The cullin, Rtt101p, provides a novel link between ubiquitination and DNA replication restart

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The cullin, Rtt101p, provides a novel link between ubiquitination and DNA replication restart

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Zusammenfassung


Rtt101p ist in chromatinhaltigen Fraktionen von Zellextrakten anzutreffen. Dies weist, zusammen mit den obenerwähnten Daten, darauf hin, dass eine E3-Ligase der Cullin-Familie das Fortsetzen der DNS-Replikation nachdem eine Replikationsgabel angehalten wurde, gewährleistet. Höchstwahrscheinlich existiert ein Protein, dass an Doppelstrangbrüchen ubiquitinisiert wird und damit die Wiederaufnahme der DNS-Synthese zur Folge hat.
Summary

Accurate DNA replication is essential for cell cycle progression and organismal survival. If the replication machinery is compromised, errors can occur which may lead to genetic instability and diseases like cancer. DNA is vulnerable to attack from exogenous agents including γ-irradiation and chemical mutagens, however the accumulation of endogenous oxidative metabolites, and naturally occurring DNA replication errors can also result in DNA damage. A cell must be equipped to deal with such replicative and damage induced stress in order to ensure error free genomic duplication.

Using the budding yeast, S. cerevisiae, as a model organism, we have found that the yeast cullin protein, Rtt101p, is required to efficiently cope with S phase problems. Specifically, we have shown that Rtt101p is involved in the re-start of collapsed replication forks, which frequently result in a double stranded break. Cells deleted for Rtt101p, are competent to repair a double stranded break indicating that repair machinery is fully intact and the cullin does directly function during repair. Furthermore, we have demonstrated that a stalled, but checkpoint stabilized replication fork is able to restart in the absence of Rtt101p. As well, Rtt101p, is localized to the chromatin fraction of cell extracts.

Rtt101p is most likely acting as an ubiquitin conjugating E3 ligase at collapsed forks, as it shows genetic and biochemical interactions with the E2 ubiquitin conjugating enzymes, Ubc4p and Ubc5p. It has been shown previously that Rtt101p can enhance the formation of ubiquitin chains in vitro (Michel et al. 2003).

Taken together these data provide the first evidence that a cullin based E3 ligase is required for replication fork re-start, and suggests that a target protein exists, most likely at a double stranded break site, that becomes ubiquitinated to allow the resumption of DNA synthesis.
Opening Remark

The work that I have done during my PhD thesis was split into two different projects, which are very different in terms of topic. The minority of (approximately 1/4) of my thesis was devoted to determining the role of the novel prefoldin protein, URI, in the activation of the amino acid sensing pathway, through the translational control of the transcription factor, Gcn4p. This work is not introduced in the general introduction, however it is covered in Chapter V. The remainder of my thesis work was focused on the role of the cullin, Rtt101p, and how this potential E3 ligase component contributes to efficient and accurate cell cycle progression, in the budding yeast, S. cerevisiae.
Chapter 1- General Introduction

**Novel roles for cullin dependent ligases**

The regulated degradation of proteins by cullin-based E3 ligases has mainly been addressed in the context of the Cullin 1 (Cul1) containing complex. Although by sequence homology it has long been known that other cullin proteins exist, their cellular functions have only recently begun to be elucidated.

We used the budding yeast, *Saccharomyces cerevisiae*, as a model system to interrogate the role of the novel and previously uncharacterized cullin protein, Rtt101p. We employed both genetic and biochemical approaches to try to learn more about the role of Rtt101p in the cell. My studies have led us to the conclusion that Rtt101p is intimately involved in both DNA replication and DNA repair following damage.

Therefore, the following introduction will provide background information both on ubiquitin ligases, focusing on cullin based ligases. I will also introduce aspects of DNA replication and repair and finally the last part of the introduction will describe what is currently know about the role of ubiquitination with regards to DNA metabolism.

**Protein degradation by the Ubiquitin/Proteasome system**

The cell cycle is driven by oscillations of cyclin dependent kinase (CDK) activity, which can in turn phosphorylate targets, thereby generating both "GO" signals, which promote entry into the next cell cycle stage, and "STOP" signals which slow the cell cycle until the previous stage is completed. CDK activity can turn these signals on, however they also need to be turned off. For example a, GO signal, must be shut off to ensure that an event is not repeated twice within the same cell cycle, whereas STOP signals need to be destroyed to allow entry into the next cell cycle stage. The best way to abolish a signal is to destroy it, thereby ensuring irreversibility. The controlled destruction of protein signals within a cell is
accomplished with great accuracy and efficiency by the ubiquitin/proteasome system. This system is based on the principal that when a ubiquitin chain is conjugated to a target, it becomes recognized as a substrate for the 26S proteasome, which consequently degrades the target into small amino acid peptides via enzymatic proteolysis (Hershko et al. 1983; Hershko and Ciechanover 1998). Specificity is key to the ubiquitin/proteasome system, ensuring that only those proteins that should be degraded become polyubiquitinated, resulting in "targeted degradation". This system helps to ensure spatial and temporal protein distribution within a single cell cycle. In addition to playing a fundamental role in cell cycle progression, ubiquitination has been shown to be involved in endocytosis, receptor down-regulation and the degradation of mis-folded proteins.

**Ubiquitin and Ubiquitin Conjugation**

Ubiquitin, a 76 amino acid protein, is the most highly conserved protein among eukaryotes, with only three amino acids substitutions from yeast to human ubiquitin (Ozkaynak et al. 1984). Many regulatory proteins are subject to rapid proteolysis following conjugation to a ubiquitin chain. The conjugation of ubiquitin requires the action of three enzymes, generically termed E1, E2 and E3, which cooperate to activate and then conjugate ubiquitin to the targeted substrate (Hershko and Ciechanover 1998). The E1, ubiquitin activating enzyme, forms a thio-ester bond between a cysteine residue in the E1 and the C-terminal glycine residue (G76) of ubiquitin in an ATP dependent manner (Ciechanover et al. 1981). Ubiquitin is then transferred via a trans-thioesterification to an active-site cysteine on an E2, ubiquitin conjugating enzyme. The last step of the reaction is carried out by an E3 or ubiquitin ligase, which is responsible for recruiting the substrate to the E2, which can subsequently transfer ubiquitin to an accepting lysine ε-amino group on the targeted protein. After repeated rounds of this reaction the substrate conjugated to a polyubiquitin chain, is recruited to the 26S proteasome which enzymatically degrades the protein. This reaction is outlined in Figure 1.
The poly-ubiquitin chain can be formed in many different manners as there are seven potential acceptor lysines within the 76 amino acids that make up ubiquitin. For example, it has become clear that K48-G76 linked ubiquitin chains primarily mark a protein for degradation (Hershko et al. 1984a; Hershko et al. 1984b; Hough and Rechsteiner 1986), whereas a different linkage may result in something other than proteolysis. Me-Ub (methyl ubiquitin), which can mono-ubiquitinate a substrate but is unable to form conjugates does not result in substrate proteolysis (Hershko and Heller 1985). Furthermore it was shown biochemically that a substrate with a long ubiquitin conjugate is more likely to be degraded than a substrate with a short conjugate (Hershko et al. 1984b). K48 linkages most likely have an increased affinity for the 26S proteasome. Although other lysine residues within ubiquitin are used in chain formation, they do not result in targeted degradation. For example, the E2 enzyme Rad6p/Ubc2p, as well as Ubc4p and Ubc5p are involved in K63 linkages (Spence et al. 1995), which has been shown to play a fundamental role in DNA repair (see
below). When K63R ubiquitin is expressed as the only source of ubiquitin, the cells are rendered DNA repair defective. K29 linked conjugates are also formed and this is also dependent on the E2s Ubc4p and Ubc5p (Spence et al. 1995). It is most likely the structure of the ubiquitin chain that determines the fate of the substrate as the differentially placed lysines leads to drastically different conjugate structures (Vijay-Kumar et al. 1987) as can be seen in Figure 2.

![Figure 2. The structure of Ubiquitin. The center and right images are two right turns about the y-axis. The C-terminal G76 is sticking up towards the top of the page and the chain forming lysines are marked accordingly. Taken from, Ubiquitin, and the biology of the cell, chapter 2, 1998.](image)

There is only one E1 protein in both yeast and human, which is responsible for activating the total cellular pool of ubiquitin. However there are many more E2s, 13 in yeast (Hochstrasser 1996), and so far an unknown number of E3s, possibly hundreds. It is the E3 ligase, which provides the specificity and determines which substrate will be degraded.

**E3 Ligases**

E3 ligases come in various forms, which has provided the major hurdle in trying to identify them. The fundamental function of the E3 is to bring the substrate into the proximity of the E2 enzyme thereby promoting ubiquitin transfer to the substrate. E3s can be composed of a single protein, however, can also be made up of a multi-subunit complex. Some E3s participate directly in the ubiquitination reaction by transiently accepting ubiquitin before it is conjugated to the substrate, whereas
others never form an E3-Ub intermediate. The different types of E3s will be described below with emphasis on the SCF and its role in cell cycle integrity.

HECT domain

HECT domain containing E3 proteins are unique in that they accept ubiquitin on a C-terminal lysine within the conserved HECT domain (Scheffner et al. 1995). This is dependent on the action of an E2 ubiquitin conjugating enzyme, which promotes the formation of a Ub-HECT intermediate. Ubiquitin is subsequently passed onto the substrate from the E3, rather than from the E2 directly. The human genome encodes for a large family of HECT domain proteins, whereas yeast has only five (Huibregtse et al. 1995). HECT proteins can bind directly to the targeted substrate; for example the yeast Rsp5p HECT containing protein interacts with the large subunit of RNA polymerase after DNA damage which marks it for proteasomal destruction (Huibregtse et al. 1997). However some HECT proteins interact with the substrate via an adaptor intermediate, as is the case with human E6, which binds to p53 through the adaptor E6-AP, and results in p53 degradation (Scheffner et al. 1993).

In contrast to ubiquitination via the N-end rule pathway, HECT dependent ubiquitin conjugation does not always lead proteolysis. The yeast general amino acid permease, Gap1p, is ubiquitinated in an Rsp5p dependent manner when grown in rich media conditions. This ubiquitination results in the internalization of Gap1p via the endocytosis pathway (Hein et al. 1995).

N-end rule E3s

The human E3α protein and the yeast Ubr1p, recognize substrates based on their N-terminal amino acid (Hershko and Ciechanover 1992). When a protein’s N-terminal amino acid is either basic or bulky and hydrophobic it can bind to E3α/Ubr1p, and then becomes ubiquitinated and degraded. This E3 also interacts with the E2, Ubc2/Rad6p, which is also conserved from yeast to man (Reiss et al. 1989; Dohmen et al. 1991).
APC/C – Anaphase promoting complex/Cyclosome

The APC was discovered on account of its ability to regulate the stability of B-type cyclins upon the exit from mitosis (King et al. 1995). It was found independently through genetic screens in yeast, and biochemical immunoprecipitations in Xenopus extracts (Irniger et al. 1995; King et al. 1995). The APC is composed of at least 12 subunits, many of which have no striking primary sequence domains (Zachariae et al. 1998). However two subunits, Apc2p and Apc11p, bear a resemblance to Cul1p/Cdc53p and Rbx1p, which are core components of the SCF (see below) (Zachariae and Nasmyth 1999).

Substrates are recognized by the APC via a conserved nine amino-acid motif called the destruction box. The deletion or mutagenesis of a substrate’s destruction box results in its stabilization, as it can no longer be ubiquitinated in an APC dependent manner. The mitotic B-type cyclins as well as the anaphase inhibitor Pds1p/securin contain destruction boxes and are targets of the APC (Cohen-Fix et al. 1996; Ciosk et al. 1998).

Once cells have replicated their DNA they condense their chromosomes and align them at the metaphase plate. At this time chromosomes are held together by the cohesin complex in order to ensure correct bipolar spindle attachment before anaphase onset, thereby minimizing the chances of chromosome mis-segregation. After correct spindle is established the APC becomes activated in a Cdc20p dependent manner and degrades Pds1p/Securin (Visintin et al. 1997; Harper et al. 2002). The destruction of Pds1p/Securin results in the release of Esp1p/separase, which cleaves the Scc1p subunit of cohesin, thereby allowing anaphase onset (Uhlmann et al. 1999) (Figure 3). The mitotic cyclin, Clb2p, is degraded to stimulate the exit from mitosis while at the same time allowing the licencing of replication origins for the next round of S phase (see below). This is accomplished by the accessory/activating factor, Cdh1p, rather than Cdc20p (Visintin et al. 1997).
SCF – Skp1-Cullin-F-Box

The SCF, Skp1-Cullin-F-Box, like the APC, is a multi-subunit E3 ligase, which also contains a ring-finger containing protein, Rbx1p, at its catalytic core. The SCF does not form an E3-ubiquitin intermediate, instead, ubiquitin is transferred directly from the E2 enzyme to the substrate. The cullin protein, forms a long rigid structure and is thought to provide a scaffolding function (Zheng et al. 2002). There are many different families of cullin proteins (Cul-1, 2, 3, 4a, 4b, 5 and 7), all of which are predicted to adopt the same general structure (Pintard et al. 2004). The highly conserved C-terminal region of the cullin binds to the ring finger, Rbx1p/Hrt1p, an interaction that is necessary to recruit the E2. The N-terminus of the Cul-1 protein is composed of three repeats of a five-helix bundle; cullin repeat 1-3 (CR1-3). Skp1p binds to the N-terminus of the cullin (CR1) as well as binding to an F-box protein, via the F-box domain (Zheng et al. 2002). F-box proteins are responsible for recognizing and recruiting the substrate to the ligase. F-box proteins generally have a C-terminal protein-protein interaction domain, eg. WD40 or leucine
rich repeats (LRR), through which they interact with the putative substrate, and an N-terminal F-box which mediates Skp1p binding (Deshaies 1999). This modular architecture is illustrated in figure 4a, where a cartoon of the SCF is shown with a well-defined target protein, Sic1p (see below). Recently the crystal structure of the human Cul-1 SCF complex containing the F-box protein, Skp-2, has been solved (Figure 4b).

Figure 4. (A) Modular schematic of the SCF\textsuperscript{Cdc4} complex: Cdc4p binds to the cullin through the F-Box domain and Skp1. At the C-terminal, Rbx1p (H), recruits the E2 (Cdc34) which transfers ubiquitin onto a phosphorylated Sic1p. How Ubiquitin is transferred is not fully clear (Deshaies, 1999). (B) The model of the SCF\textsuperscript{Skp}. The N-terminal binds to the substrate specific adaptor, Skp2p, via interaction with Skp1p. This leaves a 50-Å gap until the E2, where the substrate can fit into.
The Cullin families

Whereas the amino terminus of Cul-1 interacts with Skp1 through the cullin repeat domain, the amino termini of Cul2 and Cul3 interact with Elongin C and BTB domains, respectively (Clifford et al. 2001; Furukawa et al. 2003; Pintard et al. 2003). Although the cullin repeat domain varies in sequence between families, the overall structure is maintained, and the sequence conservation within family members is conserved. Consistent with this idea, the structure of the interacting proteins, including Skp1p, Elongin C and BTBs, is similar, as all adopt a BTB like fold, despite the fact that the primary sequences are quite variable (Pintard et al. 2004). Cul4a is missing most of CR1 and therefore does not interact with a BTB fold protein. Cul4a interacts with DDB1 through an, as of yet, undefined motif (Hu et al. 2004).

F-box proteins, interact with Skp1, to recruit the substrate to the Cul1 E3, whereas the SOCS box containing protein, VHL, provides substrate specificity within the Cul2 based ligase (Iwai et al. 1999; Pintard et al. 2004). Cul3 ligases have combined the substrate adaptor and the cullin interaction domain into a single peptide, where the BTB domain is followed by a C-terminal protein-protein interaction domain (Pintard et al. 2003; Xu et al. 2003), eg. MATH. These different cullin families are depicted in Figure 5.

Figure 5. A. F-box proteins bind to Cul1 through Skp1, which adopts a BTB fold whereas B. The SOCS box protein, VHL, provides specificity in the Cul2 complex via interaction with the Elongin C, BTB-like protein. C. In the Cul3 family the BTB domain has been combined in one protein with the substrate interaction domain, and D. Ddb1 interacts through an as of yet undefined mechanism to Cul4. All of these proteins have the ability to interact with Hrt1/Rbx1.
As previously mentioned, the human genome encodes for and expresses seven different cullins, whereas the yeast genome encodes only three. Cul1/Cdc53p as well as Cul3 are conserved from yeast to human, however, the yeast genome encodes another cullin protein, YLR047c/Rtt101p, which does not have a conserved N-terminal domain, and by sequence alone cannot be placed into an established cullin family (Michel et al. 2003). The C-terminal region of Rtt101p is highly conserved and like other cullins interacts with Rbx1p. This is the protein under investigation throughout this study.

So far all of the above mentioned cullin-based E3 ligases have been implicated in ubiquitin dependent degradation, and there are many known targets. One commonality between protein targets is that so far all known targets are covalently modified before being recognized by the E3. A well-characterized example is the yeast CKI protein Sic1p. Sic1p binds to and prevents entry into S-phase by inhibiting Clb-Cdc28p activity. At the end of G1 Sic1p is phosphorylated by Cdc28p-Cln, which promotes binding to the WD40 domain of the F-box protein Cdc4p (Schwob et al. 1994; Schneider et al. 1996). Sic1p is then conjugated to a polyubiquitin chain and targeted for degradation. It was subsequently shown that Sic1p requires six phosphorylation events to interact with Cdc4p, as none of the single sites have a high enough affinity for the Cdc4p phosphate binding pocket (Nash et al. 2001)(Figure 6). Multi-site phosphorylation is not required for the destruction of all SCF targets, as human cyclin E is multi-ubiquitinated after phosphorylation on a single site (Ferrell 2001).

![Figure 6](image_url)

Figure 6. Sic1p blocks S phase onset by inhibiting Clb-Cdc28 complexes, until it is phosphorylated on at least S or T residues, making it a substrate for SCFCdc4 (taken from Ferrell, 2001).
Interestingly, phosphorylation is not the only covalent modification that can mark a cell as an SCF target. In human cells under normoxic conditions the HIF1α protein is targeted for degradation by the Elonginc-Cul2-SOCS (ECS)(Kibel et al. 1995; Iwai et al. 1999). The SOCS protein, von Hippel Lindau (VHL) interacts with HIF1α only after HIF1α has been hydroxylated on a specific proline residue (Ivan et al. 2001). Under hypoxic conditions HIF1α is not hydroxylated and therefore stabilized and able to induce transcription of its target genes, which promote angiogenesis.

The further characterization of E3 ligases provides the next challenge to better understanding ubiquitin dependent degradation. Since the E3 provides the reaction specificity it will also be of interest to design E3 inhibitor, as these could become useful in future clinical situations. For example it has been reported that human Cul4A is amplified and up-regulated in breast carcinomas, leading to inefficient DNA replication and lesion repair (Shiyanov et al. 1999).

Cullin Regulation

Cullins are themselves targets of covalent modification, by the ubiquitin-like protein, Nedd8/Rublp. Like ubiquitin, Nedd8, is activated by an E1 activating enzyme called Uba3p and then conjugated to a conserved C-terminal lysine residue in the cullin, through the action of an E2, Ubc12p (Hochstrasser 1998). The question of whether or not a specific Nedd8 ubiquitin ligase exists is under debate and is currently being investigated.

Although the precise role of cullin neddylation is still unknown, it has become clear that the neddylation reaction is required for cell cycle control and SCF ligase activity. All know cullin proteins with the exception of APC2 (Anaphase promoting complex component) are modified by the Nedd8 protein. In C. elegans, when the neddylation machinery is disrupted, the microtubule severing protein Mei-1, accumulates and disrupts mitotic spindle formation, indication that the Cul-3 complex requires neddylation (Kurz et al. 2002). Furthermore in S. pombe, when the conserved lysine, K713, is mutated to arginine, the cells arrest at the G1/S border and the CDK1 inhibitor Rum1p accumulates, mimicking the phenotype of spCull1/Pcul1 mutants (Osaka et al. 2000). In human cells neddylation is also required for HIF1α degradation (Ohh et al. 2002).
Since the neddylation site on cullins lies in the proximity of the Rbx1p and Ubc12p binding site, it was proposed that neddylation was required to recruit the E2 enzyme into the SCF complex. In support of this theory it was shown that in vitro neddylated cullins are more efficient in recruiting a Ubc4p-Ub conjugate than non-neddylated cullin. Surprisingly, deletion of Rub1p in yeast has no discernable phenotype on its own. However when rub1Δ is combined with an SCF temperature sensitive mutant, the cells lose viability faster than the SCF mutants alone (Hochstrasser 1998). This again implicates neddylation in the activation of the SCF. Rtt101p was also found to be modified by the Rub1p ubiquitin-like modifier.

**DNA replication and repair**

As a cell enters the cell division cycle, the DNA needs to be replicated in a timely and error-free manner in order that the two resulting daughter cells each receive a copy of the genetic material. This process requires precise timing and coordination of a host of proteins involved directly and indirectly in the replicative process. The major challenges that the cell is faced with in terms of DNA replication are as follows:

1. Replication must occur, and it should occur only once per cell cycle, in order to avoid re-replication and polyploidy
2. When DNA damage arises during DNA synthesis due to replication errors or replication fork stress, it must be sensed and consequently repaired so that replication can continue until the entire genome is duplicated
3. DNA should be replicated in a timely manner in order to ensure that replication is complete before the onset of mitosis.

