Replisome progression complex links DNA replication to sister chromatid cohesion in *Xenopus* egg extracts

(姉妹染色体接着の形成における複製フォーク結合タンパク質の役割)

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Abstract

Cohesin-mediated sister chromatid cohesion is established during S-phase, and recent studies demonstrate that a cohesin protein ring concatenates sister DNA molecules. However, little is known about how DNA replication is linked to the establishment of sister chromatid cohesion. Here, I used *Xenopus* egg extracts to show that AND-1 and Tim1-Tipin, homologs of *Saccharomyces cerevisiae* Ctf4 and Tof1-Csm3, respectively, are associated with the replisome and are required for proper establishment of the cohesion observed in M-phase extracts. Immunodepletion of both AND-1 and Tim1-Tipin from the extracts leads to aberrant sister chromatid cohesion, which is similarly induced by the depletion of cohesin. These results demonstrate that AND-1 and Tim1-Tipin are key factors linking DNA replication and establishment of sister chromatid cohesion. On the basis of the physical interactions between AND-1 and DNA polymerases, I discuss a model to describe how replisome progression complex establishes sister chromatid cohesion.

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Introduction

In eukaryotic cells, chromosomal DNA is duplicated accurately during S-phase and the resulting sister chromatids are segregated during mitosis to produce the next generation. Sister chromatids remain physically connected from S-phase to the onset of anaphase to allow the chromatids to be distributed equally to the two daughter cells. The physical connection, known as sister chromatid cohesion, is generated in two ways: by catenation of sister DNA molecules during DNA replication and by the proteinaceous linkage formed by the multi-protein complex cohesin. Although DNA catenation contributes to the physical connection of sister chromatids (Vagnarelli et al. 2004; Toyoda and Yanagida 2006), it is unclear whether DNA catenation is sufficient to connect sister DNAs under physiological conditions where topoisomerases are active. The observation that cohesion proteins mediate cohesion between yeast minichromosomes in the absence of DNA catenation (Ivanov and Nasmyth 2007) indicates that the proteinaceous linkage plays a major role in sister chromatid cohesion.

Cohesin consists of at least four subunits including two structural maintenance of chromosomes (SMC) subunits, one kleisin subunit, and one non-SMC subunit (Fig. IA) (Hirano 2006). The two SMC subunits, Smc1 and Smc3, exhibit a characteristic rod-like structure bent at a central hinge domain to form an intra-molecular coiled-coil that brings the N- and C-terminal regions together to form a globular ATPase head domain (Haering et al. 2002; Hirano and Hirano 2002). Biochemical analysis revealed that the two SMC subunits bind to each other via their hinge domain and to the kleisin subunit Scc1 via their ATPase head domains (Haering *et al*. 2002). Scc1 is a member of the kleisin family of proteins that bridge the ATPase heads in different SMC complexes (Schleiffer *et al.* 2003). In the case of cohesin, Scc1 binds to the ATPase head of Smc1 at its C-terminus and to Smc3 at its N-terminus forming a tripartite ring with a diameter of about 40 nm (Haering *et al.* 2002). This unique structure is observed in purified vertebrate and yeast cohesin complexes by electron microscopy (Anderson *et al*. 2002; Haering *et al.* 2002) and by crystal structures of cohesin subcomplexes (Haering *et al.* 2002). In addition to its binding to the SMC heterodimer, Scc1 is also associated with Scc3, the fourth non-SMC subunit of cohesin (Toth *et al*. 1999). Recent studies reveal that Scc3 has an essential role in the establishment of cohesion and in the stable binding of cohesin to chromatin

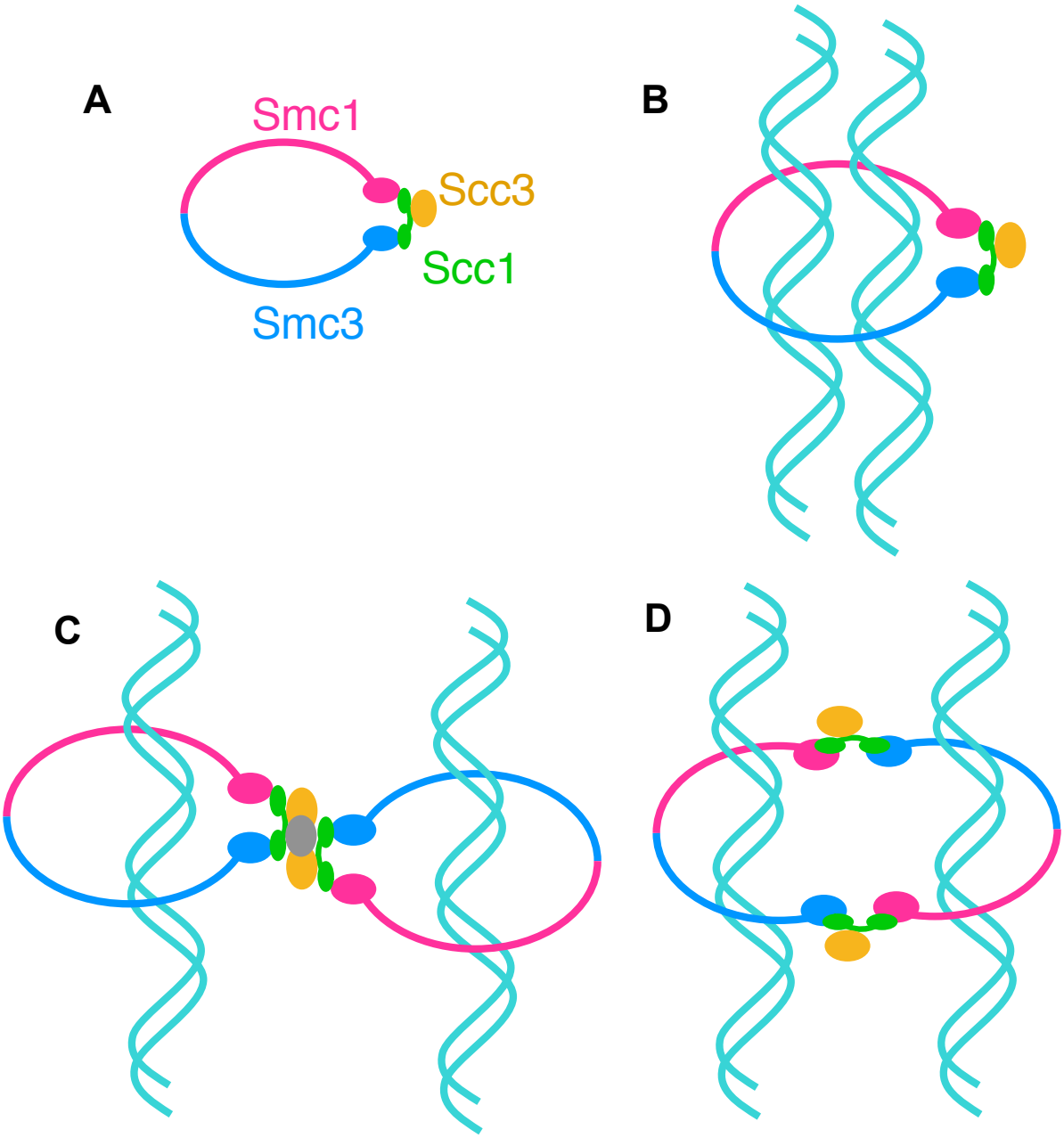

Figure I. Molecular architecture of the cohesin complex

(A) Schematic structure of the cohesin core complex. The cohesin core complex consists of the four core subunits Smc1, Smc3, Scc1, and Scc3. SMC-heterodimer and Scc1 form a ring structure. (B, C, D) Models of cohesin-DNA interaction. (B) Ring model. A single cohesin molecule encircles sister DNA molecules. (C) Cohesin dimer model. A single cohesin molecule encircles one of the sister DNA molecules and the two cohesin molecules form a dimer. (D) Oligomeric cohesin model. Two or more cohesin molecules form a large single ring that embraces the sister DNA molecules.

(Rowland *et al*. 2009), but the structure of Scc3 has not been determined. Unlike other cohesin subunits, two homologs of Scc3, SA1 and SA2, exist in somatic vertebrate cells (Losada *et al*. 2000). A cohesin complex will contain SA1 or SA2, but not both, and it is unclear whether the resulting two types of cohesin have different functions.

In addition to these four core subunits of cohesin, there are at least three proteins that bind to cohesin to modulate its function. One of these proteins is Pds5, which is conserved from yeast to human and has the HEAT-repeat protein interaction domain (Hartman *et al*. 2000; Panizza *et al*. 2000; Sumara *et al*. 2000). Vertebrate cells have two Pds5 homologs, Pds5A and Pds5B, which associate with two types of cohesin complex (Losada *et al*. 2005). Pds5 is essential for cohesion in yeast, worm, and fly through the maintenance of stable chromatin association of cohesin (Hartman *et al*. 2000; Panizza *et al*. 2000; Tanaka *et al*. 2001; Stead *et al*. 2003; Wang *et al*. 2003; Dorsett *et al*. 2005). Knockout of both Pds5A and Pds5B leads to embryonic lethality in mice (Zhang *et al*. 2009), whereas depletion of either of them by siRNA treatment in vertebrate cells has only a slight effect on cohesion (Losada *et al*. 2005). These findings indicate redundancy in the function of Pds5A and Pds5B. In contrast, depletion of both Pds5A and Pds5B causes no apparent cohesion defect in *Xenopus* egg extract (Losada *et al*. 2005). Cohesin is also associated with Wapl (Gandhi *et al*. 2006; Kueng *et al*. 2006), a human homolog of *Drosophila* wings apart-like gene (Verni *et al*. 2000). In human cells, depletion of Wapl causes accumulation of cohesin on chromatin from interphase to metaphase (Gandhi *et al*. 2006; Kueng *et al*. 2006), indicating that Wapl is required for the removal of cohesin from chromatin. However, studies in *Saccharomyces cerevisiae* reveal that the deletion of Rad61/Wpl1, the Wapl homolog, causes a mild cohesion defect (Warren *et al*. 2004) but does not cause accumulation of cohesin on chromatin (Rowland *et al*. 2009), indicating that the function of Wapl in cohesion differs among organisms. Sororin is a third cohesin binding protein and is required for the stable association of cohesin with chromatin (Rankin *et al*. 2005; Diaz-Martinez *et al*. 2007; Schmitz *et al*. 2007). Sororin has been identified only in vertebrates and the mechanism underlying its role in stabilizing cohesin is unknown.

The stable association of cohesin with chromatin is required for maintaining the connection between sister chromatids for a long time period and to allow them to endure the pulling force exerted by spindle microtubules during metaphase. However,

it is not known how cohesin connects sister chromatids. Recently, biochemical analysis of cohesin in budding yeast revealed that cohesin binds to chromatin by encircling the DNA molecule with its ring structure (Haering *et al*. 2008). This finding suggests that the cohesin complex mediates cohesion of the sister chromatids by embracing them within its ring-like structure (Fig. IB). The topological binding model for the binding of cohesin to chromatin is consistent with the experimental observation that cleavage of the cohesin subunits Scc1 or Smc3 causes dissociation of cohesin from chromatin (Uhlmann *et al.* 2000; Gruber *et al.* 2003). Further support for this model comes from the observation that cohesin dissociates from circular minichromosomes when they are linearized with restriction enzymes (Ivanov and Nasmyth 2005). This model implies that the cohesin ring must be opened to be loaded onto the chromatin; this speculation was confirmed in yeast, where cohesin cannot associate with chromatin if its subunits are genetically manipulated to fuse with each other (Gruber *et al.* 2006). This finding also suggests that the opening of the cohesin ring to allow binding to chromatin occurs at the hinge domain of Smc1 and Smc3 and not at the ATPase head of Smc1 and Smc3 (Gruber *et al*. 2006). This result is unexpected because ATP hydrolysis by Smc1 and Smc3 is required for the chromatin binding reaction of cohesin (Arumugam *et al.* 2003; Weitzer *et al*. 2003), and the ATPase heads of Smc1 and Smc3 bind to each other or dissociate depending on ATP binding and hydrolysis (Arumugam *et al*. 2003; Weitzer *et al*. 2003; Haering *et al.* 2004). Hence, these results suggest that the ATPase heads may associate with the hinge domain and open it. These experimental observations can also be explained by other models; for example, two cohesin complexes that each embrace a single sister chromatid bind to each other to form a dimeric structure (Fig. IC), or two or more cohesin complexes form an oligomeric ring that connects the sister chromatids (Fig. ID) (Huang *et al.* 2005; Nasmyth 2005; Guacci 2007; Skibbens *et al.* 2007; Zhang *et al.* 2008b). However, a monomeric cohesin molecule is obtained if subunits of cohesin covalently linked by a chemical cross-linker on the chromatin (Haering *et al*. 2008). Furthermore, oligomeric cohesin has not been found in eukaryotes. Thus, it is plausible that sister chromatid cohesion is mediated by a monomeric cohesin molecule that causes concatenation of sister DNA molecules in a ring.

