Anc1: A New Player in the Cellular Response to DNA Damage

by

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Table of Contents Page

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ABSTRACT

The continuity of living organisms depends on their ability to protect their genomes from a constant assault by internal and external sources of damage. To this end, cells have developed a variety of mechanisms to avoid and repair damage to their genetic material. In this thesis, we analyze a yeast gene that has a previously uncharacterized role in the cell's ability to survive after DNA damage. This gene, *ANC1*, is interesting for several reasons. First, Anc1 is the only common member of seven different multiprotein complexes that all function in general RNA polymerase II-mediated transcription. Second, Anc1 is evolutionarily well-conserved between yeast and humans, suggesting that its function is critically important for survival. And finally, three out of four human homologs of *ANC1* have a role in the *MLL* gene fusions associated with human acute leukemias.

Here, we show that *ANC1* falls into the same genetic pathway as several members of the postreplication repair (PRR) pathway, but has additive or synergistic relationships with other members of the pathway. Based on our epistasis data and our analysis of Anc1's role in mutagenesis, *ANC1* functions in the error-free branch of PRR. Genetically, however, *ANC1* is not in the same pathway as several canonical error-free branch members, and thus defines a new error-free branch of PRR. Similar to other genes involved in error-free PRR, *ANC1* was found to have a role in suppressing the expansion of the Huntington's Disease-associated CAG triplet repeat.

Additionally, we demonstrate a role for Anc1 in the global transcriptional response to MMS treatment: expression changes in transcripts regulated in response to environmental stress are significantly abrogated in *anc1* cells. The regulation of this transcriptional response to environmental stress has previously been attributed to the Mec1 signaling pathway. To determine if Anc1's effect on global transcription is linked to Mec1 signaling, we assayed the role of Anc1 in mediating the protein-level DNA damage response of Sml1, a downstream member of the Mec1 pathway. We observed that in the presence of MMS the Sml1 protein is abnormally degraded in *anc1* cells, indicating a possible role for Anc1 in this pathway.

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Abbreviations footnote: PRR = Postreplicative repair, TCR = Transcription Coupled Repair, RNA pol II = RNA polymerase II, TLS = Translesion Synthesis, H2A – Histone 2A, H2B = Histone IIB

INTRODUCTION

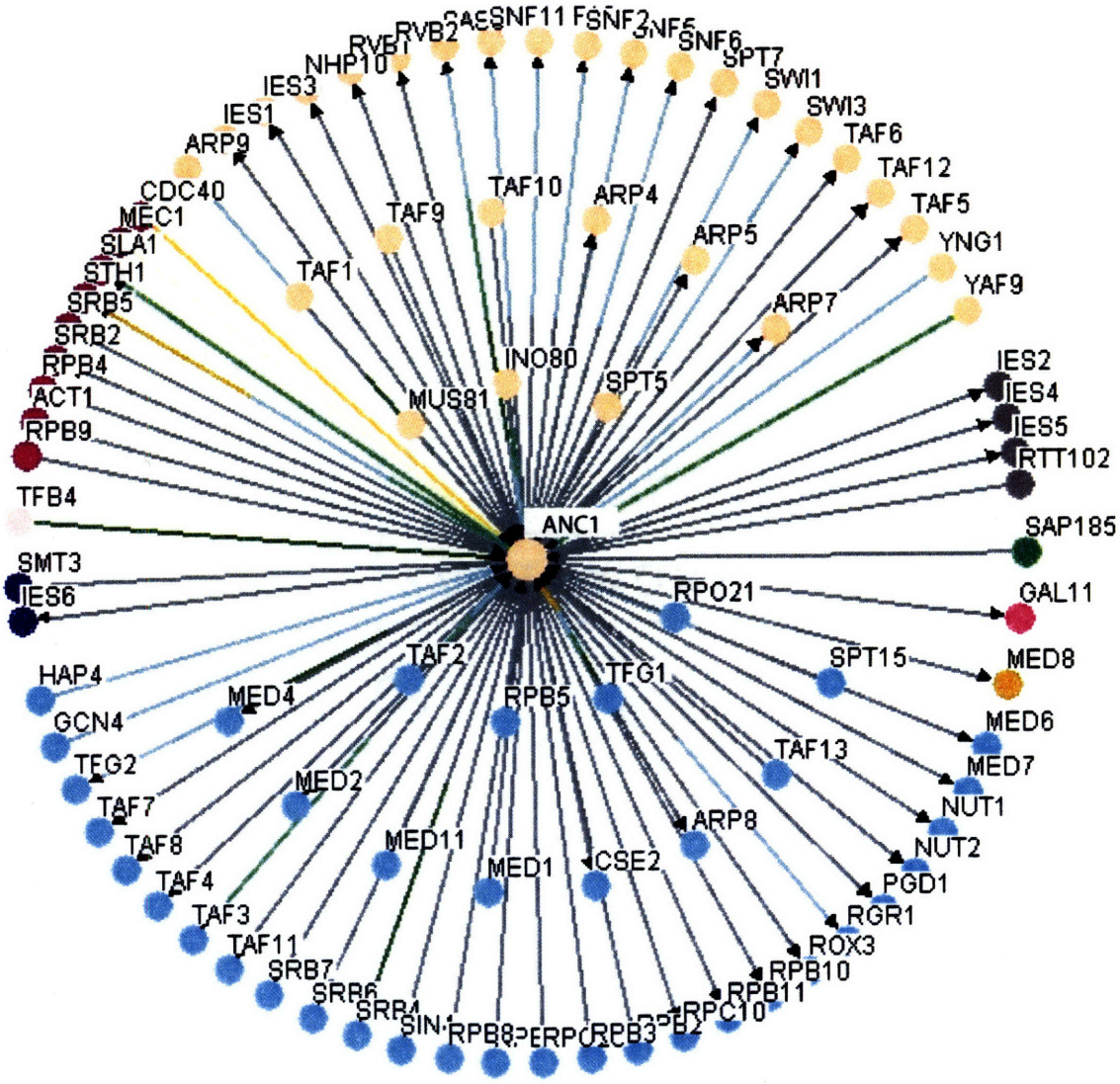
Recently, in a screen for yeast deletion strains that show sensitivity to DNA damaging agents, a target was uncovered that displayed particularly interesting characteristics. This strain, deleted for a gene called *ANC1*, showed sensitivity to three out of the four damaging agents it was screened against (UV, MMS and 4NQO, but not *t*-BuOOH), and had three human homologs associated with acute leukemias (Begley et al. 2002; Begley et al. 2004). Although several cellular roles for this protein were known or suspected (Anc1 is the only common member of seven complexes important to transcription, and is suspected to play a role in actin function), these roles did not illustrate a clear basis for the observed DNA damage sensitivity. In this thesis I will describe our efforts to characterize the underlying mechanism for *anc1*'s observed DNA damage sensitivity, and present Anc1 as an important new component of the cellular response to DNA damage.

Anc1 background

The *ANC1* gene (aka *SWP29, TAF30, TFG3, TAF14*) gets its many names from the assortment of genetic and biochemical experiments from which it has been identified. Biochemically, it encodes a member of the TFIID (*TAF30*), TFIIF (*TFG3*) and SWI/SNF (*SWP29*) complexes, and genetically, it was identified in a screen for genes that fail to complement the *act1-1* temperature sensitive allele of actin (thus *A*ctin *N*on-*C*omplementing) (Welch et al. 1993; Henry et al. 1994). At the time of this genetic screen there were several other genes whose failure to complement this actin allele had been characterized, all of which encode actin binding proteins (Welch et al. 1993). Indeed, *anc1* mutant strains do have defects in actin organization, similar to defects

observed in mutant alleles of actin itself (Welch et al. 1993). Furthermore, the *anc1* deletion fails to complement deletions in the *SAC6* and *TMP1* genes, both of which are actin-binding proteins (Vinh et al. 1993). There has been further evidence for a relationship between *ANC1* and actin since this initial discovery: INO80, a complex of which Anc1 is a member, contains Act1 along with several *ARP*s (*A*ctin *R*elated *P*roteins), and SWI/SNF, another Anc1-containing complex, also contains several *ARP*s (Cairns et al. 1996; Shen et al. 2000). Despite the compelling implication that Anc1 may have a role in actin function, this aspect of Anc1's cellular role is not well understood. In addition to its relationship to actin, several characteristics of *anc1* cells were noted in these early studies. The morphology of these slow-growing *anc1* cells was observed to be abnormally large and elongated, and the strain shows considerable resistance to formation of a mating projection in response to α-factor (and its associated arrest), possibly due to mislocalization of Spa2, a protein involved in polarity and cytokinesis (Welch and Drubin 1994). Anc1 localizes to the nucleus, a surprising quality for a protein involved in cell structure (Welch and Drubin 1994).

