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# Mta2 promotes Tipin-dependent maintenance of replication fork integrity

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Orderly progression of S phase requires the action of replisome-associated Tipin and Tim1 proteins, whose molecular function is poorly understood. Here, we show that Tipin deficiency leads to the accumulation of aberrant replication intermediates known as reversed forks. We identified Mta2, a subunit of the NuRD chromatin remodeler complex, as a novel Tipin binding partner and mediator of its function. Mta2 is required for Tipin-dependent Polymerase  $\alpha$  binding to replicating chromatin, and this function is essential to prevent the accumulation of reversed forks. Given the role of the Mta2–NuRD complex in the maintenance of heterochromatin, which is usually associated with hard-to-replicate DNA sequences, we tested the role of Tipin in the replication of such regions. Using a novel assay we developed to monitor replication of specific genomic loci in *Xenopus laevis* egg extract we demonstrated that Tipin is directly required for efficient replication of vertebrate centromeric DNA. Overall these results suggest that Mta2 and Tipin cooperate to maintain replication fork integrity, especially on regions that are intrinsically difficult to duplicate.

## Introduction

Accurate control of genome duplication is essential to preserve the physical integrity of chromosomes. DNA replication is performed by the replisome machinery, which assembles at replication origins. Once, the MCM complex is recruited to initiation sites, DNA unwinding is triggered by the S-phase kinase-dependent association of Cdc45 and GINS, with MCM (forming the CMG complex). DNA unwinding is followed by the loading of primase and polymerases onto replication forks.<sup>1</sup>

Additional factors travel with the replisome. Among these, the replication pausing complex made of Tipin, Tim1, and Claspin tethers the CMG helicase to the replicative polymerases. These proteins have a primary role in maintaining chromosomal integrity under replication stress conditions, as they prevent the CMG complex from translocating when replicative polymerases stall.<sup>2</sup> In addition, the Tipin–Tim1 sub-complex increases the processivity of all DNA polymerases by directly interacting with them.<sup>3</sup>

Cell-free systems based on vertebrate *Xenopus laevis* egg extract have been helpful to elucidate the biochemical bases of DNA replication both under physiological and stressful conditions.<sup>4–15</sup> Recently, we have successfully combined the use of the *Xenopus* egg extract with advanced imaging techniques based on transmission electron microscopy (EM) to visualize replication intermediates obtained from genomic DNA replicated in extracts depleted of particular proteins.<sup>10,16</sup>

Here we have explored the role the Tipin–Tim1 complex in DNA replication using this approach. We found that a large

fraction of replication intermediates in Tipin-depleted extracts contain reversed forks. These structures might represent replication intermediates formed in conditions in which Tipin–Tim1-mediated replication fork restart and/or stabilization are impaired.

Further investigation on the molecular basis of Tipin function led to the discovery of Tipin interaction with Mta2, the activator subunit of the nucleosome remodeling and deacetylase complex (NuRD). The NuRD complex has unique enzymatic activities, containing an ATPase and a histone deacetylase among its 6 core subunits.<sup>17</sup> The complex regulates chromatin organization, gene transcription, genomic stability, and developmental signaling.<sup>17</sup>

We show that Mta2 is required for the binding of Tipin to chromatin. Similar to Tipin depletion, Mta2 removal from egg extract results in a substantial decrease of polymerase  $\alpha$  (Pol  $\alpha$ ) binding to DNA, in the accumulation of reversed forks, and in the reduction of DNA replication efficiency. Consistent with the role of the NuRD complex in maintaining heterochromatin regions enriched for hard-to-replicate DNA sequences, we found that Tipin is required for efficient replication of centromeric DNA.

## Results

### Tipin prevents the formation of reversed replication forks

We have previously shown that beyond its role in fork stability, Tipin has a more active role in DNA replication, as it cooperates

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with And1 to guarantee the stable association of the replicative polymerases to the chromatin.<sup>8,9</sup> To gain further insight on the role played by the Tipin during DNA replication, we have visualized replication intermediates formed in interphase egg extracts depleted of Tipin (Fig. 1A) using electron microscopy (EM). Sperm nuclei were incubated for different times in mock depleted or Tipin-depleted extracts. Nuclei were isolated, and DNA was cross-linked in the presence of psoralen to stabilize replication intermediates.<sup>18</sup> Deproteinized DNA was subjected to rotary shadowing, and replication intermediates were analyzed by EM. Morphological criteria previously established were used to score replication intermediates.<sup>18</sup>

Although Tipin depletion from egg extract has no significant impact on global DNA replication efficiency,<sup>8,9</sup> the EM analysis revealed a high number of abnormal replication forks with the presence of reversed branches (Fig. 1–D). These structures were absent or sporadic in mock depleted or non-depleted extracts. As Tipin depletion is quite specific and can be rescued by Tipin recombinant protein (Errico et al., 2007) it is likely that the effects observed on replication intermediates are dependent upon Tipin deficiency.

#### Mta2 is a novel Tipin interactor

To understand the role of the Tipin in preventing the formation of aberrant replication intermediates, we looked for new Tipin binding partners. To this end we performed immunoprecipitation experiments using anti-Tipin antibody.<sup>9</sup> Proteins immunoprecipitated from interphase egg extracts were then separated on SDS-PAGE and analyzed by mass spectrometry (Fig. S1A). This analysis uncovered the interaction of Tipin with components of the NuRD complex among which the most represented one was Mta2 (Metastasis-associated protein 2) (Fig. 2A; Fig. S1B). Other proteins usually associated to the NuRD complex, such as LSD1 and CoRest2, were also found in Tipin immunoprecipitates (Fig. S1B), although the functional significance of their association to Tipin is still unclear.

