Regulation of replication-linked functions by PCNA and SUMO

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Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt habe. Ich habe weder anderweitig versucht, eine Dissertation einzureichen oder eine Doktorprüfung durchzuführen, noch habe ich diese Dissertation oder Teile derselben einer anderen Prüfungskommission vorgelegt.

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SUMMARY

Genomic integrity largely depends on the accurate replication and faithful transmission of the genetic information to the progeny each time a cell divides. To ensure the fidelity of these fundamental processes, highly sophisticated protein networks have evolved. This study investigated how the diverse mechanisms for maintaining genomic integrity are integrated and coordinated at the replication fork.

In the first part, the roles of post-translational modifications with ubiquitin and SUMO in regulating replication through DNA lesions were investigated. Previous work in *S. cerevisiae* showed that post-translational modifications of the replication factor PCNA control DNA repair activities of the replisome. PCNA is a homotrimeric ring-shaped protein that encircles DNA and confers processivity to DNA polymerases during DNA synthesis. Moreover, being devoid of enzymatic activity, PCNA is perfectly suited to act as a platform for recruitment of factors to the replication fork, including DNA repair enzymes, chromatin remodelers and replication regulators. PCNA modifications by ubiquitin govern two distinct modes of lesion bypass, either channeling the repair processes into error-prone translesion synthesis by recruiting specialized polymerases, or promoting an error-free mechanism involving a template switch to the sister chromatid. In addition, PCNA-modification by SUMO inhibits the third major bypass mechanism, namely recombinational repair, by recruiting the anti-recombinogenic helicase Srs2. In this study, the importance and universality of PCNA-modifications could be demonstrated by showing that PCNA ubiquitylation in human and chicken cells is well conserved. Interestingly, SUMO modification appeared less preserved, indicating that the pathways controlled by PCNA modifications are used to different degrees throughout species. A further finding of this thesis was the identification of DNA polymerase δ, the major replicative polymerase in *S. cerevisiae*, as a novel SUMO substrate. Importantly, it could be shown, that modification of Polδ serves as a backup pathway for Srs2 recruitment to inhibit recombination.

Next to replication-coupled DNA-repair, the major emphasis of this study was to address how the replication fork controls the correct distribution of the genetic material after replication. During S-phase, newly replicated sister chromatids are instantly tied up at the replication fork by a proteinaceous ring, called cohesin. As this process specifies and preserves the identity of sister chromatids, it is a crucial prerequisite for their proper segregation to the two daughter cells in mitosis. The conserved protein Eco1 sets up cohesion in S-phase, but the mechanism of this establishment and how it is coupled to replication remains enigmatic. In this work, Eco1 was identified as a novel PCNA interactor. Eco1 contains a conserved N-terminal PCNA-interaction domain, known as a PIP-box, and PCNA binding could be shown to be required for normal loading of Eco1 onto chromatin in S-phase. Importantly, this process is essential for establishment of cohesion, as Eco1 mutants defective in PCNA binding die due to cohesion defects. Next, PCNA SUMOylation could be shown to inhibit Eco1 binding thereby repressing cohesion. This raises the intriguing possibility that the modification might help to keep certain chromosomal regions free of cohesin. In conclusion, this work identifies sister chromatid cohesion as yet another function which is under the direct control of PCNA and SUMO.

1. INTRODUCTION

1.1 Ubiquitin and SUMO

Posttranslational modifications of proteins are easily controllable and energetically inexpensive mechanisms to regulate protein functions. Posttranslational modifiers can be small molecules, like phosphate or acetyl groups, or entire proteins. Ubiquitin, a 76-residue protein, is the founding member of a class of protein modifiers that are covalently attached to substrate proteins in a highly regulated process. Ubiquitin and the ubiquitin-related modifier SUMO are the most abundant proteins of the family, both being highly conserved throughout species.

1.1.1 Enzymology of ubiquitin and SUMO conjugation

Most of the ubiquitin-family proteins are conjugated to substrates via a related enzymatic machinery (Figure 1). Ubiquitin and SUMO are synthesized as inactive precursors that are proteolytically processed at their C-termini to yield the active form. The cleavage is achieved by special proteases, called deubiquitylating enzymes (DUBs) for ubiquitin and Ubl-specific proteases (ULPs) for SUMO and other ubiquitin-like proteins. This processing exposes a C-terminal glycine residue whose carboxyl group becomes linked to a specific substrate lysine via an isopeptidic bond (Amerik and Hochstrasser, 2004; Johnson, 2004). Ubiquitin conjugation requires a cascade of three enzymes, generally called E1 or ubiquitin-activating enzyme, E2 or ubiquitin-conjugating enzyme and E3 or ubiquitin ligase (Pickart, 2001). The activating enzyme uses energy obtained from ATP hydrolysis to form a thiol-ester bond between the cysteine in its active site and the C-terminal carboxyl group of ubiquitin. The activated ubiquitin is subsequently transferred to the active site cysteine of the E2 by a trans-esterification reaction. E3 ligases bind ubiquitin-charged E2s and facilitate formation of an iso-peptidic linkage with the ε–amino group of a substrate lysine. Depending on how E3s accomplish this task, two classes can be distinguished. The HECT E3s first transfer ubiquitin from the E2 to a cystein residue in their active site, and only then onto the substrates. In contrast, the RING class of E3 ligases function as adaptors that bring together the substrate and the ubiquitin-charged active site of the E2, thereby facilitating the direct transfer of ubiquitin from the E2 to the substrate. Although some substrates are modified with a single ubiquitin moiety, in most cases ubiquitin chains are formed on the substrate, by attaching ubiquitin to a lysine residue of a previously conjugated ubiquitin. Efficient multi-ubiquitylation often requires special ligases (E4) (Koegl *et al.*, 1999).

Corresponding to the diverse roles ubiquitin plays in cell biology, the ubiquitin-modification machinery is organized hierarchically. In *S. cerevisiae*, there is one E1, eleven E2s and probably hundreds of E3s. E3 ligases determine the specificity of ubiquitylation, as they are primarily responsible for recognizing the substrate. While ubiquitin and the E1 are essential for viability, only one E2 (Cdc34) is required for survival in *S. cerevisiae*.

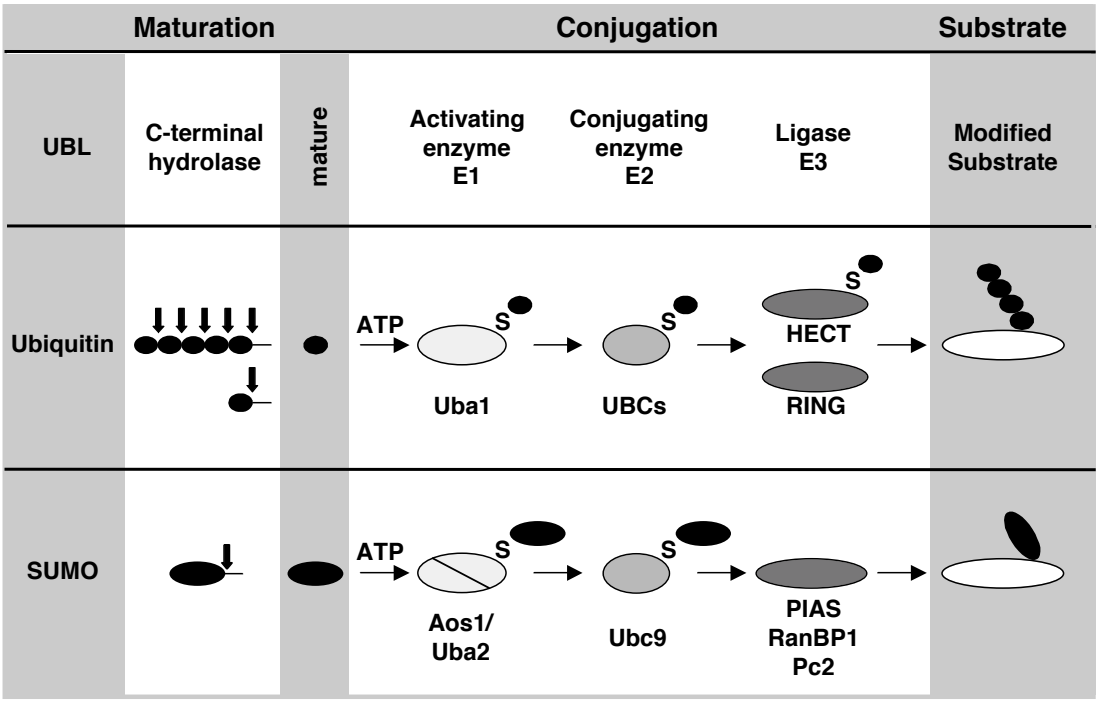

Figure 1. The conjugation systems of ubiquitin and SUMO. The modifiers mature by the action of C-terminal proteases and are covalently attached to substrates via an enzymatic cascade made up of an activating enzyme, a conjugating enzyme and a ligase. Enzymes marked with “S” form a thioesther link with the C-terminus of ubiquitin.

The SUMO conjugation pathway is analogous to that of ubiquitin, but requires a distinct set of enzymes (Johnson, 2004) The SUMO E1 is a heterodimer, each subunit bearing homology to one half (N- or C-terminal) of the ubiquitin activating enzyme. Only one E2, Ubc9, is responsible for SUMO conjugation. Ubc9 can bind substrates directly and, unlike ubiquitin-conjugating enzymes, it can modify them in the absence of an E3 ligase. Most SUMOylated lysines are located in the tetrapeptide motif ΨKxD/E (where Ψ is a hydrophobic residue, usually I, L or V), and crystallographic studies have shown that this motif is recognized directly by Ubc9, in the absence of any E3 (Bernier-Villamor *et al.*, 2002). Although the E1 and E2 are sufficient for SUMOylation, many SUMO substrates do require E3 ligase activity for efficient modification *in vivo*. Most SUMO E3 ligases belong to the Siz/PIAS family, characterized by the presence of a domain related to the RING finger

of ubiquitin ligases (Hochstrasser, 2001). Four PIAS proteins were described so far in mammals, and four in *S. cerevisiae* (Siz1, Siz2, Mms21 and Zip3). Two other types of ligases, namely RanBP2 and PC2 are present in higher eukaryotes (Kagey *et al.*, 2003; Pichler *et al.*, 2002).

Posttranslational modification with ubiquitin and SUMO are reversible processes. The same classes of enzymes required for maturation of the modifiers (DUBs and UBLs) are responsible for their removal from substrates.

1.1.2 Functions of ubiquitin

Although modification by ubiquitin or a ubiquitin-like protein (UBL) sometimes induces a conformational change in the substrate, thereby affecting its activity, in most cases, the conjugation alters the substrate ability to interact with its binding partners. This can occur by the modification itself conferring a new binding surface, or by masking a binding surface of the unmodified protein. In most cases, only a small fraction of the total pool of substrate protein is modified. Ubiquitylated substrates are often recognized by factors bearing a ubiquitin-binding domain. These domains can be found in hundreds of proteins and fall into al least nine distinct classes (Hicke *et al.*, 2005). Interestingly, it was reported that ubiquitin-binding proteins can be negatively regulated by mono-ubiquitylation, which blocks their own ubiquitin-binding domains (Hoeller *et al.*, 2006).

The best characterized role of ubiquitin conjugation is to target substrates to destruction by the 26S proteasome, a large protein complex with proteolytic activity (Glickman and Ciechanover, 2002; Pickart, 2001). Usually, this function requires the formation of multi-ubiquitin chains linked via lysine (K) 48 or K29 of ubiquitin, which are recognized by specific escort factors containing ubiquitin-binding domains, and delivered to the proteasome (Madura, 2004; Richly *et al.*, 2005). The ubiquitin/proteasome system is the most important pathway for controlling intracellular protein stability, ensuring both the elimination of aberrant proteins (Ellgaard and Helenius, 2003) and the regulated degradation of biologically active molecules, such as cell cycle regulators (Guardavaccaro and Pagano, 2006) or signal transducers (Muratani and Tansey, 2003). This makes ubiquitin modification invaluable for the coordination of a host of fundamental biological functions, and therefore essential for cellular viability.

Less prevalent types of ubiquitin modification, such as mono- or K63-linked multiubiquitylation do not signal proteasomal degradation, but rather control the target's function without affecting its stability. These modifications are involved in the regulation of many biologically relevant processes, some of which will be briefly outlined in the following. Receptor-mediated endocytosis

and subsequent lysosomal targeting are the best understood so far. In yeast, several plasma membrane-bound receptors can be mono-ubiquitylated or multi-ubiquitylated by short K63-linked chains, leading to their internalization and recognition by adaptors of the endocytosis machinery containing a ubiquitin-binding domain called UIM (ubiquitin-interacting motif) (Hicke and Dunn, 2003). Sorting and targeting of mono-ubiquitylated plasma membrane receptors to multi-vesicular bodies for lysosomal degradation requires further recognition of the modification by ESCRT (endosomal complexes required for transport) complexes (Katzmann *et al.*, 2002).

Transcriptional regulation displays ubiquitin-dependent regulation as well. Monoubiquitylation of histone H2B is a prerequisite for methylation of histone H3 which is involved in silencing of telomeric loci (Sun and Allis, 2002). Moreover, ubiquitylation can directly affect the activity of transcription factors. In *S. cerevisae*, Met4 is inactivated by ubiquitin modification, while the artificial LexA-VP16 transcription factor is only active after its ubiquitylation by the SCF^{MET30} ligase (Salghetti *et al.*, 2001).

The signaling pathways that lead to activation of the NF-κB transcription factor, essential for activation of survival genes in response to external and internal stresses, are modulated by non-degradative ubiquitin modifications at several levels. Plasma membrane receptor activation leads to formation of K63-linked multiubiquitin chains on the signal transducer TRAF6, which are recognized by the UIM-containing adaptors TAB2 and TAB3. In the alternative pathway for NF-κB activation, induced by internal stimuli like DNA damage, the transducer NEMO must be mono-ubiquitylated in order to shuttle from the nucleus to the cytoplasm and signal for the degradation of NF-κB inhibitors (Wu *et al.*, 2006). Interestingly, activation of another genome guardian protein, p53, is negatively regulated by ubiquitylation, as p53 mono-ubiquitylation by Mdm2 leads to its inactivation by shuttling it from the nucleus to the cytoplasm (Brooks and Gu, 2006).

Activation of at least two DNA damage response pathways requires degradation-independent ubiquitin signals. In response to various types of DNA damage, the Fanconi Anemia pathway component FANCD2 is mono-ubiquitylated, leading to its localization to chromatin foci, where it recruits several DNA repair enzymes (Huang and D'Andrea, 2006). In the post-replicative repair pathway, PCNA, a DNA polymerase cofactor, is mono-ubiquitylated or K63-linked multi-ubiquitylated in response to accumulation of DNA lesions (see paragraph 1.2.3). Mono-ubiquitylated PCNA recruits special repair enzymes called translesion polymerases (TLPs), while multi-ubiquitylation promotes a recombination-related DNA repair process (Hoege *et al.*, 2002; Kannouche *et al.*, 2004; Stelter and Ulrich, 2003).

1.1.3 Functions of SUMO

The number of known SUMO targets is continuously rising. Proteomic studies (Denison *et al.*, 2005; Hannich *et al.*, 2005; Panse *et al.*, 2004; Vertegaal *et al.*, 2004; Wykoff and O'Shea, 2005; Zhou *et al.*, 2004) have identified hundreds of target proteins, but in most cases the consequence of SUMOylation is unclear, as protein modifications by SUMO do not have a common functional consequence. Most of the SUMO substrates are nuclear, with the notable exception of septins, the major SUMO substrates in *S. cerevisiae*, which are localized at the bud neck. Similar to ubiquitin, SUMO is thought to be recognized by receptors bearing a SUMO recognition motif (Kerscher *et al.*, 2006). One such motif, called SBD (SUMO Binding Domain) was identified by large scale interaction studies (Hannich *et al.*, 2005; Minty *et al.*, 2000; Song *et al.*, 2004). Unlike ubiquitin-binding domains, SBD represent short sequences, typically three to four aliphatic residues followed by three to four negatively charged residues, forming a β-strand that binds to a hydrophobic surface groove of SUMO (Song *et al.*, 2005). SBDs are present also in the enzymes of the SUMO conjugation machinery and seem to be important for their activity (Takahashi and Kikuchi, 2005).

Whereas invertebrates have only one SUMO modifier, termed Smt3, four homologs, SUMO-1 to SUMO-4 are present in vertebrates. *S. cerevisiae* Smt3, and its conjugation machinery are essential for viability. Mutants in *SMT3* and *UBC9* are unable to accomplish mitosis, and they arrest at the G2/M transition with replicated DNA, short spindles and undivided nucleus (Dieckhoff *et al.*, 2004; Seufert *et al.*, 1995). *UBC9* is essential for nuclear integrity and chromosome segregation in higher eukaryotes as well, and its deletion leads to embryonic lethality in *Drosophila* and mice (Apionishev *et al.*, 2001; Nacerddine *et al.*, 2005). The SUMO target(s) essential for mitotic cell cycle progression have yet to be identified. Recently, SUMO modification and the SUMO ligase activity of Zip3 were shown to be required for normal formation of the synaptonemal complex, a structure required for the proper segregation of chromosomes during meiosis (Cheng *et al.*, 2006; Hooker and Roeder, 2006).

One early-characterized function of SUMO is its role in nucleo-cytoplasmic transport. In mammalian cells, RanGAP1 (the GTP-activating enzyme for Ran nuclear import factors) is SUMOylated by the SUMO ligase RanBP2 at the cytoplasmic face of the nuclear pore complex, and the modification is required for its transport through the pore. On the nuclear face, RanGAP1 is subsequently de-SUMOylated by ULP proteins (Mahajan *et al.*, 1997; Matunis *et al.*, 1998; Pichler *et al.*, 2002). Studies in *S. cerevisiae* and

D. melanogaster also suggested a role for SUMOylation in nuclear import, however the exact mechanism is not yet understood (Epps and Tanda, 1998; Stade *et al.*, 2002). SUMO modification is involved not only in transport to the nucleus, but as well in formation of nuclear sub-structures. The best understood example is the formation of PML (promyelocytic) bodies, which depends on prior SUMOylation of the PML proteins (Ishov *et al.*, 1999; Muller *et al.*, 1998).

Another general function of SUMO is transcriptional regulation. Although some transcription factors are activated by SUMOylation, in most cases SUMO modification leads to transcriptional repression, most likely by recruitment of chromatin remodeling factors, such as histone de-acetylases, that create a repressed chromatin state. For example, SUMOylation of the transcriptional regulators p300 and Elk-1 recruits HDAC6 and respctively HDAC2, members of the histone de-acetylase class of enzymes (Girdwood *et al.*, 2003; Yang *et al.*, 2003), while SUMOylation of histone H4 recruits HDAC1 and the structural heterochromatin component HP1 (Shiio and Eisenman, 2003). Interestingly, in *S. pombe* the SUMO E2 enzyme Ubc9 was shown to be recruited to silenced loci by the heterochromatin proteins Swi6 and Chp2, suggesting that SUMOylation is important not only for establishment, but as well for maintenance of silencing (Shin *et al.*, 2005). The role of SUMO in transcriptional repression appears to be extremely relevant for development and is implicated in many pathologies. For example, SUMOylation of the transcription factors Sox-E and MEF2 is required for proper neural crest development (Taylor and Labonne, 2005), and respectively neuronal synapse maturation (Flavell *et al.*, 2006; Shalizi *et al.*, 2006), while SUMOylation of reptin leads to transcriptional repression of the metastasis supressor *KAI1* and thus promotes cancer metastasis (Kim *et al.*, 2006).

Transcriptional regulation is not the only chromatin process which is regulated by SUMO, as SUMOylation additionally controls DNA and chromosome integrity. The SUMO ligase Mms21 is part of the DNA repair Smc5/Smc6 complex (see paragraph 1.3.1). Mms21 can SUMOylate Smc5 and the repair protein Yku70, and its activity is required for nucleolar and telomeric integrity, telomeric silencing and DNA repair (Zhao and Blobel, 2005).

Other SUMO substrates relevant for DNA repair are the homologous recombination protein Rad52 (Ho *et al.*, 2001; Sacher *et al.*, 2006), the Werner (WRN) and Bloom (BLM) helicases, regulators of recombination (Eladad *et al.*, 2005; Kawabe *et al.*, 2000), the DNA de-catenating enzyme topoisomerase I (Mao *et al.*, 2000), the replication protein PCNA (see

paragraph 1.2.3), the non-homologous end-joining repair protein XRCC4 (Yurchenko *et al.*, 2006) and the base excision repair enzyme thymine-DNA glycosylase (TDG) (Hardeland *et al.*, 2002). Interestingly, SUMOylation of TDG is one of the few cases known to lead to a conformational switch in the modified protein. This switch decreases TDG's affinity for DNA, allowing its release from chromatin after having performed its enzymatic activity (Baba *et al.*, 2005). However, a more general function of SUMOylation in DNA repair seems to be an involvement in repair foci formation, as SUMOylation of BLM, XRCC4 or topoisomerase 1 were reported to regulate their chromatin accumulation in response to DNA damage (Eladad *et al.*, 2005; Mo *et al.*, 2002; Yurchenko *et al.*, 2006).

Interestingly, there are examples of functional cooperation between ubiquitin and SUMO modification. Ubiquitylation of NEMO requires its initial SUMOylation (Wu *et al.*, 2006), while on PCNA, ubiquitylation and SUMOylation, although occuring at the same residue, do not compete with each other, but rather functionally collaborate to promote postreplicative repair (Hoege *et al.*, 2002; Pfander *et al.*, 2005). In other cases, however, the two modifications can antagonize each other. SUMOylation of IκBα, an NF-αB inhibitor, appears to inhibit its degradation by blocking the lysine required for ubiquitylation (Desterro *et al.*, 1998), and the ubiquitin-conjugating activity of E2-25K is inhibited by its SUMOylation (Pichler *et al.*, 2005).

1.2 Eukaryotic DNA replication

Faithful DNA replication is fundamental to the maintenance of genomic integrity in all organisms. In eukaryotic cells, replication is initiated at origins of replication, which are chromosomal sites that direct the assembly of a macromolecular complex called origin recognition complex (ORC) (Bell and Dutta, 2002). ORCs coordinate the loading of replication factors at origins, culminating in the loading of DNA polymerases and their co-factors, thus resulting in the formation of active replication machineries. The replication forks proceed bi-directionally, performing DNA synthesis in a tightly coordinated manner.

1.2.1 The replication machinery

DNA polymerases are enzymes that catalyze phosphoryl transfer in a template-directed mechanism, synthesizing long polymers of nucleoside monophosphates (Hubscher *et al.*, 2002; Johnson and O'Donnell, 2005; Waga and Stillman, 1998). The choice of nucleotide insertion is dictated by

the sequence of the complementary strand used as template. In addition, replicative polymerases have a 3'->5' proofreading activity, which allows them to remove misincorporated nucleotides. Eukaryotic cells have several polymerases, with well-defined functions in replication and repair DNA synthesis. The DNA polymerase α/ primase complex is the first to associate with the initiation complex at replication origins, where it synthesizes a primer consisting of 10 RNA nucleotides followed by 20 DNA bases. This is then extended by the processive polymerases δ or ε, which exchange for Polε in a process called polymerase switching. While on the leading strand (3'->5') synthesis occurs by continuous elongation of the initial primer, on the lagging strand (5'->3') it proceeds by formation of Okazaki fragments, short (200bp) DNA pieces resulting from consecutive re-initiation of synthesis by the primase and subsequent elongation by Polε or Polε. However, synthesis on the lagging and leading strand are coupled, so that the replication fork contains 2 polymerases, one for each strand. Although the exact architecture of the eukaryotic replisome is not known, it seems possible that the two polymerases, Polε and Polε, are placed on different strands at the same

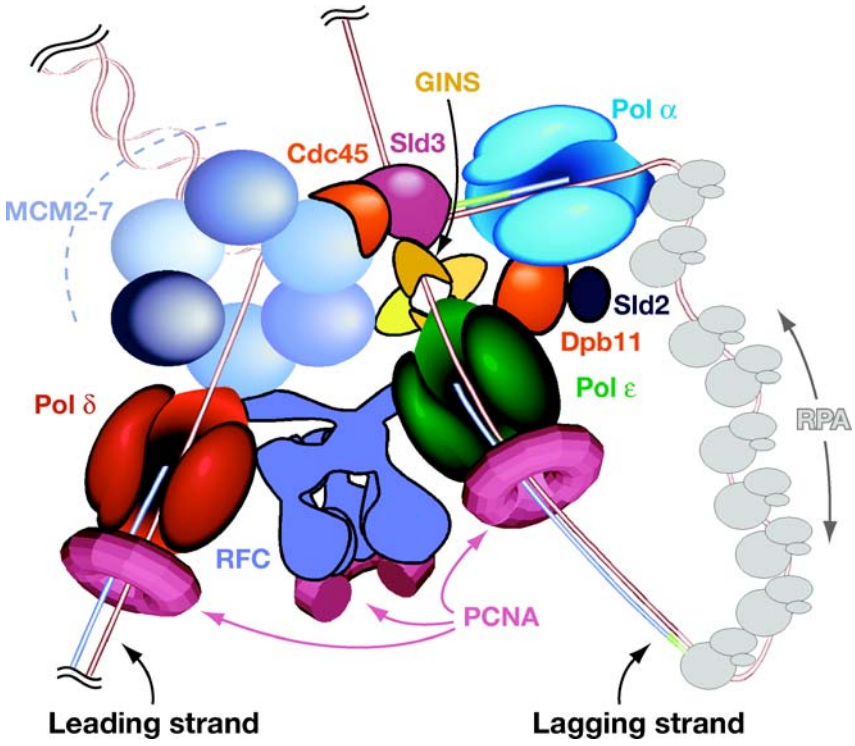

Figure 2. Model of the eukaryotic replisome. The two polymerases (in this model, Polε and Polε are shown on different strands) are tethered to DNA by PCNA. The PCNA loading complex RFC might bridge the two polymerases. The MCM helicase complex encircles the leading strand, while Polε /primase and the single strand binding protein RPA are functioning on the lagging strand. Other factors associated with the replication fork are also shown: Cdc45, Sld2-3, Dpb11 and the GINS complex. The machinery for maturation of Okazaki fragments on the lagging strand is not depicted (see paragraph 1.2.2) (Johnson and O'Donnell, 2005)

replication fork (Johnson and O'Donnell, 2005) (Figure 2). However, only Polδ, but not Polε is essential for viability in yeast, suggesting that Polε can perform also the functions of Polε. Other components of the replication fork machinery are the polymerase co-factor PCNA and, possibly, its activating complex RFC (see paragraph 1.2.2), which might be the connecting factor between the two polymerases, the helicase complex MCM2-7, the single strand binding protein RPA, and the associated proteins Cdc45, Sld2, Sld3, Dbp11 and the heptameric GINS complex (Johnson and O'Donnell, 2005).

In *S. pombe* and human cells, Polε has four subunits. In *S. cerevisiae*, it is composed of three subunits: Pol3 (essential catalytic subunit), Pol31 (essential regulatory subunit) and Pol32 (non-essential regulatory subunit) (Johansson *et al.*, 2001). While Pol3 and Pol31 are globular, Pol32 has an elongated structure and is required *in vivo* and *in vitro* for dimerization and for the interaction with the co-factor PCNA, although the other subunits can bind PCNA as well (Bermudez *et al.*, 2002; Burgers and Gerik, 1998; Gerik *et al.*, 1998; Johansson *et al.*, 2004; Johansson *et al.*, 2001). Cells lacking the non-essential subunit Pol32 show replication defects, and *in vitro* replication in the absence of Pol32 is inefficient and characterized by frequent pausing (Burgers and Gerik, 1998; Gerik *et al.*, 1998). This is consistent with the fact that in the absence of Pol32, Polε interacts much weaker with its processivity factor PCNA. Polε is as well the major polymerase performing DNA synthesis in DNA repair processes like mismatch repair (Jiricny, 2006) or base-excision repair (Showalter *et al.*, 2006).

A major drawback of replicative polymerases is that their high fidelity does not allow them to replicate through DNA lesions. Modified bases, like pyrimidine dimers or alkylated bases cannot enter the active site of polymerases, therefore the progression of the replication fork is blocked upon encountering such lesions in the DNA template (Prakash *et al.*, 2005). As prolonged replication fork stalling is extremely toxic, leading to single-strand gaps or even to dissociation of the replication machinery and double strand break formation (Barbour and Xiao, 2003), cells have evolved bypass mechanisms that allow for restarting of stalled replication forks. One such mechanism uses *RAD52*-dependent recombination (Cox, 2002), while two other are dependent on the *RAD6* pathway: the error-free bypass, which probably involves a switch of the template to the newly-replicated strand of the sister chromatid, and the error-prone bypass (Broomfield *et al.*, 2001). The latter mechanism uses special polymerases, called translesion polymerases (TLPs) (Prakash *et al.*, 2005; Rattray and Strathern, 2003) which have a lower affinity for the template and can therefore accommodate modified bases in

their active sites. However, not having a strict template requirement, they can lead to introduction of wrong nucleotides, thereby causing mutations. The switch from a replicative polymerase to a TLP is induced by replication fork stalling at a DNA lesion and is mediated by posttranslational modifications of PCNA (see paragraph 1.2.3). In parallel, a special signaling pathway, known as the replication checkpoint, is used for preventing the collapse of stalled replication forks (Lopes *et al.*, 2001; Sogo *et al.*, 2002).

In *S. cerevisiae*, the major TLPs are Polη/Rad30 (inserts preferentially adenines), Rev1 (inserts preferentially cytosines) and Polζ/Rev3-Rev7 (can extend mispaired products formed by the activity of other TLPs). Moreover, Pol32 was shown to be involved in translesion synthesis, but it is not clear whether it acts in this process together with Polδ, or as a subunit of a TLP (Huang *et al.*, 2000; Huang *et al.*, 2002). Polδ seems also to participate in double strand break repair, as it was shown to be required for extending 3' ends of strands exchanged during homologous recombination (Kawamoto *et al.*, 2005; McIlwraith *et al.*, 2005).

1.2.2 Proliferating Cell Nuclear Antigen

Proliferating cell nuclear antigen (PCNA) is a member of the DNA sliding clamp family of proteins, which are structurally and functionally conserved from viruses to humans (Maga and Hubscher, 2003; Tsurimoto, 1998). Although there is no obvious sequence similarity between the bacterial member, the β clamp, and eukaryotic PCNA, crystalographic studies have shown that they have a superimposable three-dimensional structure (Kong *et al.*, 1992; Krishna *et al.*, 1994). They form ring-shaped complexes (homodimers in bacteria and homotrimers in eukaryotes) with hexameric symmetry which encircle the DNA and are able to slide freely along it, in both directions. This topological association with DNA allows the sliding clamps to tether DNA polymerases at the replication fork, making them essential co-factors of polymerases. *In vitro*, the presence of PCNA increases the processivity of DNA polymerases from tens of nucleotides to thousands (Prelich *et al.*, 1987; Tan *et al.*, 1986). Concordantly, PCNA is essential for viability.

PCNA monomers have two similar, globular domains, linked by a long loop called inter-domain connecting loop (IDCL). Head-to-tail arrangement of three monomers form the ring, which has two non-equivalent surfaces: an inner surface, formed by α–helices, which binds DNA and an outer surface, composed of α–sheets. This outer surface is responsible for interacting with DNA polymerases, and stabilizing them on DNA. Multiple interaction sites, including the IDCL and the C-terminus of PCNA are involved in polymerase

binding (Johansson *et al.*, 2004; Majka and Burgers, 2004). The assembly of PCNA on DNA requires the activity of a family of conserved chaperone complexes, called clamp loaders or replication factor C (RFC) in eukaryotes (Majka and Burgers, 2004). RFC is a complex of five essential proteins, showing similar structure and a helical disposition. RFC can open and close PCNA rings in an ATP hydrolysis-driven mechanism. ATP binding is required for formation of a stable PCNA-RFC complex and for its loading to primer-template junctions. DNA binding activates the ATP hydrolysis activity of RFC, leading to its dissociation from the loaded clamp (Bowman *et al.*, 2004). The Rfc1 subunit of the complex has N- and C-terminal extensions in addition to a region of homology shared by the four small subunits Rfc2-5. While Rfc1 is required for PCNA loading, it is dispensable for PCNA unloading (Yao *et al.*, 2006) and can be replaced by other homologs, forming alternative complexes with specialized roles (Majka and Burgers, 2004).

Besides being required for polymerase tethering, a second essential function of PCNA in replication is coordinating the maturation of Okazaki fragments (Garg and Burgers, 2005). When the lagging strand polymerase encounters the RNA-primed DNA, a complex with the endonuclease FEN1 is formed, which is required for efficient displacement of the RNA primer. As soon as the RNA-DNA junction of the Okazaki fragment is reached, DNA ligase I is recruited to the complex to ligate the nick. PCNA interaction with both FEN1 and DNA ligase I is required for coordinating this process, and, *in vitro*, PCNA can stimulate the activity of both proteins presumably by tethering them to DNA.

In addition to the DNA synthesis roles, PCNA is involved in many replication-linked processes. Devoid of enzymatic activity, it is ideally suited to function as a recruitment platform for factors involved in DNA repair, chromatin modification or replication control (Prosperi, 2006; Sakurai *et al.*, 2005; Tsurimoto, 1999; Vivona and Kelman, 2003; Warbrick, 2000). Interestingly, most of these factors bear the conserved motif **Q**xx**h**xx**aa** (where **h** is one of the hydrophobic residues L, M or I and **a** is one of the aromatic residues F and Y), termed PIP (PCNA interacting protein)-box. Structural studies have shown that this peptide is folded into a 3_{10} helix that acts as a hydrophobic plug, docking into a hydrophobic pocket of PCNA buried under the IDCL (Bowman *et al.*, 2004; Bruning and Shamoo, 2004; Gulbis *et al.*, 1996; Matsumiya *et al.*, 2002). All RFC subunits, several subunits of Polδ and Polε, FEN-1 and DNA ligase I have PIP boxes, through which they interact with PCNA. As a PCNA ring has three hydrophobic grooves, three different PIP box proteins can bind to the clamp at the same time. The presence of a common binding site for replication proteins on PCNA

is exploited by metazoans for regulating cellular proliferation. The tumor supressor p21, which contains a PIP-box in its C-terminal domain, can bind PCNA with high affinity, effectively displacing polymerases and other replication proteins and thus blocking DNA replication (Gulbis *et al.*, 1996; Rousseau *et al.*, 1999; Waga *et al.*, 1994). The N-terminus of p21 binds to and inhibits cyclin-dependent kinases (CDKs), providing a second mechanism by which this protein represses proliferation (Rousseau *et al.*, 1999). PCNA interacts with CDKs as well, and a ternary complex CDK2-cyclinA-PCNA was proposed to be required for activating proliferation, by phosphorylating RFC and DNA ligase I (Koundrioukoff *et al.*, 2000).