The Anc1 protein has been found to interact with 85 other proteins, the large majority of which are proteins involved in RNA polymerase II (RNA pol II) transcription (Figure 1-1). Fifty-nine of Anc1's interactors are members of known transcription-related complexes (TFIID, TFIIF, Mediator, SWI/SNF, RSC, INO80 and NuA3), and 17 of the remaining proteins have roles that involve direct interaction with these Anc1-containing complexes, (12 are RNA Pol II subunits) (STARK *et al.* 2006). Five of the remaining nine proteins are directly involved in transcription activation, including Spt7 (a member of the SAGA complex), Hap4 (a member of the CCAAT binding complex), and Gcn4, Tfb4 and Mot1



Modified from BioGRID, http://www.thebiogrid.org/
(Stark et al, 2006)

Figure 1-1: Diagram of Anc1's protein-protein interactions. Anc1 has been shown to interact with 85 different proteins, 84 of which are diagrammed here (Stark et al. 2006). The characterized interaction with Dun1 is absent (Ho et al. 2002).

(transcriptional regulators) (STARK *et al.* 2006). The final four proteins, Mus81, Sap185, Smt3 and Dun1 function outside of the transcriptional machinery, and may help to define new cellular roles for Anc1, as we will see below.

In addition to its physical protein-protein interactors, an *ANC1* knockout allele is synthetically lethal with three other knockout alleles: *YAF9*, *DST1* and *SLA1* (Welch and Drubin 1994; Zhang et al. 2004; Fish et al. 2006; Stark et al. 2006). Yaf9 (*Yeast AF9*) is a homolog of Anc1 that shares a YEATS domain with Anc1, a domain they both share with their leukemogenic human homologs. Yaf9 is a subunit of the NuA4 histone acetyltransferase complex (which acetylates histones H2A and H4), and is involved in counteracting silencing near telomeres (ZHANG *et al.* 2004). NuA4 has also been implicated in double strand break repair through its acetylation of histone tails near break sites (BIRD *et al.* 2002). *DST1* encodes transcriptional elongation factor TFIIS (Transcription Factor II S), that promotes cleavage of stalled transcripts, and allows Pol II-mediated elongation to reinitiate elongation after stalling (UBUKATA *et al.* 2003). It is notable, however, that *dst1* mutants are synthetically lethal with a total of 60 genes, many of which are involved in transcription, as well as several mutants in repair pathways (i.e. *rad6* and *rad52*) (STARK *et al.* 2006). Unlike Yaf9 and Dst1, Sla1 has a structural role in the cell; it is involved in building the cortical actin cytoskeleton, and is required for normal endocytosis in *S. cerevisiae* (PIAO *et al.* 2007).

As mentioned above, Anc1's sequence has similarity to three proteins implicated in human acute leukemias, ENL, AF9 and GAS41, a connection whose importance has been of increasing relevance in the study of acute leukemias (Welch and Drubin 1994). Many additional members of this highly conserved protein family (called the "YEATS"

family) have since been identified across eukaryotic proteomes, although the connection between this sequence and leukemogenesis is still being unraveled. Currently, there are three known *S. cerevisiae* members of the YEATS family (Anc1, Yaf9 and Sas5), and four known human members (ENL/MLLT1, AF9/MLLT3, GAS41 and YEATS2), and the conserved YEATS domain shows a high degree of similarity in an alignment (Figure 1-2). Anc1 is quite a small protein, only 29 kDa, and its only recognizable domain is the domain it shares with its YEATS family homologs (http://www.sanger.ac.uk/Software/Pfam/). The sole function of the YEATS domain that has been illuminated to date is its direct binding to histones H1 and H3, as demonstrated in the ENL human homolog (Zeisig et al. 2005).

Mixed-linkage leukemia (MLL) and the human YEATS-family members

The human Mixed Linkage Leukemia gene, *MLL* (*ALL*, *HRX*, *Hrtx*), can become fused with a wide variety of other genes, resulting in either acute myeloid or acute lymphoid leukemias (accounting for its "mixed" designation) (Popovic and Zeleznik-Le 2005). In humans, three of the four known YEATS family members are involved in Mixed Linkage Leukemia (MLL)-associated leukemogenesis, which occurs primarily through a reciprocal translocation between the *MLL* gene and a fusion partner. *ENL*, and *AF9* are direct fusion partners with the *MLL* gene, and the GAS41 protein interacts directly with AF10, another *MLL* gene fusion partner (Daser and Rabbitts 2004). Of the greater than 60 *MLL* fusion partners that have been identified clinically, the YEATS-

Figure 1-2

ANC1 YEATS domain homology to other YEATS proteins

```
Anc1        RQWSIEIVLLDDE GKEIPATIFD KVIYHLHPTF ANPNRTFTDP PFRIEEQGWGGFPLDISVFL LEKAGERKIP HDLN---Fl- ---------- ---QESYEVE
Yaf9        HLWTIFVRGPQNE DISYFI---K KVVFKLHDTY PNPVRSIEAP PFELTETGWGEFDINIKVYF VEEANEKVLN FYHRLRLHpy anpvpnsdng neQNTTDHNS
Sas5        RRWQMELLMLDAT GKEVEPTILS KCIYHLHSSF KQPKRRLNSL PFFIKETGWGEFNLKIECFF IGNAGKFSIE HDLT---F-- ---------- --EDDAYAVD
AF9         HDWMVFVRGPEHS NIQHFV---E KVVFHLHESF PRPKRVCKDP PYKVEESGYAGFILPIEVYF KNKEEPRKVR FDYDLFLHLE G--------- ----------
ENL         HDWMVFVRGPEQC DIQHFV---E KVVFWLHDSF PKPRRVCKEP PYKVEESGYAGFIMPIEVHF KNKEEPRKVC FTYDLFLNLE G--------- ----------
GAS41       HQWTVYVKPYRNE DMSAYV---K KIQFKLHESY GNPLRVVTKP PYEITETGWGEFEIIIKIFF IDPNERp-VT LYHLLKLF-- ---------- --QSDTNAML
YEATS2      ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

            4444444444433 3444440006 8889999999 8888866667 7777777777766666653 2211111111 1022111111 1000000000 0000000000
```

Figure 1-2: Multiple sequence alignment of yeast and human YEATS homologs over the Anc1 YEATS domain. Amino acid sequences were obtained from SGD (www.yeastgenome.org) and NCBI (http://www.ncbi.nlm.nih.gov), and used DIALIGN multiple sequence alignment software for comparison analysis (http://bioweb.pasteur.fr/seqanal/interfaces/dialign2-simple.html) (Morgenstern 1999). The number underneath each sequence position is a measure of the degree of similarity between the sequences at that site (Morgenstern 1999). DIALIGN was unable to find homology between the YEATS2 protein and the other homologs. A region containing Anc1's YEATS domain (amino acids 52-101) is 38% identical and 58% similar to ENL, and 44% identical and 62% similar to AF9 (Welch and Drubin 1994).

associated partners are among the most common, accounting for about 35% of spontaneous human acute leukemias with *MLL* gene fusions (Daser and Rabbitts 2005).