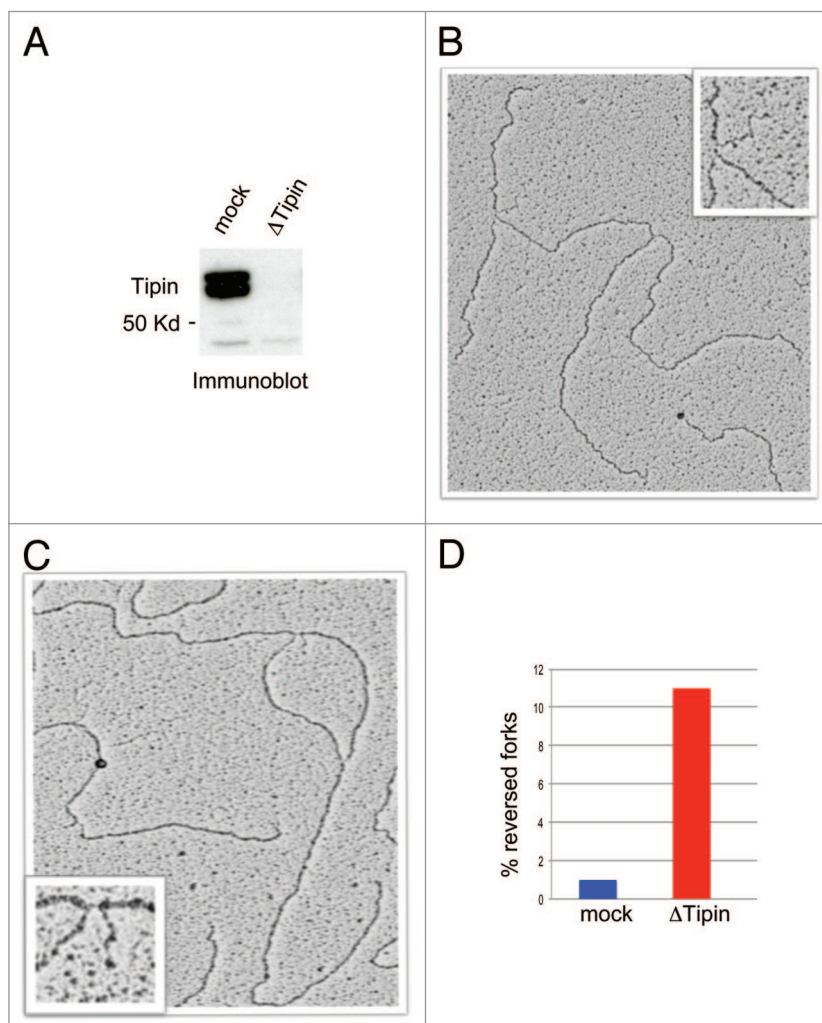
In eukaryotic cells, packaging of DNA into highly condensed chromatin is a significant obstacle to DNA metabolic processes, such as transcription, replication, and repair. Cells employ several strategies, including histone modifications and chromatin remodeling, to alter chromatin structure and allow access to nucleosomal DNA. The NuRD complex with its different subunits functions in chromatin remodeling (via CHD4), deacetylation (via HDAC1/2), and demethylation (via LSD1) and plays a vital role in regulating transcription.<sup>17,19–22</sup> It was recently shown that knock-down of Mta2, the HDAC1/2 activator, leads to the accumulation of spontaneous DNA damage and increased IR sensitivity.<sup>23</sup> Recent studies showed that the NuRD complex is also required for normal S-phase progression.<sup>24</sup>

We confirmed that Tipin interacts with Mta2 in egg extract, as Tipin immunoprecipitates contain Mta2 (Fig. 2B). We then showed that recombinant Tipin (His-Tipin) produced in bacteria (Fig. S2A) and Mta2 produced in reticulocyte extracts were able to interact (Fig. 2C). The interaction was confirmed with recombinant Mta2 protein produced in bacteria (Fig. S2B and C), indicating that Tipin directly binds Mta2.

#### Mta2 is required for efficient DNA replication

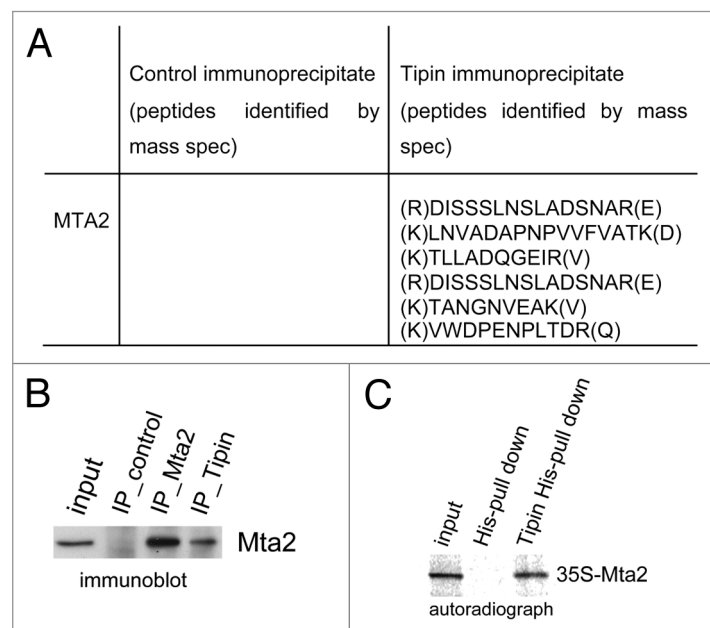
Egg extracts contain large maternal stockpiles of RNA and proteins that allow multiple rounds of cell division in the absence of transcription.<sup>25</sup> Therefore, *Xenopus* egg extract is a useful system to uncover the specific role in DNA replication of proteins such as Mta2, which also function in transcription.

