency of DNA replication that correlates with increased inter-origin distance. A possible explanation to this observation is that in the absence of dormant origins stalled forks are prone to collapse due to the absence of the replication-pausing complex. Tipin-depleted extracts are indeed not only depleted of Tim1 but are also defective in loading Claspin onto the chromatin (Errico *et al*, 2007). However, biochemical analysis of replication fork proteins Cdc45 and Sld5 did not reveal significant differences in their binding to chromatin in the absence of Tipin, indicating that most of the fork structures are intact. This suggests that if fork collapse happens in the absence of Tipin, it is likely to be minimal and, therefore, to have a minor effect on DNA replication efficiency and increased inter-origin distance. The effects on DNA replication efficiency might be better explained by the deficient association of Polα (p180) to chromatin, which we show to require Tipin under 'minimum licensing' conditions. In this case the reduced level of And1, a key factor required for Polα loading, on 'minimum licensed' chromatin (data not shown) might render Tipin function essential for this task. In support of this hypothesis, both Tipin and And1 appear to be required to promote Polα chromatin binding as shown by a defect in Polα (p180) association with the chromatin observed under 'maximum licensing' conditions when both And1 and Tipin are depleted. The yeast orthologue of And1, Ctf4, was found to interact physically and genetically with Polα (p180) (Formosa and Nittis, 1999), and data on And1 (from mammalian cells and *Xenopus*) proved its role in contributing to the regulation of Polα binding to DNA (Zhu *et al*, 2007). We confirmed that And1-depleted extracts have a moderate defect in DNA replication and also a slight decrease in Polα loading. Strikingly, we detected a more severe defect in replication by co-depleting Tipin and And1. We could not detect any change in Mcm10 level on the chromatin, confirming that Tipin and And1 function downstream of this protein (Zhu *et al*, 2007). The idea that Tipin and And1 are both involved in modulating Polα association to DNA is also corroborated by the finding that Tipin, And1 and Polα are part of a large multi-protein complex (together with Claspin, Mcm10, Mcm2–7, Cdc45 and GINS) (Gambus *et al*, 2006). It has been shown recently that Ctf4 binds directly to Polα and GINS and is required to link Polα to the replication progression complex (Gambus *et al*, 2009) in budding yeast. Our findings provide novel biochemical evidence that Tof1/Csm3 (Tim1/Tipin) interact directly with Ctf4 (And1) and Polα and further confirms Ctf4–Polα interaction in higher eukaryotes.

The effect of the depletion of Tipin and And1 seems to be partially additive. As both proteins can independently bind Polα, the formation of a complex between Tipin and And1 might help to further stabilize Polα binding to chromatin under particularly stressful conditions, for example, when the number of available origins is limited. A recent report has demonstrated the requirement for Cdc45 in Tipin–Tim1 and And1 binding to chromatin (Tanaka *et al*, 2009). In light of these and our findings, the Tipin–Tim1 complex, which interacts with many of the players found at replication forks among which Cdc45, Polα, Mcm, And1, Claspin, GINS and SMC1–3 (Chou and Elledge, 2006; Errico *et al*, 2007; Gotter *et al*, 2007; Tanaka *et al*, 2009), might create a flexible bridge linking Cdc45, Polα, the GINS and the Mcm complex (Figure 7) necessary for stable binding of Polα to chromatin. This complex might be required for progression of the replisome under normal and stressful conditions. Intriguingly, we did not observe major DNA replication defects in the absence of Tipin when inter-origin distance was artificially increased by the addition of a high number of sperm nuclei to egg extract. This suggests that once replication fork have been established, they can travel long distance even in the absence of Tipin complex. It is possible that the replication defects observed with minimum licensed chromatin are also in part due to a defect in replication fork restart, which requires Polα-mediated re-priming of leading-strand synthesis. This function might be important for Tipin-mediated checkpoint activation in response to stalled forks. In this case Tipin could play a role similar to TopBP1, which has been shown to promote Polα chromatin binding, an event that appears to be essential to recruit the 9-1-1 complex to the stalled fork and promote activation of the replication checkpoint (Yan and Michael, 2009).