In order to meet these above criteria cells have evolved complex protein signaling networks that regulate the timing and control of S-phase progression. The following section will introduce many of the regulated events of DNA replication from origin firing to damage repair, and will finish by combining what is know about the role of ubiquitin and ubiquitin-like covalent modification during these above mentioned events.

Since DNA replication is such a fundamentally important process for all organisms, it has been remarkably conserved throughout evolution. In the following introduction to replication I will mainly discuss the events in the budding yeast S.
cerevisiae, as it has provided the major paradigms to study. It will be indicated in the text if processes are not conserved or if another organism is discussed.

**Regulation of replication origins**

The yeast genome has approximately 430 replication origins (Wyrick et al. 2001), referred to as, autonomously replication sequences (ARSs), that are responsible for the assembly of the pre-replicative complex (pre-RC). The pre-RC is composed of the origin recognition complex (ORC), which remains permanently bound to ARS elements, Cdc6p, Cdt1p and the presumed replicative helicase the MCM complex (Bell and Dutta 2002). The assembly of the pre-RC onto the ARS sequences is required to license an origin, and failure to assemble this complex results in a failure to initiate DNA replication. Once an origin is licensed, the MCM complex is phosphorylated by the S-phase kinase Cdc7p-Dbf4p (Lei et al. 1997), thereby promoting the association of Cdc45p (Zou and Stillman 1998) and subsequently the rest of the replication machinery, including the DNA polymerase, polδ (Figure 7).
The licensing of a replication origin is promoted by low cyclin dependent kinase activity, and there is only a small window of opportunity at the end of mitosis when the pre-RC can be assembled (Dahmann et al. 1995; Piatti et al. 1996; Detweiler and Li 1998; Diffley 2004). CDK1 activity decreases during the exit from mitosis, by inhibition from the CKI, Sic1p, as well as by APC/C^{Cdh1} dependent degradation of the B-type cyclins (Seufert et al. 1995; Visintin et al. 1997; Baumer et al. 2000; Calzada et al. 2001; Wasch and Cross 2002). During this time of low CDK1 activity the pre-RC proteins assemble onto the ORC occupied ARS sequences. As cell size increases and G1 progresses, the G1 cyclins are synthesized and CDK1 activity begins to increase again (Figure 8). This increase in CDK1 activity has the dual effect of first, acting with Dbf4p-Cdc7p, to activate the licensed origins and second, inhibiting the re-assembly of pre-RCs to ensure that origins are not re-fired, which would result in two round of synthesis per cell cycle (Diffley 2004).

A.

B. PreRC assembly occurs in the window of low CDK1 activity, in G1.
There are multiple mechanisms by which the cell ensures that pre-RCs do not reassemble onto the chromatin, demonstrating the importance of ensuring irreversibility of the reaction. Once a replication origin has fired Cdc6p is phosphorylated by Cdc28p and then targeted for proteasomal degradation by the SCF^{Cdc4} (Piatti et al. 1995; Drury et al. 1997). Although Cdc6p levels re-accumulate throughout the rest of the cell cycle, it is unable to bind to ARS elements until the exit from mitosis, as Cdc6p is maintained inactive by its interaction with the B-type cyclin, Clb2p. However upon the exit from mitosis, Clb2p, becomes bound by Sic1p which displaces Cdc6p and allows it to then bind to a recipient origin (Mimura et al. 2004). Other members of the pre-RC are also tightly regulated after an origin has fired. While Orc2p and Orc6p become phosphorylated (Diffley et al. 1994; Aparicio et al. 1997; Tanaka et al. 1997), Cdt1p and the MCMs are exported from the nucleus following the replication initiation events at an origin (Tanaka and Diffley 2002). All of the above mentioned procedures combine to ensure that an origin becomes activated only one time per cell cycle.

The proposed idea that low CDK1 activity is the key factor in promoting pre-RC formation and high CDK1 activity activates them was strengthened by the use of genetic alleles of Cdc28p (Cdklp) that could be activated and deactivated in a temperature dependent manner. In cells arrested in G2 by nocodazole, CDK1 levels are high and pre-RC formation is inhibited, however when the cdc28 mutant was inactivated upon shift to high temperature, pre-RCs formation was able to occur (Dahmann et al. 1995; Noton and Diffley 2000). When the same cells were released back to permissive temperature, CDK1 activity was restored, and the G2 assembled origins re-fired resulting in two rounds of replication within the same cell cycle.

**Replication slow zones**

The structure of chromatin as well as non-nucleosomal protein-DNA complexes has become an area of great interest with respect to transcriptional effects and it has become clear that higher order chromatin structures can have dramatic effects on gene expression both within and around these regions. Less attention, however, has been given to the notion that the replication machinery must also deal with differentially structured chromatin regions. Until recently the majority of work done on replication fork stalling and blockage has been addressed by using exogenous
DNA damaging agents as well as DNA replication poisons such as hydroxyurea. However non-nucleosomal DNA-protein complexes cause natural replication pausing and in some cases can cause fork collapse and DNA damage (Ivessa et al. 2000; Cha and Kleckner 2002; Ivessa et al. 2002). For example the *E. coli* protein, Tus, binds to chromosome termini and prevents fork progression (Khatri et al. 1989). The Epstein-Barr virus protein, EBNA-1, also impedes replication fork movement by binding repeat sequences within its hosts genome (Dhar and Schildkraut 1991). In the budding yeast, it has been shown that natural fork pausing occurs at centromeres, telomeres, silenced regions and rDNA. All of these regions are made up of non-nucleosomal protein-DNA complexes, and can be considered replication slow zones (Ivessa et al. 2000).

In yeast, the replicative helicase, Rrm3p, facilitates replication through slow zones (Ivessa et al. 2000). It has been shown by 2D-gel analysis of replication origins that cells lacking Rrm3p have a greatly increased pausing at the natural pause sites (Figure 9), whereas the rest of the genome remains largely unaffected in terms of fork stalling. This suggests that the Rrm3p helicase is specialized for replication slow zones. Furthermore, when Rrm3p is deleted, there is increased DNA damage, and the intra-S phase checkpoint is active. The increased damage is due to the fact that stalled forks are fragile and subject to collapse.

Figure 9. Taken from Ivessa *et al.*, 2003. Diagram outlining the 2D technique. 1N-unreplicated DNA, 2N-fully replicated, BU-bubble arc, P-replication pause site. The southern blots were probed to detect the CEN3 (centromeric region). The pause is visible in the wild type but greatly increased in cells deleted for Rrm3p.
Sensing DNA damage - Checkpoints

When a stalled replication fork collapses at one of the above-mentioned non-nucleosomal proteinaceous regions, it often results in a double stranded break (DSB). DSBs are the most dangerous type of DNA damage for a cell, because if the break ends are not repaired or rejoined correctly they can lead to chromosomal loss or erroneous re-joining events (Khanna and Jackson 2001). DSBs can result from many exogenous agents, including: ionizing radiation, physical stress, and many chemotherapeutic agents. The accumulation of endogenous oxidative metabolic intermediates can also induce DSB formation. By whatever means a DSB may arise, it should be repaired rapidly and efficiently in order to avoid massive genomic instability. Before the repair of a DSB can occur it must be sensed, and the sensing proteins (checkpoint proteins) must then slow or arrest the cell cycle in order to allow time for repair to occur. These so called “checkpoint” proteins prevent the replication of damaged DNA (intra-S phase checkpoint) or the mitotic segregation of damaged chromosomes (G2/M checkpoint). The DNA damage response can be broken down into protein groups of sensors (to detect damage), transducers (to signal damage) and effector molecules which are directly involved in either cell cycle arrest, DNA repair or in the case of higher eukaryotes, the induction of apoptosis (Figure 10).

Figure 10. Taken from Khanna and Jackson, 2001. DSBs must be recognized in order to be repaired, this is accomplished through a signal transduction cascade including sensors (usually the ATR and ATM kinases), transducers (the Chk1p and Chk2p kinases) and the effectors, i.e. those proteins which are directly involved in repair, cell cycle arrest and in the case of higher eukaryotes, apoptosis.
The most upstream components of the DNA damage response are the PIKK (Phosphoinositol kinase-like kinases), Mec1p/ATR and Tel1p/ATM (Melo et al. 2001). These two kinases can be considered sensors, as they can be found at the sites of DSBs and many downstream events are dependent on their activity (Lisby et al. 2004). Mec1p and Tel1p play partially overlapping roles (Kim et al. 1999), however also have independent functions. The downstream targets of Mec1p are the transducing kinases Rad53p/Chk2 and Chklp, and upon the induction of DNA damage Rad53p and Chklp are rapidly phosphorylated in a Mec1p dependent manner (Sanchez et al. 1996; Sanchez et al. 1999). Activated Chklp induces a cell cycle arrest by inhibiting the ubiquitin dependent degradation of Pds1p/Securin through direct phosphorylation (Agarwal et al. 2003). In cultured human cells it has recently been shown that activated Chkl can also promote the degradation of the phosphatase, Cdc25A, upon DNA damage, thereby inhibiting cell cycle progression through the accumulation of an inhibitory tyrosine phosphorylation on CDK1 (Jin et al. 2003). Rad53p also inhibits Pds1p degradation via direct phosphorylation which results in decreased binding to Cdc20p and therefore increased stabilization (Agarwal et al. 2003).

The damage sensors and transducers are also involved in inducing the expression of those genes directly involved in the repair process, as well as activating their protein products in order to make them repair competent. Therefore through evolution the DNA damage checkpoint proteins have combined the activation of repair enzymes with the induction of a cell cycle delay in order to give theses enzymes enough time to function.

**Repairing Double Stranded Breaks**

There are two major pathways involved in the repair of double stranded breaks: homologous recombination (HR) and non-homologous end-joining (NHEJ). Both pathways contribute significantly to repair, albeit, in a cell cycle dependent manner (Ferreira and Cooper 2004). HR is the repair pathway of choice once the DNA has been replicated. In this manner the homologous sequence of the
undamaged sister strand is used as a template for DNA polymerase, which results in a largely error-free repair product. NHEJ, however is the predominant repair pathway utilized in G1 when a sister chromatid is not yet available. NHEJ usually results in an error-prone repair, as double stranded ends are ligated together, often after a degradation of the DSB ends, generating deletions or frame-shifts. These two pathways are outlined in Figure 11.

Figure 11. Taken from Khanna and Jackson, 2001. The processing of double stranded breaks is dependent on cell cycle stage. HR is the pathway of choice as it results in error free repair. In HR, a single stranded piece of DNA invades the undamaged sister chromatid to use it as a replicative template. NHEJ involved limited resection of the DSB ends and re-ligation in a sequence independent manner often resulting in deletions and mutation.

Recently, through the use of GFP labeled proteins, the spatial and temporal regulation of protein recruitment to double stranded breaks has been determined (Lisby et al. 2004). When a double stranded break is induced the first proteins to arrive at the broken DNA are the Mre11/Rad50/Xrs2 complex and the ATM kinase, Tel1p, followed by the single stranded binding complex, RPA. This is seen as a fluorescent focus that forms after a break is induced. If multiple breaks are induced, more foci are not seen, indicating that repair centers are established upon DSB induction (Lisby et al. 2003). These above mentioned proteins assemble at a break site independent of cell cycle position, and can be considered the early response elements. In contrast, the remaining homologous recombination machinery, including Rad51p and Rad52p, only gets recruited to the break site in the S/G2 phase of the cell cycle when a sister chromatid is available to allow strand invasion (Figure 12).
Interestingly, Rad51p and Rad52p are not recruited to Hydroxyurea (HU) stalled forks, which are stabilized due to the activation of the Rad53p checkpoint. However Rad51p and Rad52p, foci do form in response to prolonged HU exposure (e.g. 30 hours), when fork stability can no longer be maintained, moreover, they also form in the presence of HU in a rad53Δ background again due to the inability to maintain a stably paused replication fork. The early response proteins as well as Ddc1p and Mec3p, form at HU stalled forks independent of collapse. It has recently been demonstrated that CDK1 activity is required for efficient homologous recombination, as it promotes 5'–3' resection at the break site, which ensures prolonged checkpoint activation. In the absence of CDK1 activity the NHEJ pathway is up-regulated in compensation (Ira et al. 2004).
When the NHEJ pathway is activated, the Ku proteins, Ku70p and Ku80p, as well as the Mre11p/Rad50p/Xrs2p complex are targeted to the site of the double stranded break. After strand resection, perhaps via the endonuclease activity of the Mre11p complex, the DSB ends are ligated in a sequence unspecific manner (van Gent et al. 2001).

**The role of ubiquitin in DNA replication and Repair**

As discussed above, ubiquitination can be detected in many forms including mono and poly-ubiquitination, and the type of ubiquitin chain linkage is also variable from K48 to K63 linkage. Recently it has been shown that ubiquitination conjugation in all forms plays a fundamental role in S phase and DNA repair. In the following section I will outline what is known about ubiquitin and ubiquitin-like modifications with regards to replication and repair.

**Ubiquitin conjugation/degradation and Replication**

The pre-RC components have a complex regulation to prevent the re-loading and further re-activation of already fired origins of replication. Once Cdc6p is phosphorylated by CDK1, it is targeted for ubiquitination and degradation by the SCF\(^{Cdc4}\) E3 ligase, and then further prevented from pre-RC formation by interaction with Clb2, which is then itself targeted for degradation by the APC/C (Piatti et al. 1995; Drury et al. 1997). In human cells the licencing factor, Cdt1p, has been shown to be regulated in a very similar way to Cdc6p. Cdt1p is ubiquitinated and degraded following origin initiation by the Cul-4A based ligase containing DDB1 (Feng and Kipreos 2003; Zhong et al. 2003; Hu et al. 2004). As Cdt1p levels rise, it remains inhibited from licencing via interaction with Geminin, which is subsequently degraded upon the exit from mitosis by the APC/C (Wohlschlegel et al. 2000; Nishitani and Lygerou 2002; Yoshida et al. 2004), in an analogous fashion to Clb2 and Cdc6p in yeast (Figure 13).
In contrast the *S. cerevisiae*, Cdt1p is stable throughout the cell cycle and is regulated via sub-cellular localization rather than degradation (Tanaka and Diffley 2002). The fact that the cells regulates these replication events through protein degradation augments the importance of irreversibility of these reactions within the cell cycle, thus ensuring that licencing factors do not become competent for re-licencing.

As cells enter S phase one of the basic requirements to initiate replication, is the production of deoxyribonucleotides (dNTPs) to be incorporated into the new strand. The ribonucleotide reductase protein, Rnr1p, converts NTPs into dNTPs through a reduction reaction. Rnr1p is negatively regulated by an interacting protein Spd1p (Sp)/Sm11p(Sc) (Woollard et al. 1996; Chabes et al. 1999). Spd1p and Sm11p are regulated in a cell cycle dependent manner via proteasomal dependent degradation, whereby the proteins are specifically targeted for destruction at the onset of S phase, thereby de-repressing Rnr1p activity (Zhao et al. 2001; Bondar et al. 2004). In *S. pombe*, the Cul4p/Pcu4p and the substrate specific adaptor Ddb1 are required for Spd1p degradation (Liu et al. 2003; Bondar et al. 2004), whereas in *S. cerevisiae*, the E3 ligase has not yet been identified. So far a protein inhibitor of RNR has not yet been identified in the human genome.
**Ubiquitin and Repair**

The proliferating cell nuclear antigen (PCNA), is encoded by the POL30 gene in yeast. PCNA, which forms a trimeric clamp around DNA, is loaded onto Polα primed RNA-DNA primers by the clamp loader, replication factor C (RF-C) (Tsurimoto and Stillman 1989; Tsurimoto and Stillman 1991). PCNA interacts directly with Polδ and promotes replicative processivity. PCNA is post-translationally modified by both SUMO and ubiquitin on the same lysine residue (K164) (Hoege et al. 2002). Using synchronized cells it was shown that PCNA becomes sumolated at the onset of S phase. However sumolation is not required for PCNA function as K164R mutated PCNA does not have a replication defect, nor do sizIΔ cells, which are impaired for sumo ligase function (Hoege et al. 2002). In contrast K164R cells are hypersensitive to DNA damage induced either by UV light or the damaging agent, indicating that K164 is important for damage repair independent of sumolation.

When the moving replication fork encounters damage along the template strand, the E2 enzyme, Rad6p, along with the ring finger protein, Rad18p, add a single ubiquitin onto PCNA. The mono-ubiquitination does not affect the stability of PCNA however, as was demonstrated in cultured human cells, promotes a polymerase switch, to the trans-lesion polymerase, polη (Kannouche et al. 2004; Vidal et al. 2004), which is necessary to replicate past the damaged area. The E2 enzymes Mms2p and Ubc13p then add a K63-linked poly-ubiquitin chain, which is predicted to promote template switching to the undamaged sister chromatid. The multi-ubiquitinated PCNA is not degraded during this process. Therefore in the case of PCNA there appears to be a competition between ubiquitination and sumolation. Figure 14.
Figure 14. SUMO and ubiquitin compete for the same lysine. During replication PCNA is
ubiquinated on K164, however becomes ubiquitinated and then further poly-ubiquinated after DNA
damage induction.

Other targets are also degraded during DNA damage or replication stress
including the above mentioned Spd1p (Bondar et al. 2004), to increase dNTP levels
and enhance repair. Cdt1p is also degraded after exposure to UV irradiation (Hu et al.
2004). Although the regulation of many repair and replication factors has been
extensively studied, it is clear that more targets probably exist. For example, cells
expressing the PCNA K164R mutation are not as sensitive to MMS as the single
rad6, mms2Δ, or ubc13Δ cells, indication that these ligase components most likely
have other targets in response to DNA damage. In addition, Rtt101p, is also
hypersensitive to MMS (Chang et al. 2002), and further it has been shown to possess
in vitro ubiquitin conjugation activity (Michel et al. 2003), revealing that it also most
likely has one or multiple targets in the DNA damage response.
Chapter-II The yeast cullin, Rtt101p, is involved in genomic integrity and DNA repair

The work in this chapter was submitted for publication

The S. cerevisiae cullin, Rtt101p, is required for recovery after DNA damage and replication stress

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**Introduction**

Accurate replication and maintenance of an organism's genome requires a complex signaling network that ensures that DNA duplication and repair occur with high fidelity and precision. These processes must be coordinated with orderly cell cycle progression to prevent the onset of DNA-segregation during mitosis before DNA-replication and repair are completed.

Upon genotoxic stress, cells activate checkpoint pathways to slow down or arrest the cell cycle until the lesion is removed or repaired. In budding yeast, activation of DNA-checkpoints in either S or G₂ phase results in an arrest at the metaphase/anaphase transition by stabilization of the securin Pds1p (Clarke et al. 2001; Agarwal et al. 2003). Some of the most upstream components of the replication/damage checkpoints include the partially overlapping PI3-kinase-related kinases Mec1p/ATR and Tel1p/ATM (Carr 2002; Rouse and Jackson 2002). These kinases are responsible for the phosphorylation of two downstream kinases, Chklp and Rad53p/Chk2 (Gardner et al. 1999; Sanchez et al. 1999), which in turn activate targets involved in DNA-metabolism and repair. Chklp and Rad53p have also been shown to directly phosphorylate Pds1p, which prevents its degradation by the Anaphase Promoting Complex\(^{\text{Cdc20}}\) (APC\(^{\text{Cdc20}}\)) (Gardner et al. 1999; Sanchez et al. 1999; Wang et al. 2001), most likely by inhibiting the interaction with Cdc20p (Agarwal et al. 2003). Mutations that affect faithful DNA-replication or checkpoint pathways can lead to genomic instability, as seen in many human diseases including cancer.

Genomic integrity is not only dependent on the enzymes directly involved in checkpoint signaling and DNA replication and repair, but also requires covalent modification of these enzymes to ensure that they are activated at the right time and the right place. Regulated ubiquitylation and subsequent degradation of substrates triggered by the APC or cullin-based E3-ligases control many cell cycle transitions and checkpoint pathways. Cullins provide a scaffolding function within the E3-complex and interact both with the E2 ubiquitin-conjugating enzyme and the substrate via an adaptor (Cardozo and Pagano 2004). Different cullin families have been characterized based on their N-terminal interactions with different substrate specific adaptors (Pintard et al. 2004). The budding yeast genome encodes for three cullin
proteins, Cul1p/Cdc53, Cul3p and Rtt101p/Cul8p (Rtt101p from here on). Whereas Cul1p and its role in the SCF (Skp1, Cullin, F-Box) complex have been extensively studied, little is known about the role and subunits of the other two cullin proteins in *S. cerevisiae*.

