Roles of Replication-associated Factors in Cohesin Regulation

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1. ABSTRACT

Cohesin is a ring-shaped protein complex that consists of four core subunits: SMC1 and SMC3 forming a V-shape heterodimer held together at the open ends by the cohesin’s kleisin component, RAD21, and either SA1 or SA2 that associate with RAD21. Functionally, the cohesin ring mediates DNA-DNA interactions between distant loci both in cis and in trans, the latter type being important for sister chromatid cohesion, SCC. The SCC function of cohesin involves topological entrapment of one or two DNA strands into its lumen. Functionally, SCC is important for accurate segregation of chromosomes in mitosis and facilitates error-free recombination repair, overall being critical for genome integrity. Cohesin-DNA interaction is facilitated by the cohesin loader NIPBL-MAU2 and downregulated by WAPL-PDS5 that facilitates unloading of cohesin. The stabilization of cohesin on DNA against WAPL-mediated unloading is favored by cohesin acetylation at two evolutionarily conserved sites on SMC3 that is mediated by Eco1/Eso1 in budding and fission yeast, and by ESCO1 and ESCO2 in vertebrates. In addition, several replisome-associated components are involved in SCC establishment. However, their functions remain incompletely understood, especially in vertebrate cells that contain additional cohesin regulators.

Using chicken lymphoma DT40 cells suitable for genetic engineering and cytogenetic studies, I aimed to understand the roles of ESCO1/2 and replication-associated factors in cohesin regulation. Our study revealed previously unknown roles for ESCO1/2 in interphase chromatin organization and both functional separation and cooperation in SCC and proliferation. Moreover, we found that dysfunctions in replisome-associated components can lead to SCC cohesion defects that depend on WAPL but less so on ESCO1/2-mediated SMC3 acetylation. Finally, our IP-MS experiments identified novel cohesin interactors, RUVBL1 and RUVBL2, with roles in SCC. Thus, additional regulations are likely to exist in vertebrate cells to instruct cohesin abundance and its cohesive state.
ACKNOWLEDGEMENTS

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<td>ATPases Associated with diverse cellular Activities</td>
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<td>AcT</td>
<td>Acetyltransferase domain</td>
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<td>AID</td>
<td>Auxin Inducible Degron</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>Bleo</td>
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<td>BPB</td>
<td>Bromophenol blue</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>Centromere Protein</td>
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<td>Chromatin binding fraction</td>
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<td>Chromosome Transmission Fidelity Factor</td>
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<td>DEAD/H-Box helicase</td>
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<td>Dox</td>
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<td>DSB</td>
<td>Double Strand Break</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<td>Eco</td>
<td>Mycophenolic acid resistant marker casette</td>
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<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid</td>
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<td>FACS</td>
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<td>FACT</td>
<td>Facilitates Chromatin Transcription</td>
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<td>Flip-in</td>
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<td>Fork Pausing Complex</td>
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<td>Green Fluorescent Protein</td>
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<td>GINS</td>
<td>Sld5-Psf1-Psf2-Psf3 complex</td>
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<td>HR</td>
<td>Homologus Recombination</td>
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<td>Horseradish Peroxidase</td>
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<td>Hygromycin resistant marker casette</td>
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<td>KO</td>
<td>Knock Out</td>
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<td>LC-MS</td>
<td>Liquid Chromatography Mass Spectrometry</td>
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<td>MCM</td>
<td>Minichromosome Maintenance protein</td>
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<td>MFH</td>
<td>Myc-FLAG-HA tag</td>
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<td>MMS</td>
<td>Methyl methanesulfonate</td>
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<td>Nipped-B Like protein</td>
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<td>NUC</td>
<td>Nucleoplasmic fracton</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>PBAF</td>
<td>BRG1/BRM Associated Factor</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered Saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDS</td>
<td>Precocious Dissociation of Sisters</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PIM</td>
<td>PDS5 Interacting Motif</td>
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<tr>
<td>PIP</td>
<td>PCNA-interacting protein motif</td>
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<tr>
<td>PLK</td>
<td>Polo Like Kinase</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethysulfonyl fluoride</td>
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<td>PP</td>
<td>Protein Phosphatase</td>
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<tr>
<td>Puro</td>
<td>Puromycin resistant marker casette</td>
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<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<td>qPCR</td>
<td>Quantitative PCR</td>
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<tr>
<td>RBS</td>
<td>Roberts Syndrome</td>
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<td>RC</td>
<td>Replication Complex</td>
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<td>RNR</td>
<td>Ribonucleotide Reductase</td>
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<td>RSC</td>
<td>Chromatin Structure Remodeling complex</td>
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<td>RSV</td>
<td>Rous Sarcoma Virus</td>
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<tr>
<td>RT</td>
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<td>RT-PCR</td>
<td>Reverse Transcription PCR</td>
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<td>RUVBL</td>
<td>RuvB-Like protein</td>
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<tr>
<td>SA</td>
<td>Stromal Antigen</td>
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<tr>
<td>SAC</td>
<td>Spindle Assembly Checkpoint</td>
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<td>SCC</td>
<td>Sister Chromatid Cohesion</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SMC</td>
<td>Structural Maintenace of Chromosomes</td>
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<tr>
<td>SNF</td>
<td>Sucrose Nonfermenting</td>
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<tr>
<td>snoRNP</td>
<td>Small nucleolar RNAs</td>
</tr>
<tr>
<td>STED</td>
<td>Stimulated Emission Depletion microscopy</td>
</tr>
<tr>
<td>TADs</td>
<td>Topologically Associated Domains</td>
</tr>
<tr>
<td>TLS</td>
<td>Translesion DNA Synthesis</td>
</tr>
<tr>
<td>TRE</td>
<td>Tetracycline Response Element</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>---------------------------------</td>
</tr>
<tr>
<td>TS</td>
<td>Template Switch</td>
</tr>
<tr>
<td>WABS</td>
<td>Warsaw Breakage Syndrome</td>
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<tr>
<td>WAPL</td>
<td>Wings Apart-Like protein</td>
</tr>
<tr>
<td>WCE</td>
<td>Whole Cell Extract</td>
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<tr>
<td>Zf</td>
<td>Zinc-finger domain</td>
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</table>
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(*: equally contributed)
2. INTRODUCTION

2.1. Genome Organization by SMC Protein Complexes

Human genomes are 2 m in length, whereas the nuclei are about 10 µm in diameter. In such a narrow space, the genome must be replicated and propagated to daughter cells while transcription is also taking place. To make it possible, the genome architecture is made highly dynamic throughout the cell cycle (Reviewed in Yatskevich, Rhodes and Nasmyth, 2019). One outstanding case is the one of chromosome condensation during mitosis in which genomic DNA is compacted to mitotic

Model 1. Genome organization by SMC complexes. Human genome is 2 m in length. It is hierarchically organized by histone and SMC complexes including cohesin, condensin, and SMCS/6 complex.
chromosomes, shortening the length from centi-meter scale to micro-meter scale (Reviewed in (Hirano, 2015)). In turn, during interphase, the genome is organized hierarchically into different compartments that allow for specialized DNA transactions, including transcription, replication, and DNA repair (Reviewed in (Sikorska and Sexton, 2020)). SMC (Structural Maintenance of Chromosomes) protein complexes, which comprise condensin, cohesin, and SMC5/6 complex, play central roles in shaping higher-order chromatin architecture (Model 1). Condensin is essential for mitotic chromosome condensation, cohesin is important for sister chromatid cohesion (SCC) and for chromatin architecture in interphase, and the SMC5/6 complex plays critical roles in DNA replication and repair.

2.2. Sister Chromatid Cohesion

In order to preserve genetic information, sister chromosomes must be equally segregated during mitosis. Sister chromatid cohesion (SCC) is essential for accurate chromosome segregation and is established by cohesin in cooperation with DNA replication (Reviewed in (Peters, Tedeschi and Schmitz, 2008; Peters and Nishiyama, 2012; Morales and Losada, 2018)). At anaphase onset, spindles attached to the sister centromeres give bipolar tension which triggers chromosome segregation. SCC is essential for the bipolar tension at anaphase onset, therefore, cells defective in SCC elicit spindle assembly checkpoint (SAC) activation resulting in metaphase arrest, ultimately ending up in chromosome missegregation and mitotic cell death (Reviewed in (Musacchio and Salmon, 2007)). In addition, SCC ensures accurate DNA repair via homologous recombination (HR) by keeping donor DNA templates in the vicinity of damaged DNA strands (Reviewed in (Litwin, Pilarczyk and Wysocki, 2018)). Therefore, SCC is a key factor for genome integrity.
2.3. Cohesin Complex Architecture

SCC is mediated by cohesin (Guacci, Koshland and Strunnikov, 1997; Michaelis, Ciosk and Nasmyth, 1997; Losada, Hirano and Hirano, 1998). The cohesin complex consists of 4 core subunits including SMC1, SMC3, RAD21 (also called SCC1), and either SA1 or SA2 (Stromal Antigen, also called STAG1, STAG2 respectively) (Reviewed in (Morales and Losada, 2018)). SMC1 and SMC3 belong to SMC protein

**Model 2. Cohesin’s architecture.** SMC proteins has common features. Their central segments comprise hinge-domain through which they form v-shaped hetero dimers. N-lobe contains Walker-A domain, and C-lobe is composed of Walker-B domain, forming head-domain that has ATPase activity. Highly extended anti-parallel coiled-coil (∼50 nm) domain connects the hinge-domain and head-domain. RAD21, a kinesin protein, bridges the head-domains of SMC1 and SMC3 resulting in tripartite ring-shaped complex. HEAT-repeat proteins such as SA1/2, PDS5A/B, NIPBL are bound to RAD21 and regulate cohesin or further recruit other regulatory factors.
family (Strunnikov, Larionov and Koshland, 1993). SMC proteins contain hinge-domain at their central segments through which they form heterodimers (Hirano, 2002). The N-lobe and C-lobe of SMC proteins are composed of Walker-A and Walker-B domains respectively, shaping two ATPase sites at their head-domains (Strunnikov, Larionov and Koshland, 1993; Michaelis, Ciosk and Nasmyth, 1997). Between hinge-domain and head-domain, highly extended antiparallel coiled-coil domain (≈ 50 nm) connects and forms V-shaped heterodimers. RAD21, a kleisin protein, bridges the head-domains of SMC1 and SMC3, forming tripartite ring-shaped complex (Model 2) (Melby et al., 1998; Anderson et al., 2002; Haering et al., 2002, 2004; Schleiffer et al., 2003). RAD21 is largely unstructured except for its N-terminus and C-terminus where it is bound to SMC’s heads (Haering et al., 2002). In addition, either SA1 or SA2 (Scc3 in yeast) containing HEAT-repeats is bound to RAD21 and mediate interactions with additional regulators such as NIPBL and PDS5A/B (Reviewed in (Morales and Losada, 2018)).

2.4. Cohesin Loader

As cohesin is a ring-shaped protein complex and mediates SCC, it topologically entraps DNA to its lumen (Gruber, Haering and Nasmyth, 2003; Haering et al., 2008; Murayama and Uhlmann, 2014). To do so, cohesin needs to transiently open either of its interfaces. Which interfaces does cohesin open for embracing DNA? When the hinge-domain is artificially locked, cohesin’s ability to associate with chromatin is severely affected but not when the interfaces of SMC3-RAD21 and SMC1-RAD21 are locked (Gruber et al., 2006). Therefore, currently it is believed that the hinge-domain opens as the entry gate for embracing DNA (Kurze et al., 2011).

NIPBL1-MAU2 (Scc2-Scc4 in yeast) is essential for cohesin loading onto DNA (Model 3) (Furuya, Takahashi and Yanagida, 1998; Ciosk et al., 2000; Bénard et al.,...
2004; Gillespie and Hirano, 2004; Rollins et al., 2004; Takahashi et al., 2004; Seitan et al., 2006; Watrin et al., 2006). NIPBL and DNA synergistically stimulate in vitro cohesin’s ATPase activity, which is proposed to catalyze cohesin loading (Arumugam et al., 2003; Murayama and Uhlmann, 2014; Petela et al., 2018). Nevertheless, how mechanistically NIPBL promotes cohesin loading still remains ambiguous. To be noted, cohesin loading is DNA sequence independent in vitro, however, it is known

Model 3. Cohesin regulation throughout cell cycle. Telophase-G1: NIPBL-MAU2 catalyzes cohesin loading, but WAPL-PDS5 unload cohesins resulting in dynamic association of cohesins with chromatin. S-G2: CoATs (Eco1, ESCO1/2) acylate cohesin to convert cohesin into WAPL resistant form. In higher eukaryotes, Sororin binds to acylated cohesins to inhibit WAPL. Consequently, cohesin is stabilized on chromatin, mediating SCC. Prophase-Metaphase: Mitotic kinases (PLK1, Aurora B, CDK1) phosphorylate cohesin, enabling WAPL to unload acylated cohesins. Unloaded cohesins are deacetylated by HDAC8 and recycled. Cohesins on centromeric regions are protected by SGO1-PP2A complex that counteracts the mitotic kinase-mediated phosphorylation of cohesin. Metaphase-Anaphase: Upon inactivation of SAC, Separase gets activated and cleaves RAD21 that are mediating centromeric SCC.
that cohesin is loaded with preference at certain genomic loci such as centromeres and promoters of highly transcribed genes (Nonaka et al., 2002; Lengronne et al., 2004; Takahashi et al., 2004; Fernius et al., 2013; Natsume et al., 2013; Lopez-Serra et al., 2014; Murayama and Uhlmann, 2014; Zuin et al., 2014; Hinshaw et al., 2017).

SCC is mediated by cohesins that are topologically embracing DNAs, and the loader is essential for that (Haering et al., 2008; Murayama and Uhlmann, 2014). However, a number of recent studies demonstrated that cohesin mediates also intra-chromosomal interactions through which cohesin organizes higher-order genome architecture (Wendt et al., 2008; Haarhuis et al., 2017; Rao et al., 2017; Schwarzer et al., 2017; Wutz et al., 2017; Vian et al., 2018). In vitro experiments showed that cohesin has an activity to extrude DNA, termed “loop extrusion”, in ATP and NIPBL dependent manner (Davidson et al., 2019; Kim et al., 2019). Interestingly, topological entrapment seems to be unnecessary for the loop extrusion in vitro (Davidson et al., 2019). Therefore, it is suggested that the loader stimulates cohesin’s ATPase activity not only for the loading reaction, but also for the genome organization by loop extrusion.

2.5. Negative Regulators of Cohesin

While the cohesin loader, NIPBL-MAU2, catalyzes cohesin loading onto DNA, a number of proteins are implicated in negatively regulating cohesin-DNA interaction (Reviewed in (Morales and Losada, 2018)). Although SCC is essential for accurate chromosome segregation, it needs to be resolved at anaphase onset when chromosome segregation occurs. To achieve this, cohesins are stepwisely removed from DNA via two distinct pathways in vertebrates. First, cohesins on chromosome arms are unloaded during prophase to metaphase (Losada, Hirano and Hirano, 1998; Sumara et al., 2000; Waizenegger et al., 2000). In this process, called the “prophase pathway”, mitotic kinases, primarily PLK1 (Cdc5 in yeast) and Aurora B (Ipl1 in yeast) phosphorylate
SA2 and Sororin respectively to enable WAPL, a cohesin unloader, to release cohesin from DNA (Model 3) (Losada, Hirano and Hirano, 2002; Giménez-Abián et al., 2004; Hauf et al., 2005; Nishiyama et al., 2013). At this step, cohesin rings at centromeric regions are protected by the SGO1-PP2A complex (Salic, Waters and Mitchison, 2004; Tang et al., 2004; Kitajima et al., 2005). PP2A is a phosphatase which is thought to antagonizes PLK1-mediated phosphorylation of cohesin resulting in stabilization of cohesin at centromeric regions (Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006). Subsequently, separase, a cysteine protease, gets activated at anaphase onset upon inactivation of SAC and cleaves RAD21 so that SCC at centromeric regions is fully resolved (Model 3) (Uhlmann, Lottspeelch and Nasmyth, 1999; Uhlmann et al., 2000).

2.6. Sister Chromatid Cohesion Establishment by Cohesin Acetyltransferases

Although WAPL was initially characterized to displace cohesin in prophase in mammalian cells, later studies showed that WAPL is functional throughout the cell cycle for cohesin unloading (Gandhi, Gillespie and Hirano, 2006; Kueng et al., 2006; Bernard et al., 2008; Tedeschi et al., 2013). Hence, although NIPBL-MAU2 catalyzes cohesin loading, cohesin’s association with DNA is highly dynamic in the presence of WAPL (Model 3) (Gerlich et al., 2006). Nevertheless, cells need to maintain SCC for equal chromosome segregation. To this end, cohesin acetyltransferases (CoATs), ESCO1 and ESCO2 (Eco1 in budding yeast) acetylate SMC3 at K105 and K106 (K112 and K113 in budding yeast) to make cohesin resistant against WAPL-mediated cohesin unloading (Skibbens et al., 1999; Tóth et al., 1999; Ivanov et al., 2002; Hou and Zou, 2005; Ben-shahar et al., 2008; Ünal et al., 2008; Zhang et al., 2008; Rowland et al., 2009; Sutani et al., 2009; Beckouët et al., 2010). In higher eukaryotes, Sororin is shown to bind acetylated cohesin to inhibit WAPL (Model 3) (Rankin, Ayad and
Kirschner, 2005; Schmitz et al., 2007; Lafont, Song and Rankin, 2010; Nishiyama et al., 2010; Ladurner et al., 2016). The acetylated cohesin molecules persist on chromosomes until becoming phosphorylated by mitotic kinases, enabling WAPL to unload the acetylated cohesins (Hauf et al., 2005; Gandhi, Gillespie and Hirano, 2006; Kueng et al., 2006; Nishiyama et al., 2013). Although these acetylation sites are shown to be essential for cellular viability and SCC in budding yeast, whether the same principle is conserved in vertebrates is currently unclear (Ben-shahar et al., 2008; Ünal et al., 2008; Ladurner et al., 2016). Thus, further studies are required to understand how ESCO1/2 and SMC3 acetylation contribute to the maintenance of SCC.

2.7. Comparison of Eco1 and ESCO1, ESCO2

Cohesin regulation mechanism seems to be more complex in vertebrates compared with that of budding yeast. For instance, several proteins are multiplied to several orthologs such as SA1/2, PDS5A/B and ESCO1/2 (Table 1).

<table>
<thead>
<tr>
<th>Category</th>
<th>Budding yeast</th>
<th>Human</th>
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<tbody>
<tr>
<td>Cohesin</td>
<td>Smc1</td>
<td>SMC1A, SMC3, RAD21, STAG1/STAG2</td>
</tr>
<tr>
<td></td>
<td>Smc3</td>
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<td>Scc1</td>
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<tr>
<td></td>
<td>Scc3</td>
<td></td>
</tr>
<tr>
<td>Associated factors</td>
<td>Pds5</td>
<td>PDS5A/PDS5B, WAPL, CDGA5/Sororin</td>
</tr>
<tr>
<td></td>
<td>Rad61 (Wapl)</td>
<td></td>
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<tr>
<td>Loader</td>
<td>Scc2</td>
<td>NIPBL, MAU2</td>
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<td></td>
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<td>Cohesin Acetyltransferase</td>
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<td>ESCO1/ESCO2</td>
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<td>Cohesin Deacetylase</td>
<td>Hos1</td>
<td>HDAC8</td>
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<td>Mitotic regulators</td>
<td>Sgo1 (Shugoshin)</td>
<td>SGOL1 (Shugoshin)</td>
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<td>Esp1 (Separase)</td>
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<td></td>
<td>Pds1 (Securin)</td>
<td>PTTG1 (Securin)</td>
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</table>

Table 1. Component of cohesin and its regulators. Cohesin and its regulators in budding yeast and human are shown. Notably, sororin is not present in yeast. Several factors including Sec3, Pds5, and Eco1 have two orthologs in human, indicating the regulation mechanism may be more complex in human.

Structurally, Eco1 in budding yeast contains acetyltransferase (AcT) domain at its C-terminus, zinc-finger (Zf) and PIP-box at the upstream of AcT domain (Model 4)
(Ivanov et al., 2002; Moldovan, Pfander and Jentsch, 2006). Eco1 is recruited to chromatin during S-phase when SCC is being established (Kenna and Skibbens, 2003; Moldovan, Pfander and Jentsch, 2006). The PIP-box seems to be crucial for its recruitment to chromatin, consistent with the fact that SCC establishment is coupled with DNA replication (Moldovan, Pfander and Jentsch, 2006; Lyons and Morgan, 2011; Liu et al., 2020). ESCO1 and ESCO2 have elongated N-termini compared to Eco1, while their C-termini are highly conserved. It seems that N-terminal segments of ESCO1 and ESCO2 differentiate them (Minamino et al., 2015; Rahman, Mathew J K Jones and Jallepalli, 2015; Alomer et al., 2017).

**Model 4. Structure of Eco1, ESCO1, and ESCO2** Acetyltransferase domain (AcT), zinc-finger domain (Zf), and PIP-box are conserved among Eco1, ESCO1/2. N-terminus of ESCO1 comprises conserved motifs which seem to mediate the interaction with PDS5A/B. ESCO2 contains two PBMs (Pre-RC Binding Motif) that mediate the interaction with MCM helicase.

In comparison with ESCO1, ESCO2 functionally resembles Eco1. First, ESCO2 is expressed predominantly during S-phase, reminiscent of Eco1 (Hou and Zou, 2005). In addition, ESCO2’s N-terminus carries multiple motifs, namely PBM-A and PBM-B (Pre-RC Binding Motif), which interact with MCM helicases, thus recruiting
ESCO2 to the replisome in cooperation with the PIP-box (Model 4) (Higashi et al., 2012; Ivanov et al., 2018; Minamino et al., 2018; Sun et al., 2019; Bender et al., 2020). On the other hand, ESCO1 is expressed throughout cell cycle unlike Eco1 or ESCO2. ESCO1 comprises PIM (PDS5 Interacting Motif) at its N-terminus that are not present in ESCO2 (Minamino et al., 2015; Rahman, Mathew J.K. Jones and Jallepalli, 2015; Goto et al., 2017). Consistently, ESCO1-mediated SMC3 acetylation seems to be PDS5A/B dependent (Model 4) (Minamino et al., 2015). Whereas ESCO2 barely shows peaks on the genome, ESCO1 often colocalizes with cohesin and CTCF (CCCTC-binding Factor) (Rahman, Mathew J K Jones and Jallepalli, 2015). CTCF is a zinc-finger protein that anchors cohesin at CTCF-binding sites (Eric D Rubio et al., 2008; Parelho et al., 2008; Stedman et al., 2008; Wendt et al., 2008; Busslinger et al., 2017; Kojic et al., 2018). CTCF-dependent cohesin anchoring is essential for interphase chromatin organization through cohesin, therefore, is crucial for transcription regulation (Wendt et al., 2008). Notably, it was recently reported that ESCO1 is required for interphase chromatin organization during G1-phase in association with CTCF, emphasizing the importance of ESCO1 in transcription regulation (Wutz et al., 2020).

2.8. Replisome Associated Factors Implicated in SCC

SCC establishment is coupled with DNA replication with a few exceptions such as DNA damage or break-induced cohesion (Uhlmann and Nasmyth, 1998; Ström et al., 2007; Ünal, Heidinger-Pauli and Koshland, 2007). Replisome is a multi-protein complex which consists of the CMG helicase (CDC45, MCM2-7, GINS) and polymerase α, δ and ε at the core, and has additional regulatory factors (Reviewed in (O’Donnell, Langston and Stillman, 2013)). Among them, Ctf4, Tof1, Csm3, Chl1, Mrc1, Ctf18, Dec1, Ctf8 (AND-1, TIMELESS, TIPIN, DDX11, CLASPIN, CHTF18,
DSCC1, CTF8 in vertebrates, respectively) are known to participate in supporting SCC based on yeast studies (Model 5) (Mayer et al., 2001, 2004; Petronczki et al., 2004; Lengronne et al., 2006; Xu, Boone and Brown, 2007; Ansbach et al., 2008; Errico et al., 2009; Terret et al., 2009; Smith-Roe et al., 2011; Borges et al., 2013; Tong and Skibbens, 2015; Fumasoni et al., 2015). Unlike cohesin per se or Eco1, the deletion mutants of these factors are viable in budding yeast, and are called “non-essential cohesion factors”. The non-essential cohesion factors fall into two epistasis group based on genetic analysis in budding yeast (Model 5) (Xu, Boone and Brown, 2007).

The first group involves Ctf18, Dcc1, Ctf8, and Mrc1. Ctf18, Dcc1 and Ctf8 form a hetero trimer and are part of the replication factor C (RFC)-like complex (RFC

\[ \text{Ctf18} \] (Mayer et al., 2001; Naiki et al., 2001; Bermudez et al., 2003; Merkle et al., 2003). RFC

\[ \text{Ctf18} \] has activity to load PCNA onto DNA both in vivo and in vitro similar to the canonical RFC

\[ \text{Rfc1} \] complex (Bermudez et al., 2003; Shiomi et al., 2004; Lengronne et al., 2006). On the contrary, it is also reported that RFC

\[ \text{Ctf18} \] can unload PCNA in vitro when DNA is coated by RPA (Bylund and Burgers, 2005). In addition, Ctf18 plays a role in checkpoint signalling (Crabbé et al., 2010; Gellon et al., 2011; Kubota et al., 2011; García-Rodríguez et al., 2015; Okimoto et al., 2016; Stokes et al., 2020).

Interestingly, Mrc1 is also critical for checkpoint signalling, however, the connection between SCC and checkpoint is largely unknown (Kumagai and Dunphy, 2000; Alcasabas et al., 2001; Tanaka and Russell, 2001; Christiano, Chini and Chen, 2003).