Chromatin binding of cohesin ring requires the distinct heterodimeric complex adherin, which consists of Scc2 and Scc4 (Dorsett 2004). Scc2 is a

well-conserved HEAT-repeat protein in eukaryotes, including humans (Furuya et al. 1998; Ciosk et al. 2000; Gillespie and Hirano 2004; Rollins et al. 2004; Takahashi et al. 2004; Tonkin et al. 2004). By comparison, Scc4 has a less conserved amino acid sequence, and a functional homolog of Scc4 has been identified only in a limited number of organisms, including *Schizosaccharomyces pombe* and human (Ciosk *et al.* 2000; Bernard *et al.* 2006; Seitan *et al.* 2006; Watrin *et al.* 2006; Takahashi *et al.* 2008). Adherin binds to cohesin (Toth *et al.* 1999; Gause *et al.* 2008; Takahashi *et al.* 2008), and is thought to stimulate the ATPase activity of the SMC heterodimer to promote the loading of cohesin onto chromatin. Since sister chromatid cohesion can occur only with cohesin that binds to chromatin before DNA replication (Lengronne *et al.* 2006), adherin also needs to bind to chromatin prior to DNA replication. In *Xenopus* egg extracts, the loading of adherin onto chromatin is dependent upon the pre-replication complex (pre-RC) (Gillespie and Hirano 2004; Takahashi et al. 2004). Recent study reveals that adherin is recruited to the chromatin via binding to Cdc7 kinase and its activating subunit Drf1 or Dbf4 (Takahashi *et al.* 2008). The dissociation of adherin from chromatin is dependent upon M-phase CDK activity (Gillespie and Hirano 2004). In fission yeast, cohesin loading onto pericentromeric heterochromatin requires the Cdc7 homolog Hsk1 (Takeda *et al.* 2001; Bailis *et al.* 2003), indicating that replication factors may function in cohesin loading. It is not known whether chromatin loading of adherin and cohesin in other organisms requires the pre-RC. On the contrary, the pre-RC component Cdc6 is dispensable for cohesin loading in budding yeast (Uhlmann and Nasmyth 1998). In any case, the initiation of the loading of cohesin onto chromatin occurs at the time when pre-RCs are assembled, at G1-phase in budding yeast (Guacci *et al.* 1997; Michaelis *et al.* 1997) and at telophase in mammalian cells (Losada *et al.* 1998; Darwiche *et al.* 1999; Sumara *et al.* 2000; Gerlich *et al.* 2006), suggesting that cohesin loading is regulated in a cell-cycle-dependent, maybe in a replication-dependent manner.

Since cohesin is loaded onto chromatin before DNA replication and its presence on chromatin during DNA replication is required for sister chromatid cohesion, the cohesion establishment reaction may involve sliding of the replication fork through the cohesin ring. Accumulating evidence suggests that the eukaryotic replisome forms a complex composed of tens of components. At the onset of S-phase, the pre-RC at each origin is activated by S-phase promoting kinases, S-CDK and Dbf4

dependent kinase (DDK), leading to formation of the initiation complex of DNA replication (Bell and Dutta 2002; Labib and Gambus 2007). Once replication is initiated, the replication fork is formed as a result of unwinding of DNA, which is followed by replication of unwound DNA by the DNA polymerases. Current studies suggest that Mcm2-7, a central component of the pre-RC, acts as a replicative helicase and that Cdc45 and GINS are co-factors that activate Mcm2-7 by forming a ternary Cdc45/Mcm2-7/GINS (CMG) complex (Moyer *et al.* 2006). Upon unwinding of DNA, DNA synthesis is initiated by DNA polymerase α (Pol α), and the leading and lagging strands are synthesized separately by DNA polymerase ε (Pol ε) and δ, respectively (Nick McElhinny *et al.* 2008). In addition to these components essential for replication, studies by Gambus *et al* (2006) in budding yeast show that accessory factors are associated with the CMG complex to form a replisome progression complex (RPC) (Fig. II). Some non-essential components, such as the Tof1-Csm3 complex and Mrc1, appear to be involved in maintaining fork integrity and are recognized as components of the replication checkpoint (Katou *et al.* 2003). These factors, together with other components including Ctf4, are conserved from yeast to human (Chou and Elledge 2006; Zhu et al. 2007). Considering that three DNA polymerases are located separately in the vicinity of the RPC, the apparent size of the replisome formed on the replication fork may be as large as the maximum diameter of the cohesin ring. It is possible that the large size of the replisome makes it difficult for the replication fork to slide through the cohesin ring.

To establish cohesion, the replisome should interact with the cohesin molecule to catch the ring and finally slide through the ring. Thus, the cohesion establishment factors must be components of the replisome. A study of budding yeast mutants has identified a number of cohesion establishment factors, and indeed many of these factors have been detected at the replication fork (Lengronne *et al.* 2006). Eco1 is an essential S-phase factor in budding yeast and genetically interacts with proliferating cell nuclear antigen (PCNA) and with components of replication factor C (RFC) (Kenna and Skibbens 2003). Eco1 promotes cohesion establishment by acetylating the cohesin subunit Smc3 at two lysine residues located near the ATPase head domain (Ben-Shahar *et al.* 2008; Unal *et al.* 2008; Zhang *et al.* 2008a). This acetylation is essential for cohesion, whereas Smc3 mutants harboring amino acid substitutions mimicking the acetylated state survive in the absence of Eco1(Ben-Shahar *et al.* 2008;

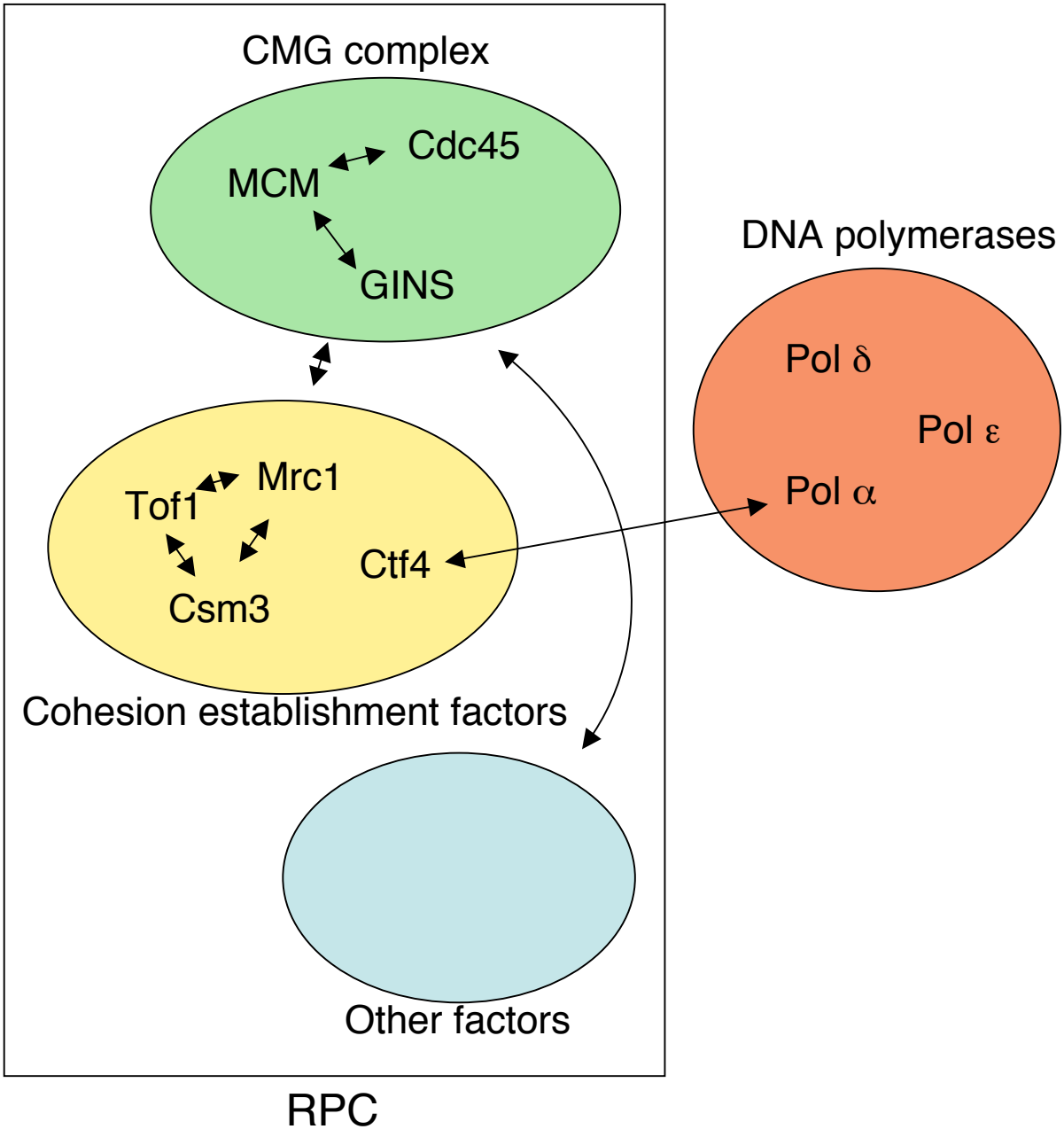

Figure II. Components of RPC in budding yeast

Yeast RPC consists of essential factors, such as the CMG complex, and non-essential factors, including non-essential cohesion establishment factors. Arrows indicate interaction between these factors.

Unal *et al*. 2008; Zhang *et al*. 2008a; Rowland *et al*. 2009; Sutani *et al*. 2009). Interestingly, Pds5 and Wapl mutants are viable in the absence of Eco1 in budding yeast (Ben-Shahar *et al*. 2008; Rowland *et al*. 2009; Sutani *et al*. 2009). Fission yeast Eso1, a homolog of Eco1, also becomes dispensable for viability in *pds5* deletion mutants (Tanaka *et al*. 2001). Similarly, the cohesion defect caused by the depletion of Esco2/Efo2, one of two human Eco1 homologs, is partially restored by depletion of Wapl (Gandhi *et al*. 2006). These findings suggest that Pds5 and Wapl may prevent establishment of cohesion, and that acetylation of Smc3 can counteract their inhibitory effect. In vertebrate cells, two Eco1 homologs are required for establishment of cohesion and have partially non-redundant functions in the cohesion process. Not Esco2/Efo2 but Esco1/Efo1 is required for Smc3 acetylation (Zhang *et al*. 2008a). However, depletion of either Esco1/Efo1 or Esco2/Efo2 causes a mild cohesion defect, and depletion of both Esco1/Efo1 and Esco2/Efo2 causes severe mitotic abnormalities (Hou and Zou 2005). It is still unclear how Esco1/Efo1 and Esco2/Efo2 function in establishment of cohesion. Ctf18-RFC, an alternative RFC complex composed of Ctf18, Ctf8, Dcc1, and Rfc2-4, is a non-essential establishment factor detected at replication forks in budding yeast (Hanna *et al*. 2001; Mayer *et al*. 2004; Lengronne *et al*. 2006). Biochemical analysis revealed that the Ctf18-RFC complex associates with PCNA, the sliding clamp, and can load it onto the chromatin (Bermudez *et al*. 2003). A homolog of Ctf18-RFC has been identified in eukaryotes (Merkle *et al*. 2003; Ansbach *et al*. 2008; Berkowitz *et al*. 2008) and vertebrate Ctf18-RFC has been shown to load PCNA (Shiomi *et al*. 2004); however, it has not been known whether Ctf18-RFC is an establishment factor in vertebrates as in yeasts.

A more precise study in budding yeast showed that some of the cohesion establishment factors are in fact non-essential components of the RPC (Gambus *et al*. 2006). The non-essential RPC component Ctf4, originally identified as an accessory factor of Pol α, is implicated in establishing sister chromatid cohesion in budding yeast (Hanna *et al*. 2001). This notion is supported by the finding that fission yeast Mcl1 and *Aspergillus nidulans* sepB, homologs of Ctf4, are required for proper segregation of sister chromatids (Harris and Hamer 1995; Williams and McIntosh 2002). Recent studies in budding yeast showed that Ctf4 is required for the stable association of Pol α with the chromatin (Tanaka *et al*. 2009), suggesting that Ctf4 may be involved in DNA replication. Similarly, human AND-1, a homolog of Ctf4, was shown to play an

essential role in DNA replication by recruiting Pol α to the chromatin, and this study suggested that the defect observed in the Ctf4 mutant of budding yeast is the result of defects in DNA replication (Zhu *et al.* 2007). Therefore, the function of the Ctf4 homologs in cohesion remains unclear. The Tof1-Csm3 complex and Mrc1 are also implicated in sister chromatid cohesion in budding yeast (Mayer *et al.* 2004; Xu *et al.* 2004). However, it is not known whether or not these proteins are required during S-phase for the establishment of cohesion. The function of these proteins in cohesion in higher eukaryotes has not been explored; however, in *Caenorhabditis elegans* the homolog of Tof1 appears to play an important role in establishing sister chromatid cohesion during meiosis (Chan *et al.* 2003).

During the course of exploring new proteins involved in DNA replication with *Xenopus* egg extracts, I became interested in the non-essential but conserved proteins of the yeast RPC. Previous studies showed that *Xenopus* Claspin is a homolog of yeast Mrc1 (Nyberg *et al.* 2002), and recent studies suggest that the *Xenopus* Tim1-Tipin complex and AND-1 are homologs of the yeast Tof1-Csm3 complex and Ctf4, respectively (Errico *et al.* 2007; Zhu *et al.* 2007). Here, I investigated the function of AND-1 and Tim1-Tipin in chromosomal replication and in the establishment of sister chromatid cohesion in *Xenopus* egg extracts. I demonstrated that AND-1 and Tim1 play a crucial role in establishing sister chromatid cohesion during S-phase.