The *MLL* gene is involved in about 10% of all pediatric leukemias, and 5% of acute adult leukemias (Daser and Rabbitts 2004). Some MLL-associated leukemias arise spontaneously (90-95%), while others are related to treatment with chemical therapies (5-10%) (Daser and Rabbitts 2004). MLL-associated leukemias are clinically found to be associated with two patient populations in particular: infants exposed to chemical insult in utero (often presenting with leukemia before they reach one year of age), and patients who have had a primary cancer early in life, and have been treated with high-dose radiation therapy and certain chemotherapeutic agents (i.e. topoisomerase II inhibitors) (Tkachuk et al. 1992; Daser and Rabbitts 2005; Eguchi et al. 2006). Despite recent advances in the treatment of childhood leukemias, the survival rate of MLL-associated infant leukemias is about 17-18%, which drops to about 5% survival in patients under three months of age (Hess 2004). The cure rate of both spontaneous and secondary *MLL*-associated leukemias is approximately 35% (Hess 2004).

Reciprocal translocations occur in several possible locations within the *MLL* gene, which spans about 90 Kb, but are most common in the breakpoint cluster region (Popovic and Zeleznik-Le 2005). The MLL protein is unusually large (about 4,000 amino acids), and contains many recognizable functional domains (Daser and Rabbitts 2004), (Figure 1-3A). Starting at the N-terminal end of the protein, MLL contains three AT hooks, which function to bind the minor groove of AT-rich regions of DNA; AT hooks

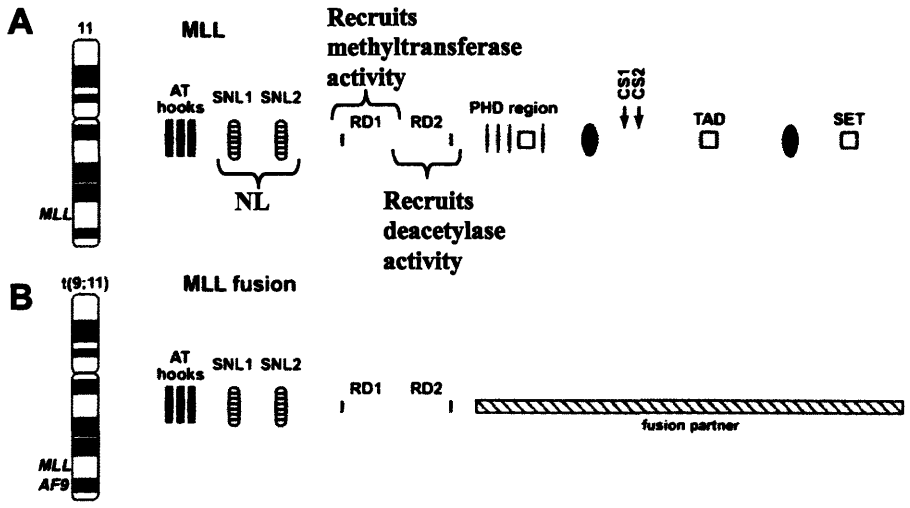


Figure 1-3: Schematic of MLL protein domains. A. Complete MLL protein. B. MLL protein fused to generic fusion partner (Daser and Rabbitts, 2004).

have been shown to mediate DNA-protein interactions or stabilize protein-protein interactions through DNA binding (Zeleznik-Le et al. 1994; Daser and Rabbitts 2004). Next there are two nuclear localization signals (SNL1 and SNL2) that direct the protein into the nucleus, and a region involved in transcriptional repression through both methyltransferase activity (RD1) and histone deacetylase recruitment (RD2) (Slany et al. 1998; Daser and Rabbitts 2004). In an *in vitro* study to determine which of these regions is required for transformation, it was determined that a deletion of MLL's AT hooks or the methyltransferase domain diminishes transformation considerably (Slany et al. 1998). The C-terminal domain of MLL is generally lost in reciprocal translocations (Figure 1-3B). In its wildtype state, however, the C-terminal domain contains three zinc-finger-PhD domains, two protease cleavage sites (CS1, 2), as well as a SET domain, involved in histone methylation (Daser and Rabbitts 2004) (Figure 1-3A). When the wildtype MLL protein is cleaved at these cleavage sites, the two resulting fragments are then reassociated with one another through the FYRN and FYRC domains (Hsieh et al. 2003). Finally, the transcriptional activation domain (TAD) interacts with the CPB acetyltransferase, resulting in transcriptional activation through binding to CREB (*C*yclic AMP *R*esponse element-*B*inding *P*rotein) (Ernst et al. 2001; Daser and Rabbitts 2004) (Figure 1-3A).

MLL is a member of the trithorax group, trx-G, (originally identified in *Drosophila*), a protein family involved in positive maintenance of gene expression during development (Tkachuk et al. 1992; Popovic and Zeleznik-Le 2005). The best understood target of trithorax group proteins is regulation of homeobox (*HOX*) gene expression. *HOX* genes are a highly conserved set of transcription factors, that are

involved in embryonic development and hematopeotic cell differentiation (Hess 2004; Daser and Rabbitts 2005). Surprisingly, the normal expression of *Hox* genes has been observed in cells containing *MLL* fusions, as well as other *MLL* defects (Daser and Rabbitts 2005). The wildtype MLL is a member of a large multi-protein complex (at least 29 proteins), containing members of several complexes, including SWI/SNF, hSNF2H, TFIID, NuRD, and Sin3A, complexes involved in RNA pol II transcription at a variety of levels (Daser and Rabbitts 2005); MLL is thus likely to be involved in chromatin remodeling, acetylation, deacetylation and histone methylation (Daser and Rabbitts 2004).

In mice, homozygous deletion of *Mll* is embryonic lethal, and, as expected from the involvement of *HOX* genes, there are numerous skeletal, segmental and hematopoetic errors in these embryos (Daser and Rabbitts 2005; Popovic and Zeleznik-Le 2005). In the absence of even a single copy of the *Mll* gene, mice have anemia and a slow growth phenotype, indicating that haploinsufficiency may play a part in human MLL-associated leukemogenesis (Popovic and Zeleznik-Le 2005). Interestingly, a null mutant in the mouse *AF9* YEATS homolog showed a misregulation of embryonic development as well, hinting that its wildtype function may also be related to *HOX* gene regulation (Collins et al. 2002). It has recently been noted that murine cells containing an inducible MLL-ENL fusion have an increased incidence of chromosomal abnormalities, although the reason for this increase has not yet been characterized (Eguchi et al. 2006).

In global analyses of MLL association, it was observed that MLL colocalizes with RNA pol II at actively transcribed genes, as well as to microRNAs that are involved in

leukemia and hematopoesis, and that a loss of function in *Mll* results in defects in RNA pol II distribution (Guenther et al. 2005; Milne et al. 2005). Several MLL fusion products, including all of the YEATS-associated fusions, were found to downregulate p21, MDM2 and Bax, all downstream targets of p53's damage response, in the presence of DNA damage (Wiederschain et al. 2005). In human nuclei, both the wildtype MLL protein and its fusion product show punctate distribution, probably corresponding to their localization to nuclear structures, and are expressed in a wide variety of tissues, including hematopoetic cells, cerebral cortex, kidney, thyroid and lymphoid tissues (Butler et al. 1997), suggesting that the fusion protein is localized normally, but misregulated.

Anc1 containing complexes

As mentioned above, Anc1 is a member of seven protein complexes: TFIID, TFIIF, Mediator, SWI/SNF, RSC, INO80 and NuA3. All of these complexes have a role in general RNA pol II transcription, although several have specialized roles outside of this function as well.