Novel PIP box containing proteins, being recently reported, expanded the repertoire of PCNA-dependent replication functions. Binding of the origin of replication licensing factor Cdt1 to PCNA is required for Cdt1 degradation after initiation of replication, thereby preventing re-replication (Arias and Walter, 2006). Furthermore, the interaction of the replication fork components Mcm10 and PCNA is essential for replication and viability in yeast, but its functional consequence remains unknown (Das-Bradoo *et al.*, 2006).

PCNA is important also for repair of injured DNA. PCNA functions not only as polymerase co-factor during repair synthesis, but was shown to directly recruit repair factors to sites of DNA lesions and to coordinate repair events. Among the many repair pathways known, mismatch repair (MMR) is one of the best characterized for PCNA involvement. The MMR system repairs misincorporation errors generated during replication. PCNA interacts with several MMR proteins, involved at different steps of the process: the sensor complexes MSH2-MSH6 (recognizes mismatches) and MSH2-MSH3 (recognizes small insertions or deletions), the effectors-recruiting complex MHL1-PMS1 and the effector exonuclease EXO1 (Clark *et al.*, 2000; Flores-Rozas *et al.*, 2000; Kleczkowska *et al.*, 2001; Lee and Alani, 2006). These interactions suggest that PCNA acts as a scaffold to coordinate the sequential recruitment of MMR factors to the mismatch site. Because of its orientation-dependent loading on DNA, PCNA was proposed to be required for discriminating between the newly synthesized and the parental strand, thereby directing the repair machinery to the newly synthesized strand (Umar *et al.*, 1996).

PCNA is also involved in other repair pathways. For example, it can stimulate the activities of several base excision repair (BER) enzymes. The *S. cerevisiae* 3'-5' exonuclease Apn2, which is involved in repair of DNA strand breaks with 3'-damaged ends, induced by oxygenic stress, has a PIP-box and is stimulated by PCNA binding (Unk *et al.*, 2002). The human homolog APE2 co-localizes with PCNA in DNA repair foci (Tsuchimoto *et al.*, 2001).

Moreover, PCNA interacts with XRCC1, a scaffold protein that organizes the BER complex (Fan *et al.*, 2004), and binds and activates at least two glycosylases: UNG2, an enzyme that initiates the repair of misincorporated uracils (Ko and Bennett, 2005), and MPG, required for excision of 3-methyladenine (Xia et al., 2005). In nucleotide excision repair (NER), the endonuclease XPG, required for excision of a small segment of single-stranded DNA containing the lesion, interacts with PCNA, and although XPG activity is not enhanced by PCNA, it is possible that the interaction might stabilize the repair complex (Gary *et al.*, 1997). Moreover, PCNA interacts with the mammalian helicase WRN (Werner syndrome helicase) and with the *S. cerevisiae* helicase Rrm3 (Lebel *et al.*, 1999; Schmidt *et al.*, 2002). Both helicases are involved in promoting repair of DNA lesion at the replication fork. Another pathway by which PCNA facilitates DNA repair involves the recruitment of the histone acetyltransferase p300 to DNA damage sites, upon which p300 can affect the local chromatin structure in order to promote DNA repair synthesis (Hasan *et al.*, 2001).

In order for replication to take place, the compact chromatin conformation must be opened. Importantly, the information encrypted in the chromatin structure must be transmitted to the sister chromatids after replication. This process, called epigenetic inheritance, takes place co-replicationally and is under direct control of PCNA. In yeast cells, PCNA recruits the chromatin assembly factor CAF1 to the replication fork in order to coordinate nucleosome deposition and heterochromatin formation (Zhang *et al.*, 2000). Moreover, in human cells, PCNA binding was shown to be required for targeting of the WSTF transcription factor to replication foci, thereby inducing chromatin remodeling by ISWI remodelling factors (Poot *et al.*, 2004). PCNA binds also to the DNA cytosine methyltransferase 1 (Dnmt1), the enzyme required for methylation of the newly synthesized strand, and thus for inheritance of methylated status (Iida *et al.*, 2002).

PCNA has also been implicated in coordinating programmed cell death, as its interaction with the tumor supressor UNG1 is required for DNA damage-induced apoptosis (Scott *et al.*, 2001). In contrast, binding of PCNA to MyD118 and Gadd45 represses their negative growth control functions (Vairapandi *et al.*, 2000).

1.2.3 Posttranslational modifications of PCNA

The number of known PCNA-controlled DNA repair pathways was further increased by the discovery of S-phase specific posttranslational modifications of *S. cerevisiae* PCNA by ubiquitin and SUMO (Hoege *et al.*, 2002) (Figure 3). PCNA ubiquitylation directs the bypass of DNA lesions by the replication

machinery. At stalled forks, PCNA is mono-ubiquitylated at the conserved lysine 164 by the E2 conjugating enzyme Rad6 and the E3 ligase Rad18, a single stranded DNA-binding protein that might recognize damaged DNA (Bailly *et al.*, 1997). Mono-ubiquitylation of PCNA promotes a switch from the replicative polymerase to a translesion polymerase, like Rad30 or Rev1, which have binding sites for both PCNA and ubiquitin and therefore a higher affinity for the ubiquitylated form of PCNA (Bienko *et al.*, 2005; Garg and Burgers, 2005; Kannouche *et al.*, 2004; Stelter and Ulrich, 2003; Watanabe *et al.*, 2004). This repair system is known as the error-prone branch of the *RAD6* repair pathway. Alternatively, PCNA can be modified at the same lysine by K63-linked ubiquitin chains. Besides Rad6 and Rad18, this modification requires the E2 heterodimer Mms2-Ubc13 and the E3 Rad5 (Hoege *et al.*, 2002; Ulrich and Jentsch, 2000). Multi-ubiquitylation promotes an error-free repair which is likely to involve a switch of the template to the newly replicated strand of the sister chromatid (Xiao *et al.*, 2000; Zhang and Lawrence, 2005).

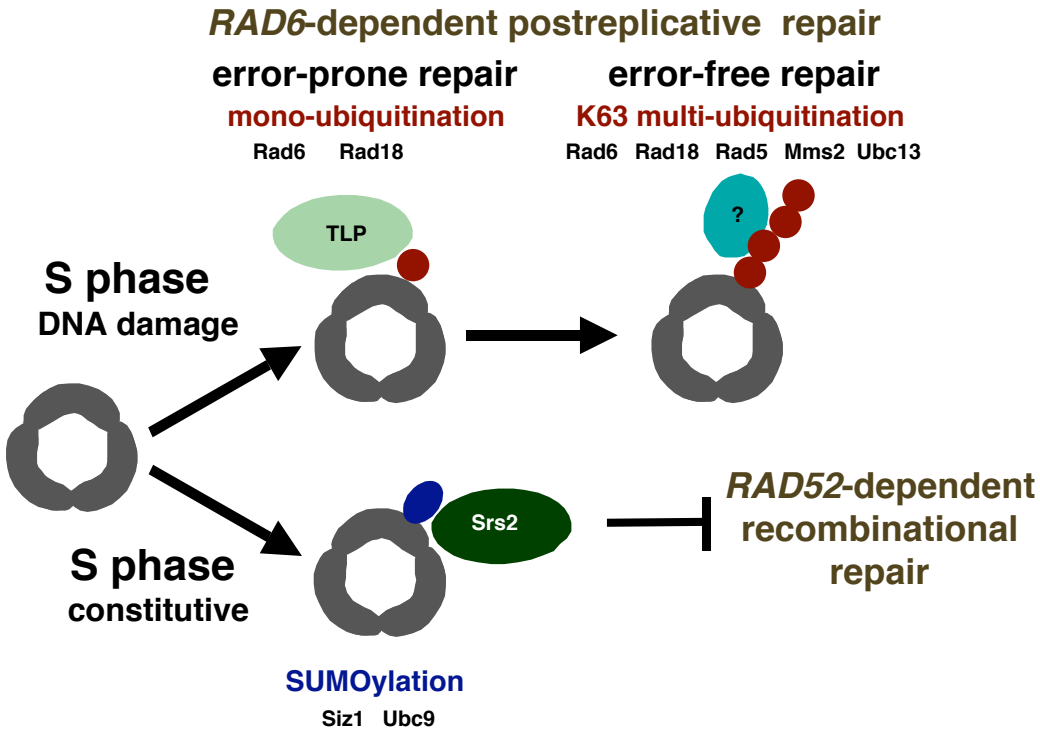

Figure 3. Posttranslational modifications of PCNA in S-phase. In response to DNA damage, the enzymes of the *RAD6* DNA repair pathway ubiquitylate PCNA at K164. PCNA is either mono-ubiquitylated, leading to recruitment of an error-prone translesion polymerase, or multi-ubiquitylated by K63-linked ubiquitin chains, which mediates an error-free repair. The factors that mediate the error-free pathway downstream of PCNA ubiquitylation are not known. Constitutively, PCNA is modified by SUMO, which recruits Srs2 to inhibit *RAD52*-dependent recombination at the replication fork.

PCNA SUMOylation occurs constitutively in S-phase (Hoege *et al.*, 2002) and represents a mechanism for protecting the replication fork from unwanted recombination events (Papouli *et al.*, 2005; Pfander *et al.*, 2005). SUMOylated PCNA recruits the helicase Srs2, which inhibits recombination by disrupting Rad51 nucleofilaments (Krejci *et al.*, 2003; Veaute *et al.*, 2003). This inhibition ensures that, upon encountering of a DNA lesion, the *RAD6*-dependent replication restart, and not *RAD52*-dependent recombination is used for processing the lesion. Attachment of SUMO to PCNA takes place at two different lysines: the bulk of SUMOylation occurring at K164, and requiring the activity of the SUMO E3-ligase Siz1. To a lesser extent, SUMO is attached also to a second acceptor site, the non-conserved K127. Unlike K164, K127 is present in a consensus site for SUMOylation (see paragraph 1.1.1) and does not seem to require an E3 ligase activity (Hoege *et al.*, 2002; Pfander *et al.*, 2005).

The importance of PCNA modifications in lesion bypass is highlighted by the fact that loss of the regulation of PCNA modifications leads to gross chromosomal rearrangements (GCRs), hallmarks of many cancers (Motegi *et al.*, 2006).

1.3 Sister chromatid cohesion

DNA replicated in S-phase must be faithfully segregated to the daughter cells during mitosis. To ensure proper segregation, the identity of sister chromatids is preserved throughout G2 by keeping them paired in close proximity to each other. This physical coupling, termed sister chromatid cohesion (Haering and Nasmyth, 2003; Nasmyth and Schleiffer, 2004) is only abrogated at the metaphase to anaphase transition, allowing migration of the sister chromatids to separate cells. Cohesion is essential for viability in *S. cerevisiae*, as in its absence cells prefer to block their cell cycle in order to avoid faulty distribution of the chromosomes. This process controls not only the accuracy, but as well the timing of DNA separation, as it allows kinetochores to be captured by opposite poles of the mitotic spindle and subsequently it resists the pulling forces of the microtubules until anaphase. The cohesion activity is strictly correlated with the chromosome cycle (Figure 4). Cohesion is established in S-phase, concomitantly with DNA replication. The sister chromatids are glued together throughout G2, until they are released at the metaphase to anaphase transition, and migrate to separate cells.

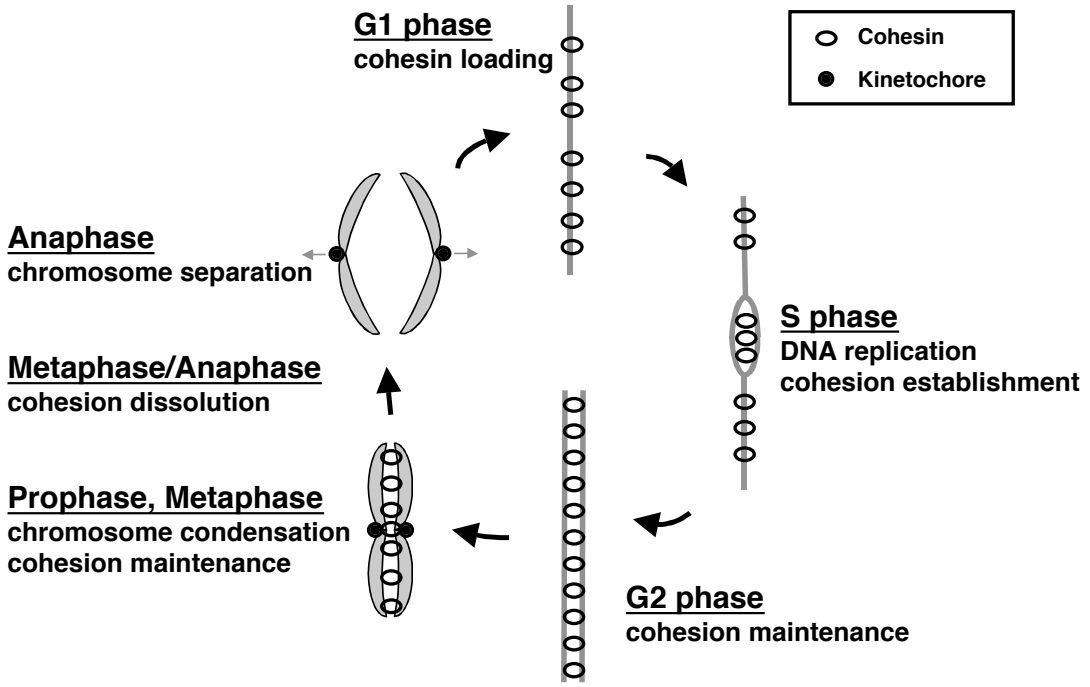

Figure 4. The mitotic cohesion cycle. Cohesin binds in G1 to unreplicated chromosomes. Cohesion is established during S.phase, concomitant with DNA replication, and maintained throughout G2 and early mitosis, when chromosomes condense. Cohesion is abolished in late mitosis, allowing sister chromatids to separate (Carson and Christman, 2001).

1.3.1 The cohesin complex

Cohesion is ensured by a protein complex called cohesin, composed of Smc1 (Chl10), Smc3, Scc1 (Mcd1) and Smc3 (Irr1) (Hirano, 2006; Michaelis *et al.*, 1997; Nasmyth and Haering, 2005). Smc1 and Smc3 belong to the SMC superfamilly, which contain as well the recombination protein Rad50, the bacterial MukB, the condensins Smc2 and Smc4 and the DNA repair proteins Smc5 and Smc6. These proteins have globular N and C-terminal domains, separated by long streches of coiled-coil, which are interrupted by central globular domains. In the cohesin complex, Smc1 and Smc3 interact with the kleisin family members Scc1 (in the mitotic cohesin complex) or Rec8 (in the meiotic cohesin complex), while in the condensin complex, Smc2 and Smc4 interact with the kleisins CAP-H or CAP-H2. Smc1 and Smc3 dimerize via their central globular (hinge) domains, and formation of intra-molecular coil-coils leads to association of the N and C-terminal globular domains (Figure 5) (Haering *et al.*, 2002). This is supposed to lead to the formation of a functional ATPase domain, from the Walker A motif of the N-terminus and the Walker B motif of the C-terminus (Lowe *et al.*, 2001). A single Scc1 molecule binds to the head domains of both Smc1 and Smc3, forming a closed ring. Scc3, the fourth cohesin subunit, binds the complex via Scc1 (Haering *et al.*, 2002).

The cohesin ring most likely acts by encircling the two sister chromatids formed by DNA replication (Figure 5). With a proposed diameter of 35nm, the ring is wide enough to accommodate two chromatin fibers of 10nm each (Haering and Nasmyth, 2003). While cohesin could not be removed from chromatin by high salt concentrations (Ciosk *et al.*, 2000), cleavage of engineered Smc subunits led to dissociation of the entire complex from DNA and destroyed sister chromatid cohesion (Gruber *et al.*, 2003). Conversely, the cohesin complex could be co-purified with circular minichromosomes (plasmids), and could be released only by cleavage of the plasmid itself, suggesting a topological association between cohesin and chromatin (Ivanov and Nasmyth, 2005).

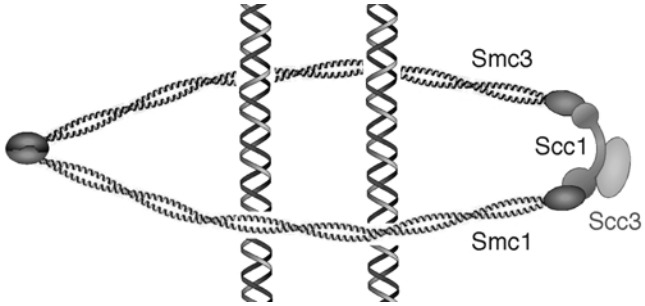

Figure 5. Model of the cohesin complex forming a ring that encircles the two sister chromatids. SMC proteins form internal coiled-coils and dimerize via their central hinge domain, while their globular N- and C-termini form a domain that bind Scc1 ends. Scc3 is associated to the complex via Scc1. (Adapted from Dr. Olaf Stemmann.)

1.3.2 Cohesion establishment

The cohesin complex is loaded on DNA immediately after chromosome separation, in telophase and early G1. This process requires the loading complex Scc2/Scc4. In its absence, the cohesin complex is formed, but is not associated with DNA (Ciosk *et al.*, 2000; Watrin *et al.*, 2006). Thus, Scc2 and Scc4 are required for establishment, but not for maintenance of sister chromatid cohesion. Although the mechanism of cohesin loading on chromsomes is not fully understood, it requires the ATP-ase activities of Smc1 and Smc3, which might be stimulated by Scc2/Scc4 (Arumugam *et al.*, 2003). Moreover, cohesion loading and replication initiation are apparently co-regulated, as Scc2 activity which loads cohesin on DNA requires licensing of the replication origins by formation of the pre-replication complexes (Gillespie and Hirano, 2004; Suter *et al.*, 2004; Takahashi *et al.*, 2004).

Cohesion between the sister chromatids is established already during DNA replication (Gerlich *et al.*, 2006; Uhlmann and Nasmyth, 1998). For proper chromosome cohesion and thus viability, the cohesin complex is needed not only during G2, but as well in S-phase (Toth *et al.*, 2000; Uhlmann

and Nasmyth, 1998). Although the mechanism of cohesion establishment, and how it is coupled to S-phase is not known, it is likely that cohesin rings tie up the sister chromatids instantly after replication (Skibbens, 2000).

In *S. cerevisiae*, the essential protein Eco1/Ctf7 is required exclusively during S-phase in order to establish sister chromatid cohesion (Skibbens *et al.*, 1999; Toth *et al.*, 2000). *ECO1* mutants arrest in the G2/M checkpoint, because inefficient cohesion in these cells leads to lack of tension across the kinetochores. The Eco1 protein contains a C2H2-type zinc finger and an acetyl transferase domain and can acetylate several substrates *in vitro*, including Scc1, Pds5 and itself (Ivanov *et al.*, 2002). However, no *in vivo* targets were discovered so far, and the importance of this activity was questioned, as mutants that loose the acetyl transferase activity *in vitro* show no obvious phenotypes (Brands and Skibbens, 2005). Although essentially nothing is known on how Eco1 mediates cohesion in S-phase, it was proposed that the protein localizes to the replication fork, where it can convert a pre-cohesion site (unreplicated DNA with pre-loaded cohesin complex) into a cohesion site (replicated DNA, the two sister chromatids being encircled by the cohesin ring) (Skibbens, 2000). This model is supported by genetic evidence showing a link between replication and cohesion: *ECO1* mutants are lethal in combination with *PCNA* mutants, and can be rescued by *PCNA* overexpression (Skibbens *et al.*, 1999).

Several Eco1 interactors were recently shown to be required for efficient establishment of cohesion, without being essential for the process. They include the helicase Chl1, an alternative PCNA loading/unloading complex composed of Ctf18, Ctf8, Dcc1, Rfc2-5, the spindle pole body subunit Mps3, the DNA polymerases ε and α and the polyA polymerase σ (Antoniacci *et al.*, 2004; Edwards *et al.*, 2003; Kenna and Skibbens, 2003; Mayer *et al.*, 2001; Read *et al.*, 2002; Skibbens, 2004; Wang *et al.*, 2000; Zhou *et al.*, 2004). Genetic studies have shown that there is an interplay between different replication-linked processes, like establishment of cohesion, DNA repair and epigenetic silencing. Thus, the polymerase σ-associated protein Ctf4, the RSC chromatin remodeling complex, the DNA helicases Srs2, Rrm3 and Sgs1 and the DNA repair complexes Mre11-Rad50-Xrs2 and Tof1-Mrc1-Csm3 are apparently all required for proper cohesion (Baetz *et al.*, 2004; Hanna *et al.*, 2001; Mayer *et al.*, 2001; Warren *et al.*, 2004). Moreover, the helicase Chl1 participates in gene silencing and DNA recombination as well (Das and Sinha, 2005), while Ctf18 is required for localization of telomeres to the nuclear periphery (Hiraga *et al.*, 2006), for the DNA replication checkpoint (Naiki *et al.*, 2001) and for unloading PCNA from DNA (Bermudez *et al.*, 2003).

Eco1 is conserved throughout species, however its domain organization varies. All homologs contain the bulk of *S. cerevisiae* Eco1 in their C-termini, but have long N-terminal extensions. The *S. pombe* Eso1 contains a domain bearing homology to the Rad30 translesion polymerase (Madril *et al.*, 2001; Tanaka *et al.*, 2000). One ortholog has been identified in *D. melanogaster* and at least two in mammals. The N-terminal domains of these proteins are not conserved between species and show no homology to known domains, except for the one present in mammalian EFO1, which shows similarity to a micronuclear linker histone identified in *Tetrahymena*. (Bellows *et al.*, 2003; Hou and Zou, 2005; Williams *et al.*, 2003). The N-terminal extensions of human Eco1 proteins (named ESCO1 and ESCO2) were proposed to be involved in DNA binding (Hou and Zou, 2005). Recently, mutations in *ESCO2* were shown to cause severe developmental diseases, the Roberts and SC phocomelia syndromes, characterized by growth retardation, microcephaly, craniofacial anomalies and reduction in limb length (Schule *et al.*, 2005; Vega *et al.*, 2005). Cells from Roberts syndrome patients, as well as from *Drosophila* mutants in the Eco1 homolog show defects in sister chromatid cohesion and chromosome segregation, demonstrating that Eco1 function is well conserved (Vega *et al.*, 2005; Williams *et al.*, 2003).

1.3.3 Cohesion maintenance

After establishment in S-phase, cohesion is maintained throughout G2 and early mitosis, when chromosomes condense, until anaphase. The protein Pds5, essential in *S. cerevisiae*, associates with the cohesin complex on chromosomes and is required for maintenance of cohesion, but not for its establishment (Hartman *et al.*, 2000). Pds5 is SUMOylated from early S-phase until the beginning of anaphase, and it appears that this modification facilitates cohesion dissolution, as overexpression of the de-SUMOylating enzyme Ulp2 can rescue the cohesion defects of *PDS5* mutants (Stead *et al.*, 2003). In *S. pombe*, Pds5 is not required for viability, and surprisingly, inactivation of *PDS5* can rescue the lethality of an *ECO1* deletion, suggesting that in this organism Pds5 has a second, cohesion inhibitory activity, that is counteracted by Eco1 (Tanaka *et al.*, 2001).

During G2, the cohesin rings were shown to be pushed by the transcriptional machinery to untranscribed intergenic regions (Lengronne *et al.*, 2004), showing that DNA-bound cohesin is mobile and thereby confirming the topological model for cohesin association with chromatin.

1.3.4 Cohesion dissolution and sister chromatid separation

In order for the sister chromatids to be separated in anaphase, the cohesin rings must be removed from the chromosomes. In the late 1990s, the anaphase-promoting complex or cyclosome (APC/C) was identified to be essential for this function (Irniger *et al.*, 1995; King *et al.*, 1995). APC/C is a ubiquitin ligase, that together with its activator Cdc20 initiates sister chromatid separation, by degrading the anaphase inhibitor securin (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1996). Securin acts by sequestering separase, a cystein protease that cleaves Scc1, thereby opening the cohesin ring and releasing the sister chromatids (Uhlmann *et al.*, 1999). Separase activity is additionally regulated by a securin-independent mechanism, as in the *Xenopus* egg extract system it was shown that the kinase Cdk1 (itself under the control of APC/C) can inhibit securin by binding and phosphorylating it (Gorr *et al.*, 2005; Stemmann *et al.*, 2001). Moreover, Scc1 cleavage by securin requires Scc1 phosphorylation by Polo kinase (Alexandru *et al.*, 2001), providing yet another level of control to the sister chromatid separation process.

In *S. cerevisiae* mitosis, virtually all cohesin is removed from chromsomes at the metaphase to anaphase transition, by the separase pathway (Clarke and Orr-Weaver, 2006; Stemmann *et al.*, 2005) (Figure 6A). In contrast, in metazoans cohesin dissociation takes place in two waves (Figure 6B). First, arm cohesin is removed in prophase, by the so-called prophase pathway. This mechanistically still uncharacterized pathway does not act via separase-dependent cleavage of Scc1, but instead requires phosphorylation of the cohesin subunit Scc3 by the Polo and Aurora B kinases (Hauf *et al.*, 2005; Sumara *et al.*, 2002). In the second step, centromeric cohesion is abolished in anaphase by separase cleavage of Scc1. How centromeric cohesin is protected from dissociation in prophase was only recently discovered. The centromer-associated protein shugoshin was shown to recruit the phosphatase PP2A, which in turn de-phosphorylates centromeric cohesin, counteracting polo kinase activity and thus protecting centromeric cohesion from the prophase pathway for cohesin removal (Kitajima *et al.*, 2004; Kitajima *et al.*, 2006; Riedel *et al.*, 2006; Tang *et al.*, 2006). Shugoshin is itself regulated by the spindle checkpoint kinase Bub1, which localizes it to the centromeres, and by the APC/C, which degrades it in anaphase (Kitajima *et al.*, 2004).

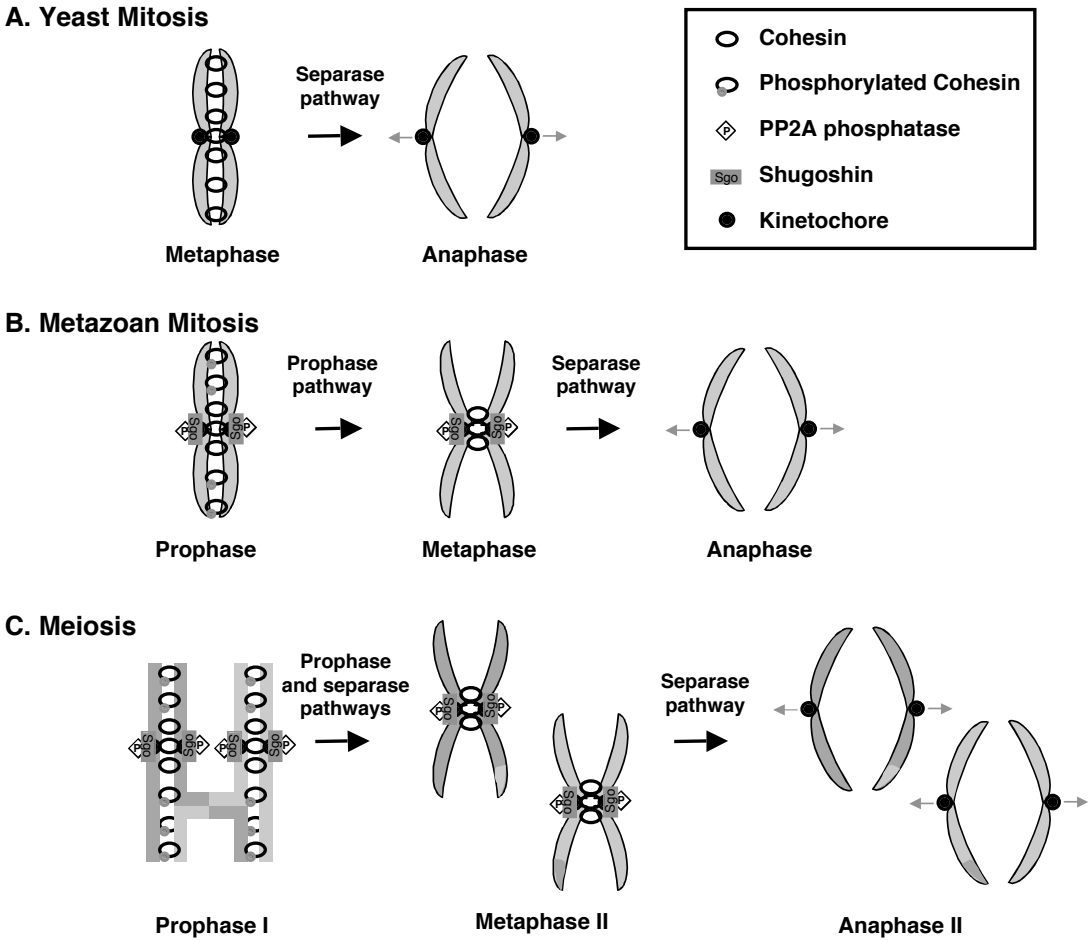

Figure 6. Schematic representation of chromosome disjunction. A. In yeast mitosis, arms and centromeric cohesin is cleaved by separase in anaphase. **B.** In metazoans, arm cohesin is phosphorylated and dissociates in prophase, while centromeric cohesin, protected in prophase by shugoshin and the PP2A phosphatase is cleaved by separase in anaphase. **C.** In meiosis, chromosomes are kept together by cohesin and chiasmata. During the first division, the prophase and separase pathways collaborate to remove arm cohesin, while centromeric cohesion is abolished by separase activity in the second division.

Regulation of cohesion is even more complicated in meiosis (Figure 6C), when a single round of DNA replication is followed by two nuclear divisions, one reductional, which separates homolog chromosomes, and one equational, which separates sister chromatids. During meiotic division, Scc1 is replaced in the cohesin complex by its homolog Rec8 (Toth *et al.*, 2000). While this alternative cohesin complex maintains cohesion between sister chromatids, homologs are held together by formation of chiasmata which result from recombinational crossing-over. During the first division, arm cohesion is resolved by the prophase pathway and by Rec8 cleavage by separase in anaphase I. Centromeric cohesion is protected by shugoshin until

anaphase II, when sister chromatids are separated (Clarke and Orr-Weaver, 2006; Megee, 2006).

Besides its role in cleaving Scc1 and Rec8, separase was shown to also have other functions that promote mitotic exit. Via the FEAR (Cdc14 early anaphase release) network, separase activates the phosphatase Cdc14. Cdc14-controlled functions include segregation of rDNA repeats, stabilization and proper orientation of the spindle (Stemmann *et al.*, 2005). Another protein recently shown to be required for proper cohesion in metazoans is sororin, which associates with the cohesion complex in interphase and is downregulated by the APC/C complex at the end of mitosis. Homologs of this protein were not identified in yeasts. It has been proposed that sororin might be required for activation of the phosphorylated cohesin that was removed from chromosomes by the prophase pathway, allowing it to be functional in the next S-phase (Rankin *et al.*, 2005).

After the mitotic division, the two sister chromatids must end up in different cells. At the centromeres, a special protein complex called kinetochore is formed, which is responsible for anchoring the microtubules emerging from the spindle pole bodies. It is essential that the kinetochores attach to spindles that emanate from opposing poles. This leads to tension across the two kinetochores, as chromatid cohesion resists the outward pulling forces of the spindle. Unattached kinetochores, or lack of tension when both kinetochores are attached to the same pole, are sensed by specific sensors, including Mad2 and Mad3, leading to activation of the mitotic checkpoint. An inhibitory complex is formed, containing Mad2, Mad3, Bub1, Cdc20 which binds stoichiometrically and inhibits APC/C (Sudakin *et al.*, 2001; Tang *et al.*, 2001). By the activity of Aurora B, the microtubules are re-oriented to form connections with the proper kinetochores (Lampson *et al.*, 2004), creating in the end tension across the kinetochores. Only then APC/C gets activated, cohesion is ablated and the spindles pull the chromatids towards the spindle pole bodies.

1.3.5 Special cohesion pathways

Cohesion at rDNA repeats. In *S. cerevisiae*, cohesion at rDNA loci was shown to be independent of cohesin. As topoisomerase II activity is required for separation of this sites, it was proposed that DNA catenation is responsible for cohesion at rDNA loci (Sullivan *et al.*, 2004). Segregation of rDNA repeats requires activation of Cdc14 via the FEAR network and the activity of Aurora B kinase and takes place late in anaphase.

Cohesion at silent loci. A direct link between silencing and cohesion was demonstrated, when it was reported that the silencing factors Sir1-4 are

recruiting cohesin to silenced DNA (Chang *et al.*, 2005). It appears that Sir factors are required for establishment, but not for maintenance of the silenced state. The chromatin fibers at repressed sites are folded into higher-order 30nm fibers, so a cohesin ring would not be large enough to accomodate two such fibers. Therefore, it was proposed that at silenced loci, the mechanism of cohesion is different, with one cohesin ring encircling one fiber.