Rtt101p can support ubiquitination *in vitro*, and like other cullins, interacts via its C-terminal domain with the ring-finger containing protein, Rbx1p (Michel et al. 2003). Cells deleted for *RTT101* are viable, but display a 60-fold increase in the transposition rate of the retrotransposon Ty1 (rtt = regulation of Ty1 transposition) with preferential insertion occurring in front of tRNA genes (Scholes et al. 2001). High-throughput screening of viable yeast deletions has revealed that *rtt101A* cells are sensitive to the genotoxic agents MMS, HU, CPT and IR, but are not sensitive to UV damage (Chang et al. 2002; Michel et al. 2003). Finally, *rtt101A* cells show a delay in anaphase progression, which is suppressed by deleting the intra-S-phase checkpoint gene *RAD9* (Michel et al. 2003). Taken together, these results indicate that the cullin Rtt101p is involved in some aspect of DNA metabolism, most likely DNA replication or repair.

In the budding yeast *S. cerevisiae*, complete DNA replication involves approximately 400 replication origins (Raghuraman et al. 2001; Wyrick et al. 2001), which are activated with different timing and efficiency during the replicative process. Replication origins are physically distributed all along the chromosomes from the telomeric to the centromeric regions. Inactivation of cyclin-dependent kinase (Cdk1p) activity at the end of mitosis promotes the licensing of replication origins for the next round of DNA replication (Noton and Diffley 2000). While the origin recognition complex (ORC) is bound to replication origins throughout the cell cycle, low CDK1 activity allows the assembly of the licensing factors: Cdc6p, Ctd1p and the likely replicative helicase, the MCM-complex (Diffley 2004). This mechanism couples mitosis and DNA-replication. Subsequent reactivation of CDK1 and the S-phase kinase Cdc7p/Dbf4p triggers origin firing followed by DNA replication (Lei et al. 1997; Zou and Stillman 1998).

Recently it has become clear that timely and accurate DNA-replication through specific chromosomal regions of the genome including telomeric, subtelomeric, centromeric, tRNA genes, rDNA and silenced regions requires specialized factors (Ivessa et al. 2002; Ivessa et al. 2003). These non-nucleosomal
proteinaceous regions form stable DNA-protein complexes, resulting in natural replication pause sites. For example, the Pif-like DNA helicase, Rrm3p (Bessler et al. 2001), is only required for the efficient replication of these genomic regions while the bulk of replication is independent of Rrm3p (Ivessa et al. 2002). Rrm3p was found to localize to these proteinaceous regions by chromatin immunoprecipitation (CHIP) experiments (Ivessa et al. 2000; Ivessa et al. 2002). In the absence of Rrm3p, replication pausing at these specific sites is dramatically increased, while no significant pausing was found throughout the rest of the genome. Interestingly, the survival of rrm3Δ cells depends on an intact intra-S phase checkpoint, which is thought to stabilize the paused replication complex when Rrm3p is absent. In addition, synthetic-lethal analysis revealed that rrm3Δ cells are inviable when combined with many replication (eg. cdc45ts) and repair mutants (eg. rad50Δ, mre11Δ), as well as with mutants involved in re-start of stalled replication forks (eg. top3Δ, sgs1Δ, srs2Δ) (Tong et al. 2004; Torres et al. 2004). These results suggest that in the absence of Rrm3p the replication machinery cannot be compromised to successfully traverse regions of high protein density. The double-stranded break (DSB) repair machinery becomes essential in an rrm3Δ background as stalled forks are known to be highly unstable and often result in a DSB upon collapse (Ivessa et al. 2000; Cha and Kleckner 2002). Since DSBs and fork stalls occur with a higher frequency in rrm3Δ cells, these cells require that a replication fork is able to restart in order to complete replication.

We are interested in the function of cullin-based ligases during cell cycle progression. Recently, we found that rtt101Δ is synthetic-lethal with rrm3Δ (Tong et al., 2004), suggesting a role of this putative E3-ligase to replicate specialized chromosomal regions. In this study, we demonstrate that rtt101Δ cells indeed exhibit a specific replication defect that results in an increased frequency of double stranded breaks and gross chromosomal rearrangements. Importantly, upon induction of replication stress or DNA damage, rtt101Δ cells were unable to recover from the checkpoint arrest in a timely manner. Taken together, our data strongly suggest that Rtt101p is required for accurate and complete DNA replication, most likely by promoting restart of replication forks that have collapsed as a result of encountering DNA damage or have been unable to be maintained in a stable manner at natural pause sites.
Results

rtt101Δ cells have a checkpoint dependent G2/M delay and accumulate with short pre-anaphase spindles

To investigate the function of the yeast cullin Rtt101p, we compared cell cycle progression of wild-type and rtt101Δ cells. Consistent with recent results (Michel et al. 2003), we found that rtt101Δ cells accumulate during mitosis with the nucleus positioned at the bud neck, a short spindle and unseparated sister chromatids (data not shown). Importantly, time-lapse movies of single wild-type and rtt101Δ cells expressing tubulin-GFP revealed that the onset of anaphase in rtt101Δ cells is severely delayed as spindle elongation starts at 120' compared to 10 minutes in wild-type cells (Fig. 1A and supplementary movie). We counted time 0' as the time when the short pre-anaphase spindle moved to the mother/bud neck and measured the time until elongation. The average elongation time was approximately 100 minutes in rtt101Δ cells (n=5) and 7 minutes in wt cells (n=4) (Fig. 1B), although the cell-to-cell variation was quite significant. The short spindle in rtt101Δ cells is very dynamic and migrated half way into the bud on several occasions, suggesting that the spindle is properly attached to chromosomes and the cortex. After the sudden elongation of the mitotic spindle, the subsequent events including cytokinesis, cell separation and exit from mitosis occurred with normal timing, indicating that the cells do not undergo an aberrant anaphase transition, which results in chromosomal damage. Taken together, these results suggest that rtt101Δ cells exhibit a severe delay at the metaphase to anaphase transition.

To determine when in the cell cycle Rtt101p was required, we expressed fully functional HA-Rtt101p in rtt101Δ cells from the regulatable GAL1,10-promoter (Figure 1C). Cells were grown in 2% galactose and arrested in G1 with α-factor for 1 hour. The culture was divided, and 2% glucose was added to one half to inhibit the expression of HA-Rtt101p. After three hours, HA-Rtt101p was no longer detectable by immunoblotting in the glucose culture, indicating that HA-Rtt101p is a rather unstable protein. The two cultures were released by washing out α-factor, and cell cycle progression was followed by morphological criteria, FACS analysis and immunoblotting of marker proteins. Rtt101p was not required for bud emergence or
the onset of the bulk part of DNA replication (data not shown). However, cells lacking Rtt101p accumulated at the anaphase-metaphase transition, and degradation of Pds1p-myc and Clb2p was significantly delayed. These results suggest that Rtt101p-activity is necessary during every S- or G2-phase of the cell cycle, and lack of Rtt101p results in a specific delay prior to the onset of anaphase. Interestingly, no Clb2p or Pds1p-myc accumulated in the G1-arrested cells after depletion of Rtt101p, demonstrating that Rtt101p is not required to maintain APC-Cdh1 activity.

Figure 1. *rtt101Δ* cells have a Mec1p dependent G2/M delay

*rtt101Δ* cells have a G2/M delay and (A) accumulate with a short pre-anaphase spindle. Tubulin-GFP was used to visualize the spindle via live cell fluorescence imaging (supplementary figure (S1)). We noticed that the spindle was delayed in the onset of elongation from the time it reached the bud-neck (time 0) until the length of the spindle spanned the length of the two cells, although the timing was quite variable from cell to cell (B). (C) Cells with a Gal regulatable HA-Rtt101p were synchronized in G1 with alpha factor and released into media containing either glucose or galactose. The degradation of Pds1p-myc and Clb2p were delayed in the glucose culture where Rtt101p was shut off. (D) Logarithmic DAPI stained cells were scored for bud-neck localization (butterfly nuclei). As expected *rtt101Δ* showed a G2/M delay that was suppressed by deleting the checkpoint protein Mec1p but not Mad2p.
Degradation of Pds1p by the APC is inhibited by either the ATR/Mec1-dependent DNA-damage checkpoint in case of unreplicated DNA or DNA-damage, or by the Mad2-dependent mitotic checkpoint in case of spindle misfunction or unattached chromosomes. To determine whether the mitotic delay of rtt101Δ cells was caused by activation of one of these checkpoint pathways, we used DAPI staining to measure the mitotic index of wild-type, rtt101Δ, rtt101Δ mec1Δ or rtt101Δ mad2Δ cells. Consistent with the mitotic delay, 23% of rtt101Δ cells accumulated with their DNA at the mother/bud neck, compared to 8% in wild-type cells (Fig. 1D). Importantly, this G2/M delay was suppressed to wild type levels by inactivating the ATR/Mec1 S phase checkpoint kinase, while deleting the spindle assembly checkpoint inhibitor Mad2p had no effect on the mitotic accumulation of rtt101Δ cells. It has previously been shown that deletion of Rad9p can also suppress the mitotic delay of rtt101Δ cells, suggesting that the G2/M delay may result from DNA damage that occurs during S phase.

**rtt101Δ cells exhibit a slower S phase than wild type cells and have fewer firing replication origins firing**

To determine if Rtt101p indeed functions during S-phase, we first compared the kinetics of DNA-replication in wild-type and rtt101Δ strains, which express the Herpes simplex thymidine kinase to allow BrdU incorporation (Lengronne and Schwob 2002). The cells were arrested in G1 by α-factor and released into the cell cycle in the presence of BrdU. Genomic DNA was then prepared and migrated via pulse-field gel electrophoresis (PFGE), so that only completely replicated chromosomes entered the gels. The chromosomes were transferred onto a membrane and visualized by an antibody against BrdU. BrdU detection was possible approximately 45 minutes after release from the G1-arrest in both wild-type and rtt101Δ cells, as shown in Figure 2A. Interestingly, while wild-type cells showed 50% BrdU incorporation after 45 minutes, BrdU incorporation in rtt101Δ cells was significantly delayed (lower graph). These data suggest that Rtt101p is required for efficient DNA-replication.
Figure 2. S phase is slow and less origins are active in rtt101Δ cells

Wild type and rtt101Δ cells expressing TK were arrested in G1 and released in the presence of BrdU. PFGE analysis revealed that rtt101Δ cells were delayed in completion of S phase compared to wild type as replicated chromosomes were slower to re-enter the gel (A) as judged by ethidium bromide staining and (upper) and BrdU incorporation (lower). (B) Cells released from G1 into BrdU were arrested with HU and chromosomes were prepared and aligned linearly onto coverslips, followed by staining with anti-BrdU (green) and anti-DNA (red). Measurements between origins (IOD) and replication track length were made (upper panel). (C) The average IOD in rtt101Δ cells was greater (68.8 kb) than the corresponding wt (54.2 kb). (D) BrdU track length is 21.7 kb in rtt101Δ cells compared to 14.2 kb in the corresponding wild type.

The slower S phase progression in rtt101Δ cells suggests that Rtt101p is either required for efficient initiation or elongation of DNA synthesis. To discriminate between these two possibilities, we have monitored the replication of individual chromosomes using DNA combing (Herrick and Bensimon 1999). For this purpose, chromosomes were first prepared from wild-type and rtt101Δ cells pulsed with BrdU after release from α-factor into 0.2M HU as described previously (Versini et al. 2003). The DNA was linearly arranged on a glass slide and probed with BrdU antibodies to visualize replicated DNA and anti-guanosine antibodies to counterstain DNA fibers (Figure 2B). Interestingly, the number of firing replication origins was significantly reduced in cells lacking Rtt101p, and the average distance between
replicating origins increased to 68.8 Kb compared to 54.2 Kb in wild-type cells (Fig. 2C). In order to measure the rate of elongation at the level of individual molecules, exponentially-growing cells were pulse-labeled with BrdU and the length of BrdU tracks was analyzed by DNA combing in wild type and rtt101Δ cells. Surprisingly, the length of replicated DNA stretches was ~50% longer in rtt101Δ cells (Fig. 2D), which is reminiscent of sgs1Δ mutants (Versini et al. 2003). We conclude that Rtt101p is either directly or indirectly needed for efficient DNA-replication. Although the replication delay is weak in rtt101Δ cells, the replication program is clearly perturbed as cells have an increased replication fork speed, most likely in order to compensate for the decreased number of active replication origins.

Rtt101p is synthetically-lethal with the replication helicase Rrm3p, but they do not share overlapping functions

Natural replication pausing sites occur at many non-nucleosomal proteinaceous areas throughout the genome. The pausing is enhanced in rrm3Δ cells which often leads to unstable replication intermediates that can be converted into toxic double stranded breaks. As a result rrm3Δ mutants confer synthetic-lethality when combined with either checkpoint mutants, DSB repair mutants, or replication restart mutants, as the replication intermediates need to be stabilized, repaired and restarted, respectively. We confirmed that rtt101Δ was also synthetically lethal when combined with rrm3Δ (Fig. 3A), as previously reported (Tong et al. 2004). To determine if the cullin, Rtt101p, was also involved in preventing extensive pausing at proteinaceous zones, we performed 2D gel electrophoresis analysis on a centromeric region, CEN4, that was shown previously to require Rrm3p to prevent pausing. We found that pausing was increased in the rrm3Δ deletion strain (Fig. 3B, black arrow), however there was no difference in the amount of pausing between wild type cells and rtt101Δ cells (open arrows).
Figure 3. Collapsed replication forks are toxic in rtt101Δ cells
(A) Tetrad analysis revealed a synthetic lethal interaction between a rtt101Δ mutation and a rrm3Δ mutation. (B) Exponentially growing cells were harvested and DNA was extracted and digested with XbaI. The after 2D gel analysis, blots were probed with a CEN4 specific probe. There was an increased replication pause in the rrm3Δ strain (black arrow) that was not seen in either of the wild type strain or the rtt101Δ strain (white arrows). (C) Genomic DNA prepared from log phase wild type, rtt101Δ, fob1Δ, and rtt101Δfob1Δ, cultures was separated by conventional gel electrophoresis. ERCs (white arrows) were detected using an rDNA specific radio-labeled probe. A shorter exposure corresponding to the rDNA array is shown (black arrows). ERC levels were quantified with a phosphoimager and expressed relative to the amount of ERCs in wild type cells. (D) Tetrad analysis shows that deletion of the HR machinery does not rescue the synthetic lethality between rtt101Δ and rrm3Δ (square boxes), and the double mutants between the HR mutants and rtt101Δ result in small colonies (triangles).

The rDNA locus is also a proteinaceous area that requires Rrm3p for efficient replication completion. Replication stress, which results in DSBs within rDNA often results in HR dependent extra-chromosomal rDNA circles (ERCs) due to the repetitive nature of the rDNA locus. Interestingly, we found that like rrm3Δ, rtt101Δ cells also have increased ERC formation (Fig. 3C). The increase in ERCs was dependent on the rDNA specific replication fork block protein, Fob1p. The RecQ like helicase, Sgs1p, is required for survival in an rrm3Δ background, and has been implicated in processing homologous recombination (HR) intermediates in
order to allow restart. Indeed, it has been demonstrated that the synthetic lethality between sgs\(\Delta\) and rrm3\(\Delta\) can be relieved by further deleting the recombination factors, Rad51p, Rad55p and to some extent Rad52p. We found that by deleting RAD51, RAD55 or RAD52, we were not able to suppress the synthetic lethality between rtt101\(\Delta\) and rrm3\(\Delta\) (Fig.3D). In fact, we found that rtt101\(\Delta\) homologous recombination double mutants gave slower growing colonies than either of the singles. Taken together these results suggest that Rtt101p is not required to prevent extensive replication pausing at replication slow zones, and like Sgs1p, is not required to metabolize toxic HR intermediates. The synthetic slow growth between rtt101\(\Delta\) and the HR mutants also indicates that it may not be involved in HR directly.

**rtt101\(\Delta\) cells are highly sensitive to the damaging agents MMS and CPT but only moderately sensitive to HU, and not sensitive to an HO induced cut**

Since rrm3\(\Delta\) cells display increased levels of DSBs and Rad53p is constitutively phosphorylated, we reasoned that DSB inducing genotoxic agents should impair the viability of rtt101\(\Delta\) cells. Consistent with this notion, we found that rtt101\(\Delta\) cells were hypersensitive to MMS and CPT, however only moderately sensitive to the dNTP-depleting chemical HU (Fig. 4A). This defect was rescued by expressing either GFP- or HA-tagged Rtt101p, demonstrating that the effect is specific. In contrast, rtt101\(\Delta\) cells are not hypersensitive to UV-damage (not shown), suggesting that not all types of DNA-damage, including cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidinone adducts, require Rtt101p activity to resolve the lesion. MMS and CPT cause various types of DNA structures that often result in a DSB after an encounter with the traveling replication fork, which consequently collapses. On the other hand HU results in stably arrested replication fork intermediates. These results suggest that rtt101\(\Delta\) cells are better able to tolerate stalled forks than forks that break and result in damage.
Figure 4. Rtt101p is required for genotoxin resistance

(A) rtt101Δ cells are highly sensitive to both MMS and CPT and moderately sensitive to HU. (B) Cells were arrested in G1 and divided. Half of the cells were released into various MMS concentrations and the other half was held in G1 in the presence of different MMS concentrations. Cells were then plated and viability assessed. rtt101Δ were approximately 2x more sensitive to MMS in S phase compared to G1. (C) Cells harbouring a Gal-HO plasmid were grown overnight in glucose containing media and diluted to 0.01 in the morning and the 15uL was plated onto either SD or Sgal plates. The number of survivors on galactose compared to glucose was compared and scored. rtt101Δ had 97% viability compared to 100% in wild type and 0% in rad52Δ cells.

Mutants that display such drug sensitivities are typically more sensitive to DNA damaging agents during S phase compared to cell cycle phases when DNA replication is not occurring as it is likely that toxic replication products are responsible for the loss of viability. To test this, cells were exposed to different concentrations of MMS for 1 hour either while arrested in G1 by α-factor, or 10 minutes after release from α-factor as the cells entered into S-phase. Cells were then plated and scored for viability. We found that rtt101Δ cells were approximately two times more sensitive to MMS in S phase compared to G1 i.e. at 0.03% MMS-concentration, rtt101Δ cells were 60% viable in G1 compared to 32% viable in S phase (Fig. 4B). As expected,
mec1Δ cells are extremely sensitive as they are impaired both in activating the intra-S phase checkpoint as well as signaling to repair enzymes. Together these results along with the rrm3Δ synthetic lethality (Fig. 3A) strongly suggest that rtt101Δ cells are sensitive to replication-induced damage, and most likely collapsed replication forks. The data further imply that rtt101Δ cells are not sensitive because they are unable to activate DNA-checkpoint pathways, as their sensitivity to genotoxins is far lower than mec1 mutants. Since HR mutants exhibit a slow growth phenotype when combined with rtt101Δ (Fig. 3D), it is likely that Rtt101p is not directly involved in the HR reaction. HO is an endonuclease that cuts at the MAT locus to promote mating type switching. Most laboratory strains are deleted for the HO endonuclease so they can be maintained as non-mating haploids. The HO endonuclease was introduced into rtt101Δ cells on a GAL-inducible plasmid, cells were grown overnight in glucose containing medium and equal number of cells was plated onto glucose and galactose containing medium, and the number of survivors on galactose compare to glucose was compared. Wild-type and rtt101Δ cells showed 100% and 98% viability respectively, whereas rad52Δ cells, which are defective for homologous recombination was needed to repair the HO-induced DSB, had 0% viability (Fig. 4C). These results suggest that Rtt101p is not required to repair DSBs via HR, as the HR machinery is in tact.

The intra-S phase checkpoint is activated and executed in rtt101Δ cells, however re-initiation of replication following MMS treatment but not HU treatment is impaired

When cells are exposed to DNA damaging agents they respond by slowing or halting the firing of late replication origins in order that the damage can be repaired before the cell tries to enter into mitosis. Rad53p/Chk2 is directly involved in this process, as cells deleted for Rad53p are unable to inhibit late origin firing upon DNA insult. Rad53p activation can be followed by its phosphorylation state, as upon DNA damage it gets phosphorylated and activated. To test whether rtt101Δ cells are able to activate Rad53p, we arrested wild-type and rtt101Δ cells with α-factor and released them in media without drug, with 0.2M HU or with 0.02%MMS. Protein extracts were prepared after 30 minutes, and the phosphorylation state of Rad53p monitored by immunoblotting with an αRad53p antibody (Figure 5A). Interestingly, cells
lacking Rtt101p, were able to efficiently signal to Rad53p in response to both HU and MMS (Fig. 5A), implying that they are not defective for damage recognition or signal transduction to Rad53p.