To test whether Mta2 is important for DNA replication, Mta2 protein was depleted from interphase egg extract using specific polyclonal antibodies, which did not affect Tipin concentration in egg extract (Fig. 3A). We have previously shown



**Figure 1.** Tipin prevents the formation of reversed forks. (A) Immunoblot to assess Tipin depletion from egg extract. (B and C) Examples of reversed replication forks in Tipin-depleted extracts. Image magnification of the reversed fork shown in the corner box. (D) Quantification of reversed forks. Experiments were repeated at least 3 times. The graph shows the results of a typical experiment, where at least 100 forks were scored in mock or Tipin-depleted extracts.

that DNA replication efficiency in egg extract in the absence of Tipin is overall not affected unless the number of MCM complexes bound to chromatin is reduced.<sup>8</sup> This condition, known as minimal licensing,<sup>26</sup> is experimentally useful to unmask subtle defects of the DNA replication machinery by reducing the number of potential replication origins that can be activated under conditions of replication stress. DNA replication was monitored in mock or Mta2-depleted extracts in normal (maximal) or minimal licensing condition. Similar to Tipin depletion, we did not observe significant reduction of DNA replication efficiency under normal conditions (Fig. 3B and C). However, when we reduced the number of MCM complexes bound to chromatin by adding geminin to mock or Tipin-depleted extract 3 min after sperm nuclei to induce minimal licensing, we found that Mta2-depleted extract had 70% reduction in the overall efficiency of DNA replication (Fig. 3C, lane 6; Fig. 3D). This defect was similar to the one observed when Tipin was depleted from egg extract under minimal licensing conditions (Fig. 3C, lane 5; Fig. 3D). Co-depletion of both Tipin and Mta2 did not result in any further decrease in DNA replication efficiency, suggesting that Mta2 and Tipin act in the same pathway to control DNA replication (Fig. 3D). Importantly, the replication defect observed in absence of Mta2 was specific, as it was fully rescued by reconstituting Mta2-depleted extract with an amount of Mta2 recombinant protein that restored physiological concentration of endogenous Mta2 (~400 nM) (Fig. 3D; Fig. S3). These results suggest that Mta2 is required for efficient DNA replication.



**Figure 2.** Tipin directly interacts with Mta2. (A) Table indicating the Mta2 peptides identified via mass spectrometry analysis in Tipin immunoprecipitates. (B) Equal amounts of extract were immunoprecipitated with either anti-Tipin, anti-Mta2 antibodies or pre-immune serum. Purified proteins were immunoblotted with the indicated antibodies to detect associated proteins. (C) Pull down assay using His-Tipin and in vitro translated <sup>35</sup>S-labeled Mta2. Mta2 was detected by autoradiograph.

### Mta2 is required for Tipin binding to chromatin

To understand the role of Mta2 during S phase, we monitored the binding of Mta2 to chromatin isolated from egg extract supplemented with either aphidicolin, to block DNA polymerization or geminin, to prevent the assembly of the replication machinery. We found that Mta2 binding to chromatin was not significantly affected by either treatment (Fig. 4A). These results suggest that Mta2 binding to DNA takes place before the replication machinery assembles onto chromatin. Consistent with these observations a time course of Mta2 loading onto chromatin confirmed that Mta2 binding to chromatin preceded the loading of Mcm7, member of the Mcm replicative helicase, and of Tipin (Fig. S4A).

We then monitored the binding of Mta2 and Tipin to DNA in Mta2 or Tipin depleted extracts. We found that Mta2 binding to chromatin was unaffected in Tipin-depleted extract (Fig. 4B). Instead, Tipin binding to chromatin was strongly decreased in Mta2-depleted extracts (Fig. 4B), suggesting that Mta2 is required for efficient binding of Tipin to chromatin. Mta2 depletion did not affect chromatin binding of other putative NuRD complex members such as CHD4 and MBD3 (Fig. S4B). In addition, Mta2 chromatin binding also preceded loading NuRD complex components such as CHD4, CHD3, HDAC1, HDAC2, and MBD3 (Fig. S4A). Importantly, reconstitution of endogenous levels of Mta2 protein by complementation of Mta2 depleted extract with recombinant Mta2 protein alone was able to fully restore the replication and the Tipin binding defects and caused by Mta2 depletion (Fig. 3D; Fig. S5). These findings suggest that the phenotypes observed depend strictly on the absence of Mta2. However, these experiments do not exclude that Mta2 might require the NuRD complex activity for its function.

### Mta2 is required to stabilize Pol α binding to replicating chromatin

We have previously shown that in the absence of Tipin Pol α binding to chromatin is decreased in minimal licensing conditions.<sup>8</sup> To uncover the mechanism of action of Mta2 in DNA replication, we examined the binding to chromatin of major DNA replication factors under minimal licensing condition. We found that when the number of replication origins is reduced, the binding of Pol α to chromatin was impaired in Mta2-depleted extract, whereas the binding of pre-replication complex proteins such as ORC1 was not affected (Fig. 5). Interestingly, the defect of Pol α binding was selective and not due to the inability to promote origin firing as shown by the efficient recruitment of Cdc45 to chromatin (Fig. 5). This phenotype is similar to what we observed in Tipin-depleted extract, reinforcing the idea that Tipin and Mta2 act in the same pathway during S phase.

### Mta2 prevents the formation of reversed forks

The requirement of Mta2 for Tipin binding to chromatin, for efficient DNA replication under minimal licensing conditions and for Pol α chromatin binding suggests that Mta2 is directly required for Tipin function. To uncover the mechanism of action of Mta2, replication intermediates were isolated from nuclei assembled in interphase egg extract depleted of Mta2 and were subjected to EM analysis. These experiments

revealed the presence of a significant number of aberrant replication intermediates with reversed branches (Fig. 6A and B). This phenotype is similar to the one observed in absence of Tipin. These data further confirm that Mta2 is required for Tipin function at the replication fork. Therefore Mta2 might play an important role in preventing the accumulation of aberrant replication intermediates, such as reversed forks.