Cohesion at centromeres. Maintenance of centromeric cohesion was shown to dependent on the activity of topoisomerase II, as TopoII mutants have decreased centromeric cohesion (Bachant *et al.*, 2002). TopoII function at the centromeres is modulated by its posttranslational modification with SUMO. Blocking of TopoII SUMOylation leads to abolished or defective chromosome segregation (Azuma *et al.*, 2005; Takahashi *et al.*, 2006), that could be due to increased cohesion at the centromeres, while hyper-SUMOylation of TopoII results in centromeric cohesion defects (Bachant *et al.*, 2002). In *Xenopus*, the SUMOylated form of TopoII was found to be easier extractable from chromatin (Azuma *et al.*, 2005), suggesting that SUMOylation might induce removal of TopoII from the centromeres. Moreover, centromeric cohesion appears to be lost prematurely in cells lacking the cohesin-interacting protein Slk19 (Zhang *et al.*, 2006), suggesting that this kinetochore-associated protein is required for maintenance of the centrosomal structure.

Cohesion at telomeres. Cohesion at telomeres was shown to be entirely dependent on the Eco1-cohesin pathway. However, telomeres do seem to have special features, as in cohesion-deficient cells, telomere separation was highly increased compared to arm separation (Antoniacci and Skibbens, 2006). This effect might be the cause of the high mobility of telomeres and might contribute to chromosome disjunction in anaphase.

Cohesion at double strand breaks. In *S. cerevisiae*, cohesion is required for efficient double strand break repair via sister chromatid recombination (Sjogren and Nasmyth, 2001; Strom *et al.*, 2004), and is established at sites of double strand breaks after the activation of the DNA damage response regulator kinases Mec1 and Tel1 (Unal *et al.*, 2004). Moreover, the Mre11-Nbs1-Rad50 complex, early sensor of DNA double strand breaks and initiator of homologous recombination, was shown to be required for recruitment of cohesin to laser-induced DNA damage in human cells (Kim *et al.*, 2002). Replication-independent establishment of cohesion at double strand breaks appears to use the canonical Eco1 pathway (Strom *et al.*, 2004). Recently, also double-strand breaks arising in S-phase by replication through a nick were shown to be repaired by a cohesin-mediated sister chromatid exchange mechanism (Cortes-Ledesma and Aguilera, 2006).

1.4 Aim of this work

The complex machinery formed at the replication fork during S-phase integrates, besides DNA replication, many other activities that ensure the proper transmission of the genetic information, including DNA repair, transfer of the correct chromatin structure, repression of deleterious recombination events. Recent studies in *S. cerevisiae* showed that posttranslational modifications with ubiquitin family proteins regulate such activities at the replication fork. Modifications of DNA polymerases co-factor PCNA with ubiquitin and SUMO coordinate the different mechanisms to bypass DNA lesions by the replisome. One aim of this study was to identify and characterize functionally other putative posttranslational modifications of replication fork proteins. Moreover, the conservation of PCNA modifications in metazoan cells was investigated.

Besides acting as a polymerase co-factor, PCNA functions as a matchmaker that regulates replication-linked processes by physical interactions. A major focus of this study was to identify novel binding partners of PCNA and to functionally characterize these interactions. Particularly, a putative role for PCNA in cohesion establishment, suggested by genetic interactions between PCNA and the cohesion protein Eco1, will be investigated. Moreover, the impact of SUMOylation, a constitutive S-phase event, on the functions of PCNA was also addressed in this study.

2. RESULTS

2.1 Characterization of PCNA modifications in higher eukaryotes

In *S. cerevisiae*, PCNA (encoded by the gene *POL30*) is modified by three posttranslational modifications: mono-ubiquitylation, multi-ubiquitylation by a non-canonical K63-linked chain and SUMOylation (Hoege *et al.*, 2002). Both types of ubiquitin modification occur at lysine K164 of yeast PCNA in response to replication fork stalling at DNA lesion sites and they each result in the activation of different mechanisms for restarting the fork (Hoege *et al.*, 2002; Kannouche *et al.*, 2004; Stelter and Ulrich, 2003). SUMOylation, on the other hand, occurs constitutively in S-phase, affecting mainly K164 and to a lesser extent K127. This modification was shown to repress recombination at the replication fork by recruiting the antirecombinogenic helicase Srs2 (Papouli *et al.*, 2005; Pfander *et al.*, 2005). The enzymes required for PCNA ubiquitylation (Rad6, Rad18, Mms2, Ubc13, Rad5) are conserved in higher eukaryotes, and inactivation of each of them renders cells sensitive to DNA damage (Andersen et al., 2005; Koken et al., 1991; Masutani et al., 1999; Tateishi et al., 2000; Unk et al., 2006), arguing for a conservation of function. Moreover, K164 (but not K127) is conserved in all PCNA homologs (Figure 7A), suggesting that posttranslational modifications of PCNA at K164 may be conserved from yeast to man.

2.1.1 PCNA modifications in human cells

In order to test this hypothesis, human cells (HeLa) were cultured in the absence or presence of the DNA damaging drug MMS, which in *S. cerevisiae* induces PCNA ubiquitylation. Cell extracts were analysed by Western blot, using anti-PCNA antibodies. While in the absence of MMS only unmodified PCNA could be detected, DNA damage led to an accumulation of a slower migrating form of PCNA, with an electrophoretic mobility as expected for mono-ubiquitylated PCNA (Figure 7B). In order to verify the identity of this band, cells were transfected with constructs encoding 6xHis-tagged versions of PCNA. When wild-type His-tagged PCNA was transfected, a slower migrating form of PCNA could be induced by DNA damage. This band was not observed when the PCNA K164R mutant was expressed, indicating that it represents a modification of PCNA at K164 (Figure 7C). A band with a similar running behaviour could be observed when 6xHis-tagged ubiquitin was

transfected. The fact that this band could be detected by anti-PCNA antibodies after NiNTA chromatography clearly indicates that it indeed represents ubiquitylated PCNA (Figure 7C).

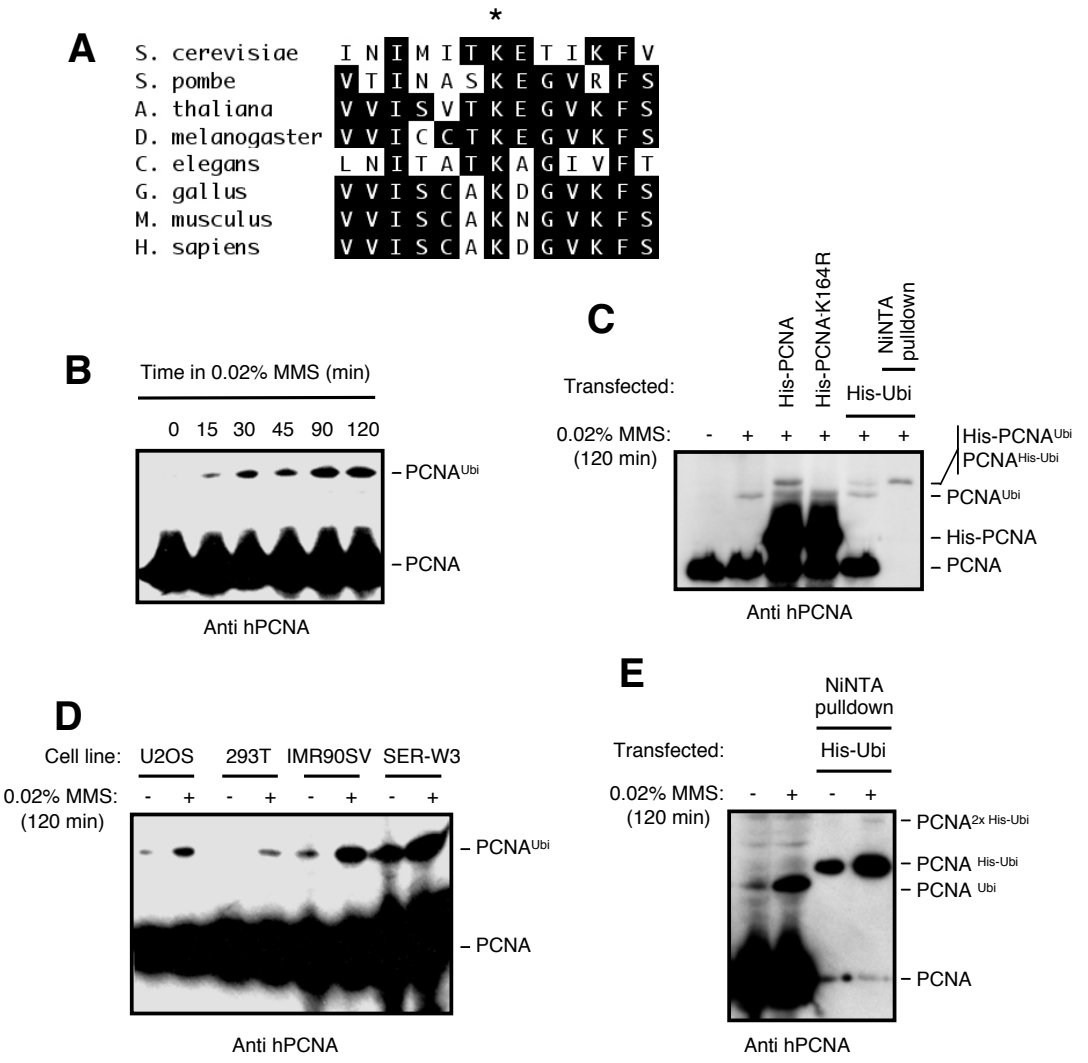

Figure 7. Mammalian PCNA is mono- and di-ubiquitylated at the conserved K164 residue. A. K164 is conserved from yeast to humans. Shown is an alignment between residues 158 and 172 of yeast PCNA. K164 is marked with an asterisk. **B**. A posttranslational modification of PCNA is induced by sub-lethal DNA damage. Extracts of HeLa cells cultivated in the presence of 0.02% MMS for the indicated periods of time wereblotted and probed with anti-PCNA antibodies. **C**. PCNA is mono-ubiquitylated at K164 on DNA damage. Extracts of cells untransfected or transiently transfected with the His-tagged PCNA variants, treated with MMS as indicated, were blotted for PCNA. For the last two lanes, a construct expressing His-tagged ubiquitin was transfected, and samples were loaded on the gel before or after NiNTA pulldown. **D**. Mono-ubiquitylation of PCNA induced by DNA damage can be detected in different cell lines. Extracts cells cultivated in the absence or presence of MMS as indicated were probed for PCNA. **E**. PCNA can be di-ubiquitylated on DNA damage. Extracts of Saos-2 cells (first two lanes) or His-ubiquitin pulldowns from these cells were blotted and probed with an antibody recognizing PCNA.

To demonstrate the universality of DNA damage-induced PCNA ubiquitylation in mammals, cell extracts from different cell lines were analysed by Western blot against PCNA. The cell lines chosen were U2OS (human osteosarcoma), 293T (human embryonic kidney cells), IMR90SV (human embryonic lung) and SER-W3 (rat testis Sertoli cells) cells. In all cases, MMS treatment led to accumulation of the mono-ubiquitylated form of PCNA (Figure 7D), arguing that this DNA-damage response is not cell-type specific, but probably occurs in all dividing cells.

In *S. cerevisiae*, DNA damage induces not only PCNA mono-ubiquitylation, but also multi-ubiquitylation, the ratio of the steady state levels of the mono- *versus* the di-ubiquitylated form being roughly one to one (Hoege *et al.*, 2002). In mammalian cells, however, no multi-ubiquitylated PCNA was detectable (Figure 7B-D). This is most likely due to a reduced efficiency of the detection method employed. To solve this problem, Saos-2 cells (human osteosarcoma) which express very high levels of PCNA were used. After transfecting 6xHis-tagged ubiquitin in these cells, and performing NiNTA chromatography to purify His-ubiquitin conjugates, not only mono-ubiquitylation, but as well di-ubiquitylation of PCNA could be observed (Figure 7E). The levels of the di-ubiquitylated form were strongly reduced compared to those of mono-ubiquitylated PCNA, thus raising the intriguing possibility that, in contrast to the situation in yeast, in mammals translesion synthesis (mono-ubiquitylation dependent) is preferred over template switch (multi-ubiquitylation dependent) when stalled replication forks are to be restarted.

Moreover, in contrast to the situation in yeast, PCNA SUMOylation could not be observed in any mammalian cell line tested, not even after transfection of SUMO ligases of the PIAS class (not shown). This does not exclude, the possibility that PCNA SUMOylation in mammalian cells is extremely transient or present at very low levels.

2.1.2 PCNA modifications in chicken cells

The mutagenic bypass of DNA lesions using translesion polymerases is controlled in yeast and mammals by mono-ubiquitylation of PCNA (Hoege *et al.*, 2002; Kannouche *et al.*, 2004; Stelter and Ulrich, 2003). Besides its crucial role in genome stability translesion synthesis (TLS) is important as well for other cell type-specific physiological functions, the most striking example being somatic hypermutation (SHM) of the immunoglobulin locus in activated B cells. Concordingly, a point mutation in K164 of PCNA in chicken DT40 cells, derived from activated B cells, led not only to DNA damage sensitivity but also resulted in reduced antibody diversification (Arakawa *et al.*, 2006), indicating that modification of PCNA at K164 is required for SHM. In

collaboration with Jean-Marie Buerstedde and Hiroshi Arakawa (GSF, Neuherberg) the different modifications of PCNA and their enzymology in DT40 cells were investigated. Either wild-type, cells homozygously mutated for K164 of PCNA or deleted for the E3 ubiquitin ligase Rad18 (obtained from J.M. Buerstedde) were employed. Cell lysates from untreated and MMS-treated cells were analysed by immunoblotting against PCNA. Besides the band corresponding to unmodified PCNA, the presence of slower migrating bands with the electrophoretic mobility expected for mono-ubiquitin- and SUMO-modified PCNA were observed (Figure 8). The identities of these PCNA modifications were confirmed by NiNTA chromatography from extracts of cells stably transfected with His-tagged ubiquitin or SUMO (Figure 8 last five lanes). As expected, both modifications were dependent on the presence of K164, and ubiquitylation, but not SUMOylation was induced by MMS treatment.

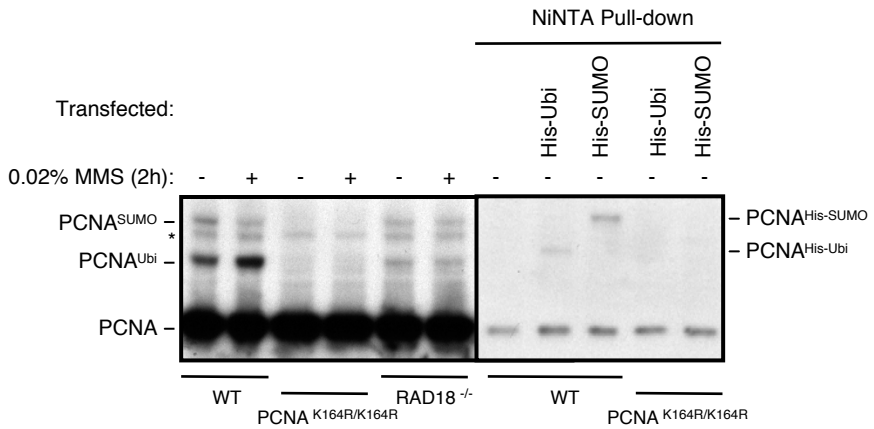

Figure 8. Chicken PCNA is mono-ubiquitylated and SUMOylated at K164. Left panel: Extracts of DT40 AID$^{R}\Psi V^{-}$ cells, either wildtype or homozygous mutants of K164 in PCNA or deleted for the E3 ligase Rad18, grown in the presence of MMS as indicated, were probed for PCNA. The asterisk denotes a cross-reactive band. Right panel: His-tagged variants of ubiquitin and SUMO were stably transfected in the indicated DT40 AID$^{R}\Psi V^{-}$ cells. Extracts were prepared and subjected to NiNTA pulldowns.

Interestingly, *RAD18*$^{-/-}$ cells retained both bands, although the band corresponding to mono-ubiquitylated PCNA is faint compared to wild-type cells and not inducible by MMS. This is in striking contrast to the situation in yeast, were no ubiquitylation of PCNA can be detected in the absence of Rad18 (Hoege *et al.*, 2002) and argues for the existence of a Rad18-independent, backup pathway for PCNA ubiquitylation in higher eukaryotes. Indeed, genetic experiments could show that *RAD18*$^{-/-}$ cells have milder SHM and DNA repair defects than *PCNA*$^{K164R/K164R}$ and the double mutant *RAD18*$^{-/-}$ *PCNA*$^{K164R/K164R}$ is phenotypically identical to the *PCNA*$^{K164R/K164R}$ single mutant

(Arakawa *et al.*, 2006). Furthermore, the finding that PCNA is SUMOylated in chicken cells is surprising, as such modification was not detectable in mammalian cells, and was described to date only in yeast and *Xenopus*.

Taken together, these data indicate that the different posttranslational modifications described for yeast PCNA are principally conserved throughout species. Their relative abundance is however differing, indicating that the preferential use of pathways controlled by PCNA modifications is species-specific.

2.2 Characterization of DNA Polymerase δ as a novel SUMO substrate

In *S. cerevisiae*, mutations of the SUMO conjugation/deconjugation machinery, as well as impairment of SUMO itself, lead to severe phenotypes that affect many cellular functions (see paragraph 1.1.3). However, only few of these phenotypes could be directly linked so far to modification of specific substrates. Therefore, identification and functional characterization of novel SUMO substrates is of major importance in order to understand the complex biology of this modification. In particular, protein SUMOylation seems to be an important mechanism for regulating DNA transactions, including replication, repair and recombination (see paragraph 1.1.3). During DNA replication, PCNA, an essential replication fork component, can be modified by SUMO and ubiquitin, modifications which coordinate three different bypasses of DNA lesions (Hoege *et al.*, 2002; Papouli *et al.*, 2005; Pfander *et al.*, 2005). Possible candidates for other factors that might be regulated by SUMOylation are DNA polymerases, the most important proteins at the replication fork.

2.2.1 SUMOylation of DNA polymerase δ subunits

In *S. cerevisiae*, the main replicative polymerase, DNA polymerase δ, contains two essential subunits, the catalytic subunit Pol3 and the regulatory subunit Pol31, in addition to one non-essential subunit, Pol32. In order to investigate their possible posttranslational modification by SUMO, yeast cells expressing individual subunits of Polδ endogenously tagged with Protein A were created. These cells were transformed with a construct over-expressing His-SUMO, and conjugates were purified by NiNTA chromatography from cells treated with different concentrations of the DNA alkylating agent MMS. Western blot using anti-Protein A antibodies revealed all three subunits of Polδ being modified by SUMO, and in all cases SUMOylation was particularly induced by treatment of the cells with 0.3% MMS for 90 minutes (Figure 9), a

lethal DNA damage condition. A similar regulation of SUMOylation was described previously for PCNA (Hoege *et al.*, 2002) (see as well Figure 9).

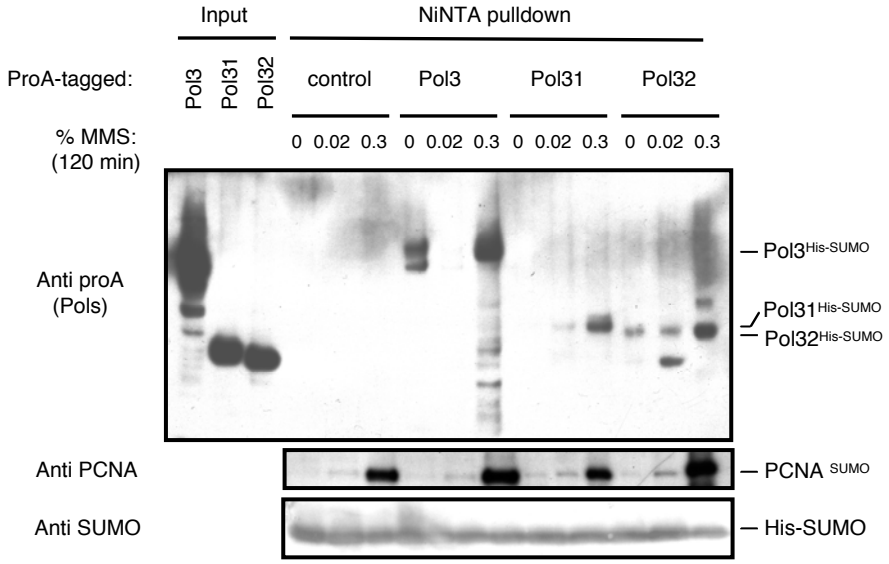

Figure 9. All three subunits of the *S. cerevisiae* polymerase delta complex are modified by SUMO *in vivo*. Cells expressing endogenous polymerase delta subunits tagged C-terminally with Protein A or control (untagged) cells were transformed with a construct overexpressing His-SUMO and grown in the presence of different MMS concentrations as indicated. Extracts were prepared and used for NiNTA pulldowns. Samples were probed for the Protein A-tagged polymerase delta subunits or against yeast PCNA, as control for the pulldown efficiency.

2.2.2 Pol32 is SUMOylated at K283 during S-phase

Of the three subunits of Polδ, Pol32 was chosen for further investigation of SUMOylation, for several reasons. First, *POL32* deletion is viable, allowing easy manipulation and investigation of mutant alleles. Second, Pol32 was previously described to be vital also for processes other than replication, the most interesting ones being in the regulation of DNA repair and recombination, functions that have been previously linked to SUMOylation as well (Huang *et al.*, 2000; Huang *et al.*, 2002; Pfander *et al.*, 2005). In order to simplify the detection and analysis of posttranslational modifications, anti-Pol32 antibodies were raised in rabbits and purified by affinity chromatography (not shown).

When SUMO conjugates were purified from cells overexpressing His-tagged SUMO and treated with 0.3% MMS, two Pol32-crossreactive bands were detected, corresponding to mono- and di-SUMOylated Pol32 species (Figure 10A). In order to confirm that the two bands represent indeed SUMOylated Pol32, the same experiment was performed in cells in which the SUMO E2 conjugating enzyme Ubc9 was mutated. As expected, both bands were absent in *ubc9-1* cells (Figure 10A).

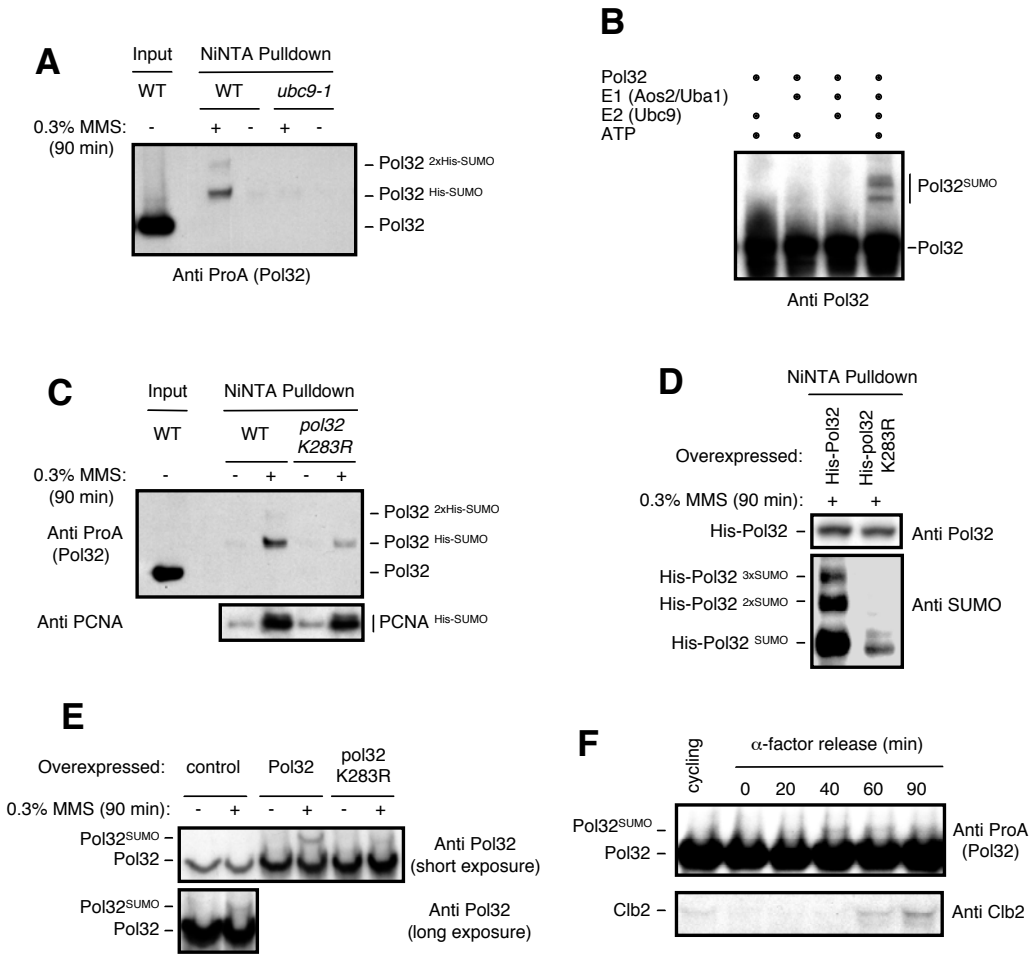

Figure 10. The polymerase delta subunit Pol32 is subjected to cell-cycle- and DNA damage-controlled SUMOylation. A. Pol32 is SUMOylated *in vivo*. Endogenous Pol32 was tagged with Protein A in wildtype cells or in mutants of the SUMO conjugating enzyme Ubc9. His-SUMO was overexpressed in these cells, and NiNTA chromatography was performed to purify modified substrates. Samples were probed for the Protein A tag of Pol32. **B**. Pol32 can be SUMOylated *in vitro* by incubation with E1, E2, SUMO and ATP. **C-E.** K283 of Pol32 is a major SUMO acceptor site. **C**. Cells expressing wildtype or K283R variants of Pol32 tagged with Protein A were transformed with His-SUMO, treated with MMS as indicated and subjected to NiNTA pulldowns. Samples were probed for Protein A to detect Pol32 or for PCNA, as control. **D**. Cells overexpressing His-tagged wildtype or K283 Pol32 variants were treated with 0.3% MMS for 90 minutes. Extracts were prepared and used for NiNTA affinity chromatography. Samples were probed with anti-Pol32 antibodies, as control for the pulldown, or with anti-SUMO antibodies to detect SUMOylated Pol32 species. **E**. Extracts of cells overexpressing wildtype or K283R mutant Pol32, or control (no overexpression) cells, treated with MMS as indicated, were bloted against Pol32. **F**. Pol32 is SUMOylated in S-phase. Cells expressing endogenous Pol32 tagged with Protein A were arrested in G1 by alpha factor treatment, and released to proceed synchronously through the cell cycle. At the indicated time points after release, samples were taken and probed against Protein A to detect Pol32. Endogenous SUMOylation of Pol32 is detected mainly at 40min and 60min time points, corresponding to S-phase. As control for the cell cycle synchronization, samples were probed against the mitotic cyclin Clb2.

Recombinant Pol32, expressed and purified from *E. coli*, could also be SUMOylated *in vitro* by the E1 complex Aos2/Uba1 and the E2 Ubc9. At least three Pol32-SUMO species were detectable in Western blots of the *in vitro* SUMOylation reaction products using anti-Pol32 antibodies (Figure 10B).

In order to identify the SUMO attachment sites in Pol32, the lysine residues positioned within the consensus SUMOylation sequence ΨKxD/E (Bernier-Villamor *et al*., 2002) were mutated to arginines. The *pol32-K283R* mutant showed reduced mono-SUMOylation and abolished di-SUMOylation, arguing that K283 represent one of the attachment sites for SUMO in Pol32 (Figure 10C). To confirm these results, His-tagged Pol32, either wild-type or mutated at K283, was overexpressed in yeast cells and purified by denaturing NiNTA chromatography. This method should allow for purification of not only the unmodified, but as well of the posttranslationally modified protein species. When the samples were blotted and probed against SUMO, several species, corresponding to SUMOylated Pol32, could be detected. In this experimental setup, not only mono- and di-, but also tri-SUMOylated Pol32 forms could be identified (Figure 10D). As expected from the previous experiment, the di- and tri-SUMOylation were not present in the purification of the pol32-K283R mutant, while the mono-SUMOylation was greatly reduced (Figure 10D). Moreover, Pol32 SUMOylation could be detected as well in extracts from cells overexpressing wild-type Pol32, but not the pol32-K283R variant (Figure 10E). As in previous experiments, the modification was induced by treating the cells with 0.3% MMS. DNA damage-induced SUMOylation of endogenous Pol32 could also be detected by probing whole cell extracts of wild-type cells with anti-Pol32 antibodies. The modification affects only a very small percentage of the endogenous Pol32 protein, and could not be detected in the absence of DNA damage.

Pol32 functions during S-phase at the replication fork (Burgers and Gerik, 1998). In order to investigate if SUMOylation is cell-cycle correlated, cells were arrested in G1 by treatment with α-factor, and released to progress synchronously through the cell cycle. Samples were harvested every 20 minutes and blotted for Pol32. SUMOylation of endogenous Pol32 was only detected when cells were in S-phase (samples harvested 40 or 60 minutes after release) (Figure 10F). Taken together, these data show that endogenous Pol32 is modified by SUMO during DNA replication or in response to lethal DNA damage treatment. This situation is reminiscent of PCNA SUMOylation, which is as well S-phase specific and inducible by 0.3% MMS.

2.2.3 SUMO-modified Pol32 recruits the recombination inhibitor Srs2

Deletion of *POL32* is known to render cells sensitive to various stress conditions that impair replication, like low temperature, DNA damage or replication inhibitors (Gerik *et al.*, 1998; Huang *et al.*, 2000), see as well Figure 11A). However, under all these conditions, cells expressing the SUMO-defective pol32-K283R mutant as the only source of Pol32 grew indistinguishably from wild-type (Figure 11A), suggesting that Pol32 SUMOylation is not required for coping with replication stress. Alternatively, as SUMOylation is only reduced in the pol32-K283R mutant, but not entirely abolished (Figure 10C,D), it is possible that the remaining modification is enough for *pol32-K283R* cells to tolerate the stress conditions tested. Therefore, identification of the additional sites of SUMOylation would be required, to obtain a protein that can no longer be SUMOylated and use such a mutant to identify the functions of Pol32 SUMOylation. One way to overcome this is to use cells that overexpress *POL32*, as the percentage of the SUMOylated form was strongly increased by overexpression of *POL32*, but not of *pol32-K283R* (Figure 10E). Thus, the functional consequence of Pol32 SUMOylation can be directly addressed experimentally by comparing the phenotypes of cells overexpressing wild-type *POL32* to those of cells overexpressing the *pol32-K283R* mutant.

While overexpression of Pol32 did not influence wild-type cells in any way, it resulted in decreased DNA damage tolerance in the DNA repair-defective *PCNA-K164R* cells (Figure 11B). In these cells, PCNA cannot be ubiquitylated anymore and therefore they are unable to use the *RAD18* post-replicative pathway of DNA repair (Hoege *et al.*, 2002). The fact that Pol32 SUMOylation sensitizes these cells to DNA damage suggests that this modification represses an alternative DNA repair pathway, whose activity is strictly required in *PCNA-K164R* cells. In fact, a similar activity was shown for PCNA SUMOylation, which inhibits *RAD52*-dependent repair and thus is toxic for mutants of the post-replication pathway (Papouli *et al.*, 2005; Pfander *et al.*, 2005). As PCNA and Pol32 SUMOylation are similarly regulated (Hoege *et al.*, 2002 and Figure 10), both are present at the replication fork and show similar phenotypes (inhibition of DNA repair - Hoege *et al.*, 2002 and Figure 11B), this raises the possibility that they activate the same downstream factors. PCNA SUMOylation is known to function by recruiting the helicase Srs2, an inhibitor of *RAD52*-dependent recombination (Krejci *et al.*, 2003; Papouli *et al.*, 2005; Pfander *et al.*, 2005; Veaute *et al.*, 2003). Interestingly, a previous report found a physical interaction between Srs2 and Pol32 (Huang *et al.*, 2000). In order to test the effect of Pol32 SUMOylation on Srs2 binding,

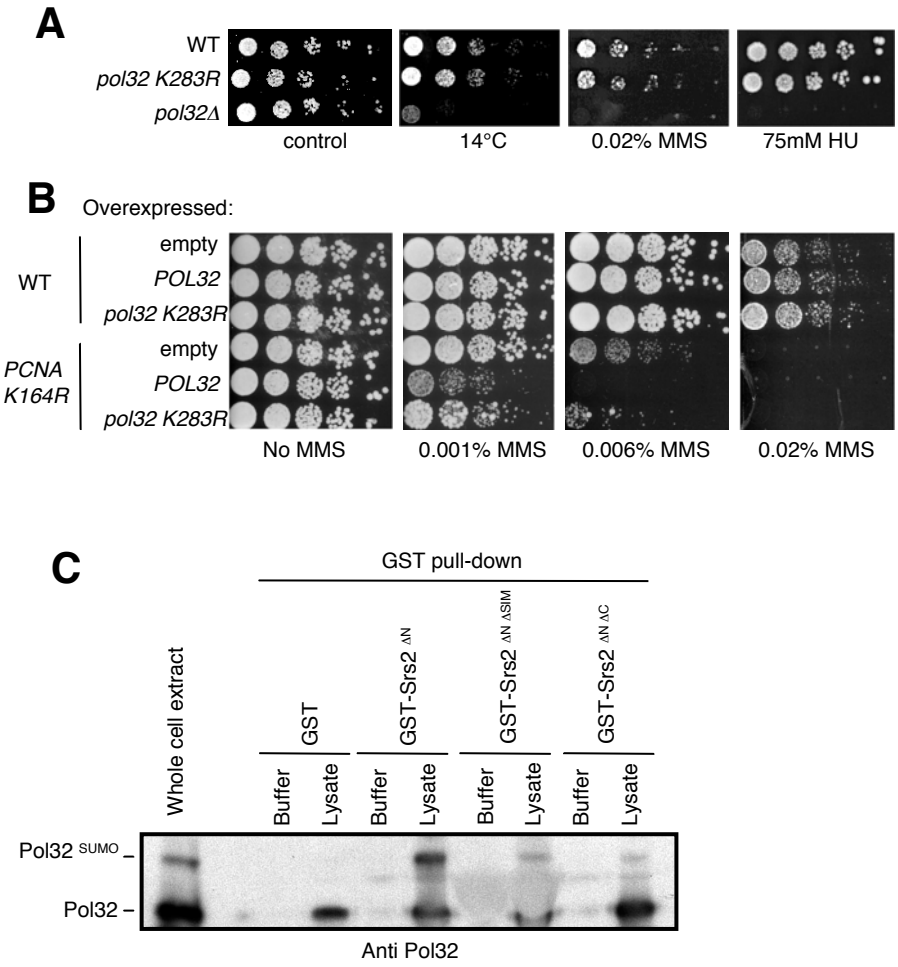

Figure 11. Pol32 SUMOylation is detrimental for DNA damage tolerance of DNA repair mutants and recruits the anti-recombinogenic helicase Srs2. A. Endogenous mutation of K283 of Pol32 does not lead to DNA replication or repair impairments. Fivefold serial dilutions of the indicated mutants were spotted on control plates or plates containing 0.02% MMS or 75mM HU to assay for DNA repair defects. Replication defects were assayed by incubating the cells at 14°C. **B**. Hyper-SUMOylation of Pol32 is detrimental for damage tolerance of DNA repair-deficient PCNA K164R mutants. Wildtype or endogenous mutants in K164 of PCNA, overexpressing wildtype or K283R Pol32 variants under the control of *ADH1* promoter were spotted in fivefold serial dilutions on plates containing the indicated concentrations of MMS. **C**. SUMOylated Pol32 interacts preferentially with Srs2. Native extracts of cells overexpressing Pol32 were subjected to GST-pulldowns using GST fusions with the C-terminal tail of Srs2 that interacts with SUMOylated PCNA ($Srs2^{\Delta N}$) or with mutants of Srs2 defective in SUMO-PCNA binding ($Srs2^{\Delta N \Delta SIM}$ and $Srs2^{\Delta N \Delta C}$). Samples were probed for Pol32.

different Srs2 fragments were expressed and purified from *E. coli* as GST-fusions, and incubated with native yeast extract. Pol32 species bound to Srs2 were identified by blotting the GST-pulldown samples with anti-Pol32 antibodies. A fragment of Srs2 encompassing the PCNA-SUMO binding site ($Srs2^{\Delta N}$, consisting of aa 783-1174 – Pfander *et al*., 2005) was able to interact with Pol32 (Figure 11B). Interestingly, this fragment showed a greater affinity for SUMOylated Pol32 compared to the unmodified form (Figure 11A). In

contrast, Srs2 fragments that lack the SUMO-PCNA binding site ($Srs2^{\Delta N\Delta SIM}$, which is similar to $Srs2^{\Delta N}$ but carries the mutation DEE1041,1042,1043AAA, and $Srs2^{\Delta N\Delta C}$ consisting of aa 783-1038– Pfander *et al.*, 2005) showed reduced interaction with SUMOylated Pol32, but could still interact normally with the unmodified protein. This demonstrates that SUMOylation of Pol32 enhances its interaction with Srs2. Together with the genetic data showing that this modification inhibits DNA repair (Figure 11B), this result suggests that Pol32 SUMOylation represent a second, backup pathway for recruitment of Srs2 at the replication fork, in order to inhibit recombination. Moreover, it is likely that SUMOylation of the other subunits of Polδ lead as well to Srs2 recruitment and inhibition of recombination.