Figure 5. Rad53p is activated but not deactivated in rtt101Δ cells following damage
(A) α-factor arrested cells were released in either rich media or media containing either 0.2M HU or 0.02% MMS and then protein extracts were prepared. Using Rad53p specific antibodies we were able to conclude that rtt101Δ cells were able to efficiently phosphorylate Rad53p following genotoxic stress. (B) rtt101Δ cells were also able to stabilize already fired origins and repress the firing of late origins after HU challenge whereas rad53 mutants were unable to do either of the above. (C) Whereas wt cells were able to dephosphorylate Rad53p, two hour following MMS release, rtt101Δ cells were unable to downregulate the checkpoint activation, PFGE analysis revealed that rtt101Δ cells also were unable to re-initiate replication as compared to wild type cells(F). (E) rtt101Δ, efficiently down regulated Rad53p phosphorylation following HU release and (F) also re-initiated DNA replication with wild type timing.
Importantly, rtt101Δ cells were not only able to activate the checkpoint signaling cascade, but also efficiently prevented the initiation of late firing origins after the addition of HU (Figure 5B). In these experiments, the firing of early (ARS306, top row) and late (ARS501, bottom row) origins was monitored by 2D gel analysis in wild-type and rtt101Δ after the addition of HU. Clearly, the early origins fire in wild-type and rtt101Δ cells (bubble), whereas the late origins remain inactive (bottom row). In contrast rad53A cells are unable to suppress the firing of the late replication origin after HU addition. Taken together, this data indicates that the intra-S phase checkpoint can be activated and executed independently of Rtt101p and the genomic instability and sensitivity to genotoxic agents is not due to a checkpoint signaling failure. Importantly, this also demonstrates that early fork stability can be maintained in response to HU treatment and doesn’t collapse as in the case of rad53Δ mutant.

We next tested whether Rtt101p may be needed for the release of the checkpoint arrest, we arrested cells in G1 with α-factor and released them into S phase in the presence of MMS for 1 hour. The MMS was then quenched and washed out and cells were released into rich media, and the phosphorylation state of Rad53p was monitored by immunoblotting with specific antibodies. In wild-type cells, Rad53p was phosphorylated upon addition of MMS, but two hours after the removal of MMS, Rad53p returned almost fully to the dephosphorylated state and as a consequence cells resumed growth (Fig. 5C). In contrast, rtt101Δ cells were unable to recover from the MMS assault, and even up to four hours after removal of MMS Rad53p remained phosphorylated. In a separate but similar experiment as the previous one, chromosomes were prepared following MMS release and analyzed by PFGE. Whereas wild type cells were able to complete replication following MMS removal, rtt101Δ cells were delayed (Fig. 5D), consistent with the Rad53p phosphorylation profile. This defect is reminiscent of rtt107Δ cells, which were previously shown to be defective for recovery from the checkpoint arrest (Rouse 2004). The experiments described for figures 5C and 5D, were repeated except using HU as a genotoxin in the place in MMS. Strikingly we found that rtt101Δ cells were able to dephosphorylate Rad53p with a similar timing as wild type cells following the removal of HU (Fig. 5E). Moreover, wild-type and rtt101Δ cells resumed replication with similar kinetics as determined by PFGE analysis (Fig. 5F). These data combined with the fact that rtt101Δ cells are less sensitive to HU than to MMS strongly suggest
that stably HU arrested forks are competent to restart independently of Rtt101p, however, broken or collapsed forks, like those induced by MMS or rrm3Δ, require the cullin, Rtt101p.

**Cells lacking Rtt101p have increased DSBs and genomic instability**

To determine if Rtt101p was required in S phase to maintain genomic integrity and stability we scored for DSBs and gross chromosomal rearrangements in rtt101Δ cells. Ddc1p-GFP is known to be recruited to sites of DSBs and forms foci that can be seen using fluorescence microscopy. Rtt101p was deleted in cells expressing a Ddc1p-GFP fusion protein, and GFP foci were scored after growth in rich media, without a DNA damaging agent. 15% of rtt101Δ cells formed Ddc1p-GFP foci, compared to 2% in wild-type and 9% in sic1Δ cells (Fig. 6A), a strain previously shown to have high rates of spontaneous DSBs. Taken together, these results confirm that in the absence of Rtt101p, the cells accumulate double strand breaks, most likely during DNA-replication.

To assess whether or not rtt101Δ cells show increased levels of genomic instability, we took advantage of a yeast strain where the right arm of chromosome V, which contains all non-essential genes, was replaced with a fragment containing a URA3- and a canavanine sensitivity marker (Fig. 6B diagram). These cells are unable to grow in the presence of canavanine and 5-FOA, but are able to survive if they lose this part of the chromosome. As shown in Figure 5B, rtt101Δ cells show a 16 fold increase in the rate of chromosome loss or instability compared to wild-type cells as measured by this assay (black arrows indicate colonies on Can, 5-FOA plates). Interestingly, sic1Δ cells exhibit a 160 fold increase in their chromosome loss rate, suggesting that although rtt101Δ cells are genetically unstable, they must be able to repair most of the damage during the checkpoint-induced arrest in mitosis.
Figure 6. Rtt101p is required for genomic integrity

(A) RTT101 was deleted in cells expressing Ddc1-GFP, and exponentially growing cells were viewed under the fluorescence microscope by taking 7 layers and projecting them maximally. 15% of rtt101Δ cells (n=500) had Ddc1-GFP foci compared to 2% in wild type (n=500) and 9% in sic1Δ (n=500) cells.

(B) Rtt101 was deleted in a strain where the right arm of chromosome V was carrying a Can1 and a Ura3 marker, a known number of cells was plated onto plates containing 5-FOA and canavanine in that survival was possible only when the markers were lost. rtt101Δ cells showed a 16 fold increase in GCR compared to wild type.
Discussion

rtt101Δ cells exhibit a G2/M cell cycle delay due to a defect during DNA-replication

Phenotypic analysis of single rtt101Δ cells by live cell imaging revealed a specific G2/M delay, which occurs in all cells but to a different extent. This metaphase-anaphase defect is caused by a delay in APC/C-induced degradation of Pds1p, an inhibitor of the Esp1p separase required to resolve sister chromatin cohesion. Several lines of evidence suggest that this defect is caused by a defect during DNA-replication. First, no defect in spindle assembly or kinetochore attachment was detected in rtt101Δ cells, consistent with the observation that the G2/M delay was not suppressed by deletion of the APC/C-inhibitor Mad2p. Second, the G2/M-delay of rtt101Δ cells was corrected in rad9Δ and mec1Δ-deficient cells, implying that the activation of the DNA-damage or replication checkpoint pathway is responsible for the arrest. Indeed, it was recently shown that unreplicated or damaged DNA triggers Pds1p stabilization through its phosphorylation by Chk1p and Rad53p, (Wang et al. 2001; Agarwal et al. 2003) both downstream kinases of Mec1p/ATR. Third, rtt101Δ cells exhibit a slight S-phase delay and a reduced number of firing replication origins as determined by PFGE analysis and molecular combing, and exhibit greatly increased number of spontaneous double strand breaks during S-phase. Finally, rtt101Δ cells are slightly sensitive to the replication inhibitor hydroxyurea (HU), but very sensitive to several DNA-damaging drugs specifically during S-phase. Taken together, these results lead to the hypothesis that the cullin Rtt101p is involved in proper S-phase progression, and cells deleted or mutated for the cullin function show a G2/M-delay due to activation of the Mec1p DNA damage/replication checkpoint.
**Rtt101p is involved in the replication of proteinaceous chromatin regions**

While Rtt101p functions during S-phase, our results clearly show that Rtt101p is not required for the bulk fraction of DNA-replication. Indeed, using PFGE analysis we found a delay of only twenty minutes for the completion of S phase when comparing synchronized wild type cells with rtt101Δ cells. However, during every S phase the replication machinery must overcome special DNA regions such as telomeres, centromeres, rDNA repeats and silenced chromatin domains. Replication through these proteinaceous, mostly non-nucleosomal regions result in replication pausing, and involves specific factors to overcome the pausing as well as stabilizing the fork. Our results strongly suggest that Rtt101p is involved in the replication of these slow zones. First, Rtt101p is required in cells lacking the Pif-like helicase Rrm3p, which are known to exhibit a high frequency of replication collapse due to increased stalling at proteinaceous DNA zones. In wild-type cells, Rrm3p ensures minimal pausing through these zones, therefore minimizing the vulnerability the paused fork (Ivessa et al. 2003). Second, we observed an accumulation of extra-chromosomal rDNA circles (ERCs) in rtt101Δ cells in a Fob1p-dependent manner. The rDNA is a tandem array of 100-200 repeats (Kobayashi et al. 1998) that causes a marked increase in replication fork pausing, most notably due to the replication fork blocking (RFB) protein, Fob1p (Kobayashi and Horiuchi 1996; Kobayashi 2003). These rDNA circles arise from homologous recombination between repetitive sequences if the paused replication fork collapses leading to the formation of a DSB. Thus, the increase in extra-chromosomal rDNA circles in rtt101Δ cells is indicative of a higher incidence of fork collapse/DSB during replication of the rDNA locus. It is interesting to note in this regard that RTT101 was initially identified because rtt101Δ cells show an increased transposition of Ty1-retrotransposons, with especially increased integration in the proximity of tRNA genes (Scholes et al. 2001). Replication of tRNA genes, like rDNA, also requires Rrm3p to avoid extensive replication pausing, and we thus speculate that collapse of such replication forks in the absence of Rtt101p may trigger Ty1-retrotransposition.
Rtt101p is not required for the Rad53p-checkpoint or DSB-repair, but specifically functions at collapsed replication forks

Our analysis shows that Rtt101p specifically functions at collapsed replication forks during DNA-replication. Short-term (30 min) hydroxyurea (HU) treatment, depletes nucleotide pools and results in replication fork stalling whereby Rfa1p, Ddc1p, Ddc2p and Rad53p are all recruited to the stalled fork (Lisby et al. 2004). Rad53p is required to stabilize the replication intermediates and prevent fork collapse, which can often result in a DSB. Rad52p, which is necessary to promote strand invasion in the HR reaction, does not get recruited to HU induced stalled forks, unless Rad53p is deleted (Lisby et al. 2004). In contrast, Rad52p forms foci at collapsed forks or DSBs. Interestingly, the number of Dcd1p-GFP and Rad52p-GFP (David Alvaro and Rodney Rothstein, personal communication) foci significantly increased in rtt101Δ compared to wild-type, suggesting that collapsed forks or DSBs accumulate during S-phase. However, our data exclude the possibility that Rtt101p may be involved in the Rad53p-checkpoint response or DSB-repair. Indeed, 2D-gel analysis in rtt101Δ mutants revealed that the Rad53p checkpoint is activated and functional, as indicated by the fact that late firing origins are efficiently repressed in response to HU-treatment. Moreover, Rtt101p is not required to repair DSBs as rtt101Δ mutants are not sensitive to overexpression of the HO-endonuclease, which causes a specific DSB at the MAT locus. The sensitivity of rtt101Δ cells to different genotoxic agents supports the notion that Rtt101p is needed after replication fork collapse. MMS mainly induces DSBs, which occur when a moving fork encounters an MMS induced DNA structure. Likewise, CPT inhibits the re-ligation step of topoisomerase-I (Top1p) resulting in a Top1p-DNA intermediate that gets converted into a double stranded break after a collision with the replication fork. HU, as described above, stalls forks, however does not result in fork collapse, unless the exposure to the drug is extended to approximately thirty hours when stabilization can no longer be maintained. rtt101Δ cells are highly sensitive to both CPT and MMS and show a moderate sensitivity to HU, while showing no sensitivity to UV light. The fact that the cells are more sensitive to damage from MMS in S phase shows that it is most likely a replication induced damage, such as fork instability that causes lethality.
Sensitivity to CPT is also indicative of an inability to re-start a collapsed fork, as a traveling fork normally induces the damage. The slight HU sensitivity supports the idea that most of the damage that is occurring in rtt101Δ cells is due to more severe problems than just fork stalling, and it would appear that the re-starting of stalled forks is not as much of a problem as the re-starting of broken forks.

**Rtt101p is required to restart collapsed but not stalled replication forks**

Strikingly, our results demonstrate that Rtt101p is required to specifically restart collapsed replication forks. While Rad53p was efficiently phosphorylated in wild-type and rtt101Δ cells upon exposure to HU and MMS, Rad53p remained phosphorylated in rtt101Δ cells after removal of MMS. In contrast, Rtt101p was not required to restart stalled replication forks after washing out HU. Consistent with this observation, 2D gels confirmed that the replication forks did not collapse upon exposure to HU arrested rtt101Δ cells. In comparison, rad53Δ cells displayed fork collapse at early origins and the inability to inhibit late origin firing upon the same HU exposure. DNA-combing experiments combined with specific labeling of replicated DNA after recovery allowed monitoring fork restart at the level of individual molecules. While in wild-type cells most of the MMS-arrested forks resumed replication 50 minutes after release, the collapsed forks in rtt101Δ cells were unable to restart DNA-replication. In contrast, Rtt101p was not required for the restart from HU-arrested cells. We therefore conclude that Rtt101p is required for efficient recovery of collapsed but not stalled replication forks. The RecQ-like helicase Sgs1p was previously shown to be required to restart collapsed forks and suppress inappropriate recombination (Fabre et al. 2002). In the absence of Sgs1p, collapsed forks in rrm3Δ cells are converted into toxic recombination-intermediates, resulting in lethality; this lethality can be suppressed by deletion of RAD55 or RAD51 and to some extent, RAD52 (Torres et al. 2004). However, there is no suppression of the rrm3Δ rtt101Δ synthetic lethality by deleting RAD51, RAD55 or RAD52, implying that Rtt101p and Sgs1p must have distinct functions during homologous recombination.
Interestingly, recent results suggest that mrc1Δ cells exhibit the opposite behavior: Mrc1p is required to restart HU stalled replication forks, while mrc1Δ cells are competent in the restarting collapsed replication forks (Philippe Pasero, personal communication). These results further highlight that stalled and collapsed replication forks are molecularly distinct, and demonstrate that different mechanisms are required to stabilize and restart these forks.

The ability to restart collapsed replication forks is important to ensure genome stability

In summary, our results indicate that Rtt101p is required to re-initiate DNA replication after a replication fork becomes destabilized and collapses. We therefore propose the following model for Rtt101p function during S-phase (Figure 7). Rtt101p is only required at replication slow zones, where natural pause sites occur, and on rare occasions form a DSB break. Normally, the replicative helicase Rrm3p ensures minimal pausing through these zones, therefore minimizing the vulnerability of the paused fork. Occasionally however, paused replication forks do collapse even in the presence of Rrm3p, and then Rtt101p is needed to re-start DNA-replication. It is unclear at present whether the restart involves rescue of the collapsed replication fork, or whether the collapsed fork needs to be disassembled and perhaps initiation of DNA-replication at a nearby origin will finish replication at the affected locus. However, in the absence of Rtt101p a DSB is created at the site of the collapsed replication fork and in this case DNA replication cannot be re-initiated. If such natural replication barriers were not present and fork pausing and collapse did not occur, it is likely that Rtt101p would not be required at all. However, the fact that almost all rtt101Δ cells accumulate DSBs and exhibit a significant delay at the metaphase/anaphase transition imply that such collapsed replication forks in slow replication zones occur virtually during every S-phase. Indeed, rtt101Δ cells show 16-fold increased rate of gross chromosomal re-arrangements (GCRs) compared to wild-type cells. While this genomic instability is significant, it is approximately 10 fold lower compared to sic1Δ cells, despite the fact of a comparable number of DSBs in the two mutant strains. It is likely that the intact Mec1p-dependent cell cycle arrest in rtt101Δ cells allows the repair of most the lesions prior to chromosome segregation.
However, when exogenous agents or cellular mutations put stress on the replication machinery, the likelihood of fork collapse is greatly increased, and in these instances Rtt101p plays an essential role in ensuring cell survival. It is interesting to note that most cancer cells are genetically unstable due to defects in DNA-damage pathways. It may thus be possible to specifically inhibit growth of tumor cells with Rtt101p inhibitors.

**Figure 7. The role of Rtt101p in replication and repair**

During DNA replication the replication machinery can encounter proteinaceous non-nucleosomal regions, which result in natural pausing. Although Rrm3p assists replication through these zones and minimizes pausing (left panel) sometimes the paused fork cannot be stabilized and will collapse. When this occurs, Rtt101p, is required to re-initiate replication and hence then cell cycle, and when Rtt101p is missing it results in genomic instability and re-arrangements. When Rrm3p is absent (right panel) or damaging agents are present, there is more breakage and damage throughout the genome, and in these cases Rtt101p becomes essential to re-initiate replication at the many damaged sites.

**Does the cullin Rtt101p function as an E3-ubiquitin ligase to restart collapsed replication forks?**

Rtt101p is a member of the cullin family of E3-ligases, which provide a scaffolding function by interacting with the E2 and substrate via an adaptor. Rtt101p can promote ubiquitination *in vitro* and interacts with its carboxy terminal domain with the ring-finger protein Rbx1p (Michel et al. 2003). Moreover, like other cullins, Rtt101p is modified at its C-terminal conserved lysine by the ubiquitin-like modifier Rub1p/Nedd8 (Laplaza et al. 2004). Different cullin families have been characterized based on their N-terminal interactions with substrate specific adaptors (Pintard et al.
2004). The amino-terminal sequence of Rtt101p most closely resembles mammalian Cul3p and Rtt101p-based E3-ligases. Cul3-based ligases have recently been shown to interact with BTB-containing proteins, while the substrate-specific adaptor of Rtt101-based ligases is unclear. Human Rtt101A and its S. pombe homologue Pcu4p interact with DDB1 and are involved in the repair of UV-damage-induced lesions. Together, these results suggest that Rtt101p is likely to function as an E3-ligase required to degrade an unknown substrate involved in restart of collapsed replication forks. However, we cannot exclude at present that Rtt101p may fulfill a structural role, or that mono- or multi-ubiquitinylation rather than proteasomal-degradation of an Rtt101p-target may regulate the activity of a component involved in replication fork restart. While the subunits of the Rtt101p complex are still unclear, it is interesting to note that the original RTT-screen identified additional components, which increase Ty1-transposition (Scholes et al. 2001). Among them features the BRCT-repeat containing protein Rtt107p, which has also been shown to be involved in restarting MMS-induced replication fork blocks (Rouse 2004). Interestingly, Rtt107p interacts with Rtt101p, via Mms22p (Ho et al. 2002), suggesting that these two proteins may function together in a complex at collapsed replication forks.

If Rtt101p indeed functions as an E3-ligase, what could its targets be? At present we do not know the mechanisms required to restart a collapsed replication fork, nor do we understand the molecular differences between a stalled and a collapsed replication fork. However, the topoisomerases I, has been shown to be unstable protein (Zhang et al. 2004), and it is conceivable that degradation of this enzymes at collapsed forks may be important for its release. Moreover, overexpressed excess histones are targets of proteasomal degradation (Alain Verreault, personal communication), and perhaps degradation of histones at collapsed forks may be needed for restart. Finally, it has been shown that a change of the polymerase accession factors is required for replication through damage zones. Perhaps degradation of these factors may be important during this process. Clearly, further work will be required to elucidate the missing Rtt101p-E3-ligase subunits and its targets involved in restart of collapsed replication forks.
Material and Methods

Strains constructions and genetic manipulations

Yeast strains are described in Table I. The genotypes of the yeast strains are: W303 (ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, ssd1-d2) and S288C (his3Δ1 leu2Δ0 met15Δ0 ura3Δ0), unless noted otherwise. The yeast k.o.-collection was purchased from Euroscarf. Standard yeast growth conditions and genetic manipulations were used as described (Guthrie and Fink 1991a). Yeast transformations were performed by lithium acetate procedure (Ito et al. 1983). YMJ1214 pds1::PDS1MYC13 was endogenously tagged using a PCR-based method described previously (Longtine et al. 1998). To construct the rtt101::NAT strain, the pMX6 NAT cassette was linearized with EcoR1, and the linearized plasmid was transformed into rtt101::kan strain. Transformants were selected for on YPD + NAT containing medium, and tested for G418 sensitivity.

DNA manipulations

Standard procedures were used for recombinant DNA manipulations (Ausubel et al. 1991). PCR reactions were performed with the Expand polymerase kit as recommended by the manufacturer (Roche). Details of plasmid constructions i.e. pGAL-HA-Rtt101 and pGAL-GFP-Rtt101 are available upon request. The Tubulin-GFP plasmid was a kind gift from Dr. Tim Stearns. Sequencing reactions were carried out by the ISREC-sequencing facility using standard procedures.

Microscopy

Proteins tagged with GFP were visualized on a Zeiss Axiophot fluorescence microscope using a Chroma GFP II filter. For time-lapse acquisitions exponentially growing cells were prepared on agarose pads as described previously(). GFP fluorescence Z-stacks of 5 layers separated by 0.5 micron were acquired every 2.5
minutes for up to 8 hours. An exposure time of 400 milliseconds was used. Spindle elongation time was determined as the time needed for the mitotic spindle to elongate once it had reached the mother/bud neck.

For DAPI staining cells were fixed 5 minutes in 70% ethanol and washed 2 times with water, cells were then re-suspended in a small volume of 50ng/mL DAPI solution in PBS, and observed with a UV filter set. To view DDC1-GFP foci, 7 z-stacks, 0.5 micron were taken and projected maximally.

**Arrest/release experiments with the Gal HA-RTT101 strains**

Exponentially growing rtt101Δ cells harbouring a Gal HA-RTT101 plasmid were grown in 2% raffinose selective media and induced with 2% galactose for 30 minutes. Cells were then arrested in G1 by the addition of 50 ug/mL α-factor (LIPAL-Biochemicals, Zurich). After 1 hour the culture was split, and half was resuspended in α-factor containing media with 2% glucose to repress the Gal HA-Rtt101 expression for a further 3 hours. Both cultures were then washed and released into fresh media containing either 2% galactose or 2% glucose. Samples for protein extracts, budding index and FACS analysis were taken at the indicated time points. Standard conditions were used for yeast cell extracts and immuno-blotting (Harlow and Lane 1988). Western blotting was performed using α-11HA (Babco), α-myc 9E11 (ISREC), α-Clb2 (kindly provided by D. Kellogg, UCSC) and anti-actin (Roche).