The second group comprises Ctf4, Chl1, Tof1 and Csm3. Ctf4 (AND-1 in vertebrates) scaffolds several factors for replication and repair such as polymerase α and Chl1 (Zhu et al., 2007; Gambus et al., 2009; Simon et al., 2014; Fumasoni et al., 2015; Samora et al., 2016). Chl1 is a 5’ to 3’ helicase implicated in Okazaki fragment maturation in cooperation with a flap end nuclease, Fen1 (Hirota and Lahti, 2000; Farina et al., 2008; Wu et al., 2012). Chl1’s ortholog in vertebrates, DDX11, is shown to have a role in
replication fork stabilization (Cali et al., 2015; Faramarz et al., 2020). Tof1 and Csm3 also function in replication fork protection that is called FPC (Fork Pausing Complex) (Katou et al., 2003; Krings and Bastia, 2004; Mohanty, Bairwa and Bastia, 2006; Errico, Costanzo and Hunt, 2007). Most recently, it was demonstrated that the second group may utilize cohesins which are already loaded onto DNA before replication to establish SCC, providing for a “conversion pathway” (Srinivasan et al., 2020). On the other hand, the first group of factors seems to rely for SCC on the cohesin loader, and is termed “de novo loading pathway” (Model 5) (Srinivasan et al., 2020). Nevertheless, how mechanistically these factors promote SCC establishment is still unclear, therefore, further analysis is required for better understanding.

2.9. Cohesin in DNA Repair and Replication

Cohesin plays roles in DNA damage response as well (Reviewed in (Litwin, Pilarczyk and Wysocki, 2018)). To be noted, the kleisin subunit of cohesin, RAD21, was first

Model 5. Non-essential cohesion factors and replisome. A number of replisome-associated factors are implicated in SCC. On the basis of genetic analysis in budding yeast, they fall into two epistasis groups. Genes belonging to Group 1 are marked in green color, and Group 2 are marked in blue color.
identified as a gene that sensitizes fission yeast to UV and γ-irradiation (Birkenbihl and Subramani, 1992). Double strand break (DSB) is the most hazardous DNA lesion which can result in chromosome loss and cell death if left unrepaird. Yet, inaccurate repair of DSBs leads to deleterious genome rearrangements which may drive cancer development. DSBs are repaired by two main pathways. One is non-homologous end joining (NHEJ) that is error-prone, the other one is homologous recombination (HR) which is error-free in principle (Reviewed in (Scully et al., 2019)). Upon induction of DSBs, cohesins accumulate at DSB sites in cohesin loader dependent manner (Ström et al., 2004; Ünal et al., 2004; Bot et al., 2017). Interestingly, the cohesins loaded after DSB induction can establish SCC without DNA replication (Ström et al., 2007; Ünal, Heidinger-Pauli and Koshland, 2007; Kim et al., 2010). The cohesin loading at DSB sites requires MRN (MRE11-RAD50-NBS1) complex, ATM, ATR and γ-H2AX (Kim
et al., 2002; Ünal et al., 2004; Ström et al., 2007; Ünal, Heidinger-Pauli and Koshland, 2007). SCC mediated by cohesin keeps sister DNAs in the proximity, thus, it is thought to facilitate the usage of sister chromatid DNA as donor for HR (Model 6) (Cortés-Ledesma and Aguilera, 2006; Covo et al., 2010). In fact, mutations on cohesin or cohesin loader result in hypersensitivity to γ-irradiation and exhibit slower chromosome recovery after the chromosome breakage (Sjögren and Nasmyth, 2001; Ström et al., 2004, 2007). These observations support the idea that cohesin facilitates HR. However, it is also known that cohesin accumulation at DSB sites constrains the mobility of damaged chromatin, which is thought to facilitate homology search in HR (Dion et al., 2013). Moreover, prolonged cohesin occupancy can interfere with the generation of a 3’ overhang that is essential for HR (McAleenan et al., 2013). Intriguingly, it was recently reported that cohesin may regulate donor choice for recombination-mediated repair (Agmon et al., 2013; Dion et al., 2013). In addition, it was recently shown that cohesin blocks NHEJ of distant DSB ends, but not those situated close to each other (Gelot et al., 2016). Collectively, cohesin’s function is likely to keep DNA ends or sister chromatids in the proximity to prevent deleterious rearrangement of chromosomes. In budding yeast, cohesin molecules recruited to DSB sites are phosphorylated by Chk1 (Heidinger-Pauli et al., 2008). This phosphorylation stimulates subsequent acetylation by Eco1 enabling cohesin’s stabilization (Heidinger-Pauli, Ünal and Koshland, 2009). In human, how cohesins are recruited to DSB sites and stabilized is largely unknown.

Cohesin is also shown to accumulate at stalled replication fork in budding yeast (Tittel-Elmer et al., 2012). As in the case of DSBs, this accumulation also requires MRX (MRN in vertebrates) and Tel1\textsuperscript{ATM}, Mec1\textsuperscript{ATR}. Methyl methanesulfonate (MMS) is an agent that creates alkylation base damages on DNA. MMS-induced damages cause replication fork stalling which is taken care by DDT (DNA Damage Tolerance)
pathway (Reviewed in (Branzei and Psakhye, 2016). DDT involves TS (Template Switch) and TLS (Trans Lesion Synthesis). TLS utilizes specialized DNA polymerases such as polymerase $\zeta$, $\eta$ to replicate through the damaged templates (Reviewed in (Waters et al., 2009)). However, TLS is an error-prone event and mutagenic, compromising genome integrity. On the contrary, TS is a recombination-based bypass of DNA lesions and is largely error-free unless it engages repeat-containing sequences (Reviewed in (Branzei and Szakal, 2016)). Cohesin facilitates TS by keeping sister DNAs in the vicinity (Model 6) (Fumasoni et al., 2015).

Consistently, artificially tethering sister DNAs is able to bypass the requirement of cohesin for generation of recombination intermediates (Fumasoni et al., 2015). Supporting the importance of cohesin for TS, it was recently shown that Irc5 (HELLS in vertebrates) chromatin remodeler promotes cohesin recruitment to the stalled replication forks and the formation of recombination intermediates (Litwin et al., 2017).

Cohesin plays roles in unperturbed DNA replication as well (Terret et al., 2009; Carvajal-Maldonado et al., 2019; Morales et al., 2020). It was shown that ESCO1/2-mediated SMC3 acetylation speeds the replication fork, which is restored by WAPL depletion (Terret et al., 2009). This suggested a role for cohesin in facilitating replication processivity. However, controversially, later studies demonstrated that WAPL depletion slows down replication fork velocity (Model 6) (Carvajal-Maldonado et al., 2019; Morales et al., 2020). It seems that the accumulation of cohesins on chromatin interferes with replication fork progression resulting in nucleolytic fork degradation by MRE11. However, it has also been suggested that cohesin plays a role in fork stabilization or protection upon fork stalling caused by HU (hydroxyurea) treatment which inhibits ribonucleotide reductase (RNR) resulting in dNTP depletion (Model 6) (Morales et al., 2020). Finally, cohesins need to be
displaced for stalled fork resumption (Benedict et al., 2020). Altogether, the interplay between replication and cohesin is highly dynamic and tightly regulated.

2.10. Determinants of Cohesin Loading

In human cells, cohesins are reloaded onto chromosomes already in telophase, while in budding yeast, cohesins are loaded in G1-phase when RAD21 is resynthesized after its proteolytic cleavage at previous anaphase (Michaelis, Ciosk and Nasmyth, 1997; Darwiche, Freeman and Strunnikov, 1999; Uhlmann, Lottspelch and Nasmyth, 1999; Ciosk et al., 2000; Sumara et al., 2000). Loci and timing of cohesin loading are determined by the chromatin receptors of the cohesin loader in vivo, although NIPBL Sec2 alone is sufficient for cohesin loading in vitro whereby naked DNA is used as substrate (Takahashi et al., 2004; Murayama and Uhlmann, 2014; Hinshaw et al., 2017; Muñoz et al., 2019). To be noted, the localization of the cohesin loader is poorly overlapping with that of cohesin in both human and budding yeast, indicating that cohesin translocates after being loaded (Lengronne et al., 2004; Lopez-Serra et al., 2014; Zuin et al., 2014). Various chromatin binding proteins are implicated in the recruitment of cohesin loader (Table 2) (Nonaka et al., 2002; Bailis et al., 2003; Takahashi et al., 2008; Gillespie and Hirano, 2004; Takahashi et al., 2004; Fischer et al., 2009; Fernius et al., 2013; Natsume et al., 2013; Hinshaw et al., 2015, 2017; Muñoz et al., 2019). For instance, in fission yeast, Swi6 (HP1a in vertebrates) functions as a receptor of the cohesin loader together with the Dbf-dependent kinase (DDK) (Bailis et al., 2003; Fischer et al., 2009). In *Xenopus laevis*, the pre-replication complex (pre-RC) recruits the cohesin loader to facilitate the cohesin loading (Gillespie and Hirano, 2004; Takahashi et al., 2004, 2008). However, this mechanism is not conserved in budding yeast, indicating that the chromatin receptors for cohesin loader are diverse (Uhlmann and Nasmyth, 1998). Instead, it has been demonstrated
that Ctf19, a subunit of the kinetochore complex, functions as a chromatin receptor of the cohesin loader in budding yeast (Hinshaw et al., 2015, 2017). As kinetochores localize to centromeres, mutations on Ctf19 reduces the cohesin amount at pericentromeric regions, but not on chromosome arms, indicating that other pathways exist for recruiting the cohesin loader. In fact, recent studies showed that the RSC chromatin remodeling complex (PBAF in human) recruits the loader complex to a set of specific genomic loci including centromeres and chromosome arms (Lopez-Serra et al., 2014; Muñoz et al., 2019). Although the RSC complex acts as a chromatin receptor for the loader, the catalytic activity is indispensable for the cohesin loading (Muñoz et al., 2019). It is noteworthy that nucleosomes inhibit the loading of cohesin in vitro, further supporting the idea that creating nucleosome-free regions may promote cohesin loading (Muñoz et al., 2019). Interestingly, several chromatin remodelers play roles in cohesin regulation. These include FACT, RSC, INO80 complexes and Irc5 (HELLS in vertebrates) in budding yeast, PBAF and SNF2, ATRX in human (Table 2) (Hakimi et al., 2002; Ogiwara, Enomoto and Seki, 2007; Kernohan et al., 2010; Brownlee et al., 2014; Lopez-Serra et al., 2014; Litwin et al., 2017; Garcia-Luis et al., 2019; Muñoz et al., 2019).

### Table 2. Cohesion loader receptors and remodelers implicated in SCC

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<tr>
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<th>Cohesion Loader Receptors</th>
<th>Chromatin Remodelers Implicated in Cohesion Regulation</th>
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<td></td>
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<tr>
<td>X. laevis</td>
<td>DDK</td>
<td>PBAF (RSC) SNF2 (ISWI) ATRX</td>
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<td>DDK</td>
<td>RSC FACT INO80 Irc5</td>
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2.11. **RUVBL1 and RUVBL2 as a Chromatin Remodeler**
Chromatin remodelers play roles in cohesin regulation via mechanisms that remain largely unknown. RUVBL1 and RUVBL2 are two highly conserved members of the AAA+ family that form ring-shaped hetero dodecamer and are components of various cellular protein complexes (Reviewed in (Mao and Houry, 2017)). Therefore, RUVBL1/2 are implicated in a variety of cellular processes such as cellular transformation, cancer metastasis, snoRNP assembly, DNA damage response, and transcription (Model 7). RUVBL1/2 are known to be part of the INO80 family remodelers that include INO80, SRCAP (SWR1 in yeast), and TIP60 (Nu4A in yeast) complexes (Reviewed in (Mao and Houry, 2017)). INO80 remodeler catalyzes sliding of nucleosomes, exchange of histone H2A.Z with H2A for regulation of transcription, DNA repair, and replication (Reviewed in (Willhoft and Wigley, 2020). Interestingly, INO80 is also shown to play a role in SCC in budding yeast (Ogiwara, Enomoto and Seki, 2007). However, the mechanism is poorly understood.

Model 7. RUVBL1/2’s roles in various cellular pathways. RUVBL1/2 form complex with various proteins, including INO80 complex. Therefore, RUVBL1/2 have various roles in different cellular pathways.

### 2.12. Interphase Chromatin Organization by Cohesin and CTCF
Cohesin was found as an essential factor for SCC, therefore it was thought to be indispensable only for proliferating cells. Nevertheless, cohesin is also present in post-mitotic cells (Wendt et al., 2008). This raised the question that cohesin may function not only for SCC. In fact, cohesin was found to often colocalize with CTCF on the genome and mediate intra-chromosomal contacts of distant loci that are important for transcription regulation, for example by mediating enhancer-promoter interaction (Model 8) (Eric D. Rubio et al., 2008; Parelho et al., 2008; Wendt et al., 2008).

Recent technical advances in chromosome conformation capture-sequencing methods such as Hi-C enabled researchers to study the higher-order structure of chromatin (Reviewed in (Dekker, Marti-Renom and Mirny, 2013)). Hi-C analysis has revealed
that interphase chromatin forms approximately 10,000 of loops, the size of which ranges from 40 kb to 3 Mb (median is 185 kb) in the human genome (Lieberman-Aiden et al., 2009; Rao et al., 2014). Among these chromatin loops, about 90% of the loop peaks are associated with CTCF binding sites in a convergent orientation (Rao et al., 2014). Consistently, those CTCF binding sites are mostly co-occupied by cohesin, and CTCF and cohesin are interdependent for loop formation (Zuin et al., 2014; Nora et al., 2017; Rao et al., 2017). Further, depletion of NIPBL causes reduction of topologically associated domains (TADs) and chromatin loops, while the compartmentalization increases (Schwarzer et al., 2017). On the contrary, depletion of WAPL increases the number of chromatin loops and the loop length, while the compartmentalization decreases (Haarhuis et al., 2017; Wutz et al., 2017).

Collectively, cohesin mediates TADs and loop formation, whereas it counteracts the chromatin compartmentalization.

Cohesin needs to translocate along with chromatin to reach CTCF sites (Busslinger et al., 2017). For cohesin’s translocation, not for its diffusion, the ATPase activity is indispensable both in vitro and in vivo (Hu et al., 2011; Petela et al., 2018; Vian et al., 2018; Davidson et al., 2019). As NIPBL stimulates cohesin’s ATPase activity together with DNA in vitro, it may promote cohesin’s translocation in vivo as well (Model 8) (Murayama and Uhlmann, 2014; Petela et al., 2018; Davidson et al., 2019; Kim et al., 2019). Besides, active transcription is also shown to be able to relocate cohesin, explaining the reason why cohesins are often enriched between convergent genes (Lengronne et al., 2004; Gullerova and Proudfoot, 2008; Hu et al., 2015; Davidson et al., 2016; Busslinger et al., 2017). Although cohesin and CTCF emerged as the key factors for interphase chromatin organization, at present little is known about how they are regulated.
2.13. **Cohesin in Cancer and Disease**

Cohesin and its regulators have been implicated in various types of cancer (Table 3) (Kandoth *et al.*, 2013; Lawrence *et al.*, 2014; Leiserson *et al.*, 2015). On the basis of early findings, these mutations on cohesin related genes were thought to elicit aneuploidy resulting in genomic instability that drives cancer development. Although it was the case in part, later studies showed that there is not clear correlation between the mutations on cohesin related genes and aneuploidy or SCC defect (Solomon *et al.*, 2011; Balbás-Martínez *et al.*, 2013; Kon *et al.*, 2013; Kleyman, Kabache and Compton, 2014; Li *et al.*, 2015; Kim *et al.*, 2016). Knowing that cohesin plays important roles in regulating transcription, one could imagine that the cohesin mutations may alter the transcription profile leading to cancer development. Among cohesin subunits and regulators, SA2 (also called STAG2) is the most frequently mutated gene (Table 3) (Reviewed in (De Koninck and Losada, 2016; Waldman, 2020). Intriguingly, the latest research demonstrated that cohesin containing SA1 (cohesinSA1) and SA2 (cohesinSA2) contribute differently to shaping chromatin structure (Kojic *et al.*, 2018; Wutz *et al.*, 2020). Although more than 50% of their binding loci are overlapping at regions where also CTCF is bound, both cohesin complexes have unique binding loci (Kojic *et al.*, 2018). Whereas the loci where only cohesinSA1 is present are still associated with CTCF binding sites, cohesinSA2 binding loci appear to often associated with tissue-specific enhancer regions (Kojic *et al.*, 2018). In general, it seems that TADs mediated by cohesin modulate the compartmentalization. In fact, cohesin depletion results in increase of compartmentalization and rewires the enhancer-promoter contacts (Lupiáñez *et al.*, 2015; Kojic *et al.*, 2018; Despang *et al.*, 2019). Notably, new enhancer-promoter contacts appear in SA2-depleted cells but not in SA1-depleted cells, correlated with their mutation frequency in cancers (Kojic *et al.*, 2018).
Mutations on cohesin related genes are also known to be implicated in developmental disorders including Cornelia de Lange Syndrome (CdLS), Roberts Syndrome (RBS), Warsaw Breakage Syndrome (WABS) that are developmental disorders known as “Cohesinopathies” (Table 3) (Reviewed in (Cucco and Musio, 2016; Avagliano et al., 2020)). Canonical CdLS is caused by mutations on *NIPBL, SMC1A, SMC3, RAD21, HDAC8*. Mutations on *ESCO2* is the cause of RBS, and WABS is caused by *DDX11* mutations. Patient cells of RBS and WABS are found to exhibit premature centromere separation (PCS), namely “rail-road” phenotype, which results in increased rate of aneuploidy (Vega et al., 2005; van der Lelij et al., 2010; Capo-Chichi et al., 2013).

![Cohesin related genes in cancers](image)

### Responsible genes for cohesinopathy

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
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<tbody>
<tr>
<td><strong>CdLS</strong></td>
<td></td>
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<tr>
<td><em>NIPBL</em></td>
<td>Cohesin loader</td>
</tr>
<tr>
<td><em>SMC1A</em></td>
<td>Cohesin subunit</td>
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<tr>
<td><em>SMC3</em></td>
<td>Cohesin subunit</td>
</tr>
<tr>
<td><em>RAD21</em></td>
<td>Cohesin subunit</td>
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<tr>
<td><em>HDAC8</em></td>
<td>Cohesin deacetylase</td>
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<tr>
<td><strong>RBS</strong></td>
<td><em>ESCO2</em></td>
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<tr>
<td><strong>WABS</strong></td>
<td><em>DDX11</em></td>
</tr>
<tr>
<td><strong>CdLS-like</strong></td>
<td></td>
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<tr>
<td><em>AFF4</em></td>
<td>Super elongation complex (SEC) subunit</td>
</tr>
<tr>
<td><em>ANKHD1</em></td>
<td>HDAC scaffold</td>
</tr>
<tr>
<td><em>BRD4</em></td>
<td>Reader of acetylated chromatin</td>
</tr>
<tr>
<td><em>EP300</em></td>
<td>Histone acetyltransferase (HAT)</td>
</tr>
<tr>
<td><em>KMT2A</em></td>
<td>Histone methyltransferase</td>
</tr>
<tr>
<td><em>SETD5</em></td>
<td>Histone methyltransferase</td>
</tr>
<tr>
<td><em>SWI/SNF</em></td>
<td>Chromatin remodeler</td>
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Table 3. **Cohesin related genes in cancer and human disease.** Upper graph: Frequency of mutations in the indicated cancer types for cohesin subunits and cohesin-related genes. (Adapted from De Konnick and Losada, 2016) Bottom table: Genes implicated in cohesinopathies and CdLS-like developmental disorders are displayed.
the contrary, mutations found in CdLS patients rarely exhibit detectable SCC defects, contradicting the idea that aneuploidy caused by the mutations drives the developmental defects (Castronovo et al., 2009). It is noteworthy that a number of transcription factors have been identified to be responsible for developmental disorders that exhibit CdLS-like phenotype, including AFF4, BRD4, EP300, KMT2A, SETD5, and SWI/SNF complex related genes (Table 3) (Reviewed in (Avagliao et al., 2020)). Mutations on these genes do not generally induce SCC defects. Collectively, similarly to mutations found in cancers, the SCC defect and aneuploidy may not be correlated with developmental defects, rather, altered transcription may be the cause of the defects.

3. RESULTS
3.1. ESCO1 and ESCO2 are required for SCC

ESCO1 has been shown to be essential for maintaining sister chromatid cohesion (SCC) in yeast (Skibbens et al., 1999; Tóth et al., 1999; Ivanov et al., 2002; Ünal, Heidinger-Pauli and Koshland, 2007). However, how its vertebrate orthologs, ESCO1 and ESCO2, divide roles in SCC establishment remain to be elucidated. In order to study ESCO1 and ESCO2’s role in SCC, we established ESCO1<sup>−/−</sup> single knock out cell line in chicken B-cell DT40 (hereafter referred as esco1) by transfecting the ESCO1 knock out constructs by which the acetyltransferase domain of ESCO1 can be deleted (Fig. 1A). ESCO1 gene disruption was verified by genomic polymerase chain reaction (PCR) and reverse transcription PCR (RT-PCR) (Fig. 1B). ESCO2<sup>−/−</sup> knock out cell line (hereafter referred as esco2) was previously established in our group (Abe et al., 2016). We tested whether the mutations on ESCO1 and ESCO2 affect SCC. For this purpose, we prepared metaphase spreads and scored chromosomes with SCC defects. Consequently, we found that both mutations on ESCO1 and ESCO2 induce a SCC defect whereby about 80% of metaphases had modest defects, that is, presenting chromosome arm separation but having the centromeres connected (Fig. 1C). Since SMC3-K105, 106 (equivalent to K112, 113 in S. cerevisiae) are shown as key substrates of Eco1 for SCC establishment, we examined if mutations on ESCO1 and ESCO2 also affect SMC3 acetylation at K105 and K106 (Ben-shahar et al., 2008; Ünal et al., 2008; Zhang et al., 2008; Rowland et al., 2009; Sutani et al., 2009). To this end, we performed western blotting to monitor the acetylation level of SMC3 by using an antibody specific to acetylated SMC3. As a result, we observed reduction in the SMC3 acetylation level in both mutants (Fig. 1D). Together, our data demonstrate that ESCO1 and ESCO2 promote SCC via acetylation of SMC3 in line with previous reports in other systems (Hou and Zou, 2005; Zhang et al., 2008; Whelan et al., 2012; Minamino et al., 2015).
3.2. **ESCO2, but not ESCO1, is critical for cellular proliferation and centromeric SCC**

**ECO1** is an essential gene in *S. cerevisiae* (Skibbens *et al.*, 1999; Tóth *et al.*, 1999; Ivanov *et al.*, 2002). Therefore, we decided to test whether disrupting *ESCO1* or *ESCO2* affects cellular proliferation. Interestingly, the results uncovered...
that *esco2* but not *esco1* cells exhibit severe growth defects, although the reduction in SMC3 acetylation was more pronounced in the *esco1* mutant (Figs. 1D, 2A). A key role of cohesin in cellular viability is via maintaining SCC, and centromeric SCC is especially important for providing bipolar tension at metaphase, thus being essential for symmetric chromosome segregation (Reviewed in (Morales and Losada, 2018)). Thereby, we asked if there is a division of labour between *ESCO1* and *ESCO2* in terms of their contribution to centromeric cohesion. To test this idea, we performed immunofluorescence to stain CENP-T as a marker of centromeres on metaphase spreads and measured the distance between paired CENP-T signals. Strikingly, this experiment revealed that the *esco2* mutant exhibits increased CENP-T distance compared to wild-type and *esco1* cells (Fig. 2B). This result points to a critical role for *ESCO2* in centromeric SCC. Since we identified centromeric SCC defects in *esco2* cells, we further examined if it results in chromosome missegregation during mitosis. For this purpose, we conducted immunofluorescence using CENP-T to visualize microchromosomes which are barely visible by DAPI staining. We then measured the frequency of chromosome missegregation at anaphase. As a result, we found that the *esco2* mutant shows more frequent chromosome missegregation, although the increase...
is modest (Fig. 5B). Altogether, our results indicate that *ESCO2* is critical for centromeric SCC, which supports accurate chromosome segregation.

### 3.3. ESCO1 overexpression suppresses the centromeric SCC defect in esco2 cells

*ESCO2* is shown to be expressed more in S-phase similar to *ECO1* in *S. cerevisiae*, whereas *ESCO1* is expressed through the cell cycle (Kenna and Skibbens, 2003; Hou and Zou, 2005; Moldovan, Pfander and Jentsch, 2006). Moreover, recent studies have reported that a conserved motif at the N-terminus of *ESCO2*, which is not present in *ESCO1*, is required for *ESCO2* association with the replisome and SCC establishment (Ivanov *et al.*, 2018; Minamino *et al.*, 2018; Sun *et al.*, 2019; Bender *et al.*, 2020). On the other hand, *ESCO1* contains conserved motifs at the N-terminus which promote the interaction with PDS5 to acetylate SMC3 (Minamino *et al.*, 2015; Rahman, Mathew J K Jones and Jallepalli, 2015). Since we found that *esco2* cells exhibit severe growth defects and centromeric SCC defects, we wondered whether *ESCO1* overexpression would rescue these defects. To test this, we ectopically expressed *ESCO1* from the beta-actin promoter in *esco2* cells. The expression level was determined by RT-PCR and western blot whereby the expression level was 6-10-fold higher than the one of endogenous *ESCO1* (Figs. 3A, 3B). We also verified that SMC3 acetylation level was compensated by *ESCO1* overexpression (Fig. 3B). We next monitored the growth rate finding that *ESCO1* overexpression is able to rescue the proliferation defect of *esco2* cells (Fig. 3C). Subsequently, we asked if the centromeric SCC defect can also be rescued by overexpressing *ESCO1*. To do so, we assessed the centromeric SCC by the same approach as we have done for *esco1* and *esco2* mutants. As a result, we found that *ESCO1* overexpression is able to rescue the centromeric SCC defect in *esco2* cells (Fig. 3D). Moreover, although the rescue was partial, we
also found that the SCC defect on chromosome arms is improved by over-expressing ESCO1 (Fig. 3E).

We have previously reported that ESCO2 and DDX11 are synthetically lethal (Abe et al., 2016). To further probe the finding that over-expressed ESCO1 could bypass the requirement of ESCO2, we decided to test if the synthetic lethality can be suppressed by over-expressing ESCO1. The experiment revealed that this is indeed the case (Fig. 3F). We further confirmed that the chromosome segregation defect is rescued by ESCO1 overexpression (Fig. 3G). Summarizing these data, our results demonstrate

Figure 3. ESCO1 overexpression suppresses the centromeric SCC defect in esco2 cells. (A) ESCO1 mRNA levels were measured by quantitative PCR. (B) ESCO1-FLAG protein level and the acetylation level of SMC3 were measured by Western blotting. (C) Growth curves as in Figure 2A. (D) The distances between CENP-T signals were measured for >400 chromosomes, as in Figure 2B. (E) Chromosomes from metaphase spreads were analyzed for cohesion defects following the classification procedure outlined in Figure 1C. (F) Growth curves as in Figure 2A. Doxycycline (Dox, 1 μg/mL final) was added at time 0 when indicated. (G) The frequency of anaphase cells with lagging chromosomes was measured. 50 anaphase cells were analyzed for each experiment. The results of two independent experiments were plotted. Auxin (500 μM final) was added 6 h before cell collection.
functional redundancy despite their differentially conserved N-terminus domains. It is tempting to speculate that ESCOs’ activity to stabilize cohesin is required more during S-phase when SCC is being established which highly relies on ESCO2 activity. Thereby, \( ESCO1 \) is able to rescue the defects in \( esco2 \) when it is overexpressed.