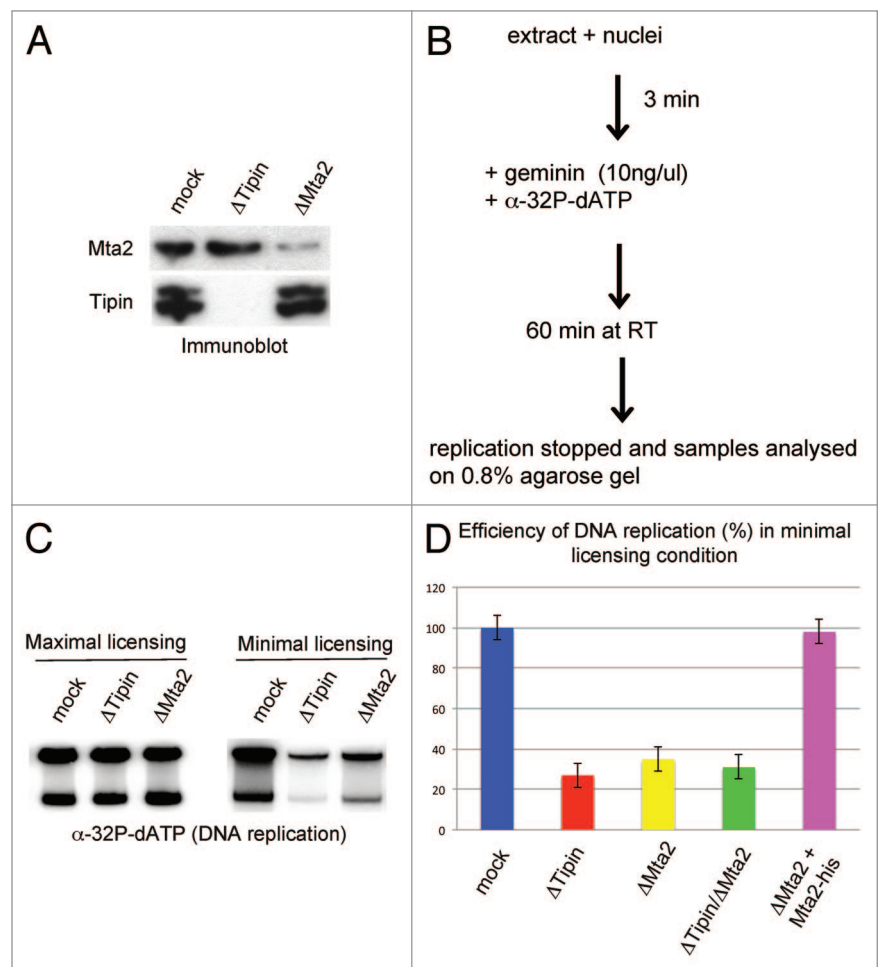
#### Efficient replication of repetitive DNA requires Tipin-dependent DNA binding of Pol $\alpha$

The fact that Tipin is required for DNA replication under minimal licensing conditions prompted us to hypothesize that Tipin is required for efficient replication of DNA regions that, due to their complex nature, are prone to spontaneous formation of stalled forks. To verify this hypothesis we set up for the first time a novel assay to monitor replication of defined genomic loci using DNA bacmids. These constructs usually contain large genomic sequences that occupy more than 90% of their length. We found that differently from small DNA plasmids, which are unable to replicate in egg extract due to the inability to form nuclear structure, large bacmids (BACs) whose size exceeds 150 Kb form nuclei similar to the ones obtained with sperm DNA when added to egg extract (Fig. 7A). The DNA inside these “bacmid-nuclei” was replicated as shown by nucleotide incorporation (Fig. 7A) with an efficiency that reached 70% of the input DNA (Aze et al., in preparation). We used this assay to test whether Tipin is required for the replication of DNA regions with repetitive sequences. We used bacmid vectors containing human centromeric DNA as source of repetitive genomic DNA. Tipin depletion severely impaired DNA replication of all the different centromeric DNA regions (Fig. 7B and C). The replication defects of centromeric DNA could be fully rescued by the addition of recombinant Tipin to Tipin-depleted extract (Fig. S6). Importantly, the binding of essential DNA replication factors to centromeric DNA templates was not affected in the absence of Tipin (Fig. 7D) except for Pol  $\alpha$ , whose loading onto repetitive centromeric DNA was not detectable even in normal licensing conditions (Fig. 7D). These data indicate that replication of genomic regions with complex structures requires Tipin-dependent stabilization of Pol  $\alpha$  binding to DNA.

## Discussion

The replication pausing complex is an important component of the replisome, as it stabilizes the replication fork and contributes

to fork restart once replication can resume. Here, we show that Tipin deficiency during DNA replication leads to the accumulation of aberrant replication intermediates characterized by the presence of 4 branches, in which the 2 newly synthesized strands anneal to form a cruciform structure, also known as reversed fork. We have previously shown that during unperturbed DNA replication ssDNA gaps accumulate in the absence of DNA repair factors such as Rad51.<sup>10</sup> This result might reflect the discontinuous nature of replication fork progression. It is indeed possible that gaps are formed by Pol  $\alpha$ -mediated re-priming downstream a replication block to overcome fork arrest. Replisome components are usually stably associated to replication forks.<sup>27</sup> Tipin is required for efficient Pol  $\alpha$  binding to chromatin, and it has been shown to increase processivity of all major DNA polymerases.<sup>3,8</sup> It is possible that the Tipin facilitates polymerase-mediated DNA



**Figure 3.** Mta2 is required for DNA replication under minimum licensing conditions. (A) Immunoblot to assess Tipin or Mta2 depletion from egg extract. (B) Scheme of minimum licensing conditions experiments and replication assays. (C) DNA replication in “maximum” or “minimum licensed” mock, Tipin or Mta2 depleted extract at 60 min. (D) The efficiency of DNA replication in “minimum licensed” mock (blue), Tipin (red), Mta2 (yellow), and Tipin/Mta2 (green) co-depleted extracts at 60 min from nuclei addition to egg extract was tested. The defect in DNA replication observed in the Mta2 depleted extract (yellow) was rescued by the addition of 400 nM Mta2 recombinant protein (purple). Three independent experiments are averaged in the bar graphs. The error bars are standard deviation from the mean value.