The physical and functional interaction between Tipin and And1 is not only limited to DNA replication. Genetic data from the yeast orthologues suggest that Ctf4 (And1) and Csm3 (Tipin) are also important for sister chromatid cohesion (Xu *et al*, 2007). We showed that in *Xenopus* egg extract depletion of Tipin, Tim1 and And1 causes a general loosening of the pairing of sister chromatids. Similar results were also reported by Tanaka *et al* (2009). Our experiments indicate that the cohesion defect was generated in S-phase. Importantly, we did not observe any striking alteration in the amount of cohesins present on interphase chromatin assembled in the depleted extracts. Thus, it is most likely that the problem stems from the process of cohesion establishment that is coupled to replication fork progression (Skibbens *et al*, 1999; Skibbens, 2005; Lengronne *et al*, 2006). Two different models have been proposed for the establishment of cohesion. One model foresees that cohesion is established by the sliding of the fork through the cohesin ring. In an alternative model, cohesin has to dissociate from DNA to allow the passage of the replication fork, but is held in close proximity of the fork to re-associate with DNA

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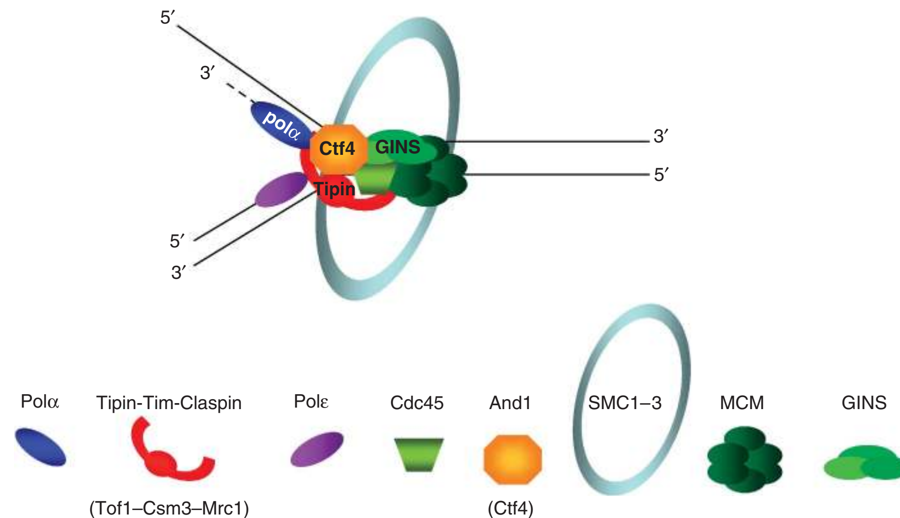

Figure 7 Model for Tipin–And1–Polα function. Tipin binds directly to And1 and Polα. And1 also binds Polα and GINS directly. Tipin/Tim1/And1 might create a flexible bridge between replisome components such as Cdc45, GINS, Polα and the MCM complex necessary for the stable binding of Polα to the replication fork. The cohesin ring is also represented.

immediately after, thus ensuring pairing of the replicated chromatids (Lengronne *et al*, 2006). Although depletion of Tipin, Tim1 and/or And1 does not affect the loading of cohesin on DNA, it is possible that their absence alters the topology of the replication fork, preventing it from sliding through the ring. Alternatively, if cohesins have to briefly dissociate from DNA to allow the passage of replication fork, it is possible to envisage a situation in which the interaction between cohesin subunits and members of the replication complex can help in holding cohesin in proximity of the fork to facilitate its re-association with the two sister chromatids after fork passage. In support of the latter, it has been shown that in *Caenorhabditis elegans* Tim1 interacts with Smc1 (Chan *et al*, 2003), and that human And1 interacts with cohesin in human cells (Yoshizawa-Sugata and Masai, 2009). Data obtained from *Xenopus* showing interaction of SMC1 and Tim1 also support a role for Tipin complex and SMC1 interaction in cohesion (Tanaka *et al*, 2009).