3.4. \textit{ESCO1 and ESCO2 are redundant for promoting SCC}

As we found functional redundancy between \( ESCO1 \) and \( ESCO2 \), we were interested to study further their genetic interaction. Accordingly, we asked if \( ESCO1 \) is essential in the absence of \( ESCO2 \). To this end, we first tried to establish \( esco1 \) \( esco2 \) double knockout mutants from \( esco1 \) by disrupting \( ESCO2 \) using Cre-loxP system (Arakawa, Lodygin and Buerstedde, 2001). However, we were not able to obtain any positive clones, indicating that \( ESCO1 \) and \( ESCO2 \) are synthetic lethal as \( ECO1 \) is essential in \( S. \) cerevisiae (Skibbens \textit{et al.}, 1999; Tóth \textit{et al.}, 1999; Ivanov \textit{et al.}, 2002). To verify this assumption, we employed a proteolysis based conditional knock out system called Auxin Inducible Degron (AID) (Nishimura \textit{et al.}, 2009; Morawska and Ulrich, 2013) (Fig. 4A). Essentially, a subunit of the ubiquitin ligase complex called \( TIR1 \) from \( O. \) sativa is expressed in our cell lines. \( TIR1 \) recognizes proteins fused to the AID-tag in the presence of auxin and ubiquitylates AID-tagged proteins to cause their degradation by the proteasome (Fig. 4A). For our purpose, we introduced 3xmAID-6xFLAG-tag at the C-terminus of \( ESCO2 \) in \( esco1 \) cells (thereafter referred as \( esco1 \) \( esco2\)-aid) background by using the Flip-in system (Kobayashi \textit{et al.}, 2015) (Figs. 4B, 4C). We then tested whether AID-tagged \( ESCO2 \) can be degraded upon auxin treatment by western blot. The result showed that the amount of \( ESCO2\)-3xmAID-6xFLAG is reduced to undetectable levels in 3 hours after auxin addition, confirming that the AID system works in our cell lines (Fig. 4D). Using the conditional double knock out \( esco1 \) \( esco2\)-aid cells, we first asked how concomitant depletion of \( ESCO1 \) and \( ESCO2 \)
affects SCC. The result uncovered that ESCO2 depletion in esco1 cells further aggravates the SCC defect in which the majority of chromosomes exhibit severe SCC defect, with separation of sister chromatids extended also at the centromeric level (Fig. 4E). In addition, we monitored the SMC3 acetylation level and found that SMC3 acetylation is nearly abolished 6 hours after auxin addition (Fig. 4F). These results support the notion of compensatory roles of ESCO1/2 in promoting SCC, possibly via SMC3 acetylation.

Figure 4. ESCO1 and ESCO2 are redundant for promoting SCC. (A) Schematic representation of Auxin Inducible Degron (AID) conditional knock out system. (B) Scheme of the conditional double mutant’s establishment. (C) Schematic representation of ESCO2 gene locus and ESCO2-3xmAID-6xFLAG FLP-In construct. (Closed boxes) Exons; (red box) Stop codon; (gray box) 3’-UTR; (dashed line) Back bone of plasmid. (D) Depletion of ESCO2-3mAID-6xFLAG was assessed by Western blotting using the anti-FLAG antibody. α-tubulin was used as a loading control. (E) Chromosomes from metaphase spreads were analyzed as in Figure 1C. Auxin (500 μM final) was added 6 h before cell collection. (F) The acetylation level of SMC3 was measured by Western blotting. The average of three independent experiments were plotted.
3.5. Concomitant depletion of ESCO1 and ESCO2 leads to chromosome missegregation

Centromeric SCC is crucial for accurate chromosome segregation, thus it is essential for cellular proliferation. In addition, we have shown that esco1 esco2-aid cells exhibit severe SCC defects characterized by centromeric separation (Fig. 4E). For this reason, we were interested to test how simultaneous depletion of ESCO1 and ESCO2 affects cellular proliferation. Hence, we monitored the growth rate of esco1 esco2-aid in the presence of auxin. This experiment revealed that concomitant depletion of ESCO1 and ESCO2 leads to synthetic lethality (Fig. 5A). This is similar to the ECO1 essential function in S. cerevisiae. Since we observed synthetic lethality between ESCO1 and ESCO2, we wondered if the lethality is caused by chromosome missegregation. To assess this, we carried out immunofluorescence using CENP-T to mark microchromosomes and counted anaphase cells with chromosomes missegregation, which is defined by remaining signals of DAPI and/or CENP-T between segregated anaphase chromosomes.

![Image of immunofluorescence](image)

**Figure 5. Concomitant depletion of ESCO1 and ESCO2 leads chromosome missegregation.** (A) Growth curves as in Figure 2A. Auxin (500 μM final) was added at time 0 when indicated. (B) Lagging chromosomes in anaphase cells. The analysis was performed as in Figure 3G. Auxin (500 μM final) was added 6 h before cell collection.
As a result, we found that concomitant depletion of ESCO1 and ESCO2 leads to massive chromosome missegregation, which is likely the cause of synthetic lethality (Fig. 5B). Altogether, our data point out compensatory roles for ESCO1 and ESCO2 in supporting cellular proliferation by ensuring accurate chromosome segregation.

3.6. SMC3 acetyl-mimicking is not able to rescue the synthetic lethality of esco1 esco2-aid cells

Next, our question was directed at identifying the ESCO1/2 substrate responsible for the observed defects. Previous reports proposed that SMC3-K105, 106 (K112, 113 in S. cerevisiae) are the key substrate of ESCO1/2 and Eco1 (Ben-shahar et al., 2008; Ünal et al., 2008; Zhang et al., 2008; Rowland et al., 2009; Sutani et al., 2009). In fact, the lethality, SCC defect and condensation defect caused by mutation on ECO1 in S. cerevisiae are rescued by point mutations SMC3-K113Q or N, which mimic the acetylated form of the lysine residue (Ben-shahar et al., 2008; Ünal et al., 2008). In addition, recent studies reported that the acetyl-mimicking mutation stabilizes the cohesin complex on chromatin in human cells (Ladurner et al., 2016). Therefore, we constructed the knock-in construct to induce point mutation on SMC3-K105, 106 endogenously (Fig. 6A). The mutation was confirmed by sequencing and western blot (Fig. 6B, 6C). We then asked whether the acetyl-mimicking mutation is able to suppress the SCC defect in esco1 esco2-aid mutant. Surprisingly, our result uncovered that the acetyl-mimicking mutation is not able to rescue the SCC defect, indicating SMC3-K106, 106 are not solely responsible for the SCC defect (Fig. 6D). Subsequently, we tested if the synthetic lethality can be rescued by the acetyl-mimicking mutation. However, the experiment revealed that the acetyl-mimicking is not able to rescue the synthetic lethality (Fig. 6E). Moreover, we also monitored the chromosome segregation accuracy, and found that it was not rescued either (Fig. 6F).
In conclusion, our results suggest that SMC3-K105, 106 is not the sole critical substrate of ESCO1/2 for supporting SCC, cellular proliferation or chromosome segregation.

3.7. SMC3-K105, 106 are not critical for either viability or SCC

Our data and recent studies have indicated that SMC3-K105, 106 are not essential for SCC and viability, whereas SMC3-K112, 113R in *S. cerevisiae* induces severe growth defects that are nearly lethal (Ben-shahar *et al.*, 2008; Ünal *et al.*, 2008; Rowland *et al.*, 2009; Sutani *et al.*, 2009; Ladurner *et al.*, 2016). In order to test whether the
acetylation at these two sites play a role in supporting cellular proliferation, we established smc3-aid cells by disrupting one allele of SMC3, and by introducing the 3xAID-6xFLAG-tag to the other allele. We first verified the degradation of SMC3-AID by western blot where we found SMC3-AID amount reduced to undetectable levels in 6 hours after auxin addition (Fig. 7B). We also monitored if the SCC and viability are affected by SMC3 depletion. As SMC3 is an essential gene, we found that

Figure 7. SMC3-K105, 106 are not critical for neither viability nor SCC. (A) Schematic representation of SMC3 structure. (B) Depletion of SMC3-3xAID-6xFLAG and the expression of different SMC3 variants were verified by Western blotting. α-tubulin was used as a loading control. (C-F) Growth curves as in Figure 2A. Auxin (500 μM final) was added at time 0 when indicated. (G) Chromosomes from metaphase spreads were analyzed as in Figure 1C with one additional category, “Separated”. Auxin (500 μM final) was added 6 h before cell collection. The results of two independent clones were plotted.

3xAID-6xFLAG-tag to the other allele. We first verified the degradation of SMC3-AID by western blot where we found SMC3-AID amount reduced to undetectable levels in 6 hours after auxin addition (Fig. 7B). We also monitored if the SCC and viability are affected by SMC3 depletion. As SMC3 is an essential gene, we found that
SMC3 depletion induces lethality and severe SCC defect as the majority of sister chromatids are totally separated (Fig. 7C, 7G). To complement smc3-aid cells with different SMC3 variants, we constructed SMC3-HA expressing constructs including SMC3-K105, 106R (RR), SMC3-K105, 106Q (QQ), SMC3-K105, 106A (AA), SMC3-K38I (K38I) as a control defective in ATP-binding (Fig. 7A) (Arumugam et al., 2003). The expression of SMC3 variants was verified by western blot (Fig. 7B). We first tested how different variants affect the viability in the smc3-aid background. The result showed that except for SMC3-K38I, none of the SMC3 variants exhibited considerable growth defects (Figs. 7D-7F). Subsequently, we performed metaphase spread analysis to assess SCC in these SMC3 variants. As a result, we found that all variants were able to support SCC except for SMC3-K38I (Fig. 7G). Altogether, these results indicate that the acetylation at K105 and K106 is not essential for SCC and cellular proliferation, while the ATP-binding is required for both functions.

3.8 Major acetylation sites on SMC3 are not responsible for cellular viability

It has been reported that SMC3 is acetylated at other lysine residues (Choudhary et al., 2009; Kulemzina et al., 2016). Other than K105, 106, two other sites were identified with relatively high scores by acetylome, including K140 which is near the Q-loop, and K1190 positioned near the other side of ATPase site (Fig. 7A). In order to test whether the acetylation at these two sites play roles in supporting cellular proliferation, we complemented smc3-aid cells with either SMC3-K105, 106, 140, 1190Q (4KQ) where all the lysine residues identified to be acetylated are acetyl-mimicked or SMC3-K105, 106, 140, 1190R (4KR) which is acetylation deficient at all lysine residues. We then monitored the growth rate of both 4KQ and 4KR in the presence of auxin and found that both of them exhibit no growth defect or only mild growth defects (Figs.
These data indicate that the relevant substrate of ESCO1 and ESCO2 is not solely SMC3.

3.9. **WAPL depletion rescues the SCC defect in esco1 esco2-aid cells**

In previous studies, suppressor screens of temperature sensitive mutants of ECO1 have identified several suppressors such as SMC3, WAPL, PDS5 and SCC3 (Ben-shahar et al., 2008; Rowland et al., 2009; Sutani et al., 2009). As described above, mutations on SMC3 mimicking acetylation of K105, 106 do not rescue any defects observed in the esco1 esco2-aid mutant. Thus, we next decided to test other suppressors. Pds5 and Sec3 are subunits of cohesin complex which interact with other regulatory factors of cohesin including Wapl which facilitates cohesin dissociation from DNA (Kueng et al., 2006; Rowland et al., 2009). Besides, it was shown that the suppressor mutations on PDS5 and SCC3 reduce the interaction between cohesin and Wapl, the main negative regulator of cohesin (Metson and Nasmyth, 2012; Beckouët et al., 2016). Based on these facts, the reason of suppression by mutations on WAPL, PDS5, SCC3 could simply be explained by Wapl deficiency. Therefore, we decided to test if WAPL deletion can rescue the defects in esco1 esco2-aid cells. Since WAPL is known to be crucial for genome integrity by promoting accurate chromosome segregation, we employed the AID system as we did for ESCO2 (Gandhi, Gillespie and Hirano, 2006; Kueng et al., 2006; Tedeschi et al., 2013). To this end, we disrupted an allele of WAPL.
and introduced 3xmAID-6xFLAG-tag to the other allele (thereafter referred as *wapl-aid*) (Figs. 9A, 9B). First, we verified that both WAPL-3xmAID-6xFLAG and ESCO2-3xmAID-6xFLAG are degraded within 3 hours after auxin treatment (Fig. 9C).

We then carried out immunofluorescence to examine whether WAPL depletion stabilizes cohesin on mitotic chromosomes. The result showed that SMC3 persists on

**Figure 9.** WAPL depletion rescues the SCC defect in esco1 esco2-aid cells. (A) Scheme of the conditional triple mutant’s establishment. (B) Schematic representation of WAPL gene locus and the knock out construct (Closed boxes) Exons, (Marker) drug resistance genes. (C) Depletion of WAPL-3xmAID-6xFLAG and ESCO2-3xmAID-6xFLAG was verified by Western blotting using anti-FLAG antibody. α-tubulin was used as a loading control. (D) Immunofluorescence using the anti-SMC3 antibody and DAPI staining. Metaphase spread samples were prepared as in Figure 2B. (E, G) Chromosomes from metaphase spreads were analyzed as in Figure 3C with one additional category, “Unseparated”. Auxin (500 μM final) was added 6 h before cell collection. The results of two independent clones were plotted. (F) Depletion of WAPL-3xmAID-6xFLAG and SMC3-3xmAID-6xFLAG was confirmed by Western blotting. Auxin (500 μM final) was added 6 h before cell collection.

ESCO2-3xmAID-6xFLAG are degraded within 3 hours after auxin treatment (Fig. 9C).
mitotic chromosomes when WAPL is depleted in both wild-type and esco1 esco2-aid cells, while it is barely visible when WAPL is present (Fig. 9D). Since we observed that cohesin persists on chromosomes upon WAPL depletion, we next asked if it is reflected to SCC. We analyzed metaphase spreads and found that the SCC defect was reversed when WAPL is simultaneously depleted in esco1 esco2-aid, with sister chromatids even more tightly cohered than in wild-type, which is defined as “unseparated” sister chromatids (Fig. 4E). In order to make sure that the tight cohesion is mediated by cohesin, we also established smc3-aid wapl-aid (Fig. 4F). Subsequent metaphase spreads analysis revealed that smc3-aid wapl-aid cells exhibits separated sister chromatids (Fig. 4G). Summarizing our data, we confirmed that WAPL depletion stabilizes cohesin on chromosomes and is able to induce tight SCC in esco1 esco2-aid cells.

3.10. **WAPL depletion does not rescue but rather aggravates the segregation defect of esco1 esco2 cells**

Since it is known that WAPL depletion in ecol mutant in S. cerevisiae suppresses the lethality, we wondered if WAPL depletion rescues not only the SCC defect but also the synthetic lethality in our cell lines (Rowland *et al.*, 2009; Sutani *et al.*, 2009). Accordingly, we monitored the growth rate of esco1 esco2-aid wapl-aid triple conditional mutant with or without auxin treatment. The experiment unveiled that WAPL depletion in esco1 esco2-aid cells does not rescue the synthetic lethality but aggravates it, while WAPL depletion alone induces only mild growth defects (Fig. 10A). The aggravation was also observed by analyzing cell cycle progression by propidium iodide (PI) staining, in which the majority of the esco1 esco2-aid wapl-aid cells is arrested in G2 to M-phase 12 hours after auxin addition (Fig. 10B). Because
we found that the synthetic lethality gets aggravated in the triple mutant, we wondered if it is accompanied by a defect in chromosome segregation. Hence, we performed

**Figure 10. WAPL depletion does not rescue the segregation defect but aggravates.** (A) Growth curves as in Figure 2A. Auxin (500 μM final) was added at time 0 when indicated. (B) Cell cycle profile for indicated cell lines was analyzed by propidium iodide staining. Samples were taken at indicated time points. Auxin was added at time point 0. (C) Chromosome bridges and missegregation in anaphase. The frequency of chromosomes bridges and missegregation was measured as in Figure 3G. Auxin (500 μM final) was added 6 h before cell collection.

immunofluorescence using CENP-T to assess the accuracy of chromosome segregation. In line with previous studies, *wapl-aid* cells showed connected chromosome arms at the anaphase, likely because of excess cohesin persists on chromosomes (Fig. 10C) (Haarhuis et al., 2013; Tedeschi et al., 2013). Moreover, strikingly, we found a unique phenotype in *esco1 esco2-aid wapl-aid* cells whereby chromosome arms remain tightly connected and CENP-T signals lag in the middle (Fig. 10C). This phenomenon is likely the cause of the aggravated lethality in the *esco1*
esco2-aid wapl-aid triple mutant. Altogether, our data denote that WAPL depletion is not able to rescue the synthetic lethality, whereas it apparently reverses the SCC defect.

3.11. Reducing the amount of cohesin improves growth defects of esco1 esco2 cells

We have shown that WAPL depletion rescues the SCC defect but aggravates the synthetic lethality. On the other hand, the acetyl-mimicking mutation of SMC3 does not rescue either of these defects. These findings suggest that WAPL-dependent cohesin removal and SMC3 acetylation have different outcomes, differently from the situation of S. cerevisiae. These thoughts attracted us to test the effect of combining two mutations, SMC3-K105, 106Q and wapl-aid, on cellular viability (Fig. 11A). We then monitored the growth rate of esco1 esco2-aid smc300- wapl-aid cells to compare with esco1 esco2-aid wapl-aid mutant. Notably, we found that SMC3-QQ mutation rescued the drastic lethality in esco1 esco2-aid wapl-aid cells, although the rescue is partial (Fig. 11B). We next asked whether the defect in chromosome segregation is improved. To do so, we carried out immunofluorescence to evaluate the accuracy of chromosome segregations. Interestingly, this experiment revealed that the frequency of missegregation was reduced when SMC3-QQ mutation is present (Fig 11C). Furthermore, surprisingly, we found that the number of chromosome bridges also decreased in esco1 esco2-aid smc300- wapl-aid (Fig. 11C). Notably, esco1 esco2-aid smc300- wapl-aid cells exhibits unseparated sister chromatids, ensuring that the rescue of chromosome bridges is not because of incomplete WAPL depletion (Fig. 11D). These results imply the possibility that WAPL and SMC3 acetylation are working in two distinct pathways and both are important for cellular viability by ensuring accurate chromosome segregation. However, the fact that esco1 esco2-aid smc300- wapl-aid mutant showed the rescue in chromosome bridges led us wonder if the amount of cohesin present in the cells is critical for the observed rescue. In fact, one allele of
SMC3 is disrupted in esco1 esco2-aid smc3<sup>30D/−</sup> wapl-aid cells, so the amount cohesin is expected to be reduced to half. In order to test this, we established esco1 esco2-aid smc3<sup>30D/−</sup> wapl-aid

**Figure 11. Reducing cohesin amount improves the synthetic lethality.** (A) Scheme of the conditional quadruple mutant’s establishment. (B, E) Growth curves as in Figure 2A. Auxin (500 μM final) was added at time 0 when indicated. (C) The frequency of chromosomes bridges and missegregation was measured as in Figure 3G. Auxin (500 μM final) was added 6 h before cell collection. (D) Chromosomes from metaphase spreads were analyzed as in Figure 1C. Auxin (500 μM final) was added 6 h before cell collection.

and esco1 esco2-aid smc3<sup>30D/−</sup> wapl-aid to test the effect of reducing cohesin amount and having acetyl-mimicking without reducing cohesion amount. We monitored their
growth rate and found that reducing cohesin amount improved the synthetic lethality, although the rescue is milder than when it is combined with the acetyl-mimicking mutation (Fig. 11E). On the other hand, the experiment revealed that acetyl-mimicking without the reduction of cohesin amount did not rescue the lethality (Fig. 11E). These results indicate that reduction of the cohesin amount is a critical factor in the observed rescue in esco1 esco2-aid smc3<sub>Q0/Q</sub> wapl-aid cells. In conclusion, our data suggest that over-stabilized cohesin in the absence of ESCO1/2 and WAPL is highly toxic for cells, whereas we cannot rule out a role for the SMC3 acetylation in supporting cellular proliferation. One possible explanation is that non-acetylated cohesin is toxic if it persists on chromosomes, thus WAPL depletion accelerates cell death in esco1 esco2-aid background, which is rescued by reducing the amount of cohesin. However, the key substrate of ESCO1/2 in this process remains to be identified.

3.12. **CPC is diffused over metaphase chromosomes upon WAPL depletion in esco1 esco2 cells**

We found that WAPL depletion reverses the SCC defect in esco1 esco2-aid mutant by stabilizing cohesin on chromosomes, whereas it does not rescue the synthetic lethality. These facts led us to consider if other key factors important for chromosome segregation are influenced by depletion of ESCO1/2 and/or WAPL, such as the chromosome passenger complex (CPC). CPC is a multi-protein complex composed of INCENP, Aurora B, Borealin and Survivin (Reviewed in (Carmena et al., 2012)). It is enriched at the centromeric region during metaphase and promotes chromosome segregation by regulating downstream pathways including cytokinesis. The enrichment of CPC at the centromeric region is highly dependent on phosphorylation of Histone H3-T3 mediated by Haspin kinase, and Haspin kinase localization is regulated by cohesin (Fig. 12A) (Rosasco-Nitcher et al., 2008; Kelly et al., 2010;
Wang et al., 2010; Yamagishi et al., 2010; Goto et al., 2017; Zhou et al., 2017). In fact, we previously reported that Aurora B and pH3T3 localizations are altered in the esco2 mutant, and that diffusion of CPC precedes the SCC defect (Abe et al., 2016).

Therefore, we decided to investigate if the CPC localization is altered in esco1 esco2-aid wapl-aid and wapl-aid cells. To do so, we performed immunofluorescence on

Figure 12. CPC is spread over metaphase chromosomes upon WAPL depletion. (A) Schematic representation of pathway related to cohesin at metaphase. (B) Immunofluorescence using anti-Histone H3-pT3 and DAPI staining on metaphase spreads. Sample were prepared as in Figure 2B Auxin (500 μM final) was added 6 h before cell collection. (C) Immunofluorescence using anti-Aurora B and DAPI staining on metaphase spreads. Sample were prepared as in Figure 2B. Auxin (500 μM final) was added 6 h before cell collection.
metaphase spreads using anti-Aurora B and anti-Histone H3-pT3. The result revealed that both signals are diffuse and faint in esco1 esco2-aid cells, indicating that CPC localization is defective in the absence of ESCO1 and ESCO2 (Figs. 12B, 12C). Moreover, intriguingly, we found that CPC signals were spread over chromosomes with strong signal upon WAPL depletion (Figs. 12B, 12C). We observed this phenotype in both esco1 esco2-aid wapl-aid and wapl-aid cells, indicating that this phenomenon alone does not explain the massive chromosome segregation defect observed in the triple mutant.

3.13. Condensin localization is altered upon WAPL depletion

One of the key substrates of Aurora B kinase is a kleisn subunit of condensin I, CAP-H, besides Histone H3-S10 (Fig. 13A) (Ono et al., 2004; Lipp et al., 2007; Nakazawa et al., 2011; Tada et al., 2011). These phosphorylations are known to facilitate condensin I association to mitotic chromosomes. Therefore, we wondered if condensin regulation is altered in the absence of WAPL and/or ESCO1/2. To visualize condensin by immunofluorescence, we introduced the 6xHA-tag to the C-terminus of SMC2, a subunit of the condensin complex. We verified the tagging of SMC2 by western blot (Fig. 13B). Subsequently, we performed immunofluorescence to stain SMC2 on metaphase spreads. Strikingly, the experiment revealed that SMC2 signal is enriched between sister chromatids upon WAPL depletion, whereas it is enriched on the axis of each sister chromatid in case of wild-type (Fig. 13C). This may be associated with unresolved sister chromatid junctions. However, it is not clear yet if it is caused by cohesin persistence. Therefore, it is worth testing how condensin localization is affected in wapl-aid smc3-aid double conditional mutant where both cohesin and WAPL are absent. Moreover, it would be also interesting to test whether
overexpressing TOP2 can rescue the condensin mis-localization and/or the chromosome bridges by decatenating DNA.

![Image](image_url)

**Figure 13. Condensin localization is altered upon WAPL depletion.** (A) Schematic representation of downstream pathway of Aurora B relevant for condensin regulation. (B) 6xHA-tagging of SMC2 was confirmed by Western blotting using anti-HA antibody. Histone H3 was used as a control. (C) Immunofluorescence using anti-HA antibody and DAPI staining on metaphase spreads. Sample were prepared as in Figure 2B. Auxin (500 μM final) was added 6 h before cell collection.