2.3 PCNA directs establishment of sister chromatid cohesion during S-phase

Faithful inheritance of the genetic material demands both correct DNA replication and its equal distribution to the daughter cells. DNA replicated in S-phase must be faithfully segregated to the daughter cells during mitosis. To ensure this, the identity of sister chromatids is preserved during G2 by keeping them in close proximity to each other (Nasmyth and Schleiffer, 2004). Sister chromatid cohesion is essential for cellular viability and must be established in S-phase (Gerlich *et al.*, 2006; Uhlmann and Nasmyth, 1998), by the activity of the acetyl transferase Eco1/Ctf7 (Skibbens *et al.*, 1999; Toth *et al.*, 1999). The mechanism of sister chromatid cohesion establishment by Eco1 and how it is coupled to S-phase remains unclear.

2.3.1 PCNA mutants have cohesion defects

A previous report (Skibbens *et al.*, 1999) identified an *ECO1* mutation, $eco1^{ctf7\text{-}203}$, which leads to temperature sensitivity due to inefficient establishment of cohesion at the restrictive temperature. The growth deficiency of this mutant could be partially supressed by overexpression of PCNA. Moreover, the $eco1^{ctf7\text{-}203}$ mutation was found to be lethal in combination with the PCNA mutation *pol30-104* (Skibbens *et al.*, 1999) suggesting a functional link between Eco1 and PCNA.

In order to test if PCNA plays a role in sister chromatid cohesion, cells expressing the PCNA A251V mutant, encoded by the *pol30-104* allele, as the only source of PCNA were assayed for cohesion defects, using cohesion tester strains (Bhalla *et al.*, 2002). In these cells, a tandem array of 256 copies of the Lac operator is inserted on the arm of Chromosome IV, either in a position more proximal (650kb) or more distal (950kb) to the centromere.

Expression of a GFP-LacI repressor fusion leads to fluorescent labelling of the chromosome, due to the specific binding of the LacI receptor to the Lac operator. If cohesion is proper, during the G2 phase of the cell cycle the two sister chromatids will be kept in close vicinity to each other, thus only one GFP signal is detected. If however cohesion is defective, the sister chromatids will diffuse away from each other, splitting the GFP signal in two. This system allows for quantitative measuring of cohesion efficiency. Wild-type or *PCNA A251V* cells were arrested in G2/M by incubation with the microtubule-depolymerizing drug nocodazole, and the number of GFP signals in each cells was analyzed by direct fluorescence microscopy on living cells As reported before, cohesion is established efficiently in wild-type cells, such that more than 90% of cells show only one GFP signal. *PCNA A251V* cells showed at least two times more cohesion defects than wild-type (Figure 12), arguing that PCNA is directly involved in sister chromatid cohesion.

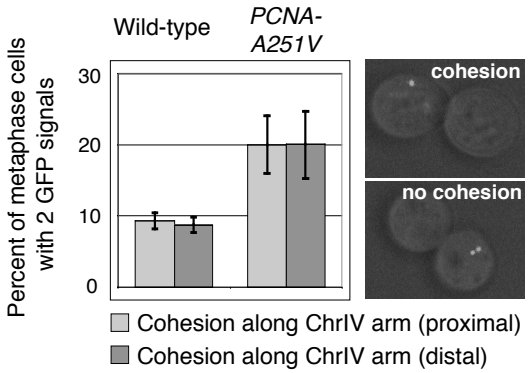

Figure 12. PCNA A251V mutants have cohesion defects. Quantification of cohesion defects of the indicated strains were assayed using yeast strains GFP-tagged on two different positions on ChrIV. Cells were arrested in metaphase as diploid, large-budded cells and the number of GFP foci present in each cell was scored, indicating either normal cohesion (one signal), or a cohesion defect (2 signals; see examples in micrographs). Each strain was assayed 4-10 times, and for each experiment at least 50 cells were counted. Error bars represent standard deviations.

2.3.2 PCNA interacts physically with Eco1

The implication of PCNA in sister chromatid cohesion made it an obvious candidate for the long sought-after factor that couples sister chromatid cohesion to replication. In order to test this, it was investigated whether PCNA and Eco1 are present in the same complex. Indeed, PCNA could be immunoprecipitated by antibodies against Eco1 (Figure 13A). Moreover, 3myc-tagged Eco1 could immunoprecipitate 3HA-tagged Eco1, arguing that Eco1 multimerizes *in vivo*. Furthermore, PCNA and Eco1 also interacted in a

two-hybrid assay (Figure 13B). In order to address whether the binding is direct, an *in vitro* system was compiled. Recombinant GST fusions of Eco1, eco1$^{ctf7-203}$ and of the helicase Chl1, also required for efficient cohesion, were purified from *E. coli* (Figure 14A) and employed for interaction studies, using recombinant or endogenous PCNA. Eco1 interacted directly with recombinant PCNA, showing that the interaction is not mediated by other factors, (Figure 14B). Chl1 was also found to bind directly to PCNA in this assay. GST-tagged Eco1 and Chl1 also interacted with endogenous PCNA in cell extracts, as verified by Western blot using PCNA antibodies (Figure 14B). Interestingly, PCNA binding to GST-eco1$^{ctf7-203}$ was reduced compared to wildtype (Figure 14B), suggesting that the temperature sensitivity of the mutant might partly be a consequence of a defective interaction with PCNA.

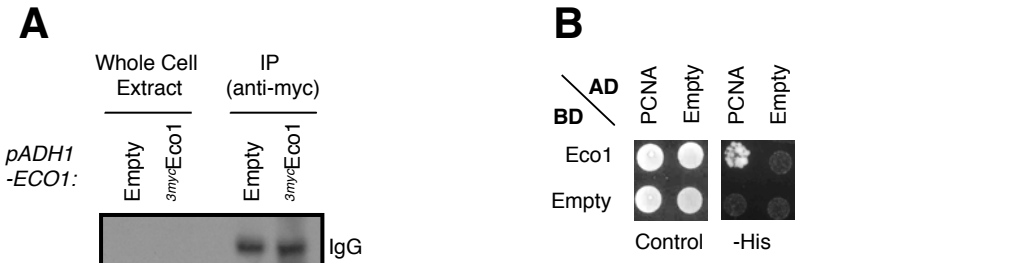

Figure 13. Eco1 interacts with PCNA *in vivo*. A. PCNA co-immunoprecipitates with Eco1. 3mycEco1 expressed from the ADH1 promoter was immunoprecipitated with anti-myc antibodies. Samples were analyzed by Western blot for myc or PCNA reactivity. When cells expressing 3HA-tagged endogenous Eco1 where used, the blot against HA showed that endogenous Eco1 is precipitated as well (lower panel). The asterisk denotes a cross-reactive band. **B.** Yeast two-hybrid assay showing interaction of Eco1 with PCNA.

S. cerevisiae Eco1 is a polypeptide of 281 aminoacids, encompassing a C2H2-type zinc finger (aa 33-57) and an Acetyl Tranferase (ACT) domain (aa 111-266) (Brands and Skibbens, 2005; Ivanov *et al.*, 2002), (Figure 15A). In order to map the region of Eco1 involved in binding to PCNA, different truncations of Eco1 were expressed in *E. coli* and purified as GST fusions (Figure 15A,B). These fusions were assayed for their ability to interact with recombinant or endogenous PCNA (Figure 15C). Constructs spanning the ACT domain (GST-eco$^{104-281}$) or the C2H2 finger (GST-eco$^{33-141}$) failed to pulldown PCNA. In contrast, constructs containing the amino-terminal 33 aminoacids (GST-Eco1, GST-eco$^{1-33}$ and GST-eco$^{1-141}$) were able to interact with PCNA (Figure 15C). Hence, Eco1 seems to contain a PCNA-binding site

in the region encompassing the amino-terminal 33 aminoacids. Indeed, a truncation lacking only the first 33 aminoacids (GST-eco$^{33-281}$) could not interact with PCNA in this assay (Figure 15C).

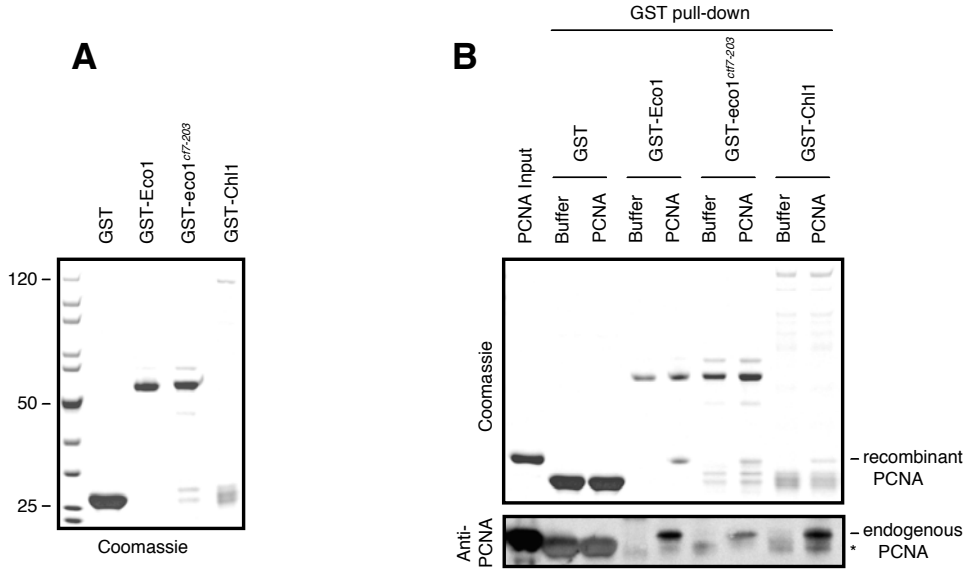

Figure 14. Eco1 and PCNA interact directly *in vitro*. A. Recombinant GST fusions of Eco1 and Chl1 were expressed and purified from *E. coli*. **B.** GST pull-downs with recombinant GST-tagged Eco1 fragments and recombinant or endogenous PCNA from yeast native extracts. Asterisks denote cross-reacting proteins.

The mapping of the PCNA-interacting region to the amino-terminus of Eco1 could be validated in two-hybrid experiments. In this experimental setup, the first 33-aminoacids of Eco1 were sufficient to interact with PCNA, and truncation of this sequence completly abolished the interaction (Figure 15D). Altogether, these data demonstrate that Eco1 bears in its N-terminal 33 aminoacids a PCNA-binding domain, which is sufficient and crucial for the interaction with PCNA.

2.3.3 The PCNA-interacting region is required for Eco1's essential function

The *S. cerevisiae* Eco1 protein comprises at least three domains: a C2H2 Zn-coordinating finger, an acetyl transferase domain (Ivanov *et al.*, 2002) and a PCNA-interacting region (see above). In order to address which domain is crucial for its function, a yeast strain was created in which the only source of Eco1 was expressed from an autonomously replicating centromeric vector containing the *URA3* marker. In such a setup, constructs encoding Eco1 mutants can be transformed, and their viability can be tested by plating the transformants on medium containing 5-fluoro-orotic acid (5-FOA), which counterselects for the *URA3* encoding plasmid. Consequently, the plasmids

encoding Eco1 mutants will remain the only source of Eco1 in the cells plated on 5-FOA-containing medium.

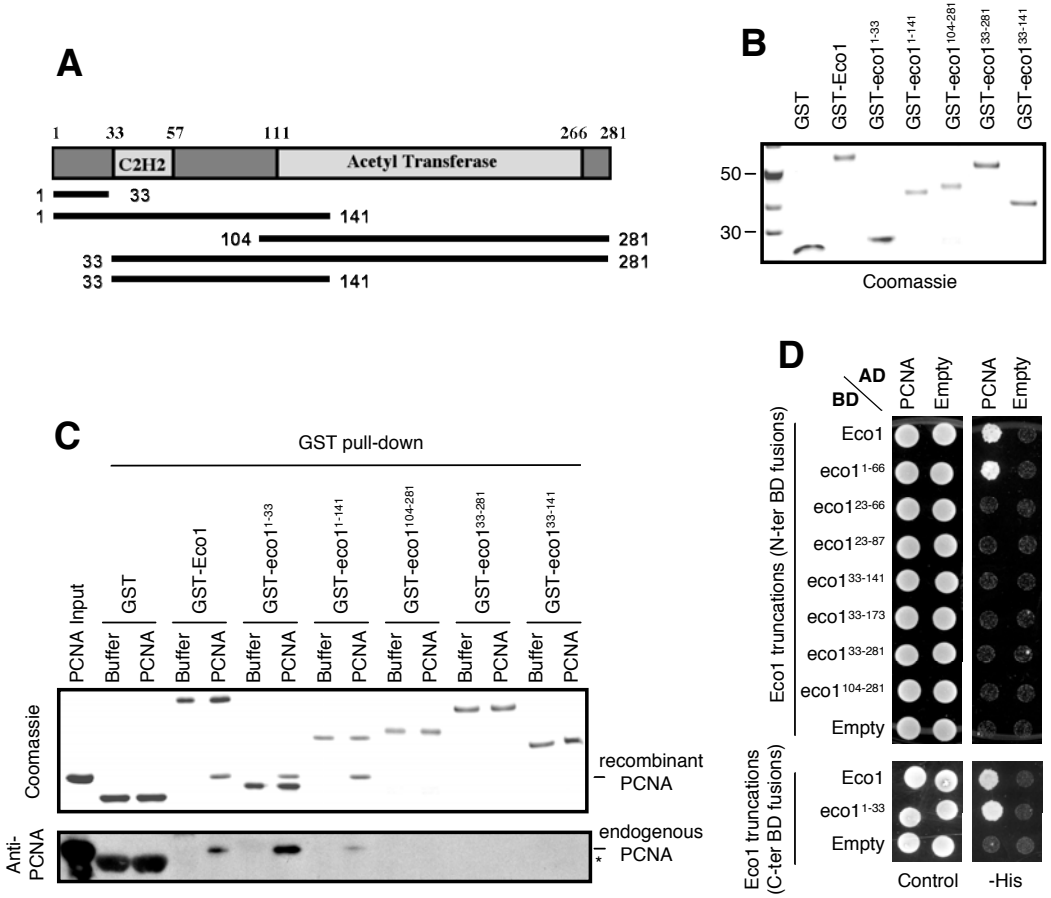

Figure 15. The PCNA interaction site lies within the amino-terminal 33 aminoacids of Eco1. A. Diagram showing Eco1 domains and the truncations used for GST pull-downs. **B.** Eco1 truncations were purified from *E. coli* as recombinant GST fusions **C.** GST-pull-downs with recombinant Eco1 truncations and recombinant or endogenous PCNA. **D.** Mapping of the binding site of PCNA by two-hybrid analysis, using truncations of Eco1.

In this assay, both the ACT domain with its activity, and the zinc finger were required for Eco1's essential function (Figure 16A). Indeed, mutations abolishing the activity of the ACT domain and the folding of the zinc finger were previously shown to lead to chromosome transmission defects (Brands and Skibbens, 2005; Ivanov *et al.*, 2002). Importantly, also the N-terminal 32 residues of Eco1 are essentially required because expression of a truncated variant that lacks this domain (eco1$^{33-281}$) does not support growth of the *eco1* deletion mutant (Figure 16A). Interestingly, when strongly overexpressed from the ADH1 promoter, the PCNA interaction-defective eco1$^{33-281}$ was partially able to restore viability (Figure 16B), suggesting that this truncation is defective in localizing to its sites of action.

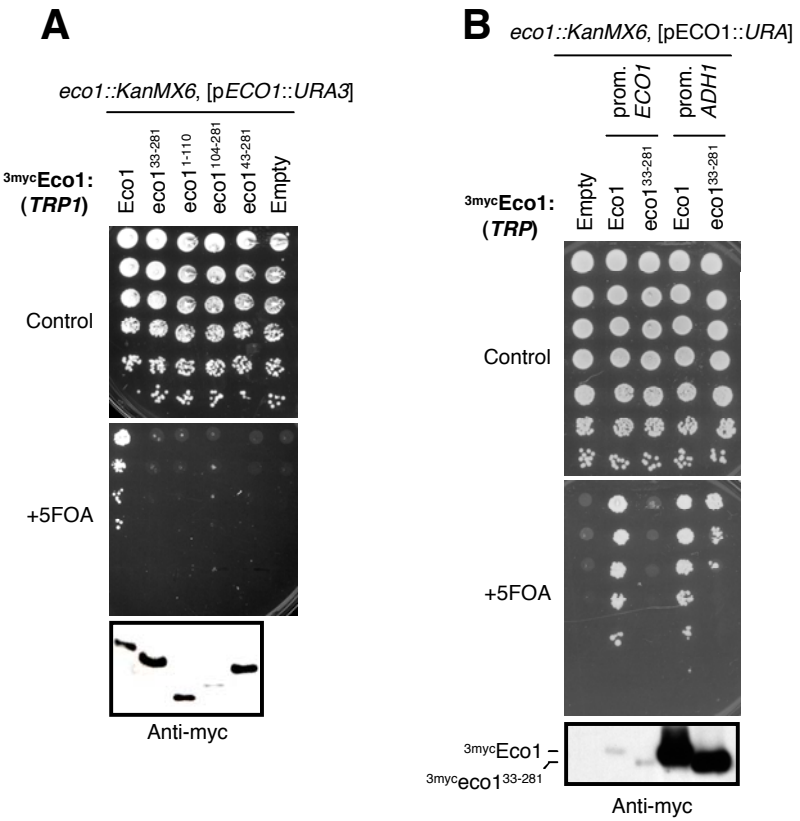

Figure 16. The PCNA-interaction motif, the zinc finger and the acetyl transferase domain are all required for the essential function of Eco1. A. Different Eco1 truncations where assayed for their ability to support viability in the absence of WT *ECO1*, which is expressed from a plasmid that can be counter-selected by plating the cells on 5-fluoro-orotic acid (5FOA)-containing medium. **B.** Overexpression of eco1$^{83-281}$ under control of the ADH1 promoter can partly restore viability to cells lacking endogenous Eco1.

Eco1 was shown to posses acetyl-transferase activity *in vitro*, and mutations that abolish this activity resulted in cohesion defects (Ivanov *et al.*, 2002). In order to test if the PCNA-interacting domain is required for this activity, recombinant Eco1 and eco1$^{33-281}$ were incubated with Acetyl Coenzyme A (AcCoA) in the presence of recombinant Scc1, one of the known *in vitro* substrates of Eco1. Acetylated products were detected by Western blot using antibodies recognizing acetylated lysins. The eco1$^{33-281}$ protein fully retained its acetylation activity, being able to acetylate both itself (auto-acetylation) and Scc1 to the same extent as wild-type Eco1 (Figure 17), showing that the truncated protein is not aberrantly folded. In conclusion, the PCNA-interacting domain of Eco1 is required for the essential function of Eco1, but does not affect its enzymatic activity.

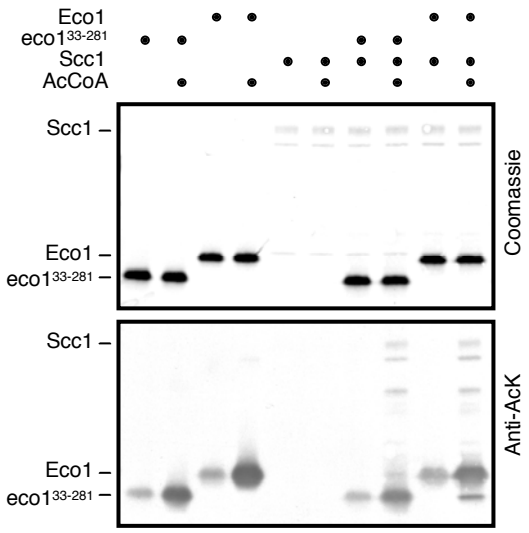

Figure 17. Deletion of the PCNA interacting region of Eco1 does not afffect its acetyl-transferase activity. Eco1 and eco1$^{ctf7\text{-}203}$ show similar levels of acetylation activity *in vitro*. Self-acetylation and acetylation of Scc1 were visualized by Western blot using an antibody recognizing acetylated lysine residues (AcK).

2.3.4 The Eco1-PCNA interaction is crucial for establishment of cohesion

To characterize the essential feature of the N-terminal domain in greater detail, an alanine-scan mutagenesis approach was performed. Residues conserved between *S. cerevisiae* and *S. pombe* were mutated to alanine and analyzed for their ability to interact with PCNA in two-hybrid assays and to confer cellular viability in 5-FOA shuffling assay. The most drastic phenotype was observed with a mutant protein in which the residues Q18, K20, and L21 had been replaced (eco1^{Q18A,K20A,L21A}). Cells that express the corresponding mutant gene as the only source of Eco1 were inviable (Figure 18A), and binding to PCNA was absent with this mutant (Figure 18B). This confirms the results of the Eco1 truncation studies (Figure 16A), that the Eco1-PCNA interaction is required for cellular viability.

Notably, an Eco1 mutant protein (eco1^{S12A,K13A}) that conferred viability to cells, but was nevertheless significantly defective in PCNA binding (Figure 18A,B) was also identified. As this mutant was viable, it could be used to study the effects of a defective Eco1-PCNA interaction on cohesion. Cohesion tester strains expressing eco1^{S12A,K13A} as the only source of Eco1 were created and analyzed by fluorescence microscopy. These cells exhibited significant deficiencies in sister chromatid cohesion (Figure 19A). The inability of *eco1*^{S12A,K13A} cells to establish proper cohesion argues for the Eco1-PCNA interaction playing an important role in the establishment of sister chromatid cohesion. This makes it tempting to speculate that the cause for the lack of viability of *eco1*^{Q18A,K20A,L21A} cells is the loss of the crucial Eco1-PCNA interactions, making it impossible to establish sister chromatid cohesion. For confirmation, and to rule out the possibility that *eco1*^{Q18A,K20A,L21A} cells are inviable because of the inactivation of another putative function of Eco1, the

eco1^{Q18A,K20A,L21A} mutant was expressed in *eco1$^{ctf7-203}$* cells. As these cells are not viable at the restrictive temperature due to cohesion defects, they should be rescued by co-expression of eco1^{Q18A,K20A,L21A} should this mutant be defective in another putative function of Eco1. Wild-type Eco1 could complement both the cohesion defect and the inviability of *eco1$^{ctf7-203}$* cells at restrictive temperatures (Figure 19B,C). In contrast, eco1^{Q18A,K20A,L21A} could not aleviate the cohesion defects or restore viability to *eco1$^{ctf7-203}$* cells (Figure 19B,C), demonstrating that this mutant is definitely defective in establishment of sister chromatid cohesion.

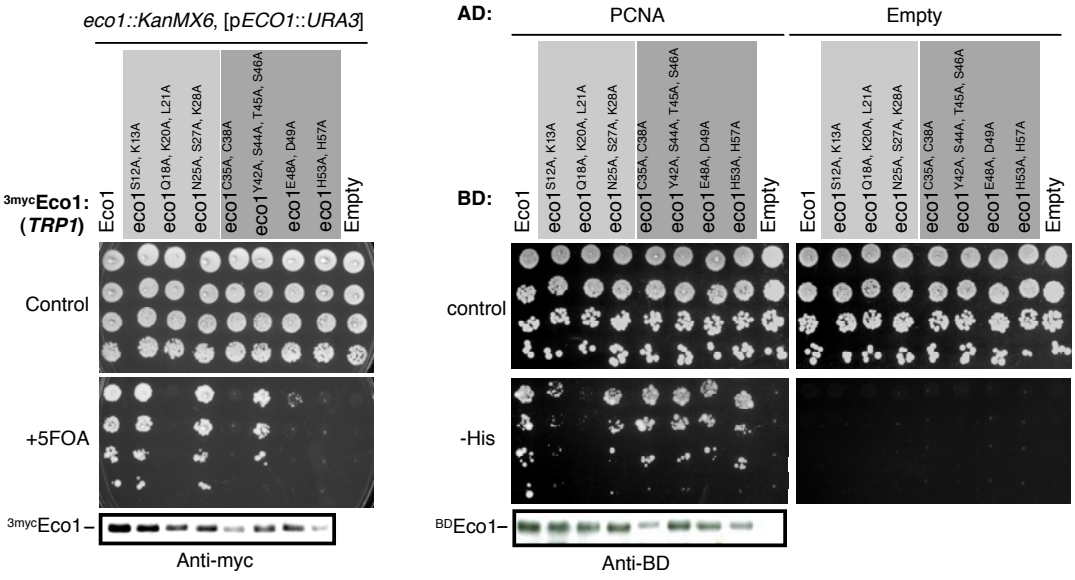

Figure 18. The ability to interact with PCNA and the zinc finger are required for the essential function of Eco1. A. Viability of *eco1* mutants defective in the PCNA-interaction domain (marked with a light grey box) or in the zinc finger (dark grey box) were assayed by shuffling out WT *ECO1* upon plating on 5FOA-containing medium. **B.** Two-hybrid analysis of the interaction of eco1 mutant proteins with PCNA, showing that the N-terminus, but not the zinc finger is required for this interaction.

Four multiple point mutations in the C2H2 finger, including double alanine mutations of either the cysteins (eco1^{C35A,C38A}) or the histidines (eco1^{H53A,H57A}) involved in Zn coordination were also created and investigated. As expected, none of the mutations in the C2H2 finger affected binding to PCNA (fig 18A). On the other hand, double mutations in the cysteins or histidines involved in zinc coordination led to inviable mutants (Figure 18B), confirming that the C2H2 finger is essential for Eco1 function.

In conclusion, these data demonstrate that Eco1-PCNA binding is essential for chromatid cohesion, and that an impairment in the interaction results in cell death due to the loss of cohesion.

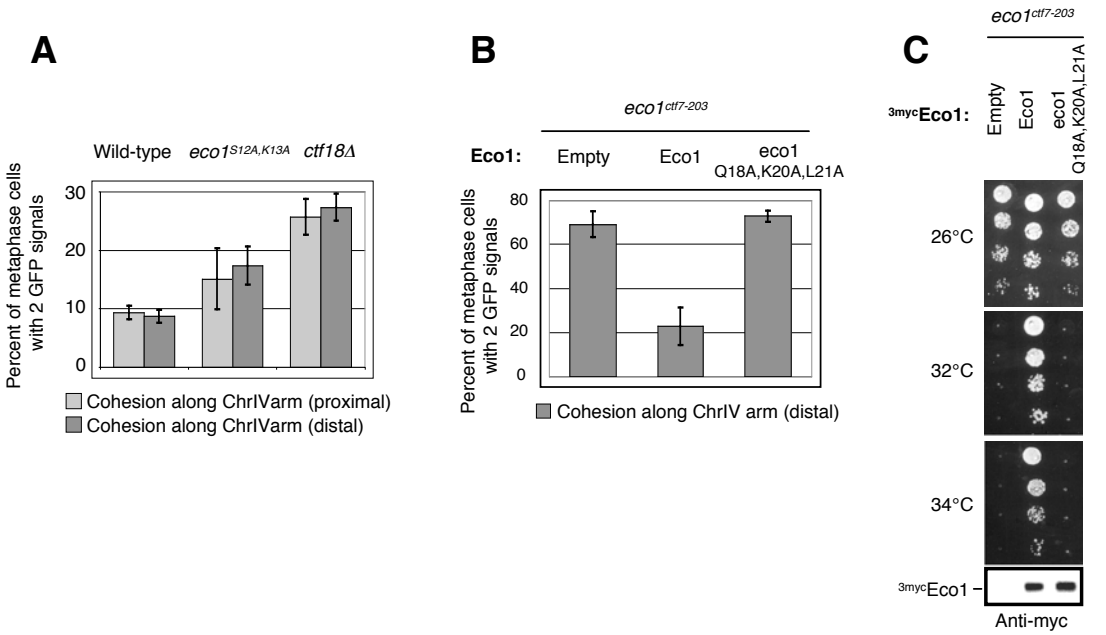

Figure 19. The PCNA-Eco1 interaction is required for efficient sister chromatid cohesion. A. The mutant *eco1*^{S12A,K13A}, partially defective in PCNA-binding, shows cohesion defects. The cohesion assays were done using GFP tagging of ChrIV. *ctf18Δ* cells, impaired in establishment of cohesion (Mayer *et al.*, 2001), were used as control. Each strain was assayed 4-10 times, with 50 cells being counted in each assay. Error bars represent standard deviations. **B.** An *eco1* mutant, which expresses a variant defective in PCNA binding (eco1^{Q18A,K20A,L21A}) cannot complement the cohesion defects of *eco1*$^{ctf7-203}$. *eco1*$^{ctf7-203}$ cells with tagged ChrIV were arrested in G1 by α–factor treatment, and released into medium containing nocodazole at the restricted temperature (34°C) of the mutant. G2/M-arrested cells were microscopically assayed for cohesion defects. For each strain, 3-6 experiments were done in which at least 50 cells were counted. Error bars represent standard deviations. **C.** Endogenous expression of wild-type Eco1, but not of eco1^{Q18A,K20A,L21A} can rescue the viability of *eco1*$^{ctf7-203}$ mutants at restrictive temperatures.

2.3.5 Eco1 and its human homolog ESCO2 bind PCNA via a conserved PIP-box variant

Eco1 is highly conserved across species. In humans, two Eco1 homologs, ESCO1 and ESCO2 have been described (Bellows *et al.*, 2003; Hou and Zou, 2005). Mutations in *ESCO2* were linked to severe hereditary diseases, Roberts and SC phocomelia syndromes (Schule *et al.*, 2005; Vega *et al.*, 2005). The conserved region between all Eco1 family members comprises the yeast Eco1 protein, including the zinc finger and the ACT domain. Within the N-terminal 33 residue PCNA-binding region of Eco1, the sequence QxxL/I (residues 18-21) is conserved among all Eco1 family members. This sequence resembles an element of two previously characterized PCNA-binding motifs, the PIP and KA boxes (Maga and Hubscher, 2003) (Figure 20A) suggesting that the conserved Q18 and L21 residues may be crucial for PCNA binding. Indeed, replacement of these aminoacids by alanine led to

Eco1 mutant proteins (eco1^{Q18A}, eco1^{L21A}, eco1^{Q18A,L21A}) that were deficient in PCNA binding and incapable of supporting cell viability (Figure 20B,C). As expected, these mutants could not rescue the temperature sensitivity of *eco1$^{ctf7-203}$* (Figure 21), demonstrating that they are incapable of establishing chromatid cohesion.