replication re-priming, ensuring discontinuous progression of DNA replication. The inability to restart halted DNA replication in absence of Tipin might lead to the formation of reversed forks at some genomic loci prone to form secondary structures, which stall replication fork progression (Fig. 8). Formation of these structures might be facilitated by the ADP-ribosylation activity of PARP1 and by the action of enzymes such as SMARCAL1, which has been shown to convert stalled forks into these intermediates in vitro.<sup>28,29</sup> In turn, reversed forks could help to bypass the obstacle to replication fork progression or engage with the DNA repair machinery, potentially leading to the formation of DNA rearrangements. Restart of reversed forks might be facilitated by RecQ1 helicase.<sup>28</sup>

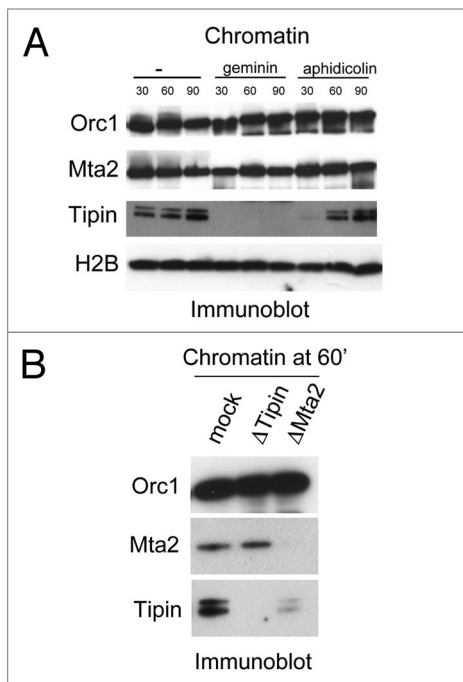
Overall these results indicate that Tipin has an essential role in maintaining the structural integrity of the replication fork by preventing spontaneous formation of aberrant replication intermediates. However, it is unclear where on the genome Tipin and in general the replisome pausing complex is more needed during DNA replication. We have shown that Tipin is not required for overall genome replication. It is instead likely that Tipin is required in areas of the genome such as heterochromatin, which is particularly rich in DNA sequences giving rise to secondary structures that impair fork progression.

The NuRD complex plays important roles in the organization of heterochromatin, ensuring proper DNA replication, cellular proliferation, and protection of genome integrity.<sup>23,30-33</sup> Rapidly proliferating cells accumulate a high local concentration

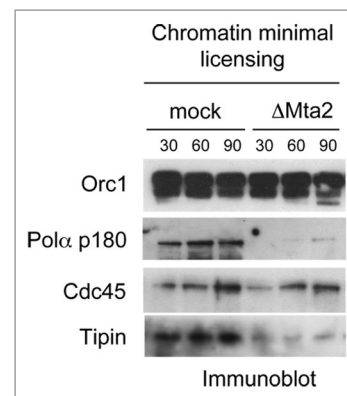
of NuRD complex at pericentromeric heterochromatin (NuRD foci) during S phase.<sup>33</sup> These foci contain proteins usually associated with active replication forks such as PCNA and the chromatin assembly factor CAF1, suggesting a role for NuRD in the replication and/or post-replicative chromatin assembly of these regions.<sup>33</sup>

Our data indicates that Tipin function requires NuRD subunit Mta2, which contributes Tipin binding to chromatin. Importantly, Mta2 and Tipin act in the same pathway, as depletion of either protein shows a similar defect in the overall efficiency of DNA replication, with no additive effects in the Tipin/Mta2 double-depleted extract. In addition, depletion of Mta2 from *Xenopus* egg extract results in an impaired binding of both Tipin and Pol  $\alpha$  to DNA and in the formation of reversed forks. These results indicate that Mta2 likely acts upstream Tipin.

The NuRD complex has both nucleosome remodeling and histone deacetylase activity. The acetylation/deacetylation state of histone affects chromatin accessibility and, as such, several DNA-based processes. Recently, it was also shown that a large number of chromatin proteins among which Tim1, And1, and Clasp1 are acetylated.<sup>34</sup> Mta2 might regulate their acetylation status, promoting their chromatin binding. Alternatively, as Mta2 is enriched on heterochromatin it might promote Tipin binding to heterochromatic genomic loci containing complex and repetitive DNA sequences where Tipin function might be required for their correct duplication (Fig. 8). Consistent with this hypothesis, we have shown here that Tipin is required for efficient replication of centromeric genomic DNA. However, direct requirement for Mta2 in centromeric DNA replication remains to be demonstrated. Overall, these data suggest that Mta2 and Tipin act to preserve replication fork integrity, especially on regions that are intrinsically difficult to replicate. When Mta2 and Tipin function are impaired, DNA replication generates dangerous intermediates, such as reversed forks, most likely on repetitive sequences. These structures might represent a serious threat to genome stability if not properly resolved.



**Figure 4.** Mta2 is required for Tipin loading on the chromatin. (A) Immunoblot analysis to detect the level of Mta2, Tipin, Orc1, and Histone H2B on the chromatin at different time points (30, 60, 90 min) in extract untreated or treated with geminin or aphidicolin. (B) Immunoblot to detect the levels of Mta2, Tipin, and Orc1 on the chromatin at 60 min from nuclei addition.



**Figure 5.** Mta2 is required for the efficient association Pol  $\alpha$  to the chromatin in minimum licensing conditions. Sperm nuclei were added to mock or Mta2 depleted *Xenopus* egg extract. Geminin was supplemented 3 min post-nuclei addition to generate a minimum licensing system and chromatin was harvested at different times. Chromatin-bound proteins were analyzed by SDS-PAGE and immunoblot analysis was performed with the indicated antibodies.