### 3.14. ESCO1/2 regulate interphase chromatin via cohesin

We have unveiled that the synthetic lethality in esco1 esco2-aid mutant cannot be simply explained by the SCC defect because WAPL depletion was not able to rescue the lethality whereas the SCC defect was reversed. This finding led us speculate that ESCO1/2 have roles outside SCC establishment. In fact, recent studies have shown that cohesin is essential not only for SCC but also for interphase chromatin organization, the latter often in association with *CTCF* (Parelho *et al.*, 2008; Wendt *et al.*, 2008; Busslinger *et al.*, 2017; Rao *et al.*, 2017; Wutz *et al.*, 2017). Therefore, we were attracted to test if depletion of ESCO1 and ESCO2 affect the interphase chromatin organization. For this purpose, we performed immunofluorescence to stain
Figure 14. ESCO1/2 regulate interphase chromatin via cohesin regulation. (A) Immunofluorescence using the anti-SMC3 antibody and DAPI staining. Samples were prepared by the cytospin method. After centrifugation, cells were fixed with 4% PFA in PBS containing 0.1% Triton X-100. Auxin (500 μM final) was added 6 h before cell collection when indicated. (B) Immunofluorescence using the anti-SMC3 antibody and DAPI staining. Images were taken by stimulated emission depletion (STED) microscopy. Samples were prepared as in A. Auxin (500 μM final) was added 6 h before cell collection when indicated. (C) DAPI staining and immunofluorescence with the anti-SMC3 antibody following nuclear permeabilization. Samples were prepared by the cytospin method. Cells were treated with 0.3% Triton X-100 for 5 min before the centrifugation. The signal intensities versus distance along the indicated line, A to B, were plotted using ImageJ’s analytical functions. For merged graphs, each signal intensity was normalized to the mean, and normalized intensity versus micrometer were plotted. Auxin (500 μM final) was added 6 h before cell collection.
SMC3 in esco1 esco2-aid mutant. From this experiment, we did not observe remarkable differences between wild-type and esco1 esco2-aid cells (Fig. 14A). Next, we tested esco1 esco2-aid wapl-aid cells to see the effect on the cohesin status. Strikingly, we found that concomitant depletion of ESCO1/2 and WAPL induces anomalous cohesin staining pattern (brain-like pattern), whereas WAPL depletion alone does not change the SMC3 staining pattern (Fig. 14A). This phenotype, previously defined as “vermicelli”, has been observed in WAPL depleted mouse embryonic fibroblast (MEF), HeLa cells and HAP1 cells, although it is observed only when ESCO1/2 and WAPL are co-depleted in our system (Tedeschi et al., 2013; Haarhuis et al., 2017; J. Rhodes et al., 2017). This difference could depend on cell lines and/or organisms, although the reason behind is not known. In fact, it was recently reported that U2OS cells do not exhibit a clear vermicelli phenotype, potentially because cohesion is more dynamic in proliferating cells (J. D. P. Rhodes et al., 2017). To assess the cohesin staining pattern more carefully, we next employed Stimulated Emission Depletion (STED) microscopy. We confirmed that the vermicelli phenotype is induced only in esco1 esco2-aid wapl-aid, while WAPL single depletion induces modest alteration (Fig. 14B). In order to test that the staining pattern of cohesin is associated with altered chromatin structure, we analyzed whether the DAPI signal and SMC3 signal are overlapping. The result verified that the signals are overlapping (Fig. 14C). The transcriptional effect of ESCO1/2 depletion has also been investigated, that preliminary data shows potential widespread changes and that is under further investigation. In conclusion, our results suggest that ESCO1 and ESCO2 have a role in interphase chromatin organization likely by regulating the dynamics of cohesin occupancy.
3.15. Cohesin amount increases on chromatin in esco1 esco2-aid wapl-aid cells

Our data have indicated a role of ESCO1/2 in interphase chromatin organization by regulating cohesin. However, the mechanism underlying this remains to be elucidated.

**Figure 15. Cohesin amount increases on chromatin in esco1 esco2-aid wapl-aid cells.** (A, B) Immunofluorescence using the anti-SMC3 antibody and DAPI staining without (A) or following (B) pre-extraction with 0.3% Triton X-100 to allow visualization of cohesin in nuclei (A) or cohesin bound on chromatin (B). Auxin (500 μM final) was added 6 h before cell collection. For analysis, the nucleic area was defined by the DAPI signal. The SMC3 signals that overlapped with DAPI signals were measured and normalized to the SMC3 signal intensity in wild-type and then the median values were averaged for three independent experiments, then plotted. >100 nuclei were examined in each experiment. P-values were calculated by Student’s t-test. (**) P < 0.05; (****) P < 0.01. (C) Left panel: chromatin binding assay. Western blotting was performed with fractionated protein samples from each of indicated cell lines. SMC3 and acSMC3 were detected. α-tubulin and Histone H3 were used as control. WCE = whole cell extract, NUC = nucleoplasmic fraction, CHIR = chromatin bound fraction. Right panel: quantification of SMC3 and acSMC3 in each fraction. The signal intensity of each fraction was normalized to WT, and plotted. Auxin (500 μM final) was added 6 h before cell collection.
To tackle this question, we first asked if the amount of cohesin is affected upon depletion of ESCO1/2 and/or WAPL. For this purpose, we carried out immunofluorescence with or without pre-extraction where cells were treated with 0.3% of Triton X-100. Consequently, we found that the amount of cohesin stayed at the same levels in all the mutants without the pre-extraction (Fig. 15A). However, consistent with the known role for WAPL, the immunofluorescence with pre-extraction revealed that the cohesin amount on chromatin increases upon WAPL depletion (Fig. 15B) (Tedeschi et al., 2013). Moreover, strikingly, we discovered that esco1 esco2-aid wapl-aid cells showed even more increase (Fig. 15B). This finding was surprising since ESCO1 and ESCO2 have been shown as a factor that stabilizes cohesin on DNA. Therefore, we next conducted chromatin fractionation and performed western blot to assess the amount of chromatin-bound cohesin to make sure our finding is true. The result showed that the cohesin amount on chromatin increases when WAPL was depleted and this was further increased in esco1 esco2-aid wapl-aid cells, confirming the role of ESCO1 and ESCO2 in negatively regulating cohesin (Fig. 15C). Taken together, our results emphasize an unexpected role of ESCO1/2 in negatively regulating cohesin binding during interphase.

3.16. ESCO1 and ESCO2 are redundant for interphase chromatin organization

We have identified ESCO1/2’s role in interphase chromatin organization. Next, we asked how the contributions of ESCO1 and ESCO2 are balanced in the interphase chromatin organization. To do so, we established esco1 wapl-aid and esco2 wapl-aid cells. We then performed immunofluorescence to stain SMC3 and found that neither of mutants exhibited a clear vermicelli phenotype, indicating their functional redundancy in the organization of interphase chromatin (Fig. 16A). To strengthen the idea that ESCO1 and ESCO2 are redundant in this process, we also complemented
escol esco2-aid wapl-aid cells with ESCO2 fused to HA-tag (thereafter referred as 
esco1 esco2-aid wapl-aid $^{HA}$ESCO2). The expression of HA-ESCO2 was verified by 
western blot (Fig. 16B). We also verified that expressed HA-ESCO2 is functional by 
confirming the rescue of SMC3 acetylation (Fig. 16B). We then conducted 
immunofluorescence using escol esco2-aid wapl-aid $^{HA}$ESCO2 cells and found that 
complemented HA-ESCO2 was able to rescue the vermicelli phenotype (Fig. 16C). In 
summary, our data indicate the redundant role of ESCO1/2 in interphase chromatin 
organization.

**Figure 16. ESCO1 and ESCO2 are redundant for interphase chromatin organization.** (A, C) 
Immunofluorescence of SMC3 and DAPI staining as in Figure 14A. Auxin (500 μM final) was added 6 h 
before cell collection. (B) Expression of HA-ESCO2 and the acetylation level of SMC3 were monitored by 
Western blotting. Auxin (500 μM final) was added 6 h before cell collection.

### 3.17. SMC3 acetylation is not critical for interphase chromatin organization

We have found that ESCO1/2 play roles in interphase chromatin organization in 
limiting cohesin amount on chromatin. To understand the mechanism how ESCO1/2 
regulate the interphase chromatin, we next decided to test whether SMC3 acetylation
plays a role in it. To test this idea, we expressed different SMC3 variants in smc3-aid wapl-aid background, including SMC3-K105, 106R (wapl-aid smc3-RR), SMC3-K105, 106Q (wapl-aid smc3-QQ), SMC3-K105, 106A (wapl-aid smc3-AA). If SMC3 acetylation plays a critical role in interphase chromatin organization, wapl-aid smc3-
RR and wapl-aid smc3-AA cells should also exhibit the vermicelli phenotype as esco1 esco2-aid wapl-aid mutant does. The expression of SMC3 variants were verified by western blot (Fig. 17A). Using these cell lines, we performed immunofluorescence using SMC3 antibody to test the vermicelli phenotype, but we did not find any remarkable change in any mutant, suggesting that the acetylation of SMC3 is not critical in interphase chromatin organization (Fig. 17B). However, because of limited resolution of images, this result needs to be tested in a different experiment. For instance, wapl-aid smc3-RR shows slightly different staining pattern compared with the others (Fig. 17B). To investigate this idea in another way, we also tested esco1 esco2-aid smc3QQ- wapl-aid for SMC3 staining. We should observe the suppression of vermicelli phenotype if SMC3 acetylation plays a role. We then conducted immunofluorescence and analyzed by both widefield microscopy and the STED microscopy to have better resolution to detect even small changes. This experiment revealed that esco1 esco2-aid smc3QQ- wapl-aid mutant shows partial rescue of the vermicelli phenotype (Figs. 17C, 17D). Together, our results are compatible with a potential role of SMC3 acetylation in chromatin organization, when cohesin levels are reduced, although it is still inconclusive. We cannot exclude that simply the amount of cohesin is the reason of the vermicelli rescue phenotype since the cohesin amount varies when SMC3 expression constructs are randomly integrated, and it is reduced to half in case when the SMC3-QQ mutation is knocked in on the genome.

3.18. **CTF18 mutation is synthetic lethal with DDX11 knock out**

CTF18, DCC1, and CTF8 comprise a hetero trimer (18-1-8) that forms an RFC-like complex with RFC2-5 (Mayer et al., 2001; Naiki et al., 2001; Bermudez et al., 2003; Merkle et al., 2003). Mutating DCC1 was shown to reduce the acetylation level of SMC3 (Terret et al., 2009). Consistently, in budding yeast, it has been shown that
 deleting either subunit of 18-1-8 elicits SCC defects (Mayer et al., 2001; Petronczki et al., 2004; Ansbach et al., 2008). We have previously reported that ESCO2 mutation and DDX11 depletion are synthetic lethal (Abe et al., 2016). Therefore, we thought to test the genetic interaction between CTF18 and DDX11. For this purpose, we

**Figure 18. CTF18 mutation is synthetic lethal with DDX11 knock out.** (A) Degradation of CTF18 fused to AID-tag was confirmed by Western blotting. α-tubulin was used as a loading control. (B) Growth curves as in Figure 2A. Auxin (500 μM final) was added at time 0 when indicated. (C) Chromosomes from metaphase spreads were analyzed as in Figure 1C. Auxin (500 μM final) was added 6 h before cell collection. The results of two independent experiments were plotted. (D) Frequency of chromosome misalignment was measured by immunochemistry. CENP-T was used to mark micro chromosomes barely visible by DAPI staining. Auxin (500 μM final) was added 6 h before cell collection. (E) Lagging chromosomes in anaphase cells. The analysis was performed as in Figure 3G. Auxin (500 μM final) was added 6 h before cell collection.
introduced the AID-tag to both allele of *CTF18* in *ddx11* background where *TIR1* is ectopically expressed. We first verified the degradation of *CTF18*-AID by western blotting (Fig. 18A). Then, we monitored the viability of cells in the presence or absence of auxin (Fig. 18B). Interestingly, the experiment revealed that depleting *CTF18* in the absence of *DDX11* is lethal (Fig. 18B). Consistently, simultaneous gene disruption of *CHL1* (*DDX11* in vertebrates) and *CTF18* has been shown to cause synthetic lethality in budding yeast (Tong *et al.*, 2004; Xu, Boone and Brown, 2007). Since both *CTF18* and *DDX11* are implicated in SCC, we next assessed the effect on SCC by analyzing the metaphase spreads. The experiment showed that co-depletion of *DDX11* and *CTF18* result in severe SCC defect (Fig. 18C). Next, we asked whether the severe SCC defect results in chromosome segregation errors. To this end, we performed immunofluorescence to score the frequency of chromosome misalignment and missegregation. The results revealed drastic misalignment and mis-segregation of chromosomes specifically when both *CTF18* and *DDX11* were absent, revealing the likely cause of the observed synthetic lethality (Figs. 18D, 18E).

### 3.19. *CTF18* and *ESCO2* may function in distinct cohesion pathways

Given that *DCC1* gene disruption results in the reduction of SMC3 acetylation level, one would imagine that 18-1-8 and *ESCO2* may function together (Terret *et al.*, 2009; Borges *et al.*, 2013). Supporting this idea, in budding yeast, it has been reported that acetyl-mimicking SMC3 masks the SCC defect caused by knocking out *CTF18* (Liu *et al.*, 2020). Therefore, we decided to test whether restoring SMC3 acetylation is able to rescue the synthetic lethality. For this purpose, we over-expressed *ESCO1* and *ESCO2* in *ctf18-aid ddx11* double conditional mutant. *ESCO1* over-expression restored reduced acetylation of SMC3 in *esco2* cells, rescued its growth defect and the synthetic lethality observed in double conditional mutant of *ESCO2* and *DDX11* (Figs.
We determined the mRNA levels of ESCO1 and ESCO2 by qPCR and found that their expression levels increased more than 12 times compared to the endogenous levels (Figs. 19A, 19B). SMC3 acetylation level was also monitored by western blot, confirming the over-expression of either of ESCO1 and ESCO2 restores the acetylation level to the same level of wild-type (Fig. 19C). However, to our surprise, we found that restoration of overall SMC3 acetylation is not able to rescue the synthetic lethality (Figs. 19D, 19E). This result suggests that the underlying

Figure 19. CTF18 and ESCO2 may function in distinct pathways. (A) mRNA level of ESCO1 was determined by qPCR as in Figure 3A. (B) mRNA level of ESCO2 was determined by qPCR. (C) SMC3’s acetylation level was monitored by Western blotting. Expressions of ESCO1-FLAG and HA-ESCO2 were also verified. (D), (E) Growth curves as in Figure 2A. Auxin (500 μM final) was added at time 0 when indicated.
mechanism of synthetic lethality in ctf18-aid ddx11 cells is different from that of esco2-W615G tetoff-ddx11 double conditional mutant. Collectively, our data raise the possibility that CTF18 and ESCO2 may function in distinct pathways for SCC.

3.20. **WAPL depletion rescue the synthetic lethality of DDX11 CTF18 double mutant**

WAPL is a negative regulator of cohesin, and its depletion restores SCC defects in various mutants. Accordingly, we wondered if stabilizing cohesin by WAPL depletion is able to rescue the synthetic lethality of ctf18-aid ddx11 double mutant. To this end,

![Figure 20. WAPL depletion rescue the synthetic lethality of ctf18-aid ddx11 double mutant.](image)

Figure 20. WAPL depletion rescue the synthetic lethality of ctf18-aid ddx11 double mutant. (A) Chromosomes from metaphase spreads were analyzed as in Figure 1C. Auxin (500 µM final) was added 6 h before cell collection. The results of two independent experiments were plotted. (B), (C) Growth curves as in Figure 2A. Auxin (500 µM final) was added at time 0 when indicated. (D) Chromosome bridges and missegregation in anaphase. The frequency of chromosomes bridges and missegregation was measured as in Figure 3G. Auxin (500 µM final) was added 6 h before cell collection.

we established ctf18-aid ddx11 wapl-aid triple conditional mutants. We then determined the SCC status, and found that WAPL depletion restores the SCC defects
observed in ctf18-aid ddx11 cells, confirming that WAPL depletion stabilizes cohesin on chromosomes (Fig. 20A). Subsequently, we monitored the cellular proliferation rate in the presence or absence of auxin. Strikingly, the experiment revealed that WAPL depletion rescues the synthetic lethality caused by simultaneous depletion of CTF18 and DDX11 (Fig. 20B), a situation opposite to that of esco1 esco2-aid cells. To make sure that the rescue depends on WAPL depletion, we complemented WAPL in ctf18-aid ddx11 wapl-aid cells and confirmed that WAPL’s presence induces synthetic lethality (Fig. 20C). Since simultaneous depletion of CTF18 and DDX11 causes massive chromosome missegregation, we also tested whether the chromosome segregation error is improved. Consistently, we found greatly reduced frequency of lagging chromosomes in ctf18-aid ddx11 wapl-aid triple mutant compared to ctf18-aid ddx11 double mutant (Fig. 20D). Interestingly, not only the frequency of lagging chromosomes, but also the frequency of chromosome bridges appeared to be reduced in the triple mutant compared with wapl-aid single mutant, suggesting mutual suppression (Figs. 20A, 20D). Therefore, our results indicate roles of DDX11 and CTF18 in SCC establishment, although we cannot rule out their potential roles in SCC maintenance by counteracting WAPL.

3.21. Exploring novel cohesin interactors

Our data demonstrated that the phenotype observed in the esco1 esco2-aid mutant cannot be simply explained by SMC3 acetylation at K105 and K106. This led us to explore other targets of ESCO1 and ESCO2 responsible for the defects we have observed. To investigate new substrate of ESCO1/2, we decided to employ immunoprecipitation-mass spectrometry (IP-MS) to explore interactors of cohesin. To do so, we introduced Myc-FLAG-HA-tag (MFH) to the C-terminus of RAD21 (also known as SCC1, MCD1), a kleisin subunit of cohesin complex, to immunoprecipitate
the cohesin complex (Fig. 21A). For sequential immunoprecipitation, RAD21-MFH was first immunoprecipitated by the anti-FLAG antibody, then immunoprecipitated by anti-HA antibody subsequently (Fig. 21B). The samples were analyzed by mass spectrometry and we identified only 37 proteins including known subunits of cohesin complex (Fig. 21C). Moreover, of interest, we found RUVBL1 and RUVBL2 which were not previously reported as cohesin interactors.

![Diagram of cohesin complex](image)

**Figure 21. Exploring novel cohesin interactors.** (A) Schematic representation of cohesin complex and Myc-FLAG-HA-tag fused to the C-terminus of RAD21. (B) Left panel: Scheme of the sequential immunoprecipitation. Right panel: Coomassie staining. Samples were prepared by sequential immunoprecipitation from each cell line as markers indicate. The detail is described in Materials and Methods part. (C) Scatter plot of the results of LC-MS/MS analysis. The vertical axis shows the peptide count number in RAD21\(^{MFLH}\) sample in logarithm scale. Core complex of cohesin and proteins of interest were labelled. The horizontal axis shows the intensity ratio between RAD21\(^{MFLH}\) and no-tag sample in logarithm scale.

### 3.22. **RUVBL1/2 are novel cohesin interactors**

RUVBL1/2 has been reported as a multifunctional protein involved in various cellular pathways such as chromosome decondensation, chromatin remodeling, DNA repair.
and splicing (Reviewed in Mao and Houry, 2017). Recent studies have reported that several chromatin remodelers such as RSC, FACT and SNF2h facilitate cohesin loading by distinct mechanisms (Hakimi et al., 2002; Lopez-Serra et al., 2014; Garcia-Luis et al., 2019; Muñoz et al., 2019). We decided to study the role of RUVBL1/2 in regulating cohesin because RUVBL1/2 is also known as a component of chromatin remodelers, such as INO80 complex (Reviewed in Willhoft and Wigley, 2020)). Before proceeding into this direction, we first conducted co-IP experiment using the same condition for the IP-MS to verify the interaction between cohesin and RUVBL1/2. The result proved the interaction between cohesin and RUVBL1/2 (Fig. 22A). We next introduced the MFH-tag to the C-terminus of RUVBL1 to carry out co-IP experiment the other way around. We then performed the co-IP using the same

**Figure 22. RUVBL1/2 are novel cohesin interactors.** (A) Co-IP experiment. Protein samples were prepared from the cell lines of indicated genotype. RUVBL1 and RUVBL2 were detected by Western blotting. SMC3 was detected as a positive control. Immunoprecipitation was performed as in Figure 21B. The detail is described in Materials and Methods part. Saturated signal was marked with red color. (B) Co-IP experiment as in A. RAD21 and SMC3 were detected by Western blotting. RUVBL2 was detected as a positive control. (C) Co-IP experiment as in B with small modification. Samples were treated with benzonase during immunoprecipitation when indicated.
setup for cohesin, and confirmed that the cohesin complex is co-immunoprecipitated with RUVBL1 (Fig. 22B). To exclude the possibility that the interaction is mediated by DNA, we performed co-IP again with or without benzonase treatment. The result confirmed that the interaction is not mediated by DNA (Fig. 22C). Taken together, our results demonstrate that RUVBL1/2 interacts with cohesin.

### 3.23. Cohesin depletion does not affect RUVBL1 localization

It has been shown that RUVBL1/2 localization is dynamic and RUVBL1/2 is critical for accurate chromosome segregation (Gentili et al., 2015). Moreover, it is also shown to be essential for chromosome decondensation after mitosis (Magalska et al., 2014). In addition, cohesin is also known to be critical in both events (Reviewed in (Batty and Gerlich, 2019)). Therefore, we were attracted to test whether cohesin depletion affects RUVBL1’s localization during mitosis. To this end, we performed immunofluorescence in smc3-aid cells using anti-RUVBL1 antibody. This experiment confirmed the dynamic localization of RUVBL1 during mitosis in DT40 cells similar to mammalian cells (Fig. 23) (Gentili et al., 2015). However, the depletion of cohesin did not induce remarkable changes in the RUVBL1 localization (Fig. 23). In summary, our result indicates that cohesin is not required for RUVBL1’s localization during mitosis.

### 3.24. RUVBL1 depletion causes SCC defects

We have identified RUVBL1/2 as a novel cohesin interactor. To study RUVBL1/2 roles in cohesin-mediated transactions, we decided to establish a conditional RUVBL1 mutant since RUVBL1 is essential in various organisms (Reviewed in (Mao and Houry, 2017)). We introduced the AID-tag to the C-terminus of RUVBL1 and disrupted the other allele of RUVBL1 by knockout construct (thereafter referred to as ruvbl1-aid)
We first monitored the degradation of RUVBL1-3xAID-6xFLAG by western blot and found that the RUVBL1 protein level reduced to undetectable level 24 hours after auxin addition (Fig. 24C). We also tested if the depletion of RUVBL1 leads to lethality and found that cells stop proliferating and start dying 24 hours after auxin addition, confirming that the AID system is working (Fig. 24D).
RUVBL1/2 was identified as an interactor of cohesin, therefore, we wondered if the depletion of RUVBL1 results in SCC defects. To test this, we performed metaphase spread analysis. The result revealed that RUVBL1 depletion causes severe SCC defect (Fig. 24E). However, we also found the SCC defect without RUVBL1 full depletion, although it is further aggravated after auxin treatment, indicating C-terminally AID-tagged protein is hypomorphic (Fig. 24E). In fact, we observed also a growth defect without adding auxin (Fig. 24D). Nevertheless, our data suggest that RUVBL1 plays a role in promoting SCC. Since we found that adding an AID-tag at the C-terminus of RUVBL1 makes the protein hypomorphic, we decided to introduce the AID-tag at the N-terminus of RUVBL1. To do so, we employed the Flip-in system again (Fig. 24F). In this approach, the endogenous promoter cannot be used, so we tried three different promoters to choose the best one for our purpose, including the TRE3G promoter which can be shut off by doxycycline, CMV promoter which is weak and the RSV promoter which is strong. Among these three promoters, TRE3G and CMV were not strong enough to fulfil the function of RUVBL1 in supporting cellular proliferation. In contrast, we were able to establish $RUVBL1^{pRSV-3xAID}$ cells (thereafter referred as $aid$-$ruvbl1$). We first monitored the kinetics of the AID-RUVBL1 degradation by western blot and found that RUVBL1 protein level reduces to undetectable levels within 24 hours after auxin addition (Fig. 24G). We also tested for RUVBL2 protein amount since they form a complex. Indeed, the result showed that RUVBL2 amount was also reduced (Fig. 24G). Using $aid$-$ruvbl1$ cells, we next monitored the growth rate and did not find any delay without auxin treatment (Fig. 24H). In contrast, after addition of auxin, the $aid$-$ruvbl1$ mutant started dying at 24 hours (Fig. 24H). This result indicates that N-terminal AID-tagging does not make RUVBL1 protein hypomorphic. Next, we assessed how SCC is affected upon RUVBL1 depletion in the $aid$-$ruvbl1$ mutant. Therefore, we conducted metaphase spread analysis, and found that
Figure 24. RUVL1 depletion causes SCC defect. (A) Scheme of the RUVL1 conditional mutant’s establishment. (B) Schematic representation of RUVL1 gene locus and the knock out construct. (Closed boxes) Exons; (green box) Start codon; (yellow box) ATPase domain of RUVL1. (C) Depletion of RUVL1-3xmAID-6xFLAG was verified by Western blotting. Histone H3 was used as control. (D, H) Growth curves as in Figure 2A. Auxin (500 μM final) was added at time 0 when indicated. (E, I) Chromosomes from metaphase spreads were analyzed for cohesion defects following the classification procedure outlined in Figure 1C. (F) Schematic representation of RUVL1 gene locus and pRSV-3xmAID-RUVL1 FLP-In construct. (Closed boxes) Exons; (green box) Start codon; (yellow box) ATPase domain of RUVL1. (pRSV) RSV promoter. (G) Depletion of 3xmAID-RUVL1 was verified by Western blotting. Histone H3 was used as control.
RUVBL1 depletion results in severe SCC defect which was not induced in the absence of auxin (Fig. 24I). Altogether, our data demonstrate the role of RUVBL1 in facilitating SCC.

3.25. Cohesion defect caused in ruvbl1-aid cells is rescued by WAPL depletion

We have proved a role of RUVBL1 in SCC establishment. To test the genetic interaction with other cohesin regulators, we next decided to combine the wapl-aid mutation with ruvbl1-aid. Therefore, we established ruvbl1-aid wapl-aid cells, and monitored the SCC by analyzing metaphase spreads. The result showed that the SCC defect in ruvbl1-aid mutant is rescued by depleting WAPL, supporting the idea that SCC defect caused by RUVBL1 depletion is related to cohesin dysregulation (Fig. 25).

3.26. Cohesin amount on chromatin is barely reduced after RUVBL1 depletion

It has been reported that several chromatin remodelers are involved in cohesin regulation such as the RSC complex (Lopez-Serra et al., 2014; Muñoz et al., 2019). Moreover, RUVBL1 is known as a subunit of the INO80 complex which is also a chromatin remodeler implicated in SCC (Ogiwara, Enomoto and Seki, 2007). Therefore, we decided to test the cohesin amount on chromatin in aid-ruvbl1 cells. To this end, we monitored the acetylation status of SMC3 as a marker of chromatin bound cohesin. The result showed that acetylation of SMC3 was not reduced 12 hours after...
auxin addition and it was slightly reduced within 24 hours (Figs. 26A, 26B). Importantly, cohesin amount itself was not reduced (Figs. 26A, 26B). However, according to the cell cycle analysis, RUVBL1 depletion induces cell cycle arrest at M/G1-phase and this was prominent at 24 hours after auxin addition (Fig. 26C). Thus, the reduction of SMC3 acetylation is likely caused by the alteration of cell cycle profile in which the population of S/G2-phase cells is reduced. Together, our results suggest that RUVBL1 does not play a role in cohesin loading or stabilization in global manner but facilitates SCC.