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Results

Xenopus AND-1, Claspin and Tim1-Tipin are S-phase specific chromatin binding proteins

To investigate the function of these proteins in *Xenopus* egg extracts, I raised antibodies against *Xenopus* AND-1, Tim1, Tipin, and Claspin. Western blot analysis of egg extracts using antibodies against each protein specifically recognized a single band corresponding to the calculated molecular mass of each protein (Fig. 1). Since yeast homologs are implicated in S-phase and M-phase activity, I first examined chromatin binding of these proteins during S- and M-phase. When sperm chromatin was incubated in S-phase egg extracts, pre-RC was assembled within 15 min, judging from the chromatin binding of Orc2 and Mcm3 (Fig. 2A). DNA replication was then initiated within 30 min after starting the incubation (data not shown), and proteins essential for DNA replication such as Cdc45 and Pol α were loaded onto chromatin. AND-1, Tim1, and Tipin were bound to chromatin at similar timing with those of Cdc45 and Pol α and their amounts were increased during replication, and decreased when the replication was completed (about 120 min incubation). Claspin behaved similarly with AND-1, Tim1, and Tipin, but showing some delay in its initial binding; I could not detect Claspin binding at 15 min incubation. At 120 min after the incubation, chromatin binding of Tim1, Tipin, and Claspin was almost completely abolished, and small amount of AND-1 was detected on chromatin fraction by western blotting, which was similar to background level detected in the control prepared in the absence of sperm chromatin (-sperm). Observation under fluorescent microscopy confirmed that AND-1 bound to replicating chromatin (Fig. 2B), but only weak AND-1 signal detected in nuclei when the replication has been almost completed (Fig. 2B, 60 min). These results show that *Xenopus* AND-1, Claspin, Tim1, and Tipin specifically bind to chromatin during DNA replication.

I next investigate the chromatin binding of various proteins during M-phase by adding non-degradable cyclin B (ΔN 106-cyclin B) to S-phase extracts after the completion of DNA replication. Within 30 min after the addition of cyclin B, the nuclear envelope was broken down and chromatin structure was dramatically altered into condensed state (Fig. 2B, 240 min). In accord with the changes in chromatin structure, condensin was loaded onto chromatin, which was detected as chromatin

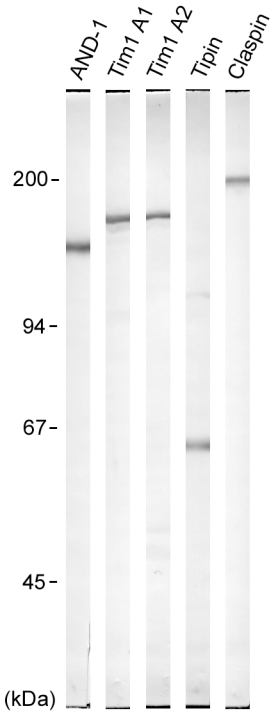


Figure 1. Specificity of the anti-sera against *Xenopus* AND-1, Tim1, Tipin and Claspin

Egg extract was resolved by SDS-PAGE and immunostained with anti-sera raised against AND-1, Tim1, Tipin and Claspin.

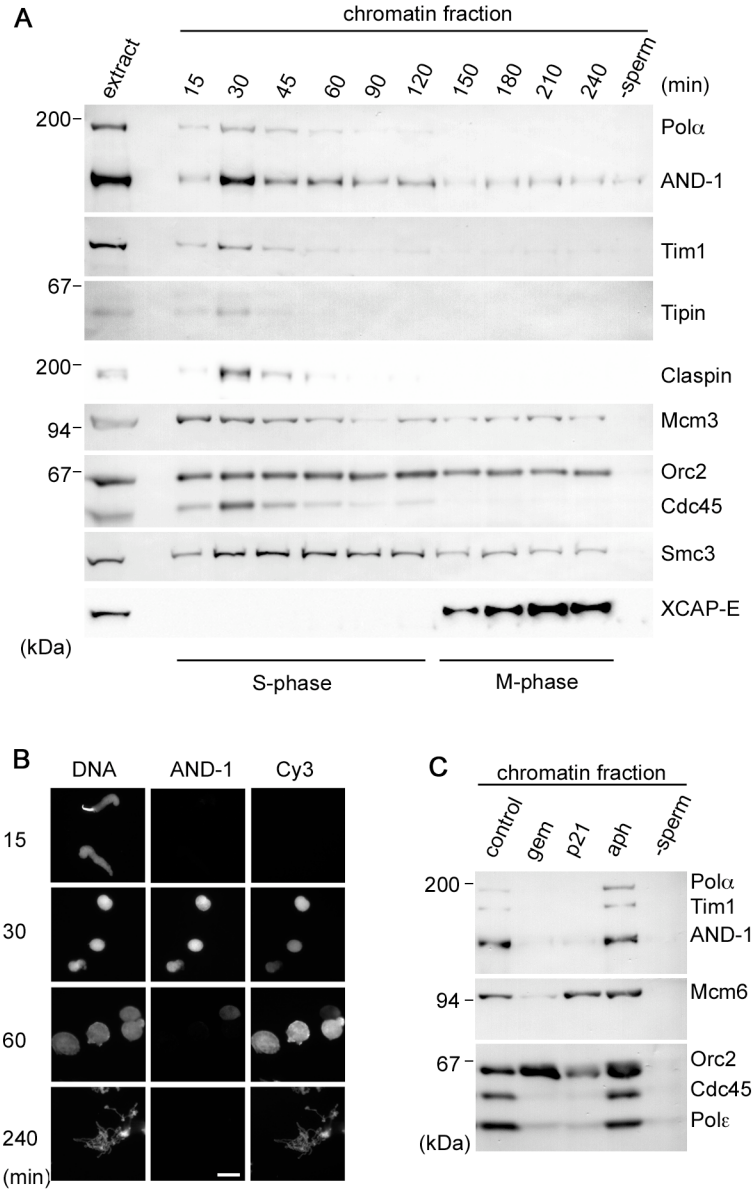


Figure 2. ***Xenopus*** **AND-1, Claspin, and Tim1-Tipin are S-phase-specific chromatin-binding proteins**

(A) Time-course analysis of chromatin binding of various proteins involved in replication, cohesion, and condensation. Sperm chromatin was incubated with egg extracts for 120 min at 23 ºC. Then ΔN106-Cyclin B (final concentration, 130 μg/mL) was added to each extract, which was then incubated for a further 120 min to induce chromatin condensation. Chromatin fractions were isolated at the times indicated. Egg extract and chromatin fractions were analyzed by SDS-PAGE and immunostained with antibodies indicated on the right panel. As a negative control, the chromatin fraction was isolated from the egg extract and incubated without sperm chromatin (-sperm). (B) Immunofluorescent analysis of the chromatin binding of AND-1. Chromatin was fixed at indicated times with 3.7% formaldehyde and immunostained with anti-AND-1 antibody. DNA was stained with Hoechst33258. Bar, 25 μm. (C) Effect of cell cycle inhibitors on the binding of AND-1 and Tim1 to chromatin. Geminin (gem, final 15 μg/mL), GST-p21 and roscovitine (p21, final concentrations 50 μg/mL and 100 μM, respectively), or aphidicolin (aph, final concentration 10 μM) was added to the extracts, and incubated with sperm chromatin for 60 min at 23 ºC. Chromatin fractions were isolated, analyzed by SDS-PAGE, and immunostained with antibodies indicated on the right panel.

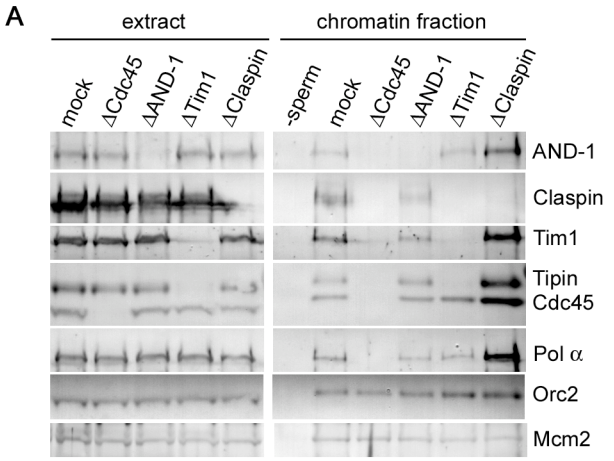
binding of XCAP-E, a condensin subunit, by immunoblot (Fig. 2A). On the contrary, chromatin binding of Smc3, a cohesin subunit, was decreased upon chromatin condensation as previously reported (Losada *et al*. 1998). Tim1, Tipin, and Claspin were not detected on M-phase chromatin by western blotting, and immunofluorescence observation of AND-1 verified that AND-1 was not present on M-phase chromatin.

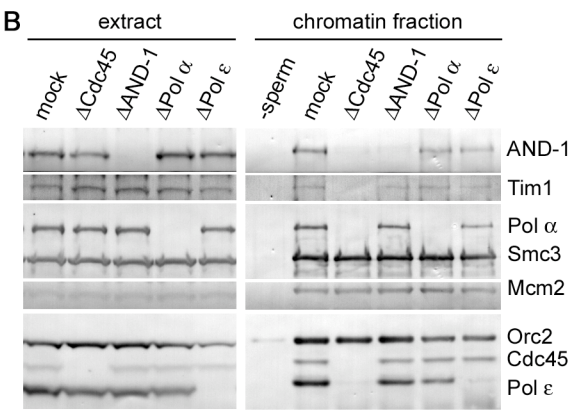
I further examined the effect of various cell cycle inhibitors on the chromatin binding of AND-1 and Tim1 (Fig. 2C). The chromatin binding of AND-1 and Tim1 was completely suppressed by geminin, which inhibits the pre-RC formation, and also by p21 plus roscovitine, which completely inhibit CDK activity in the extracts and thus inhibit the initiation of DNA replication. However, the binding was not significantly affected by the addition of aphidicolin, which inhibits DNA polymerase activities but not the initiation of the replication. Similar results were also obtained with the binding of Tipin and Claspin (data not shown). Previous studies on *Xenopus* Claspin, Tipin and AND-1 show that these proteins bind to chromatin in S-phase extracts, and CDK activity and/or pre-RC formation, but not DNA polymerase activity is required for these proteins to bind chromatin (Lee *et al*. 2003; Errico *et al.* 2007; Zhu *et al.* 2007) . I confirmed that the specific binding of *Xenopus* AND-1, Claspin, Tim1, and Tipin to chromatin depends on the initiation of DNA replication in S-phase and does not occur in M-phase.

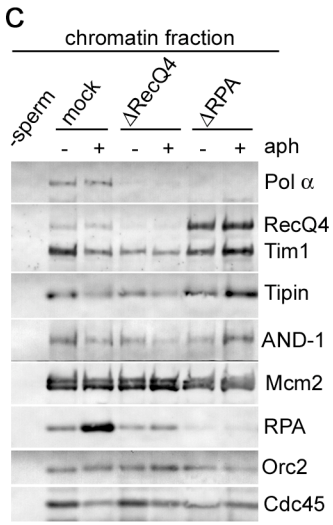
Cdc45-dependent binding of AND-1 and Tim1 to chromatin

The similar behavior of AND-1, Tim1, Tipin and Claspin prompted me to explore the factors involved in their binding to chromatin. I first examined whether the binding of each of these proteins to chromatin depends on the other proteins (Fig. 3A and 3D). In accordance with the initiation-dependent binding of these proteins to chromatin, depletion of Cdc45 from the egg extract abolished the binding of AND-1, Tim1, Tipin, and Claspin to chromatin. Tim1, Tipin, and Claspin bound to chromatin in the absence of AND-1, and AND-1 bound to chromatin in the absence of Tim1 or Claspin. It should also be noted that Tipin and Claspin failed to bind to chromatin in the absence of Tim1. These data demonstrate that AND-1 and Tim1 bind independently to chromatin and that the binding of these proteins to chromatin requires Cdc45.

Since AND-1 and Tim1 bound to chromatin in the presence of aphidicolin (Fig. 2C), I examined whether the binding requires the recruitment of DNA polymerases







D

	chromatin binding protein	depleted protein							
		Cdc45	AND-1	Tim1	Claspin	RecQ4	RPA	Pol α	Pol ε
licencing	Mcm2	+	+	+	+	+	+	+	+
pre-initiation	Cdc45	-	+	+	+	+	+	+	+
	AND-1	-	-	+	+	+	+	+	+
	Tim1	-	+	-	+	+	+	+	+
	Tipin	-	+	-	+	+	+	+	+
	Claspin	-	+	-	-	n.d.	+[a]	n.d.	n.d.
initiation	Pol ε	-	+	+	+	+[b]	+[c]	+	-
	Pol α	-	+	+	+	-	-	-	+

Figure 3. Cdc45-dependent chromatin binding of AND-1 and Tim1-Tipin

(A) Independent chromatin binding of AND-1 and Tim1-Tipin. Egg extracts were immunodepleted with pre-immune, anti-Cdc45, anti-AND-1, anti-Tim1 (A1) or anti-Claspin anti-sera. Sperm chromatin was incubated with the depleted extracts for 60 min at 23 ºC. Depleted extracts and chromatin fractions were analyzed by SDS-PAGE, and immunostained with antibodies indicated at right side of the panel. As a negative control, the sample was prepared from the egg extract without sperm chromatin (-sperm). (B) Requirement of Pol α and ε for the chromatin binding of AND-1 and Tim1. Egg extracts were immunodepleted with pre-immune serum or anti-Cdc45, anti-AND-1, anti-Pol α, or anti-Pol ε antibody. Chromatin fractions were prepared as in (A). (C) Requirement of RecQ4 and RPA for the chromatin binding of AND-1 and Tim1-Tipin. Egg extracts were immunodepleted with pre-immune serum or anti-RecQ4 or anti-RPA antibody. Sperm chromatin was incubated in the depleted extracts with or without aphidicolin (aph, final 10 μM) for 45 min at 23 ºC. Chromatin fractions were isolated, analyzed by SDS-PAGE, and immunostained with the indicated antibodies. (D) Summary of the requirement of AND-1, Tim1-Tipin, Claspin, and various replication proteins for chromatin binding of these proteins. +: bound to chromatin, -: not bound to chromatin, n. d. : not tested in this study. [a]Lee *et al*. 2003. [b]Matsuno *et al*. 2006. [c]Mimura *et al*. 2000.

onto the chromatin. AND-1 and Tim1 bound to chromatin in the absence of Pol ε or Pol α (Fig. 3B). In addition, the binding of Pol ε and Pol α to the chromatin was unaffected by AND-1 depletion. My finding that the binding of Pol α to chromatin was unaffected by AND-1 depletion is not consistent with the findings of a previous report (Zhu *et al*. 2007). To further explore the critical events in the binding of AND-1 and Tim1 to chromatin, I investigated the role of RecQ4 and RPA, both of which are required for replication after the binding of Cdc45 (Sangrithi *et al*. 2005; Matsuno *et al*. 2006). RPA is required for stabilizing the single-stranded DNA formed upon DNA unwinding and is required for the loading of Pol α onto the chromatin. The precise function of RecQ4 is unknown, but it is required for the loading of Pol α onto the chromatin after the binding of Cdc45. Upon depleting RecQ4 or RPA from the extract, the replication activity was diminished in the depleted extract (data not shown), while both AND-1 and Tim1 bound to chromatin in the absence of RecQ4 or RPA, irrespective of the presence of aphidicolin in the extracts (Fig. 3C). It should be noted that the binding of RecQ4 to chromatin increased in RPA-depleted extract compared with the mock-depleted extract.