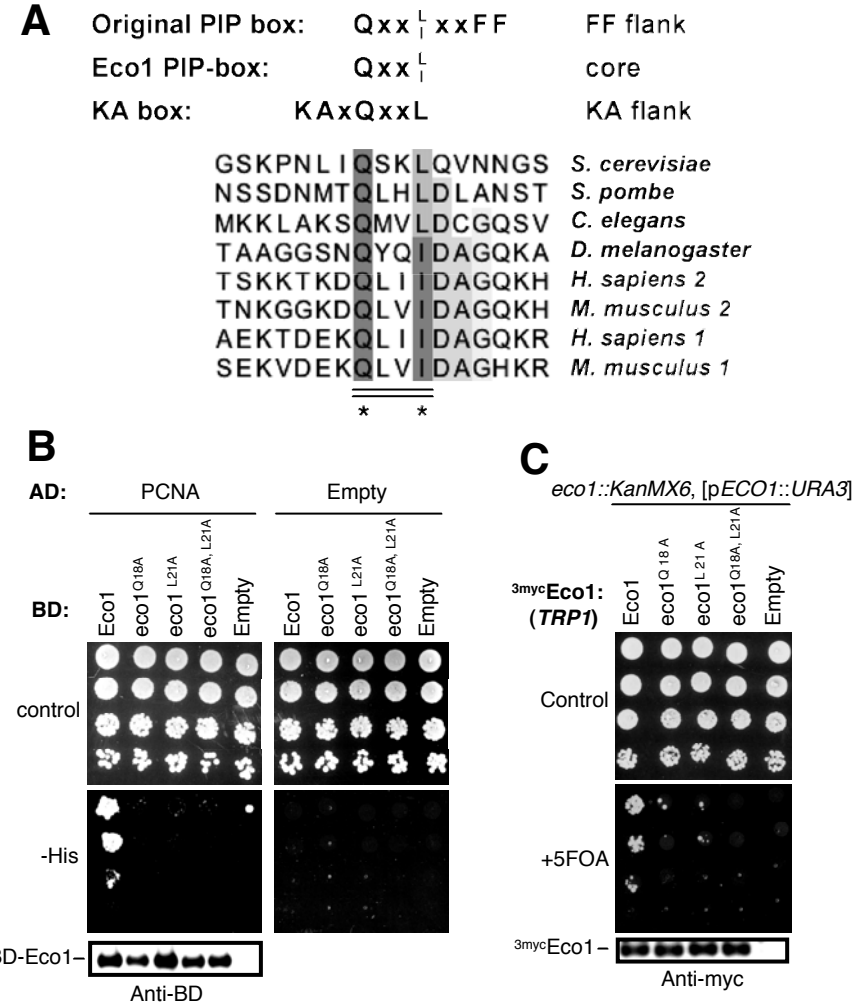

Figure 20. The PCNA-binding site represents a variant PIP-box. A. A conserved PCNA interaction motif is present in all Eco1 family members. Alignment of homologous sequences to *S. cerevisiae* Eco1 from residues 11 to 27. The positions occupied by the conserved amino acids Q and L/I, marked with an asterisk, matches the core of the PCNA-interaction motifs PIP-box and KA-box. **B.** The Eco1 PIP-box is required for interaction with PCNA in two-hybrid assays. **C.** The Eco1 PIP-box is required for cellular viability.

Although homologs of Eco1 from other species have long amino-terminal extensions, they all contain the novel Eco1 PIP box (Figure 20A, 22A), suggesting that PCNA binding is probably a conserved feature in all members of the Eco1 family. Indeed, the full-length human ESCO2 protein and an N-terminal fragment (ESCO2$^{1-386}$) that contains the conserved QxxI

sequence, interacted with both human and yeast PCNA (Figure 22B). In summary, these findings demonstrate that Eco1 protein family members contain a core PIP-box required for PCNA interaction.

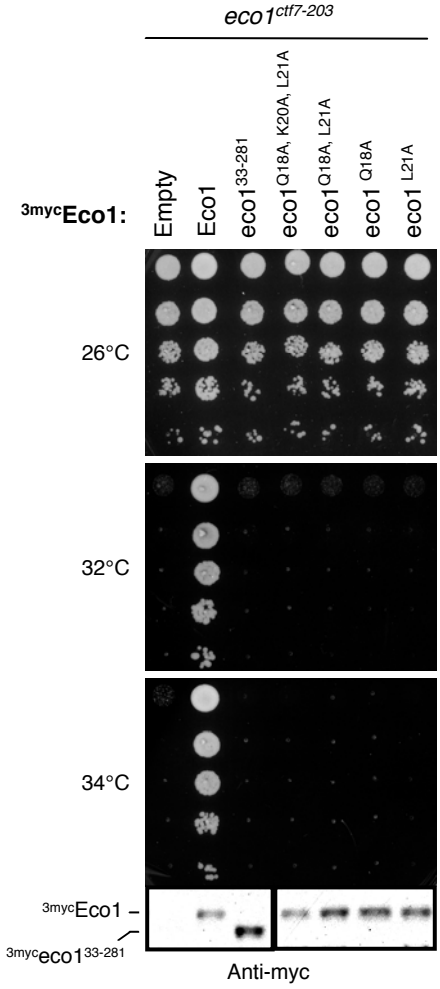

Figure 21. The PIP box of Eco1 is required for normal establishment of cohesion. Endogenous expression of wildtype Eco1, but not of variants lacking the PCNA interaction domain or mutated in the PIP box can rescue the viability of cohesion-defective *eco1*$^{ctf7-203}$ cells at restrictive temperatures.

2.3.6 PCNA interaction is required for normal chromatin association of Eco1

PCNA was previously shown to be required for the loading of the silencing factor Cac1 onto chromatin (Zhang *et al.*, 2000). In order to test if the outcome of its interaction with Eco1 might be similar, the chromatin association of wild-type Eco1 and of mutants defective in PCNA interaction was tested. Chromatin was purified from cells expressing different 3myc-tagged Eco1 variants under the control of the *ECO1* promoter and analyzed by Western blotting using anti-myc antibodies. The localization of the Eco1 PIP-box mutants eco1^{Q18A,K20A,L21A} and eco1^{Q18A,L21A} to chromatin was found to be significantly diminished, arguing that the interaction with PCNA is required for efficient loading of Eco1 onto chromatin. In contrast, the localization of eco1^{S12A,K13A}, which is only partially defective in binding PCNA, was found to be only weakly affected (Figure 23). As controls, the DNA-associated proteins

PCNA and Histone 2B were found in the chromatin fraction (H2B as well in the soluble fraction), while the cytoplasmic Dpm1 was not (Figure 23A).

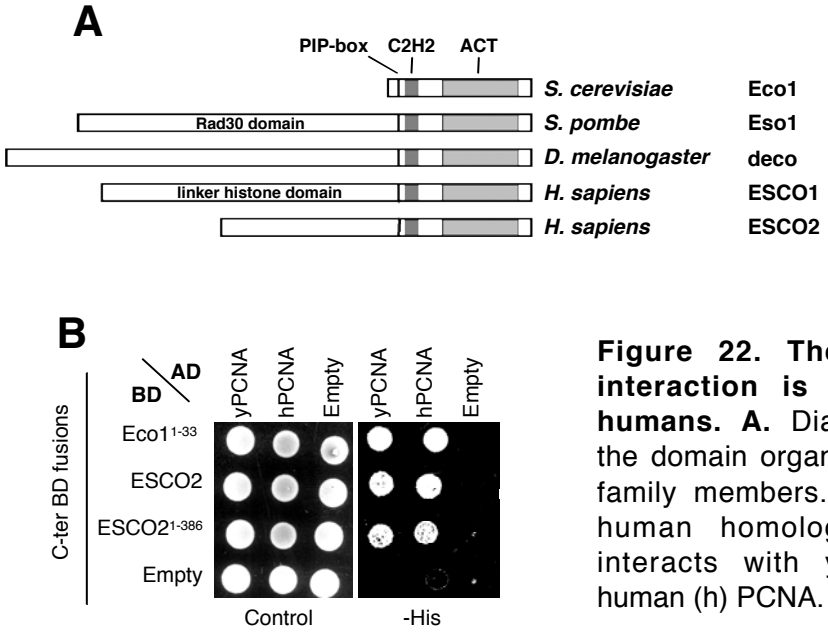

Figure 22. The Eco1-PCNA interaction is conserved in humans. A. Diagram showing the domain organization of Eco1 family members. **B.** ESCO2, a human homologue of Eco1, interacts with yeast (y) and human (h) PCNA.

Both PCNA and Eco1 function during S-phase. In order to test if their chromatin association is influenced by the cell cycle, chromatin was purified from cells synchronized by α-factor arrest/release. Consistent with their replication-linked roles, both were found to be recruited to chromatin preferentially in S-phase (Figure 24). Interestingly, this activity was still present in the Eco1 mutant lacking the N-terminal PCNA-binding region, although the overall levels of chromatin-bound protein were reduced (Figure 24). Overall, these data show that PCNA recruits Eco1 to chromatin by binding to the Eco1 PIP box.

2.3.7 Eco1-dependent cohesion is repressed by PCNA SUMOylation

In S-phase, PCNA can be modified by mono-ubiquitin, a multi-ubiquitin chain, and SUMO (Hoege *et al.*, 2002). Ubiquitylation, triggered by DNA damage, occurs at lysine 164 of PCNA and promotes two types of replicative DNA damage bypasses (Hoege *et al.*, 2002; Stelter and Ulrich, 2003). SUMO is attached to both K164 and K127 independent of DNA damage and recruits the helicase Srs2 to prevent homologous recombination during S phase (Hoege *et al.*, 2002; Papouli *et al.*, 2005; Pfander *et al.*, 2005). Given the importance of these modifications for PCNA functions, their possible effect on Eco1-dependent cohesion was investigated.

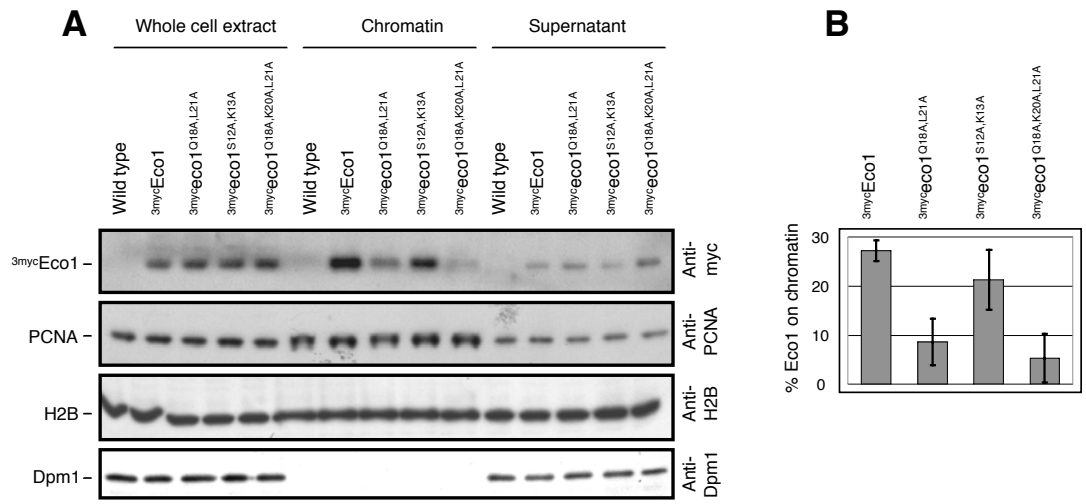

Figure 23. Chromatin association of Eco1 depends in part on PCNA interaction. **A.** Chromatin fractionation experiment using cells expressing 3myc-tagged wild-type Eco1, or mutants with defective PCNA interaction, shows that mutations in the PCNA-interacting domain of Eco1 reduce its ability to bind chromatin. PCNA and H2B (controls) are chromatin associated, whereas the cytoplasmic Dpm1 protein was used as a negative control. 10% of the extract and 5% of the supernatant were loaded. **B.** Quantification of chromatin-association activity of Eco1 variants. Bars represent the percentage of chromatin-bound Eco1, averaged from 3 independent experiments. Error bars represent standard deviations.

The temperature sensitivity of an *ECO1* mutant, *ctf7-203*, was previously shown to be alleviated by overexpression of PCNA (Skibbens *et al.*, 1999). Surprisingly, overexpression of the PCNA mutant *PCNA-K127R* and especially of the mutant that lacks both lysine residues (*PCNA-K127,164R*) suppressed the temperature sensitivities of two different *eco1* mutants (*ctf7-203 and eco1-1*) (Toth *et al.*, 1999) significantly stronger than wild-type PCNA (Figure 25A). Moreover, suppression by wild-type PCNA was considerably stronger in cells deficient in Siz1, the SUMO ligase responsible for PCNA SUMOylation at K164 (Hoege *et al.*, 2002; Pfander *et al.*, 2005). These results suggest that PCNA SUMOylation is toxic for $eco1^{ctf7\text{-}203}$ and *eco1-1* cells.

Suppression of the *eco1* mutant phenotype was not restricted to overexpression of PCNA mutants defective in SUMOylation, but also was observed when the lysine mutants of PCNA were expressed from the genome to normal levels, or in *siz1* mutant cells (Figure 25B). The *PCNA K164R* or *siz1Δ* mutations showed a weaker suppression compared to the *PCNA K127R* mutation, while the *PCNA KK127,164RR* mutant presented the strongest rescuing abilities (Figure 25B), indicating that SUMOylation at both lysins, but especially at K127 are inhibitory for cohesion.

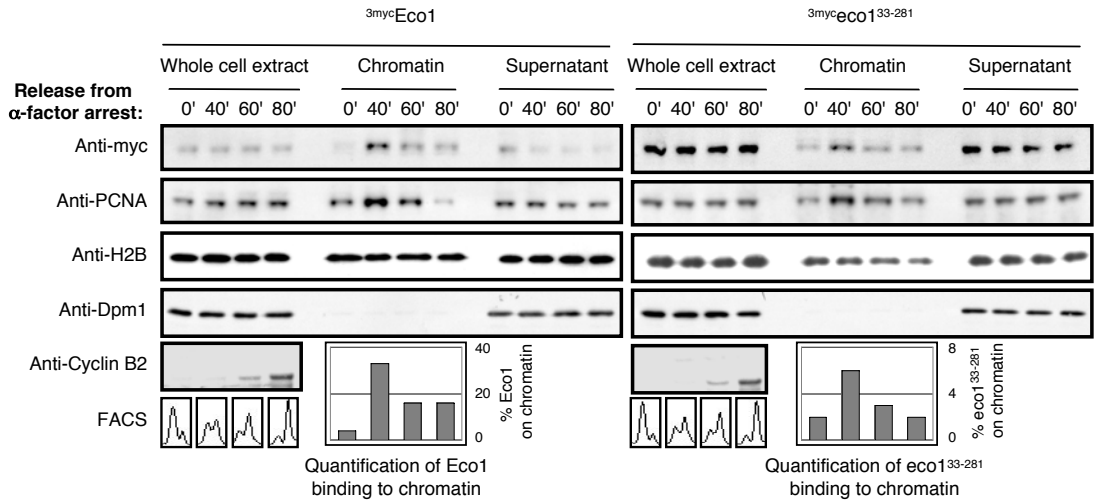

Figure 24. Eco1 and PCNA are recruited to chromatin in S-phase. Analysis of chromatin from cells synchronized in different cell cycle phases by α-factor arrest/release shows a cell cycle-dependent chromatin association of PCNA and Eco1, and strong binding in S-phase. The binding of eco1$^{33-281}$ is significantly reduced. 10% of the extract and supernatant fractions were loaded. FACS analysis and the levels of the mitotic cyclin Clb2 are used to indicate the cell cycle phase. The quantifications show the amounts of ^{3myc}Eco1 an ^{3myc}eco1$^{33-281}$ on chromatin. Bars represent the percentage of chromatin-bound Eco1.

As expected, loss of endogenous PCNA SUMOylation led to a partial rescue of the cohesion defect of *eco1$^{ctf7-203}$* (Figure 25C). PCNA SUMOylation was shown to inhibit recombination through Srs2 recruitment (Papouli *et al.*, 2005; Pfander *et al.*, 2005), however, this pathway is not responsible for the effect observed, as *srs2Δ* mutants do not suppress the temperature sensitivity of *eco1$^{ctf7-203}$* (Figure 25B). Together, these findings indicate that modification of PCNA by SUMO is detrimental for Eco1 function.

Eco1 was shown to possess *in vitro* acetyl transferase activity, and mutations in the ACT domain of *Drosophila* and human orthologues of Eco1 impair its function (Vega *et al.*, 2005; Williams *et al.*, 2003). In *S. cerevisiae,* the *eco1-1* temperature sensitive mutant encodes a variant with a single aminoacid substitution (G211D) in the ACT domain which inactivates the *in vitro* acetyl transferase activity of Eco1 (Ivanov *et al.*, 2002). In order to find out if the temperature sensitivity of *eco1$^{ctf7-203}$* cells was as well linked to deficient acetylation activity, this allele was sequenced and found to encode a protein with three point mutations (D168V, I231F and G259R), localized to the conserved motifs (Dyda *et al.*, 2000) of the ACT domain (Figure 25D). *In vitro* acetylation assays showed that eco1$^{ctf7-203}$ is unable to self-acetylate itself or to acetylate Scc1 (Figure 25E and data not shown). These results argue that that the acetyl transferase activity is essential for Eco1 function and that PCNA SUMOylation is toxic in Eco1 mutants defective in this activity.

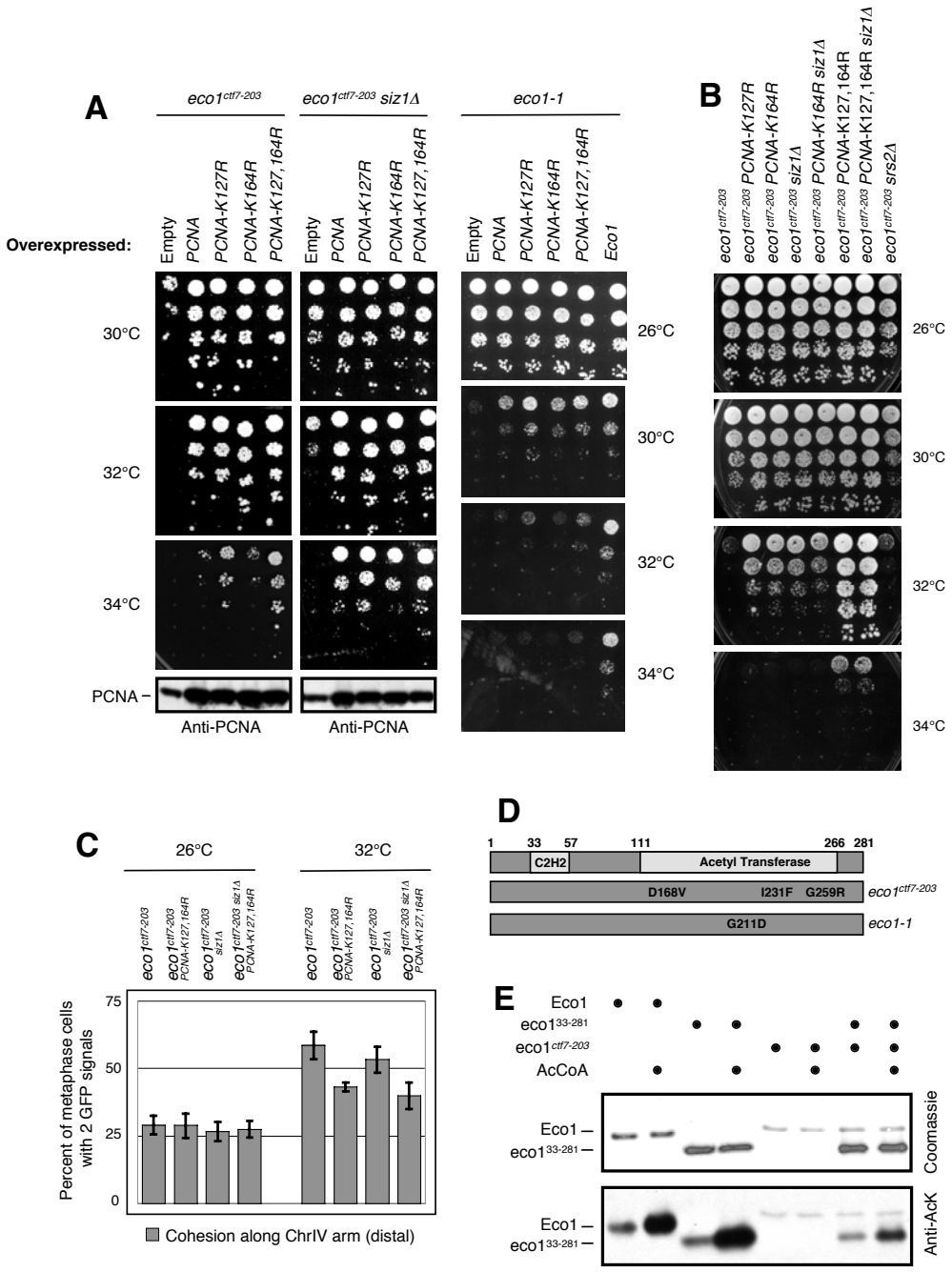

Figure 25. SUMOylation of PCNA is detrimental for Eco1 function. A. Overexpression of SUMOylation-deficient PCNA mutants leads to a stronger suppression of the temperature sensitivity of *eco1-1* and *eco1*$^{ctf7-203}$ mutants compared to WT PCNA. In cells deficient in the SUMO ligase Siz1 overexpression of WT PCNA leads to a stronger suppression as well. **B.** Loss of SUMOylation of endogenous PCNA, but not inactivation of the helicase Srs2, suppresses the temperature sensitivity of *eco1*$^{ctf7-203}$. **C.** Loss of PCNA SUMOylation partially rescues the cohesion defects of *eco1*$^{ctf7-203}$ at the restrictive temperature (32°C). Sister chromatid cohesion was quantitatively assayed using yeast strains GFP-tagged on ChrIV. Cells were grown either at permissive temperature (26°C) or shifted for 3 hours at 32°C. Each strain was assayed 3-5 times at both temperatures with at least 50 cells being counted in each experiment. Error bars represent standard deviations. **D.** Diagram showing the point mutations identified in the *eco1*$^{ctf7-203}$ and *eco1-1* alleles **E.** The eco1$^{ctf7-203}$ mutant protein is devoid of acetylation activity *in vitro*.

Although loss of PCNA SUMOylation led to activation of cohesion in *ECO1* mutants (Figure 25A,B), it had no detectable effect on cohesion of wild-type cells (Figure 26A). However, the *PCNA A251V* mutant, which is lethal in conjunction with the *eco1*$^{ctf7\text{-}203}$ mutation (Skibbens *et al.*, 1999) and which exhibits strong cohesion defects (Figure 12), encodes for a PCNA variant that is hyper-SUMOylated (Figure 26B). This hyper-SUMOylation is not a general effect explaining the synthetic interactions with Eco1, as other mutants that are lethal in combination with *eco1*$^{ctf7\text{-}203}$, like *chl1Δ* (Figure 26B) or *ctf18Δ* (not shown) have normal SUMOylation. Although *PCNA A251V* is a mutant with pleiotropic phenotypes, that may also be defective in other interactions, its cohesion defective phenotype correlates well with its hyper-SUMOylation state, suggesting that increased PCNA SUMOylation is inhibitory for cohesion even in cells wild-type for *ECO1*.

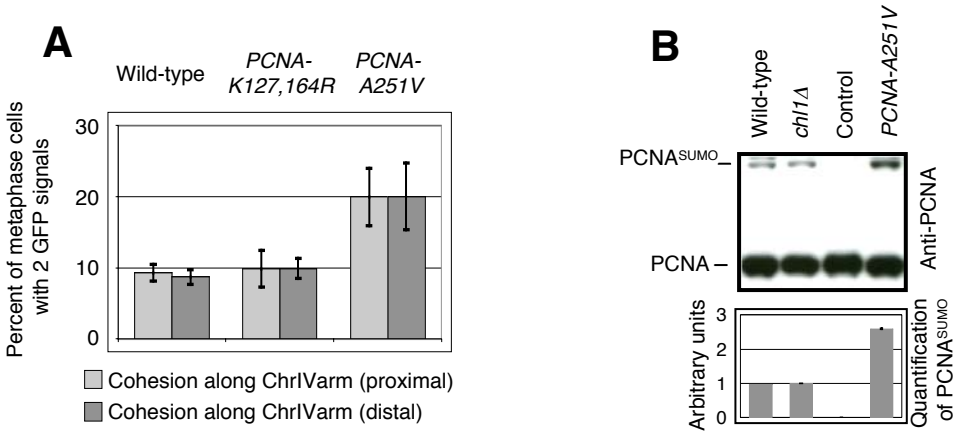

Figure 26. Increased PCNA SUMOylation is detrimental for cohesion. A. Loss of PCNA SUMOylation does not affect cohesion, but increased SUMOylation leads to cohesion defects. The assays for the cohesion defects of the indicated strains were done using GFP tagging of ChrIV. Bars represent averages of 3-10 independent experiments. Error bars represent standard deviations. **B**. The cohesion-defective *PCNA A251V* mutant shows increased levels of SUMOylation *in vivo*. Another mutant that is synthetically lethal with *eco1*$^{ctf7\text{-}203}$, *chl1Δ*, shows normal levels of PCNA SUMOylation. Cells deficient in PCNA SUMOylation were used as control. The quantification of PCNA^{SUMO} levels was done by normalization to the band corresponding to unmodified PCNA and represents an average of 3 independent experiments. Error bars represent standard deviations.

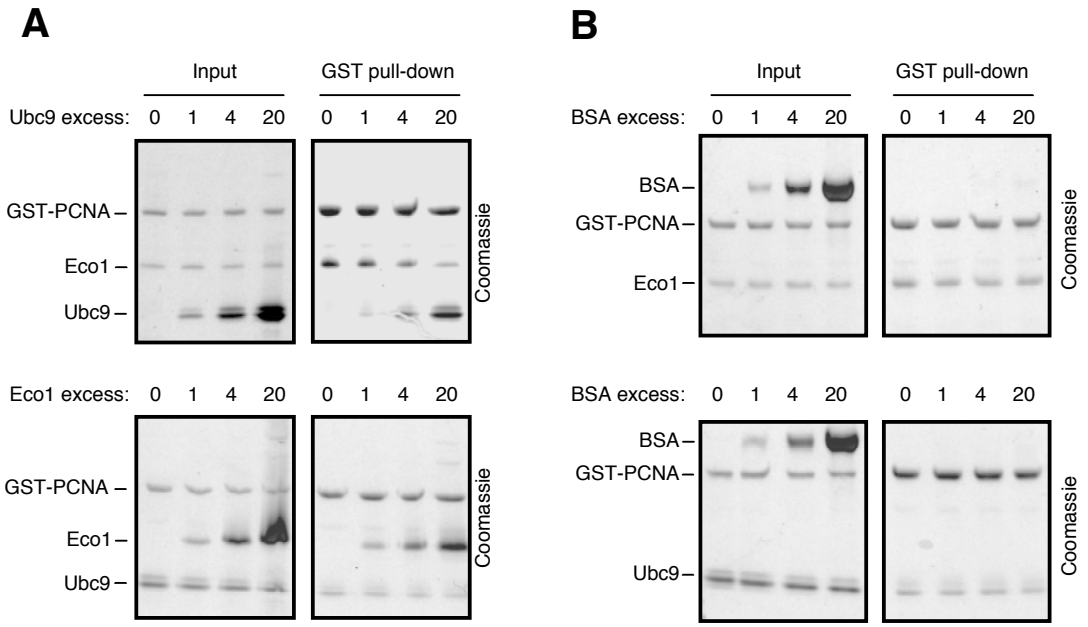

Figure 27. Ubc9 and Eco1 are rivals for PCNA binding. A. GST-PCNA was incubated with constant amounts of Eco1 and increasing amounts of Ubc9 (top panel), or with constant amounts of Ubc9 and increasing amounts of Eco1 (bottom panel), and subjected to GST pull-downs. **B.** Increasing amounts of BSA do not interfere with binding of either Eco1 (top panel) or Ubc9 (bottom panel) to GST-PCNA.

The PIP-box-containing proteins p21 and RFC1 were shown to interact with an area on the surface of PCNA that contains PCNA's interdomain-connecting loop. Because Eco1 also interacts with PCNA via its PIP-box (Gulbis *et al.*, 1996; Matsumiya *et al.*, 2002), it is likely that it binds to the interdomain-connecting loop as well. Interestingly, lysine 127 is located in the interdomain-connecting loop (Hoege *et al.*, 2002), and SUMOylation at this site is particularly detrimental for survival of the cohesion-defective *eco1*$^{ctf7\text{-}203}$ mutant (Figure 25A,B). Alltogether, this raises the possibility that PCNA SUMOylation sterically hinders Eco1 binding to PCNA, thereby repressing cohesion. Because of the low level of PCNA SUMOylation *in vivo* and of its transient nature, it could not be tested directly whether SUMOylation of PCNA inhibits Eco1-binding *in vivo*. However, K127 is present in a consensus site that interacts directly with the SUMO-conjugating enzyme Ubc9 (Bernier-Villamor *et al.*, 2002; Hoege *et al.*, 2002). This suggests that Ubc9 and Eco1 are alternative binding partners for PCNA. Indeed, when GST-PCNA was mixed with constant amounts of Eco1 and increasing amounts of Ubc9, Ubc9 could efficiently displace Eco1 form PCNA in a concentration-dependent manner (Figure 27A), Eco1 displaced Ubc9 less potently in a similar assay (Figure 27A), while a control, BSA, had no effect on the binding of Eco1 or

Ubc9 to PCNA (Figure 27B). Together with the genetic data on the suppression of *eco1* mutants by SUMOylation-defective PCNA (Figure 25), this finding clearly supports Eco1-PCNA-dependent sister chromatid cohesion being inhibited by PCNA SUMOylation, and that Eco1 competes with the SUMOylation machinery for PCNA binding.

2.3.8 PCNA SUMOylation functionally antagonizes PIP-box proteins

The fact that Ubc9 and PIP-box factors bind to the same surface area of PCNA suggests that not only Eco1, but all PIP-box containing proteins compete physically and functionally with PCNA SUMOylation. The effect of this modification on the functions of two PIP-box-containing interactors of PCNA, Rfc1 and Cac1 was investigated by genetic assays.

Rfc1 is the large subunit of the RFC complex, required for PCNA loading on chromatin during S-phase (Majka and Burgers, 2004). The *rfc1-1* mutation (McAlear *et al.*, 1994) renders cells sensitive to low temperatures. The cold sensitivity of *rfc1-1* mutant could be supressed by overexpression of PCNA (Figure 28A). Importantly, overexpression of PCNA mutants defective in SUMOylation, especially of PCNA K127R, could rescue the cold tolerance of *rfc1-1* much better than overexpression of wild-type PCNA (Figure 28A), suggesting that PCNA SUMOylation at K127 is inhibitory for Rfc1 function.

Cac1 is a subunit of the chromatin remodeling complex CAF-1, which interacts with PCNA during S-phase to re-establish the silencing state of the newly replicated DNA (Zhang *et al.*, 2000). The function of Cac1 can be directly assayed using silencing tester strains, which contain the *URA3* marker integrated at the telomere of ChrVII. This region shows normally a variegated expression, with some cells expressing it, and others spontaneously silencing it, which ensures that wild-type cells can grow on both medium lacking uracil and medium containing 5-FOA, which counter-selects cells expressing the *URA3* gene. In contrast, silencing defective *cac1Δ* cells cannot turn off gene expression at telomeres and are unable to grow in the presence of 5-FOA (Zhang *et al.*, 2000 and Figure 28B). Interestingly, cells defective in PCNA SUMOylation at K127 grow normally on 5-FOA-containing medium, but show reduced growth on medium lacking Uracil (Figure 28B). This suggests a defect in expression of the *URA3* gene at the telomeres, which could be attributed to an increased silencing at this locus. As Cac1-binding is required for the type of silencing measured by this assay (Zhang *et al.*, 2000), these results argue that the PCNA K127R mutant interacts stronger with Cac1 than wild-type PCNA.

Taken together, these experiments suggest that PCNA SUMOylation is detrimental to the function of both PIP-box proteins analyzed, Rfc1 and Cac1.

In light of the effect of PCNA SUMOylation on cohesion and Eco1 binding to PCNA, it is reasonable to speculate that this posttranslational modification geberally blocks the binding of PIP-box containing proteins to PCNA, thereby antagonizing their function.

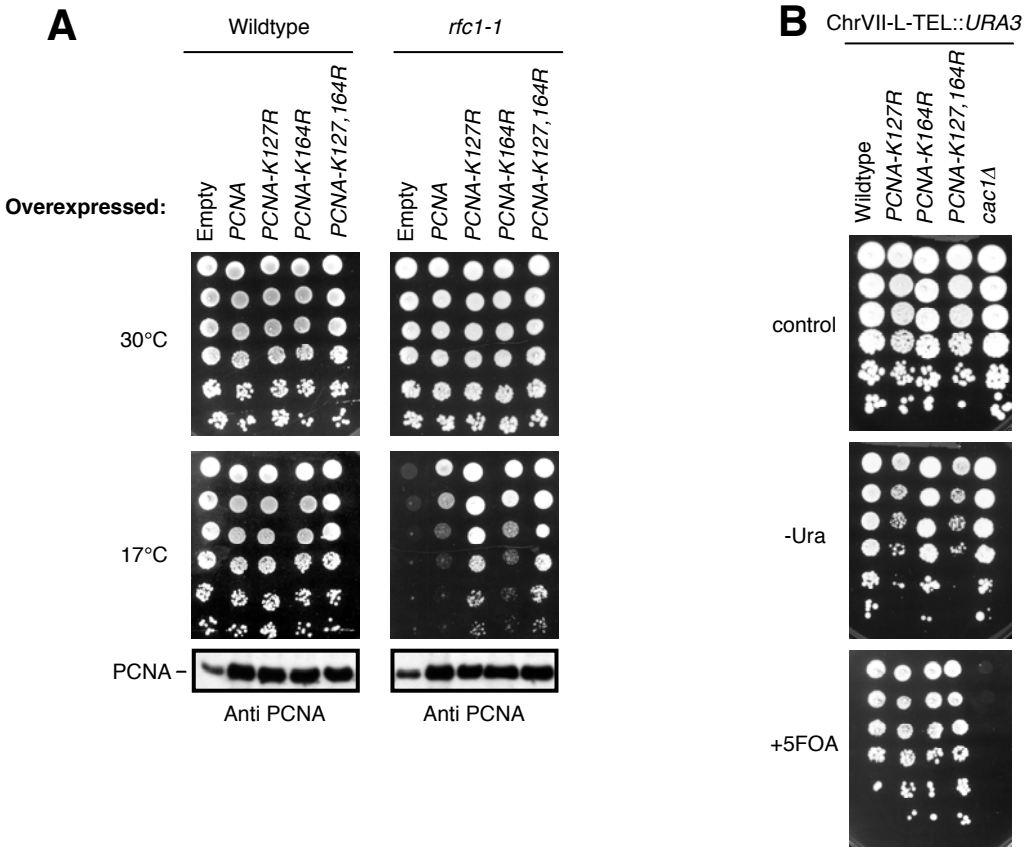

Figure 28. PCNA SUMOylation at K127 inhibits the functions of PIP-box-containing PCNA-interacting proteins. **A.** Overexpression of *PCNA K127R* mutant rescues the cold sensitivity of *rfc1-1*. Wildtype or lysine mutants of PCNA, as indicated, were overexpressed in *rfc1-1* cells or in the isogenic wildtype cells. **B**. Lack of SUMOylation at K127 leads to increased silencing of telomeric genes. Cells containing the only copy of the URA3 gene inserted at the telomere of ChrVII –long arm, and containing either wildtype PCNA, or lysine mutants of endogenous PCNA or being mutated for *CAC1*, as indicated, were spotted in fivefold serial dilutions on control (YPD) plates or on plates lacking Uracil or containing 5-FOA.