Figure 26. Cohesin amount on chromatin is barely reduced after RUVBL1 depletion. (A, B) SMC3 amount and its acetylation level were monitored by Western blotting. The intensity of SMC3 or acSMC3 were normalized to α-tubulin, then normalized to WT without auxin treatment and plotted. Auxin (500 μM final) was added 12h (A) or 24 h (B) before sample collection. (C) Cell cycle profile was analyzed by propidium iodide staining as in Figure 10B.
4. DISCUSSION

4.1. Substrates of ESCO1 and ESCO2

We found that ESCO1 and ESCO2 deletions are synthetic lethal and this lethality coincided with nearly abolished SMC3 acetylation at K105 and K106 (Kawasumi et al., 2017). However, our subsequent experiments revealed that the acetyl-mimicking QQ mutation of SMC3-K105, K106 is not able to rescue the lethality, severe SCC defects and chromosome segregation errors. Moreover, complementation experiments uncovered that acetyl-deficiency at SMC3-K105, K106 causes little or no effect on the cellular proliferation rate and SCC (Kawasumi et al., 2017). Taken together, these observations raise the possibility that ESCO1 and ESCO2 have additional substrates besides SMC3-K105, K106.

Although the substrates of ESCO1 and ESCO2 are not limited to cohesin genes, the fact that simultaneous depletion of ESCO1 and ESCO2 leads to severe SCC defects, not bypassed by SMC3-K105, K106 acetyl-mimicking mutations, indicates that the relevant substrates may still be related to factors that promote sister chromatid synapsis (Kawasumi et al., 2017). Interestingly, a number of acetylation sites have been identified within cohesin related genes (Choudhary et al., 2009; Kulemzina et al., 2016). Of those, we hypothesized and tested whether additional two lysine residues on SMC3-K140, K1190, which have relatively high PTM scores and are situated near ATPase sites, play roles in SCC. However, none of the two lysines were critical for SCC or cellular proliferation (Kawasumi et al., 2017).

Other interesting acetylation sites within the cohesin complex are those located on SMC1’s hinge domain (K536, K648), although the PTM scores are not high (Models. 9A) (Choudhary et al., 2009). Both lysines are situated at the lumen of the hinge domain (Model. 9B) (Kurze et al., 2011; Shi et al., 2020). Importantly, the hinge lumen comprises basic patches that are essential for chromatin association of cohesin (Kurze
et al., 2011; Srinivasan et al., 2018). Coincidentally, one of the acetylated lysine, SMC1-K648, is equivalent to Smc1-K661 in budding yeast which is shown to be critical for cohesin’s chromatin association, suggesting that the acetylation of SMC1-K648 may be critical in cohesin loading (Model. 9B) (Kurze et al., 2011; Srinivasan et al., 2018). Moreover, on the basis of recent structural studies, SMC1-K648 and K661 are in the vicinity of SMC3-D594 and E584, respectively, when the hinge is in closed state or “South open” state (Model. 9B) (Kurze et al., 2011; Shi et al., 2020). Since their interactions may rely on their charge, neutralizing the positive charge of SMC1-K648, K661 may weaken their interaction resulting in dynamic conformational change in the hinge. “North open” form was observed in complex with DNA or other cohesin subunits including SA1 and NIPBL, but not SMC1/3 alone (Model. 9B) (Shi et al., 2020). Collectively, the hinge seems to be highly dynamic depending on the state of cohesin, and acetylation of lysine residues situated in the hinge could be a key factor for that process.

SMC1 and SMC3 are also acetylated at their coiled-coil domains (Choudhary et al., 2009; Kulemzina et al., 2016). Recently, in budding yeast, it was reported that acetyl-deficient mutants of Smc1 and Smc3 are defective in their interaction between coiled-coil and coiled-coil, and in Rad21 association (Kulemzina et al., 2016). The authors further showed that in vitro deacetylation elicits the dissociation of coiled-coil interaction. Therefore, although there is no evidence that these acetylations are mediated by Eco1 at present, they could be potential targets of ESCO1/2.

Among cohesin’s regulatory factors, PDS5A-K1146 and PDS5B-K1136 were found to be acetylated with highest PTM scores (Model. 9C) (Choudhary et al., 2009). C-termini of PDS5A and PDS5B are unstructured, unlike other segments of PDS5 containing HEAT-repeat domains (Ouyang et al., 2016). Therefore, the structure is not resolved at present. Nevertheless, interestingly, PDS5B-K1136 was found to be
crosslinked to SMC3-K500 which is situated at the transition of coiled-coil to hinge (Model. 9C) (Huis In’t Veld et al., 2014). Consistently, recent studies have shown the interactions between the hinge and HEAT-repeat proteins (Huis In’t Veld et al., 2014;
Murayama and Uhlmann, 2014, 2015; Bürmann et al., 2019; Shi et al., 2020). Based on structural studies, one of the most accepted models for cohesin’s translocation activity is called the “inchworm model” (Bürmann et al., 2019). Essentially, SMC proteins contain an “elbow” in the middle of their coiled-coils where the probability of forming coiled-coil are lower. According to the “inchworm model”, the elbow goes cycles of folded and stretched conformations, presumably coupled with ATP hydrolysis (Bürmann et al., 2019). Therefore, if HEAT-repeat proteins determine the head-hinge interactions of SMC proteins, acetylation of PDS5B-K1136 might affect the cohesin’s translocation activity.

PDS5A is acetylated at K974 as well. This lysine is equivalent to PDS5B-K964. Pds5 proteins have hook-like shape and the groove seems to be the main interface with Rad21 (Models. 9D, 9E) (Lee et al., 2016; Muir et al., 2016; Ouyang et al., 2016). In fact, mutation on the conserved tyrosine on budding yeast Pds5-Y458 reduces interaction with Rad21 (Lee et al., 2016; Muir et al., 2016). Intriguingly, although it is conserved only in higher eukaryotes, PDS5B-K964 is found to be situated in the groove (Model. 9F) (Ouyang et al., 2016). As acetylation of cohesin seems to stabilize the interaction between cohesin and PDS5, PDS5B-K964 may also be an interesting candidate as substrate of ESCO1/2.

Finally, in mice oocyte meiosis and porcine oocyte meiosis, it has been shown that ESCO1 acetylates α-tubulin to maintain microtubule stability, and ESCO2 acetylates Histone.H4-K16 to regulate SAC activation (Lu et al., 2017, 2018, 2019). Thus, the substrates of ESCO1 and ESCO2 are not limited in the cohesin related genes. It will be exciting to explore novel substrates of ESCO1/2, for instance, by SILAC experiments using our esco1 esco2-aid double conditional mutant.
4.2. Role of SMC3 acetylation at K105 and K106

Although our results pointed out that the acetylation of SMC3-K105, K106 is not essential for SCC or cellular proliferation in our system, it does not rule out the possibility that these acetylation sites play a role in cohesin regulation (Kawasumi et al., 2017). In budding yeast, replacing both lysines with arginines causes lethality and SCC defects (Ben-shahar et al., 2008; Ünal et al., 2008; Rowland et al., 2009). Recent studies reported interesting consequences of the acetyl-mimic (QQ) or acetyl-deficiency (RR) in higher eukaryotes as well. Both mutations seem to enhance the interaction with Sororin, and QQ mutation bypasses the requirement of ESCO1 and ESCO2 for cohesin’s stable association with chromatin in the G2-phase, which still requires Sororin (Nishiyama et al., 2010; Ladurner et al., 2016). Therefore, although mutating these residues does not result in detectable SCC and growth defects, these lysines contribute to cohesin regulation.

Recent studies have illustrated that SMC3-K105, K106 are situated at the inner surface of SMC3’s head domain (Model. 10A) (Shi et al., 2020). Interestingly, cryo-EM analysis has further uncovered that these two lysine residues are in the vicinity of DNA and NIPBL-E1899, E1900 (Model. 10A) (Shi et al., 2020). In addition, DNA and NIPBL synergistically stimulate cohesin’s ATPase activity (Murayama and Uhlmann, 2014; Petela et al., 2018; Davidson et al., 2019; Kim et al., 2019). Hence, it is tempting to speculate that acetylation of these sites neutralizes the positive charge and modulates the interaction with DNA and/or NIPBL-E1899, E1900, potentially weakening the ATPase activity of cohesin. Although several studies have reported that neither in vitro acetylation nor acetyl-mimic mutation (QQ) shows detectable change in the cohesin ATPase activity, it could be because the ATPase activity was measured in the absence of NIPBL in those experimental setups (Ladurner et al., 2014).
Therefore, it will be informative to test whether the acetylated SMC3 or SMC3-QQ show reduced ATPase activity in the presence of NIPBL.

Although there is no evidence that the acetylation of SMC3-K105, K106 modulates its ATPase activity, several biochemical experiments emphasized the importance of the above acetylation on cohesin’s biochemical characteristics. First, substitution of either
lysine to glutamine greatly affected the topological entrapment of DNA (Murayama and Uhlmann, 2015; Kanke et al., 2016). Mutating both lysines resulted in nearly abolished topological entrapment (Murayama and Uhlmann, 2015; Kanke et al., 2016). Similar consequence was observed also in vivo (Hu et al., 2015). The authors showed that the acetyl-mimic mutant is tolerant of WAPL-mediated unloading, imitating the cohesin’s stabilization in vivo (Murayama and Uhlmann, 2015). Collectively, it seems that acetyl-mimic mutation inhibits both loading and unloading reactions, indicating that these lysine residues need to be regulated dynamically in vivo (Beckouët et al., 2010; Borges et al., 2010; Xiong, Lu and Gerton, 2010; Deardorff et al., 2012). Since ATP-binding and/or ATP hydrolysis are crucial, if not essential, for both cohesin loading and unloading, it will be important to understand whether the acetylation of SMC3-K105, K106 affects the ATPase activity (Murayama and Uhlmann, 2014, 2015).

On the contrary, in case of cohesin’s diffusion on DNA, the acetylation of SMC3-K105, K106 was shown to increase the diffusion coefficient (Kanke et al., 2016). Cohesin diffusion was observed in the absence of ATP and was inhibited by addition of the unhydrolyzable ATP analogue, AMP-PCP (Model. 10B) (Davidson et al., 2016; Kanke et al., 2016; Stigler et al., 2016). Thus, it seems that constitutive engagement of the ATPase heads slows the diffusion, possibly suggesting that the diameter of the cohesin’s pore is smaller in the ATP-binding state. If the diameter of the pores is the determinant of the diffusion coefficient, it may indicate that the acetylation of SMC3-K105, K106 elicits conformational change of the cohesin complex to create a larger pore (Model. 10C). Of note, despite the fact that cohesin’s central pore is approximately 35 nm in the absence of DNA, it has been shown that cohesin cannot pass through obstacles larger than 20 nm in diameter, suggesting that cohesin might not exhibit an open-ring shape when it is topologically bound to DNA (Davidson et
al., 2016; Stigler et al., 2016). It is noteworthy that a recent study has resolved the structure of the cohesin complex with the C-terminus of NIPBL (from M1193 to L2628) by cryo-EM and found that NIPBL creates a narrow path for DNA in association with the engaged ATPase heads (Model. 10A) (Shi et al., 2020). Since SMC3-K105, K106 is situated in the vicinity of both NIPBL-E1899, E1900 and DNA, weakening the interaction by neutralizing the charge of lysines may create a larger pore that increases the diffusion coefficient (Model. 10C).

Alternatively, the fact that higher salt concentration increases the diffusion coefficient might indicate that weaker interaction between DNA and cohesin can increase the diffusion coefficient (Davidson et al., 2016; Kanke et al., 2016; Stigler et al., 2016). In this scenario, rather than inducing conformational change of cohesin, the acetylation may weaken the cohesin-DNA interaction which results in increased diffusion coefficient (Model. 10C).

Both in vitro and in vivo, a number of studies have shown that cohesin can be moved by the transcription machinery (Lengronne et al., 2004; Gullerova and Proudfoot, 2008; Hu et al., 2015; Davidson et al., 2016; Busslinger et al., 2017). Intriguingly, ChIP-seq experiments revealed that acetylated SMC3 are preferentially enriched downstream of transcribed genes (Deardorff et al., 2012). Collectively, the acetylation of SMC3 could be a critical determinant for cohesin’s translocation ability that may impact on transcription regulation. However, the underlying mechanism needs to be addressed in future studies.

4.3. ESCO1 and ESCO2 in interphase chromatin organization

We have demonstrated that ESCO1/2 contributes to interphase chromatin organization (Kawasumi et al., 2017). In line with our finding, it was recently reported that ESCO1 stabilizes cohesinSA1 in cooperation with CTCF in G1-phase, leading to the formation
of long loops (Wutz et al., 2020). To be noted, in vertebrates, the acetylation of SMC3 promotes stable chromatin association of cohesin by recruiting Sororin (Lafont, Song and Rankin, 2010; Nishiyama et al., 2010; Ladurner et al., 2016). However, Sororin is nearly absent in G1-phase, thus there must be a mechanism by which acetylated cohesins are stabilized (Nishiyama et al., 2010).

Interestingly, a recent study has uncovered that CTCF directly binds to RAD21-SA2 interface where WAPL also competes for binding to, counteracting WAPL-mediated cohesin unloading (Model. 11A) (Li et al., 2020). Given that ESCO1 and CTCF act together in the stabilization of cohesinSA1, one could envision that ESCO1-mediated cohesin’s acetylation facilitates CTCF-dependent stabilization of cohesin. In addition, PDS5A/B have also been shown to regulate anchoring cohesin at CTCF sites, and ESCO1 is recruited to cohesin through the interaction with PDS5A/B (Minamino et al., 2015; Wutz et al., 2017). Moreover, immunofluorescence experiments have shown that depletion of either CTCF, PDS5A/B or ESCO1 in WAPL-depleted cells aggravates the vermicelli phenotype (Busslinger et al., 2017; Kawasumi et al., 2017; Wutz et al., 2017). If the aggravated vermicelli phenotype is reflecting mis-localization of cohesin and altered loop formation, it may suggest that PDS5A/B-ESCO1-CTCF axis is a key pathway for the regulation of cohesin binding at CTCF sites (Model. 11A).

The fact that we observed a clear vermicelli phenotype only when ESCO1/2 and WAPL were simultaneously depleted suggest that ESCO2 also plays a role in interphase chromatin organization, similar to and jointly with ESCO1 (Kawasumi et al., 2017). Because ESCO2-mediated acetylation of cohesin does not seem to be PDS5 dependent, it may hint that PDS5A/B’s role might not be in the recruitment of ESCO1, but PDS5A/B per se might be targeted by ESCO1/2 for its role in regulating cohesin’s localization at CTCF sites (Minamino et al., 2015; Wutz et al., 2017). Importantly,
turnover of PDS5 has been shown to increase upon Eco1 inactivation in budding yeast, supporting the idea that CoATs (ESCO1/2) may target PDS5 for stabilizing its association with cohesin (Chan et al., 2012). Altogether, it is tempting to speculate that ESCO1/2 and PDS5A/B interdependently control cohesin’s stable binding to CTCF sites.

Model 11. ESCO1/2’s roles in chromatin organization. (A) Left panel: Schematic representation of cohesin’s interaction network. Right panel: Enlarged image of SA2-RAD21-CTCF interface (PDB: 6QNX). (B) Hypothetical model of loop formation and stabilization by cohesin regulatory factors.
Pds5 has been shown to compete with Scc2\textsuperscript{NIPBL} for binding of Rad21 in budding yeast (Petela \textit{et al.}, 2018). The authors also showed that Scc2 is replaced by Pds5 after cohesin is loaded onto DNA (Model. 11A). Additionally, in vitro experiments have revealed that Pds5 inhibits cohesin’s ATPase activity stimulated by DNA and Scc2 (Murayama and Uhlmann, 2015; Petela \textit{et al.}, 2018). Since the ATPase activity is critical for both loading and loop extrusion, replacement of Scc2 by Pds5 could be a key step for switching cohesin’s biochemical characteristics. If the acetylation of cohesin enhances the interaction between Pds5 and Rad21, its loss may strengthen the interaction of Rad21 with Scc2 that leads to excessive cohesin loading. This potentially explains the reason why we observed increased amount of chromatin bound cohesin in \textit{esco1 esco2-aid wapl-aid} triple conditional mutant (Kawasumi \textit{et al.}, 2017).

Supporting this idea, it has been shown that PDS5A/B depletion increases the cohesin amount on chromatin in G1 phase (Wutz \textit{et al.}, 2017).

Although topologically bound cohesin can translocate passively with the transcription machinery, cohesin can also translocate actively and catalyze loop extrusion in NIPBL and ATP dependent manner (Model. 11B) (Davidson \textit{et al.}, 2019). Since Pds5 can replace Scc2 after loading of cohesin, it is possible that the loop extrusion activity is in fact negatively regulated by Pds5. In this scenario, Pds5 should inhibit loop expansion. Indeed, a recent study in in budding yeast has demonstrated that Pds5 depletion results in increased loop length more than \textit{WAPL} knock out (Dauban \textit{et al.}, 2020). Interestingly, the authors further showed that Eco1 depletion also results in increased loop length, and concomitant depletion of Wap1 and Eco1 resembled Pds5 depletion. These observations further support the hypothesis that CoATs regulate cohesin’s loop extrusion activity in association with PDS5 (Model. 11B).
Taken together, although we still do not know the mechanism, it is apparent that CoATs (ESCO1/2) and PDS5 are key factors for cohesin mediated chromatin organization, in cooperation with CTCF in case of higher eukaryotes.

4.4. CTF18’s role in SCC establishment

We have investigated the genetic interaction between DDX11 and CTF18. Strikingly, we revealed that the synthetic lethality of ctf18 and ddx11 can be rescued by WAPL depletion, but not by restoring the level of SMC3’s acetylation level through overexpressing either ESCO1 or ESCO2 (Figs. 20D, 20E, 21B). Whereas, ESCO1 overexpression rescues the synthetic lethality caused by concomitant inactivation of ESCO2 and DDX11 (Kawasumi et al., 2017). Therefore, although Ctf18 has been hypothesized to promote SCC by loading PCNA to recruit Eco1 to replication forks, our data raised the possibility that CTF18 may contribute to SCC independently of ESCO1/2 (Model. 12A) (Liu et al., 2020).

Recent study has reported that ELG1 deletion rescues the SCC defect in CTF18 knock out cells in budding yeast, suggesting that Ctf18’s function for promoting SCC is via PCNA loading activity (Model. 12A) (Liu et al., 2020). In fact, this was the reason why CTF18’s role in SCC establishment is thought be Eco1 dependent, since Eco1 has a PIP-box that is crucial for its function (Moldovan, Pfander and Jentsch, 2006). Combining with our data, one could envision that PCNA loaded by CTF18 may enhance the function of other proteins involved in SCC establishment, but not of Eco1. Which protein could be the candidate for that? Good candidates should be factors relevant for SCC establishment. Intriguingly, of cohesin related genes, NIPBL and PDS5A are found to have potential PIP-box (Model. 12B) (Kumar et al., 2019). Thus, testing whether those potential PIP-boxes contribute to the establishment of SCC will be an exciting question to be asked.
Although Ctf18 is shown to play a role in SCC establishment, it has also been shown that Ctf18 is required for efficient intra-S checkpoint activation (Crabbé et al., 2010; Kubota et al., 2011; García-Rodriguez et al., 2015). In fact, CTF18 deletion elicits late-origin firing, which is generally repressed by the intra-S checkpoint in the presence of HU-induced replication stress, reminiscent of MRC1 deletion (Crabbé et al., 2010; Kubota et al., 2011). However, whether the intra-S checkpoint plays a role in SCC establishment is not well understood.

Interestingly, recent studies have proposed that replication stress induces WAPL-mediated cohesin removal from stalled replication forks to facilitate efficient recovery (Model. 12C) (Benedict et al., 2020). The authors also proposed that WAPL-mediated cohesin removal upon replication stress might be a cause of SCC defects (Model. 12C) (Masamsetti et al., 2019; Benedict et al., 2020). Moreover, it has been shown that accumulation of cohesin can interfere with replication fork progression, further
supporting the idea that cohesin needs to be mobilized or unloaded under replication stress (Carvajal-Maldonado et al., 2019; Morales et al., 2020). To be noted, although whether CTF18 plays a role in intra-S checkpoint is not fully understood in mammalian cells, it has been shown that CTF18 is critical for CDT2-dependent CDT1 degradation to prevent re-replication in unperturbed condition (Shiomi et al., 2012). Therefore, loss of CTF18 may induce replication stress by excess origin firing which may lead to WAPL-mediated cohesin unloading (Model. 12C). If the SCC defect observed in CTF18 deleted cells is in fact the consequence of replication stress induced cohesin removal by WAPL, similar outcomes should be expected upon inhibition of key factors for intra-S checkpoint such as CHK1 and ATR in DDX11 knock out background. The fact that CLASPIN is required for both SCC and S-phase checkpoint similar to CTF18 further supports this idea (Alcasabas et al., 2001; Tanaka and Russell, 2001; Xu, Boone and Klein, 2004; Xu, Boone and Brown, 2007; Smith-Roe et al., 2011). Whether the SCC defects associated with CLASPIN mutation or concomitant dysfunction in DDX11 and CLASPIN can be suppressed by WAPL removal is testable and will provide more insights on the observed synthetic lethality and the relationship to specific types of replication stress.

4.5. RUVBL1 and RUVBL2 in cohesin regulation

Our IP-MS experiments have successfully identified novel cohesin interactors, RUVBL1 and RUVBL2, which are known to play multiple roles in various cellular pathways (Fig. 22C). Of those, its roles as part of INO80 remodeling complex might be relevant for its function in cohesin regulation. Supporting the idea that RUVBL1 may be a regulator of cohesin, we have found that the depletion of RUVBL1 results in SCC defects (Figs. 25D, 25H). Besides, in budding yeast, it has been reported that INO80 deletion causes SCC defects (Ogiwara, Enomoto and Seki, 2007). As several
chromatin remodelers, including the RSC complex, have been implicated in cohesin loading, RUVBL1/2 may also facilitate cohesin loading as part of the INO80 complex (Model. 13) (Hakimi et al., 2002; Brownlee et al., 2014; Lopez-Serra et al., 2014; Garcia-Luis et al., 2019; Muñoz et al., 2019). However, our data indicated that the amount of chromatin bound cohesin is barely reduced, despite the fact that RUVBL1 depletion causes SCC defects (Figs. 27A, 27B). Consistently, INO80 deletion in budding yeast does not reduce the amount of chromatin bound cohesin (Ogiwara, Enomoto and Seki, 2007). Instead, the authors found that the recruitment of Ctf18 to replisome was attenuated, partially explaining the underlying mechanism of SCC defects observed in INO80 knock out cells. Nevertheless, the fact that RUVBL1 depletion does not reduce cohesin amount on chromatin despite the SCC defects may indicate that RUVBL1 regulates cohesin at specific loci that are crucial for SCC. One such region could be the centromere (Model. 13). Because cohesin loading is preferentially taking place at nucleosome-free regions that are created by chromatin remodelers in vitro, it will be interesting to assess the nucleosome occupancy in aid-ruvbl1 conditional mutant (Kanke et al., 2016; Muñoz et al., 2019). In addition, since some of the remodelers could function as receptors of the cohesin loader, it will be
worth checking whether there is a physical interaction between RUVBL1/2 and the cohesin loader.
5. MATERIALS AND METHODS

5.1. General buffer compositions

**PBS:**
- 137 mM NaCl
- 2.7 mM KCl
- 8 mM Na$_2$HPO$_4$
- 2 mM KH$_2$PO$_4$

**PBS-T:**
- 137 mM NaCl
- 2.7 mM KCl
- 8 mM Na$_2$HPO$_4$
- 2 mM KH$_2$PO$_4$
- 0.05% Tween 20 (v/v)

**4x Laemmli buffer:**
- 40% Glycerol (v/v)
- 200 mM Tris-HCl pH 6.8
- 8% SDS (w/v)
- 200 mM dithiothreitol (DTT)
- 0.1% bromophenol blue (BPB)

**1x Transfer buffer:**
- 196 mM Glycine
- 25 mM Tris-HCl pH 8.6
- 20% MeOH (v/v)
- 0.1% SDS (w/v)

**CSK buffer:**
- 10 mM PIPES-KOH pH 6.8
- 100 mM NaCl
300 mM Sucrose
1 mM EGTA
1 mM MgCl₂
0.3% Triton X-100 (v/v)
1x Complete protease inhibitor cocktail (Roche)
1 mM phenylmethylsulfonyl fluoride (PMSF)
*DTT, Complete, PMSF were added immediately before using as they were required.