The results from FRAP analyses of GFP-tagged cohesin in vertebrate cells suggest that the binding mode of cohesin to chromatin changes upon DNA replication so that cohesin is stabilized and its exchange rate is dramatically decreased (Gerlich *et al*, 2006). Cohesin-interacting factors such as Pds5, Wapl and Sororin do not affect the amount of cohesin loaded on chromatin, but do affect its chromatin-binding behaviour (Losada *et al*, 2005; Rankin *et al*, 2005; Kueng *et al*, 2006). For example, depletion of Sororin has been shown to decrease the fraction of stably chromatin-bound cohesin and to result in cohesion defects (Schmitz *et al*, 2007). Thus, passage of the replication fork might modify cohesin and the way it interacts with its closely associated factors. In this regard, acetylation of Smc3 by Eco1/Ctf7 has recently been proposed to be a key step in cohesion establishment (Ben-Shahar *et al*, 2008; Zhang *et al*, 2008; Rowland *et al*, 2009; Sutani *et al*, 2009). The acetyltransferase travels with the fork and its activity could depend, at least partially, on other replisome components such as Tipin and And-1. These data provide new biochemical insights directly linking replication-fork components such Tipin, Tim1 and And1 to establishment of sister chromatid cohesion and maintenance of genome stability.

Materials and methods

Plasmids and recombinant protein

Expression plasmids for Tipin were previously described (Errico *et al*, 2007). Human Tim1 recombinant protein was a kind gift from FM Pisani (IBPE, Naples, Italy). The plasmid (pCMV-SPORT6) carrying *X. laevis* Polα p180 full-length cDNA was obtained from ImaGenes (Germany), IRAKp961B05331Q.

Antibodies

Polyclonal Tipin antiserum has been previously described (Errico *et al*, 2007). Monoclonal xAnd1 antibody was purchased from Acris (clone 23-5-14). Additional antibodies used in this study included antibodies against Mcm7 (Santa Cruz Biotechnology), polα p180 (Abcam) and Smc3 (Abcam). Rabbit polyclonal TopBP1, Cdc45 and Sld5 were a kind gift from H Takisawa (University of Osaka, Japan). Monoclonal ORC1 antibody TK15 was generated previously in the Tim Hunt's laboratory (Tugal *et al*, 1998); the Mcm10 antibody was a kind gift from J Walter (Harvard Medical School). Antibodies against xSmc1, xSa1 and xCAP-E were previously described (Rivera and Losada, 2009).

Xenopus *egg extracts and chromatin isolation*

To isolate the chromatin fractions, sperm nuclei (4000 nuclei per microlitre) were added to 40 μl of egg extracts for appropriate time points (30, 60 or 90 min). For immunoblotting, samples were diluted with 10 volumes of EB (100 mM KCl, 2.5 mM $MgCl_2$, and 50 mM HEPES–KOH (pH 7.5)) containing 0.25% NP-40 and centrifuged through a 30% sucrose layer at 10 000 *g* at 4°C for 5 min. Pellets were suspended in sample buffer loaded on a SDS–PAGE.

DNA replication assay

Sperm nuclei (4000 or 10 000 nuclei per microlitre) were added to 20 μl of egg extract (mock or depleted). Samples were supplemented with 0.1 μl of α^{32}P-dATP and incubated at 23°C for 2 h. Replication was stopped and analysed by agarose gel electrophoresis and autoradiography. For quantitation, the intensity of the radioactive band was measured with the phosphoimager analysis programme (Amersham).