3. DISCUSSION

Faithful replication and transmission of the genome is a fundamental prerequisite for cell division and warrants proper genetic inheritance. The replication fork integrates several activities that ensure the proper transmission of the genetic information, including DNA replication, DNA repair, transmission of the silenced chromatin state and repression of deleterious recombination events. How these genome stability mechanisms are orchestrated by the replisome is thus far still poorly understood. To shed some light on the regulation of these important processses, the present study addressed, in its first two parts, posttranslational modifications with proteins of the ubiquitin family, that regulate replication through DNA lesions. In the third part, this work investigated how the replication fork controls the correct distribution of the replicated genetic material, by ensuring the cohesion of newly replicated sister chromatids. In particular, a direct coupling of cohesion to replication could be revealed for the first time in this study.

3.1 Posttranslational modifications of PCNA with ubiquitin and SUMO are conserved from yeast to man

As elaborated before, PCNA is a highly conserved protein, both structurally and functionally. Moreover, K164 of PCNA, which is the attachment site for ubiquitylation and for the bulk of SUMOylation was preserved throughout evolution (Figure 7A). Thus, although the posttranslational modifications of PCNA by ubiquitin and SUMO were originally identified in *S. cerevisiae* (Hoege *et al.*, 2002), it came as no surprise that these modifications were retained in higher eukaryotes as well.

3.1.1 Conservation of PCNA ubiquitylation

PCNA ubiquitylation is an S-phase specific DNA damage response, mediated by the activity of the *RAD6* pathway of DNA repair. When the replication fork encounters a DNA lesion, PCNA gets ubiquitylated at K164 in order to prevent prolonged stalling of the replication machinery at the damaged site. Mono-ubiquitylation of PCNA by Rad6 and Rad18 recruits translesion polymerases, special enzymes that are able to perform DNA synthesis through the lesion in a template-independent mechanism, thereby often introducing mutations (Bienko *et al.*, 2005; Kannouche *et al.*, 2004; Watanabe *et al.*, 2004). Alternatively, PCNA modification by K63-linked ubiquitin chains, which requires as well Rad5, Ubc13 and Mms2, governs an error-free lesion bypass

mechanism. This error-free repair has not been mechanistically characterized so far, but it is likely to involve a recombination-like event with the undamaged sister chromatid. It is plausible that special factors recognize the K63-multiubiquitin chains on PCNA and activate the error-free mechanism (Zhang and Lawrence, 2005).

Homologs of all members of the *RAD6* pathway of DNA repair, including Rad6, Rad18, Rad5, Ubc13, Mms2 and TLPs were found in mammals, and their inactivation leads to sensitivity to DNA damaging agents (Andersen et al., 2005; Koken et al., 1991; Masutani et al., 1999; Tateishi et al., 2000; Unk et al., 2006). Indeed, in both chicken and human cells, treatment with the DNA alkylating agent MMS leads to induction of PCNA ubiquitylation (Figures 7 and 8) by the activity of the RAD6 pathway (Figure 8 and Watanabe *et al.*, 2004). Paralleling the situation in yeast, ubiquitin is attached to the conserved K164 of PCNA (Figures 7 and 8), and in chicken cells, endogenous mutation of K164 sensitizes cells to a variety of mutagens, without affecting the replication function of PCNA (Arakawa *et al.*, 2006). Thus, the PCNA-ubiquitin-dependent DNA repair mechanism is conserved from yeast to humans.

Interestingly, the relative amounts of PCNA mono- or multi-ubiquitylation are different in metazoans compared to yeast. While in *S.cerevisiae* MMS treatment induces roughly similar levels of mono- and di-ubiquitylation (Hoege *et al.*, 2002), in metazoans the mono-ubiquitylated form is prevalent (Figures 7 and 8). Probably due to its very low amounts, multi-ubiquitylation could only be detected in Saos-2 cells which express PCNA at high levels (Figure 7E). This finding suggests that the use of the error-prone pathway has been up-regulated during evolution. Indeed, higher eukaryotes have more TLPs than yeast, and it can be envisaged that a tight regulation of their recruitment, based on their specificities to insert certain nucleotides, might even ensure bypass of lesion without introducing mutations.

Surprisingly, in *RAD18*$^{-/-}$ DT40 cells, basal levels of PCNA ubiquitylation are still detected (Figure 8). This is in stark contrast with the situation in *S. cerevisiae*, where *RAD18* deletion completely abolishes this modification. Thus, metazoans seem to have evolved a backup pathway, *RAD18*-independent, for PCNA ubiquitylation. As the residual PCNA ubiquitylation in *RAD18*$^{-/-}$ cells is not induced by exogenous DNA damage (Figure 8), it remains equivocal whether this backup pathway is a general DNA damage response mechanism, or rather a cell type specific activity.

Again in contrast to baker's yeast, in chicken DT40 and mammalian Saos-2 and SER-W3 cells (Figures 7D,E and 8), mono-ubiquitylation could also be detected in the absence of exogenous DNA damage, but was further

inducible by treatment with MMS. It is likely that the ubiquitylation detected in the absence of MMS simply represents a response to endogenous DNA damage, as a similar situation was reported for PCNA ubiquitylation *in S.pombe* (Frampton *et al.*, 2006).

Importantly, in metazoans the PCNA-ubiquitin pathway is exploited for diversification of the antibody repertoire. In immature B cells, the gene loci encoding for immunoglobulins (Igs) are subjected to intensive recombination of the V, D and J segments to obtain a basal level of antibody diversification (Neuberger *et al.*, 2000). To produce antibodies with higher specificities, a second round of diversification takes place in activated B cells via somatic hypermutation (SHM) and gene conversions (Neuberger *et al.*, 2003). SHM consists of random aminoacid substitution in the V segment, while gene conversion is a process which involves recombination with a non-functional pseudo-V variant genes. Both mechanisms rely on DNA repair pathways, which are induced by the Ig locus-specific activity of activation-induced deaminase (AID), an enzymes that deaminates cytosines (Arakawa *et al.*, 2002). The mismatched uracils formed are removed by the uracil glycosylase UNG-2, leading to abasic sites which are repaired in an error-prone mode by either SHM or gene conversion (Di Noia and Neuberger, 2002). For SHM to occur, PCNA mono-ubiquitylation is required to recruit TLPs, like Rev1 and Polη, to the abasic site generated by UNG-2 (Arakawa *et al.*, 2006; Diaz and Lawrence, 2005). It is not clear how PCNA ubiquitylation is coupled to AID–UNG-2 processing of cytosines, but it is likely that certain factors bind to the abasic site and protect it from the repair machinery until S phase. When the replication fork encounters such sites, PCNA is mono-ubiquitylated and TLPs are recruited to bypass the structure, producing mutations.

Recently, PCNA was found to be ubiquitylated at K164 in *X. laevis* egg extract (Leach and Michael, 2005). Multi-ubiquitylation occurred in *X. laevis* in response to DNA damage and it is very likely that it represents a conserved DNA repair event. Interestingly, PCNA mono-ubiquitylation was observed in S phase in the absence of DNA damage, and was not further induced by UV light treatment. Moreover, inhibition of PCNA ubiquitylation greatly inhibited replication fork progression, arguing that, in contrast to the situation *in S. cerevisiae*, this modification is required for DNA replication in *Xenopus* egg extract. It is not clear, however, how DNA replication is affected by loss of PCNA ubiquitylation. It is possible that TLPs are used extensively for DNA replication in this *in vitro* system, because of DNA lesions induced during manipulation, and the slow-down of replication fork progression merely reflects the inability to target TLPs to chromatin in the absence of PCNA ubiquitylation. Alternatively, it is possible that PCNA mono-ubiquitylation-

dependent translesion polymerases are specifically used for DNA synthesis during early embryonic cell cycles in order to ensure a fast replication and to avoid checkpoint activation, which would interfere with the developmental program.

In conclusion, PCNA ubiquitylation at K164 in response to DNA damage was identified in all species investigated (Table 1): *S. cerevisiae* (Hoege *et al.*, 2002), *S.pombe* (Frampton *et al.*, 2006), *X. laevis* (Leach and Michael, 2005), *G. gallus* (this study, Figure 8) and *H. sapiens* (this study, Figure 7). The regulation and amounts of different ubiquitin modification varies from species to species, and novel functions have been acquired in some cells, however the PCNA-ubiquitin pathway for bypass of DNA lesions appears highly conserved from yeast to man. The importance of this pathway is highlighted by the observations that mutations of human Polη, which requires PCNA ubiquitylation for its recruitment to DNA lesions, are associated with increased susceptibility to cancer (Masutani *et al.*, 1999), and that loss of PCNA ubiquitylation greatly reduces the antibody repertoire of B cells (Arakawa *et al.*, 2006).

3.1.2 Conservation of PCNA SUMOylation

In *S. cerevisiae*, PCNA SUMOylation is a constitutive event, occuring during S-phase and affecting mainly K164, and to a minor extent K127 of PCNA. SUMOylated PCNA recruits the helicase Srs2 to the replication fork thereby preventing *RAD52*-dependent unwanted recombination events (Papouli *et al.*, 2005; Pfander *et al.*, 2005). *S. pombe* PCNA is apparently not SUMOylated (Frampton *et al.*, 2006), and in addition, *S. pombe* Srs2 lacks the C-terminal tail which interacts with SUMOylated PCNA, arguing that the SUMO-Srs2 pathway for inhibition of recombination is not conserved among yeast species. Moreover, the homolog of Srs2 could not be identified thus far in metazoans.

One can anticipate, however, that other helicases, perhaps members of the RecQ family (WRN or BLM) have an activity similar to Srs2, to inhibit recombination at the replication fork, in PCNA-SUMO dependent manner. PCNA SUMOylation could not be detected in human cells (Figure 7), albeit it was present in chicken B cells (Figure 8), as well as in *Xenopus* egg extract (Leach and Michael, 2005). In this system, it could be shown that the S-phase constitutive PCNA SUMOylation does not influence DNA replication, as addition of a dominant negative Ubc9 could inhibit the modification, but did not affect replication of sperm chromatin. However, possible functions of SUMOylation in recombination inhibition were not addressed in the study by Leach *et al.*

During Ig gene diversification, the abasic site produced by the activities of AID and UNG-2 can be repaired in an error-prone mode either by SHM, via PCNA ubiquitylation, or via pseudo-V gene conversion (see paragraph 3.1.1). Interestingly, gene conversion (Diaz and Flajnik, 1998) is a process mechanistically similar to the *RAD52*-dependent intrachromosomal recombination which is inhibited by PCNA SUMOylation in *S. cerevisiae* (Pfander *et al*., 2005). This modification is present in metazoan B cells (Figure 8). It is an attractive possibility that PCNA SUMOylation functions in B cells analogously to yeast, to coordinate the choice of SHM *versus* gene conversion repair of abasic sites at the Ig locus.

In conclusion, the levels of PCNA SUMOylation appear to vary depending on the organism. This heterogeneity might be due to the different approaches and techniques used to detect the modification in the different organisms, or due to its presence at very low, undetectable levels. Alternatively, PCNA SUMOylation could be a cell type-specific event. Indeed, this modification was for example detectable in B cells, but not in fibroblasts (Figures 7 and 8). Moreover, in *X. laevis*, PCNA SUMOylation was observed in egg extracts, but not in tissue culture cells (Leach and Michael, 2005). Early, undifferentiated embryonic mammalian cells were not analyzed so far. It is possible that PCNA SUMOylation is present in metazoans only in specific cell types, like embryonic or B cells, where the regulation conferred by this modification is required. However, the absence of a clear homolog of the helicase Srs2 in metazoans makes it difficult to assert unequivocally the role of SUMOylation in these organisms.

	Mono-ubiquitin	**Multi-ubiquitin**	**SUMO**
S. cerevisiae (Hoege *et al*., 2002)	Error-prone lesion bypass	Error-free lesion bypass	Inhibition of recombination
S. pombe (Frampton *et al*., 2006)	Error-prone lesion bypass	Error-free lesion bypass	Not detected
***X. laevis* egg extract** (Leach *et al*., 2005)	DNA replication	Error-free lesion bypass	Unknown function
Chicken B cells (Figure 8)	Error-prone lesion bypass	Not detected	Unknown function
Human fibroblasts (Figure 7)	Error-prone lesion bypass	Error-free lesion bypass	Not detected

Table 1. Conservation of posttranslational modifications of PCNA. So far, PCNA modifications have been investigated in *S. cerevisiae*, *S. pombe*, *X. laevis* egg extract, chicken DT40 cells and several mammalian tissue culture lines. Although overall the modifications are conserved, it appears that there are some differences from species to species regarding the presence and the function of some modifications.

3.2 SUMOylation of DNA polymerase δ - a backup pathway for inhibiting recombination at the replication fork

During DNA synthesis, PCNA forms a complex with DNA polymerases and other replication proteins at the replication fork. SUMOylation of PCNA results in the recruitment of Srs2 to the complex, mediated by a SUMO binding site in the C-terminus of Srs2 (Papouli *et al.*, 2005; Pfander *et al.*, 2005). Srs2 is able to quickly disassemble Rad51-nucleofilaments, effectively blocking any intrachromosomal recombination. This ensures that upon encountering of a DNA lesion, it is the *RAD6* pathway and not *RAD52*-dependent homologous recombination that is used for bypass of the DNA lesion. In the absence of the *RAD6* pathway, PCNA SUMOylation becomes toxic as cells have no means to restart replication forks stalled at DNA damage sites. Intriguingly, SUMOylation of PCNA can be induced by treating the cells with lethal concentrations of the DNA alkylating agent MMS (Hoege *et al.*, 2002), but the significance of this induction is not known.

Interestingly, all three subunits of DNA polymerase δ are modified by SUMO (Figure 9). The regulation of Polδ SUMOylation appears identical with that of PCNA: both are S-phase specific, and both are induced by lethal DNA damage (Figures 9 and 10). These observations already suggest a functional analogy. Indeed, similarly to PCNA SUMOylation, Pol32 SUMOylation enhances the interaction with Srs2 (Figure 11C). It is difficult to assess to what extent the Pol32-SUMO-Srs2 pathway contributes to inhibition of recombination under normal conditions. It is clear, however, that in the absence of *RAD6*-dependent lesion bypass, Pol32 SUMOylation is detrimental for DNA damage tolerance (Figure 11B), suggesting that it becomes vital for *RAD52* repression.

PCNA SUMOylation appears to be prevalent over Polδ SUMOylation, both in terms of relative amounts of modified protein and of phenotypes. This suggests that the primary SUMO target at the replication fork is PCNA, the subunits of Polδ being maybe modified simply because they are in close proximity to the clamp. Once recruited to the replication fork by PCNA, Ubc9 might modify lysine targets in other proteins, especially if they lie within consensus sites. Indeed, Srs2 becomes itself SUMOylated in the PCNA-SUMO-Ubc9 complex (Boris Pfander, unpublished data). Large scale proteomic studies showed in fact that in multiprotein complexes, several subunits are modified by SUMO at the same time. Examples of complexes containing multiple SUMOylated subunits are RNA polymerases I, II and III, the ribosome and the machineries for mRNA capping and poly-adenylation

(Hannich *et al.*, 2005; Panse *et al.*, 2004). It can be envisioned that SUMO acts as a molecular glue, that helps the formation of a stable macromolecular complex. Indeed, although both Pol32 and PCNA interact with Srs2 as well in the absence of SUMO modifications (Huang *et al.*, 2000; Pfander *et al.*, 2005). SUMOylation greatly enhances the binding (Pfander *et al.*, 2005, and Figure 11C). This scenario might explain why, in some cases, for example Pol32, inactivation of the SUMO acceptor sites in a protein does not lead to phenotypes, as long as other proteins in the complex are still SUMOylated.

In conclusion, cells have developed redundant mechanisms for the recruitment of the recombination inhibitor Srs2 to the replication fork. At least two replication proteins, PCNA and Pol32, bind directly to Srs2. The interactions are strengthened by SUMOylation of these proteins, and probably of other subunits of the complex, as the modifier is recognized by a SUMO-binding domain of Srs2. In general, the combination of SUMO modification and SUMO-binding domains might represent a widespread mechanism for assembling and stabilizing large macromolecular complexes.

3.3 Control of sister chromatid cohesion by PCNA and SUMO

Maintaining sister chromatids in close proximity to each other until anaphase is essential to ensure a correct segregation of the genetic material to the two daughter cells. Early studies have shown that cohesion must be set up during DNA replication (Gerlich *et al.*, 2006; Uhlmann and Nasmyth, 1998). The exact mechanism of cohesion establishment is enigmatic, but the conserved protein Eco1 is known to be essential for this process (Skibbens *et al.*, 1999). Eco1 is required exclusively during S-phase (Skibbens *et al.*, 1999), but how its activity is coupled to replication remained a mystery.

PCNA is ideally suited to play a role as a matchmaker for replication-linked functions because it is directly associated with the replication machinery and it does not have direct enzymatic activity. Known examples of activities that are regulated through direct interaction with PCNA-binding proteins, besides replication, include chromatin assembly via CAF-1, prevention of re-replication through Cdt1 degradation, p21-mediated cell cycle arrest and DNA mismatch repair.

3.3.1 A variant PIP-box in Eco1 mediates PCNA binding

In this study, Eco1 was identified as a novel PCNA interactor (Figure 13). Mutational analysis and binding assays revealed that Eco1 possesses a PCNA-binding site within its N-terminal region (Figures 15 and 18A).

Most PCNA interactors contain PIP-boxes, which are PCNA interaction motifs defined by the sequence QxxL/I/MxxFF/Y. This motif is accommodated in a hydrophobic groove of PCNA formed by the IDCL and the C-terminus (Gulbis *et al.*, 1996; Matsumiya *et al.*, 2002). A second PCNA-binding motif, containing the KAxQxxL sequence, was identified by *in vitro* peptide screening, but its *in vivo* relevance has not been investigated (Xu *et al.*, 2001). The latter sequence was named KA-box and, interestingly, it partially overlaps with the PIP-box sequence. The present study established that the QxxL sequence in Eco1 is crucial for PCNA binding (Figures 18A, 22B). This suggests that the PIP and KA boxes represent in fact variants of the same binding motif, composed of a common core, the QxxL sequence, and alternative flanks, KA or FF (Figure 20A).

The identification of a direct Eco1-PCNA interaction also suggests that the previously reported weak binding of Eco1 to all five subunits of the PCNA clamp loader (Kenna and Skibbens, 2003) may be indirect and bridged by PCNA.

Notably, all known members of the Eco1 protein family possess this variant PIP-box (QxxI/L, Figures 20A, 22A), suggesting that PCNA-binding is a conserved feature of these proteins. Indeed, human ESCO2, the Eco1-related protein that is defective in patients suffering from Roberts syndrome, binds PCNA as well (Figure 22B). Thus, this work identified Eco1 cohesion factors as a new class of PIP-box proteins

3.3.2 PCNA-dependent loading of Eco1 on chromatin is crucial for cohesion

It has been proposed that sister chromatids must be captured by the cohesin ring directly following replication (Skibbens, 2005), but how this might be achieved has remained unclear. The finding of a PIP box domain in Eco1 (Figure 20) suggests that the coupling of establishment of sister chromatid cohesion with replication is brought about by a direct physical coupling of this essential establishment of cohesion factor with the replication protein PCNA. This hypothesis was confirmed by the observation that PCNA binding is crucial for the essential function of Eco1, as deletions or mutations of the PCNA-interacting region in Eco1 abrogated cellular viability (Figures 16A, 18A, 20B). Indeed, Eco1 mutants in the conserved PIP-box are unable to perform establishment of cohesion (Figure 19B), while mutations in the vicinity of the PIP-box, which only partially impede PCNA binding, lead to cohesion defects (Figure 19A).

PCNA is thought to control replication functions by acting as a switchboard in recruiting different factors to the replication fork. In fact, experimental evidence for PCNA-dependent chromatin localization was only described so far for the Cac1 subunit of the CAF-1 silencing complex (Zhang *et al*., 2000). It is likely that PCNA has a similar effect on Eco1, that is to correctly localize it to DNA. This is supported by the observation that cells expressing at endogenous levels an Eco1 variant lacking the PCNA-interacting region (eco1$^{33\text{-}281}$) are not viable, however when this variant is over-expressed, under control of the *ADH1* promoter, it can partly restore viability to *eco1Δ* cells (Figure 16B).

Direct evidence for PCNA-dependent localization of Eco1 was obtained by analyzing the chromatin localization of Eco1 mutants defective in PCNA binding (Figure 23). While 27% of the wild-type Eco1 protein is bound to chromatin in cycling cells, this percentage is reduced to 5% and 9% in the case of the inviable Eco1 variants mutated in the PIP-box. Thus, when PCNA binding is abolished, cells become inviable because Eco1 cannot be recruited to chromatin to establish cohesion. On the other hand, a mutation (*eco1*^{SK12,13AA}) that only partially hinders the interaction with PCNA (Figure 18A) reduces the chromatin localization of Eco1 from 27% to 21% (Figure 23). As a consequence of this mild reduction, cells expressing this mutant are viable, but show cohesion defects (Figures 18B, 19A).

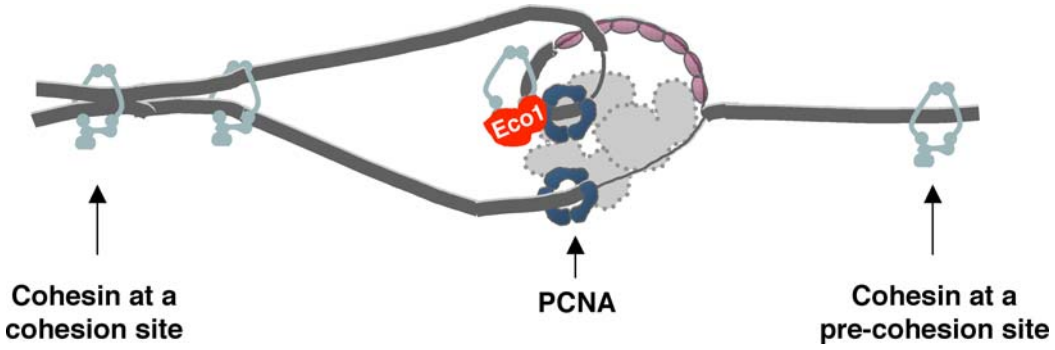

Figure 29. **Model for the coupling of cohesion establishment to DNA replication**. The replication protein PCNA recruits Eco1 to the replication fork. When the replication fork encounters a pre-cohesion site (cohesin pre-bound on un-replicated DNA), Eco1 activity transforms it into a cohesion site (cohesin rings encircling the two sister chromatids). (Adapted from Georgios Karras.)

In conclusion, these results clearly demonstrate that PCNA binding is required for normal Eco1 loading onto chromatin. Moreover, Eco1 is found on chromatin preferentially in S-phase (Figure 24). We propose that PCNA recruits Eco1 to the replication fork, where Eco1 enables cohesin rings to

encircle the two sister chromatids, thereby establishing sister chromatid cohesion (Figure 29). PCNA interaction is crucial, as inability to localize Eco1 to chromatin leads to cohesion defects, which impair cellular proliferation. Thereby, this work exposed sister chromatid cohesion as yet another function that is under direct PCNA control.

3.3.3 Three domains of Eco1 mediate its essential function in cohesion establishment

Eco1 comprises at least three different domains (Figure 22A): a PCNA-interacting motif and a C2H2-type zinc finger in its N-terminus and an acetyl-transferase (ACT) domain in its C-terminal half.

The PCNA-binding domain mediates chromatin localization of Eco1 and is crucial for cohesion establishment (see paragraphs 2.3.4 and 2.3.6). Intriguingly, elimination of the PCNA-interacting region did not abolish the S-phase peak of chromatin loading of Eco1, although the overall binding was reduced fivefold (Figure 24). This observation argues for the presence of a PCNA-independent mechanism that activates Eco1 in S-phase, making it available for chromatin loading. Such a mechanism might affect the nuclear localization of Eco1, for example by inducing the import of Eco1 into nucleus during S-phase. Alternatively, it can be envisaged that a nucleoplasmic factor inhibits Eco1 outside S-phase, maybe by binding to its PIP-box.

The zinc-finger of Eco1 must also be essential for its function, as mutations in this domain abolished viability without affecting PCNA binding (Figure 18). Moreover, a previous study identified single point mutants in the zinc finger which confer chromosome transmission defects (Brands and Skibbens, 2005). C2H2-type zinc fingers are known to mediate protein-DNA interactions. The low level of expression of zinc finger mutants makes it difficult to address their influence on chromatin loading of Eco1 (data not shown). It is possible that the DNA binding activity still observed in PIP-mutants is due to the C2H2 finger. Alternatively, the zinc finger might mediate protein-protein interactions required for cohesion establishment.

The exact function of the ACT domain remains enigmatic as well. *In vitro*, its activity is required for the acetylation of Scc1, Scc3, Pds5 and itself (Ivanov *et al*., 2002). Modification of these substrates could not be confirmed *in vivo*, and the overall acetylation proteome does not appear to be affected by mutations that inactivate ACT activity (data not shown). Moreover, some ACT mutants identified *in vitro* show no chromosome transmission defects, making the *in vivo* importance of this domain questionable (Brands and Skibbens, 2005). However, the absence of a known *in vivo* target renders the above-described observations inconclusive, as it is possible that the mutants

employed in the study by Brands and Skibbens still retain some activity *in vivo*. Indeed, there is more evidence for an important function of the ACT domain, then against it. For example, deletion of the ACT domain of the *Drosophila* homolog deco and a point mutant in the ACT of the human homolog ESCO2 were shown to lead to cohesion defects in both cases (Vega *et al.*, 2005; Williams *et al.*, 2003). Furthermore, in *S. cerevisiae*, the *eco1-1* temperature sensitive allele codes for an Eco1 variant with a single aminoacid substitution (G211D) in the ACT domain, which inactivates the *in vitro* acetyl transferase activity (Ivanov *et al.*, 2002). In this study, another temperature-sensitive allele, $eco1^{ctf7\text{-}203}$, was shown to encode an acetylation-defective mutant, with three point mutations in the ACT domain that inactivate ACT activity (Figure 25D,E). Both *eco1-1* and $eco1^{ctf7\text{-}203}$ cells have cohesion defects at restrictive temperatures, strongly arguing that the acetyl transferase activity is indeed important for Eco1 function. Identification of the relevant *in vivo* targets of Eco1 ACT activity will be invaluable to understanding the mechanism of cohesion establishment. The observations that Eco1 dimerizes (Figure 13A) and has auto-acetylation activity (Ivanov *et al.*, and Figure 17) might suggest a regulation of its own function by acetylation.

Moreover, the observation that PCNA overexpression augments viability of Eco1 mutants defective in acetylation (Figure 25A) suggests that this activity might be as well required for the stability of the association at the replication fork. Alternatively, PCNA may activate, in some way, the enzymatic activity of these mutants, or might enable a pathway that partially bypasses the requirement for acetylation.

Notably, Eco1 homologs from other species have extra domains, located N-terminally (Hou and Zou, 2005; Madril *et al.*, 2001; Williams *et al.*, 2003). These domains are variable and include a linker-histone domain in the human protein ESCO1, and a translesion polymerase domain in Eso1 from *S. pombe*. It seems plausible that these domains might direct the proteins to specific regions or functions.

In conclusion, Eco1 encompasses three domains required for its activity in cohesion establishment. The N-terminal PCNA-interacting domain is needed for its localization to the replication fork, while the function of the other two, a C2H2 zinc finger and an acetyl-transferase domain is less clear. How Eco1 mediates cohesion at the replication fork is unknown. Identification of novel Eco1 interactors should shed light on the mechanism of this important process. Establishment of cohesion is likely to proceed by opening and re-closure of the cohesin ring. As these processes require ATP binding and hydrolysis by the SMC proteins (Arumugam *et al.*, 2003), it is likely that Eco1 might regulate these activities at the replication fork.

3.3.4 Repression of cohesion, a novel function of PCNA SUMOylation

The repertoire of PCNA-linked functions is further amplified through PCNA modifications by ubiquitin and SUMO (see paragraph 1.2.3). Mono- and multi-ubiquitylation of PCNA activate two different bypasses for replication across DNA lesions and SUMOylation inhibits homologous recombination through recruitment of the helicase Srs2.

The present study established that PCNA SUMOylation also inhibits establishment of cohesion. Loss of SUMO modification of PCNA could be shown to partially rescue the viability and cohesion defects of two different *ECO1* mutants (Figure 25). Moreover, hyper-SUMOylation of PCNA was found to be correlated with cohesion defects (Figure 26). Biochemical experiments showed that the SUMOylation machinery can efficiently remove Eco1 from PCNA (Figure 27), suggesting that cohesion inhibition by SUMO is the result of a sterical constraint that impedes Eco1 binding.

At a first glance, it appears paradoxical that PCNA SUMOylation, which is triggered in S-phase, concomitant with cohesion establishment, is in fact inhibiting cohesion. However, SUMOylation of PCNA affects only a small portion of PCNA molecules and may thus be a modulator of Eco1 function. It was reported previously that cohesion is not uniform along the chromosomes, but it is rather clustered to intergenic regions (Hakimi *et al.*, 2002). This is in part the result of transcriptional activity during G2 (Lengronne *et al.*, 2004). However, it is tempting to speculate that already in S-phase, certain chromosomal regions (e.g. heavily transcribed regions) are kept free of cohesion, and SUMO might inhibit establishment of cohesion on those particular regions.

3.3.5 SUMO as a “reset button” for PCNA functions

PCNA is a fundamental regulator of DNA replication and replication-linked activities, acting as a docking site for proteins required at the replisome (Maga and Hubscher, 2003; Tsurimoto, 1999; Warbrick, 2000). Most of the proteins that bind PCNA contain a common motif, the PIP-box, which represents a PCNA-docking peptide. Proteins shown so far to use their PIP-box for PCNA interaction include DNA polymerases δ, ε and η, FEN-1, DNA ligase I, RFC, CAF-1, WSTF, Dnmt1, MSH3, MSH6, MLH1, EXO1, APE2, UNG2, MPG, XPG, Gadd45, p21, Topoisomerase II, ING1, WRN, Rrm3, UNG2, Cdt1, Mcm10 (see paragraph 1.2.2) and Eco1 (this study). All these proteins bind to a hydrophobic groove of PCNA, formed by the IDCL and the C-terminal part (Gulbis *et al.*, 1996; Matsumiya *et al.*, 2002), and they are likely to compete with each other for PCNA binding. Indeed, p21 binding to PCNA inhibits cell

proliferation by blocking the access of DNA polymerases (Gulbis *et al.*, 1996; Waga *et al.*, 1994).

Even though a PCNA trimer has three docking sites for PIP peptides, the sheer number of interactors suggests that cells have to regulate the competition of PIP-box proteins. One way to achieve this is by altering the sequences of the PIP-boxes. Indeed, the affinity of a PIP-box binding to the hydrophobic cleft of PCNA can be dramatically altered by even modest changes in the identity of the non-conserved aminoacids in the QxxL/I/MxxFF/Y box, as well as of those N- or C-terminal of the this sequence (Bruning and Shamoo, 2004). These sequence alterations result in a range of PCNA binding affinities calculated to vary at least 200-fold. The p21 PIP-box apparently has the highest affinity for PCNA, explaining its potent ability to effectively block replication. Therefore, the *in vivo* dynamics of PCNA interactions might be regulated by fine-tuning the affinities of PIP-boxes. (Bruning and Shamoo, 2004). Another model implies the kinase Cdk2 in regulating PCNA interactions (Prosperi, 2006). Interestingly, Cdk2 is one of the few PCNA interactors lacking a PIP-box, and was found in trimeric complexes comprising PCNA and PIP-box proteins (Riva *et al.*, 2004). Cdk2 phosphorylation of PIP-box proteins like RFC-1, FEN1 and DNA ligase I was shown to trigger their dissociation from the complex (Henneke *et al.*, 2003), and presumably this allows a new interactor to bind PCNA. Interestingly, a large-scale proteomic study (Ubersax *et al.*, 2003) identified Eco1 as a Cdc28 (the *S. cerevisiae* homolog of Cdk2) target, suggesting that the Cdk-dependent regulatory mechanism might apply as well to the PCNA-Eco1 interaction.

Genetic and biochemical analyses presented in the study at hand evidence that in *S. cerevisiae*, PCNA modification by SUMO inhibits cohesion by blocking the binding of Eco1 to PCNA (see paragraph 3.2.4). Intriguingly, PCNA SUMOylation appears to inhibit PCNA-linked functions more broadly. Genetic assays revealed that PCNA SUMOylation also interferes with the functions of two other PIP-box-containing PCNA interactors, Rfc1 and Cac1 (Figure 28). Remarkably, K127, one of the SUMO acceptor lysine residues for PCNA SUMOylation resides within the IDCL, an area of PCNA used for PIP-box binding. Moreover, K127 is encompassed in a “consensus” site for SUMOylation, defined by the sequence ΨKxD/E, which is known to directly bind Ubc9, the E2 SUMO-conjugating enzyme. Indeed, Ubc9 binds PCNA with high affinity and can efficiently displace Eco1 from PCNA (Figure 27). Therefore, it is conceivable that Ubc9 binding and PCNA SUMOylation might in general interfere with the function of proteins that bind PCNA via this region. Thus, SUMO modification of PCNA might constitute yet a third

mechanism for coordinating PIP-box binding to PCNA. SUMO may function as a “reset button” that clears PCNA from its binding partner in order to facilitate another round of engagement, with a new co-factor.