5.2. Cell line list

<table>
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<tr>
<th>No.</th>
<th>Genotype</th>
<th>Detail</th>
<th>Reference</th>
</tr>
</thead>
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<td>WT</td>
<td>DT40 CL18</td>
<td>(Buerstedde and Takeda 1991)</td>
</tr>
<tr>
<td>#262</td>
<td>esco2-W615G ddx11 +tetoff-DDX11</td>
<td>ESCO2(KO-Bsr/KI-W615G-Puro), DDX11(KO-Bleo/KO-Hyg), +pTRE-GgDDX11-HA(Eco), Tet-off(Neo)</td>
<td>(Abe et al. 2016)</td>
</tr>
<tr>
<td>#483</td>
<td>esco1</td>
<td>ESCO1(KO/KO/KO), +Cre(Neo)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>#521</td>
<td>esco2</td>
<td>ESCO2(KO-Puro/KO), +Cre(Neo)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>#525</td>
<td>esco1</td>
<td>ESCO1(KO/KO/KO), ESCO2(KO-Puro/cFLP-3mAID6FLAG-His), +Cre(Neo), +pBACT-OsTIR1-9Myc(Bsr)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>#526</td>
<td>esco2-aid</td>
<td>ESCO1(KO/KO/KO), ESCO2(KO-Puro/cFLP-3mAID6FLAG-His), +Cre(Neo), +pBACT-OsTIR1-9Myc(Bsr)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>#540</td>
<td>esco1-aid wap1-aid</td>
<td>ESCO1(KO/KO/KO), ESCO2(KO-Puro/cFLP-3mAID6FLAG-His), WAPL(KO-Eco/cFLP-3mAID6FLAG-Bleo), +Cre(Neo), +pBACT-OsTIR1-9Myc(Bsr)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>#546</td>
<td>ctf18-aid</td>
<td>CTF18(cFLP-3mAID6HA-Bleo /cFLP-3mAID6FLAG-His), +pBACT-OsTIR1-9Myc(Bsr)</td>
<td>Lab collection</td>
</tr>
<tr>
<td>#548</td>
<td>wap1-aid</td>
<td>WAPL(KO-His/cFLP-3mAID6FLAG-Bleo), pBACT-OsTIR1-9Myc(Bsr)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>#</td>
<td>Description</td>
<td>Details</td>
<td>Lab Reference</td>
</tr>
<tr>
<td>----</td>
<td>------------------------------------------</td>
<td>-------------------------------------------------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>#558</td>
<td>ctf18-aid ddx11</td>
<td>CTF18(cFLP-3mAID6HA-Bleo/cFLP-3mAID6FLAG-His), DDX11(KO-Hyg/KO-Puro), +pBACT-OsTIR1-9Myc(Bsr)</td>
<td></td>
</tr>
<tr>
<td>#563</td>
<td>esco2 +ESCO1</td>
<td>ESCO2(KO-Puro/KO), +pBACT-GgESCO1-FLAG(His), +Cre(Neo)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>#564</td>
<td>esco2-W615G ddx11 +tetoff-DDX11 +ESCO1</td>
<td>ESCO2(KO-Bsr/KI-W615G-Puro), DDX11(KO-Bleo/KO-Hyg), +pTRE-GgDDX11-HA(Eco), Tet-off(Neo), pBACT-GgESCO1-FLAG(His)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>#573</td>
<td>RAD21-MFH</td>
<td>RAD21(+/+/cFLP-Myc-FLAG-HA-Bleo)</td>
<td>This study</td>
</tr>
<tr>
<td>#578</td>
<td>esco1 esco2-aid smc3-QQ</td>
<td>ESCO1(KO/KO/KO), ESCO2(KO-Puro/cFLP-3mAID6FLAG-His), SMC3(KO-Eco/KI-K105Q,K106Q), +Cre(Neo), +pBACT-OsTIR1-9Myc(Bsr)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>#583</td>
<td>smc3-aid</td>
<td>SMC3(KO-His/cFLP-3mAID6FLAG-pRSV-OsTIR1-9Myc-Bsr)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>#590</td>
<td>smc3-aid</td>
<td>SMC3(KO-His/cFLP-3mAID6FLAG-pRSV-OsTIR1-9Myc-Bsr)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>R002</td>
<td>ruvbl1-aid</td>
<td>RUVBL1(KO-Puro/cFLP-3mAID6FLAG-His), pBACT-OsTIR1-9Myc(Bsr)</td>
<td>This study</td>
</tr>
<tr>
<td>R003</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R011</td>
<td>smc3-aid</td>
<td>SMC3(KO-His/cFLP-3mAID6FLAG-pRSV-OsTIR1-9Myc-Bsr), +pBACT-GgSMC3- HA- K105R,K106R(Neo)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>R012</td>
<td>+SMC3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R013</td>
<td>smc3-aid</td>
<td>SMC3(KO-His/cFLP-3mAID6FLAG-pRSV-OsTIR1-9Myc-Bsr), +pBACT-GgSMC3- HA- K105Q,K106Q(Neo)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>R014</td>
<td>+SMC3-RR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R015</td>
<td>smc3-aid</td>
<td>SMC3(KO-His/cFLP-3mAID6FLAG-pRSV-OsTIR1-9Myc-Bsr), +pBACT-GgSMC3- HA- K105Q,K106Q(Neo)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>R016</td>
<td>+SMC3-QQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R029</td>
<td>smc3-aid</td>
<td>SMC3(KO-His/cFLP-3mAID6FLAG-pRSV-OsTIR1-9Myc-Bsr), +pBACT-GgSMC3- HA- K105A,K106A(Neo)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>R030</td>
<td>+SMC3-AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R051</td>
<td>RUVBL1-MFH</td>
<td>RUVBL1(cFLP-Myc-FLAG-HA-Bleo/cFLP-Myc-FLAG-HA-His)</td>
<td>This study</td>
</tr>
<tr>
<td>R055</td>
<td>smc3-aid</td>
<td>SMC3(KO-His/cFLP-3mAID6FLAG-pRSV-OsTIR1-9Myc-Bsr), +pBACT-GgSMC3- HA- K38I(Neo)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>R056</td>
<td>+SMC3-K38I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R059</td>
<td>smc3-aid</td>
<td>SMC3(KO-His/cFLP-3mAID6FLAG-pRSV-OsTIR1-9Myc-Bsr), +pBACT-GgSMC3- HA- K105Q,K106Q, K140Q,K119Q( Neo)</td>
<td>This study</td>
</tr>
<tr>
<td>R060</td>
<td>+SMC3-4KQ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R061 R062</td>
<td>smc3-aid +SMC3-4KR</td>
<td>SMC3(KO- His/cFLP-3mAID6FLAG -pRSV- OsTIR1-9Myc-Bsr), +pBACT-GgSMC3-HA-K105R,K106R, K140R,K1190R(Neo)</td>
<td>This study</td>
</tr>
<tr>
<td>R103</td>
<td>smc3-aid wapl-aid</td>
<td>SMC3(KO- His/cFLP-3mAID6FLAG -pRSV- OsTIR1-9Myc-Bsr), WAPL(KO- Eco/cFLP-3mAID6FLAG-Bleo), +Cre(Neo), +pBACT-OsTIR1-9Myc(Bsr)</td>
<td>(Kawasumi et al., 2017)</td>
</tr>
<tr>
<td>R139 R140</td>
<td>esco1 esco2-aid smc3QQ-wapl-aid</td>
<td>ESCO1(KO/KO/KO), ESCO2(KO-Puro/cFLP-3mAID6FLAG-His), SMC3(KO- Eco/K1-K105Q,K106Q), WAPL(KO-Hyg/cFLP-3mAID6FLAG-Bleo), +Cre(Neo), +pBACT-OsTIR1-9Myc(Bsr)</td>
<td>This study</td>
</tr>
<tr>
<td>R149</td>
<td>smc3-aid wapl-aid +SMC3</td>
<td>SMC3(KO- His/cFLP-3mAID6FLAG -pRSV- OsTIR1-9Myc-Bsr), WAPL(KO- Eco/cFLP-3mAID6FLAG-Bleo), +pBACT-GgSMC3-HA(Puro)</td>
<td>This study</td>
</tr>
<tr>
<td>R153</td>
<td>smc3-aid wapl-aid +SMC3-QQ</td>
<td>SMC3(KO- His/cFLP-3mAID6FLAG -pRSV- OsTIR1-9Myc-Bsr), WAPL(KO- Eco/cFLP-3mAID6FLAG-Bleo), +pBACT-GgSMC3-HA-K105Q,K106Q(Puro)</td>
<td>This study</td>
</tr>
<tr>
<td>R155</td>
<td>smc3-aid wapl-aid +SMC3-RR</td>
<td>SMC3(KO- His/cFLP-3mAID6FLAG -pRSV- OsTIR1-9Myc-Bsr), WAPL(KO- Eco/cFLP-3mAID6FLAG-Bleo), +pBACT-GgSMC3-HA-K105R,K106R(Puro)</td>
<td>This study</td>
</tr>
<tr>
<td>R157</td>
<td>smc3-aid wapl-aid +SMC3-AA</td>
<td>SMC3(KO- His/cFLP-3mAID6FLAG -pRSV- OsTIR1-9Myc-Bsr), WAPL(KO- Eco/cFLP-3mAID6FLAG-Bleo), +pBACT-GgSMC3-HA-K105A,K106A(Puro)</td>
<td>This study</td>
</tr>
<tr>
<td>R171 R172</td>
<td>esco1 esco2-aid smc3QQ+wapl-aid</td>
<td>ESCO1(KO/KO/KO), ESCO2(KO-Puro/cFLP-3mAID6FLAG-His), SMC3(KO- Eco/K1-K105Q,K106Q), WAPL(KO-Hyg/cFLP-3mAID6FLAG-Bleo), +Cre(Neo), +pBACT-OsTIR1-9Myc(Bsr)</td>
<td>This study</td>
</tr>
<tr>
<td>R201 R202</td>
<td>esco1 esco2-aid smc3+/wapl-aid</td>
<td>ESCO1(KO/KO/KO), ESCO2(KO-Puro/cFLP-3mAID6FLAG-His), SMC3(KO-Puro/cFLP-3mAID6FLAG-His), WAPL(KO- Eco/cFLP-3mAID6FLAG-Bleo), +Cre(Neo), +pBACT-OsTIR1-9Myc(Bsr)</td>
<td>This study</td>
</tr>
<tr>
<td>R259</td>
<td>ruvbl1-aid wapl-aid</td>
<td>RUVBL1(KO-Puro/cFLP-3mAID6FLAG-Eco), WAPL(KO- His/cFLP-3mAID6FLAG-Bleo), +pBACT-OsTIR1-9Myc(Bsr)</td>
<td>This study</td>
</tr>
</tbody>
</table>
| R269  | ctf18-aid  
ddx11  
wapl-aid | CTF18(cFLP-3mAID6HA-Bleo  
cFLP-3mAID6FLAG-His),  
DDX11(KO-Hyg/KO-Puro),  
WAPL(KO-Neo/cFLP-3mAID6FLAG- 
Eco),  
+pBACT-OsTIR1-9Myc(Bsr) | Lab collection |
|-------|-----------------------------|------------------------|
| R270  | esco1  
esco2-aid    
wapl-aid   
SMC2-6HA  | ESCO1(KO/KO/KO),  
ESCO2(KO-Puro/cFLP-3mAID6FLAG- 
His),  
WAPL(KO/cFLP-3mAID6FLAG-Bleo),  
SMC2(+/cFLP-6HA-Eco),  
+Cre(Neo),  
+pBACT-OsTIR1-9Myc(Bsr) | This study |
| R283  | esco1  
esco2-aid    
wapl-aid   
SMC2-6HA  | ESCO1(KO/KO/KO),  
ESCO2(KO-Puro/cFLP-3mAID6FLAG- 
His),  
WAPL(KO/cFLP-3mAID6FLAG-Bleo),  
SMC2(+/cFLP-6HA-Eco),  
+Cre(Neo),  
+pBACT-OsTIR1-9Myc(Bsr) | This study |
| R285  | SMC2-6HA  | SMC2(+/cFLP-6HA-Eco) | This study |
| R287  | wapl-aid    
SMC2-6HA  | WAPL(KO-Neo/cFLP-3mAID6FLAG- 
Bleo),  
SMC2(+/cFLP-6HA-Eco),  
pBACT-OsTIR1-9Myc(Bsr) | This study |
| R298  | ctf18-aid  
ddx11  
wapl-aid   
+WAPL   | CTF18(cFLP-3mAID6HA-Bleo  
cFLP-3mAID6FLAG-His),  
DDX11(KO/KO-Puro),  
WAPL(KO-Neo/cFLP-3mAID6FLAG),  
+pRSV-GgWAPL-HA(Eco),  
+pBACT-OsTIR1-9Myc(Bsr) | Lab collection |
| R299  | wapl-aid    
SMC2-6HA  | WAPL(KO-Neo/cFLP-3mAID6FLAG- 
Bleo),  
SMC2(+/cFLP-6HA-Eco),  
pBACT-OsTIR1-9Myc(Bsr) | This study |
| R320  | esco1  
esco2-aid    
wapl-aid   
+ESCO2  | ESCO1(KO/KO/KO),  
ESCO2(KO-Puro/cFLP-3mAID6FLAG- 
His),  
WAPL(KO/cFLP-3mAID6FLAG-Bleo),  
+Cre(Neo),  
+pBACT-OsTIR1-9Myc(Bsr) | This study |
| R321  | wapl-aid    
SMC2-6HA  | WAPL(KO-Neo/cFLP-3mAID6FLAG- 
Bleo),  
SMC2(+/cFLP-6HA-Eco),  
pBACT-OsTIR1-9Myc(Bsr) | This study |
| R346  | esco1  
wapl-aid    | ESCO1(KO-Puro/KO-Puro),  
WAPL(KO-Hyg/cFLP-3mAID6FLAG- 
Bleo),  
+pRSV-OsTIR1-9Myc(Neo) | This study |
| R355  | esco2  
wapl-aid    | ESCO2(KO-Puro/KO-Puro),  
WAPL(KO-Hyg/cFLP-3mAID6FLAG- 
Bleo),  
+pRSV-OsTIR1-9Myc(Neo) | This study |
| R372  | aid-ruvbl1  | RUVBL1(KO-Puro/nFLP-pRSV-3mAID- 
His),  
pRSV-OsTIR1-9Myc(Neo) | This study |
| R404  | ctf18-aid  
ddx11  
ESCO2    | CTF18(cFLP-3mAID6HA-Bleo  
cFLP-3mAID6FLAG-His),  
DDX11(KO-Hyg/KO-Puro),  
+pRSV-GgESCO2(Eco),  
+pBACT-OsTIR1-9Myc(Bsr) | This study |
| R405  | ctf18-aid  
ddx11  
ESCO1    | CTF18(cFLP-3mAID6HA-Bleo  
cFLP-3mAID6FLAG-His),  
DDX11(KO-Hyg/KO-Puro),  
+pBACT-GgESCO1-FLAG(Neo),  
+pBACT-OsTIR1-9Myc(Bsr) | This study |
5.3. **Cell culture**

Cells were cultured at 39.5°C in D-MEM/F-12 GlutaMAX supplement (Gibco) supplemented with 10% fetal bovine serum, 2% chicken serum (Sigma), Penicillin/Streptomycin mix, and 10 µM 2-mercaptoethanol (Gibco). 500 µM Auxin or 1 µg/mL Doxycycline was added when required.

To plot growth curves, cells were cultured in 24 well-plate and passaged every 12 h or 24 h. Cell density was determined by FACS analysis at each time point.

5.4. **Transfection and Selection**

1 x 10^7 cells were collected and resuspended with 700 µL of medium containing 30 µg of linearized required constructs. Resuspended cells were transferred to 4 mm cuvette (BioRad), then cells were electroporated using Gene Pulser (700 V, 25 µF) (BioRad). After electroporation, cells were immediately transferred to pre-warmed 15 mL of medium in 10 cm dishes. After over-night incubation at 39.5°C, cells were diluted with 45 mL of medium and appropriate reagents for selection (see the list below) were added. Then, cells were dispensed to 96-well plates 150 µL each. 5-10 days after selection, appeared colonies were picked up into 24-well plates filled with 1 mL of medium without selection reagents. After cells became confluent (about 1 x 10^6 cells/mL), 900 µL of cells were used for genomic DNA isolation for PCR check or for Western blotting sample preparation. Remaining cells were diluted with 900 µL of medium for further expansion.

- **Selection reagents list**-
  
Puromycin (final: 500 ng/mL, Sigma), Blastcidin S (final: 30 µg/mL, Thermo), Neomycin (final: 1 mg/mL, Thermo), Histidinol D (final: 1mgmL, Sigma), Hygromycin B (final: 2.5 mg/mL, Invitrogen), Bleomycin (final: 1 mg/mL, InvivoGen), Mycophenolic acid (final: 10 µg/mL, Sigma)
5.5. **Plasmid construction**

**ESCO1 knock out construct**

ESCO1 KO-Bsr, ESCO1 KO-Puro and ESCO1 KO-His were generated from genomic PCR products combined with Blasticidin S, Puromycin and Histidinol D selection marker cassettes. Genomic DNA sequences were amplified using primers 5’-CTATAGGGCGAATTGGAGCTGCTTTGCGTAAGACTTGTGATCG-3’ and 5’-GCCGCCACCGCGGTGGAGCTGTTCTCGAGAATTGAGCGGAG-3’ (for the right arm of the KO construct); and 5’-CTCGAGGGGGGGCCCGGTACCTCCATCTGTACTGGGAGACTTCTG-3’ and 5’-AAGGGAACAAAAAGCTGGTACCGTAGAGATCGGAGAGCTCGAG-3’ (for the left arm of the KO construct). Amplified PCR products were purified by gel extraction and cloned into pre-cut pLoxP vectors (SacI and KpnI) by using GeneArt Seamless PLUS Cloning and Assembly Kit (invitrogen) according to the manufacturer’s protocol.

**WAPL knock out construct**

WAPL KO vectors were generated from genomic PCR products combined with mycophenolic acid and Histidinol D selection marker cassettes. Left arm and right arm of WAPL KO vectors were amplified using the primers 5’-AGTGAGCTCCCAGTGTCCCAGTAAGTAGTCTG-3’ (SacI) and 5’-AGTGCGGCGGAGCAGCTTTAAGACTCCATCAT-3’ (NotI) (for the left arm of the KO construct); and 5’- AGTATCGATACGGTGTGAGTAGAATCGAGGCACGAG-3’ (Clal) and 5’- AGTGGTACCGTAGCTCTTGACAGATCTC-3’ (KpnI) (for the right arm of the KO construct). Amplified PCR products were cut and cloned into pLoxP vectors using attached restriction sites.
**SMC3 knock out construct**

Left arm and right arm of SMC3 KO vectors were amplified using the primers 5’-CCTCTCCTTGCTCTGCTATAGGACGATGC-3’ and 5’-GAGGATCCGCCACCCCGACCCTATCAGTAC-3’ (BamHI) (for the left arm of the KO construct); and 5’- GGATCCTTAAACAAGGTATGGTCGTGACCTTGAC-3’ (BamHI) and 5’- GGTCAGAAGAACCCTTCAGAGAGGAATCC-3’ (for the right arm of the KO construct), and cloned into vector with a Histidinol D marker using attached restriction sites.

**RUVBL1 knock out construct**

Left arm and right arm of RUVBL1 KO vectors were amplified using the primers 5’-GCATGAGCTCGGTGGCATGGGCAAAGGAGAAAGGTGAC-3’ (SacI) and 5’-GCATACTAGTCTCATGTAACAGCACTCCCACCAGGGAATCC-3’ (SpeI) (for the left arm of the KO construct); and 5’- GCATATCGATGAGACCAAGGAGGTGTATGAAGGAGAAG-3’ (ClaI) and 5’- GCATGGTACCGTTCAGACATCCACTCCCACCTGCTTAGCAA-3’ (KpnI) (for the right arm of the KO construct), and cloned into vector with a Puromycin marker using attached restriction sites.

**3mxAID-6xFLAG FLP-In at C-termini of WAPL, ESCO2, SMC3, RUVBL1**

To add 3xmAID-6xFLAG tag by FLP-In system, we generated p3xmAID-6xFLAG vectors by combining pHyg-AID*-6xFLAG, pTRE2-hygro vector (Clontech) and pLoxP vectors. 2-3 kb upstream DNA sequences of stop codons of WAPL, ESCO2, SMC3 and RUVBL1 were amplified with the primers 5’-AAGTGTCGACAGCCAAAGGAGACCTGGGAATTGG-3’ (Sall) and 5’-
GATCAGGTGTTTCAAGTTTCAATCCACCATAG-3’ (NheI) (for WAPL); and 5’- CTGACAGCTCAGTGCACCACAGTCATAC-3’ (SalI) and 5’- TTTACTAGTTTGCATAAAACAAAGTTTGAG-3’ (NheI) (for ESCO2); and 5’- TTTGCTAGCACCATGCGTGTGTCATCCTCTACAAAAG-3’ (SalI) and 5’- TTTGCTAGCACCATGCGTGTGTCATCCTCTACAAAAG-3’ (NheI) (for SMC3); and 5’- AGTCGTCAGCATTGGGTCTCCCATTTTCTACAAAAG-3’ (SalI) and 5’- AGTCGTCAGCATTGGGTCTCCCATTTTCTACAAAAG-3’ (NheI) (for RUVBL1). These amplified DNA fragments were cloned into p3xmAID-6xFLAG vectors at SalI and NheI restriction enzyme sites containing appropriate selection marker cassette. The FLP-In vectors were then linearized at one restriction enzyme site in the middle of the homology region prior to transfection.

**pRSV-3xmAID FLP-In at N-terminus of RUVBL1**

2 kb downstream of RUVBL1 start codon was amplified by PCR using primers, 5’-GCATGATATCAAGATCGAGGAGGTGAAGAGCA-3’ (EcoRV) and 5’-GCATGCTAGCCTAGAGGATGCTGCCAGTGTTAATG-3’ (NheI), then digested with EcoRV and NheI. Fragment was cloned into p3xmAID-6xFLAG containing Histidinol D marker pre-digested with SmaI and SpeI. The FLP-In vector was linearized by SacI prior to transfection.

**6xHA FLP-In at C-terminus of SMC2**

To add 6xHA tag to SMC2, we first amplified 6xHA sequence from p3xmAID-6xHA with primers 5’-AAAAGCGGCCGCTATCACCCGAAATACCGAC-3’ (NotI) and 5’-TTTGGCAGAGGGAAAAAGATCTCAGTGG-3’ (BglII). Then the fragment digested by NotI and BglII was cloned into p9xMyc pre-digested with NotI and BglII (thereafter referred as p6xHA). Subsequently, 2 kb upstream of SMC2 stop
codon was amplified using primers 5’-GTCGTCGACTTTGGTCCATCTTCTCGATGCTTCTACC-3’ (Sall) and 5’-GTCGCTAGCCTCATCCTTTGGTTGGCTGGCTCAC-3’ (NheI). The PCR product was digested with Sall and NheI and cloned into p6xHA containing mycophenolic acid resistant marker (Eco) pre-cut with Sall and NheI.

**SMC3, ESCO1, ESCO2 cDNA cloning**

For SMC3 and ESCO1, ESCO2 cDNA cloning, we used primers 5’-TAGACGCGTATGTACATCAAGCAGGTAATCATTCAGGG-3’ (MluI) and 5’-TAGGCTAGCTTAAGCCTGAGCTCAGAAAGGAAATGTCTGC-3’ (NotI) and 5’-AGTGCGGCCACCATGCGTGTTCCTTTTGATTCCATAGCCTGGTGC TGCCCGTTGAGGAAG-3’ (NotI + FLAG) (for ESCO1 cDNA); and 5’-GACTGCTAGCCACCACATGTATCCATATGACGTTCCAGATTACGCTGCAGCT GTGAGCTCGCAGAA-3’ (NheI + kozack + HA) and 5’-GACTGGCCGCCTCAAGTGCATCGGTTCAGGCTCCAGAAGGAAATGTCTGCAGCTGTGAGCTCGCAGAA-3’ (AscI) (for ESCO2 cDNA). For SMC3 and ESCO1, after digesting with indicated restriction enzymes, the PCR products were cloned into pBACT vector. In case of ESCO2, the PCR product was cloned into pRSV-9xMyc after restriction enzyme digestion.

**Site directed mutagenesis on SMC3**

To make SMC3 point mutants (SMC3 K38I, K105 106Q, K105 106R, K105 106A, K140R, K1190R), we used WT SMC3 cDNA (pBACT-SMC3-HA) as a template and inserted mutations using the primers 5’-ATCAGGAATAAGCACAATTCTTTACGCAAT-3’ and 5’-
AGTTGCTTATTCCTGATCCATT TTCCTTCCCA-3’ (for K38I); and 5’-
CAGGACCAATATTTCCTTAGACAAGAAAATGGTGAC-3’ and 5’-
CTGGGCTCCAATGACTCTGCGAAGTGAGACTTC-3’ (for K105Q, K106Q); and
5’-AGGGACCAATATTTCCTTAGACAAGAAAATGGTGAC-3’ and 5’-
CCTGGCTCCAATGACTCTGCGAAGTGAGACTTC-3’ (for K105R, K106R); and
5’-GCGGCGGACCAATATTTCCTTAGACAAGAAAATGGTGACG-3’ and 5’-
GGCTCCAATGACTCTGCGAAGTGAGACTTC-3’ (for K105A, K106A); and 5’-
AGACAAGGGAAGATCAAACCAGATGGCCACACGC-3’ and 5’-
GACAATATAATATGGGATTACTGCGAGAAAATC-3’ (for K140R); and 5’-
AGATTCAGAAATAAGGTTAGTCATATTGTGATGATC-3’ and 5’-
TACACCATAGACTTTGTCAGCCGATTC-3’ (for K1190R). After PCR, they were
treated with DpnI for digesting template plasmid DNA for overnight at 37 °C. PCR
products were then purified by gel extraction, and self-ligated by Quick Ligation Kit
(NEB) according the manufacturer’s protocol.

**SMC3-K105, 106Q knock in construct**

To establish the SMC3-K105Q, K106Q (QQ) Knock-In construct, 2 kb of SMC3
homology sequence with K105Q, K106Q mutation and BamHI site on the center of
the homology sequence was synthesized, Bleo marker cassette was inserted to the
BamHI site, and the left arm was extended by a DNA fragment amplified using the
primers 5’-AAAGGTACCTGAATACAGTGCAGGTAGAGAATTTC
(KpnI) and 5’-AAAGGTACCGTCAATATCTTATTTGCTCATTGTGATGAC-3’ (KpnI).

**5.6. PCR**

PCR reaction was performed using PrimeSTAR GXL DNA polymerase (TaKaRa)
according the manufacturer’s protocol. Detailed condition is described below.
-50 µL scale (minimum 6 µL scale)-

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>32.5 µL</td>
</tr>
<tr>
<td>5x PrimeSTAR GXL buffer</td>
<td>10 µL</td>
</tr>
<tr>
<td>dNTP mix (2.5 mM each)</td>
<td>4 µL</td>
</tr>
<tr>
<td>Forward primer (10 µM)</td>
<td>1 µL</td>
</tr>
<tr>
<td>Reverse primer (10 µM)</td>
<td>1 µL</td>
</tr>
<tr>
<td>PrimeSTAR GXL</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Genomic DNA (100 ng/uL)</td>
<td>1 µL</td>
</tr>
</tbody>
</table>

- Reaction -

98 °C, 1 min : 1 cycle
98 °C, 15 sec – 68 °C, min/kbp : 35 cycle
68 °C, 2 min : 1 cycle
4 °C, continuous

5.7. Reverse transcription PCR

Total RNA isolation was performed using the TRIzol RNA Isolation Reagents kit (Thermo) according to the manufacturer’s protocol. Isolated RNA was used for cDNA preparation by SuperScript™ III Reverse Transcriptase (Thermo) according to the manufacturer’s protocol.