TFIID, TFIIF and Mediator complexes

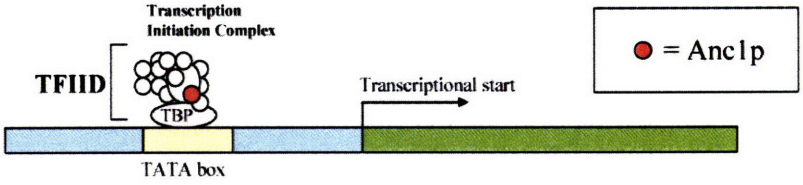
RNA pol II is the major transcriptional polymerase in all eukaryotes. Unlike the prokaryotic transcriptional polymerase, the RNA pol II enzyme is unable to initiate transcription *in vitro* unless several cofactors, called general transcription factors are present (Alberts et al. 2002). These general transcription factors include the Anc1-

containing complexes TFIID and TFIIF, among others. The TFIID complex contains a subunit called *T*ata *B*inding *P*rotein (TBP), which binds to the thymine- and adenine-rich TATA box upstream of the transcriptional start site, initiating transcription, and seeding the formation of the transcription initiation complex (Figure 1-4A). The TFIID complex also contains a histone acetyltransferase that allows it to aceylate histones H3 and H4 (Mizzen et al. 1996). It has a demonstrated function in regulating the state of cellular growth through the G1/S cyclin genes (Walker et al. 1997). In human cells it has been shown that a partial loss of function in TAF1 can lead to activation of the ATR damage signaling pathway (Buchmann et al. 2004).

While the TFIID complex is involved primarily in transcriptional initiation, the TFIIF complex functions in both transcription initiation and elongation. TFIIF joins the transcription initiation complex after it has already bound to RNA pol II, and is positioned, with RNA pol II, directly over the transcriptional start site (Lodish et al. 2000) (Figure 1-4B). TFIIF is necessary for RNA pol II's binding to the TFIIB-promoter complex (Lodish et al. 2000). The TFIIF complex remains in contact with RNA pol II through at least the beginning of elongation (and possibly longer), and suppresses RNA pol II pausing during nucleotide addition through an unknown mechanism, though it is possible that it's role in elongation is limited to the first few bases after initiation (called "promoter escape") (Shilatifard et al. 2003) (Figure 1-4B). Recently, it was shown in human cells that TFIIF is involved in the bypass of oxidative lesions during transcriptional elongation (Charlet-Berguerand et al. 2006), although this finding has been challenged (Kuraoka et al. 2007). Although there is currently no direct role known for TFIIF in DNA repair, TFIIF recruits TFIIE, which, in turn, recruits TFIIH to the RNA

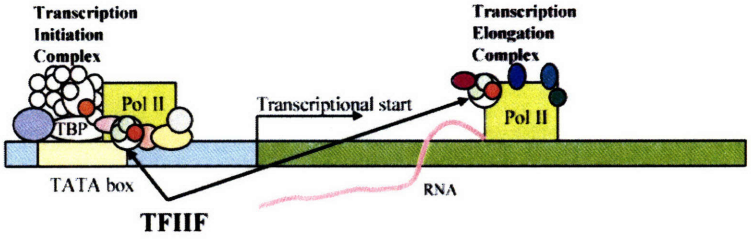
A.

TFIID:



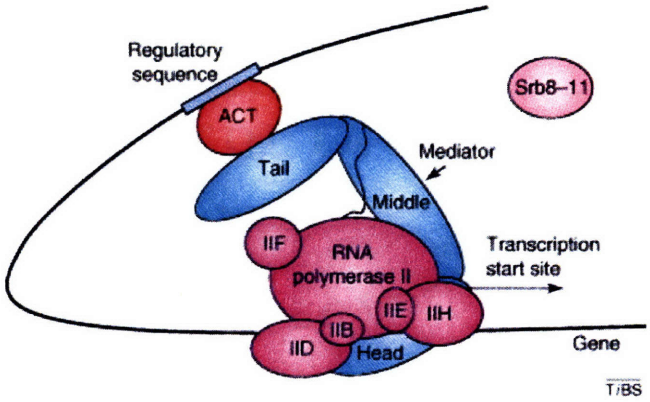
B.

TFIIF:



C.

Mediator:



Bjorklund and Gustafsson, 2005

Figure 1-4: Anc1-containing complexes involved in general transcription. A. Schematic of Anc1 in TFIID complex, showing its place in transcription initiation Anc1 is marked in red. B. Schematic of Anc1 in TFIIF complex, shoing its place in transcription initiation and elongation. Anc1 is marked in red. C. Model of Mediator function bridging activators (in red) with general RNA pol II transcription complex (purple). Activators interact with the tail region of Mediator, and the RNA pol II complex interact with the head and middle (Bjorklund and Gustafsson 2005).

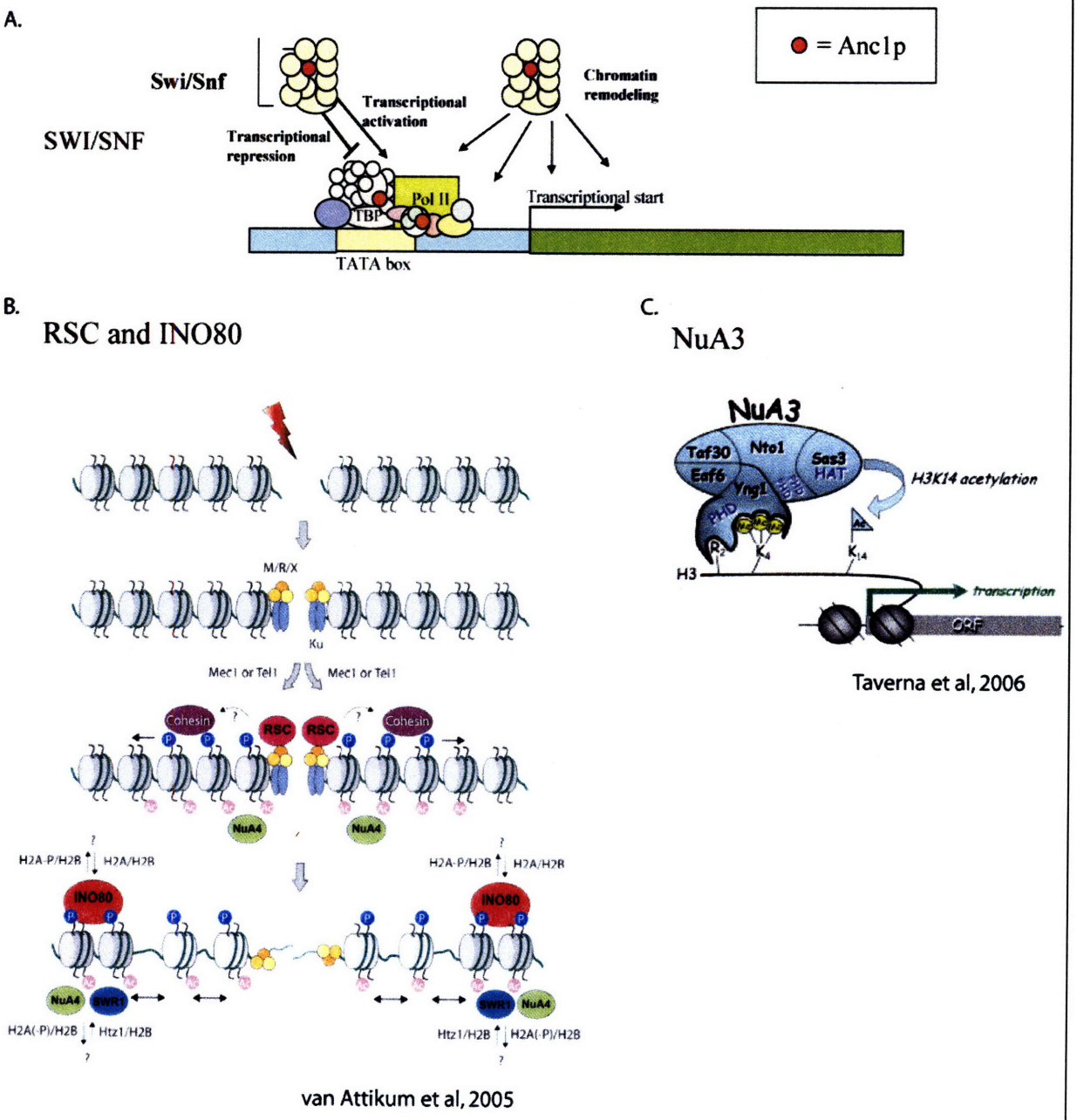
pol II holoenzyme, and TFIIH is known to function in Transcription Coupled Repair (TCR), a DNA repair that targets DNA damage specifically on the transcription template strand.