**PFGE analysis and BrdU detection**

For PFGE analysis, cells were embedded in LMP agarose plugs (5.10^7 cells/ml) and genomic DNA was extracted as described (Lengronne et al. 2001). Yeast chromosomes were separated by PFGE for 32 hours on a Gene Navigator (GE Healthcare) and were stained with SybrGold (Molecular Probes). The amount of DNA entering the gel was quantitated with ImageQuant after scanning with a Typhoon (GE Healthcare). BrdU was quantitated with a Typhoon after transfer on a nitrocellulose membrane and detection with a monoclonal antibody against BrdU.
(DakoCytomation, clone Bu20a) and a secondary antibody coupled to Alexa 488 (Molecular Probes).

2D gel analysis and ERC isolation

Neutral/neutral 2D agarose gels were performed essentially as described (Brewer and Fangman 1987). To quantify ERC levels, genomic DNA was prepared as described previously (Versini, 2003) and was separated on a 0.7% agarose gel for 22 h at 1 V/cm. Ribosomal DNA species were detected after Southern blotting and hybridization with a 35S probe. Quantification of autoradiograms was performed with a Typhoon (GE Healthcare). ERC levels were normalized against the chromosomal rDNA.

Dynamic molecular combing

Genomic DNA plugs (800 ng DNA) were stained with YOYO-1 (Molecular Probes) and resuspended at 150 ng/ml in 50 mM MES pH 5.7. DNA combing was performed on silanized coverslips as described previously (Michalet, 1997). Combed DNA fibers were denatured with 1 N NaOH and BrdU was detected with a rat monoclonal antibody (AbCys, clone BU1/75) and a secondary antibody coupled Alexa 488 (Molecular Probes). Since denaturation eliminates YOYO-1 staining, DNA molecules were counterstained with an anti-guanosine antibody (Argene) and an anti-mouse IgG coupled to Alexa 546 (Molecular Probes). Images were recorded with a Leica DMRA microscope coupled to a CoolSNAP HQ CCD camera (Roper Scientific) and were processed as described (Pasero et al. 2002). Signals were measured with MetaMorph v6.1 (Universal Imaging Corp.)
### Table 1. Strain List

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Chapter-III Other components of the Rtt101p pathway

Using Genetic approaches to find other components of the Rtt101 pathway

Many phenotypes associated with rtt101Δ cells are very robust and provide the opportunity to perform systematic screens in order to find other pathway components. Since RTT101 is a non-essential gene, we reasoned that other players in the same genetic pathway would also be non-essential. Therefore we used a collection of viable deletion mutants and screened them for phenotypes that resembled the rtt101Δ strain. From this, we found a list of potential candidates that we are currently testing further.

As there are only thirteen genes in the yeast genome that code for E2 ubiquitin conjugating enzymes, we looked at these genes apart from the screen directly. We found that the E2s, Ubc4p and Ubc5p, are most likely working together with the cullin, Rtt101p.

The redundant E2s, Ubc4p and Ubc5p, interact genetically and physically with Rtt101p

It was previously shown that Rtt101p could support in vitro ubiquitin chain formation, however it was far less efficient than what was observed for the same reaction conditions when Cdc53p was used as the cullin instead of Rtt101p (Michel et al. 2003). This in vitro reaction was performed using the E2, Cdc34p, as the conjugating enzyme and it was speculated that a non-specific E2 was being used in the reaction, and that this was accounting for the low efficiency. We screened through all of the viable E2 deletions and searched for those that had an MMS sensitivity phenotype. We found that only four of the non-essential E2 deletions were sensitive to MMS, including ubc13Δ, mms2Δ, rad6Δ, and ubc4Δubc5Δ (Figure 1.). In the case of ubc4Δ and ubc5Δ, it was necessary to analyze the phenotype of the double mutant, as these genes have a 95% primary sequence identity and can substitute for each other.
This narrowed down the number of possible E2s that could be functioning in the potential Rtt101p complex. Another characteristic phenotype of rtt101Δ cells is that they have an increased number of cells with DNA at the mother-bud neck in exponentially growing cells. We then examined the E2 deletions that were MMS sensitive and scored them for “% butterfly nuclei”, by analyzing fixed DAPI stained cells. The results are summarized in Figure 2. As rad6Δ cells and ubc4Δ ubc5Δ cells are the only E2 deletions that have nuclei positioned like rtt101Δ, we reasoned that one of these two E2s must function with Rtt101p.

By spotting the cells onto camptothecin (CPT), a topoisomerase poison that induces DSBs, we were able to determine that the most likely E2 in the Rtt101p based
E3 ligase is the redundant Ubc4p/Ubc5p pair. Strikingly the mms2Δ and ubc13Δ strains were not at all sensitive to CPT, although they were both highly MMS sensitive. The cells deleted for Rad6p were mildly CPT sensitive, as previously reported, whereas the ubc4Δubc5Δ double mutant was sensitive to CPT to the same extent as the rtt101Δ (Figure 3).

Figure 3. At 10 μM CPT the rad6Δ displays moderate sensitivity, while the ubc4Δubc5Δ is highly sensitive
rtt101=cul4

We confirmed this genetic interaction by showing that a bacterially produced GST-Ubc4p fusion protein was able to pull down Gal-overexpressed HA-Rtt101p from yeast extracts, while GST alone interacted only with barely detectable amounts of HA-Rtt101p (Figure 4.).

Figure 4. Bacterially produced GST-Ubc4 and GST was combined with a yeast extract from Gal HA-Rtt101p producing yeast. GST was then affinity purified using glutathione beads. Western blots were performed and blotted with anti-HA and anti-GST antibodies. Rtt101=cul4, figure provided by Céline Tafoir

Ubc4p and Ubc5p are two E2s that are known to be able to support both K63 linked ubiquitin chains as well as K29 linked chains (Arnason and Ellison 1994). A K29 conjugated chain is competent to target a protein to the proteasome for degradation (Johnson et al. 1995), whereas K63 linked ubiquitin is not known to induce degradation (Arnason and Ellison 1994). Since we have previously shown
that Rtt101p is required to re-start collapsed replication forks, and because we know
that the Rtt101p ligase is using the E2s, Ubc4p and Ubc5p, we reasoned that if we
inhibited proteasomal function and followed DNA replication re-start, we could
determine if the ligase was functioning through the usage of K63, K48 or K29 linked
ubiquitination. We found that by using the proteasomal inhibitor, MG132, in an
\textit{erg6A}, background, replication was able to re-start as in wild-type as assayed by
following Rad53p phosphorylation (Figure 5). This suggests that the Rtt101p E3
ligase is functioning through K63 linked ubiquitination pathway, or another pathway
that used a non-degradative lysine linkage. To control that the proteasome inhibitor
had functioned correctly, we looked at the protein, Gic2p, which has previously been
reported to accumulate as higher migrating, ubiquitin conjugated forms, in the
presence of proteasome inhibitor (Jaquenoud et al. 1998).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{The experiment was carried out as depicted above. We noticed that inhibition of the
proteasome did not prevent cells from recovering from MMS treatment. Open arrows correspond to
full-length Gic2p, and the accumulated upper bands correspond to the ubiquitinated forms in the
MG132 treated sample, \textit{cul4A}=rttl01.}
\end{figure}
The N-terminal region of Rtt101p is essential for function indication a conserved role in substrate adaptor binding

As previously mentioned the N-terminal cullin repeat domain is implicated in binding to the substrate specific adaptor (Figure 6A). In order to address the question of whether or not the N-terminal region of Rtt101p was playing a similar role, we generated a series of 50 amino acid N-terminal truncation mutants under the control of the GAL inducible promoter (Figure 6B). Interestingly, N-terminal deletions of the cullin mimic the null mutation, as already a 50 amino acid truncation results in a completely non-functional protein (Figure 6C). This indicates that the N-terminal region is important, most likely to interact with a substrate specific adaptor.

Figure 6. A. Modular schematic of the SCF complex where the substrate adaptor interacts with the N-terminal cullin repeat domain. B. 50 amino acid truncation deletions of Rtt101p were generated and expressed under an inducible promoter. C. Deletion constructs were expressed in an rtt101Δ strain and tested for complementation of GAL+MMS containing media (cul4=rtt101).
Apart from DDB1 which is the substrate specific adaptor for human Cul4A all other known adaptors contain a BTB fold motif. The yeast *S. cerevisiae* genome encodes for at least three genes that encode BTB domains as predicted by SMART (primary sequence analysis tool): YIL001w, YLR108c, and YDR132c. The deletion mutants of these strains were spotted onto YPD + CPT, to assess whether or not they had an *rtt101Δ* like phenotype. Figure 7 shows that none of the BTB single mutations, nor different combinations of the double mutations, nor the triple (not shown) are sensitive to CPT. Furthermore, Rtt101p, does not interact with Skp1p, by yeast 2-hybrid analysis.

Taken together, these results indicate that the substrate specific adaptor in the Rtt101p complex is most likely not a BTB containing protein. This is also consistent with the fact that the N-terminal region of the cullin is divergent from the BTB interaction domain of other cullin proteins. Nonetheless, the N-terminal domain of the cullin is essential for function suggesting that an adaptor-cullin interaction is taking place.

Since there is no apparent DDB1 homologue in *S. cerevisiae*, we decided to perform a genetic screen to find the adaptor in the Rtt101p complex.

A genomic wide screen to find other Rtt101p interactors

The non-essential knockout collection was used as a tool to screen for deletions that gave *rtt101Δ*-like phenotypes. We used the same approach as with the
E2s, i.e. doing a screen and then multiple secondary screens. However this time we used CPT as our primary screening drug, as it seemed to give more specific results as described previously with the E2s. Using a 96-well pinning apparatus, we used a robot to spot the liquid knockout collection in an arrayed format onto plates containing 20uM CPT. After 3 days of growth at 30°C we removed the plates and scored for any colonies that gave a slow growth phenotype, or completely failed to form a colony. We expected to pick up not only CPT sensitive but slow growth mutants that we would later rule out in confirmation screening. We found 501 CPT sensitive mutant candidates. An example of one of the plates is shown in Figure 8, where the cullin mutant is circled.

These 501 mutants were then individually picked from the collection and spotted by hand after serial dilution onto different drugs, all of which resulted in a slow grow phenotype in the *rtt101Δ* mutant. This had the dual effect of ruling out false positive interactions as well as applying secondary screens to the candidates. We spotted these cells onto plates containing HU, MMS, AMSA (topoisomerase II inhibitor) and CPT (Figure 9), and scored them as being hypersensitive (HS), sensitive (S), or medium sensitive (M). Through the application of these secondary screens we were able to narrow the number of candidates down to approximately 30.
eliminate the HR machinery we expresses HO in all of these mutants and looked for cells like rtt101Δ that were able to survive HO induction. Figure 10 shows that all of the strains were able to survive a single DSB at the mating locus, except, rad52Δ, rad54Δ, rad57Δ and rad55Δ. We are currently testing the remaining strains for synthetic lethal interactions with rrm3Δ.

We also tested all of the deletions strains in order to see which ones had increased rDNA circles as in the rtt101Δ (data not shown). Surprisingly, only top3Δ, and dia2Δ cells showed increased rDNA circle accumulation. This is interesting as Dia2p is an F-box protein that could potentially act in and SCF-type ligase. A current list of the candidates that came out of our screen and could be working with Rtt101p as an E3 ligase are presented in Table 1 along with their respective phenotypes. Interestingly, we found four previously uncharacterized genes in our screen, including: MMS22, RTT107, MMS1 and RTT109. Strikingly, MMS22 and RTT107 were previously show to be able to interact with Rtt101p through affinity precipitation, which leaves these two genes as prime candidates for being Rtt010p interactors and possible substrate specific adaptors (see general discussion).
Figure 10. When HO was expressed in those cells that were found to be sensitive to CPT, only rad52Δ, rad55Δ, rad57Δ, and rad54Δ strains were unable to survive the single cut at the MAT locus. Cul4 = Rtt101

Table 1. Results of the CPT screen and potential Rtt101p interactors

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<th>si with cul4</th>
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Modification and regulation of the cullin Rtt101p

As with all other cullins studied so far, Rtt101p, is modified on a conserved lysine at its C-terminus by the ubiquitin like modifier, Rub1p/Nedd8 (Figure 11) (Laplaza et al. 2004).

Consistently, an epitope tagged version of Rtt101p, migrates on SDS gels as a distinct doublet, the slower migrating form corresponding to the Rub1p-modified protein and the faster migrating form, unmodified. As previously reported (Laplaza et al. 2004) we have noticed that by deleting Rub1p, we can almost completely abolish the slower migrating form, however upon long exposure we notice that there is still a modified form that persists. The Rub1p modification on Rtt101p is not essential for it function, as a rub1Δ strain is not sensitive to MMS, and does not show any of the other phenotypes that rtt101Δ cells (Figure 12).

Figure 11. The lysine that becomes modified by Rub1p/Nedd8 is conserved in all cullin proteins. ScCul4=Rtt101p

Figure 12. Rtt101p is modified by Rub1p. Upper arrow points to the modified form. There is still a faint modification in the rub1Δ strain that becomes more visible with longer exposures. The bottom panel shows that the rub1Δ strain does not share phenotypes with rtt101Δ, showing that rubbylation is not essential for function

Cul4 = Rtt101
Surprisingly, when we mutated the K791 residue to either an alanine or an arginine residue we found firstly that the faint modification that we see in a *rub1Δ* strain is completely undetectable, and secondly, that this mutation no longer compliments an *rtt101Δ*. These findings suggest that this residue is essential for the cullin function and that the second modification is most likely playing a significant role in activation of Rtt101p (Figure 13). This is reminiscent of the regulation of PCNA where a single lysine residue is modified by either ubiquitin (under damaging conditions) or the small ubiquitin-like modifier, SUMO (during S-phase) (Hoege et al. 2002).

Figure 13. K791R completely abolishes the modification on Rtt101p. It also fails to complement the phenotype of the cullin deletion. 
Cul4 = Rtt101
Chapter-IV General Discussion

Overview

The work in my thesis has attempted to elucidate the function and composition of the Rtt101p cullin complex, with respect to cell cycle progression. We have made use of biochemical, and genetic approaches to address these questions.

It has become clear that Rtt101p is required for accurate replication and repair of chromosomal DNA. Our results have allowed us to postulate a model whereby the cullin, Rtt101p, is required to re-start the cell cycle following DNA damage. This has provided a new link between cullin dependent ubiquitination and replication recovery following damage.

In the following section I will attempt to validate the model that we have proposed in chapter II, based on the results that we have obtained. Furthermore I will speculate about the other components of the hypothetical Rtt101p complex.

The cell cycle contribution of Rtt101p

We started our investigation using a reverse genetic approach by evaluating the phenotype of the rtt101Δ strain. We were able to see that the cell cycle was slightly perturbed in these cells and that the time needed for a single cell division was increased with a greater proportion of time spent at the G2/M boundary. This indicated that the cullin Rtt101p was contributing, somehow, to the cell division cycle, and gave us our first indication that a cullin other than Cul1p/Cdc53p is involved in S. cerevisiae cell proliferation.

Checkpoints and cell cycle arrest

The most important role of checkpoint proteins is to ensure that after cell division both mother and daughter cell have an accurately replicated copy of the genome. This is accomplished by checkpoint dependent "wait" signals i.e. checkpoints halt future cell cycle events until the previous one is correctly and completely executed. In yeast, a common waiting place is the G2/M transition point. Checkpoint proteins ensure that DNA replication is completed before the onset of
mitosis, as well they ensure that microtubules are correctly attached to kinetochores before spindle elongation. These proteins provide security for the cell in the occasional instances that something goes wrong, giving the cell time to repair or recover, before the cell cycle drives on.

**Checkpoint inactivation to reveal a pathway**

As previously mentioned, specific checkpoint proteins are needed to halt the cell cycle when abnormalities arise. We reasoned that by inactivating various checkpoint proteins in the \( rtt101 \Delta \) background we would be able to suppress the G2/M cell cycle delay providing that we disrupted the checkpoint corresponding to the process that Rtt101p is involved in. This genetic approach revealed that by deleting either Mec1p or a downstream effector of Mec1p, Rad9p, we were able suppress the G2/M accumulation. However deletion of the spindle assemble checkpoint protein, Mad2p, did not alter the \( rtt101 \Delta \) phenotype. This along with live cell imaging revealed that spindle assembly was in tact and microtubules were being correctly attached to kinetochores.

Since both Mec1p and Rad9p are involved in responding to unreplicated and damaged DNA (Gardner et al. 1999; Cha and Kleckner 2002) we speculated that Rtt101p was playing a role in DNA metabolism. However these results did not tell us if Rtt101p was involved directly in DNA synthesis, replication initiation or repair of damage, as these checkpoints are activated in response to all of the above mentioned processes.

**Is the cullin, Rtt101p, involved in replication initiation?**

Replication initiation can be defective for a number of reasons, however the primary reasons would be inability to license replication origins, or an inability to activate an already licensed origin. We believe that these processes are both functional in cells deleted for Rtt101p.

Replication origin licencing is dependent on low CDK1 activity levels (Diffley 2004). This is achieved on multiple levels by downregulating the B-type cyclins at the end of the mitotic process. By using synchronized cell cultures we have
observed that Clb2p is degraded efficiently by the APC\(^{Cdh1}\) at mitotic exit, albeit later than in wild type. Furthermore we have seen that after a promoter shut-off of Rtt101p, in G1 arrested cells, Clb2p, levels do not re-accumulate, again suggesting that the APC\(^{Cdh1}\) is active. This indicates that there is a window of opportunity when origins can be licensed by the licencing factors, Cdc6p, Cdt1p and MCM.

Even though we see that Clb2 eventually does get correctly degraded in \(rtt101\Delta\) cells, one could argue that there is less time to license origins and therefore less origins are licensed. This line of argument would also be supported by what we see with the DNA combing results, in that less origins are fired. However it is very unlikely that this can account for the phenotypes that we see in \(rtt101\Delta\) cells. Firstly, it has been shown that many replication origins can be deleted from cells and this has almost no effect on replication timing (Newlon et al. 1993), which is not consistent with the 20 minute S phase delay that we observe in culin deletion cells. Secondly, \(cdh1\Delta\) cells, are defective for Clb2p degradation, although they are not sensitive to the DNA damaging agents, MMS, and CPT as is the case with \(rtt101\Delta\) cells. Finally, providing \(rtt101\Delta\) with more time to license origins by deleting the S phase cyclins Clb5p and Clb6p does not rescue the increased DSB phenotype seen in \(rtt101\Delta\) cells. It has been previously shown that a \(sic1\Delta\) strain, which also has less time to license origins due to the inability to inhibit the S phase cyclins, can be suppressed by introducing \(clb5\) and \(clb6\) mutations (Lengronne and Schwob 2002). Taken together, this strongly supports the notion that replication initiation is not the primary defect in \(rtt101\) mutants. Although there are less active origins as seen by DNA combing, this is most likely a secondary effect of slight checkpoint activation, which actively suppresses the firing of late replicating origins, due to accumulation of collapsed replication forks (see below).

**Does Rtt101p play direct role in DNA repair?**

It has previously been shown that the repair of some DNA lesions requires ubiquitination. For example PCNA must be ubiquitinated in order to promote recruitment of the trans-lesion polymerase (Kannouche et al. 2004). We have seen however that cells lacking the \(RTT101\) gene are repair competent.
The HO endonuclease induces a double stranded break in the MAT locus, and promotes mating type switching after cytokinesis. When the HO endonuclease is induced in a strain that is defective for DSB repair, eg. rad52Δ, or rad55Δ, it induces approximately 100% lethality, and these mutants are completely incapable of repairing the break. In contrast, when HO is induced in rtt101Δ cells, they retain 97% viability. Therefore a HO cut is efficiently repaired in the absence of Rtt101p.

Furthermore, cells deleted for rtt101, are resistant to UV light (Michel et al. 2003), and are more sensitive to MMS in G1 than in S phase. These results again suggest that the damage itself can be repaired, and the latter result indicates that it is replication interacting with DNA damage together that are causing increased toxicity in rtt101Δ cells.

The fact that we detect Ddc1p-GFP foci in the rtt101Δ strain indicates that the DSB machinery gets localized to sites of damage. Together, we can conclude that in rtt101 deletions the DSB machinery is in tact and regulated correctly in terms of localization. Furthermore the HR reaction is not impaired and it is likely that all repair pathways are intact in rtt101Δ cells.