Protein interactions of AND-1, Tim1-Tipin, and Claspin

The similar chromatin binding profiles of AND-1 and Tim1 in various depleted extracts suggested that AND-1 and Tim1 recognize a similar intermediate for the formation of the replisome. To identify targets of AND-1 and Tim1, I first examined whether these proteins interacted with Mcm2-7 or cohesin, two major chromatin-binding proteins in the egg extracts (Fig. 4A and Table 1). Tim1, Tipin, and Claspin did not co-precipitate with AND-1, being consistent with my finding that AND-1 and Tim1 bind independently to chromatin. Immunoprecipitation of Tim1 with the Tim1 A2 antibody, but not the Tim1 A1 antibody, resulted in the co-precipitation of AND-1, Mcm2, and Mcm6 from the extracts. Smc3 also co-precipitated with both of Tim1 antibodies. Tim1, AND-1 and Mcm6 co-precipitated with Claspin. I also found that Tim1 and Tipin co-precipitated with each other in a similar manner, i.e., the amount of Tim1 and Tipin in the immunoprecipitates were similar irrespective of the antibody used (Fig. 4A). Consistent with this finding, I demonstrated that depletion of Tim1 resulted in almost complete depletion of Tipin from the extracts (Fig. 3A and 11B).

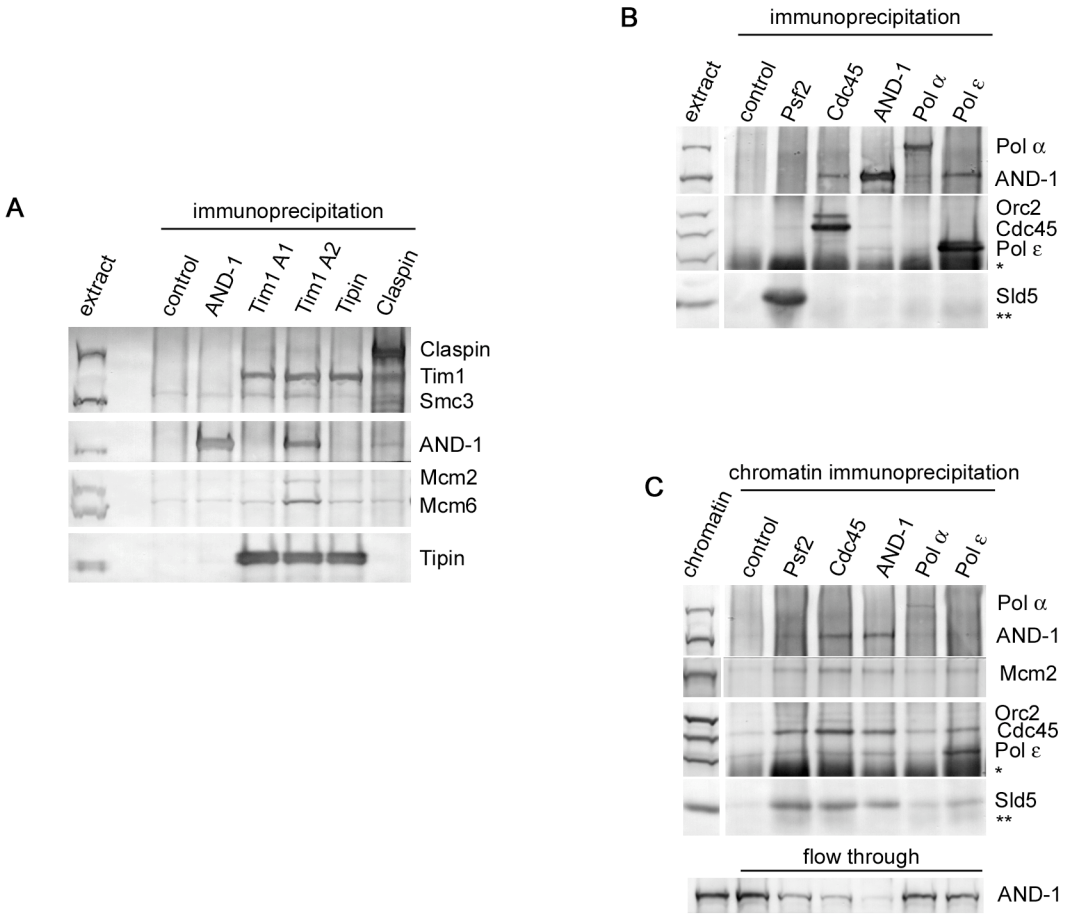


Figure 4. Interactions between AND-1 and Tim1 and various replisome components

(A) Immunoprecipitation of AND-1, Tim1, Tipin, and Claspin from egg extracts. Egg extract was immunoprecipitated with pre-immune serum (control) or the antibodies indicated at the top of the panel. Egg extracts and immunoprecipitates were analyzed by SDS-PAGE and immunostained with antibodies indicated at right side of the panel. (B) Co-immunoprecipitation of AND-1 and replication proteins from egg extracts. Immunoprecipitation was performed as described in (A). The band above Cdc45 was non-specific band. (C) Co-immunoprecipitation of AND-1 and replication proteins from replicating chromatin. The replicating chromatin fraction was isolated from egg extracts incubated with sperm chromatin for 35 min at 23 ℃. Isolated chromatin was digested by MNase to solubilize the chromatin-binding proteins. Solublized proteins were then immunoprecipitated and analyzed as in (A). Flow-through fractions collected after the chromatin immunoprecipitation were also analyzed. Asterisk indicates the IgG heavy chain. Double asterisks indicate the IgG light chain.

Table 1. Interaction of AND-1, Tim1-Tipin, Claspin, and replication proteins

Immunoprecipitation from egg extract. Data from Fig.4.

	antibody used for immunoprecipitation								
	Claspin	Tipin	Tim1 A1	Tim1 A2	AND-1	Cdc45	Pol ε	Pol α	Psf2
Claspin	■					⧹	⧹	⧹	⧹
Tim1	■	■	■	■		⧹	⧹	⧹	⧹
Tipin		■	■	■		⧹	⧹	⧹	⧹
Smc3			■	■		⧹	⧹	⧹	⧹
MCM	■			■		⧹	⧹	⧹	⧹
AND-1	■			■	■	■	■	■	
Cdc45	⧹	⧹	⧹	⧹	■	■			
Pol ε	⧹	⧹	⧹	⧹	■		■		
Pol α	⧹	⧹	⧹	⧹				■	
Sld5	⧹	⧹	⧹	⧹					■

Immunoprecipitation from replicating chromatin. Data from Fig.4 and Fig. 5.

	antibody used for immunoprecipitation						
	AND-1	Cdc45	Psf2	Pol ε	Pol α	Tim1 A2	Tipin
Pol α					■	⧹	⧹
Pol ε	■	■	■	■		⧹	⧹
AND-1	■	■	■			⧹	⧹
Sld5	■	■	■	■	■	⧹	⧹
Cdc45	■	■	■	■	■	■	■
MCM	■	■	■	■	■	■	■
Tim1	⧹	⧹	⧹	⧹	⧹	■	■
Tipin	⧹	⧹	⧹	⧹	⧹	■	■

Red box: precipitated, open box: not precipitated, shaded box: not tested in this study.

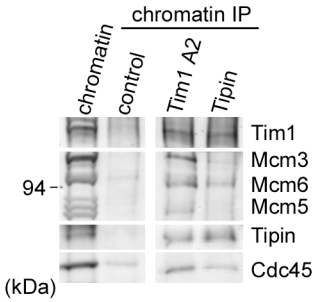


Figure 5. Interaction of Tim1-Tipin with components of the CMG complex

The replicating chromatin fraction was isolated from the egg extract after incubation with sperm chromatin for 35 min at 23 ℃. The chromatin isolated was digested by MNase to solubilize the chromatin binding proteins. Solublized proteins were immunoprecipitated with pre-immune serum or anti-Tim1 A2, or anti-Tipin antibody. Immunoprecipitates were analyzed by SDS-PAGE and immunostained with antibodies indicated on the right panel.

I next examined a possible target of replisome components for the binding of AND-1, because there was no apparent interaction between AND-1 and Mcm2-7 or cohesin in the extracts. To identify candidate proteins that interact with AND-1 I screened replication proteins in the egg extract by immunoprecipitation assays (Fig. 4B). Previous studies with budding yeast suggest that Ctf4 interacts with PolI (Pol α) and GINS (Miles and Formosa 1992; Gambus et al. 2006). AND-1 co-precipitated with Pol α but did not co-precipitate with GINS. By comparison, Sld5, a subunit of GINS, co-precipitated with the antibody to Psf2, the other GINS subunit, but not with AND-1. I found that Cdc45 and Pol ε co-precipitated with AND-1. It should be noted that the depletion of AND-1 scarcely affected the amount of Pol ε or Cdc45 in the extracts (Fig. 3B).

I next performed immunoprecipitation assays to examine the interaction between AND-1 and a component of the replisome on the chromatin (Fig. 4C). Unlike with the egg extracts, I did not detect any robust interactions between AND-1 and the DNA polymerases using replicating chromatin fractions. One reason may be the lower level of polymerases in the fragmented chromatin fractions. By comparison, AND-1 and Cdc45 efficiently co-precipitated from the chromatin fractions and more than 50% of the AND-1 bound to chromatin was recovered by immunoprecipitation with the anti-Cdc45 antibody (Fig. 4C, flow through fraction). I also detected co-precipitation of AND-1 and Cdc45 in the chromatin fractions with Psf2. Again, I found that the amount of AND-1 in the flow through fraction was markedly reduced by immunoprecipitation with Psf2. In addition, Mcm2 was detected in immunoprecipitates of Psf2, Cdc45, and AND-1. These results showed that the interactions among GINS, Cdc45, AND-1, and Mcm2-7 were more stable on chromatin than in the egg extracts. In contrast to the findings for AND-1, I did not detect tight association between Tim1-Tipin and Mcm2-7 or Cdc45, but I detected co-precipitation of Tim1-Tipin with Mcm3, 5, 6, and Cdc45 in the chromatin fractions (Fig. 5).

Role of AND-1 and Tim1 in DNA replication and Chk1 activation

Chromatin binding and chromatin immunoprecipitation experiments suggest that AND-1 is assembled into a replisome complex before the start of DNA synthesis and stably associates with the replisome during DNA replication. To understand the

function of AND-1, I first examined the replication activity of AND-1–depleted extracts (Fig. 6A). The extracts were subjected to the anti-AND-1 antibody three times to generate AND-1–depleted extracts. Following this process, AND-1 was not detected in samples with an extract volume five times greater than that of the mock-treated extract, and comparison of the depleted extracts with various dilution of the mock-depleted extracts suggest that the amount of AND-1 in the AND-1–depleted extracts was as much as few percent of the mock-depleted one (Fig. 7). Chromatin binding of AND-1 was abolished in the AND-1–depleted extracts (Fig. 3, and see also Fig. 6B). In an average of seven independent experiments DNA replication activity was not significantly decreased in AND-1–depleted extracts, compared with that in the mock-depleted extract, and recombinant AND-1 did not affect DNA replication activity when added to the AND-1–depleted extracts. I further examined DNA replication activity in extracts exposed to the anti-AND-1 antibody to interfere with AND-1 function, but although I used 1/20 volume of anti-sera or up to 60 μg of affinity purified antibody per mL extract I did not detect any effect on replication activity (Fig. 8 and data not shown).

The yeast *ctf4* mutant shows sensitivity to DNA damaging agents and synthetic lethality with deletion of components of the replication fork protection complex, such as Mrc1 (Tong *et al.* 2004; Ogiwara *et al.* 2007). Therefore, I examined whether AND-1 affects Chk1 activation upon the inhibition of DNA replication. Activation of Chk1 was detected by examining the phosphorylation status of Chk1 in nuclear fractions (Kumagai *et al.* 1998). The amounts of Orc2, Pol α, and Claspin in the nuclear fractions were not markedly affected by AND-1 depletion, whereas the level of AND-1 was diminished in the AND-1–depleted extracts (Fig. 6B). Addition of recombinant AND-1 to the AND-1–depleted extracts resulted in the accumulation of AND-1 in the nuclear fractions. In the absence of aphidicolin, Chk1 phosphorylation was not detected in the mock-depleted extracts, and a low level of Chk1 phosphorylation was detected in the AND-1–depleted extracts; this level diminished upon the addition of recombinant AND-1. By comparison, in the presence of aphidicolin I detected a similar level of Chk1 phosphorylation in mock- and AND-1–depleted extracts. These results suggest that AND-1 is not essential for the activation of Chk1 upon inhibition of replication activity with aphidicolin.