Immunodepletion and immunoprecipitation

For depleting 1 ml of *Xenopus* egg extract, 30 μg of anti-Tipin or 100 μl of monoclonal And1 antibodies were used. For immunopre-

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cipitations, antibodies (10 μg) were conjugated with 30 μl of protein A–Sepharose FF (Amersham) and added to 200 μl of *Xenopus* egg extract. After 1 h of incubation, beads were washed and harvested. Samples were analysed by SDS–PAGE, transferred to a PVDF membrane and immunoblotted.

GST pull-down assays

GST-tagged recombinant proteins were purified using glutathione fast-flow beads (GE Healthcare). Tipin–6His was purified using Ni–NTA beads (Qiagen). Polα p180 was produced *in vitro* using the Sp6 TNT-quick coupled transcription/translation system (Promega). Binding reactions contained equal amount of recombinant proteins or 2 μg of GST-tagged recombinant protein and 15 μl of the *in vitro* translated protein in 1 ml of binding buffer (20 mM Tris–HCl (pH 7.5)/200 mM NaCl/0.5% NP-40). The reactions mixtures were incubated for 3 h at 4°C, followed by five washes in binding buffer. Complexes were resolved by SDS–PAGE and probed with the indicated antibodies or exposed to the phosphoimager for detection of the ^{35}S signal.

DNA combing

Sperm nuclei were incubated at 2000 nuclei per microlitre in egg extract (mock or Tipin depleted) supplemented with digoxygenin–dUTP (Roche). Biotin–dUTP (Roche) was added at different time points (40 and 60 min) and DNA replication was allowed to proceed to completion (up to 120 min). Digoxigenin–dTP or biotin–dTTP analogues were directly added to the extract since they can be efficiently incorporated into the replicating DNA and they can be then detected with fluorescent probes. At 120 min the reaction was stopped by adding the same volume of 1% LMP agarose (Lonza) and transferring to a casting mould to prepare the plugs. Plugs were then treated with 2 mg/ml of proteinase-K (Roche) at 50°C overnight. The treatment was repeated, changing the proteinase-K solution, the day after, both over day and overnight. Subsequently plugs are washed several times in TE (with 50 mM EDTA). The TE buffer was replaced with 50 mM MES (pH 5.7) (3 ml/each plug) and plugs were incubated at 65°C for 15 min. Once melted, plugs were treated with β-agarase (3 units/plug; New England Biolab) at 42°C overnight. The resulting solution was used for stretching DNA fibres on silanized slides (Montpellier DNA Combing Facilities) at a constant speed of 18 mm/min. Slides were then dried at 65°C for 30 min and stained as described by Marheineke and Hyrien (2004).

Cohesion assay

The assay was performed as described by Losada *et al* (1998). Briefly, interphase extract supplemented with 1/100 volume of 1 mM biotin–dUTP (Roche) and 2000 nuclei per microlitre were incubated at 23°C for 2 h. The extract was then driven into mitosis by adding 1 volume of CSF extract and incubated at 23°C for 100 min. Samples were fixed with 2% PFA (0.25% Triton-X-100) for 10 min at RT and spun onto coverslips. Coverslips were blocked with 3% BSA in PBS/0.05% Tween (PBST) and incubated with primary antibody (1 μg/ml anti-XCAP-E in blocking) for 1 h at RT. After several washes in PBST, coverslips were incubated with secondary antibodies, streptavidin–AlexaFluor-594 conjugated (Molecular Probes, Invitrogen) and anti-Rabbit, AlexaFluor-488 conjugated (Molecular Probes, Invitrogen) (1:200 in blocking) 1 h at RT. After several washes in PBST, coverslips were mounted and analysed by fluorescence microscopy using the Volocity software (Improvision). The average distance between sister chromatids was measured in chromosomes with paired morphology. About 50 chromosomes assembled in control extracts or depleted extracts were randomly selected from three independent experiments. Distance between the chromatids was measured along the entire length of the chromosomes. The average distance among chromatids (in the different samples) was calculated using Excel together with its standard deviation.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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