In addition to the general transcription factors required for RNA pol II to transcribe RNA *in vitro*, there are additional factors that are required for RNA pol II transcription within the context of the cell. The Anc1-containing Mediator complex is one such factor. Mediator interacts with the unphosphorylated C-terminal domain (CTD) of RNA pol II, and dissociates as the tail becomes phosphorylated during elongation (Bjorklund and Gustafsson 2005). Mediator has been shown to bridge the general RNA pol II holoenzyme with transcriptional activators that are sequence specific (Bjorklund and Gustafsson 2005) (Figure 1-4C). It has also been shown to acetylate histones, through its Nut1 subunit, indicating that Mediator may help to keep chromatin acetylated and open for transcription factors (Lorch et al. 2000; Wang et al. 2005).

SWI/SNF, RSC and INO80 complexes

The Swi2/Snf2 family of DNA-dependent ATPases is a family of chromatin remodelers with an important role in gene transcription, and is conserved through all eukaryotes. This family includes several Anc1-containing complexes, including SWI/SNF, RSC, and INO80. The Rad5 protein, a member of the postreplicative repair pathway, is also a member of the SWI/SNF family. The SWI/SNF complex is recruited to promoter regions by DNA-bound activators and repressors in a sequence dependent manner for chromatin remodeling (Martens and Winston 2003) (Figure 1-5A). The

Figure 1-5



van Attikum et al, 2005

Taverna et al, 2006

Figure 1-5: Anc1-containing complexes involved in chromatin remodeling and histone acetylation. A. Schematic of Anc1 in the SWI/SNF complex, showing its place in transcription activation, repression and chromatin remodeling. Anc1 is shown in red. B. Model of NHEJ repair, showing the putative binding of the RSC complex to the Ku70/80 complex at the DNA ends, and the later binding of the INO80 complex to the phosphorylated histones a short distance away from the break (van Attikum and Gasser 2005). C. Model of NuA3 binding to H3K4me3 through its PhD domain, and H3K14ac (Taverna et al. 2006).

chromosome is remodeled by a repositioning of the nucleosome that allows for better access to the DNA (Sudarsanam and Winston 2000). The SWI/SNF ATPase subunit, like others in the family, contains a bromodomain motif that allows for binding to acetylated lysines in histone tails, which is required for its stable association with chromatin, and for localization to some of its promoter targets (Martens and Winston 2003). In mice, it has recently been shown that the components of SWI/SNF are downregulated in response to the activation of TCR, resulting in a change in gene expression patterns, which may hint at a regulator role for SWI/SNF in TCR (Lee et al. 2007).

Like the SWI/SNF complex, the RSC complex is involved in both activation and repression of transcription, but unlike SWI/SNF, the RSC complex is essential for mitotic growth (Martens and Winston 2003). In addition to activation and repression, RSC may also have function in transcription elongation (Govind et al. 2005). It was recently shown that RSC and, to a lesser extent, SWI/SNF, decrease the accumulation of arrested RNA products and increase the yield of full-length transcript; it is thought that this may relate to their ability to recognize acetylated histones and regulated accessibility of the regions to be transcribed (Carey et al. 2006). Finally, both RSC and SWI/SNF have been implicated in the homologous recombination repair of double-strand breaks (DSBs), with SWI/SNF being recruited to the break site before strand-invasion, and helping to extend the invading strand, and RSC recruited after synapsis has already occurred (Chai et al. 2005). RSC has also been implicated in the non-homologous end-joining of DSBs; NHEJ is impaired in the absence of certain RSC

subunits, and RSC is recruited to the site of the break for chromatin remodeling activity (Shim et al. 2005; Shim et al. 2007) (Figure 1-5B).

The INO80 complex, along with SWR1 complex, belongs to a subclass of the SWI/SNF family characterized by a split ATPase domain in the core ATPase subunit (Bao and Shen 2007). In addition to the DNA-dependent ATPase activity that defines this family, the INO80 complex also has a 3'-5' helicase activity (Bao and Shen 2007). Like SWI/SNF and RSC, the INO80 complex has roles in both transcriptional regulation and DNA repair. When *INO80*, the ATPase subunit of the INO80 complex, is deleted, the expression of a subset of genes is significantly reduced, particularly genes related to cell structure (Ebbert et al. 1999). It was recently discovered that the INO80 complex localizes to the phosphorylated histone γ-H2AX that forms near the site of DSBs (Morrison et al. 2004; van Attikum et al. 2004). To assay INO80's role, H2 was modified such that γ-H2AX could not form at DSBs: it was found that single-strand DNA production at the break site was diminished, hinting that INO80 may function in this crucial stage of homologous recombination (van Attikum et al. 2004; Bao and Shen 2007). A potential role for INO80 in non-homologous end-joining (NHEJ), a separate pathway that also repairs DSBs, has been indicated through both gene expression analysis, and survival after an induced double strand break (van Attikum et al. 2004) (Figure 1-5B).

NuA3 complex

The NuA3 complex is the least well understood of the Anc1-containing complexes. It has been shown to acetylate histone H3 through its catalytic subunit Sas3; acetylated chromatin is associated with areas of active transcription (John et al. 2000). Recently, it was shown that NuA3 is recruited to the areas it acetylates by the *SET1*- and *SET2*-dependent methylation of H3 (Martin et al. 2006b) (Figure 1-5C). This recruitment takes place through recognition of H3 methylation by a PhD domain in one of its subunits (Martin et al. 2006a; Taverna et al. 2006) (Figure 1-5C).

The Postreplicative repair pathway

Upon the occurrence of DNA damage, there are a panoply of DNA repair mechanisms that the cell may employ in order to survive the insult. The pathway the cell chooses depends on the type of damage, its extent, the position of the cell within the cell cycle and the state of the DNA that has become damaged. To this end, eukaryotic pathways have evolved that promote direct reversal of damage, mismatch repair, nucleotide excision repair, base excision repair, homologous recombination, non-homologous end-joining, transcription-coupled repair and postreplication repair. In the absence of successful DNA repair, a cell may not be able to replicate its DNA for cell division, or it may make errors in its DNA replication or transcription that lead to death for the cell, or even the entire organism. Although each pathway has its preferred substrates, in many cases a substrate may be acted upon by several different repair pathways; this redundancy leads to competition for substrates between pathways and further protects the cell from the consequences of DNA damage.

The postreplicative repair pathway (PRR) is highly conserved from yeast to humans, and functions at sites where unrepaired lesions in the DNA block the replication machinery, creating stalled replication forks. The PRR pathway, sometimes called the "damage avoidance" pathway, resolves the replication blockage without the removal of the lesion. If the stalled replication fork were allowed to persist, it could lead to cell cycle arrest, and, ultimately, cell death (Ulrich 2006). This pathway consists of at least one error-prone branch, characterized by specialized polymerases that are capable of replicating past DNA lesions without fixing them, and at least one error-free branch, in which lesions are avoided for the purposes of replication through a poorly understood recombination mechanism (Zhang and Lawrence 2005; Friedberg et al. 2006). The error-prone branch is so named because in the absence of its members, the mutation rate decreases; thus, the members of the error-prone pathway, when intact, promote mutagenesis. Conversely, when members of the error-free branch are absent, the mutation rate of the cell increases, indicating that these genes have a role in suppressing mutagenesis. The classification of branches and sub-branches in this pathway has proven difficult, as its outcome differs depending on the type of DNA damage employed (i.e. UV-treatment vs. MMS-treatment vs. spontaneous damage), the assay utilized (i.e. survival vs. mutagenesis) and other technical details (i.e. chronic vs. short-term exposure to damage, liquid treatment vs. treatment on agar, etc.) (Cejka et al. 2001; Ulrich 2001; Broomfield and Xiao 2002; Barbour and Xiao 2003; Minesinger and Jinks-Robertson 2005).