Lysine 164 of PCNA is located further away from the PIP-box binding site, and it is therefore not expected to markedly affect the binding of PIP-box proteins. Indeed, although K164 is the acceptor site for most of PCNA SUMOylation (Hoege *et al.*, 2002), mutation of this lysine had only minor effects on cohesion (Figure 25A,B) and Rfc1-dependent replication (Figure 28A), and no detectable effect on Cac1-dependent cohesion (Figure 28B). In contrast, the low levels of SUMOylation at K127, located in the IDCL, strongly suppress the functions of Eco1, Rfc1 and Cac1 in genetic experiments (Figures 25, 28). Previously, PCNA SUMOylation was shown to inhibit recombination by recruiting the helicase Srs2 (Papouli *et al.*, 2005; Pfander *et al.*, 2005). Srs2 binding to PCNA and recombination inhibition is proportional to the amount of SUMOylated PCNA, thus K164 is much more relevant than K127 for this function (Pfander *et al.*, 2005). Altogether, these observations show that SUMOylation at K127 and K164 of PCNA are not totally equivalent. Because only K164 SUMOylation depends on the SUMO E3 ligase Siz1 (Pfander *et al.*, 2005), this enzyme may delicately balance these two SUMO-dependent PCNA functions. Understanding the mechanism of Siz1 regulation should unravel how SUMO coordinates the numerous events that are linked to replication.

Intriguingly, K127 is not conserved in metazoans. Besides *S. cerevisiae*, SUMOylation of PCNA was reported so far in *Xenopus* egg extract (Leach and Michael, 2005) and in chicken DT40 cells (Figure 8), and in both cases it affected K164. Therefore it is possible that the K127-SUMO mechanism for regulating the dynamics of PIP-box interactions is not conserved. On the other hand, as in *S. cerevsiae* the levels of SUMOylation at K127 are very low, it cannot be excluded that this regulation is present in other eukaryotic cells. Perhaps a lysine from the IDCL or its vicinity is modified by SUMO in metazoans, to levels undetectable with the technologies currently employed.

In conclusion, this study identified inhibition of sister chromatid cohesion establishment as a novel function of PCNA SUMOylation. Most likely, SUMO performs this activity by blocking the access of the cohesion factor Eco1 to PCNA, thereby inhibiting an interaction essential for cohesion establishment. Repression of cohesion can be added to the list of three previously known functions of PCNA modifications (Figure 30): two types of DNA repair, (1) error-prone and (2) error-free, mediated by ubiquitin modification and (3) recombination inhibition, mediated by SUMOylation.

Especially relevant for cohesion inhibition is SUMO modification at K127 of PCNA. Moreover, K127 SUMOylation may represent a more general mechanism to regulate PCNA functions, by removing a bound cofactor and thus resetting PCNA for a new interaction.

PCNA modifications with ubiquitin and SUMO show how handy these posttranslational modifications can be for regulating protein functions, as they can act both by enabling and by inhibiting protein-protein interactions. By employing local control on the conjugation/deconjugation machineries, cells can turn on and off the pathway controlled by modification in a fast and energetically cheap way. Thus, it is of no wonder that more and more proteins are found to be posttranslationally modified by ubiquitin and/or UBLs, and it is becoming clear that cells use such modifications for regulation of many biological functions. PCNA serves as a perfect example for the versatility of the ubiquitin/UBL system and constitutes an excellent model for studying cellular regulation by posttranslational protein modifications.

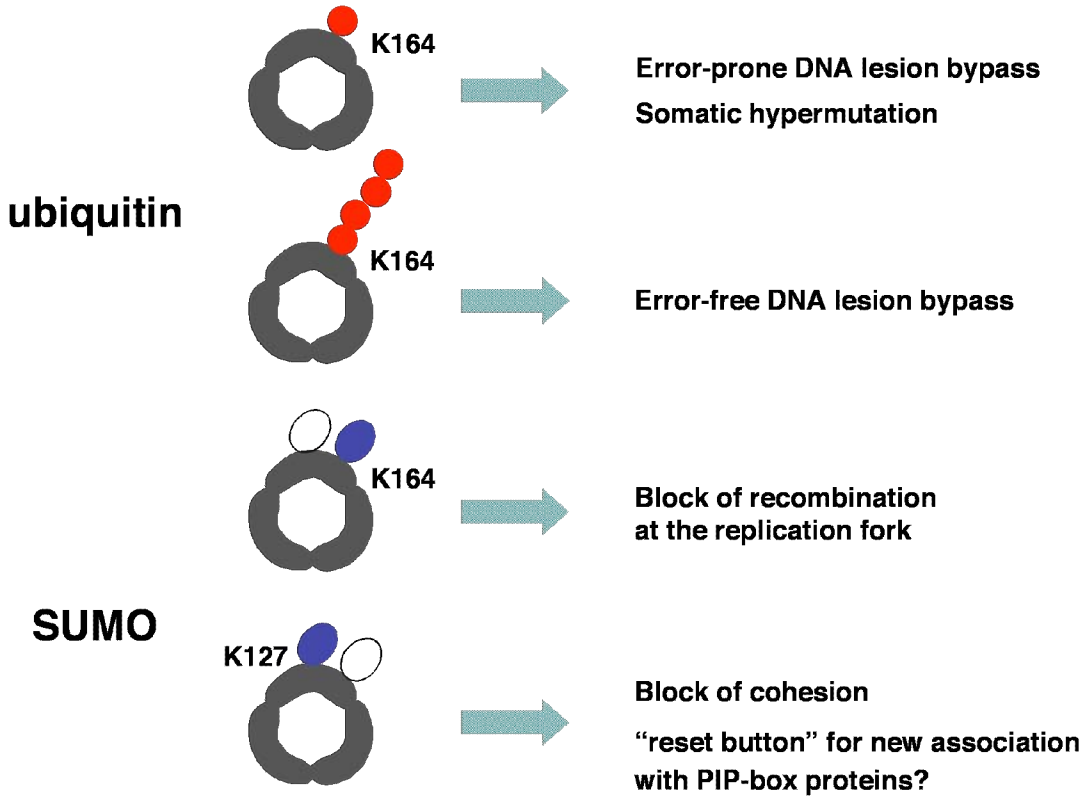

Figure 30. The ubiquitin/SUMO switch. PCNA functions are modulated by posttranslational modifications. While two types of ubiquitylation activate DNA repair pathways, modifications by SUMO inhibit recombination, cohesion and possibly other PCNA functions.

4. MATERIALS AND METHODS

The following microbiological, molecular biological and biochemical methods are based on standard techniques (Ausubel *et al.*, 1994; Sambrock *et al.*, 1989) or on the manufacturers' instructions. For all methods described, de-ionized sterile water, sterile solutions and sterile flasks were used. Unless otherwise mentioned, chemicals and reagents were obtained from Amersham-Pharmacia, Applied Biosystems, Biomol, Biorad, Difco, Fluka, Invitrogen, Kodak, Merck, New England Bioloabs, Promega, Roth, Roche, Riedel de Haen, Serva, or Sigma.

4.1 Computational analyses

For database searches (sequence search and comparison, literature research) electronic services provided by Saccharomyces Genome Database (htttp://www. yeastgenome.org/) and National Center for Biotechnology Information (htttp://www.ncbi.nlm.nih.gov/) were used. DNA and protein sequence analyses (DNA restriction enzyme maps, DNA sequencing analyses, DNA primer design, protein sequence comparison) were done with the DNA-Star software (DNA Star Inc.). Western-Blot films were digitalized with a scanner (AGFA Arcus II) and processed with the Adobe Photoshop software (Adobe Systems Inc.). For quantification of immunoblots, the chemiluminescence signals were detected by a CCD camera (LAS 1000, Fujifilm) and processed with the programs Image Reader LAS 1000 V1.1 (Fujifilm), Image Gauge V3.01 (Fujifilm), and Adobe Photoshop (Adobe Systems Inc.). For the presentation of texts, tables, graphs and figures, the Microsoft Office software package (Microsoft Corp.) was used.

4.2 Microbiological and genetic techniques

4.2.1 *E. coli* techniques

***E. coli* strains**

XL1-Blue:	hsd R17 rec A1 end A1 gyrA46 thi-1 sup E44 relA1 lac [F' pro AB $lacI^qZ\Delta$ M15 Tn10 (Tet^r)] (Stratagene)
BL21 (DE3)/RIL:	B F^- ompT hsdS($r_B^- m_B^-$) dcm+ Tet^r galλ (DE3) EndA Hte [argU ileY leuW Cam^r] (Stratagene)

***E. coli* vectors**

pet28a-c	(Novagen)
pQE9 and 32	(Qiagen)
pGEX-2T, -4T and -5X	(Amersham)

***E. coli* plasmids**

pET28–*ECO1*, pET28–*POL32*, pET28-*SCC1*, pGEX-2TK–*ECO1*, pGEX-4T1–*CHL*1 and pGEX-4T1–*POL32* were created by PCR from a yeast

genomic DNA extract or by subcloning. *ECO1* truncations were cloned into pGEX-4T1 by PCR. pGEX-2TK–*eco1*$^{ctf7\text{-}203}$ and pET28–*eco1*$^{ctf7\text{-}203}$ were created by site-directed PCR mutagenesis from the respective wild-type constructs. Other *E. coli* plasmids used were pGEX-4T1–*srs2ΔN* and pQE32–*SMT3* plasmids (obtained from Boris Pfander), pGEX-5X1–*POL30* and pET28–*POL30* (from Carsten Hoege) and pQE9–*UBC9* (from Wolfgang Seufert).

***E. coli* media**

LB-medium / (plates):	1%	Trypton (Difco)
	0,5%	yeast extract (Difco)
	1%	NaCl
	(1,5%	agar)
		sterilized by autoclaving

Cultivation and storage of *E. coli*

Liquid cultures were grown in LB media at 37°C (or 23°C for protein expression experiments), with shaking at 200rpm. Solid cultures were grown on agar plates at 37°C. The selection of transformed bacteria was done by adding antibiotics to the media. The antibiotics used are ampicillin (50*µ*g/ml), chloramphenicol (24*µ*g/ml) or kanamycin (30*µ*g/ml). The culture density was determined by measuring the absorbance at a wavelength of 600 nm (OD600). Cultures on solid media were stored at 4°C up to 7 days. For long-term storage, stationary cultures were frozen in 15% (v/v) glycerol solutions at –80°C.

Preparation of competent bacteria

E. coli vectors were transformed into competent cells either by calcium chloride transformation or by electroporation. For the preparation of competent cells, 1l liquid LB medium was inoculated with 10ml of an overnight culture derived from a single *E.coli* colony and grown to an OD600 of 0.6-0.8 at 37°C. The cultures were chilled in ice-cold water for 1h and cells were harvested by centrifugation (15min, 5000g, 4°C). All following steps were performed at 4°C, with prechilled sterile materials and solutions. For the preparation of electrocompetent bacteria, sedimented cells were washed once with 1l water centrifuged and a second time with 0,5l water containing 10% (v/v) glycerol. After another centrifugation step, cells were resuspended in 3ml 10% (v/v) glycerol and stored in 100*µ*l aliquots at –80°C. For the preparation of chemically competent cells, sedimented cells were carefully resupended in 200ml $MgCl_2$ solution (100mM). The cells were re-pelleted by centrifugation, resuspended in 400ml CaCl2 solution (100mM) and incubated in ice-cold water for 20min. Finally, the competent cells were pelleted again by centrifugation, resuspended in 20ml 100mM CaCl2 solution containing 10% (v/v) glycerol and stored in 100*µ*l aliquots at –80°C.

Transformation of plasmid DNA into bacteria cells

Shortly before transformation, competent cells were thawed on ice. For electroporation, 25*µ*l competent cells were mixed with 10ng plasmid DNA or

2µl ligation sample dialyzed against water. The suspension was electroporated in a pre-chilled cuvette (0.1cm electrode gap) with a pulse of 1.8kV and 25µF at a resistance of 200Ω. Cells were recovered in 1ml LB medium, incubated on a shaker at 37°C for 1h and plated on antibiotic-containing LB agar plates overnight at 37°C.

For chemical transformation, 50µl competent cells were mixed with 10ng plasmid DNA and incubated on ice for 30 min. A 42°C heat shock was performed for 45s, followed by a 2min incubation on ice. For recovery, 1ml pre-warmed LB medium without antibiotics was added, and cells were incubated on a shaker at 37°C for 1h. Transformed cells were selected by plating the cell suspension on antibiotic-containing LB agar plates and incubating the plates over-night at 37°C.

Expression of proteins in *E. coli*

For the expression of recombinant proteins, the *E. coli* strain BL21(DE3)/RIL was used. Liquid LB medium was inoculated at a dilution of 1:100 with an overnight culture of a freshly transformed colony. Generally, the culture was incubated at 23°C and expression of the protein was induced by addition of IPTG to 1mM final concentration, when the culture reaches an OD600 of 0.6. Cells were harvested 3-6h after IPTG addition by centrifugation (10min, 5000g, 4°C), washed in ice-cold PBS and stored at –80°C after shock freezing in liquid nitrogen. Expression of the protein of interest was assayed by analyzing samples taken before and after induction by SDS PAGE and coomassie staining.

4.2.2 *S. cerevisiae* techniques

S. cerevisiae strains

The yeast strains used in this study are shown in the following table. All strains are isogenic to DF5, with the exception of PJ69, Y1122 and Y1123.

Name	Relevant genotype	Reference
DF5	*trp1-1 ura3-52 his3Δ200 leu2-3,11 lys2-801*	(Finley *et al.*, 1987)
YLM090	*POL3-ProA::kanMX6*	this study
YLM091	*POL31-ProA::kanMX6*	this study
YLM092	*POL32-ProA::kanMX6*	this study
YLM210	*POL32-ProA::kanMX6 ubc9::klTRP1 bar1::HIS3MX6 ubc9-1::LEU*	this study
YLM117	*pol32::kanMX6*	this study
YLM162	*pol32:: klTRP1*	Boris Pfander
Y1192	*PCNA-K164R*	(Hoege *et al.*, 2002)
Y1194	*PCNA-K127,164R*	(Hoege *et al.*, 2002
YLM312*	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::URA3*	this study
YLM055**	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::LEU2*	this study
YLM392*	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::URA3 PCNA-A251V*	this study
YLM403**	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::LEU2 PCNA-A251V*	this study
YLM034	*ECO1-HA::kanMX6*	this study
YLM065	*eco1::kanMX6 YCplac33-ECO1*	this study
YLM394*	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::URA3 eco1::kanMX6 Ylplac128-* $^{3myc}eco1^{SK12,13AA}$	this study
YLM410**	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::LEU2 eco1::kanMX6 YCplac22-eco1*^{SK12,13AA}	this study

YLM343*	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::URA3 ctf18::kanMX6 PDS5-3myc::klTRP1*	this study
YLM344**	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::LEU2 ctf18::kanMX6 PDS5-3myc::klTRP1*	this study
YLM086	*eco1::kanMX6 YCplac22-eco1$^{ctf7-203}$*	this study
YLM428**	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::LEU2 eco1::kanMX6 YCplac22-eco1$^{ctf7-203}$*	this study
YLM430**	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::LEU2 eco1::kanMX6 pol30-K127,164R YCplac22-eco1$^{ctf7-203}$*	this study
YLM308	*eco1::kanMX6 YCplac22-eco1^{eco1-1}*	this study
YLM240	*eco1::kanMX6 YCplac22-eco1$^{ctf7-203}$ PCNA-K127R*	this study
YLM074	*eco1::kanMX6 YCplac22-eco1$^{ctf7-203}$ PCNA-K164R*	this study
YLM078	*eco1::kanMX6 YCplac22-eco1$^{ctf7-203}$ siz1::HIS3MX6*	this study
YLM489	*eco1::kanMX6 YCplac22-eco1$^{ctf7-203}$ PCNA-K164R siz1::HIS3MX6*	this study
YLM076	*eco1::kanMX6 YCplac22-eco1$^{ctf7-203}$ PCNA-K127,164R*	this study
YLM490	*eco1::kanMX6 YCplac22-eco1$^{ctf7-203}$ PCNA-K127,164R siz1::HIS3MX6*	this study
YLM242	*eco1::kanMX6 YCplac22-eco1$^{ctf7-203}$ srs2::HIS3MX6*	this study
YLM494**	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::LEU2 eco1::kanMX6 YCplac22-eco1$^{ctf7-203}$ siz1::NatMX*	this study
YLM487**	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::LEU2 eco1::kanMX6 PCNA-K127,164R YCplac22-eco1$^{ctf7-203}$ siz1::NatMX*	this study
YLM245	*chl1::HIS3MX6*	this study
YLM381	*PCNA-A251V*	this study
YLM052*	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::URA3 PCNA-K127,164R*	this study
YLM054**	*pCUP1-GFP12-LacI12::HIS ChrIV::LacO::LEU2 PCNA-K127,164R*	this study
YLM008***	*ChrVII-L-TEL::URA3*	this study
YLM010***	*ChrVII-L-TEL::URA3 PCNA-K127R*	this study
YLM011***	*ChrVII-L-TEL::URA3 PCNA-K164R*	this study
YLM012***	*ChrVII-L-TEL::URA3 PCNA-K127,164R*	this study
YLM007***	*ChrVII-L-TEL::URA3 cac1::klLEU2*	this study
PJ69	*trp-901-, leu2-3,112 ura3-53 his3-200 gal4 gal80 GAL1::HIS GAL2-ADE2 met2::GAL7-lacZ*	(James *et al*., 1996)
Y1122	*ade2 his4 ura3-52*	(Beckwith and McAlear, 2000)
Y1123	*ade2 his4 ura3-52 rfc1-1*	(Beckwith and McAlear, 2000)

* These strains are derivatives of NBY291 (Bhalla *et al*., 2002)
** These strains are derivatives of NBY292 (Bhalla *et al*., 2002)
*** These strains are derivatives of AEY1017 (Meijsing and Ehrenhofer-Murray, 2001)

S. cerevisiae vectors

CEN plasmids: pYCplac33, pYCplac22, pYCplac111
2μ plasmids: pYEplac195, pYEplac112, pYEplac181
integrative plasmids: pYIplac211, pYIplac204, pYIplac128 (Gietz and Sugino, 1988)
Yeast-Two-Hybrid vectors: pGBT9, pGAD424 (Bartel *et al.*, 1993) pGAD-C1-3, pGBD-C1-3 (James *et al.*, 1996)

S. cerevisiae plasmids

The *POL32* ORF, with an N-terminal His-tag, was amplified by PCR from a genomic DNA preparation and cloned 3' of the *ADH1* promoter in pYIplac211,

yielding pYIplac211–[prom*ADH1*]^{His}*POL32*. For construction of pYIplac128–[prom*POL32*]*POL32-ProA,* the promoter, ORF and protein A tag sequence were amplified by PCR from a genomic DNA preparation of YLM92 cells, in which the chromosomal *POL32* locus was fused with the Protein A tag sequence. Site directed mutagenesis of these plasmids was used to construct pYIplac211–[prom*ADH1*]^{His}*pol32*^{K283R} and pYIplac128–[prom*POL32*] *pol32*^{K283R}*-ProA.* The promoter, ORF and terminator of *ECO1* were amplified by PCR from a genomic extract and cloned into pYCplac22 and pYCplac33. For amplification of the *eco1*$^{ctf7\text{-}203}$ allele, a preparation of genomic DNA from *ctf7-203* cells (obtained from Robert V. Skibbens) was used. These constructs were used as source of Eco1 ORF for further plasmid constructions. For generation of pYIplac128–[prom*ADH1*]^{3myc}*ECO1* and pYIplac128–[prom*ECO1*]^{3myc}*ECO1*, a 3xmyc tag was inserted in frame, 5‘ of the *ECO1* ORF. Plasmids encoding Eco1 truncations were obtained similarly. Plasmids containing full-length *ECO1* ORF were subjected to site-directed mutagenesis to create mutants in the N-terminal PCNA-binding region and in the C2H2 finger. Constructs for overexpression of *POL30* variants were obtained from Carsten Hoege, while pYIplac128–[prom*ADH1*]^{His}*SMT3* was provided by Boris Pfander.

To construct N-terminal BD fusions for Yeast Two-Hybrid experiments, the *ECO1* full-length ORF or truncations were obtained by PCR and cloned in pGBD-C1. PCR-amplified full-length (without the STOP codon) or truncated *ECO1* were inserted in frame, 5’ of the BD sequence in pYEPlac195–[prom*ADH1*], to create C-terminal BD fusions. The *ESCO2* ORF was purchased from Origene and was cloned as C-terminal BD fusion in pYEPlac195–[prom*ADH1*]. The ORF encoding human PCNA was subcloned in pGAD-C1 from pcDNA3.1/GS–PCNA (Invitrogen). pGAD-C2–*POL30* was obtained from Carsten Hoege.

S. cerevisiae media and solutions

YPD / YPGal [plates]:
1% (10 g/l) yeast extract (Difco)
2% (20 g/l) bacto-peptone (Difco)
2% (20 g/l) D-(+)-glucose or galactose
[2% (20 g/l) agar]
sterilized by autoclaving

YPD G418/NAT plates: After autoclaving, YPD medium with 2% agar was cooled to 50°C, and G418 (geneticine disulphate; Sigma) to 200 mg/l or NAT (nourseothricin, HKI Jena) to 100mg/l was added.

SC-media [plates]:
0.67% (6,7 g/l) yeast nitrogen base (Difco)
0.2% (2 g/l) drop out amino acid mix
(according to the requirements)
2% (20 g/l) carbon source
(glucose, raffinose, or galactose)
[2% (20 g/l) agar]

SC-5'FOA plates:	0.67%	(6,7 g/l)	yeast nitrogen base (Difco)
	0.2%	(2 g/l)	drop out amino acid mix (according to the requirements)
	3%	(30 g/l)	adenine
	3%	(30 g/l)	uracil
	2%	(20 g/l)	glucose
	2%	(20 g/l)	agar

After autoclaving, the mixture was cooled to 50°C, and 5'FOA was added to the final concentration of 1g/l.

drop out amino acid mix:	20	mg	Ade, Ura, Trp, His
	30	mg	Arg, Tyr, Leu, Lys
	50	mg	Phe
	100	mg	Glu, Asp
	150	mg	Val
	200	mg	Thr
	400	mg	Ser

Sporulation medium: 2% (w/v) potassium acetate (in sterile water)

SORB:	100	mM	LiOAc
	10	mM	Tris-HCl, pH 8.0
	1	mM	EDTA, pH 8.0
	1	M	sorbitol

sterilized by filtration

PEG:	100	mM	LiOAc
	10	mM	Tris-HCl, pH 8
	1	mM	EDTA, pH 8.0
	40 %	(w/v)	PEG-3350

sterilized by filtration, stored at 4°C

Zymolase 20T solution:	0.9	M	sorbitol
	0.1	M	Tris-HCl, pH 8.
	0.2	M	EDTA, pH 8.0
	50	mM	DTT
	0.5	mg/ml	zymolase 20T (ICN Biochemicals)

Cultivation and storage of *S. cerevisiae*

Liquid cultures were inoculated with a single yeast colony from freshly streaked plates and grown overnight. In general, the main culture was inoculated with this starter culture at a dilution of 1:100 – 1:1000 and grown until the culture had reached the mid-log phase growth (1-3x10^7 cells/ml). Liquid cultures were grown at 30°C (temperature sensitive strains at 23°C), in an incubator with shaking at 150-250rpm. The culture density was determined photometrically (OD600 of 1 is equal to 1.5x10^7 cells/ml). Cultures on agar

plates were stored at 4°C up to 1-2 months. For long-term storage, stationary cultures were frozen in 15% (v/v) glycerol solutions at –80°C.

Preparation of competent yeast cells
Cells from a mid-log phase growing culture were harvested by centrifugation (500g, 5 min, room temperature), washed first with 1/5 volume sterile water and then with 1/10 volume SORB solution and resuspended in 360µl SORB solution. After addition of 40µl carrier DNA (salmon sperm DNA, 10mg/ml, Invitrogen), competent cells were stored in 50µl aliquots at –80°C.

Transformation of yeast cells
For transformation, 0.2µg of circular or 2µg linearized plasmid DNA or PCR product was mixed with 10µl or 50µl competent cells, respectively. 6 volumes of PEG solution were added and the cell suspension was incubated at 30°C for 30min. Subsequently, DMSO (final concentration 10%) was added and a heat shock performed at 42°C for 15min. Cells were sedimented by centrifugation (400g for 3min at room temperature) resuspended in 100µl sterile water and plated on the respective SC medium plates. If G418 or Nat were used for selection, transformed cells were first shaken for 3h in liquid YPD medium before plating. Selection of transformants was carried out for 2-3 days at 30°C (or 23°C for temperature sensitive strains). If necessary, transformants were replica-plated on selection plates to remove the background.

Genomic integration by homologous recombination
The YIplac vector series (Gietz and Sugino, 1988) can be used for the integration of a gene in the yeast genome (integrative transformation). These vectors contain no autonomous replication elements, thus only vectors stably integrated are propagated in yeast. The ORF of the gene of interest was cloned in the multiple cloning site of integrative vectors together with a promoter (endogenous, conditional, or constitutive) and a terminator. Before transformation, vectors were linearized with the help of a restriction enzyme which cuts only in the auxotrophy marker gene. Liniarized plasmids are inserted in the endogenous locus of the marker gene by homologous recombination.

Chromosomal gene deletions or insertions of epitope tags were performed by a PCR strategy (Knop *et al.*, 1999; Longtine *et al.*, 1998). The oligonucleotides used contain sequences for amplification of special cassettes which contain marker genes and target complementary sequences, which allow homologous recombination within the endogenous locus. For gene deletions, the forward primer contains 55bp of the promoter sequence 5' of the start ATG, while the reverse primer has 55bp of the terminator sequence 3' of the stop codon. For the insertion of C-terminal epitope tags, the forward primer contains 55bp 5' of the stop codon instead. After amplification of the cassette, the PCR product was purified by ethanol precipitation and transformed into competent cells. Recombination leads to replacement of the ORF by a marker gene in the case of gene deletions, while in the case of epitope tag insertion, the STOP codon of the target gene is replaced by the

epitope sequence and a marker gene. The correct recombination event was identified by PCR for gene deletions and Western blot for epitope tagging.

A similar, recombination-based strategy was used for the introduction of the mutant *pol30-104* allele at the endogenous *POL30* locus. For this, YCH193 cells (obtained from Carsten Hoege) were employed. These cells harbor their only source of Pol30 encoded on an *URA3*-containing plasmid, while at the endogenous locus, *POL30* is replaced by a marker gene. A PCR product containing the *pol30-104* allele and homology regions to the chromosomal sequences 5' and 3' of the ORF was transformed in these cells and transformants were selected on plates containing the pyrimidine analogue 5'FOA (5'-fluoroorotic acid). The presence of this drug in cells results in a toxic intermediate only when *URA3* is expressed. Cells in which the PCR product got stably integrated in the endogenous *POL30* locus could remove the *URA3*-containing plasmid and thus grow on 5'FOA. The stable integration was verified by PCR and sequencing.

Mating type analysis of haploid strains

For mating type identification, the tester strains RC634a and RC75-7α were used. These strains are hypersensitive to the pheromone secreted by the opposite mating type strain. 50μl of an aqueous cell suspension of each tester strain was mixed with 5ml molten agar (1% w/v), which has been cooled to 45°C, and poured over a YPD plate. Plates containing cultures to be analyzed were replica plated on the a- and α-tester plates. The tester cells cannot grow in proximity of cells of different mating type. Therefore, after 1-2 days of incubation, a halo of clear agar appears around the colony, if the mating type of the tester strain is different. Diploid cells do not secrete any mating type pheromones, therefore no halo is formed on any mating type tester plate.

Mating of haploid *S. cerevisiae* strains

Haploid strains of opposite mating types (MATa, MATα) grown to mid-log growth phase were mixed by spotting 10μl of each on a pre-warmed YPD plate and grown overnight. Cells were streaked on YPD or selection plates and diploids were identified by mating type analyses.

Sporulation and tetrad analysis of diploid *S. cerevisiae* strains

Diploid cells of a 36h stationary culture (500μl) were harvested by centrifugation (500g, 3min), washed 4 times with sterile water, resuspended in 4ml sporulation medium and incubated on a shaker at 23°C. After 3 days, 10μl of the sporulation culture was mixed with an equal volume of zymolase-20T solution and incubated at 23°C for 10min. The spores were dissected in tetrads with a micromanipulator (Singer MSM Systems). Germination and growth of the spores were carried out on non-selective YPD plates for 2-3 days. Tetrads were analyzed genotypically by replica plating on selection plates and for known phenotypes, where applicable.

Analyses of protein-protein interactions with the Two-Hybrid System

The two proteins analyzed for interaction were fused to the DNA-binding and, respectively, the activation domain of the Gal4 transcription factor. The

expression constructs of the fusion proteins were transformed in PJ69-7A cells (James *et al.*, 1996). An interaction between the two proteins results in the reconstitution of the Gal4 transcription activator. Thus, the expression of reporter genes under the control of Gal4 (i.e. HIS3, ADE2) is turned on, and cells can grow on the respective selection media.

Phenotype analyses by growth tests

The phenotypes of different mutant strains can be characterized by comparing their growth when spotted in equal amounts on plates. Temperature sensitivity phenotypes were identified by incubating the plates at temperatures higher (32°C, 34°C or 36°C) or lower (14°C, 18°C) than the standard growth temperature, while DNA repair defects were analyzed by growing cells on plates containing different concentration of the DNA alkylating agent MMS or the replication inhibitor hydroxyurea (HU). Moreover, this method allows the identification of mutants of an essential gene, which are unable to support viability. In this case, cells expressing the wild-type gene only from a centromeric plasmids with the *URA3* auxotrophy marker were transformed with different mutant alleles and plated on 5'FOA-containing plates. This drug counter-selects the *URA3*-expressing plasmids, and cells can survive only if the mutant alleles can confer viability.

To perform such growth tests, yeast overnight cultures were diluted with sterile water to an OD600 of 1, and 6 five-fold serial dilutions were prepared in water. The cell suspensions (5μl) were plated onto the respective plates and analyzed after 2-5 days.

Cell cycle synchronization of *S. cerevisiae* cells

Treatment of MAT-a yeast cells with the mating pheromone α-factor arrests them in the G1-phase of the cell cycle. For cell cycle synchronization, logarithmic cultures (OD=0.3) were incubated with 10μM α-factor for 3h at 23°C with shaking. The arrest was verified microscopically. In order to release them from the arrest, cells were centrifuged for 5min at 500g at 23°C, washed with YPD medium and grown in fresh medium at 23°C. Once released, cells progress synchronously through at least 2 cell cycles. Samples were taken every 20min and the cell cycle phase was estimated by Western blot analyses of Clb2 expression or FACS analyses. Alternatively, cells were arrested in G2/M by treatment with 15μg/ml of the microtubule de-polymerizing drug Nocodazole at 30°C for 3h.

FACS analyses

To confirm their synchronous progression through the cell cycle, cells released from α-factor arrest were analyzed for the DNA content by FACS assays. For this, cells from 1ml of culture were harvested by centrifugation at the time points of interest, washed once with PBS and fixed in 5ml ethanol 70% for 1h at 4°C. After fixation, cells were washed again with PBS, resuspended in 1ml PBS and sonicated for 20s with a Sonopuls HD2200 sonicator (Bandelin). The cellular RNA was removed by incubation with 1mg/ml RNase A for 1h at 37°C. Cells were washed again with PBS and the DNA was quantitatively stained by incubation for 4h at 4°C with 50μg/μl PI

(propidium iodide, Sigma), a fluorescent agent that intercalates between DNA bases. The samples were subsequently analyzed with a FACSCalibur flow cytometer (BD Bioscience) and the signal was processed with the CellQuest software (BD Bioscience).

Cohesion assays
The strains used for sister chromatid cohesion assays are derivatives of NBY291 and NBY292 (Bhalla *et al.*, 2002), obtained from Aaron Straight, and contain a tandem repeat of 256 copies of the *Lac* operator sequence inserted proximal (650kb) or distal (950kb) from the centromere of ChrIV and an inducible GFP-Lac repressor fusion protein, which binds the *Lac* operator leading to fluorescent tagging of ChrIV arm. If cohesion was properly established, as is the case in over 90% of wild-type cells, only one GFP signal is visible, as the two sister chromatids are kept in very close proximity to one another, otherwise two distinct GFP signals are detected. For cohesion assays, cells were arrested in metaphase by treatment with nocodazole (15μg/ml) and the number of GFP signals was scored by direct fluorescent microscopy in live cells, using a Zeiss Axioplan II microscope. For some experiments, a pre-synchronization step was performed, by arresting cells first in G1-phase by α-factor and releasing them in Nocodazole-containing medium.

Silencing assays
Silencing assays (Meijsing and Ehrenhofer-Murray, 2001) represent a special growth test in which strains having the *URA3* marker integrated at telomeres (obtained from Ann Ehrenhoffer-Murray) were used. Due to variegated expression of this chromosomal site, wild-type cells were able to grow both on medium lacking uracil (cells that expressed telomeric genes) and on medium containing 5'FOA (cells that repressed them). Strains defective in silencing were identified by their inability to grow on 5'FOA, while strains with hyper-silencing phenotypes grew poorly on synthetic medium lacking uracil.

4.3 Tissue culture methods

Cell lines

HeLa	human cervix adenocarcinoma cells
U2OS	human osteosarcoma cells
293T	human embryonic kidney cells
IMR90SV	human embryonic lung cells
SER-W3	rat testis Sertoli cells
Saos-2	human osteosarcoma cells
DT40	chicken B cells (the cultivation, storage and genetic manipulation of DT40 cells was performed by Hiroshi Arakawa, GSF, Neuherberg)

Mammalian expression vectors

pcDNA3.1/GeneStorm	(Invitrogen)
pBluescript SK	(Stratagene)

Mammalian plasmids
The mammalian expression plasmid pcDNA3.1/GS-PCNA, encoding human PCNA with a His and a V5 N-terminal tags, was purchased from Invitrogen. Site directed point mutagenesis was performed on this plasmid, to obtain pcDNA3.1/GS-PCNA^{K164R}. For human ubiquitin expresion, the plasmid pBSSK-^{His}Ubiquitin (pMT107), obtained from Mathias Treier, was used.