One caveat with the above conclusions is that when we induced a DSB with the HO endonuclease it is only a single break. It is possible that it is the accumulation of DSB intermediates that causes toxicity, and one break does not produce enough intermediates. We ruled this possibility out, however, by showing that the synthetic lethality of an rtt101Δrrm3Δ strain, where there are many DSBs, is not rescued by further deleting RAD51 or RAD55 which would abolish the formation of HR intermediates. Sgs1p is the S. cerevisiae homologue of bacterial RecQ and human Bloom and Werner’s helicases, which are both implicated in cancer development when mutated. In contrast to rtt101Δrrm3Δ cells, the synthetic lethality of an sgs1Δrrm3Δ strain is completely rescued by subsequent deletion of either RAD51 or RAD55, as it is known that Sgs1p is required for complete processing of HR intermediates (Torres et al. 2004). This indicates, that HR intermediates are properly metabolized as well as establishing that sgs1Δ and rtt101Δ are synthetic lethal with rrm3Δ for different reasons. Interestingly, these findings place Rtt101p in a restart pathway other than that of Sgs1p. Even though sgs1Δ cells are sensitive to DNA damaging agents to the same extent as rtt101Δ cells and are also synthetically lethal
with *rrm3Δ* mutations, *sgs1Δ rtt101Δ* double mutants are more sensitive to genotoxins than either of the respective single mutants.

Furthermore, we have seen synthetic effects and enhanced growth defects between *rtt101Δrad55Δ, rtt101Δrad51Δ, rtt101Δrad52Δ* and *rtt101Δmre11Δ*, compared to any of the respective single mutants, suggesting that defective homologous recombination is not causing the lack of re-start seen in the *rtt101Δ* mutant.

**So what is Rtt101p doing?**

As previously discussed Rtt101p is not required to repair damaged DNA through homologous recombination, nor is it required for APC activity or replication initiation. We have found however that Rtt101p is needed to re-initiate DNA replication following replication fork collapse when a traveling fork encounters a lesion.

Replication stress and DNA damage result in the arrest of DNA replication, through the inhibition of fork progression. What has recently become clear, however, is that not all stalled forks are equal, and different drugs lead to drastically different replication intermediates (Lisby et al. 2004). HU, for example, which results in depleted nucleotide pools, was shown to arrest replication forks albeit in a stable manner. When HU was added to cultures it was found that many components of the repair machinery were recruited to the stalled fork, however Rad52p foci failed to appear. This implies that a DSB did not incur. In contrast, when HU was added to a *rad53Δ* strain Rad52p foci readily formed, implying that when forks are destabilized due to checkpoint failure, it results in complete DSB processing. Furthermore, when HU is applied for extended periods of time eg. 30 hours Rad52p foci also occur, indicating that long-term fork stabilization is not possible.

As mentioned previously we have seen that *rtt101Δ* cells are sensitive to HU, MMS and CPT, although the sensitivity to HU is far less pronounced than the sensitivity to MMS and CPT. We also found that when these cells were released from HU they were able to resume growth and complete DNA replication. *rtt101Δ* cells released from MMS, were not able to resume DNA replication. This led us to the simple conclusion that, Rtt101p, is required to restart collapsed replication.
intermediates (produced by MMS), but is not required to re-start stalled and checkpoint stabilized replication forks. This would explain the increased sensitivity to MMS and CPT compared to HU as well as explaining the synthetic lethality with \textit{rrm3}\textDelta, where there are increased numbers of DSBs caused by fork instability at pause sites.

Interestingly, other genes have been implicated in fork restart and these genes also confer synthetic lethality when deleted in combination with Rrm3. For example, Sgs1p is needed to process HR intermediates (already discussed) and Mrc1p, has been shown to be required to maintain coupling between replication factors and replication. When a fork is stalled in an \textit{mrc1}\textDelta strain, the replication machinery, including Cdc45p and MCM, continue to move along the unreplicated template, however DNA replication does not occur (Katou et al. 2003). Interestingly when \textit{mrc1}\textDelta cells are released from HU treatment they are not able to restart DNA synthesis (Pasero, personal communications). The fact that \textit{mrc1}\textDelta cells are also synthetic lethal with \textit{rrm3}\textDelta, suggests that Mrc1p is also required for restart of collapsed forks. Therefore, Rtt101p, is not required to maintain the coupling of replication with the replication factors, as the \textit{rrt101}\textDelta cells are restart competent after HU exposure.

From the above data we have formulated the following model of how, and where Rtt101p is functioning: During replication Rtt101p is chromatin associated (data not shown), either traveling with the replication machinery, or tethered at another chromosomal position. When the replication machinery pauses, due to natural pausing sites or DNA structures it is usually dealt with by the, Rrm3p helicase. However, from time to time these fragile pause sites are not able to be maintained in a stabilized manner and result in a double stranded break. We believe that in these instances Rtt101p is required, not to repair the break, but to allow replication to re-start (Figure 1A). However when the replication pause sites are destabilized and more frequently result in DSBs (eg. in an \textit{rrm3}\textDelta or in the presence of genotoxic agents) Rtt101p is required at more places throughout the genome (Figure 1B). This would explain why Rtt101p is only essential in the former conditions, as rare breakage may lead to chromosome instability and re-arrangements, but not necessarily lethality. Increased DSB frequency increases the chance that the break will be in an essential gene or cause a lethal rearrangement.
Figure 1. A. During normal replication natural pausing sites occur at approximately 1400 places throughout the genome. Normally these pauses result in a stalled but checkpoint stabilized replication fork. Sometimes a break can occur and then the DSB machinery will repair it and Rtt101p ensures replication re-initiation. B. When more forks are destabilized, e.g. when Rrm3p is deleted or in the presence of genotoxins, Rtt101p plays a larger and more important role as multiple breaks need to be repaired throughout the genome. The DSB machinery is also recruited to these sites but has been omitted due to space restrictions.

Although the target of the Rtt101p complex remains elusive it is tempting to speculate that it may be one of the DSB repair proteins that needs to be ubiquitinated after it has performed its function at the break site. Consistent with this idea it has recently been demonstrated by FRAP (fluorescent recovery after photobleaching) experiments that human single stranded binding protein, Rpa2, is rapidly turned over at break sites (Solomon et al. 2004). It has also been recently shown that histone H3 and H4 can be found polyubiquitinated when produced in excess (Alain Verreault, personal communication). PCNA becomes ubiquitinated after DNA damage in order to recruit the trans-lesion polymerase, Pole. Although Rtt101p is not involved in PCNA ubiquitination (data not shown), it may be involved in the re-recruitment of Polδ, which would explain why replication does not re-initiate.

Whatever the target of the Rtt101p complex is, it is most likely a protein that gets recruited to broken fork but not stalled ones. Accordingly it will be important to better characterize the differences between these two types of replication intermediates, which may shed some light on possible Rtt101p substrates. Once the target is known we will be able to characterize the effects of ubiquitin conjugation on the corresponding protein.
Is Rtt101 functioning as a ubiquitin ligase?

Although we have not answered this question directly several indications suggest that Rtt101p is taking part of an E3 ubiquitin ligase.

As previously mentioned all cullin proteins adopt a long rigid structure and interact with Rbx1p at the C-terminal and a substrate specific adaptor at the helical N-terminal region (Zheng et al. 2002). It has previously been reported that C-terminal deletions which result in loss of Rbx1p interaction result in non-functional cullin complexes (Patton et al. 1998). Consistently Rtt101p C-terminal truncations that no longer interact with Rbx1p fail to compliment the G2/M delay, typical of rtt101Δ cells.

Furthermore N-terminal truncations, which disrupt the cullin repeat (CR) structure also produce non-functional Rtt101p variants. This indicates that like other cullins, the N-terminal CR region of Rtt101p is important for function, most likely to interact with some type of substrate specific adaptor as is the case for all other cullin proteins studies so far. Due to the divergent nature of the Rtt101p N-terminal domain, the substrate adaptor is likely not a classical BTB fold containing protein, and may contain a novel cullin interacting domain. All of the Rtt101p N-terminal deletion proteins were still found in the chromatin fraction of cell extracts indicating that their localization was not affected by the deletion mutations and this did not account for their loss of function.

Rtt101p is able to form ubiquitin chains in an in vitro reaction when combined with the E2, Cdc34p, and the ring finger containing protein, Rbx1p. In these conditions the reaction proceeded with very low efficiency (Michel et al. 2003). This indicated that perhaps a different E2 was taking part in the in vivo complex. Indeed, through genetic screening combined with biochemical interaction studies we have shown that the E2 enzymes, Ubc4p and Ubc5p are functioning in the Rtt101p complex. Further studies on the ubc4Δubc5Δ double mutant will be required to verify these possibilities.

Although this all points strongly towards the notion that this cullin is taking part in an E3 ligase complex, we do not believe that protein degradation is playing a role. We have seen that cells treated with the proteasome inhibitor, MG132, are able to down regulate the Rad53p/Chk2 checkpoint following MMS release. In contrast rtt101Δ cells are unable to down regulate Rad53p phosphorylation even up to 5 hours
post MMS release. This suggests that the proteasome may not be required for recovery from MMS induced damage, whereas Rtt101p is. It is therefore possible that Rtt101p is involved in the formation of non-degradative ubiquitin chains. This would be consistent with the idea that Rtt101p interacts with Ubc4p, as it has been reported that Ubc4p and Ubc5p support K63 linked polyubiquitin formation, which is not associated with subsequent degradation (Spence et al. 1995).

When taken together: the interaction with Rbx1p, the interaction with Ubc4p, in vitro ubiquitination, and requirement of the N-terminus for function, these data strongly suggests that Rtt101p is functioning in an E3 ubiquitin ligase complex.

**Are there other components?**

The CPT screen and the subsequent secondary screens have left us with a list of potential candidates that could be functioning in the Rtt101 genetic pathway. Many of these genes including MMS1, MMS22, RTT107 and RTT109 are uncharacterized and have unknown function. Strikingly, Mms22p and Rtt107p were found to Co-immunoprecipitate with Rtt101p in large scale IP/Mass.Spec. experiments (Ho et al. 2002). Although Mms2p has no known primary sequence domains, nor does it have any recognizable homologues in other organisms it is nonetheless a prime candidate to be working in the Rtt101p complex. Due to the divergent N-terminal region of Rtt101p we do not expect that it will interact with a BTB-like folded protein, moreover, none of the known yeast BTB containing proteins, including Skp1p and Elc1p, are able to interact with the cullin by 2-hybrid experiments. Furthermore the deletion of the BTB genes in various combinations do not render cells MMS sensitive. A network of genes physically with RTT101 is provided below, Figure 2. The two hybrid data (in blue) reveals interactions between Rtt101p and Opi3p (phospholipids methyltransferase), Snz3p (unknown function), and Bzz1 (actin polarization). None of these genes however have been implicated in DNA replication or repair pathways and the corresponding deletions are not sensitive to genotoxin. The IP interactions (pink) however provide more intriguing links. Rtt107p, has also been shown to indirectly interact with Rtt101p via Mms2p. Interestingly, Rtt107p, has 4 BRCT repeats, a domain that is frequently mutated in various typed of breast cancers. BRCT domains have been shown to bind to
phospho-epitopes, and often get recruited to sites of DNA damage (Au and Henderson 2004). Furthermore is has recently been demonstrated that Rtt107p becomes phosphorylated in a Mec1 dependent manner following genotoxic stress. Strikingly, rtt107Δ cells are also unable to re-start replication following MMS exposure (Rouse 2004), reminiscent of rtt101Δ cells. It is tempting to speculate that a Mec1p phosphorylated Rtt107p may get recruited to damaged sites and be involved in bringing Rtt101p to the site of damage to restart DNA synthesis. There is less known about Mms2p and the other proteins that came out of the screen including, Mms1p and Rtt109p, however it will be interesting to determine if they are also playing a role in replication restart and if they are working in the same or complementary genetic pathways. Preliminary results demonstrate that the double mutant, rtt101Δ rtt107Δ, is more sensitive to damaging agents than either one of the single mutations, suggesting that they have only partially overlapping roles. The biochemical interactions between these proteins also needs to be better characterized as well.

Figure 2. Pink lines represent interactions that were determined through affinity precipitation experiments and blue represent 2-hybrid results.
**Is Rtt101p regulated?**

As is the case with all other cullins in all other organisms, Rtt101p, is modified by the ubiquitin-like protein, Rub1p/Nedd8, on a conserved C-terminal lysine residue, K791 (Laplaza et al. 2004). Nedd8 modification is required for the function of cullin ligases in all organisms, except in *S. cerevisiae*. *rub1A* strains are viable, and degrade Sic1p in a timely manner, in contrast to SCF mutants, which accumulate at the G1/S border with increased Sic1p levels. *rub1A* cells do however show synthetic effects with SCF ts (temperature sensitive) mutants suggesting a minor role may be played in complex activation (Lammer et al. 1998). We have not yet investigated synthetic enhancement phenotypes in *rtt101Δ* cells.

In general, this modification is dispensable for the function of the protein, as the *rub1Δ* strain shares none of the same phenotypes as the *rtt101Δ* strain. Interestingly, mutation of K791 to either alanine or arginine, renders Rtt101p non-functional. This suggests that either there is another modification on Rtt101p that is required to activate the protein or the K791R and K791A mutations render Rtt101p non-functional and unable to interact with Rbx1p. We have noticed that when Rub1p is deleted, a high migrating form of Rtt101p is still slightly detectable. Mutation of K791 abolishes this band however. This suggests that a modification that competes for the same lysine as Rub1p is required for cullin function, and is very reminiscent of the regulation of PCNA which is also modified on the same lysine by either ubiquitin or the ubiquitin-like modifier, SUMO (Hoege et al. 2002). It could be a common regulation mechanism among DNA replication/damage proteins, that they switch between different functions or different activational states depending on the ubiquitin-like modification. Perhaps only one of the two forms is able to recruit the E2 or Rbx1p? Further investigation is certainly required to better characterize this potentially novel and complex regulatory mechanism.

**Is this a conserved process?**

The involvement of cullin proteins has recently shown to play an important role in the DNA damage response. It has been convincingly demonstrated that in
human cells, the licensing factor, Cdt1, is targeted for degradation following exposure to UV light (Hu et al. 2004). Ubiquitination of Cdt1 depends on the activity of the Cul4A complex, whereby Ddb1 acts as the substrate adaptor and bridges the interaction between the cullin and Cdt1. It is proposed that this inhibits the replication of damaged DNA. Furthermore it was demonstrated in C. elegans, that massive re-replication occurs after RNAi of Cul-4 due to accumulation of Cdt-1 (Feng and Kipreos 2003; Zhong et al. 2003).

*S. cerevisiae*, has neither an obvious Cul4A homolog, nor a Ddb1 homolog by sequence analysis alone, Rtt101p, can not be placed into any of the known cullin family categories. Furthermore, in the budding yeast, Cdt1p is a stable protein where protein levels do not fluctuate throughout the cell cycle nor do they vary following DNA damage (Tanaka and Diffley 2002). Furthermore, the deletion of the inhibitor of ribonucleotide reductase, Sml1p, does not rescue the phenotypes associated with a rtt101Δ, as is the case in *S. pombe*, in that a spd1Δ, rescues many of the *pcu4*-phenotypes (data not shown). However the question of whether or not the role of Rtt101p is conserved in other organisms is not clear, as replication re-start following damage has not yet been addressed in other organisms in the context of cullin-based E3 dependent ubiquitination. Through many of the experimental approaches mentioned here it will be possible to start to address these questions in cultured human cells. For example through the use of cullin specific RNAi and synchronized cell cultures, it should be possible to look for replication re-start defects by BrdU detection as outlined in chapter II. Furthermore RNAi of cullins in human cells could also help to elucidate which of the cullins are required for the repair of DSB and suppression of chromosomal translocations. Through these approaches one will be better able to address which of the human cullins is orthologous to the yeast, Rtt101p.

**Rtt101p and the cancer connection**

The extensive investigation of the role of Rtt101p throughout the cell cycle has led to the clear conclusion that this cullin and most likely its associated cullin ligase activity are required to maintain genomic stability and replication fidelity. We have seen that mutant *rtt101Δ* cells frequently lose a piece of chromosome V (and perhaps others) and have an increased number of double stranded breaks even in the
absence of genotoxic agents. It has been shown that in human cells a single DSB within an essential gene or a gene that triggers apoptosis is enough to kill a cell (Cox 2001). Alternatively when a DSB is not able to be repaired, the broken ends may be fused in a sequence unspecific manner erroneously within the genome. This is supported by recent work, which has demonstrated that an endonuclease induced DSB increases chromosomal translocation rates. Translocations clearly lead to increased carcinogenesis. For example if a translocation occurs where a strong promoter lands in front of an oncogene, tumorogenesis can occur. A translocation that results in the mutation of a tumor-suppressor gene could equally lead to cancer development. Many lymphoid cancers are know to arise from erroneously resolved VDJ recombination products which requeite an intact DSB machinery (Cox 2001; Khanna and Jackson 2001).

Although we have seen that Rtt101p is likely not directly involved in DSB repair, it is likely involved in re-starting replication following repair. The inability to restart replication leads to checkpoint stabilized replication intermediates. These fragile intermediates have a chance of breaking and inducing more DSBs, which in turn puts the cells at risk of a translocation event. In this respect it will be important to further characterize the components and the targets of the Rtt101p complex as they are potential candidates in the tumorogenic process.

Concluding Remarks and Outlook

This study has started to shed some light on the interplay between the ubiquitin pathway and replication restart following fork collapse through a novel cullin protein, Rtt101p. Through this mainly genetic approach we have been able to identify other potential members of this process. Using the approaches that were outlined in this study it should be possible to further characterize the genetic interactors and determine their role, if any, in the restart pathway. Determining the substrate of the Rtt101p complex will also be instrumental in figuring out the exact molecular mechanism through which Rtt101p is working.

The detailed regulation of this complex could clearly help to better understand how damaged DNA is processed with respect to cell cycle progression. This knowledge could eventually be applied to tumor cells where these processes are often non-fuctional and deregulated.
Chapter- V Control of nutrient-sensitive transcription programs by the unconventional prefoldin URI

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Abstract

Prefoldins (PFDs) are members of a recently identified small-molecular weight protein family able to assemble into molecular chaperone complexes. Here we describe the discovery of a unusually large member of this family, termed URI, that forms complexes with small molecular weight PFDs and RPB5, a shared subunit of all three RNA polymerases. Functional analysis of the yeast and human orthologs of URI revealed that both are targets of nutrient signaling and participate in TOR-controlled gene expression. Thus, URI represents a component of a signaling pathway that coordinates nutrient availability with gene expression.

Introduction

The evolutionary conserved phosphoinositide (PI) 3-kinase related kinase TOR (target of rapamycin) pathway occupies a central role in the integration and transduction of nutritional cues into a coherent cell growth and proliferative response. Nutrient-rich conditions sustain TOR activity, which in turn, fuels cell growth whereas nutrient-deplete environments (or treatment with the immunosuppressant rapamycin) cause inhibition of TOR resulting in the activation of a response program which includes the induction of nutrient sensitive gene expression (Hardwick et al. 1999; Schmelzle and Hall 2000; Raught et al. 2001; Peng et al. 2002). Here we report the discovery of an evolutionary conserved member of the PFD family, termed URI, that participates in the regulation of nutrient-sensitive, TOR-dependent transcription programs.

Results

While searching for proteins associated with the substrate recognition component of the cell cycle regulatory SCF$^{SKP2}$ ubiquitin ligase, the F-box protein SKP2 (Lisztwan et al. 1998; Sutterlüty et al. 1999), we identified a new member of the prefoldin (PFD) family of small molecular weight (14-23 kDa) proteins. PFD
family members are comprised of N- and C-terminal α-helical coiled-coil structures connected by either one (β class PFD) or two (α class PFD) β-hairpins (Siegert et al. 2000). Yeast and human PFD1-6 assemble into α2β4 hexameric complexes, referred to as the prefoldin/GimC complex, that functions as a molecular chaperone in actin and tubulin folding (Vainberg et al. 1998; Siegers et al. 1999; Martin-Benito et al. 2002). Since the newly identified protein is an α class PFD that associates with Skp2 in vivo, we termed it STAP1 (for SKP2-associated alpha PFD) (Fig. S1).

We reasoned that STAP1, which is not part of prefoldin/GimC, may be a component of a yet unknown ‘prefoldin-like’ complex. Indeed, mass spectrometric identification of STAP1-associated proteins from HeLa cells (Fig. 1A) revealed two β-class PFDs, PFD2 and PFD4-related (PFD4r), three proteins of as yet unknown function(s) and four proteins, whose functions have been linked to transcription, in particular RPB5, a subunit shared by RNA pol I, II and III (Woychik et al. 1990), the ATPases TIP48 and TIP49 (Kanemaki et al. 1997; Wood et al. 2000) and RMP known to bind RPB5 (Dorjsuren et al. 1998). We refer to RMP thereafter as URI.

Upon gel-filtration STAP1 eluted with URI as a single peak at ~1 MDa (Fig. S2). RPB5 and TIP49/48 were present in multiple fractions including those containing STAP1 and URI. A fraction of total SKP2 protein and its partner proteins CUL1 and SKP1 eluted together with STAP1. Thus, STAP1 and URI may reside in vivo in a single high-molecular weight complex that contains the aforementioned components and subunits of SCF^{SKP2}. In support of this, URI, RPB5, TIP49 and TIP48 were only detected in anti-STAP1 immunoprecipitates derived from those fractions of the gel-filtration column that also contained the majority of STAP1 (Fig. S2). These proteins co-immunoprecipitated with each other from whole cell extracts (Fig. S3). In
addition, depletion of cellular URI by two sequential immunoprecipitations with URI antibodies co-depleted STAP1 and PFD4r (Fig. S3).