Both the error-prone and error-free pathways are encoded by the RAD6 epistasis group, whose members are: *RAD6, RAD18, REV1, REV3 REV7 RAD30, RAD5, MMS2*

and *UBC13* (Friedberg et al. 2006). *SRS2*, an additional gene involved in the PRR pathway, suppresses the extensive sensitivity of *rad6* cells to UV light (Lawrence and Christensen 1979). Several members of the PRR pathway are involved in ubiquitin-mediated signaling, although a relationship between their roles in ubiquitination and DNA repair has been somewhat elusive (Friedberg et al. 2006).

The Rad6 protein has been implicated in a variety of cellular processes, ranging from sporulation to α-factor sensitivity to DNA repair (Welch and Drubin 1994; Alberts et al. 2002). Its deletion results in massive DNA damage sensitivity to a variety of agents (UV, MMS, γ-rays, cross-linking agents), cell cycle defects and decrease in chemically-induced mutagenesis (although an increase in spontaneous mutagenesis) (Friedberg et al. 2006). The damage sensitivity phenotype of *rad6* cells is suppressed in *srs2* cells, as is the cell cycle defect, in part, but the defects in mutagenesis and sporulation are not suppressed, demonstrating that these aspects of Rad6 function can operate independently from one another (Schiestl et al. 1990). Rad6 was identified as a functional member of the ubiquitin conjugating enzyme (E2) family, which, in combination with an E3 ubiquitin ligase, transfers activated ubiquitin to a target protein (i.e. H2A, H2B and PCNA, in the case of Rad6) (Jentsch et al. 1987; Hoege et al. 2002). The role of Rad6's ubiquitin function in DNA repair is a matter that is still being explored. When the active site cysteine of Rad6, conserved in all E2s, is mutated, all Rad6 function appears to be disrupted (i.e. the phenotype is like that of *rad6* mutants) (Sung et al. 1990). Surprisingly, however, if just the acidic tail is deleted, a region necessary for the ubiquitination of histones, Rad6's DNA repair function is unchanged (Morrison et al. 1988; Sung et al. 1990).

Rad6's other known ubiquitination target, PCNA, plays an important role in determining whether the error-prone or error-free branches of PRR will be utilized at a stalled replication fork (Hoege et al. 2002). PCNA acts as a homotrimer ring that encircles DNA and tethers polymerases to it; PCNA is necessary for DNA synthesis (Moldovan et al. 2007). PCNA can be mono- or poly- ubiquitinated in a Rad6/Rad18-dependent fashion at lysine 164. Monoubiquitination at this site is associated with initiation of the error-prone branch, and polyubiquitination, dependent on Rad5's recruitment of the Mms2/Ubc13 heterodimer for polyubiquitination at lysine 63, is associated with the error-free branch (Ulrich and Jentsch 2000; Hoege et al. 2002). Monoubiquitinated PCNA has been specifically associated with several TLS polymerases (Bienko et al. 2005; Wood et al. 2007), though the link between the error-free pathway and polyubiquitination is still poorly understood (Moldovan et al. 2007).

The Rad18 protein forms a stable heterodimer with Rad6 (Bailly et al. 1994), and *rad18* cells display a similar range, but not extent, of DNA damage sensitivity as *rad6* cells; it is sensitive to MMS, UV, γ-rays, etc, and its spontaneous mutation rate in increased over that of wildtype (Friedberg et al. 2006; Ulrich 2006). The Rad18 protein is an E3 ubiquitin ligase that binds directly to single-stranded DNA, which may be involved in the targeting of PRR to sites of locally single-stranded DNA at stalled replication forks (Bailly et al. 1997). Like Rad6, Rad18 is necessary for both the error-free and the error-prone branches of PRR.

Rev1, Rev3 and Rev7 and Rad30 are all involved in translesion polymerase activity in the error-prone branch of PRR. Rev3 and Rev7 function as a heterodimer, polymerase ζ, while Rad30 (polymerase η) acts alone, and Rev1, whose role is still

largely uncharacterized, may gain some of its activity through interactions with translesion synthesis (TLS) polymerase ζ (Nelson et al. 1996; Ulrich 2006). When translesion polymerases are deleted, a marked decrease in mutagenesis is observed, although some polymerases show this phenotype in a broader spectrum of mutagenesis assays (i.e. polymerase ζ), than others (i.e. polymerase η) (Friedberg et al. 2006). These polymerases are characterized by low processivity (i.e. they often dissociate from the DNA after only a few bases), presumably to allow a more accurate and processive polymerase a chance to resume normal activity (Friedberg et al. 2005).

Although Rad5 is generally characterized as belonging to the error-free branch of postreplicative repair, where its role is better understood, it has several characteristics that hint it may play a role in the error-prone branch as well (and possibly even non-homologous end-joining) (Ahne et al. 1997). In epistasis assays, *rad5* mutants shows a synergistic relationship with mutant TLS polymerases, indicating that they may function in partially overlapping or parallel pathways (Ulrich 2006). Although *rad5* cells show an increase in mutagenesis in most assays, certain markers have shown a decrease in mutagenesis, as would be expected from involvement in the error-prone pathway (Schurer et al. 2004). It should be noted that a Mms2/Ubc13-independent role for Rad5 has been characterized, and may involve histone H2B, a Rad6/Rad18 substrate (Martini et al. 2002; Gangavarapu et al. 2006). Rad5 is a member of the SWI/SNF DNA-dependent ATPase family; it has both chromatin remodeling and helicase domains, though helicase activity has not yet been demonstrated (Ulrich and Jentsch 2000). Rad5 binds to DNA, has single-strand ATPase activity, and appears to mediate contact between the Rad6/Rad18 and the Mms2/Ubc13 heterodimers (Johnson et al. 1992;

Johnson et al. 1994; Ulrich and Jentsch 2000) (Figure 1-6). Based on its placement in the pathway, and its interaction with Mms2/Ubc13, it is possible that Rad5 functions as an E3 ubiquitin ligase (Ulrich 2006).

Like Rad6 and Rad18, Mms2 and Ubc13 form a stable heterodimer that consists of an E2 ubiquitin-conjugating enzyme (Ubc13), and its E3 partner (Mms2) (Ulrich and Jentsch 2000). Interestingly, the unique polyubiquitination created by this heterodimer (K63 instead of K48) seems to be specifically indicative of DNA damage, although its target is as yet unknown (Spence et al. 1995; Friedberg et al. 2006). The main physical contact of this heterodimer with the other members of the PRR pathway is through Rad5, and only occurs in the presence DNA damage, when Mms2 and Ubc13 localize to the nucleus from the cytosol (Ulrich and Jentsch 2000; Friedberg et al. 2006) (Figure 1-6).

Srs2, although not technically a member of the Rad6 epistasis group, also functions in the PRR pathway. Mutations in the *SRS2* gene suppress the DNA damage sensitivity of *rad6* cells, and of mutants in the error-free branch of PRR, but not the error prone branch (Ulrich 2001; Broomfield and Xiao 2002). Srs2 is a 3'-5' helicase, and it has been shown to direct stalled replication forks to the PRR pathway by stripping the Rad52-pathway promoting Rad51 filament from single-stranded DNA (Krejci et al. 2003; Veaute et al. 2003). As mentioned earlier, the competition between multiple repair pathways, in this case, homologous recombination and PRR, helps to ensure that even under bad genetic or environmental conditions, damage can be handled by the cell, promoting cell survival.