A previous study revealed that Tipin is not essential for replication but is

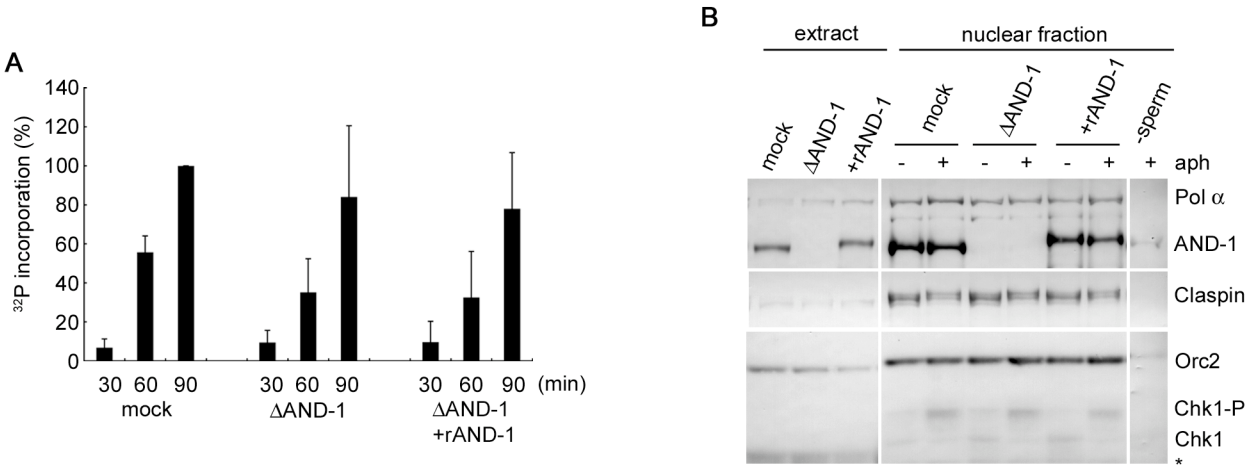


Figure 6. Role of AND-1 in DNA replication and Chk1 phosphorylation

(A) Effect of AND-1 depletion on the replication activity of egg extract. Sperm chromatin was incubated at 23 ºC for the indicated time periods with mock- or AND-1-depleted extract with or without recombinant AND-1 (final 20 μg/mL), in the presence of [α-^{32}P] dCTP. The replication products were analyzed by agarose gel electrophoresis and the amounts of ^{32}P incorporated into the DNA were quantified by autoradiography. The replication activities were normalized against that of the mock-depleted extract after incubation for 90 min. Error bars indicate mean ± S. D. of seven independent experiments. (B) Effect of AND-1 depletion on the activation of Chk1 induced by aphidicolin. Sperm chromatin was incubated for 120 min at 23 ºC in mock- and AND-1-depleted extract with or without recombinant AND-1 (final concentration, 20 μg/mL), in the presence or absence of aphidicolin (final 10 μM). Depleted extracts and nuclear fractions isolated from the extracts were analyzed by SDS-PAGE and immunostained with the antibodies indicated at the right side of the panel. Chk1-P indicates hyperphosphorylated Chk1 detected with the Chk1 antibody. Asterisk indicates nonspecific signal.

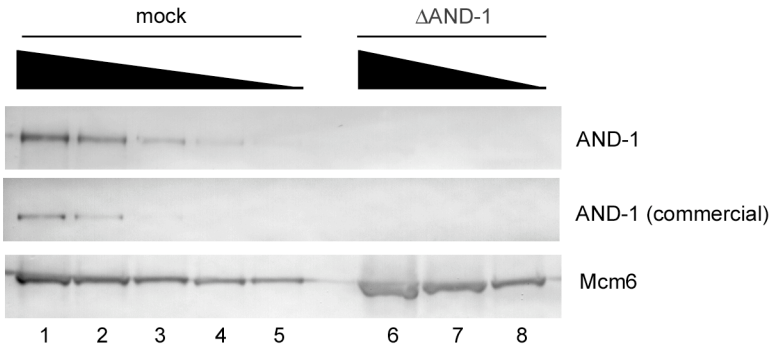


Figure 7. Efficiency of AND-1 depletion

Egg extracts were immunodepleted with pre-immune serum or anti-AND-1 anti-body. Depleted extracts were analyzed by SDS-PAGE and immunostained with anti-AND-1 and anti-Mcm6 antibodies indicated at right side of the panel. AND-1 was also detected with monoclonal antibody to nucleoplasmic Protein AND-1 from PROGEN (AND-1 (commercial)). The amount of the mock-depleted extracts applied on each lanes was 0.4, 0.3, 0.2, 0.1, 0.04 μL (lanes 1-5), and that of AND-1-depleted extracts was 2, 1, 0.4 μL (lanes 6-8).

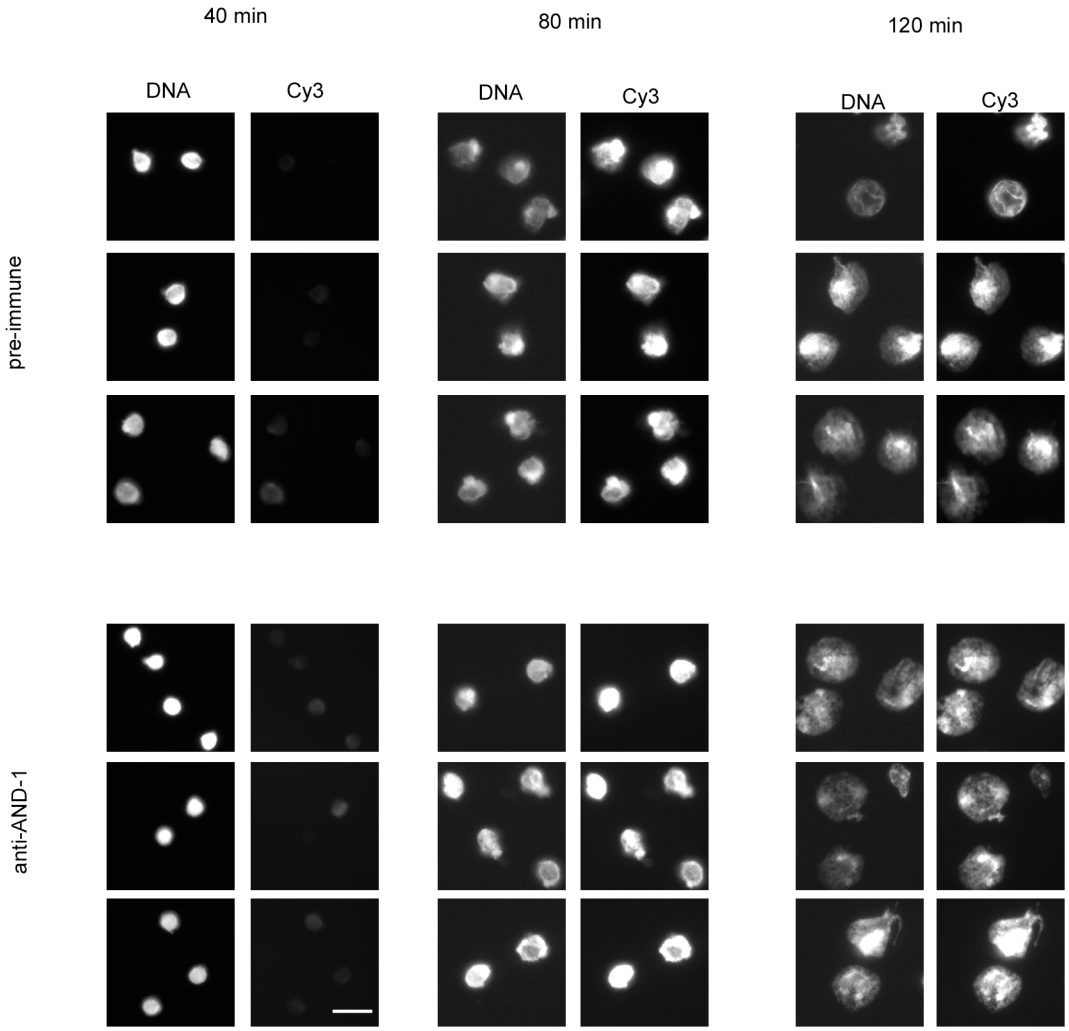


Figure 8. DNA replication is unaffected by the addition of Anti-AND-1 antibody to the egg extract
Immunoneutralization of AND-1 with anti-AND-1 anti-sera. Egg extracts were incubated with 1/20 volume of pre-immune or anti-AND-1 anti-sera for 10 min on ice. Sperm chromatin was incubated at 23 ^{o}C for the indicated time periods with the immunoneutralized extracts containing 1 µM Cy3-dCTP and then fixed with 3.7% formaldehyde. DNA was stained with Hoechst33258. Bar, 20 µm.

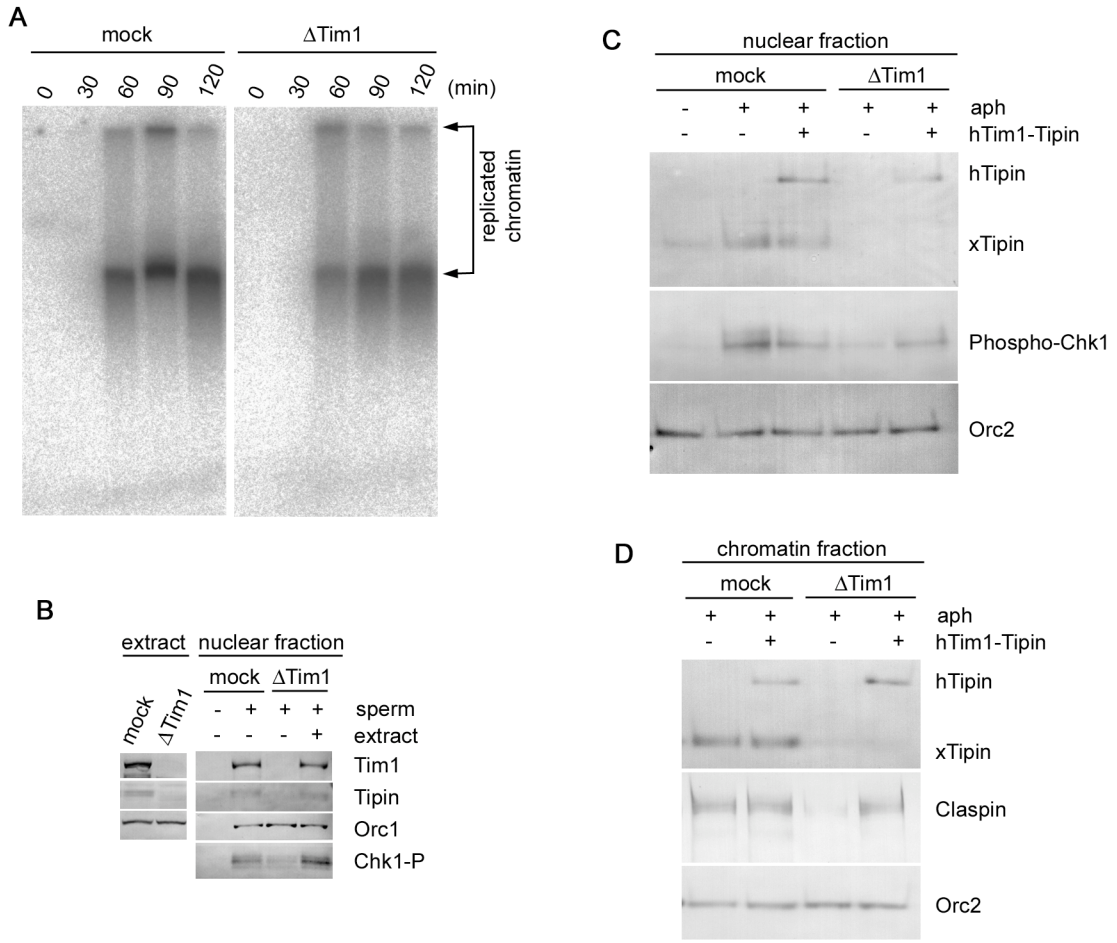


Figure 9. Role of the Tim1-Tipin complex in DNA replication and Chk1 phosphorylation

(A) Effect of Tim1 depletion on replication activity of egg extract. Sperm chromatin was added to mock- and Tim1-depleted extracts containing [α-^{32}P] dCTP and incubated for the indicated time periods at 23 ºC The replication products were subjected to agarose gel electrophoresis and the amounts of ^{32}P incorporated into the DNA were detected by autoradiography. (B) Effect of Tim1 depletion on activation of Chk1 induced by aphidicolin. Sperm chromatin was incubated for 120 min at 23 ºC with the mock- or Tim1-depleted extract in the presence of aphidicolin (final 10 μM). Nuclear fractions were isolated and analyzed by SDS-PAGE and immunostained with antibodies indicated at the right of the panel. As a control, nuclear fraction was prepared from the mock-depleted extract without sperm chromatin (-, -) or from the Tim1-depleted extract supplemented with 1/10 volume of untreated egg extract (+, +). (C) Effect of human Tim1-Tipin on activation of the replication checkpoint in *Xenopus* egg extract. Sperm chromatin was incubated for 120 min at 23 ºC with the mock-depleted or Tim1-depleted extract with or without aphidicolin (aph, final 10 μM). Recombinant human Tim1-Tipin complex was added to the mock- and Tim1-depleted extracts before incubation with sperm chromatin (hTim1-Tipin, final concentrations 3 μg/mL and 10 μg/mL, respectively). Nuclear fractions were isolated and analyzed by SDS-PAGE and immunostained with the antibodies indicated at the right of the panel. (C) Effect of human Tim1-Tipin on chromatin loading of Claspin in *Xenopus* egg extracts. Sperm chromatin was incubated for 120 min at 23 ºC with mock-depleted or Tim1-depleted extract with aphidicolin (aph, final concentration 10 μM). Recombinant human Tim1-Tipin complex (hTim1-Tipin, final concentrations 3 μg/mL and 10 μg/ml, respectively) was added to the mock- and Tim1-depleted extract before incubation with sperm chromatin. Chromatin fractions were isolated and analyzed by SDS-PAGE and immunostained with antibodies indicated at the right of the panel.

required for the activation of Chk1 in response to aphidicolin (Errico *et al.* 2007). Tipin forms a complex with Tim1, but it remains to be determined whether the defect observed with Tipin-depleted extracts is due to depletion of the Tim1-Tipin complex or Tipin alone. I therefore examined the role of Tim1 in the phosphorylation of Chk1 induced by aphidicolin. Upon depletion of Tim1, most of the Tipin was removed from the extracts, but the replication activity of Tim1-depleted extracts was similar to the level of replication activity in mock-depleted extracts (Fig. 9A). In contrast, Chk1 phosphorylation was markedly reduced in Tim1-depleted extracts compared with that in mock-depleted extracts (Fig. 9B). This decrease in Chk1 phosphorylation was recovered by adding human recombinant Tim1-Tipin complex to the Tim1-depleted extracts (Fig. 9C). In addition to the recovery of Chk1 phosphorylation, the binding of Claspin to the chromatin was recovered by adding recombinant Tim1-Tipin to the Tim1-depleted extracts (Fig. 9D).