Primers list for knock out check

ESCO1 fw: 5’-ACGTAAGGAATGCTCTTGGCACAACCGTG-3’
ESCO1 rv: 5’-CTCTGTAGCCCCATTGGATATGTTCTGC-3’
ESCO2 fw: 5’-CTCAGTCGAACTCCGAGGTGC-3’
ESCO2 rv: 5’-GATCCTGCTGACACCACACACTGCAGGC-3’
5.8. Quantitative PCR

Quantitative PCR was performed using GoTaq qPCR Master Mix (Promega) according to the manufacturer’s protocol. Analysis was performed by LightCycler 96 Instrument (Roche).

Primers list for mRNA level check

ESCO1 fw: 5’-CAGAATGTCGCAGCGAAAGC-3’
ESCO1 rv: 5’-AGAGCTGGTCACTTCTACAC-3’
ESCO2 fw: 5’-GTGTGTTGGAAAGAAAGACGGG-3’
ESCO2 rv: 5’-CTTGCTTTGAAGCCCAGTTCATTAC-3’
ACTB fw: 5’-CGTGCTGTGTTCCCATCTATCGTG-3’
ACTB rv: 5’-TACCTCTTTTGCTTGGCTTCATC-3’

5.9. Genomic DNA isolation

1 x 10^6 cells were collected into 1.5 mL tube. Pelleted cells were resuspended with 10 µL of PrepMan Ultra Sample Preparation Reagent (Thermo). After resuspending completely by vortex, samples were incubated at 95°C for 10 min. Then, 70 µL of MQ was added. After vortex, samples were centrifuged at 14000 rpm, 4°C for 10 min and supernatant was transferred to new 1.5 mL tube.

5.10. Miniprep

Plasmid DNA was isolated from 1.5 mL of E. coli (DH5α) using Wizard Plus SV Minipreps DNA Purification System (Promega) according to the manufacturer’s protocol with small modification. Plasmid DNA was eluted with 100 µL of MQ water.
5.11. Midiprep

Plasmid DNA was isolated from 100 mL of *E. coli* (DH5α) using NucleoBond Xtra Midi (Macherey-Nagel) according to the manufacturer’s protocol.

5.12. Cell cycle analysis

Cells were centrifuged at 300 xg for 5 min and resuspended with remaining media after removing supernatant roughly. Cells were then fixed with 1 mL of 70% EtOH for more than 1 h at 4°C. After fixation, cells were centrifuged at 500 xg for 5 min and washed with PBS containing 1% of BSA. Cells were then resuspended with staining solution (PBS with 100 µg/mL RNase A, 50 µg/mL Propidium Iodide) for 1 h at 37°C. Stained samples were analyzed by flow cytometry (FACS Calibur, BD).

5.13. Western blotting analysis

Protein samples were boiled at 95 °C for 10 min with 1x Laemmli buffer. Samples were then run on NuPAGE™ 4-12% Bis-Tris Protein Gels (Invitrogen) for 1 h at 140 V. After running, proteins were transferred to 0.45 µm PVDF membrane (Amersham) in 1x transfer buffer on ice at 100 V for 90 min. Membranes were blocked with 5% skim milk in PBS-T for 30 min, then incubated with primary antibody diluted with PBS containing 1% BSA and 0.1% sodium azide for overnight. After washing with PBS-T for 10 min, membranes were incubated with secondary antibody diluted with PBS-T contain 1% skim milk for 40 min. Membranes were then washed with PBS-T for 10 min twice. Proteins were detected by either SuperSignal West Dura Extended Duration Substrate (Thermo) or SuperSignal West Femto Maximum Sensitivity Substrate using ChemiDoc Imaging Systems (BioRad).
-**Antibodies list**-

Histone H3 (1:2000, ab1791, Abcam), α-tubulin (1:3000, T5168, Sigma), FLAG (1:2500, F3165, Sigma), HA (1:500, 11867431001, Roche), miniAID (1:500, M214-3, MBL), GFP (1:1000, TP401, OriGene), acSMC3 (1:1000, kindly gifted by Dr. Katsuhiko Shirahige, IQB, the University of Tokyo), SMC3 (1:1000, kindly gifted by Dr. Ana Losada, Spanish National Cancer Research Centre [CNIO]), SMC3 (1:1000, 5696S, CST), RAD21 (1:1000, kindly gifted by Dr. Ana Losada, Spanish National Cancer Research Centre [CNIO]), RUVBL1 (1:1000, sc393905, Santa Cruz), RUVBL2 (1:1000, sc374135, Santa Cruz), Rabbit IgG HRP-linked (1:3000, 7074S, CST), Mouse IgG HRP-linked (1:3000, 7076S, CST), Rat IgG HRP-linked (1:3000, 7077S, CST).

5.14. **Chromatin fractionation assay**

The chromatin-binding assay was performed using the subcellular protein fractionation kit for cultured cells (Thermo) according to the manufacturer’s protocol.

5.15. **Metaphase spreads and SCC measurement**

Cells were cultured in medium containing 100 ng/mL of colcemid (Gibco) for 2 h before harvesting. Collected cells were resuspended with remaining medium (approximately 200 µL) by P200-tip, then 10 mL of 75 mM KCl solution was added gently. Cells were kept for 10 min at RT, then 2 mL of Carnoy’s solution was added. Samples were centrifuged at 500 xg for 5 min. After removing supernatant roughly, pellets were resuspended with remaining solution by cut P200-tip, then dropped onto slide glasses. Slides were kept in the dark for overnight, then counterstained with DAPI at 0.2 µg/mL in PBS.
5.16. **Immunofluorescence following nuclear membrane permeabilization or metaphase spread**

For pre-extraction, cells were transferred to microtubes and incubated for 3 min on ice. Triton X-100 was added to a final concentration of 0.3%, incubated for 5 min on ice, and then spun onto slides with a cytocentrifuge.

For metaphase spread, cells were treated with 100 ng/mL of colcemid (Gibco) for 1 h then collected into microtubes. Cells were resuspended with left media (approximately 50 µL), then 1 mL of 75 mM KCl solution was added gently. After 10 min of incubation, cells were spun onto slides with a cytocentrifuge.

After centrifugation, cells were fixed by 4% paraformaldehyde in PBS for 10 min, then permeabilized by 0.5% Triton X-100 in PBS for 10 min. After permeabilization, slides were incubated in 0.5% BSA/ PBS for 10 min, then incubated with primary antibodies for 1 h. After washing with PBS-T for 10 min three times, slides were incubated with secondary antibodies for 30 min. Slides were then counterstained with DAPI at 0.2 µg/mL.

**Antibodies list**

CENP-T (1:1000, kindly gifted by Dr. Tatsuo Fukagawa, Osaka University), SMC3 (1:200, kindly gifted by Dr. Ana Losada, Spanish National Cancer Research Centre [CNIO]), Aurora B (1:200, kindly gifted by Dr. Tatsuo Fukagawa, Osaka University), Histone H3-pT3 (1:200, 07-424, Millipore), RUVBL1 (1:200, sc393905, Santa Cruz), Rabbit IgG Alexa488-linked (1:300, 711-545-152, Jackson ImmunoResearch ), Mouse IgG Cy3-linked (1:400, 715-165-150, Jackson ImmunoResearch), Rat IgG Cy3-linked (1:400, 712-165-150, Jackson ImmunoResearch).
5.17. Data quantification and analysis for immunofluorescence

Signal intensities obtained in fluorescent microscopy experiments were quantified using ImageJ. DAPI signal was used to select nuclei and measure the fluorescence intensity of target proteins inside the nuclei. Quantifications were processed with Microsoft Excel.

5.18. Super resolution microscopy (STED)

Cells were spun onto slides with a cytocentrifuge and fixed by 4% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were then permeabilized by 0.5% Triton X-100 in PBS for 10 min at room temperature, rinsed with 0.5% BSA, and incubated for 1 h at room temperature with the anti-SMC3 antibody (a gift from Dr. Ana Losada, Spanish National Cancer Research Centre [CNIO]). Binding of primary antibody was then detected with Atto674-conjugated goat anti-rabbit IgG (Sigma) at an appropriate concentration in PBS/0.5% BSA. Chromosomes and nuclei were counterstained with DAPI at 0.2 µg/mL. Immunofluorescence images were collected with a 100x NA 1.40 objective lens together with a filter wheel by SP8 confocal microscopy equipped with STED (Leica) at room temperature. Subsequent analysis and processing of images were performed using LAS-X software (Leica) and ImageJ (National Institutes of Health). All images were scaled and processed identically. Immunofluorescence images were collected on a Leica TCS SP8 STED 3x superresolution microscope using the HC PL APO 100x/1.40 oil immersion objective. After acquisition, the images were deconvolved with SVI Huygens Professional software.
5.19. *mRNA sequencing*

1 µg of Total RNA was poly-A selected using the Dynabeads mRNA Direct Micro Purification kit (Thermo), according to manufacturer’s protocol. After polyA selection RNA were used to prepare strand-specific barcoded RNA libraries with the Ion Total RNA-Seq kit v2.0 (Thermo) following manufacturer’s protocol. The library qualities were checked by running on a BioAnalyzer HS Dna 2100 and the concentrations were determined from the analysis profiles. Two bar-coded libraries were pooled together on an equimolar basis and run using three PIV3 chips on an Ion Torrent Proton using HiQ200 chemistry.

5.20. *RNA-seq data bioinformatics analysis*

Sequence reads were demultiplexed and were processed for adapter trimming by Ion Proton. These sequences were analysed using the CLC Genomics Workbench (Qiagen) as previously described (Arakawa, 2016). Briefly, the sequence reads were aligned with the Ensemble chicken genome database (International Chicken Genome Sequencing 2004) by the RNA seq analysis toolbox using the alignment parameters of a length fraction of 80 % and a similarity fraction of 80 %. Expression levels of each gene were normalized by Reads Per Kilobase of exon model per Million mapped reads (RPKM). The differentially expressed genes between the different mutants were hierarchically clustered and visualized as a heatmap. The number of genes identified under the parameters of fold change of ≥1.5 and false discovery rate (FDR) p-value of ≤ 5 %, are displayed in Venn Diagrams.

5.21. *Immunoprecipitation, IP-MS*

1 x 10^8 cells were collected into 50 mL tube, washed with 10 mL of cold-PBS, resuspended with 1 mL of cold-PBS and transferred to 2 mL tube. After the
Supernatant was removed, 1 mL of 0.3% Triton 100-X in CSK buffer (treated with protease inhibitor cocktail and PMSF) was added and cells were resuspended. 5 min after the resuspension, samples were centrifuged at 4 degrees, 500 xg for 5 min. Supernatant was removed and pellet was resuspended with 0.3% Triton 100-X in CSK buffer (treated with protease inhibitor cocktail and PMSF) and sonicated at 10% power, 10 seconds, 5 times. Samples were centrifuged at 4 degrees, 15000 rpm for 5 min. Supernatant was transferred to new 2 mL tube and incubated with 300 µL of anti-FLAG M2 Magnetic agarose beads (Sigma Aldrich) at 4 degrees for overnight, samples were continuously rotated. Beads were washed with 1 mL of 0.3% Triton 100X in CSK buffer 3 times and incubated with 250 µL of 3xFLAG peptides two times. Eluted samples were combined and incubated with 200 µL of anti-HA affinity matrix (Roche) for 3 hr at 4 degrees with rotation. Beads were washed with 1 mL of 0.3% Triton 100X in CSK buffer 3 times and boiled with 30 µL of 2x laemmli buffer for 10 min. Samples were used for either immunoblotting or LC-MS/MS analysis by IFOM mass spectrometry facility.
6. APPENDIX

During my PhD, I worked on a project of the lab aiming at elucidating the role of AND-1 (Ctf4 in yeast) in replication. This work was published in Nature Communications, 2018;9(1):3091.

Title: AND-1 fork protection function prevents fork resection and is essential for proliferation

Abstract: AND-1/Ctf4 bridges the CMG helicase and DNA polymerase alpha, facilitating replication. Using an inducible degron system in avian cells, we find that AND-1 depletion is incompatible with proliferation, owing to cells accumulating in G2 with activated DNA damage checkpoint. Replication without AND-1 causes fork speed slow-down and accumulation of long single-stranded DNA (ssDNA) gaps at the replication fork junction, with these regions being converted to DNA double strand breaks (DSBs) in G2. Strikingly, resected forks and DNA damage accumulation in G2, but not fork slow-down, are reverted by treatment with mirin, an MRE11 nuclease inhibitor. Domain analysis of AND-1 further revealed that the HMG box is important for fast replication but not for proliferation, whereas conversely, the WD40 domain prevents fork resection and subsequent DSB-associated lethality. Thus, our findings uncover a fork protection function of AND-1/Ctf4 manifested via the WD40 domain that is essential for proliferation and averts genome instability.

The manuscript is given in attachment to the thesis.
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8. ATTACHMENT
AND-1 fork protection function prevents fork resection and is essential for proliferation

Takuya Abe1,2, Ryotaro Kawasumi1, Michele Giannattasio1,3, Sabrina Dusi1, Yui Yoshimoto2, Keiji Miyata2, Koyuki Umemura2, Kouji Hirota2 & Dana Branzei1,4

AND-1/Ctf4 bridges the CMG helicase and DNA polymerase alpha, facilitating replication. Using an inducible degron system in avian cells, we find that AND-1 depletion is incompatible with proliferation, owing to cells accumulating in G2 with activated DNA damage checkpoint. Replication without AND-1 causes fork speed slow-down and accumulation of long single-stranded DNA (ssDNA) gaps at the replication fork junction, with these regions being converted to DNA double strand breaks (DSBs) in G2. Strikingly, resected forks and DNA damage accumulation in G2, but not fork slow-down, are reverted by treatment with mirin, an MRE11 nuclease inhibitor. Domain analysis of AND-1 further revealed that the HMG box is important for fast replication but not for proliferation, whereas conversely, the WD40 domain prevents fork resection and subsequent DSB-associated lethality. Thus, our findings uncover a fork protection function of AND-1/Ctf4 manifested via the WD40 domain that is essential for proliferation and averts genome instability.
faithful DNA replication is essential to prevent accumulation of mutations and genome rearrangements, which are leading causes of genome instability. DNA replication is carried out by the replisome, minimally composed of the replisome progression complex (RPC) and DNA polymerases. RPC consists of the CMG complex, comprising Cdc45, the MCM helicase, and DNA2, and accessory factors that help efficient DNA replication by removing nucleosomes ahead of the replication forks, resolving DNA topological problems, and assisting in the bypass of DNA damage.

**Results**

**AND-1 is essential for proliferation.** To establish AND-1 conditionally depleted cells, we applied the auxin-inducible degron (AID) system, which enables rapid degradation of target proteins by the proteasome in chicken DT40 cell lines. We used a cell line that stably expresses TIR1, an essential component in the auxin degron system, from the β-actin promoter. We C-terminally tagged the endogenous AND-1 genes with the 3AID-6FLAG and 3AID-6HA tags, respectively, using the flip-in system for insertion of epitope tags. By sequential transfection of the epitope tags, we obtained AND-1 3AID-6FLAG/3AID-6HA cells expressing TIR1 (hereafter referred to as and-1-aid) cells. The correct insertion of the AID tags to the carboxyl termini of AND-1 was confirmed by western blotting. Both auxin addition, AND-1 3AID-6FLAG and AND-1 3AID-6HA proteins disappeared within 2 h. In the absence of auxin, and-1-aid cells behaved similarly with WT in regard to proliferation, replication fork speed and cell cycle distribution. We found that AND-1, visualized by anti-HA antibodies, co-localizes with replication foci marked by EdU. Next, to address if AID-tagged AND-1 protein is functional, we examined AND-1 recruitment to replication factories. We found that AND-1, visualized by anti-HA antibodies, co-localizes with replication foci marked by EdU.

We next examined the consequences of AND-1 depletion for cellular proliferation. and-1-aid cells stopped proliferating immediately after auxin addition. DNA fiber analysis revealed reduced DNA replication speed in AND-1 depleted cells, but intriguingly, overall replication monitored by EdU incorporation was not affected. This latter result was consistent with the one reported in human cells in which AND-1 depletion caused a reduction in replication fork speed.

We next addressed if replication fork slow-down in AND-1 depleted cells is caused by defective Tipin and/or Claspin localization to the fork, as these proteins interact with AND-1 and are required for fast replication fork speed. To these ends, we tagged endogenous Tipin and Claspin (encoded by the CLSPN gene) C-terminally with 9Myc in chicken DT40 cell lines. We used a cell line that stably expresses 3AID-6HA cells. The and-1-aid cells behaved similarly with WT in regard to proliferation, replication fork speed and cell cycle distribution. Using immunofluorescence microscopy, we found that the binding of Claspin to chromatin was not reduced upon AND-1 depletion.

As overall replication monitored by EdU incorporation was not affected in the absence of AND-1, we further checked if deregulated origin firing and/or fork collapse may diminish the inter-origin distance to support fast replication. In line with previous reports, we found reduced inter-origin distance in claspin mutants, but no significant differences in AND-1 depleted cells. Thus, AND-1 depleted cells have slow replication fork speed not associated with altered origin firing dynamics, and complete bulk replication before stopping their proliferation.

**AND-1 depletion causes DSBs and checkpoint activation in G2.** Since AND-1 depleted cells completed bulk replication with kinetics similar to control cells (Fig. 1f), we further checked if deregulated origin firing and/or fork collapse may diminish the inter-origin distance to support fast replication. In line with previous reports, we found reduced inter-origin distance in claspin mutants, but no significant differences in AND-1 depleted cells. Thus, AND-1 depleted cells have slow replication fork speed not associated with altered origin firing dynamics, and complete bulk replication before stopping their proliferation.
cells to gradually accumulate and subsequently arrest in G2/M (Fig. 2a). To address if the G2/M accumulation is the result of checkpoint activation and/or if AND-1 depleted cells fail cell division and stop during M phase, we added caffeine 12 h after auxin addition to inhibit the DNA damage checkpoint, as previously reported\(^{38,39}\). In the presence of caffeine, a large fraction of AND-1 depleted cells were released from the G2 arrest and proceeded to the next G1 phase, with concomitant increased accumulation of sub-G1 cells (Fig. 2b). Moreover, substantiating the notion that in AND-1 depleted cells spontaneously occurring DNA damage causes checkpoint activation and G2 arrest, we found that AND-1 depletion induces Chk1 phosphorylation (Fig. 2c). Chk1 phosphorylation could be detected in AND-1 depleted cells 4 h after auxin addition, with the signal maximizing...
Fig. 2 AND-1 depleted DT40 cells display spontaneous accumulation of DNA double strand breaks (DSBs) and DNA damage checkpoint activation. **a** Cell cycle distribution of AND-1 depleted cells. and-1-aid cells were incubated with auxin for indicated times, stained with propidium iodide (PI), and DNA content was analyzed by flow cytometry. **b** and-1-aid cells were incubated with or without auxin for 12 h, caffeine was added, and further incubated for the indicated times. DNA content was analyzed by flow cytometry. **c** Total cell lysates were prepared from and-1-aid cells at the indicated time points and analyzed by Western blotting. **d** and-1-aid cells were incubated with or without Auxin for 12 h, caffeine and colcemid were added, and further incubated for 2 h. Metaphase spreads were prepared from and-1-aid cells. Examples of intact and damaged chromosomes (the chromosomes are identified by shape in DT40) are shown. Scale bars represent 10 μm in left panel, 1 μm in enlarged picture (chromosome 2). **e** and-1-aid cells were incubated with auxin for 8 h and γH2AX or RAD51 foci were visualized by immunostaining with specific antibodies. Scale bars represent 25 μm in upper panels, 5 μm in enlarged pictures. **f** DSB detection via PFGE. and-1-aid cells were incubated with auxin for the indicated times, collected into agarose plugs and their DNA was separated by size on an agarose gel. Under the electrophoresis conditions used, high molecular weight genomic DNA remains in the well, whereas lower molecular weight DNA fragments (several Mb to 500 kb) migrate into the gel and are compacted into a single band.
8–10 h after auxin addition when most AND-1 depleted cells were arrested in G2/M. We did not observe major effects on total Chk1 levels (Supplementary Fig. 3a).

To address the nature of the spontaneously arising DNA damage that causes checkpoint activation, we analyzed chromosome abnormalities of AND-1 depleted cells. To allow AND-1 depleted cells to reach metaphase, we first released them from the checkpoint-mediated G2 arrest using caffeine (see Fig. 2b), and then induced metaphase arrest by addition of colcemid. Notably, under these conditions, we observed a drastically increased number of chromatid and chromosome breaks, with the latter class being especially prominent (Fig. 2d).

In line with the notion that AND-1 depleted cells have increased levels of DNA damage, we detected γH2AX and RAD51 foci accumulation in these cells (Fig. 2e). Furthermore, pulse-field gel electrophoresis (PFGE) analysis revealed higher levels of broken DNA in AND-1 depleted cells (Fig. 2f). Taken together, the results indicate that in the absence of AND-1, cells can complete bulk DNA synthesis, but DSBs accumulate, triggering DNA damage checkpoint activation and subsequent G2 arrest.

AND-1 loss causes long ssDNA stretches at replication forks.

To address the timing of DSB formation versus active replication, AND-1 depleted cells were co-stained with γH2AX and EdU. Interestingly, most replicating cells marked by EdU staining (shown by white arrow) did not have γH2AX foci (Fig. 3a). To address the mutually exclusive staining patterns of γH2AX and EdU quantitatively, we employed FACS and used camptothecin (CPT)-treated cells as control, as it was reported that H2AX increases quantitatively, we employed FACS and used camptothecin (CPT)-treated cells as control, as it was reported that H2AX phosphorylation predominantly in S phase, and found that auxin-treated and-1-aid cells displayed strong phosphorylation predominantly in S phase (Fig. 3a). Untreated, auxin-treated, or CPT-treated and-1-aid cells were co-stained with γH2AX antibody, EdU, and propidium iodide (PI). From γH2AX and PI staining, we confirmed that CPT-treated cells display strong H2AX phosphorylation predominantly in S phase, and found that auxin-treated and-1-aid cells displayed strong phosphorylation predominantly in G2/M (Supplementary Fig. 3b). Moreover, in line with this result, the γH2AX/EdU staining revealed that CPT treatment induced H2AX phosphorylation specifically in EdU positive cells, whereas conversely, AND-1 depletion induced γH2AX in EdU negative cells (Fig. 3b). To further address the observed inverse correlation between ongoing replication and γH2AX foci, we incubated cells with auxin and low concentration of HU. In this condition, the number of γH2AX and RAD51 foci observed upon AND-1 depletion was greatly reduced (Supplementary Fig. 3c), probably as a consequence of slowing down the replication speed and enriching cells in S phase (Supplementary Fig. 3d).

The nature of the replication fork-related DNA lesions, if any, induced by AND-1 depletion during normal replication are not known. Here, we used in vivo psoralen cross-linking and transmission electron microscopy (TEM) to visualize DNA replication intermediates generated after a single S phase without AND-1. We observed increased percentage of replication forks having abnormally long single stranded (ss) DNA at the fork branching point (gapped forks) in auxin-treated and-1-aid cells compared to untreated and-1-aid controls (Fig. 3c, d). Specifically, while 94% of the forks were normal, with no or relatively short stretches of ssDNA at the fork junctions in control and-1-aid cells, addition of auxin caused a drop in the normal forks and the appearance of 34% gapped forks. Gapped forks in AND-1 depleted cells were characterized by the presence of a long ssDNA discontinuity with an average length of 300 nt on one of the two replicated strands connected to the fork branching point (Fig. 3c–e). This asymmetric ssDNA discontinuity at the fork branching point was much shorter, with an average length of 150 nt, in the forks of the control cells (Fig. 3e). Taken together, the results indicate that the chromosome breaks observed in AND-1 depleted cells occur in the G2 phase and may originate from the long ssDNA stretches formed during DNA replication.

AND-1 prevents nucleolytic processing of replication forks.

The accumulation of gapped forks observed upon AND-1 depletion may imply a role for AND-1 in preventing fork resection, either by itself or via its ability to interact with replication fork protection factors that inhibit nucleolytic processing of the fork. As ssDNA gaps at the fork junction in AND-1 depleted cells correlate with increased RAD51 and γH2AX foci in G2, we reasoned that these foci may form as a consequence of the ssDNA accumulation at the fork junction, possibly as a result of nucleolytic processing of the fork. We asked if treatment of cells with mirin, an inhibitor of MRE11 previously implicated in fork resection, could rescue RAD51 and γH2AX focus formation in AND-1 depleted cells. When asynchronous and-1-aid cells were treated with auxin and mirin for 8 h, we found that both RAD51 and γH2AX foci were reduced upon treatment with mirin (Supplementary Fig. 4a). However, mirin treatment also caused cells to be more enriched in S phase under these conditions (Supplementary Fig. 4b), and this S phase enrichment could potentially explain the reduction in the RAD51 and γH2AX foci (see Fig. 3a, b and Supplementary Fig. 3c-d). Release of and-1-aid cells from nocodazole arrest in the presence or absence of mirin, indicated that mirin addition does not affect the release, but delays S-phase entry and possibly S phase progression (Supplementary Fig. 4c).

To overcome mirin-induced S phase delays, we used a different experimental set-up in which cells reach G2/M in response to different treatments. Specifically, cells were arrested with nocodazole for 7.5 h, then released into medium with or without auxin. Mirin was added 3 h after release to half of the cultures, and samples were collected at different time points afterwards for PI FACS (Fig. 4a). The time points labeled as G2/M in the PI FACS analysis for the different conditions, namely, untreated (7 h), auxin (8 h), mirin (11 h), auxin plus mirin (11 h), were further processed for focus formation analysis. Also in this new set up, mirin treatment significantly reduced the accumulation of RAD51 and γH2AX foci in AND-1 depleted cells (Fig. 4a).

To address if the effects of mirin originate at the fork, we used TEM to examine the replication fork structure in AND-1 depleted cells treated or not with mirin (Fig. 4b, experimental scheme). Strikingly, addition of mirin strongly reduced the percentage of gapped replication forks in AND-1 depleted cells, from 32% observed in AND-1 depleted cells to 4% in AND-1 depleted cells additionally treated with mirin (Fig. 4b), and significantly reduced the average length of ssDNA gaps at the fork junction (Fig. 4c).