## Material and Methods

### Plasmids and recombinant proteins

Expression plasmids for Tipin were previously described.<sup>9</sup> The plasmid (pCMV-SPORT6) carrying *Xenopus laevis* Mta2 full-length cDNA was obtained from ImaGenes, IRBHp990G0784D. Mta2 cDNA was cloned into Pet 16b vector containing His Tag for protein expression. His-Mta2 and His-Tipin proteins were expressed in BL21 CodonPlus (Stratagene). Pellets were collected from 20 L cells grown at the stationary phase following induction with 1 mM IPTG for few hours and processed for protein purification using Ni-NTA beads according to manufacturer instructions (Qiagen). His-Geminin plasmid and protein purification were previously described.<sup>9</sup>

### Antibodies

Polyclonal Tipin antiserum has been previously described.<sup>9</sup> Polyclonal Mta2 antibody was purchased from Novus biological (NB100-56483). Antibodies against Mcm7 were purchased from Santa Cruz Biotechnology. Antibodies against Pol  $\alpha$  p180 subunit were obtained from Abcam. Rabbit polyclonal Cdc45 was a kind gift of H Takisawa (University of Osaka). Monoclonal ORC1 antibody TK15 was previously generated in Tim Hunt's lab.<sup>35</sup> Anti-HDAC1 Cat N. ARP32358, HDAC2 Cat N. ARP32618 and CHD4 Cat N. ARP34372 were from Aviva Systems Biology. Anti-MBD3 Cat N. 3896 and anti-CHD3 Cat N. 4241 were from Cell Signaling.

### *Xenopus* egg extracts and chromatin isolation

Egg extract was prepared as previously described.<sup>9</sup> To isolate the chromatin fractions, sperm nuclei (4000 nuclei/ $\mu$ L) were added to 40  $\mu$ L of egg extracts for 30, 60, or 90 min. For immunoblotting, samples were diluted with 10 volumes of EB (100 mM KCl, 2.5 mM MgCl<sub>2</sub>, and 50 mM HEPES-KOH, pH 7.5) containing 0.25% NP40, and centrifuged through a 30% sucrose layer at 10 000  $\times$  g at 4 °C for 5 min. Pellets were suspended in sample buffer loaded on a SDS/PAGE. For Mta2 add-back experiments the volume of chromatin binding reactions was reduced to half and Mta2 depleted extracts were reconstituted with 400 nM of recombinant His-Mta2.

### DNA replication assay

Sperm nuclei (4000 nuclei/ $\mu$ L) were added to 20  $\mu$ L egg extract (mock or depleted). Samples were supplemented with  $\alpha$ <sup>32</sup>P-dATP or  $\alpha$ <sup>32</sup>P-dCTP and incubated at 23 °C for 2 h. Replication was stopped and analyzed by agarose gel electrophoresis and autoradiography. Phosphoimager was used for the quantification of the signals (Amersham). DNA replication in minimal licensing was performed as previously described (Errico et al., 2009). Reaction volume was reduced to facilitate reconstitution of Mta2-depleted extract with 400 nM His-Mta2 recombinant protein.

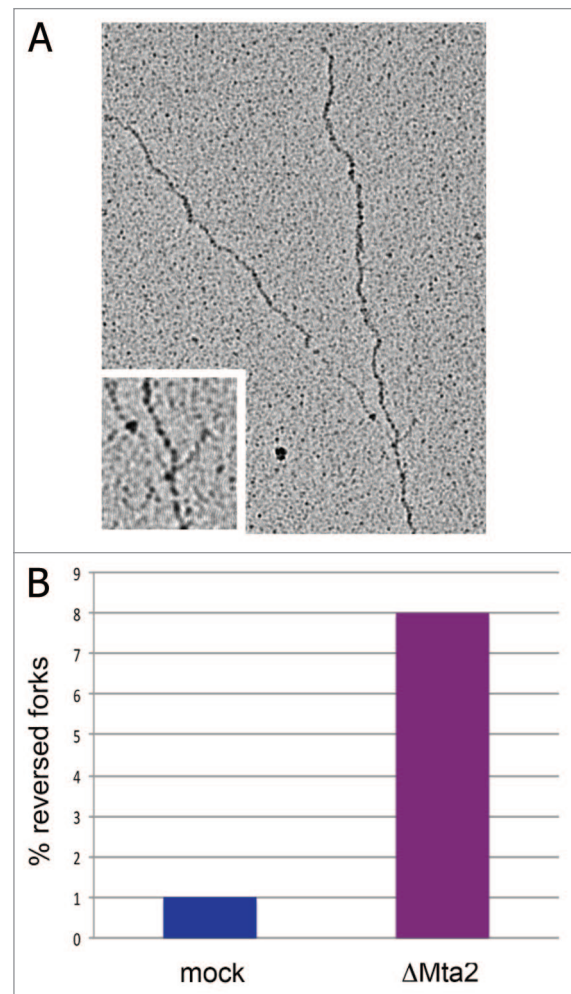
### Immunodepletion and immunoprecipitation

For depleting 1 ml *Xenopus* egg extract, 30  $\mu$ g of purified anti-Tipin or 100  $\mu$ L Mta2 antibodies were used. Anti-Tipin antibodies were conjugated to protein-A-Sepharose FF (Amersham) and removed by low-speed centrifugation. The procedure was repeated twice for complete removal of Tipin. Anti-Mta2 antibodies were instead conjugated to protein A-Dynabeds (Dyna) and removed by magnetic separation. The procedure was