In search of homologous proteins encoded by the yeast genome we recognized a homology between STAP1 and the uncharacterized open reading frame (ORF) YFL023w (Murakami et al. 1995). Conceptual translation of YFL023w predicts a 796 amino acid protein (referred to as scUrip) with an N-terminal segment that contains all the features of an α class PFD. Subsequent database searches uncovered orthologs of YFL023w in the genomes of A. thaliana, D. melanogaster, C. elegans and H. sapiens (Fig. S4). Notably, the human ortholog of YFL023w is identical to the RPB5-binding protein RMP, identified as a STAP1-associated protein (see Fig. 1A). We noticed that the published cDNA encoding RMP is rearranged at the N-terminus. The corrected cDNA sequence encodes a protein of 534 amino acids with an α-class PFD-homology domain at its very N-terminus. In addition, a large-scale yeast two-hybrid screen revealed scUrip as an interaction partner for scRpb5p and scPfd6p (Ito et al. 2001). Indeed, URI and scUrip bound efficiently their respective PFD and RPB5 partners in GST-pull down assays (Fig. 1B) in a RPB5 and PFD domain-dependent manner (Fig. S5). Therefore, we refer to this polypeptide as URI/scUrip (for Unconventional prefoldin RPB5 Interactor).

To probe the function of scUrip, we deleted the corresponding gene in yeast. URI deletion resulted in viable cells. Genome-wide expression analysis identified 39 genes whose expression was consistently affected by the loss of scUrip (Table S1). The products of these genes function primarily in amino acid metabolism (Fig. 2A). URI deletion resulted also in the downregulation of genes encoding different tRNA species. Similar effects were seen in a sigma (Σ) yeast strain deleted for URI (Table S2). Thus, part of the normal function of scUrip is to contribute, directly or indirectly, to the expression of both RNA pol II and pol III transcripts, specifically those involved in the nutrient response pathway. Loss of scUrip caused also cell elongation.
(Fig. 2B) and agar penetration (Fig. 2C), hallmarks of invasive growth, induced by nutrient limitation (Gimeno et al. 1992; Kron and Gow 1995). Moreover scUrip levels decrease when cells are either grown in amino acid-deprived medium (Fig. 2D), in a low quality nitrogen source (urea media) (Fig. 2D) or treated with rapamycin (data not shown). They also decrease when cells enter stationary phase (Fig. 2D). Down-regulation of scUrip protein levels may therefore provide a mechanism to fine-tune the expression of scUrip-dependent genes during long-term nutrient starvation.

Exposure of yeast cells to rapamycin or amino acid starvation results in a rapid and rich transcriptional response (Hardwick et al. 1999; Gasch et al. 2000). Rapamycin affected the expression of 710 ORFs. Amino acid starvation altered the expression of 587 ORFs out of which 331 ORFs were identical to the ones affected by rapamycin. In fact, 29 out of the 36 ORFs affected in the uriΔ mutant overlapped with the group of 331 ORFs (Fig. 3A). Thus, the majority of genes affected by loss of URI are particularly those that are common transcriptional targets of both amino acid starvation- and rapamycin-sensitive signaling.

Next we used hierarchical cluster analysis to compare transcriptional responses between wild-type and uriΔ cells following rapamycin treatment (Fig. 3B) or amino acid starvation (Fig. 3C). While loss of scUrip had little impact on the ability of rapamycin to repress genes (n=325), it did affect the induction of rapamycin-sensitive genes (Fig. 3B, Table S3). In response to amino acid starvation, loss of scUrip affected the extent and kinetic of both gene activation and repression (Fig. 3C, Table S3). Thus, the function of scUrip is critical for adaptive transcriptional responses originating from distinct nutrient signals.

To identify underlying transcriptional pathways engaged upon scUrip loss, we analyzed the 5' promoter regions of scUrip-dependent genes. A large fraction of genes activated in uriΔ cells contain a consensus-binding sites for Gcn4p (Fig. 4A), a transcription factor known to activate amino acid biosynthesis genes in response to
amino acid deprivation (Natarajan et al. 2001). A role of GCN4 in gene activation in uriΔ cells is evidenced by the fact that these genes failed to be induced in the URI/GCN4 double mutant (Fig. S7). However, not all scUrip-dependent genes are also GCN4-dependent including certain tRNA genes and GAP1, an established rapamycin-induced gene.

Consistent with the above, Gcn4p levels were upregulated under conditions of amino acid sufficiency in uriΔ cells (Fig. 4B). This upregulation of Gcn4p was at least in part Gcn2p-independent. A lacZ-reporter under the control of the wild-type, but not mutated GCN4 5’leader sequence was dramatically activated in these cells (Fig. 4C) suggesting that the increase in Gcn4p in uriΔ cells involves a relief of translational repression. This activation was again, in part, GCN2-independent (Fig. 4C). Similar results were obtained in the prototrophic yeast strain Σ (Fig. S8). No changes in Gcn4p protein stability were detected in uriΔ cells (Fig. S8). Hence, scUrip may contribute directly or indirectly, to eIF2-GTP-tRNAiMet ternary complex formation and consequently, to the suppression of GCN4 protein translation and GCN4-dependent transcription programs under nutrient-rich conditions.

It is notable that only those Gcn4p target genes are induced in uriΔ cells that are also repressed by the TOR pathway. The latter is known to repress the translation of GCN4 and the activation of the transcription factors Gln3p and Gat1p. Because Gcn4p can facilitate the induction of certain Gln3p target genes, this negative effect of TOR on GCN4 translation is critical to prevent the expression of genes under dual control of GLN3/GAT1 and GCN4 in rich media (Chen et al. 1995; Cherkasova and Hinnebusch 2003). GLN3 and GAT1 are indeed induced in uriΔ cells in a Gcn4p-dependent manner (Fig. 4D), providing a potential explanation for the activation of TOR-controlled genes in uriΔ cells. Consistent with this view, Gcn4p is expressed in uriΔ cells at levels similar to those of rapamycin treated wild-type cells (Fig. 4E). Moreover, rapamycin did not affect Gcn4p levels in uriΔ cells (Fig. 4E). Upon amino
acid starvation however, Gcn4p can be induced in uriΔ cells like in wild-type cells (Fig. 4F). Combined, these results suggest a model in which loss of scUrip triggers the activation of genes under dual control of Gcn4p and Gln3p/Gat1p, at least in part, via the translational derepression of Gcn4p.

The conservation in structure between yeast and human URI proteins, led us to question if human URI might also participate in mTOR signaling in mammalian cells. URI is phosphorylated in vivo (Fig. 5A). Serum-starvation of HEK293 cells caused dephosphorylation of URI (Fig. 5B). Insulin reverted this effect and produced hyperphosphorylated URI (Fig. 5B), which could be blocked by rapamycin (Fig. 5B) and the PI3-K inhibitor wortmannin (data not shown). Thus, the phosphorylation state of URI is dependent on signals affecting mTOR activity. Consistent with this view, transfection of a rapamycin-resistant allele of mTOR (Chenet et al. 1995) recovered URI phosphorylation in the presence of rapamycin while wild-type mTOR did not (Fig. 5C). These results imply a role for URI in mTOR signaling in mammalian cells.

Next we created HeLa cells that had been stably silenced for URI expression by siRNA (Fig. 5D) and compared their transcriptional profiles in response to rapamycin (Fig. 5E). Following treatment of control cells for two hours with rapamycin, the expression of 194 genes was changed. Of these, the transcription pattern of 28 genes, whose products are primarily involved in the regulation of cell growth/maintenance and cell communication, was significantly altered in URI-silenced HeLa cells. This suggests a functional link between the presence of URI and the implementation of a proper rapamycin-sensitive transcriptional response in human cells.

**Discussion**

The results presented in this report describe the discovery of a unconventional member of the PFD family, termed URI, that is part of an ~1 MDa multiprotein
complex in human cells. URI appears to occupy a central role therein as a scaffolding protein able to assemble through its PFD-homology and RPB5-binding domains a new prefoldin-like complex that contains other PFDs and proteins with roles in transcription and ubiquitination. Functional analysis of the yeast and human orthologs of URI established a critical role for URI in TOR-controlled transcription pathways.

TOR coordinates nutrient availability with cell growth and proliferation at least in part by controlling the transcription of distinct sets of nutrient metabolism genes. scUrip appears to play a critical role in this process in that it contributes to TOR-mediated suppression of a select class of genes under dual control of the transcriptional activators GCN4 and GLN3/GAT1 under nutrient-rich conditions. In this regard, scUrip protein levels are down-regulated in response to various nutritional signals known to inhibit TOR activity such as nitrogen starvation or rapamycin. Thus, scUrip is a new component of a TOR-controlled downstream effector pathway that modulates nutrient-sensitive gene expression.

In mammalian cells URI is a phosphorylation target of the mTOR pathway and, like in yeast, contributes to rapamycin-sensitive transcription. This implies an evolutionary conserved role for URI proteins in TOR signaling. Further investigations on the function(s) served by URI and other prefoldin family members in human diseases in which rapamycin-sensitive mTOR signaling has been implicated are, therefore, clearly warranted.
Fig. 1. Isolation of a novel PFD-like complex. (A) Immunoprecipitates from HeLa cell extracts using control mouse IgG or STAP1 mAb 105-128. Polypeptides that yielded unambiguous MS/MS spectra are indicated. (B) In vitro translated (IVT) human and yeast URI proteins were tested for binding to indicated GST fusion proteins. Input lanes display 20% of the input IVT protein.
Fig. 2. Loss of URI affects the expression of nutrient-sensitive genes in S. cerevisiae. (A) Functional categorization of yeast ORFs, whose expression is changed in uriΔ cells (yBM80) compared to wild-type cells (yBM79). (B) Phase contrast images of wild-type or uriΔ cells grown to log phase in YPD. The percentage of elongated cells was determined from at least 200 cells. (C) Wild-type and uriΔ cells were grown for 2 days at 30°C on rich media and photographs were taken before and after gently washing cells off the agar. (D) Immunoblotting of yeast extracts prepared from cells expressing myc-scUrip, subjected to either amino acid or nitrogen starvation, or diauxic shift.
Fig. 3. scUrip is required for adaptive transcriptional responses. (A) Venn diagram depicting the overlap of genes whose mRNA expression is changed in response to rapamycin (left) or amino acid starvation (right) in wild-type cells with mRNA genes whose expression is altered in urιΔ cells shown in Table S1 (bottom). (B) Genes affected by rapamycin in wild-type cells were clustered according to their expression profile in wild-type and urιΔ strains using the program dCHIP. Mean expression values are displayed in white. Values above the mean in red and values below the mean as blue bars. scUrip-dependent clusters are indicated (RC1-RC4). (C) Standardized expression values of 587 ORF's changed upon amino acid starvation in wild-type cells were analyzed and displayed as in (B). scUrip dependent gene clusters are indicated (AC1-AC6).
Fig. 4. scUrip represses GCN4-dependent transcription. (A) Conserved motifs within the promoter region of scUrip-dependent genes. The consensus site for Gcn4p is underlined. Numbers in parenthesis refer to the number of allowed mismatches. P-values correspond to the false positive probabilities for each motif. (B) Extracts from indicated strains were analyzed by Western blotting using anti-myc antibody 9E10. (C) Indicated strains harbouring GCN4uORF-lacZ or the mutant, GCN4uORF4only-lacZ, were analyzed for lacZ activity. (D) Standardized mRNA expression values of GAP1, GCN4, GLN3 and GAT1 in the indicated strains relative to wild-type were obtained from genome wide transcription analysis. (E) Wild-type and uriΔ mutant cells were analyzed for Gcn4p-myc expression following 100 nm rapamycin treatment. (F) Wild-type and uriΔ mutant cells were analyzed for Gcn4p-myc expression following amino acid starvation.
Fig. 5. URI participates in mTOR signaling. (A) HEK 293 cells were serum-starved, treated with insulin, processed for lambda phosphatase treatment and analyzed by Western blotting for URI. (B) Serum-starved HEK293 cells were induced for the indicated time with insulin in the absence or presence of rapamycin and analyzed by Western blotting for URI. (C) Untransfected HEK293 cells or cells transfected with HA-mTOR(wt) or rapamycin resistant mutant HA-mTOR(RR) plasmids were serum-starved, treated with insulin and rapamycin and analyzed by immunoblotting. (D) Cell extracts of HeLa cells stably transfected with either pSuper or pSuper-si-URI were analyzed by immunoblotting for URI. (E) Gene expression profile of pSuper-HeLa cells and pSuper-si-URI cell lines following rapamycin treatment. Silencing of URI affected the rapamycin response of 28 genes shown in the cluster analysis.
References


23. Acknowledgments:
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SUPPORTING ONLINE MATERIAL

Materials and Methods

Yeast two-hybrid screen and plasmids. A standard two-hybrid screen was carried out with the yeast reporter strain Y153, using a HeLa cDNA library (pGAD-GH plasmid; Clontech) and a Gal4-fused SKP2 encoding amino acids 113-435 of human SKP2 expressed from pAS2-1 plasmid as a bait. Positive clones were analyzed by direct DNA sequencing. All were found to contain a sequence encoding a predicted ORF of 157 amino acids. One was used to construct a mammalian expression plasmid encoding an untagged version of STAP1 in pcDNA3 (InVitrogen). To generate pcDNA3-HA-STAP1, pcDNA3-STAP1 was digested with BamHI and EcoRI and the resulting fragment cloned into pcDNA3 containing an HA-tag previously introduced at the HindIII/BamHI site.

URI peptide sequences obtained from MS/MS sequencing, were used to identify overlapping EST sequences and human genomic sequences encoding full-length URI. Based on this information, primers were designed, and used in PCR reactions to amplify the first 426 bp of URI coding sequence from a human lymphoid library (Strubin et al. 1995). The resulting PCR product was digested with BamHI/EcoRI and subcloned into pcDNA3. The commercially available cDNA clone IMAGE clone 3609351, containing the 3' part of the URI cDNA, was digested with EcoRI and the resulting fragment
ligated into the EcoRI site of pcDNA3 containing the 426bp fragment of the 5’ region of URI. The resulting plasmid is referred to as pcDNA3-URI. From this plasmid a Flag-epitope tagged version was generated, resulting in the plasmid pcDNA3-FT-URI. Human RPB5 and p17 cDNAs were PCR amplified from a HeLa cDNA library (Clontech) and subcloned into pGEX and pcDNA3. cDNAs encoding the yeast proteins scPfd6, scRpb5 and scUrip were amplified from yeast cDNA libraries using a one-step PCR protocol and subcloned into pGEX and pBS-KS, respectively. For stable silencing of URI in human cells we cloned the following oligonucleotides into pSuper (Brummelkamp et al. 2002) to obtain pSuper-siURI:

forward:
GATCCCATGGAGAAGATACGACATCTTCAAGAGAGATGTCGTATCTTCTCCATTTTTTGGAAA;

reverse:
AGCTTTTCCAAAAAATGGAGAAGATACGACATCTCTTGAAGATGTCGTATCTTCTCCATGGG.

Antibodies. Mouse monoclonal antibodies 4H7 and 8WG16 directed against RPB5 and RPB1, respectively, have been described previously (Thompson et al. 1989; Nguyen et al. 1996). Polyclonal antibodies directed against SKP2 and CUL1 were described previously (Lisztwan et al. 1998). Mouse monoclonal antibodies recognizing SKP1 (90.122b), SKP2 (95.60.2), STAP1 (105.72 and 105.128) and URI (179.30 and 179.63) were raised against the respective full-length human proteins expressed as glutathione S-transferase (GST)-fusion protein. Rabbit polyclonal antibodies recognizing STAP1 were generated against a maltose-binding protein (MBP)-fusion protein. Monoclonal antibodies directed against URI(179.63.1, 179.58.1) have been raised against full-length human proteins expressed as GST-fusion proteins. Polyclonal antibodies recognizing URI were provided by Dr. Seishi Murakami.
Rabbit polyclonal anti-TIP48 and TIP49-antibodies were raised against peptides corresponding to their respective C-termini (TIP48 peptide: MKEYQDAFLFNELKGETMDTS; TIP49 peptide: LFYDAKSSAKILADQQDKYMK). Peptides were coupled to keyhole limpet hemocyanin by glutaraldehyde coupling and injected into rabbits. Rabbit polyclonal antibodies specific for human PFD3 and p17 (PFD4r, Accession number: Q9NUG6) were raised against bacterially produced GST-fusion protein. Affinity-purification of the different polyclonal rabbit sera was achieved by incubation with first a GST-affinity-column followed by a GST-fusion protein column, prepared by covalently cross-linking the respective proteins to glutathione-Sepharose with dimethylpimelimidate (Harlow and Lane 1999). Anti-peptide antibodies were affinity-purified as described (Lisztwan et al. 1998). Monoclonal antibodies HA-11, 12CA5, 9E10 and anti-actin mAb were purchased from Babco, Boehringer Mannheim, Santa Cruz and Chemicon International respectively. Anti-eIF2α antibodies were kindly provided by Dr. Ron Wek.

**Immunoprecipitation, immunoblotting, gel filtration, phosphatase assay.** Immunoprecipitations and immunoblotting were performed essentially as described (Lisztwan et al. 1998). Ponceau S (Sigma) staining of western blot membranes has been performed according to the procedures of the manufacturer. For Superose 6 gel-filtration 3-4 x 10⁹ HeLa cells were lysed in 10 ml lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 0.5 mM PMSF, 20 mM NaF and 1 x protease inhibitor cocktail (Roche)] for 30 min on ice, lysates were pre-cleared by centrifugation for 30 min at 4°C at 3000 g and filtered through a 0.45 μm filter prior to loading on a Superose 6 column (FPLC; Pharmacia). Gel filtration was performed at 4°C at 0.5 ml/min flow rate using gel-filtration buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM MgCl₂ and 0.5 mM DTT] and 4 ml fractions were collected. Blue
Dextran 2000 (2 MDa), thyroglobuline (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa) were used as molecular-weight standards. For phosphatase experiments HEK293 cells have been starved for 24h in DMEM w/o serum and induced with 10μM insulin for 1h prior to lysis in PL buffer [50 mM Tris-Hcl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, 250 mM NaCl, 0.5 % NP40] for 30 min on ice. 200 μg of protein extracts have incubated with 400 U of λ-PPase (NEB) in 50 μl 1x λ-PPase buffer [50 mM Tris-Hcl (pH 7.5), 0.1 mM EDTA, 5 mM DTT, 250 mM NaCl, 0.01 % Brij 35] for 30 min at 30° C. Reactions have been stopped by adding 20 μl Laemmli-buffer and boiling for 10 min before loading on 6% SDS-PAGE gels.

Purification of STAP1-associated proteins and mass spectrometric analysis. For purification of STAP1-associated proteins, 4 ml of packed HeLa cells were lysed for 30 min on ice in 50 ml TNN-buffer [50 mM Tris-HCI (pH 7.5), 250 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na3VO4, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin], dounce homogenized (10 strokes with a B pestle) and lysates cleared by centrifugation at 18000 x g for 20 min. Supernatants were collected and incubated for 2 hrs at 4°C with anti-STAP1 mAb 105.128 covalently coupled to protein A-Sepharose using a dimethylpimelimidate coupling procedure. Immunobeads were washed 4 x with TNN-buffer, proteins eluted with 300 μl 0.2 M glycine (pH 2.5), neutralized with 100 μl 1M K2HPO4, concentrated on a Centricon concentrator (Amicon) and resolved on a 6%-15% gradient SDS-polyacrylamide gel. The gel was silver stained, protein bands were excised, digested with trypsin and subjected to sequence analysis. Peptides were sequenced using nano-electrospray tandem mass spectrometry (NanoESI-MSMS) on a API 300 mass spectrometer (PE Sciex, Toronto, Ontario, Canada) and identified as described (Mann and Wilm 1994). Identities of STAP1-associated proteins were confirmed in an independent
experiment using MALDI-TOF analysis on a TofSpec 2E (Micromass, Manchester, UK) according the direction of the manufacturer. The full dataset of this analysis is available upon request.

**In vitro binding reactions.** STAP1, hRPB5, scRpb5 or scPfd6 were expressed as GST-fusion proteins in *E. coli*, purified on glutathione-Sepharose beads and subsequently incubated with ^35^S-methionine-labeled in vitro translation products of either URI or scUrip generated by the TNT system (Promega) as previously described (Krek et al. 1994). Bound proteins were analyzed on SDS-PAGE and detected by fluorography.

**Yeast strains and techniques.** The following yeast strains have been used in this study: yBM80 (W303): uri::His3 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 psi+. yBM79 (W303):ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 psi+. yBL45 (K699): ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11, 15, ura3, psi+, ssd1-d2 bar- uri::U13myc His3. yBL284 (W303): ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11, 15, ura3, psi+ gcn4::kan. yBL285 (W303): ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11, 15, ura3, psi+, gcn4::kan uri::His3. YBL295 (W303): ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11, 15, ura3, psi+, ssd1-d2, uri::His gcn2::kan. yBL282 (W303): ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11, 15, ura3, psi+, uri::U13myc-HIS3. yBL225 (Σ):ura3. yBL234 (Σ):ura3-52 uri::KAN. yMJ205 (W303): cdc34-2 ade2-1, trp1-1, can1-100, leu2-3, 112, his3-11, 15, ura3, psi+, ssd1-d2.