Notably, mirin did not rescue the replication for speed delay characteristic of the AND-1 depleted cells (Fig. 4d), indicating that the functions of AND-1 in fork speed and fork protection are uncoupled. Moreover, addition of mirin, although toxic by itself, partly alleviated the proliferation defect in AND-1 depleted cells (Fig. 4c), and increased the percentage of cells that recover from auxin-induced G2 arrest (Supplementary Fig. 4d). Altogether, these results uncover a fork protection function of AND-1 that is critical in preventing fork resection and subsequent damage-induced lethality.

AND-1 facilitates fast fork speed via its HMG domain.

AND-1 has several distinguishable domains including WD40 repeats, SepB and HMG. The roles of its different domains in binding and stimulating the helicase activity of the human CMG complex has
been in part addressed\textsuperscript{15}. AND-1 binding to polymerase α is mediated by its SepB domain\textsuperscript{18}; in addition, a contribution for the HMG domain was proposed in mammalian cells\textsuperscript{16}. However, the critical domains of AND-1 in DNA replication, cell survival or its localization remain unknown. To address the latter questions, we amplified full-length chicken AND-1 cDNA containing the HA tag by RT-PCR and cloned it to an expression vector in which AND-1-HA is expressed from the chicken β-actin promoter.

![Image](https://example.com/image.png)
Fig. 4 AND-1 prevents nucleolytic processing of the forks and subsequent damage accumulation. a Foci assay of γH2AX and RAD51. Upper panels: scheme of the experiment and cell cycle distribution at the indicated time points, when mirin was added and samples were collected for PI FACS and immunostaining. Cells untreated or treated with either or both mirin and auxin at indicated time points were stained with propidium iodide (PI), and DNA content was analyzed by flow cytometry. Bottom panels: γH2AX and RAD51 foci in G2/M cells untreated or treated with mirin, auxin, or both. Results of two experiments are shown. n represents the number of cells analyzed in the two experiments.

b EM analysis of the replication intermediates purified from and-1-aid cells treated with auxin with or without mirin for 4 h as in Fig. 3d, e. Percentage or normal and gapped forks (b) and distribution of ssDNA length at the fork branching point (c). The reported gapped forks have ssDNA > 300 nt. Molecules derive from two independent experiments, for a total number (n) of molecules shown in c. n is the total number of replication forks analyzed, A is the average length of ssDNA, and M is the median length of ssDNA. In the box plots in c, the middle line indicates the median value; the box shows the 25th and 75th percentiles; the bars, the 5th and 95th percentiles. P values were calculated by Student’s t-test. ** P ≤ 0.01. d Fork speed measured as in Fig. 1e for and-1-AID cells untreated or treated with auxin, or auxin and mirin for 4 h as indicated in the scheme in Fig. 4b. ****P ≤ 0.0001. e Growth curves of and-1-aid cells in the indicated conditions. 10^5 cells were inoculated in 1 mL of medium and passaged every 24 h. Error bars represent SD obtained from three independent experiments.

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From the full-length AND-1-HA, we made ΔSepB, ΔWD40 and ΔHMG mutants, and transfected them to and-1-aid cells (Fig. 5a). As SepB is the most highly conserved domain of AND-1 that binds to Polymerase α and Sld5, we first analyzed the and-1-ΔSepB mutant. As expected based on its high conservation, this domain was essential for all AND-1 functions, judging from the fact that the growth defects (Supplementary Fig. 5a), replication fork slowing (Supplementary Fig. 5b), accumulation of spontaneous γH2AX and RAD51 foci (Supplementary Fig. 5c-d), cell cycle arrest (Supplementary Fig. 5e) characteristic of AND-1 depleted cells failed to be suppressed by the expression of the AND-1 ΔSepB variant, whereas all these phenotypes were suppressed by the expression of AND-1 WT (see below).

Next, we analyzed and-1 ΔWD40 and ΔHMG mutants. The growth curve of these mutants revealed that the WD40 domain, but not the HMG box, is essential for proliferation, although proliferation also becomes slower in the absence of the HMG box (Fig. 5b). Conversely, when we monitored the replication fork speed of these mutants, we found that and-1-ΔHMG, but not and-1-ΔWD40, had shorter DNA replication tracts (Fig. 5c). It is reasonable to assume that AND-1 firmly binds to DNA via its HMG box, since the HMG domain is known as a DNA binding motif. Thus, our results suggest that reduced DNA binding of AND-1 causes replication fork slowing and results in mild proliferation defects.

To address this hypothesis further, we examined the capability of AND-1 protein and variants to engage in replication factories. We found that AND-1 focus formation was reduced in the and-1-ΔHMG and and-1-ΔSepB mutants, but not in the and-1-ΔWD40 mutant (Fig. 5d). The same trend was observed via chromatin fractionation assay, in which we found reduced chromatin binding for all AND-1 mutant variants, but stronger reduction was observed with the ΔHMG mutant compared to the ΔWD40 variant. Very little binding was observed for the ΔSepB variant, although in this case, the expression levels were lower to begin with, likely due to reduced protein stability in the absence of the SepB domain, making the direct comparison with the other variants difficult (Fig. 5e).

To investigate further the role of the HMG domain of AND-1 in replication fork speed and to compare it with those caused by mutations in Claspin and Tipin, we constructed and-1 knockout cell lines expressing the ΔHMG variant (Supplementary Fig. 6a-b). In this case there is no residual AND-1 protein caused by incomplete AND-1 depletion. The and-1 knockout cells expressing the ΔHMG variant cells showed reduced replication fork speed, but the defects were modest in comparison with those of tipin and clasin mutants (Supplementary Fig. 6c). Similarly, the proliferation defects of ΔHMG cells were milder than those of tipin and clasin (Supplementary Fig. 6d) and showed similar trends with those of AND-1 depleted cells expressing the ΔHMG variant (Fig. 5b).

These results suggest that robust binding of AND-1 to DNA mediated by its HMG box is required for fast replication fork speed, but it is not essential for proliferation, likely because the basic and essential amount of AND-1 on chromatin is supported by SepB- and WD40-mediated protein interactions.

AND-1 WD40 repeats prevent resected forks and DSBs in G2. Next, we monitored cell cycle distribution, checkpoint activation, and γH2AX focus formation in and-1-aid mutants. Similar to cells depleted for AND-1, and-1-ΔWD40, but not and-1-ΔHMG cells gradually accumulated in the G2 phase after auxin addition (Fig. 6a). and-1-ΔWD40 mutants also showed spontaneous Chk1 phosphorylation, although to a reduced extent compared to AND-1 depleted cells (Fig. 6b). Spontaneous formation of γH2AX foci was also more frequently observed in and-1-ΔWD40 than in and-1-ΔHMG mutants, although both variants had higher levels of γH2AX than WT AND-1 complemented and-1-aid cells (Fig. 6c). Notably, RAD51 foci were specifically increased in and-1-ΔWD40 cells, and not in and-1-ΔHMG, to similar levels as in AND-1 depleted cells (Fig. 6c). These results indicate that AND-1 roles in averting DSB formation in G2 and subsequent checkpoint activation-mediated cell cycle arrest are primarily mediated by the WD40 repeats and its SepB domain.

The phenotype of G2 arrest in AND-1 depleted cells correlates with accumulation of ssDNA stretches proximal to replication fork branching points. The ssDNA accumulation proximal to replication fork junctions may be directly related with replication fork speed, which is reduced in AND-1 depleted and and-1-ΔHMG cells, or rather represent chromosome lesions caused by loss of AND-1 and subsequently triggering DSB formation and checkpoint activation. The AND-1 variants generated here with specific defects either for replication fork speed or for proliferation, offered the possibility to test these hypotheses. When we compared the length of ssDNA at replication forks and the percentage of normal versus gapped forks in different and-1 mutants, we specifically observed long ssDNA at replication forks in the and-1-ΔWD40 mutant, but not in the and-1-ΔHMG mutant or and-1-aid cells complemented with WT AND-1 (Fig. 6d, e). Thus, the WD40 domain, and not the HMG domain necessary for physiologically fast levels of DNA replication and robust enrichment of AND-1 in replication factories, is essential to prevent ssDNA formation at replication fork junctions.

Considering that mirin treatment could also significantly reduce gapped fork formation (Fig. 4b) and RAD51 focus accumulation in G2 (Fig. 4a), the results imply that the fork protection function of AND-1 against nucleolytic cleavage by MRE11 is largely mediated via its WD40 domain. As RAD51 foci are increased in AND-1 depleted cells and in and-1-ΔWD40 mutants, and reduced by mirin treatment, these results suggest that RAD51 is recruited to the ssDNA gaps at the fork junction after these are formed via MRE11-mediated nucleolytic processing. This could explain why AND-1 depleted cells are not protected by RAD51 against MRE11-mediated cleavage. Moreover, the results indicate that the DSBs formed in AND-1 depleted cells derive in part from long ssDNA formed proximal to the replication fork junction as both are prevented by the WD40 repeat domain of AND-1 and by treating cells with mirin.

Discussion AND-1/Ctf4 is a critical component of the replisome, bridging polymerase α with the replicative CMG complex, and acting as a scaffold for various proteins that are recruited to the replication fork. Intriguingly, although the bridging function of Ctf4-AND-1 is common in all the organisms studied to date, and likely shared with MCM10, its requirement for proliferation function is not conserved across species.

Here we set out to identify the type of replication stress caused by defective AND-1-mediated bridging between the CMG helicase and the polymerase α and how this impacts proliferation in vertebrate cells. To these ends, we established and-1-aid conditional mutants in genetically amenable chicken DT40 cells. This system allows fast and complete degradation of AND-1, permitting analysis and dissection of the phenotypes induced by its loss.

We uncovered that cells can complete bulk DNA replication without AND-1, but newly synthesized DNA presents long ssDNA gaps proximal to the replication fork junction. We present evidence that lethal DSBs that activate the DNA damage checkpoint and stall cell cycle progression arise after completion of DNA replication and most likely originate from the ssDNA...
stretches formed during replication. This conclusion is not only correlative, based on the timing of the observed phenotypes—ssDNA first, at the replication fork junction, and then DSBs after the bulk replication is complete—but also it has a genetic basis as AND-1 variants defective in suppressing the ssDNA accumulation at replication forks are also specifically defective in proliferation and show prominent G2 arrest. Moreover, addition of mirin, which inhibits the MRE11 nuclease activity, reverses not
only ssDNA gap accumulation at the fork junction, but also the formation of DNA damage foci and prominent G2 arrest in AND-1 depleted cells. Thus, prevention of persistent ssDNA accumulation during replication underpins the critical function of AND-1 in proliferation.

Interestingly, yeast Ctf4 is also important to prevent ssDNA accumulation at replication forks in the presence of DNA damage and is required for viability under damaging conditions12. Thus, it seems that AND-1/Ctf4 has evolutionally conserved roles in averting ssDNA accumulation at replication forks. We posit that the different requirement for proliferation of vertebrate AND-1 and budding yeast Ctf4 is related to the frequency of the replication-related events causing ssDNA formation, with this being much higher in vertebrate cells. An implication of our findings is that a common function of AND-1/Ctf4 conserved across organisms is related to the prevention and effective management of those gapped ssDNA containing regions, often dealt with by subsequent recombination events12.

Our work reveals that in vertebrate cells lacking AND-1 the gapped forks arise in large part from unscheduled nucleolytic events, thus identifying a role for AND-1 in replication fork protection. The findings that RAD51 is bound to chromatin in AND-1 depleted and and-1-ΔWD40 mutants, but does not protect against nucleolytic cleavage, suggest that RAD51 is recruited after ssDNA gap formation took place. Alternatively, it is possible that the mode of binding of RAD51 in AND-1 depleted cells is defective and does not offer the same level of fork protection44, as is the case with certain RAD51 variants45. This latter hypothesis may explain the subsequent formation of DSBs, which is the case with certain RAD51 variants46. This latter hypothesis may explain the subsequent formation of DSBs, which cause prominent cell cycle arrest in G2, likely due to improper repair.

Importantly, our results indicate that the WD40 repeat domain of AND-1, and not the HMG box, is essential in preventing ssDNA and DSB accumulation coupled with cell cycle arrest. In many organisms, replication fork restart and protection strongly rely on HR factors and mediators42–44. Notably, DSB accumulation in G2/M has also been observed upon depletion or inactivation of HR factors in vertebrate and mammalian cell systems45,47,48, and critical interactors of the WD40 domain of the PALB2 recombinase protein involves binding to other HR factors, such as RAD51C, RAD51D, and BRCA249. Although RAD51 binding appears proficient in AND-1 depleted cells, our results indicate that this binding is not sufficient to prevent nucleolytic processing of the forks in cells lacking AND-1 and is also not sufficient to promote repair in G250. We thus propose that AND-1/Ctf4 essential function, executed in large part via its WD40 repeats, is to provide fork protection, and thereby to promote genome integrity.

Methods

Cell lines. The cell lines and the targeting constructs are described in the Supplementary Table 1.

Cell culture. Cells were cultured at 39.5 °C in D-MEM/F-12 medium (Gibco) supplemented with 10% fetal bovine serum, 2% chicken serum (Sigma), penicillin/streptomycin mix, and 10 μM 2-mercaptoethanol (Gibco) in the presence or absence of 300 μM auxin. To plot growth curves, each cell line was cultured in three different wells of 24-well plates and passed every 12 h or 24 h. Cell number was determined by flow cytometry using plastic microbeads (07312-5; Polysciences).

Antibodies and Western blots. The antibodies used for immunoblotting were the ones against HA (1:2000, 11176423001, Roche), Myc (1:2000, clone 9E10, produced by IFO laboratory), Cdc45 (ac11000, Santa Cruz), Cdk1-P (1:1000, 2341, Cell Signaling Technology), RPA (1:3000-244A, Abcam), Histone H3 (1:1000, 06-570, Millipore), a-tubulin (1:3000, TS168, Sigma) and FLAG (1:2500, F3165, Sigma). All uncropped blots in the main figures are shown in Supplementary Fig. 7.

Plasmid construction and transfection. To add 3xMyc-6xFLAG and 3xMyc-6xHA to AND-1 by Flip-in system, 2.3 kb upstream DNA sequences of stop codons of AND-1 was amplified with the primers 5'- TTTTGTGGAACCCGGTGTCTGCACTGTCGCTGCCGTCACGATGGACAAGTGGTTTGAGCTATCGTGTTAATCTGTCCAG-3' and 5'- TTTGCTGCTGCTACTGCTGCT-3' (SalI) and 5'- TTTTACTAGTATGCAATTCGTTCTGGAATGCTGCT-3' (SalI) respectively, and cloned into pcDNA3.1 vector (Invitrogen, ABI7919, Abcam), Histone H3-P (1:1000, 06-570, Millipore), a-tubulin (1:3000, TS168, Sigma) and FLAG (1:2500, F3165, Sigma). All uncropped blots in the main figures are shown in Supplementary Fig. 7.

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Fig. 6 Identification of AND-1 domains critical for proliferation and prevention of spontaneous DSB formation. a Cell cycle distribution of AND-1 deletion mutants. Cells of the indicated genotypes were incubated with auxin for indicated times, stained with propidium iodide (PI), and DNA content was analyzed by flow cytometry. b Total cell lysates were prepared from cells of the indicated genotypes and analyzed by Western blotting. c Cells of the indicated genotypes were incubated with auxin for 8 h and γH2AX and RAD51 foci were visualized by immunostaining with specific antibodies. Results of two experiments are shown. n represents the number of cells analyzed in the two experiments. d, e EM analysis of the replication intermediates purified from the cell lines of the indicated genotype as in Fig. 3d, e. Molecules derive from two independent experiments, for a total number (n) of molecules shown in e. n is the total number of replication forks analyzed, A is the average length of ssDNA, and M is the median length of ssDNA. Error bars in d represent SDM obtained from two independent experiments. In the box plots in e, the middle line indicates the median value; the box shows the 25th and 75th percentiles; the bars, the 5th and 95th percentiles. P values were calculated by Student’s t-test. ***P ≤ 0.001
vectors were then linearized with NotI restriction enzyme before transfected to ART40 cells.

**DNA fiber analysis.** DNA fiber analysis was performed as previously described with small modifications. Cells (5 x 10^5 in 1 mL of medium) were pulse-labeled with 25 μM chlorodeoxyuridine (CdU; Sigma) and then sequentially pulse-labeled with 230 μM iododeoxyuridine (IdU; Sigma). Cells were resuspended in ice-cold PBS and then washed on glass slides. Cells were lysed with DNA fiber lysis buffer (0.5% SDS, 200 mM Tris-HCl, pH 7.4, 50 mM EDTA), and then glass slides were tilted to extend DNA. For fixation, glass slides were immersed in Carnoy fluid (MeOH:AcOH, 3:1) for 3 min, 70% EtOH for 1 h. After washing with PBS, glass slides were immersed in 2.5 M HCl for 10 min to denature DNA molecules and subsequently in 0.1 M sodium tetraborate for 3 min to neutralize. After washing with PBS, the slides were treated with rat anti-β3-IdU antibody (1:200; Abcam) and mouse anti-β3-UdR antibody (1:50; BD Biosciences), which reacted against CdU and IdU, respectively. C3-conjugated anti-rat IgG (1:400; Jackson Immunoresearch Laboratories) and Alexa Fluor 488 anti-mouse IgG (1:100; Invitrogen) were used as the secondary antibodies. The first and second antibodies were incubated for 1 h each at room temperature. Washing of antibodies was performed with 0.05% Tween 20 in PBS. Coverslips were mounted with PermaFluor mounting medium (Lab Vision). Images were captured with a fluorescence microscope. Fiber lengths were measured using ImageJ. DNA replication elongation rates were calculated as CdU fiber length divided by pulse-labeling time.

**Detection of DSBs by pulse-field gel electrophoresis (PFGE).** PFGE analysis was performed as previously reported with small modifications. Cells were harvested and washed with ice cold PBS once. Then cells were incubated with ice cold 0.075 M KCl for 5 min. After centrifugation, 0.7% agarose plugs containing 10^7 cells were prepared with a CHEF-disposable plug mold (Bio-Rad). The cells were lysed by the addition of 3.5 M MgCl2 in 0.5 M EDTA, 0.1% Lauroylsarcosine, 10 mM Tris-HCl (pH 8.0) for 24 h at 50 °C and then washed with small modiﬁcations. DT40 cells. Vectors were then linearized with NotI restriction enzyme before transfected to ART40 cells.

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**References**


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40. Kolijin, V., Bunch, T. A., R.K., and D.B. analyzed the data; T.A. and R.K. made the figures; D.B. wrote the paper and all authors contributed suggestions.

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Author contributions

T.A., R.K., and D.B. designed the research; T.A. and R.K. performed the experiments; M.G. and S.D. performed TEM sample acquisition and analysis; Y.Y., K.M., K.U., and K. H. contributed to experiments on molecular combing and pulse field gel electrophoresis; T.A., R.K., and D.B. analyzed the data; T.A. and R.K. made the figures; D.B. wrote the paper and all authors contributed suggestions.

Additional information

Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-05586-7.

Competing interests: The authors declare no competing interests.

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AND-1 fork protection function prevents fork resection and is essential for proliferation

Abe, Kawasumi et al
**Supplementary Table 1.** Cell lines used in the study.

<table>
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<th>Selective marker</th>
<th>Reference</th>
<th>Strain No.</th>
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<td>-</td>
<td>(26)</td>
<td>8</td>
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| ANDRAND-1^{3mAID}  
+TIR1 9xMyc  
+AND-1^{1HA}  
\Delta SepB  
\Delta WD40  
\Delta HMG  
CLASPN  
+TIPIN  
\Delta HA  
\Delta HMG  | AND-1/AND-1::3mAID-6xHA  
FLP-In-His/3mAID-6xFLAG  
FLP-In-Bleo, +TIR1-9xMyc::Bsr  
AND-1/AND-1::3mAID-6xHA  
FLP-In-His/3mAID-6xFLAG  
FLP-In-Bleo, +TIR1-9xMyc::Bsr, +AND-1-1::3mAID-6xHA  
AND-1/AND-1::3mAID-6xHA  
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AND-1/AND-1::3mAID-6xHA  
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Supplementary Figures

Supplementary Figure 1. Comparison between WT and and-1-AID cells.

a Growth curves of WT and and-1-aid cells. $10^5$ cells were inoculated in 1 mL of medium and passaged every 12 h. b Cell cycle distribution of WT and and-1-aid cells.
Cells were stained with propidium iodide (PI), and DNA content was analyzed by flow cytometry. DNA replication elongation rates of WT and and-1-aid cells, derived from the lengths of CldU tracks (only the ones clearly connected with IdU tracks were considered). Scale bar on representative image represent 5 kb. Middle line = median; box = 25th and 75th percentiles; bars = 5th and 95th percentiles. M indicates median values, n, the number of fibers analyzed in each condition. Similar trend was observed in an independent experiment.
Supplementary Figure 2. Chromatin binding of Claspin in AND-1 depleted cells and inter-origin distance in AND-1-depleted and Claspin-inactivated cells.
a Schematic representation of the flip-in constructs for CLSPN-9Myc and TIPIN-9Myc. The CLSPN gene encodes Claspin. Black boxes indicate exons containing stop codons of each genes, and “Eco” indicates drug resistance gene markers. b Upper panel: Presentation of experimental set-up and immunoblot of total lysate and chromatin fraction from and-1-aid Claspin-9Myc at indicated time points. Blotting was performed to address AND-1, Claspin, RPA, Histone H3. Bottom panel: Quantification of chromatin bound Claspin in control and AND-1 depleted cells. Ratio of chromatin bound/versus total Claspin-9Myc. Measured chromatin bound Claspin-9Myc amount normalized to the total Claspin-9Myc in the cell lysate of two independent experiments were averaged and plotted. Error bar represents SDM obtained by two independent experiments. c Inter-origin distance measured by molecular combing in control, AND-1 depleted and conditional claspin mutants. The number n of molecules analyzed from one experiment is included in the graph. Middle line = median; box = 25th and 75th percentiles; bars = 5th and 95th percentiles. P values were calculated by Student’s t-test. **** indicates a P value ≤0.0001.
Supplementary Figure 3. DSBs accumulate in AND-1 depleted cells after completion of bulk replication.

a Total cell lysates were prepared from cells with indicated genotype at the indicated time points and analyzed by Western blotting. b and-1-aid cells were cultured with Auxin or 10 µM CPT for 5 min, and incubated with Propidium Iodide (PI). Cells with DSBs were detected by immunostaining with anti-γH2AX antibody. c and-1-aid cells were cultured with Auxin for 8 h in the absence or presence of 100 µM HU.
γH2AX- and RAD51-foci were visualized by immunostaining with anti-γH2AX and anti-RAD51 antibodies. Scale bars represents 10 µm. n represents the numbers of cells analyzed in the indicated conditions. d Cell cycle distribution of and-1-aid cells after treatment with or without Auxin and HU for 8 h.
Supplementary Figure 4. Mirin effects on RAD51, γH2AX foci and cell cycle distribution.

a and-1-aid cells were incubated with Auxin with or without Mirin for 8 h. γH2AX foci and RAD51 foci were visualized by immunostaining with specific antibodies. Results of two experiments are shown. n represents the number of cells analyzed in the two experiments. b Cell cycle distribution of and-1-aid cells treated with Auxin with or without Mirin for 8 h. Cells were stained with propidium iodide (PI), and DNA content was analyzed by flow cytometry. c Cell cycle distribution of and-1-aid cells treated with
or without Mirin after nocodazole (NOC) arrest and release. Cells were harvested at indicated time points and stained with propidium iodide (PI). DNA content was analyzed by flow cytometry. d Cell cycle distribution by PI FACS of \textit{and-1-aid} cells treated with or without Mirin after nocodazole (NOC) arrest and release. Mirin was added 3 hours after addition of Auxin. Auxin was added immediately after the release from Nocodazole arrest.
Supplementary Figure 5. The SepB domain of AND-1 is required for proliferation, normal replication fork speed and genome integrity. a Growth curves of and-1-aid cells expressing AND-1 variant with deletion of the SepB domain in the presence or absence of Auxin. $10^5$ cells were inoculated in 1 mL of medium and passaged every 12 h. Error bars represent SDM obtained from three independent experiments. b and-1-aid cells
expressing or not AND-1 variants truncated for the SepB domain were cultured with or without Auxin for indicated times, and DNA replication elongation rates were calculated as CldU fiber length divided by pulse-labeling time. n indicates the number of fibers analyzed for the indicated condition and genotype. Middle line = median; box = 25th and 75th percentiles; bars = 5th and 95th percentiles. c-d and-1-aid cells expressing or not AND-1 variant truncated for the SepB domain were incubated with Auxin for 8 h and γH2AX and RAD51 foci were visualized by immunostaining with specific antibodies. n represents the numbers of cells analyzed. e and-1-aid cells and-1-aid cells expressing or not AND-1 variant truncated for the SepB domain were incubated with Auxin for indicated times, stained with propidium iodide (PI), and DNA content was analyzed by flow cytometry.
Supplementary Figure 6. Effects of the HMG domain of AND-1 on proliferation and fork speed and comparison with *tipin* and *claspin* mutants.

**a** Flow chart of *and-1-aid +and-1 ΔHMG* cell’s establishment. **b** Schematic representation of *AND-1* gene locus and knock out construct. Black boxes indicate exons, and “Bleo” and “Bsr” indicate drug resistance markers. **c** DNA replication elongation rates of cells with indicated genotypes; constitutive *tipin* and *claspin* knockout cells were used. DNA replication elongation rates, derived from the lengths of CldU tracks (only the ones clearly connected with IdU tracks were considered), are shown. Middle line = median; box = 25th and 75th percentiles; bars = 5th and 95th percentiles. M indicates median values, n, the number of fibers analyzed in each condition. Similar trend was observed in independent experiment. **d** Growth curves of cells with indicated genotypes; here constitutive *tipin* and *claspin* knockout cells were used as controls. $10^5$ cells were inoculated in 1 mL of medium and passaged every 24 h. Error bars present SDM obtained from three independent experiments.
Supplementary Figure 7. Uncropped Western blots corresponding to different panels. Boxes with dashed lines in red color indicate the areas cropped and used in main figures.