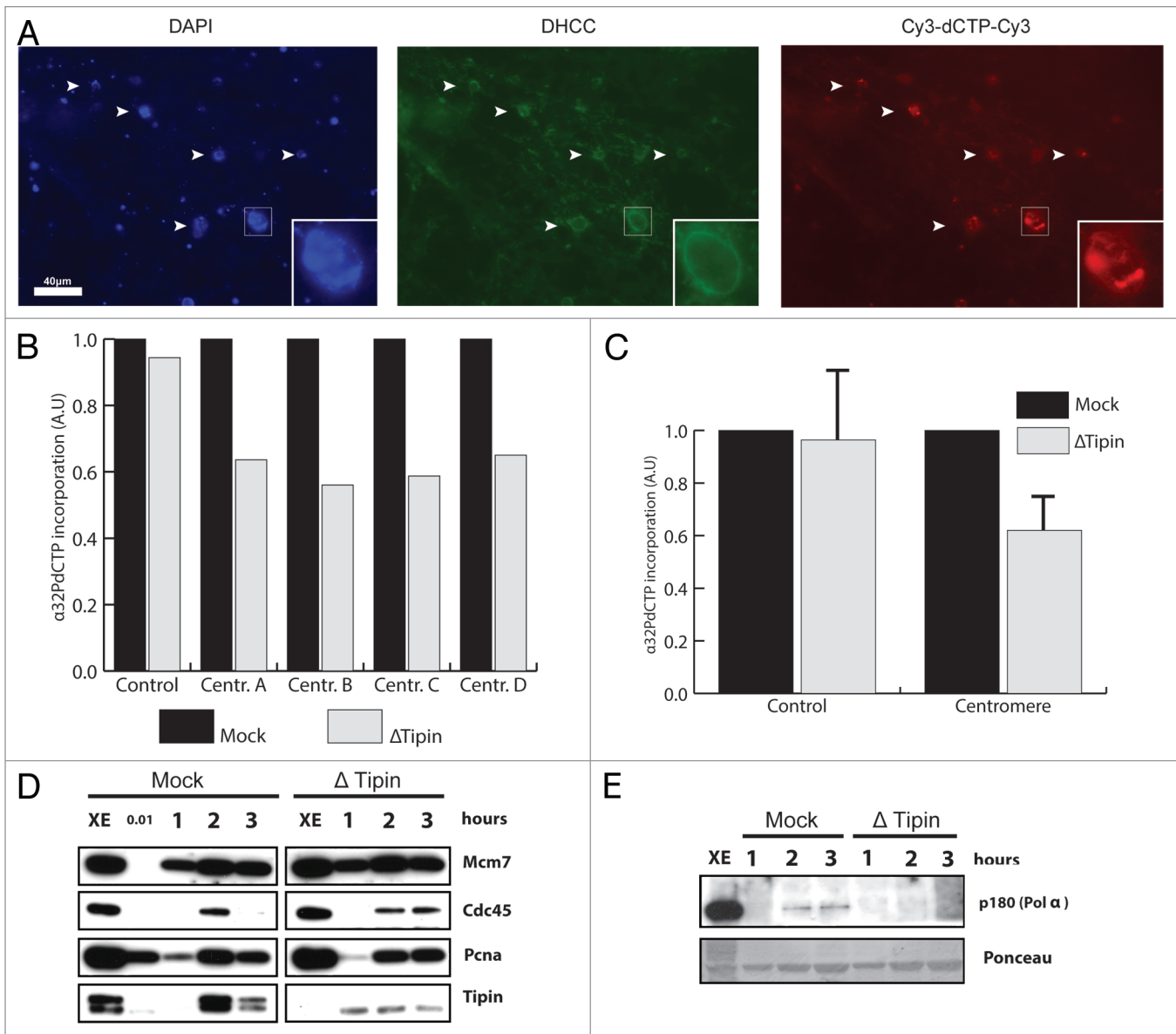
repeated 4 times for complete removal of Mta2. For immunoprecipitations and mass spec analysis, 10  $\mu$ g of purified antibodies were conjugated to 30  $\mu$ L of protein A-Sepharose and added to 200  $\mu$ L of *Xenopus* egg extract. After 1 h incubation, beads were washed and harvested. Samples were analyzed by SDS-PAGE, which was transferred to a PVDF membrane and immunoblotted or stained with Sypro Ruby (Invitrogen) and cut into slices to be processed by the mass spec facility of the LRI Clare Hall laboratory.

### His-pull down assays

For pull down assays, recombinant His-Mta2 and His-Tipin were purified using Ni-NTA beads. Mta2 was also produced in vitro using the Sp6 TNT-quick coupled transcription/translation system (Promega). Binding reactions contained 2  $\mu$ g his-tagged recombinant protein and 15  $\mu$ l the in vitro translated protein in 1 ml binding buffer (20 mM TRIS-HCl pH 7.5 200 mM NaCl 0.5% NP40). The reactions were incubated for 3 h at 4 °C, followed by 5 washes in binding buffer. Complexes were resolved by



**Figure 6.** Mta2 prevents the formation of reversed forks. (A) Example of reversed replication fork in Mta2 depleted extract. (B) Quantification of replication intermediates with reversed forks in mock or Mta2-depleted extract performed as in Figure 1D. Experiments were repeated at least 3 times. The graph shows the results of a typical experiment where at least 100 forks were scored in mock or Mta2-depleted extracts.



**Figure 7.** Tipin is required for replication of centromeric DNA regions. (A) BACs were incubated in egg extracts and analyzed for nuclei morphology (DAPI), membrane formation (DHCC staining), and replication (Cy3-dCTP incorporation). (B and C) Control and centromeric sequences containing BACs and were incubated in mock or Tipin-depleted extracts supplemented with  $\alpha^{32}\text{P}$ -dCTP to monitor DNA replication, which was measured following 3 h incubation. The graph in (B) shows the results of one representative experiment using 4 different centromeric BACs with the relative incorporation of  $\alpha^{32}\text{P}$ -dCTP. The graph in (C) shows the average efficiency of DNA replication obtained from 3 independent experiments using control and centromeric bacmid. Errors bars indicate standard deviation. (D and E) Chromatin isolated from mock or depleted extracts at the different times after centromeric BAC addition to egg extract and analyzed by western blotting with the indicated antibodies.