AND-1 is required for proper establishment of sister chromatid cohesion in M-phase extracts

Previous reports show that Ctf4 is involved in the proper establishment of cohesion (Hanna *et al*. 2001). Therefore, I investigated whether AND-1 is required for sister chromatid cohesion. To examine the sister chromatid cohesion with egg extracts, I first incubated sperm chromatin in the treated extracts containing Cy3-dCTP, which is incorporated into the replicated region of chromatin. After 120 min incubation when DNA replication has been completed, ΔN106-Cyclin B was then added to the extracts to induce S- to M-phase transition and incubated for a further 120 min to complete the condensation of chromatin. In a mock-depleted extract, a pair of condensed and replicated chromatids was observed by Cy3 fluorescence and was found to be closely aligned with a regular interval of cohesive structures along the entire chromosome length (Fig. 10A). To quantify the cohesive structure of condensed chromatin, I evaluated the distance between paired chromosome molecules by measuring the distance between peaks of fluorescence signals for each chromosome axis visualized by immunostain with anti-XCAP-E antibody (Fig. 10B). In mock-depleted extracts, the distribution of distances showed a peak and the average of mean distance measurements taken from three independent experiments was 0.57 ± 0.030 μm. In AND-1–depleted extracts, pairs of replicated chromosomes displayed irregular and

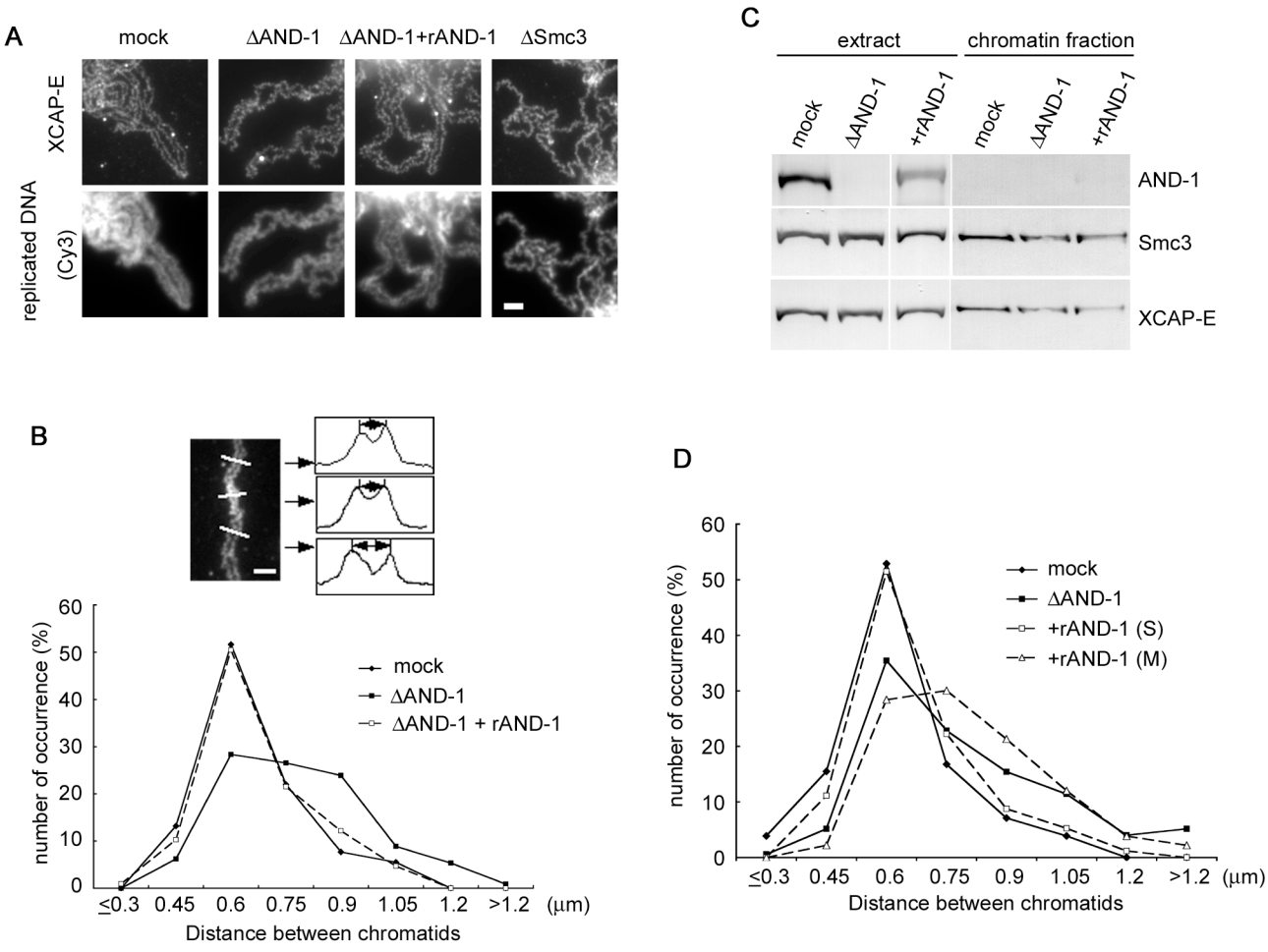


Figure 10. Sister chromatid cohesion in the AND-1–depleted extract

(A) Assay of sister chromatid cohesion in the egg extract. Sperm chromatin was incubated for 120 min at 23 ºC with mock-, AND-1- or Smc3-depleted extracts containing 1 μM Cy3-dCTP. Recombinant AND-1 (final 20 μg/mL) was added to the AND-1-depleted extract before incubation of the extract with sperm chromatin (ΔAND-1 + rAND-1). After the incubation, ΔN106-Cyclin B (final 130 μg/mL) was added to the extracts, which were further incubated for 120 min to induce chromatin condensation. Condensed chromatin was fixed with 3.7% formaldehyde and immunostained with anti-XCAP-E antibody to visualize the chromosome axis. Bar, 1 μm. (B) Measurement of distances between sister chromatids. To measure the distance between sister chromatids, regions of chromatin aligned in parallel were selected and the distances between peaks of fluorescence signals from anti-XCAP-E were measured (upper panel). The results obtained with mock- and AND-1–depleted extracts with or without recombinant AND-1 were plotted (lower panel). (C) Chromatin binding of cohesin, condensin and AND-1 at M-phase. M-phase chromatin was prepared as in (A); depleted extracts and condensed chromatin fractions isolated from extracts were analyzed by SDS-PAGE and then immunostained with antibodies indicated at the right of the panel. (D) Assay of the temporal requirement of AND-1 for sister chromatid cohesion. Sperm chromatin was incubated for 120 min at 23 ºC with the mock- and AND-1–depleted extracts containing 1 μM Cy3-dCTP. Recombinant AND-1 (final concentration, 20 μg/mL) was added to the AND-1–depleted extract before incubation with sperm chromatin (+ rAND-1 (S)) or after DNA replication (+ rAND-1 (M)). Distances between sister chromatids were measured as in (B)

partly separated structures revealed by Cy3 fluorescence. The distances between paired chromosome axes were widely distributed and the line plot of the distribution showed a broad peak with an average of mean distances of 0.72 ± 0.059 μm. This disordered chromosome structure was restored by adding recombinant AND-1 to the AND-1–depleted extracts. Chromosomal structures in the AND-1–depleted extracts with added recombinant AND-1 appeared to be similar to those observed in mock-depleted extracts and had a similar distribution of distances (average of mean distances, 0.61 ± 0.038 μm). Rescue of the disordered structures observed in AND-1–depleted extracts by the addition of recombinant AND-1 suggested that these structural defects were due to the absence of AND-1 from the egg extracts.

In Smc3-depleted extracts, the paired structures of replicated chromosomes were almost completely abolished and most of the replicated chromatin formed clumps, making it difficult to distinguish paired chromosome structures by microscopy. The chromatin structural defects that I observed are in good agreement with the finding of a previous report that cohesin is essential for sister chromatin cohesion in CSF-arrested extracts (Losada *et al.* 1998). I hereafter describe the defect in AND-1–depleted extracts as a defect in sister chromatid cohesion. The most likely reason underlying impaired cohesion is dissociation of the cohesin complex from M-phase chromatin in AND-1–depleted extracts. To test this possibility, I compared M-phase chromatin isolated from mock-depleted extracts with that from AND-1–depleted extracts, in the presence and absence of recombinant AND-1. Western blot analysis of egg extracts showed that AND-1 was absent in the depleted extracts, but Smc3, a subunit of cohesin, remained in both extracts. Both Smc3 and XCAP-E bound to M-phase chromatin in the absence of AND-1, and the ratio of Smc3 to XCAP-E in the chromatin fractions was similar in mock- and AND-1–depleted extracts (Fig. 10C). Since cohesin loaded onto the chromatin in the S-phase extracts, irrespective of the presence of AND-1 (Fig. 3B), my results suggest that the cohesion defect in AND-1–depleted extracts is not due to a defect in the loading of cohesin onto the chromatin in S-phase extracts or to dissociation of cohesin from the chromatin during M-phase.

Since AND-1 is tightly associated with the replisome components, I investigated the requirement for AND-1 in the establishment step of cohesion during DNA replication. Thus, I examined sister chromatid cohesion following the addition of recombinant AND-1 to the AND-1–depleted extracts before and after replication (Fig.

10D). The average distances between sister chromatids in mock- and AND-1–depleted extracts were 0.57 and 0.73 μm, respectively. The cohesion defect observed in AND-1–depleted extracts was almost completely restored by the addition of recombinant AND-1 before the start of DNA replication (average of mean distances, 0.61 μm); this is the same condition as shown in Fig. 10A. By comparison, the cohesion defect was not rescued when AND-1 was added after the completion of DNA replication (120 min after the start of incubation) and incubated for a further 120 min in the M-phase extracts. The average distance between sister chromatids was 0.75 μm, giving a distribution similar to that observed for AND-1–depleted extracts (Fig. 10D). These results suggest that AND-1 is required for the proper establishment of cohesion during DNA replication but not after DNA replication.

Tim1-Tipin is involved in the proper establishment of sister chromatid cohesion

Previous studies with budding yeast suggest a possible role for the fork protection complex (Tof1-Csm3-Mrc1) in establishing sister chromatid cohesion (Mayer *et al.* 2004; Xu *et al.* 2004). However, little is known about the exact nature of the cohesion defect. Taking advantage of the egg extracts that allowed me to directly observe sister chromatid cohesion, I investigated whether Tim1-Tipin and Claspin are involved in sister chromatid cohesion. Depletion of Tim1 or Claspin from the extracts did not affect the chromatin binding of AND-1 (Fig. 3A) or Smc3 (Fig. 11B). The establishment of cohesion was monitored in the depleted extracts by using a similar approach to that used for AND-1–depleted extracts. The distribution of distances between the sister chromosome axes was unaltered by depletion of Tim1 or Claspin from the extracts, but some regions of the chromatids showed a constantly open configuration in Tim1-depleted extracts compared with mock-depleted extracts (Fig. 11A). In order to distinguish such subtle structural change in sister chromatid cohesion, the distribution of distances between the chromatids was displayed at the interval of one pixel length (0.065 μm). Examination of the histogram of the distribution revealed that the peak position shifted to two pixel lengths wider in the Tim1-depleted extracts than in the mock-depleted extracts, and the average distance increased to 0.59 μm from 0.53 μm for the mock-depleted extract (Fig. 11A). By comparison, the peak shifted only one pixel length for Claspin-depleted extracts, and the overall distribution of distances was not markedly altered (average distance, 0.54 μm).