Details about strain constructions will be provided upon request. If not stated otherwise standard yeast growth conditions and genetic manipulations were used (Guthrie and Fink 1991b). For protein half-life analysis cells were grown at 25 °C until early-log phase in synthetic media. Cells were then shifted to a non-permissive temperature of 37 °C for 3 hours. At time 0 cyclohexamide was added to a final concentration of 50 ug/ml and samples were taken at the
indicated times, protein extracts were analyzed by Western blotting. Band intensities were analysed with Quantity One quantitation software, version 4.2 from Biorad. Intensities minus the lane background were determined and expressed as a ratio (intensity of GCN4-myc/control intensity), where time 0 was always normalized and set to a value of 100. Standard deviations were determined from three individual experiments and plotted on a scatter graph. For expression of GCN4p-myc yeast cells have been transformed with plasmid p3330 (kindly provided by Alan Hinnebusch). LacZ assays were performed on yeast strains transformed with the reporter plasmids GCN4µORF-lacZ or GCN4µORF4only-lacZ, where the ATGs of µORF1-3 have been mutated (Mueller and Hinnebusch 1986), as described (Breeden and Nasmyth 1985). Yeast cells were tested for invasive growth by washing yeast plates under the tap water after growth on yeast extract/peptone/dextrose (YPD) plates for two days at 30°C. In nutrient starvation experiments, overnight cultures of yeast cells grown in YPD medium were diluted to OD600 of 0.1 and grown in YPD to early log-phase (OD600 = 0.4), washed twice with H2O prewarmed at 30°C and resuspended in either amino acid starvation medium (3.4 g/l yeast nitrogen base, 3% glucose), or in urea medium (3.4 g/l yeast nitrogen base, 5 µM urea, 3% glucose, appropriate supplements for auxotrophs Ura, Leu, Trp, Ade). In diauxic shift experiments yeast cells were grown to early log phase in YPD (= 0 hrs time point) and aquisitions were taken at different time points thereafter for total protein extraction.

Global gene expression analysis. Three colonies of wild-type strain (yBM79) and uriΔ (yBM80) were inoculated in 10 ml YPD, grown overnight at 30°C on a shaker at 225 rpm, diluted to OD600 of 0.1 and continued to grow to early log phase (OD600=0.3). Yeast cells were collected by centrifugation for 5 min at 3000 rpm and total RNA extracted by the acid-phenol method
from wt (yBM79) and uriΔ (yBM80) strains for microarray analysis (Schmitt et al. 1990). In order to define global gene expression affected by loss of URI, RNA from six independent replicate cultures of yBM79 and yBM80 were analyzed. For the rapamycin time-course experiments (Fig. 5B), two separate replicate cultures of yBM79 and yBM80 cells were grown to early log-phase (OD<sub>600</sub> of 0.3) and rapamycin was added to a final concentration of 100 nM in YPD. Aliquots of 50 ml cell suspension were harvested at indicated time points by centrifugation for 5 minutes at 3000 rpm and pellets were snap-frozen in liquid nitrogen before RNA isolation. For measuring global gene expression following amino acid starvation two independent replicate cultures of yBM79 and yBM80 yeast cells were grown to early log phase (OD<sub>600</sub> of 0.3), collected by centrifugation at 3000 rpm at room temperature and washed twice with H<sub>2</sub>O at 30°C before resuspending them in amino acid starvation medium at 30°C, (0.34 % YNB, 3% glucose). Aliqots of 50 ml cell suspension were harvested at indicated time points for extraction of total RNA as described above. Microarray analyses were performed using YG_S98 GeneChips™ (Affymetrix, Santa Clara, USA). Ten μg of total RNA was reverse transcribed using the SuperScript Choice system for cDNA synthesis (Life Technologies) according to the protocol recommended by Affymetrix. The following oligonucleotide was used for priming: 5'-ggccagtgaattgtaatacgactcactatagggaggcg-(t)<sub>24</sub>-3'. Double-stranded cDNA was extracted by phenol:chloroform and the aqueous phase removed by centrifugation through Phase-lock Gel (Eppendorf). In vitro transcription was performed on 1 μg of cDNA template using the Enzo BioArray High Yield RNA transcript labelling kit (Enzo Diagnostics, USA) following the manufacturer's protocol. The cRNA was cleaned using RNAeasy clean-up columns (Qiagen). To improve the recovery from the columns, the elution water was spun into the matrix at 27 x g and then left for one minute prior to the standard 8000 g centrifugation recommended by Qiagen. This low speed wetting step gave
nearly double the yield of eluted RNA (E. O., unpublished observation). The cRNA was fragmented by heating in 1x fragmentation buffer [40 mM Tris-acetate (pH 8.1), 100 mM KOAc, 30 mM MgOAc] as recommended by Affymetrix. Ten μg of fragmented cRNA were hybridised to a YG_S98 GeneChip (Affymetrix) using standard procedures (45°C, 16 hours). Washing and staining was performed in a Fluidics Station 400 (Affymetrix) using the protocol EukGE-WS2v4 and scanned in an Affymetrix GeneChip scanner. The complete data sets for all array experiments performed are communicated upon request. Chip analysis was performed using the Affymetrix Microarray Suite v4 for determining average difference (AvgDiff) values and dCHIP v1.0 (www.biostat.harvard.edu/complab/dchip/) to calculate fold changes and to perform cluster analysis. The AvgDiff values for those genes whose AvgDiff values remained negative or very low have been set to the 50th percentile of the expression values called absent across all arrays. Fold change calculations were based on normalized signals to account for variations in overall fluorescence intensity between individual GeneChips and include only genes whose absolute fold changes were greater than 50, that passed the lower 90% confidence bound for fold change and had P values for fold change smaller or equal to 0.05. Yeast ORFs were assigned to functional groups using the tools provided at http://cgsigma.cshl.org/jian. GeneSpring 4.2 (Silicon Genetics) was used to analyze promoter regions for the presence of conserved sequence motifs shown in Fig. 4A. For global transcription analysis of human cells one million cells of the respective HeLa cell line were seeded onto a 10cm dish 24h prior treatment of the cells with 20 nM rapamycin. Total RNA was prepared from two 10cm dishes at the indicated time points using the TRIZOL reagent (Life Technologies). RNA samples were processed for the analysis on HG-U133 oligonucleotide microarray chip from Affymetrix essentially as described above. Analysis of the chips was performed using MicroArraySuite v5 and GeneSpring 5.0 (Silicon Genetics). Changes in gene
expression were determined by looking for concordant changes between replicates that pass a signed Wilcoxon rank test. The "change" p-values threshold was <0.003 for increase and <0.997 for decrease and was used to identify a list of 194 genes that change their expression during the two hour rapamycin treatment in non silenced control cells. After applying a parametric Welch t-Test (p<0.05) we found that 28 out of the 194 rapamycin sensitive genes revealed statistically significant changes in rapamycin sensitive gene expression in two independent HeLa cell clones (siURI-5 and siURI-11) with stably silenced URI when compared to non silenced control HeLa cells. This list of 28 genes has been used for further gene clustering by GeneSpring v5. Gene ontology analysis was performed with the GO-analysis tool provided at the Affymetrix server (www.affymetrix.com/analysis/query/go_analysis.affx).

**Cell culture.** HEK293, HeLa and U2OS cells were cultivated in DMEM supplemented with 10% FCS (Gibco) at 37°C in a 10% CO2-containing atmosphere. For growth of HeLa cells in suspension, cells were seeded in Spinnerflasks containing Joklik-modified medium including 10% FCS. Transfections by the calcium phosphate precipitation method have been described (Lisztwan et al. 1998). To establish cell lines with stably silenced URI expression HeLa cells were transfected with 9 μg of pSuper or pSuper-siURI together with 1 μg of pBabe-puro using Fugene (Roche). Puromycin (0.5 μg/ml) was added 24 hours after transfection and stable puromycin resistant clones were isolated after two weeks of selection. Out of twelve puromycin resistant clones we could isolate three independent clones with silenced URI expression. Once cell lines were established, they were grown in the absence of puromycin and silencing of URI was found to be stable under these conditions as monitored by Western blotting.
Fig. S1. STAP1 is a member of the α class PFD family and part of a multi-protein complex. (A) Amino acid sequence alignment of STAP1 with members of the prefoldin protein family. Secondary structure elements were deduced from the crystal structure of M. thermoautotrophicum PFDα subunit (Siegert et al. 2000) and are represented as blue cylinders (α helices) and grey arrows (β strands). (B) Characterization of anti-STAP1 antibodies. Whole cell extracts of U2-OS cells (lanes 1 and 3) or U2-OS cells expressing untagged STAP1 (lanes 2 and 4) were analyzed by Western blotting with anti-STAP1 mAb 105-72 or affinity-purified polyclonal anti-STAP1 antibody. (C) Nuclear extracts of 293 cells were subjected to immunoprecipitation with control antibody (mouse IgG, lane 1) or anti-STAP1 mAb 105-128 (lane 2) coupled to protein A-Sepharose and the immunoblot probed with anti-SKP2 or anti-STAP1. (D) Top: Extracts from untransfected U2-OS cells (lanes 1 and 2) or from U2-OS cells transfected with an HA-STAP1 expression plasmid (lanes 3 and 4) were immunoprecipitated with control antibody (preimmune serum, lanes 1 and 3) or polyclonal anti-SKP2 antibody (lanes 2 and 4) followed by Western blotting using anti-HA antibody. Bottom: Extracts were immunoblotted with anti-HA antibody before immunoprecipitation.
Fig. S2. STAP1 and URI are part of a ~1MDa complex in human cells. (A) A whole cell lysate of HeLa cells was fractionated on a Superose 6 gel-filtration column and individual fractions processed for Western blotting using antibodies to the indicated proteins. (B) Fractions shown in (A) were processed for immunoprecipitation with anti-STAP1 antibody followed by immunoblotting with antibodies directed against the proteins indicated on the right.
Fig. S3. Analysis of the URI complex by co-immunoprecipitation. (A) Aliquots of HEK293 cell extracts were immunoprecipitated using anti-URI polyclonal (lanes 2), anti-TIP48 polyclonal (lane 4) coupled to proteinA sepharose or control antibody (lanes 1 and 3) and analyzed by immunoblotting with antibodies directed against indicated proteins. (B) Prefoldin subunits STAP1 and PFD4r are stoichiometrically associated with URI. Whole cell extracts prepared from HEK293 cells were subjected to immunoprecipitation using monoclonal antibodies directed against human URI. Lysates before (lane 1) and after sequential immunoprecipitations (lanes 2 and 4) and immunoprecipitates (lanes 3 and 5) were analyzed by Western blotting using antibodies to the proteins indicated.
Fig. S4. URI is an evolutionary conserved protein. Alignment of the predicted amino acid sequences of human URI with A. thaliana, D. melanogaster, C. elegans and S. cerevisiae homologs. Secondary structure elements characteristic for α-class PFDs found at the N-terminus are represented as in Fig.S1A. The red rectangle delineates the portion of human URI involved in RPB5 binding whereas the open rectangle highlights a portion of URI proteins rich in acidic amino acids. The orange box denotes a short conserved stretch (URI box).
Fig. S5. Interaction of URI proteins with PFD and RPB5 subunits require conserved domains within human and budding yeast URI orthologs. (A) Left panel, various $^{35}$S-labeled, in vitro translated Flag-epitope tagged URI polypeptides were tested for binding to GST, GST-RPB5 or GST-STAT1 fusion proteins. Input lanes display 20% of the input of each in vitro translated URI protein. Right panel, schematic of URI mutants used in this analysis. The 'blue box-arrows-blue box' region denotes the prefoldin-homology domain; the red box indicates the RPB5 binding domain, the open box denotes the acidic region and the yellow area denotes the URI-box. (B) Left panel, various $^{35}$S-labeled, in vitro translated scUrip polypeptides were tested for binding to GST, GST-scRpb5p or GST-scPfd6p fusion proteins. Input lanes display 20% of the input of each in vitro translated HA-tagged scUrip protein. Right panel, schematic of scUrip mutants used in this analysis. Domains are denoted as in (A). (C) Schematic overview of the conserved PFD and RPB5 interaction domains found in human and budding yeast URI.
Fig. S6. Similar amounts of proteins have been analyzed in starvation experiments shown in Fig. 2D. Westernblot panels shown in Fig. 2D have been analyzed for equal loading of protein using either western blotting with anti actin antibodies (A, B) or have been stained with ponceau S (C).
Fig. S7. Changes in gene expression caused by loss of URI can be partially rescued by an additional deletion of the yeast transcription factor GCN4. Wild type yeast, or isogenic strains deleted for URI (uriΔ), GCN4 (gcn4Δ) or both URI and GCN4 (uriΔgcn4Δ) were grown to early log phase and their RNA expression profile from two independent experiments was analyzed with affymetrix gene chip arrays. Standardized expression values for 39 genes affected by URI loss (see Table S1) have been used for hierarchical cluster analysis using the analysis software dCHIP. Genes whose expression is dependent on URI but independent on the presence of GCN4 are indicated on the right side of the panel.
Fig. S8. Increased Gcn4p levels in uriΔ cells can also be observed in the prototrophic Δ strain deleted for URI and are not caused by increased protein stability. (A) Wild type Δ and uriΔ mutant Δ cells expressing a myc-tagged version of Gcn4p (Gcn4p-myc) were harvested at log phase and analyzed by Western blotting using anti-myc antibody 9E10. (B) Loss of URI causes upregulation of GCN4 translation. Wild type and uriΔ Δ yeast cells harboring a GCN4pORF-lacZ reporter plasmid have been grown to mid log phase and lacZ activity has been measured from three independent experiments. (C) Yeast cells were grown to early log phase at 25 °C and shifted to 37 °C for 3 hrs before addition cyclohexamide to a final concentration 50 μg/ml. GCN4p-myc levels were quantified densitometrically after Western blotting of extracts with anti-myc antibodies (9E10) prepared at the indicated time points from wild type, uriΔ and cdc34-2 yeast strains expressing GCN4p-myc. Mean protein abundance relative to time 0 (=100) and standard deviation was determined from three individual experiments.
Appendix I

Materials and Methods

This materials and methods section contains those methods used that were not covered to completeness in either chapters II or Appendix I.

Determination of DNA content by FACS analysis

0.5-1.0 OD_{600} units of cells were harvested and fixed by shaking in 70% ethanol at room temperature for 1 hour (alternatively, incubation was extended overnight). Cells were washed in 1 mL 50 mM Tris-HCl (pH = 7.9) once, resuspended in 0.5 mL 50 mM Tris-HCl (pH = 7.9) containing 20 µl of 10 µg/ul RNAse A and incubated with shaking over night at 37°C. After centrifugation the cell pellets were resuspended in 0.5 ml of 5 mg/ml Pepsin dissolved in 55 mM HCl, followed by a 30 min incubation at 37°C. Cells were washed in 1 ml FACS buffer (200 mM Tris-HCl (pH = 7.5), 211 mM NaCl, 78 mM MgCl₂) and resuspended in 0.5 ml FACS buffer containing 55 ul of 5 mg/ml propidium iodide. After a brief sonification 10⁴ cells were scored for their DNA content as judged by propidium iodide fluorescence using a FACScan device from Beckton and Dickinson. (10ul was taken in 500uL of 1x PBS for analysis).

DNA prep for 2D gel analysis

Haploid yeast grown to: 100ml to 1x10⁷ cells/ml or 50 ml to 2x10⁷ cells/ml

Wash once with TP1 and spin for 3’ at 4000 rpm and remove SN. The pellet was placed at -80°C. Cold NIB and H₂O mq were placed on ice and EDTA was added to 2 mM to the NIB. Cells washed once with cold NIB. SN removed and resus in 2 ml H₂O and transferred to polypropylene tube. Spun 4000 for 3’ and SN removed. Pellet then resus in 0.8 ml NIB (with EDTA) and an equal volume of the pellet (500ul) acid washed glass beads (Sigma, 425-600µM diameter) to the cell suspension. Shaken 1’ and then iced 30 sec.....lysis checked by microscopy. The SN was collected in a 2ml eppindorf. The glass beads were rinsed 2x with 0.8ml ice-cold NIB
and transferred to the same eppendorf. The broken cells were spun at 4°C 6500g for 10’ and SN removed.

The pellet was dissolved in 1.8ml Qiagen Buffer G2 containing 200ug RnaseA/ml. A homogeneous lysate was generated by gently inverting the tube 20 times (no vortex). The lysates were incubated at 37°C for 30 minutes and then 100ul of proteinase K (10 mg/ml) was added and incubated for an additional 60 minutes. During this hour, the eppen was gently inverted. The lysate was again spun at 6500g at 4°C for 10’. The SN was carefully removed to a white polyprop tube.

An equal volume of Qiagen Buffer (QBT) (1.8ml) was added, and a qiaugen column (20/G) was equilibriated with 1 ml of Buffer QBT. The sample was loaded on the column and allowed to pass through by gravity flow. The column was then washed 2 times with 1ml Qiagen Buffer QC. The DNA was eluted twice with 0.9ml Qiagen buffer QF pre-warmed to 50°C.

1.26 ml room temp isopropanol was added to the sample mixed well and the solution was divided into two 1.5ml eppis. Followed by 15 min spin at full speed to precipitate the DNA. The SN was removed and 500ul of 70% ethanol (RT) was added to each tube. The pellets were dis-lodged and pooled into one tube and the DNA was again pelleted. All 70% EtOH was removed.

The DNA was then re-suspended in 40ul of TE pH 8.0 at RT (O/N).

2D gel

For the first dimension a 0.4% gel in a medium size horizontal gel apparatus (300ml agarose, 2 liters 1x TBE) was used. The combs were 1.5 mm from the bottom support plate and teeth on the combs were 4mm wide and 1.2mm thick. Alternate lanes of the gel were loaded. The electrophoresis was carried out in 1x TBE at 0.7 to 1 V/cm for 15 to 30 hours at room temperature. The lanes of interest were excised with a clean razor blade. The lanes were placed on a clean new support and 90° to the direction of electrophoresis. 1.2% agarose was prepared and poured over the excised lanes containing 0.3ug/ml ethidium bromide. Electrophoresis was performed in the cold room at 5V/cm for 4-8 hours. DNA was transferred onto a nylon membranb and specific probes detected the origins as described in materials and methods, Chapter II.
**Preparation of BrdU-substituted DNA plugs**

Cells were washed twice in 10mM Tris-HCl pH 7.5, 50mM EDTA and cells were counted using the CASY. Pellet was resuspended in 2x spheroblasting Buffer (100mM phosphate buffer, 40mM DTT, 0.2M EDTA) at 1x10⁹ cells/ml (5.0x10⁷ cells/plug). 1% LMP agarose was prewarmed at 42°C and mixed with 90ul cells. agarose was allowed to polymerase at 4°C for 30 min and then incubated ON at 37°C in spheroblasting buffer. Plugs were incubated for 24h at 50°C in proteinase K Buffer washed and stored in 10mM Tris, 50mM EDTA, pH 7.5.

**PFGE**

A 1% agarose gel was poured on special PFGE plates and allowed to polymerize 20’ and then placed in cold room for an additional 10’. The gel holder was then removed and the agarose plugs were placed into the wells. The gel was placed in the tank and the cryostat set to 8°C. A program was used with 32 hours and 2 phases, first phase 90 sec pulses during 16 H and second phase was 60 second pulses 16 H (180 V) At the end of the electrophoresis the gel was stained either with ethidium bromide of Sybrgold. DNA was transferred onto nitrocellulose filters and BrdU was quantified and detected as described in chapter, II.
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2001-2002 PhD student, ISREC, Lab of Matthias Peter, Lausanne Switzerland, novel prefoldin-like protein involved in nutritional signaling in *S. cerevisiae*.
2002-Present PhD student ETH Zürich, Lab of Matthias Peter, Institute of biochemistry, the role of the *S. cerevisiae* cullin, Culc, in DNA synthesis and repair

Awards

2002 Rotary International Ambassadorial Scholarship for studies abroad
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2000 Ontario Graduate Scholarship, R. Samuel McGlaughlin Fellowship
1999 Placed on Dean's Honour list (top 1% of faculty)

Conferences Attended

2001 Cancer and the Cell Cycle, Lausanne
Swiss Cell Cycle workshop, Vue des Alpes
Swiss yeast meeting, Geneva

2002 Swiss Cell Cycle workshop, Vue des Alpes
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ETH PhD summer School
Cancer and the Cell Cycle, Lausanne
International yeast meeting, Göteborg
ETH PhD summer School

2004
ETH department of BIOL conference, Davos

**Publications**

Control of nutrient-sensitive transcription programs by the unconventional prefoldin URI.

Thimo Kurz, Nurhan Ozl̄üt, Sean O'Rourke, Brian Luke, Anthony A Hyman, Bruce Bowerman and Matthias Peter
Identification of a potential E3 for cullin neddylation in C. elegans and S. cerevisiae
Submitted

The yeast cullin, Rtt101p, is involved in genomic integrity and DNA repair.
Submitted

**Language**

English- Mother Language

French- good written, oral and understanding

German- good understanding and basic communication and writing