Figure 1-6

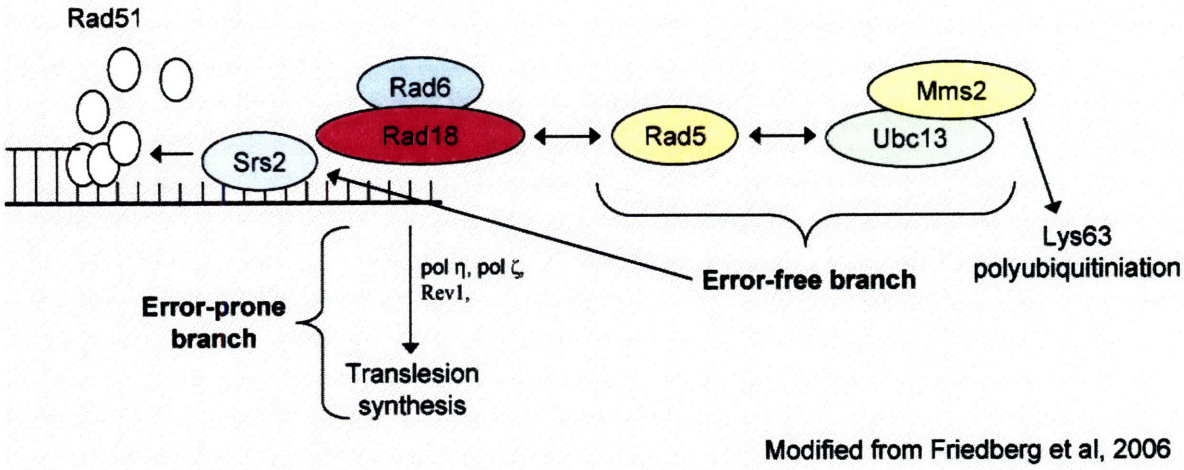


Figure 1-6: Model of the Postreplication Repair Pathway. The proteins involved in the error-prone and error-free branches are shown, with error-free pathway members shown in color. Srs2 strips the Rad51 filament from single-stranded DNA, where it would sequester the DNA for homologous recombination, allowing the Rad6/Rad18 heterodimer to bind. Rad5 mediates the interaction between the Rad6/Rad18 heterodimer and the Mms2/Ubc13 heterodimer. Rad6/Rad18 functions in both the error-prone and error-free branches.

The aforementioned qualities of Anc1 – its roles in transcription and its homology to human genes associated with leukemia – make its mechanism of promoting survival after DNA damage particularly fascinating. In this thesis, we will explore the connection between what is known of Anc1's function in transcription and its characterized sensitivity to DNA damage. We will also explore Anc1's involvement in the DNA damage cell cycle checkpoint, DNA repair pathways, the global transcriptional response to DNA damage and the damage signaling pathway. Our results will provide a new understanding of Anc1's role in cell survival.

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CHAPTER 2: *ANC1*, A MEMBER OF SEVEN RNA POLYMERASE II-ASSOCIATED COMPLEXES, DEFINES A NOVEL BRANCH OF THE POSTREPLICATIVE DNA REPAIR PATHWAY

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ABSTRACT

S. cerevisiae strains lacking Anc1, a member of the YEATS protein family, are sensitive to several DNA damaging agents. The YEATS family includes two human genes, *ENL* and *AF9*, that are common fusion partners with *MLL* in human acute leukemias. Anc1 is a member of seven multi-protein complexes involved in RNA polymerase II-mediated transcription, and the damage sensitivity observed in *anc1* cells is mirrored in strains deleted for some other non-essential members of several of these complexes, including SWI/SNF, INO80, Mediator, and RSC. Here we show that *ANC1* is in the same epistasis group as *SRS2* and *RAD5*, members of the postreplicative repair (PRR) pathway, but has additive or synergistic interactions with several other members of this pathway. Although PRR is traditionally divided into an "error-prone" and an "error-free" branch, *ANC1* is not epistatic with all members of either established branch, and instead defines a new error-free branch of the PRR pathway. Like several genes involved in PRR, an intact *ANC1* gene significantly suppresses spontaneous mutation rates, including the expansion of $(CAG)_{25}$ repeats.

INTRODUCTION

Understanding the role of all genes that function to provide resistance upon chemical exposure will provide a systems level view of how cells respond to changing environments, and an understanding of what happens to the cell and the organism when this system is impaired. Recently, we screened all of the non-essential yeast genes to identify those that provide resistance to DNA damaging agents (BEGLEY *et al.* 2002; BEGLEY *et al.* 2004). Based on the genes of known function whose deletion resulted in sensitivity, we identified several unexpected cellular processes that were overrepresented among damage sensitive mutants (BEGLEY *et al.* 2002; BEGLEY *et al.* 2004). RNA polymerase II (Pol II)-mediated transcription was among the many pathways that were significantly overrepresented (BEGLEY *et al.* 2002; BEGLEY *et al.* 2004).

The product of the *ANC1* gene (also known as *TAF14* and *TFG3*) is a member of at least seven multi-protein complexes that have distinct but related cellular roles, the common theme being their involvement in RNA Polymerase II-mediated transcription. Anc1-containing complexes include two members of the RNA Pol II holoenzyme, TFIID and TFIIF, the chromatin remodeling complexes RSC, SWI/SNF, and INO80, the histone acetyltransferase complex, NuA3, and the transcriptional activation adapter complex, Mediator. (Henry et al. 1994; Poon et al. 1995; Cairns et al. 1996; John et al. 2000; Le Masson et al. 2003; Zhang et al. 2004; Kabani et al. 2005). The sensitivity of *anc1 S. cerevisiae* strains to UV light, γ-irradiation the DNA alkylating agents methane methylsulfonate (MMS) and 4-nitroquinoline 1-oxide (4NQO), and to hydroxyurea (HU)

was recently reported (Bennett et al. 2001; Begley et al. 2002; Begley et al. 2004; Zhang et al. 2004). How Anc1 promotes survival after exposure to DNA damage and replicative stress has not, until now, been explored.

The Anc1 protein contains a highly conserved YEATS domain that is present in three yeast (Yaf9, Anc1 and Sas5) and four human (ENL/MLLT1, AF9/MLLT3, GAS41 and YEATS2) proteins. Three of the four YEATS family human proteins are associated with the human mixed linkage leukemia) gene: *MLL* gene fusions occur in ~3% of AML (acute myeloid leukemia) and 8–10% of ALL (acute lymphoid leukemia) (DASER and RABBITTS 2005). Both *ENL* and *AF9* are common translocation partners with *MLL* in these cancers, and GAS41 has been shown to interact directly with the product of the *AF10* gene, another *MLL* fusion partner (DEBERNARDI *et al.* 2002). Together, *ENL*, *AF9* and *AF10* fusions with *MLL* account for about 35% of spontaneous human acute leukemias with *MLL* gene fusions (DASER and RABBITTS 2005). The function of the YEATS domain is still largely unknown, although it was recently reported that tagged ENL binds specifically to histones H1 and H3 via its YEATS domain (ZEISIG *et al.* 2005). Moreover, the wildtype MLL protein is a member of a large multiprotein complex that contains many members of the human TFIID and SWI/SNF transcription complexes. Similar to MLL, Anc1 is a member of yeast TFIID and SWI/SNF complexes, and is thus intriguingly similar to MLL itself (NAKAMURA *et al.* 2002). This, along with the fact that Anc1 and several of *MLL*'s leukemogenic fusion partners share the YEATS domain makes Anc1 a particularly interesting candidate for mechanistic analysis.

During DNA replication, template nucleotides that have been chemically modified or lack a base altogether, frequently block advancement of the replication fork, and can

even cause fork collapse. Unless a stalled replication fork is enabled to restart, the cell cannot properly complete DNA replication, resulting in cell cycle arrest and cell death. The post-replication repair (PRR) pathway, exemplified by the *RAD6* epistasis group in *S. cerevisiae*, employs a variety of mechanisms for restarting stalled replication forks. It is the least well characterized of the DNA repair pathways, and is generally divided into error-prone and error-free branches, although there is some disagreement as to the number and sub-branches therein (Xiao et al. 2000; Cejka et al. 2001; Gangavarapu et al. 2006; Ulrich 2006). The error prone branch employs specialized translesion DNA polymerases (i.e. Rev1, Pol ζ, Pol η) that individually, or in collaboration, allow replication past and beyond replication-blocking DNA lesions, usually in an error-prone manner. Such DNA lesion bypass enables continued replication, albeit at the cost of increased mutation, and renders the lesion available for subsequent DNA repair (FRIEDBERG *et al.* 2006). The error-free branch of PRR, still largely uncharacterized, competes with Rad52-mediated homologous recombination for substrates, and likely repairs these substrates by recombination between sister-strands, through either template strand switching or copy choice mechanisms (ZHANG and LAWRENCE 2005). The error-free branch of PRR is associated with a subset of the Rad6 epistasis group, including Rad6, Rad18, Srs2, Rad5, Ubc13 and Mms2 (ULRICH 2006).