To explore the possible interplay between AND-1 and Tim1, I examined the effect of depleting both AND-1 and Tim1 from the egg extracts on sister chromatid cohesion. Depletion of both AND-1 and Tim1 did not alter the amount of cohesin in the egg extracts or that bound to the chromatin after completion of DNA replication (Fig. 11B). The replication activity of double-depleted extracts was lower than that of the mock-depleted extracts (Fig. 12); this may have been the result of poor nuclear formation in the double-depleted extracts. In order to assess the completion of replication, I examined condensed chromatin uniformly labeled with Cy3-dCTP. In mock-depleted extracts, induction of chromosomal condensation after DNA replication led to the formation of sister chromatid cohesion, which was detected as a close alignment of a pair of replicated chromosomes with a regular interval of cohesive structures (Fig. 11C, mock). Cohesive structure of the replicated chromosomes was difficult to detect in double-depleted extracts, as most of the replicated chromatin detected by Cy3 fluorescence showed regions of clamping and dispersal; in the latter there was an irregular and separated configuration of chromatin fibers, which is similar to the chromatin structure found in Smc3-depleted extracts (Fig. 10A). These cohesion defects were completely rescued by the addition of recombinant *Xenopus* AND-1 and human Tim1-Tipin to the depleted extracts before the start of replication. Most of the replicated chromatin showed cohesive structures similar to that observed in mock-depleted extracts (Fig. 11C). Therefore, I conclude that the aberrant sister chromatid cohesion in extracts depleted in AND-1 and Tim1 was due to the absence of both AND-1 and Tim1-Tipin.

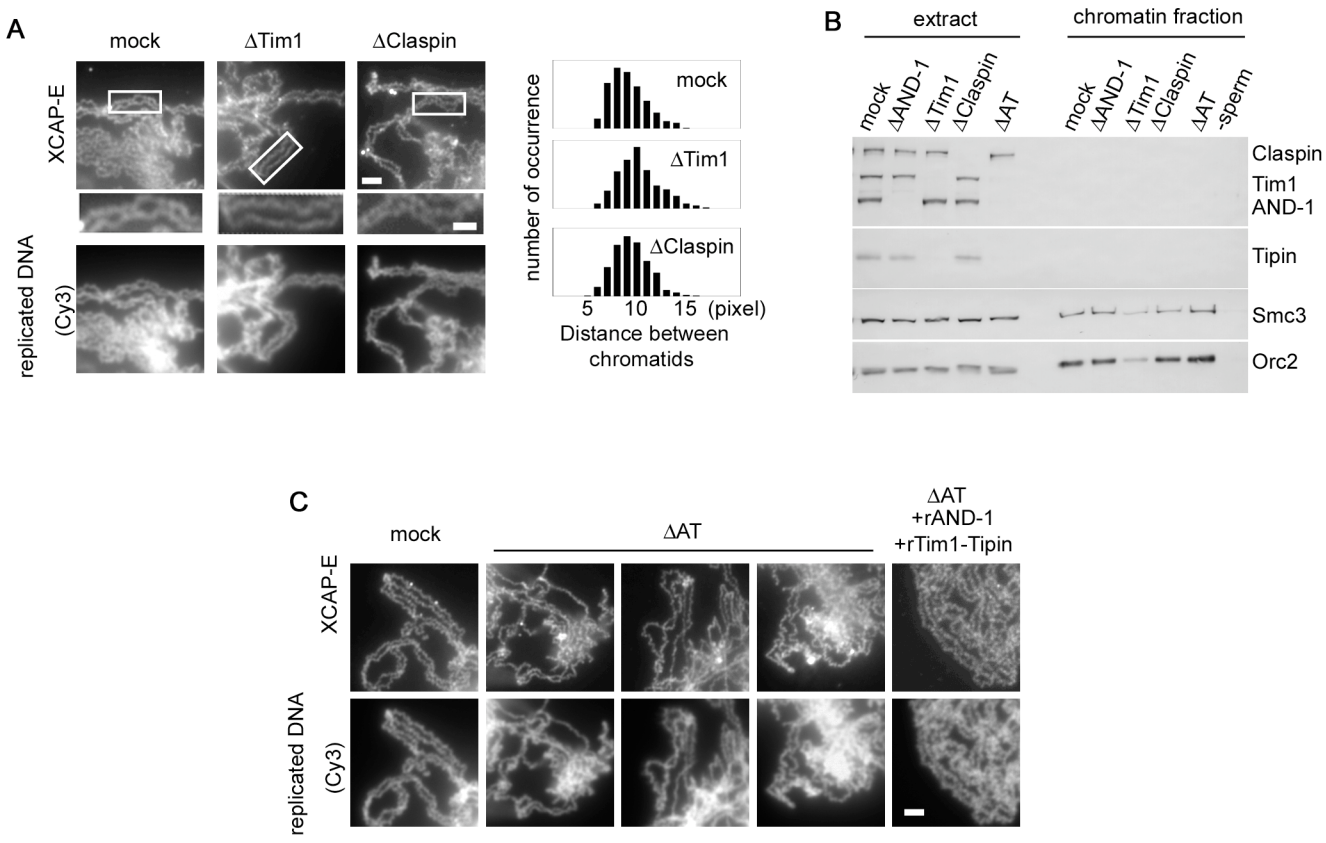


Figure 11. Tim1-Tipin and AND-1 together are required for proper establishment of sister chromatid cohesion

(A) Effect of Tim1 and Claspin depletion on sister chromatid cohesion in the egg extracts. Condensed chromatin in mock-, Tim1-, and Claspin-depleted extracts was prepared and visualized as described in the legend to Figure 10. Bar, 1 μm. Boxed areas indicate enlarged regions shown on the middle panels. Bar, 0.5 μm. Right panel shows the distribution of distances between sister chromatids, measured as described in the legend to Figure 10B, except that measurements were accumulated at each 1 pixel (= 0.065 μm). (B) Chromatin binding of cohesin after DNA replication in the absence of AND-1, Tim1, or Claspin. Sperm chromatin was incubated for 120 min at 23 ºC with the mock-, AND-1-, Tim1-, Claspin-, or AND-1 and Tim1–depleted extracts (mock, ΔAND-1, ΔTim1, ΔClaspin, and ΔAT, respectively). Depleted extracts and chromatin fractions isolated from the extracts were analyzed by SDS-PAGE, and immunostained with the antibodies indicated at the right of the panel. (C) Aberrant sister chromatid cohesion in the absence of both AND-1 and Tim1. Sperm chromatin was incubated for 120 min at 23 ºC with the mock and with double-depleted extracts containing 1 μM Cy3-dCTP. Recombinant AND-1 (final concentration, 20 μg/mL) and recombinant human Tim1-Tipin (final 3 μg/mL and 10 μg/mL, respectively) were added to the double-depleted extracts before incubation with sperm chromatin (ΔAT+rAND-1 + rTim1-Tipin). Bar, 1 μm.

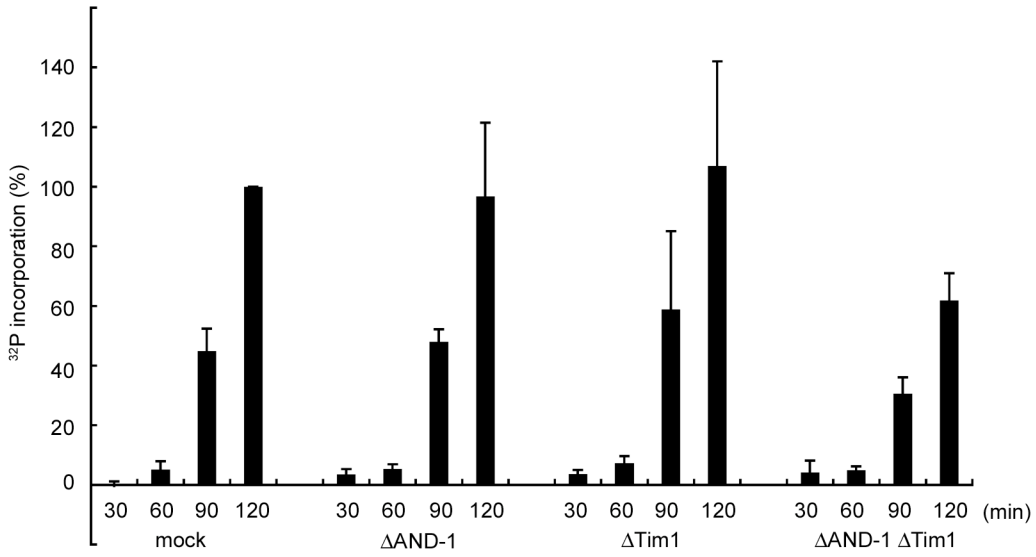


Figure 12. Effect of AND-1 and Tim1 depletion on DNA replication of the egg extracts

Sperm chromatin was incubated at 23 ºC for the indicated time periods with mock-, AND-1-, Tim1-, or AND-1 and Tim1-depleted extract in the presence of [α-^{32}P] dCTP. The replication products were analyzed by agarose gel electrophoresis and the amounts of ^{32}P incorporated into the DNA were quantified by autoradiography. The replication activities were normalized against that of the mock-depleted extract after incubation for 120 min. Error bars indicate mean ± S. D. of three independent experiments.

Discussion

I showed that AND-1 and Tim1-Tipin were required for the sister chromatid cohesion observed in *Xenopus* egg extracts. Factors involved in sister chromatid cohesion have been extensively studied in budding yeast as a model organism. Yeast Ctf4 is one of the factors involved in sister chromatid cohesion (Hanna *et al*. 2001), but the exact function of Ctf4 remains unknown. Tof1 and Csm3 are implicated in the same pathway as Ctf4 for the establishment of cohesion (Xu *et al*. 2007). Since these proteins are putative components of the RPC (Gambus *et al*. 2006), it is plausible that the replisome plays an important role in establishing sister chromatid cohesion. However, no evidence has been presented for the requirement of these factors during S-phase. Recent studies in budding yeast have demonstrated that cohesion is formed by embracing sister chromatids with a single cohesin molecule (Haering *et al*. 2008). This novel finding formed the basis of my investigations into the molecular mechanisms underlying the cohesion establishment reaction catalyzed by the replisome. I found here that AND-1 and Tim1-Tipin, vertebrate homologs of Ctf4 and Tof1-Csm3, respectively, formed a complex with replisome components in *Xenopus* egg extracts and were required for establishing cohesion during DNA replication. This is the first report showing the role of replisome components such as AND-1 and Tim1 in establishing the sister chromatid cohesion during S-phase.

Formation of replisome progression complex in *Xenopus* egg extracts

Previous studies show that Cdc45, but not RPA, is required for the binding of Claspin to chromatin, and that Mcm10 is required for the binding of AND-1 to chromatin (Lee *et al*. 2003; Zhu *et al*. 2007). I confirmed here that AND-1, Tim1-Tipin, and Claspin are S-phase–specific chromatin-binding proteins, and that the binding of these proteins to chromatin depends on Cdc45, but not RPA or the DNA polymerases. In addition, I found that Tim1-Tipin is required for the binding of Claspin to chromatin; this is consistent with the results of a previous report that Tipin is required for the binding of Claspin to chromatin (Errico *et al*. 2007). Apparent chromatin binding of Tim1-Tipin in the absent of RecQ4 further suggests that the fork protection complex is assembled before the formation of replication fork. The observed independent binding of Tim1-Tipin and AND-1 to chromatin, and the strict dependence of this binding on

Cdc45, suggests that Tim1-Tipin and AND-1 are assembled onto chromatin after formation of the CMG complex but before the loading of DNA polymerases.

The chromatin immunoprecipitation assays for AND-1 and Tim1-Tipin showed that these proteins were associated with Cdc45, GINS, and Mcm2-7 on digested chromatin fractions. In particular, AND-1 appeared to form a tight complex with Cdc45 and GINS, because the immunoprecipitations of Cdc45 and GINS led to a marked decrease in AND-1 in the flow-through fractions. By comparison, I did not detect a tight association between AND-1 and Pol α on the chromatin; however, the chromatin binding of AND-1 slightly decreased in the absence of Pol α (Fig. 3B). These results suggest that Pol α plays an important role in stabilizing AND-1 in the RPC. In addition, I found that AND-1 and Pol ε could be reciprocally immunoprecipitated from digested chromatin fractions and egg extracts. Taken together, these data suggest that a complex, like the RPC of budding yeast, is formed during DNA replication, and that AND-1 and Tim1-Tipin are components of this complex.

Functional implications of AND-1 and Tim1-Tipin in DNA replication and Chk1 activation

Previous studies show that Tipin and AND-1 are not essential for DNA replication in *Xenopus* egg extracts, but instead play an important role in maintaining the replication fork. Tipin is required for the stalled replication fork to resume DNA replication after the removal of aphidicolin (Errico *et al.* 2007), whereas AND-1 is required for stabilizing Pol α on the chromatin (Zhu *et al.* 2007). In addition, Tipin is required for the activation of Chk1 following the inhibition of DNA polymerases by aphidicolin (Errico *et al.* 2007). We found here that the Tim1-Tipin complex is required for the activation of Chk1 and the association of Claspin with chromatin. The ability of Tim1-Tipin in recruiting Claspin to chromatin thus suggests that the activation of Chk1 is mediated by Claspin recruited onto the replicating fork via Tim1-Tipin. In contrast, AND-1 is not essential for the activation of Chk1. A previous report showed that AND-1 is required for efficient DNA replication in *Xenopus* egg extracts (Zhu *et al.* 2007). It is unclear why I did not detect an effect of AND-1 depletion on DNA replication. It is possible that the procedures I used did not adequately deplete AND-1. However, I detected a defect in the establishment of cohesion with AND-1 depletion

and rescued the defect with recombinant AND-1. Differences in the antibodies used in the experiments in the previous study and mine may also account for my failure to detect an effect on DNA replication with AND-1 depletion. The antibody used in the previous report is a neutralizing antibody, which inhibits replication when added to the egg extracts. However, the antibody I used here did not inhibit replication. Thus, it is possible that immunodepletion with the neutralizing antibody results in release of the antibody from the conjugated beads into the extracts, leading to the apparent inhibition of replication.