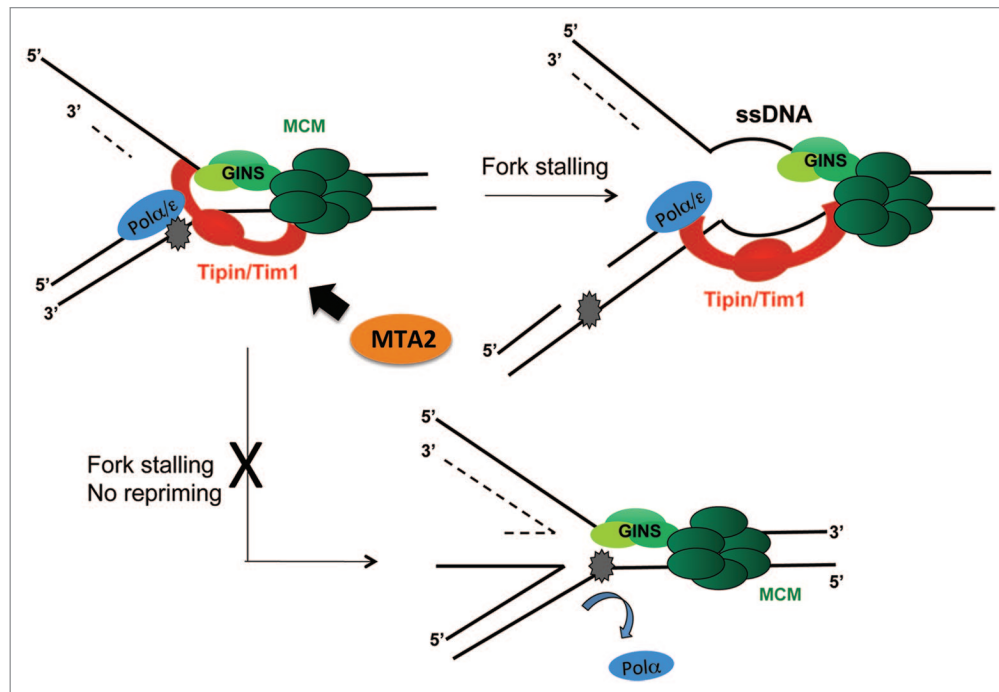
SDS/PAGE and probed with the indicated antibodies or exposed to the phosphoimager for detection of the  $^{35}\text{S}$  signal. For the in vitro pull down shown in Figure S2C 2  $\mu\text{g}$  recombinant His-Tipin protein were incubated for 1 h at 4  $^{\circ}\text{C}$  in binding buffer with either recombinant His-MTA2 (500 ng) or His-Geminin (1  $\mu\text{g}$ ) used as negative control. The mixture was then supplemented with 1  $\mu\text{g}$  mouse anti-Tipin antibody or mouse IgG as control. One hour later, 15  $\mu\text{l}$  protein A/G agarose beads (Santa Cruz) were added to reaction and left incubating for 1 h at 4  $^{\circ}\text{C}$ . Next, beads were washed 4 times with coupling buffer and once more with PBS before eluting proteins in Laemmli buffer.

Samples were then resolved on a SDS-PAGE and analyzed by western blotting with anti-MTA2 antibody.

#### Electron microscopy

Demembrated sperm nuclei (4000 n/ $\mu\text{l}$ ) were incubated in 200  $\mu\text{l}$  egg extracts for 60 min, diluted with 400  $\mu\text{l}$  EB-EDTA buffer, layered onto 500  $\mu\text{l}$  EB buffer plus 30% (w/v) sucrose and centrifuged at 3000 g for 10 min at 4  $^{\circ}\text{C}$ . Pellets were resuspended in 100  $\mu\text{l}$  EB-EDTA buffer and transferred to a 96-well plate (100  $\mu\text{l}$  per well). 4,5',8-Trimethylpsoralen (TMP) was added at 10  $\mu\text{g}/\text{ml}$  to each well. Samples were incubated on ice for 5 min in the dark and irradiated with 366-nm UV light for





**Figure 8.** Model for Tipin–Mta2 function in maintaining fork integrity. (A) Tipin–Tim1 complex might create a bridge between replisome components such as Cdc45, GINS, Pol  $\alpha$ , and the MCM complex necessary for the stable binding of Pol  $\alpha$  to the replication fork. Mta2 might facilitate Tipin function at specific genome loci containing repetitive DNA sequences. The presence of Tipin might promote Pol  $\alpha$ -mediated replication restart downstream lesions or structures that halt polymerases progression. (B) In the absence of Mta2 and/or Tipin inefficient re-priming and inability to resume fork progression leads to fork reversal.

7 min on a precooled metal block. The procedure from TMP addition to irradiation with UV light was repeated 3 more times. Then, the genomic DNA was purified following proteinase K (1 mg/ml) and RNase A (167  $\mu$ g/ml) treatment, phenol–chloroform extraction, and isopropanol precipitation. The purified DNA was digested with NdeI endonuclease (100 units) for 5 h, and the replication intermediates were further purified on a BND cellulose column and were processed for the observation with EM as previously described.<sup>18</sup> EM analysis was performed at the EM facilities of IFOM and London Research Institutes.

#### BACs DNA

Protocols concerning bacmid (BAC) isolation will soon be published elsewhere. Control BAC (RP11-115L10) and centromeric BACs DNA (RP11-5B18, RP11-449A3, RP11-285M22 and RP11-643N23) were purchased from (<http://bacpac.chori.org/home.htm>).

#### Fluorescence microscopy analysis

Sixty nanograms (60 ng) BACs DNA were incubated in 20  $\mu$ l *Xenopus* egg extracts supplemented with Cy3dCTP for 2 h. Two microliters (2  $\mu$ l) reaction was then mounted between slide and coverslip in a fixing solution (45 mM PIPES pH 7.2, 45 mM NaCl, 240 mM KCl, 10% formalin, 50% glycerol, 2  $\mu$ g/ml Hoechst, and DHCC 3,3'-dihexyloxycarbocyanine [Fisher]). Slides were analyzed by fluorescence microscopy.

#### BAC DNA replication and chromatin binding

BAC DNA was incubated to a final concentration of 8 ng/ $\mu$ l egg extract. DNA replication and chromatin isolation were performed as described above with the difference that DNA

replication reactions were performed for 3 h. For the reconstitution of Tipin-depleted extract 200 nM of recombinant Tipin were used.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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#### Author Contribution

A.E. performed the experiments and co-wrote the manuscript. A.A. set up the bacmid replication assays and performed the experiments for the revision. V.C. planned the experiments and co-wrote the manuscript.

#### Supplemental Materials

Supplemental materials may be found here: [www.landesbioscience.com/journals/cc/article/29157](http://www.landesbioscience.com/journals/cc/article/29157)

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