General solutions

PBS	137	mM	NaCl
	2.7	mM	KCl
	8	mM	Na_2HPO_4
	1.4	mM	KH_2PO_4
			pH 7.4, sterilized by autoclaving

Cultivation of mammalian cells
All mammalian cell lines were grown in cell culture dishes (Falcon), at 37°C with 7.5% CO_2 and 96% humidity. The growth medium used was Dulbecco's Modified Eagle Medium (GIBCO-BRL), which was complemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL) and 1% Penicillin/Streptomycin mixture (GIBCO-BRL). Cells were split when they reached a confluence of 80-100%. For this, cells were washed once with PBS, and removed from the culture dish by incubation for 5min at 37°C with 2ml / 150cm^2 Trypsin/EDTA solution (GIBCO-BRL). The cell suspension was recovered in medium and centrifuged for 4min at 400g at 23°C. The cell pellet was resuspended in fresh medium and inoculated in new culture dishes at a 1:5-1:10 dilution. The number of cells was estimated by using a counting chamber (Primat).

Storage of mammalian cells
For long term storage, cell cultures were frozen in liquid nitrogen. For this, cells were grown to a confluence of 80%, trypsinized, centrifuged and resuspended in 10% DMSO in fetal bovine serum. The cell suspension was distributed in cryo-vials (Nalgene) and transferred to pre-cooled isopropanol-filled cryo-containers (Nalgene) and frozen at –80°C. After two days, the cryo-vials were transferred to liquid nitrogen. For thawing of the cells, the cryo-vials were incubated on a water bath at 37°C. To remove the DMSO, cells were centrifuged and resuspended in fresh medium in a cell culture flask.

Transient transfection of mammalian cells
Cells were transfected using the Lipofectamine Plus Transfection kit (Invitrogen), according to the instructions of the manufacturer. In general, 0.5μg plasmid DNA was used for transfection of 3x10^7 cells.

4.4 Molecular biology methods

General buffers and solutions

TE buffer:	10	mM	Tris-HCl, pH 8.0

	1	mM	EDTA sterilized by autoclaving
TBE buffer 5x:	90	mM	Tris
	90	mM	boric acid
	2.5	mM	EDTA, pH 8.0 sterilized by autoclaving
DNA loading buffer 6x:	0.5%	(w/v)	SDS
	0.25%	(w/v)	bromophenol blue or orange G
	0.25%	(v/v)	glycerol
	25	mM	EDTA, pH 8.0

4.4.1 Isolation of DNA

Isolation of plasmid DNA from *E. coli*
Plasmid DNA was isolated using commercially available kits from either Qiagen (Plasmid Mini Kit) or Macherey-Nagel (Nucleospin Plasmid Quick Pure) according to the manufacturers' instructions. For small DNA preparations (minipreps) 4ml overnight culture was used, while 500ml cultures were employed for large preparations (maxipreps).

Isolation of plasmid DNA from *S. cerevisiae*

Lysis buffer:	1%	(v/v)	SDS
	10	mM	Tris-HCl, pH 8.0
	1	mM	EDTA, pH 8.0

A fast and easy protocol was used for isolation of plasmid DNA from a transformed yeast strain and its direct propagation in *E. coli*. A single yeast colony was resuspended in 50µl lysis buffer, and, after addition of 50µ phenol/chloroform/isoamyl alcohol (24:24:1 v/v/v; Roth), the liquid volume was filled with acid-washed glass-beads (∅ 425-600µm; Sigma). Cells were lysed by vortexing 1-2min at highest speed. The DNA was recovered by centrifugation at high speed for 3min at room temperature. 0.5µl of the aqueous phase, containing the DNA, were subsequently transformed into *E. coli*.

Isolation of chromosomal DNA from *S. cerevisiae*

Breaking buffer:	2%	(v/v)	Triton X-100
	1%	(v/v)	SDS
	100	mM	NaCl
	10	mM	Tris-HCl, pH 8.0
	1	mM	EDTA, pH 8.0

Yeast genomic DNA was prepared to be used as template for amplification of genes via PCR. Cells from a stationary (36h) culture (10ml) were pelleted by centrifugation (1500g, 5min, 23°C), washed once in 0.5ml water and resuspended in 200µl breaking buffer. Subsequently, 200µl

phenol/chloroform/isoamyl alcohol (24:24:1 v/v/v; Roth) and 300mg acid-washed glass beads (∅ 425-600µm; Sigma) were added, and the mixture was vortexed 5min. The lysate was mixed with 200µl TE buffer, centrifuged for 5min at 14000rpm at 23°C and the aqueous layer transferred to a microcentrifuge tube. DNA was precipitated by addition of 1ml ethanol (100%) followed by centrifugation at 14000rpm for 3min at 23°C. The pellet was resuspended in 0.4ml TE buffer and RNA contaminants were destroyed by treatment with 30µl of DNase-free RNase A (1 mg/ml) for 5min at 37°C. Afterwards, DNA was re-precipitated by mixing with 10µl ammonium acetate (4M) and 1ml ethanol (100%). After a brief centrifugation, the pellet was resuspended in 100µl TE buffer. The yield of isolated DNA was determined photometrically.

Precipitation of DNA
For ethanol precipitation, 1/10 volume sodium acetate (3M, pH 4.8) and 2.5 volumes ethanol were added to the DNA solution and incubated at –20°C for 30min. The mixture was then centrifuged at 16000g, at 4°C for 20min. The DNA pellet was washed once with 0.5ml 70% ethanol. After centrifugation, the DNA was air-dried and resuspended in an appropriated volume of TE buffer or sterile water.

Determination of DNA concentration
The DNA concentration was determined photometrically, by measuring the absorbance at λ=260nm. An OD260 of 1 represents a concentration of 50µg/ml double-stranded DNA.

4.4.2 Molecular cloning methods

Restriction digest of DNA
Restriction enzymes were employed for sequence-specific cleavage of DNA according to standard protocols (Sambrock *et al.*, 1989) and the instructions of the manufacturer (New England Biolabs). For the digestion of 1µg DNA, 5 to 10 units of restriction enzyme were usually used. Reaction samples were incubated at the recommended temperature for 2h. To avoid the re-circularization of linearized vectors, the 5' end of vector DNA was dephosphorylated by incubation with 5-10 units of Calf Intestinal Phosphatase (New England Biolabs) at 37°C for 30min.

Separation of DNA fragments by gel electrophoresis
For isolation of DNA fragments, 0.8-2% agarose gels, containing 0.5µg/ml ethidium bromide were used. DNA samples were mixed with 6x DNA loading buffer and electropheritically separated at 120 volts in TBE buffer. Due to the intercalation of ethidium bromide into DNA, DNA fragments could be visualized by using an UV transilluminator (324nm). The size of the fragments was estimated by migration on the same gel of standard size markers (1kb DNA Ladder, Invitrogen).

Purification of DNA fragments from agarose gels
The DNA fragment was excised from the gel, after electrophoresis, using a sterile razor blade. Next DNA purification from the cut agarose block was performed using kits from the companies Qiagen (QIAExII, QIAquick Gel Extraction Kit) or Macherey-Nagel (Nucleospin Extract II) according to the manufacturers' instructions

Ligation of DNA fragments
The amounts of linearized vector and insert required for the ligation reaction were estimated by gel electrophoresis of the purified fragments. A ratio of 1:3–1:10 of vector to insert was used. The 10μl ligation reaction sample contained 100ng of vector DNA and 10 units T4 DNA ligase (New England Biolabs). The ligation was performed at 16°C for 4-12h. Before electroporation of the ligation products into *E. coli*, the sample was dialyzed against de-ionized water for 15min using a nitrocellulose filter (pore size 0.05μm, Millipore).

DNA sequencing
DNA sequencing reactions were carried out by the Core Facility of the Max Planck Institute, using an Abi-Prism 377 sequencer. The sample contained 0.5μg plasmid DNA and 5pmol primer. The sequencing reaction and the subsequent sample preparation were done with the DYEnamic ET terminator cycle sequencing kit (Amersham-Pharmacia), according to the instructions of the manufacturer

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4.4.3 Polymerase chain reaction (PCR)

The PCR technique was used for cloning, for direct yeast transformation and for analysis of chromosomal recombination events. The PCR reactions were performed in a volume of 50μl, containing 50ng plasmid DNA or 0.2μg genomic DNA preparation, 0.6μM of the forward and reverse primers, 1.75μl deoxynucleotide mix (each 10mM, New England Biolabs) and 0.2-5 units DNA polymerase, either Pfu Turbo (Stratagene), or a mixture of 4:1 Taq/Vent polymerase (New England Biolabs). The mixture was compiled in the buffer required by the polymerase used (Pfu-buffer, Stratagene or Thermopol-buffer, New England Biolabs). A PCR Mastercycler (Eppendorf) was used for the reaction. The temperatures for primer annealing and primer extension were been optimized on a case by case basis, taking into consideration the quality of template DNA, the length and the G/C content of the primers. In general, the following program was used:

initial denaturation	94°C	180s
10 amplification cycles	94°C	60s
	45°C	50s
	68°C	100s
20 amplification cycles	94°C	60s
	54°C	50s
+20s / cycle	68°C	100s
cooling	4°C	

For verification of gene deletions and other chromosomal integrations, the colony PCR method was used. A single yeast colony was resuspended in 20µl NaOH 20mM, the liquid volume was filled with glass beads (∅425-600nm, Sigma), and boiled in a thermomixer at 1400rpm for 5min. After a brief 15s centrifugation, 4µl of the supernatant was removed and used as template for the PCR reaction. The reaction was carried out in a volume of 50µl, containing 0.6µM of each primer, 1.75µl deoxynucleotide mix (each 10mM, New England Biolabs) and 2 units Taq polymerase. The mixture was made in Thermopol buffer (New England Biolabs), and the reaction was performed in a Mastercycler (Eppendorf) using the following program:

initial denaturation	94°C	300s
30 amplification cycles	94°C	30s
	55°C	30s
	68°C	60s
final extension	68°C	300s
	54°C	50s
cooling	4°C	

4.4.4 Site-directed mutagenesis

The method used for insertion of point mutations, was a PCR-based strategy based on the QuickChange protocol (Stratagene). Two complementary oligonucleotide primers containing the mutated codon in the middle, flanked by 15 bases of the target sequence on each side were used. The 25µl PCR reaction mixture contained 50ng DNA template (plasmid), 62.5ng of each primer, 0.625µl deoxynucleotide mix (each 10mM, New England Biolabs), and 5 units Pfu Turbo (Strategene) in Pfu-buffer (Stratagene). The reaction was performed in a Mastercycler (Eppendorf), with the following program:

initial denaturation	94°C	30s
30 amplification cycles	94°C	30s
	55°C	60s
	68°C	120s / kb plasmid
cooling	4°C	

To eliminate the template DNA, the reaction mixture was incubated at 37°C for 1h with DpnI, a restriction enzyme that cuts specifically methylated DNA. After dialysis, the mixture was transformed in *E. coli* and mutated plasmids were identified by DNA sequencing.

4.5. Protein biochemistry methods

4.5.1 Gel electrophoresis and immunoblot techniques

General buffers and solutions

HU sample buffer	8	M	Urea
	5	%	SDS

	1	mM	EDTA
	1.5	%	DTT
	1	%	Bromphenolblue
	200	mM	Tris-HCl pH 6.8
Laemmli sample buffer	2	%	SDS
	20	%	glycerol
	100	mM	DTT
	1	%	Bromphenolblue
	60	mM	Tris-HCl pH6.8
MOPS buffer	50	mM	MOPS
	50	mM	Tris base
	3.5	mM	SDS
	1	mM	EDTA
Coomassie solution	0.1	%	Coomassie Brilliant Blue R-250
	20	%	methanol
	10	%	acetic acid
destaining solution	20	%	methanol
	10	%	acetic acid
transfer buffer	250	mM	Tris base
	1.92	M	glycine
	0.1	%	SDS
	20	%	methanol
TBST	25	mM	Tris-HCl, pH 7.5,
	137	mM	NaCl,
	2.6	mM	KCl,
	0.1	%	Tween 20

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

For the experiments presented in this study, 4-12% gradient Bis-Tris polyacrylamide gels were used. These gels allow a good resolution over a large range of molecular sizes and do not require a stacking gel. The gels were either purchased from Invitrogen or poured using the Mighty Small gel system (Hoefer). Protein samples were prepared in Laemmli or HU samples buffers, denatured by heating (10min at 95°C for Laemmli buffer or at 65°C for HU buffer) and were then run at a constant voltage of 140 V in MOPS buffer. Protein size was estimated by running on the same gel the standard size marker PEQ Gold (PEQLabs).

Gel composition:	4%	12%
Water	11.85 ml	6.2 ml
65% saccharose	-	1.2 ml

2.5M BisTris-HCl pH7.5	2.4 ml	2.4 ml
30% acrylamide/0.8% bis-acrylamide	2.2 ml	6.6 ml
10% SDS	82.5 µl	82.5 µl
TEMED	16.5 µl	16.5 µl
10% ammonuim peroxidsulphate	82.5 µl	82.5 µl

Coomassie staining of protein gels
In order to visualize protein bands separated by electrophoresis, the gels were stained with Coomassie solution for 30min and the background was cleared by intensive washings with destaining solution.

Western blot
Proteins separated by gel electrophoresis were transferred to a polyvinylidene fluorid (PVDF) membrane (Millipore) in a wet (tank) blot system. The blotting was done in transfer buffer, at a constant voltage of 70V for 90min at 4°C.

Immunological detection of membrane-transferred proteins
The PVDF membrane with immobilized proteins was blocked with 5% milk in TBST for at least 30min and incubated over-night with primary antibodies diluted in blocking solution. Afterwards, the membrane was washed 6 times for 5min with TBST and incubated for 1h with secondary antibodies coupled to horse radish peroxidase (Dianova) diluted 1:5000 in TBST with 5% milk. Subsequently, the membrane was washed again as described. The detection of the protein of interest was carried out using the chemiluminiscence detection kits ECL or ECL-Advanced (Amersham), according to the instructions of the manufacturer, followed by exposure to ECL Hyperfilm (Amersham) or to a CCD (charged-coupled device) camera (LAS, Fuji).

The following antibodies were used in this study:

Anti-hPCNA (human PCNA)	mouse monoclonal	Abcam
Anti-ProteinA	HRP-coupled	DAKO
Anti-HA	mouse monoclonal	Santa Cruz Biotech.
Anti-Myc	rabbit polyclonal	Santa Cruz Biotech.
Anti-AcK (Acetylated lysines)	mouse monoclonal	Cell Signaling Tech.
Anti-Pol32	rabbit polyclonal	this study
Anti-SUMO (yeast Smt3)	rabbit polyclonal	from Carsten Hoege
Anti-Clb2 (yeast cyclin B2)	rabbit polyclonal	Santa Cruz Biotech.
Anti-PCNA (yeast Pol30)	rabbit polyclonal	from Carsten Hoege
Anti-BD (Gal4 DNA binding domain)	rabbit polyclonal	Santa Cruz Biotech.
Anti-H2B (yeast histone 2B)	rabbit polyclonal	Abcam
Anti-Dpm1	mouse monoclonal	Molecular Probes
Anti-rabbit IgG	HRP-coupled	Dianova
Anti-mouse IgG	HRP-coupled	Dianova

For generation of anti-Pol32 antibodies, recombinant his-tagged Pol32 expressed in E. coli was used for immunization of one rabbit. For this, 650µg of the purified protein (diluted to 1µg/µl in PBS), was mixed with an equal volume of complete Freud adjuvant (Sigma) until a homogeneous emulsion formed, which was injected subcutaneously. After six, nine and twelve weeks,

respectively, additional boost immunizations were performed, using 500µg protein, emulsified in incomplete Freud adjuvant. Ten days after the third boost, the rabbit was bled and the serum was recovered by incubating the blood at 37°C for 1h, cooling it on ice and centrifugation for 30min at 20000 g, at 4°C. The serum was stored at –80°C and was either used directly for immunoblots or further purified by affinity chromatography.

4.5.2 Preparation of cell extracts

Determination of protein concentration
The concentration of protein samples was determined using the Bradford method (BioRad). As standard, IgG solutions of known concentrations were employed.

Preparation of denatured yeast extracts
In order to avoid de-conjugation of posttranslational modifications during lysis, yeast cells were lysed under denaturing conditions. Usually, cells from 1ml of a yeast culture of OD600=1 were harvested by centrifugation, resuspended in 1ml water and lysed by incubation with 150µl 1.85M NaOH/ 7.5% β-mercapto-ethanol for 15min on ice. Proteins were precipitated by addition of 150µl 55% trichloroacetic acid (TCA) followed by incubation on ice for 10min and centrifugation at 20000g for 20min at 4°C. The pellet was resuspended in 50µl HU sample buffer.

Preparation of native yeast extracts
Native protein extracts were employed in binding studies. Logarithmically growing cells were harvested by centrifugation, washed once with cold PBS and resuspended in an equal volume of PBS containing protease inhibitors (20mM NEM, 5mM benzamidine, 6µg/ml antipain, 6µg/ml leupeptin, 4.5µg/ml aprotinin, 5µg/ml trypsin inhibitor, 2mM PMSF, 5µg/ml pepstatin, 6µg/ml chymostatin –all from Sigma, and Complete cocktail from Roche). The liquid volume was filled with glass beads (∅ 425-600µm, Sigma) and cells were lysed by vortexing 4-6 times for 4min at 4°C, using a bead beater (Retsch). The cell lysate was collected by centrifugation, after the bottom of the tube was perforated with a needle. The extract was cleared by incubation with 1% Triton and 0.05% SDS for 30min at 4°C followed by centrifugation at 20000g for 15min at 4°C. The protein concentration in the extract was determined by Bradford assays. The cleared lysate was used for GST-pulldowns or co-immunoprecipitation studies.

Preparation of denatured extracts from mammalian and chicken cells
Mammalian or chicken cells were harvested, washed once with PBS and resuspended in Laemmli sample buffer. In general, 100µl sample buffer was used for lysis of 10^6 cells. The protein samples were denatured by boiling for 15min at 95°C, and cleared by sonication for 2min in a Sonopuls HD2200 sonicator (Bandelin), followed by centrifugation at 20000g for 5min.

4.5.3 Protein purification and binding experiments

Purification of recombinant protein from *E. coli*

Proteins were expressed in *E. coli* BL21 DE3/RIL cells, with a GST or a His tag. Recombinant proteins were purified by affinity chromatography for the respective tag.

For purification of GST fusion proteins, cells from 1l bacterial culture were resuspended in 30ml PBS containing 0.1mM EDTA, and protease inhibitors, and lysed by high pressure in an Emulsiflex C5 cell disruptor. For clearing, the lysate was incubated with TritonX-100, added to the final concentration of 1%, for 30min at 4°C and centrifuged for 30min at 20000g at 4°C. The supernatant was afterwards incubated with Gluthathion Sepharose (Amersham) for 3h at 4°C. The sepharose was pelleted by centrifugation at 500g for 2min at 4°C, and washed once with PBS containing 300mM NaCl and 1% Triton and twice with PBS with 0.1% Triton. Finally, one last wash with PBS was performed, and the beads were resuspended in an equal volume of PBS. The quality of purification was assayed by gel electrophoresis and Coomassie staining. The sepharose was kept at 4°C and used directly for GST-pulldowns. Alternatively, the GST-tagged protein was eluted from the beads by repeated incubations (5-10 times) with equal volumes of 50mM Tris pH 8 containing 25mM reduced Gluthathione and 0.1% TritonX-100. The elution fractions were analyzed by gel electrophoresis and Coomassie staining. The fractions containing the recombinant protein were pooled, dialyzed two times overnight against PBS at 4°C and frozen in liquid nitrogen.

For purification of His-tagged recombinant proteins, cells from 1l of bacterial culture were resuspended in NiNTA lysis buffer and lysed in an Emulsiflex C5 cell disruptor followed by sonication for 2min using a Sonopuls HD2200 sonicator (Bandelin). The extract was centrifuged for 30min at 4°C at 20000g, and the cleared supernatant was incubated for 3h with NiNTA Agarose (Qiagen). The agarose was washed extensively with NiNTA washing buffer and bound proteins were eluted by repeated incubations with NiNTA elution buffer and treated as described above. All NiNTA buffers contain 300mM NaCl in 50mM NaH_2PO_4 pH 8, but they differ in the amount of imidazole: 10mM in the lysis buffer, 20mM in the washing buffer and 250mM in the elution buffer.

NiNTA Chromatography from yeast denatured extracts

NiNTA chromatography under denaturing conditions was used for characterization of posttranslational modifications of Polδ with SUMO. Either the modifier was His-tagged, and the substrates were detected by Western blot after the chromatography, or the substrate (Pol32) was His-tagged and its modifications detected by anti-SUMO blot. In general, 200OD cells were lysed in 4ml 1.85M NaOH / 7.5% β-mercaptoethanol for 5min on ice. The proteins were precipitated by addition of 4ml 55% TCA and incubation for 15min on ice. Subsequently, a centrifugation for 15min at 3000g was performed, and the protein pellet was washed two times with acetone. The pellet was afterwards solubilized in buffer A (6M guanidinium hydrochloride, 0.1M NaH_2PO_4, 0.01M Tris-HCl, pH 8, 20mM imidazole) containing 0.05% Tween-

20. Insoluble aggregates were removed by centrifugation for 20min at 13000g and the protein solution was incubated overnight with 100µl NiNTA Magnetic Agarose Beads (Qiagen). The beads were afterwards washed three times with buffer A containing 0.05% Tween-20 and four times with buffer C (8M Urea, 0.1M NaH_2PO_4, 0.01M Tris-HCl, pH 6.3) with 0.05% Tween-20. Bound proteins were eluted by incubation with 20µl 1% SDS at 65°C, dried in a SpeedVac (Eppendorf), solubilized in 10µl water and 25µl HU sample buffer and analyzed by gel electrophoresis and immunoblot.

NiNTA Chromatography from denatured extracts of mammalian cells
This technique was applied for confirmation of PCNA ubiquitination in mammalian cells. In general, 10^7 cells transfected with an expression construct encoding His-tagged ubiquitin were washed with PBS and lysed by boiling for 15min in 1ml PBS containing 1% SDS. The extract was cleared by centrifugation at 20000g for 15min, and the supernatant was diluted tenfold in PBS and incubated with 100µl NiNTA Magnetic Agarose Beads (Qiagen) overnight. The agarose was washed five times with 0.1% SDS in PBS, eluted with 20µl Laemmli sample buffer and bound proteins were analyzed by Western blot with antibodies recognizing human PCNA.

NiNTA Chromatography from denatured extracts of chicken cells
In order to investigate posttranslational modifications of PCNA in chicken cells, 10^7 DT40 cells expressing His-tagged ubiquitin or SUMO-1 were washed with PBS and lysed in 1ml buffer A. The extract was cleared by sonication for 2min with a Sonopuls HD2200 sonicator (Bandelin) followed by centrifugation for 15min at 20000g. The supernatant was incubated with 25µl NiNTA Magnetic Agarose Beads (Qiagen) overnight. Subsequently, the beads were washed four times each with 0.05% Tween 20 in Buffer B (8M Urea, 0.1M NaH_2PO_4, 0.01M Tris-HCl, pH 8) and respectively in Buffer C, and eluted in 20µl HU sample buffer at 65°C for 15min. Bound proteins were analyzed by Western blot with antibodies recognizing human PCNA.

Co-Immunoprecipitation from native yeast extracts
For identification of *in vivo* protein-protein interactions, native yeast extracts were subjected to immunoprecipitations. For this, native extracts (in general 500µl of 10µg/µl) of yeast cells expressing ^{3myc}Eco1 under the control of the *ADH1* promoter, or an empty vector were incubated with 25µl monoclonal anti-myc antibodies coupled to agarose beads (Santa Cruz Biotechnology) for 3 hours at 4°C. Unspecifically bound material was removed by washing 4 times with PBS containing 1% TritonX-100 and 0.05% SDS. Proteins bound to beads were eluted by boiling in sample buffer and identified by Western blot

GST-pulldowns from native yeast extract
For the pulldown assay, 50 µg of GST or GST-Srs2ΔN fusion bound to beads were incubated with 5 mg of yeast native lysate for 3 h at 4 °C. The beads were then washed four times with the incubation buffer and eluted in HU sample buffer. Bound proteins were identified by Western blot. As input sample, 50 µg of the lysate was loaded.

In vitro binding studies

To test the interaction of PCNA with cohesion proteins in solution, 50μg recombinant GST fusions of Eco1 variants or Chl1 were pre-bound to 50μl glutathione sepharose and incubated with 50μg His-PCNA in PBS containing 5% glycerol and 0.1% Triton-X100 for 1 hour at 4°C. The beads were washed four times with the incubation buffer and bound proteins were eluted in HU sample buffer and analyzed by gel electrophoresis followed by Coomassie staining. In order to verify if PCNA interactions with Eco1 and Ubc9 are mutually exclusive, 20μg each of GST-PCNA and His-Eco1 were incubated with 0, 20, 80 or 400μg His-Ubc9, or vice versa, for 1h at 4°C in the same buffer described before, and bound to glutathione beads for another 1h at 4°C. The sepharose was washed four times with the incubation buffer and incubated for 10min at 65°C with HU sample buffer to elute bound proteins. As controls, similar competition experiments were performed with BSA (Sigma). Eluted proteins were analyzed by gel electrophoresis and Coomassie staining

Chromatin binding assays

To investigate the chromatin association activities of Eco1 variants, chromatin was purified from yeast cells as described (Kai *et al.*, 2001). Cells from 25 ODs of logarithmic phase cultures were harvested by centrifugation, washed in SP1 buffer (1.2M sorbitol, 50mM $MgSO_4$, 100mM K_3PO_4 pH 7.4) and shperoblasts were generated by treatment with Zymolyase 100T (Seikagaku) in SP1 buffer, for 15min at 30°C. The reaction was stopped by addition of buffer SP2 (1M sorbitol, 5mM $MgSO_4$, 1mM EDTA, 25mM MES pH 6.4). Spheroplasts were washed with 1.2M sorbitol and lysed with 1% Triton in lysis buffer (50mM potassium acetate, 2mM $MgCl_2$, 20mM HEPES pH 7.9, 1M sorbitol, protease inhibitors) and chromatin was precipitated by high spin centrifugation (12,000*g*). To remove unspecifically bound proteins, the chromatin pellet was washed with lysis buffer containing 150mM NaCl, and chromatin-bound proteins were released by digestions with DNase (Roche). Whole cell extract, supernatant and chromatin fractions were analyzed by blotting against the myc-epitope (for detection of myc-tagged Eco1), PCNA, H2B, and Dpm1. For cell cycle synchronization, logarithmic cells grown at 23ºC were arrested in G1 by 10μM α-factor for 2.5h, washed, and resuspended in fresh media. At the indicated time points, 25 ODs were harvested and processed as described earlier. Cell cycle synchronization was confirmed by FACS analysis and blotting against cyclin Clb2.

Affinity purification of polyclonal antibodies

High affinity anti-Pol32 antibodies were purified by affinity chromatography from the serum obtained from rabbits immunized with recombinant His-tagged Pol32. For the purification, the serum was first run three times through a pre-column, to which a whole cell lysate of *E. coli* cells that expressed His-Pol30. Unspecific antibodies, recognizing bacteria proteins, and antibodies recognizing the His tag were retained on this column. The serum pre-cleaned this way, was afterwards run three times through the main column, containing

a whole cell lysate of *E. coli* cells, in which GST-Pol32 was expressed. The antibodies recognizing Pol32 were specifically retained on this column.

As matrix for the columns, CnBr sepharose 4CLB (Amersham-Pharmacia) was used. For coupling, the bacterial extracts were first dialyzed against coupling buffer (100mM $NaHCO_3$, 0.5M NaCl, pH 8.3) and subsequently incubated with the matrix, in coupling buffer. Free binding sites were blocked by incubation with 0.2M glycine, pH 8.0 for 2h. The columns were subsequently washed extensively (for both columns: 4 volumes 100mM NaAc pH 4.0, 0.5M NaCl; 2 volumes PBS; 2 volumes PBS, 1% SDS; denaturation at 65° for 40min; 2 volumes PBS, 1% SDS; pre-column only: 2 volumes PBS, 1% Triton X-100; main column only: 2 volumes PBS, 1% Triton X–100; 2 volumes PBS, 1% Triton X-100, 1% BSA). After washing, the serum was passed through the columns as described before. The main column was afterwards washed with 2 volumes PBS, 3 volumes PBS containing 1% Triton-X100 and again with 4 volumes PBS. Spefically bound IgGs were eluted in fractions, first with glycine buffer (0.2M glycine pH 2.5, 1mM EGTA), immediately neutralized with 1M Tris-HCl, pH 8.0 and afterwards with guanidinium hydrochloride (4M guanidinium hydrochloride pH 7.0). Positive fractions of the eluates were pooled and dialyzed against PBS. After addition of glycerol to a final concentration of 50%, the purified IgGs were stored at –80°C.

4.5.4 Enzymatic reactions *in vitro*

In vitro acetylation

In vitro acetylation assays with Eco1 variants were done as described for other enzymes (Kobet *et al*., 2000). Recombinant His-tagged Eco1 or $eco1^{33-281}$ (5μg) were incubated with His-tagged Scc1 (5μg) and 10μM acetyl coenzyme-A in 50mM Tris/HCl pH 8, 10% glycerol, 0.1 mM EDTA, 1mM DTT, 10mM sodium butyrate for 3 hours at 30°C. The reaction was stopped by boiling in sample buffer, and the reaction products were identified by Western blotting with an antibody recognizing acetylated lysins.

In vitro SUMOylation

In this work, Pol32 was SUMOylated *in vitro* using recombinant proteins. For this, Pol32 (200ng) was incubated with Aos1/Uba2 (Boston Biochem, 1μg), Smt3 (2μg), Ubc9 (500ng) and ATP (10nM) in SUMOylation buffer (100mM NaCl, 5mM $MgCl_2$, 0.1mM DTT, 50mM Tris-HCl, pH 7.5) for 2h at 30°C. The reaction was stopped by boiling in sample buffer, and the products were identified by Western blot.

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ABBREVIATIONS

Ψ	aliphatic aminoacid
ΨV	pseudo V
μ	micro
5-FOA	5-fluoroorotic acid
AcCoA	Acetyl Coenzyme A
ACT	acetyl transferase
AD	Gal4 Activation Domain
ADP	adenosine 5'-diphosphate
AID	Activation-Induced Deaminase
Amp	ampicillin
APC/C	Anaphase Promoting Complex / Cyclosome
ATP	adenosine 5'-triphosphate
BD	Gal4 DNA Binding Domain
BER	base excision repair
bp	base pairs
BSA	Bovine Serum Albumin
CAF-1	Chromatin Assembly Factor-1
CCD camera	Charged-Coupled Device camera
Cdk	cyclin-dependent kinase
cDNA	complementary DNA
Chr	chromsome
C-terminal, C-ter	carboxy-terminal
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
dNTP	deoxy nucleoside triphosphate
DSB	double-strand break
DTT	dithiothreitol
DUB	de-ubiquitylating enzyme
E1	ubiquitin activation enzyme
E2	ubiquitin conjugation enzyme
E3	ubiquitin ligase
E4	multiubiquitylation factor
EDTA	ethylenediaminetetraacidic acid
FACS	Fluorescence Activated Cell Sorting
FEAR	Cdc14 Early Anaphase Release
g	gram, gravitational constant
GCR	gross chromosomal rearrangement
GFP	Green Fluorescent Protein
GST	gluthathion S-transferase
h	hours
H2B	histone 2B
HA	hemagglutinin
HECT	homologous to E6-AP C-terminus
HRP	Horse Radish Peroxidase
HU	hydroxyurea

IDCL	Inter-Domain Connecting Loop
Ig	immunoglobulin
IP	immunoprecipitation
IPTG	isopropyl-1-thio-β-D-thiogalactopyranoside
k	kilo
Kan	kanamycine
kb	kilo base pairs
kDa	kilo Daltons
LB	Luria-Bertani
m	milli
M	molar
MAT	mating type
min	minutes
MMR	mismatch repair
MOPS	3-N-Morpholinopropane sulfonic acid
mRNA	messenger RNA
MW	molecular weight
n	nano
NEM	N-ethylmaleimide
NER	nucleotide-excision repair
NTA	nitrilotriacetic acid
N-terminal, N-ter	aminoterminal
OD	optical density
ORC	Origin Recognition Complex
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	phosphate-buffered saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIAS	Protein Inhibitor of Activated STAT
PIP	PCNA-Interacting Protein
PML bodies	promyelocytic bodies
PMSF	phenylmethylsulfonyl fluoride
Pol	polymerase
ProA	Protein A
RFC	Replication Factor C
RING	Really Interesting New Gene
RNase	ribonuclease
RPA	Replication Protein A
rpm	rounds per minute
RT	room temperature
s	seconds
S	sedimentation coefficient (Svedberg)
SBD	SUMO Binding Domain
SCF	Skp1-Cullin-F-Box complex
SDS	sodium dodecylsulfate
SHM	somatic hypermutation

SMC	Structural Maintenance of Chromosomes
SUMO	Small Ubiquitin-related Modifier
TBS	tris-buffered saline
TCA	trichloro acidic acid
TEMED	N,N,N´,N´-tetramethylethylene diamine
TLP	translesion polymerase
TLS	translesion synthesis
Tris	Tris (hydroxymethyl) aminomethane
U	unit
UBA	ubiquitin-associated domain
UBC	ubiquitin conjugating enzyme
UBL	ubiquitin-like
UBP	UBL-specific protease
UIM	Ubiquitin-Interacting Motif
UNG	Uracil DNA Glycosylase
UV	ultraviolet light
V	Volt
v/v	volume per volume
w/v	weight per volume
WT	wild-type
YPD	yeast bactopeptone dextrose

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