AND-1 and Tim1-Tipin are required for the proper establishment of sister chromatid cohesion

My results showed for the first time that AND-1 was required for proper establishment of sister chromatid cohesion in higher eukaryotes. The depletion of AND-1 from *Xenopus* egg extracts resulted in pairs of replicated chromatids with a more separated structure than in the controls, and this defect was recovered by adding recombinant AND-1 to the AND-1–depleted extracts before DNA replication. Unreplicated DNA should form a physical link between sister chromatids; thus incomplete DNA replication may lead to incomplete resolution of the chromatids. On the contrary, I detected separated structures of replicated DNAs instead of a cohesive structure. Therefore, my results suggest that AND-1 is required for proper formation of sister chromatid cohesion, and incomplete DNA replication in the absence of AND-1 is not the underlying cause of the defect.

Morphological defects in cohesion were also observed in Tim1-Tipin–depleted extracts. Again, I confirmed that the depletion had no marked effect on the replication activity of the extracts (Fig. 9). Thus, the defect in Tim1-depleted extracts is the result of the absence of Tim1 and not the inhibition of DNA replication. There were no detectable cohesion defects in Claspin-depleted extracts. Since the binding of Tim1-Tipin to chromatin is required for the binding of Claspin to chromatin, but not vice versa, the defects in cohesion in Tim1-Tipin–depleted extracts are not the result of the absence of Claspin on the chromatin and suggest a distinct role for Tim1-Tipin in establishing sister chromatid cohesion.

A severe defect in sister chromatid cohesion was observed by the combination of AND-1 and Tim1 depletion. The irregular morphology of the replicated chromatin

in the double-depleted extracts was similar to that observed in the Smc3-depleted extracts. Since Smc3 is a component of cohesin, the observed defect in the double-depleted extracts suggests that AND-1 and Tim1-Tipin are essential for the proper establishment of sister chromatid cohesion. The requirement for AND-1 and Tim1 in the establishment of cohesion is consistent with previous work in fission yeast and *C. elegans* (Williams and McIntosh 2002; Chan et al. 2003). In budding yeast, Ctf4 and Tof1 or Csm3 are reported to have a redundant role in the establishment reaction (Xu *et al.* 2007), and conversely, another report shows that the depletion of both genes is synthetic lethal (Tong *et al.* 2004). The cause of the lethality is unknown, and I could not directly correlate my results with the synthetic lethality observed in budding yeast, but my results showed that both AND-1 and Tim1 are required for the proper establishment reaction.

Models for the cohesion establishment reaction mediated by RPC

I found novel morphological defects in sister chromatid cohesion along the entire length of the chromosome in the absence of AND-1 and Tim1-Tipin. Although it is difficult to speculate on the molecular function of AND-1 and Tim1-Tipin in the establishment reaction on the basis of morphological defects, I propose models for the molecular mechanism underlying the establishment reaction. There are two possible models to explain the behavior of the cohesin molecule bound to unreplicated DNA during DNA replication. In the first model, the cohesin ring remains intact throughout replication, whereas in the second model the ring embracing the DNA opens during replication to allow the passage of the RPC. In the first model, the cohesin molecule bound to the DNA might be an obstacle for the progression of the replisome. Although I do not know the exact diameter of the replisome, it may be as large as 30 nm, which is close to the maximum diameter of the cohesin ring. When the replisome encounters the ring, if the ring is not pushing away, the replication fork may be stalled by the ring. If the helicase alone could slide through the ring, it would be necessary to stop the helicase from moving forward and leaving the polymerases at the ring. In this scenario, Tim1-Tipin may have a role in stabilizing the stalled fork. In order to slide through the ring, the replisome structure needs to be as compact as possible. Since AND-1 interacts with Pol ε and α, it is possible that AND-1 tethers both polymerases on the leading and lagging strands of the DNA and also stabilizes the replisome by interacting with the

helicase. If the ring is opened transiently during DNA replication, according to the second model, it would be difficult to catch the sister chromatids with a cohesin molecule after replication. The replicated sister chromatids would physically separate soon after replication, such that catching both chromatids with a cohesin ring would be increasingly difficult. In this situation, the replisome may function as a tether of the cohesin molecule to allow re-embracing of the replicated sister chromatids soon after replication. The physical interaction that I found here between Tim1 with Smc3 may contribute to such a tethering process. In either case, my study suggests that components of the RPC play a crucial role in the establishment reaction, and further investigation of the functions of these proteins may help to elucidate the molecular mechanism underlying the cohesion establishment reaction.

Experimental procedures

Cloning and protein expression

Full-length *Xenopus* AND-1 was constructed as follows: The ORF encoding the N-terminal amino acids 1-571 of AND-1 was PCR amplified from the cDNA clone (I.M.A.G.E. ID 4970671, from ATCC) by using the 5' primer CGCGGATCCATGCCAGCTATAAAGAAG and the 3' primer ACCTCTATGATAGACCAC, and then digested with BamHI and NcoI. The cDNA clone XL021113 (supplied from NIBB), which contains the ORF encoding the C-terminal amino acids 321-1127, was digested with BamHI and NcoI, and ligated with the PCR-amplified N-terminal fragment.

The full-length AND-1 was then cloned into pGEX 6P-3 (GE Healthcare) for expression of the GST-tagged AND-1 protein. Full-length AND-1 was sub-cloned into the BamHI-XhoI site of pGEX 6P-3. The GST-tagged AND-1 protein was expressed in DH5α at 23 ºC for 4 h after induction with 0.1 mM IPTG. Cells were harvested and then lysed by using french pressure cell. Purification of the GST-tagged AND-1 protein and removal of the GST-tag with PreScission Protease (GE Healthcare) were performed in accordance with the manufacturer's instructions. For His-tagged AND-1 protein expression, I used the Bac-To-Bac Baculovirus expression system (Invitrogen). His-tagged AND-1 protein was expressed in Sf9 insect cells and purified with Ni-NTA agarose (Qiagen) in native conditions in accordance with the manufacturer's instructions. The expression vector GST-ΔN106-cyclin B was a generous gift from Dr. K. Ohsumi (Iwabuchi *et al.* 2002). GST-ΔN106-cyclin B was expressed in *E. coli* BL21 DE3 and affinity purified with Glutathione Sepharose 4B (GE Healthcare) in accordance with the manufacturer's instructions.

To prepare anti-*Xenopus* Tim1 antibodies, the N-terminal fragment of Tim1 (1-1113 bp) was PCR amplified using the 5' primer ATAGAATTCATGGACTTGTACATGATGAATTG and the 3' primer CTATCTCGAGTTATAAAGAACAGCGCAACACC and sub-cloned into the EcoR1-Xho1 site of pGEX 6P-3. The GST-tagged N-terminal fragment of Tim1 was expressed in *E. coli*. The recombinant protein was recovered as insoluble pellets and further purified SDS-PAGE.

To prepare anti-*Xenopus* Tipin antibodies, full-length Tipin cDNA was PCR

amplified using the 5' primer ATAGAATTCATGATGGATCCTTTGGACAACGG and the 3' primer TATCTCGAGTTCAATATTCTTCTTTAGTGTTTGCACAAGC and then sub-cloned into the EcoR1-Xho1 site of pGEX 6P-1. GST-tagged full-length Tipin expressed in *E. coli* was purified by standard procedures using PreScission protease. The Protein complex of full-length human Tim1-Tipin was a generous gift from Dr. H. Masai (Yoshizawa-Sugata and Masai 2007).

Antibodies

Anti-AND-1 antibody was raised against full-length AND-1 expressed in *E. coli.* Anti-Tim1 and Tipin antibodies were raised against the N-terminal fragment of *Xenopus* Tim1 and full-length *Xenopus* Tipin, respectively. All of these polyclonal antibodies were raised in the rabbit (Hokudo Inc., Japan). XCAP-E and *Xenopus* Smc3 antibodies were raised against the C-terminal peptides SKTKERRNRMEDVK (Hirano *et al*. 1997) and EQAKDFVEDDTTHG (Losada *et al*. 1998), respectively (OPERON Biotechnologies). Phosphorylation of *Xenopus* Chk1 at Ser344 was detected with human Phospho-Chk1 (Ser345) monoclonal antibody from Cell Signaling Technologies. Other antibodies were prepared as described previously (Hashimoto *et al*. 2006).

Immunodepletion and DNA replication assays

Xenopus egg extracts and permeabilized sperm nuclei were prepared as described previously (Kubota and Takisawa 1993), with slight modifications. For preparation of egg extract, a second centrifugation was carried out at 40,000 × *g* for 10 min. Egg extract was supplemented with 5% glycerol and 20 μg/mL cycloheximide and then frozen in liquid nitrogen until required. Immunodepletion and DNA replication assays were carried out as described previously (Mimura and Takisawa 1998) with slight modifications. For the immunodepletion assay rProtein A sepharose Fast Flow (GE Healthcare) was used instead of Affi-Prep protein A matrix (Bio-Rad), and 10-μL anti-sera were used instead of antibodies. For double-depletion assay anti-AND-1 and anti-Tim1 anti-sera were mixed and bound to rProtein A sepharose Fast Flow (GE Healthcare), and used for immunodepletion as described above. For the DNA replication assay the autoradiograms were quantified with Image Gauge software and a BAS2500 image analyzer (Fujifilm).

Preparation of chromatin and nuclear fractions

To isolate the chromatin fraction, sperm nuclei were incubated at 23 ºC in 50 to 100 μL of egg extracts (4000 nuclei/μL) for the indicated periods of time in the Figures. The samples were diluted with 10 volumes of extraction buffer (EB; 100 mM KCl, 2.5 mM $MgCl_2$, 50 mM HEPES-KOH; pH 7.5) containing 0.25% NP-40 (Wako), incubated for 2 min on ice, and then centrifuged at 10,000 × *g* for 10 min through the layer of EB containing 10% sucrose . The upper layer containing the diluted extract was removed by aspiration and the remaining extract was washed by adding EB to the lower layer and repeating centrifugation at 10,000 × *g* for 10 min. The pellets were washed once with EB, solubilized with SDS-PAGE sample buffer, and then filtered through a 0.45-μm filter (Millipore, Ultrafree-MC) to remove insoluble matrix. To isolate the nuclear fraction, sperm nuclei were incubated at 23 ºC in 50 μL of egg extract (4,000 nuclei/μL) in the presence or absence of 10 μM aphidicolin for the indicated periods of time. The samples were diluted with 450 μL EB, incubated for 2 min on ice, and then centrifuged at 10,000 × *g* for 5 min through the layer of EB containing 1 M sucrose . The pellets were washed with EB containing 1 M sucrose, solubilized with SDS-PAGE sample buffer, and then filtered through a 0.45-μm filter (Millipore, Ultrafree-MC) to remove insoluble matrix.

Immunoprecipitation and chromatin immunoprecipitation

Immunoprecipitation from egg extracts was carried out as described previously (Mimura and Takisawa 1998), except that 10 μL anti-sera were used instead of antibodies. Chromatin immunoprecipitation was carried out with the digested chromatin fraction in the absence of aphidicolin, as described previously (Mimura *et al.* 2000).

Cohesion assay and immunofluorescent staining

Egg extracts containing 1 μM Cy3-dCTP (GE Healthcare) were incubated at 23 ºC for 2 h with sperm chromatin (2000 nuclei/μL) to complete DNA replication. GST-ΔN106-cyclin B (final 130 μg/mL) was then added to the egg extracts to condense the replicated chromatin. The egg extracts were incubated at 23 ºC for a further 2 h. The samples were then diluted and fixed for 10 min on ice with 10 volumes

of EB containing 3.7% formaldehyde, and the condensed chromatin was recovered on polylysine-coated coverslips by centrifugation at 1200 × *g* for 5 min through EB containing 1 M sucrose. The coverslips were washed and incubated overnight at 4 ºC with anti-XCAP-E antibody as the primary antibody, following by incubation for 1 h at room temperature with Alexa488–labeled anti-rabbit IgG (Molecular Probes) as the secondary antibody. The coverslips were washed and mounted on glass slides with mounting solution (15 mM PIPES [pH 6.9], 15 mM NaCl, 80 mM KCl, 3.7% formaldehyde, and 50% glycerol) containing Hoechst33258 (Wako) for DNA staining. Images of the condensed chromatin were collected by OpenLab 3.0.9 software (Improvision) from a cooled CCD camera (CoolSNAP HQ, Photometrics) with a microscope (BX50, Olympus) using a UPlanFl objective lens (100×, 1.30 NA, oil immersion, Olympus). Distances between sister chromatids were measured as the lengths between peaks of fluorescent signals of each sister chromosome axis, by using ImageJ software (NIH). The distances were measured at regular intervals at a rate of more than 100 measurements per sample. Average distances between sister chromatids were calculated from mean distances of at least three independent experiments, with standard deviation (± S.D).

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Acknowledgements

First and foremost, I would like to express my sincere gratitude to Professor Haruhiko Takisawa for his unwavering support and invaluable advice during my years at Osaka University. I would also like to deeply thank Associate Professor Yumiko Kubota for her kind and supporting help and critical reading of my manuscript.

I am very grateful to Dr. Tatsuya Hirano for providing antibodies against XRad21 and XCAP-E, Dr. Keita Ohsumi for the expression vector of GST-ΔN106-cyclin B, and Dr. Tatsuro S. Takahashi and Dr. Masato Kanemaki for helpful discussions. I would like to thank all members of Nuclear Function Laboratory. I would especially thank Dr. Maya Kumano and Tsuyoshi Tsujimura for cloning of XTim1-Tipin and valuable suggestion.

Finally, I would like to thank my parents for always supporting me and believing in me for long years. Without their support, I would not be where I am today.