Rad6, an E2 ubiquitin conjugating enzyme, forms a heterodimer with Rad18, a ubiquitin ligase and single-strand DNA-dependent ATPase. Under appropriate conditions the Rad6/Rad18 heterodimer monoubiquitinates PCNA at lysine 164. PCNA thus modified activates the error-prone PRR pathway by recruiting translesion polymerases to the replication fork (HOEGE *et al.* 2002; STELTER and ULRICH 2003).

Alternately, monoubiquitinated PCNA can serve as a substrate for polyubiquitination by the Rad5/Mms2/Ubc13 complex, leading to activation of the error-free pathway instead (HOEGE *et al.* 2002; STELTER and ULRICH 2003). Like Rad18, Rad5 is a single-strand DNA-dependent ATPase. Rad5 appears to play a complex role in these pathways, with evidence for its participation in error-prone translesion synthesis, and at least one putative branch of the error-free pathway, although its primary role is considered to be in the error-free branch (Schiestl and Prakash 1990; Cejka et al. 2001; Minesinger and Jinks-Robertson 2005; Gangavarapu et al. 2006; Ulrich 2006). Srs2 ("*S*uppressor of *R*ad*6*"), a DNA-dependent ATPase and helicase, strips Rad51 from single-stranded DNA, preventing Rad51 from sequestering the DNA for homologous recombination, and allowing PRR pathway members to access the substrate instead (KREJCI *et al.* 2003). Thus, Srs2 acts as the gatekeeper to all of postreplicative repair, although it only suppresses damage-induced sensitivity and mutagenesis in mutants of the error-free branch of PRR (ULRICH 2001).

In this study we investigate the basis of *anc1*'s sensitivity to alkylating agents. We show that *ANC1* defines a new branch a new branch in the PRR pathway, one that is error-free, promotes cell survival in the presence of DNA damaging agents, and suppresses both induced and spontaneous mutation, including the expansion of CAG triplet repeats.

RESULTS

Analysis of Anc1-containing complexes

As discussed earlier, Anc1 is a member of several RNA Pol II-related multi-protein complexes, namely TFIID, TFIIF, RSC, SWI/SNF, INO80, NuA3 and Mediator. Given these associations, we set out to determine whether Anc1's role in providing alkylation resistance could be assigned to one or more of these complexes, bearing in mind that Anc1 might provide resistance independently of these complexes. We therefore checked the sensitivity of mutants deleted for the non-essential members for each complex. We reasoned that if deletion mutants for other members of a particular protein complex share *anc1*'s damage sensitivity profile, this would pinpoint the complex via which Anc1 helps cells survive after chemical damage.

Using data from our genome-wide DNA damage sensitivity phenotyping screen, the non-essential members of Anc1's constituent complexes were checked for MMS, 4NQO and UV sensitivity (BEGLEY *et al.* 2002; BEGLEY *et al.* 2004) (Figure 2-1). For two of the seven Anc1-containing complexes, namely TFIID and TFIIF, Anc1 is the only non-essential member, so these complexes could not be interrogated. Of the five complexes containing non-essential members in addition to Anc1, four have a majority of subunits that, when deleted, share *anc1*'s sensitivity to MMS, 4NQO or UV; these are Mediator, SWI/SNF, INO80, and RSC excluding only NuA3 (Figure 2-1). The damage sensitivity of strains deleted for several subunits in four out of five complexes demonstrates that Anc1's role in survival after DNA damage is likely to be tied to the functions of at least four of its protein complexes.

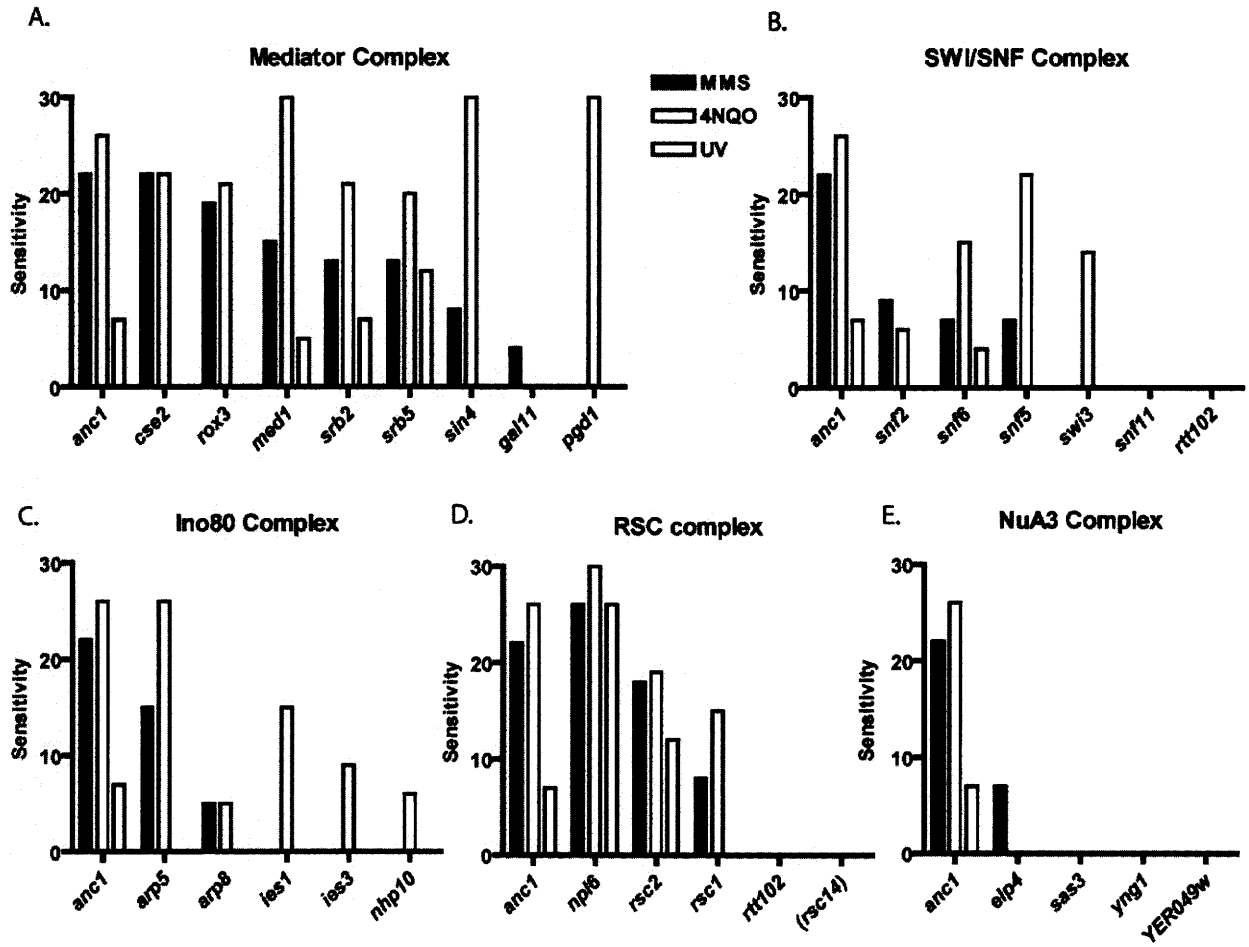

Figure 2-1: Sensitivity of strains deleted for non-essential members of Anc1-containing complexes to MMS and 4NQO and UV. Values for increasing sensitivities from 2-30 were calculated as described in Materials and Methods, by Begley et al, 2004 and as displayed at http://genomicphenotyping.mit.edu/newpages/source2.html. A) Mediator complex, B) SWI/SNF complex, C) Ino80 complex, D) RSC complex - although *RSC14* is not essential, there is no sensitivity data available, E) NuA3 complex.

Alkylation damage induces normal cell cycle arrest in ANC1 deficient cells

Many cell cycle-related genes are critical for survival after alkylation damage; indeed, ~45% of known cell cycle regulation genes were found to be MMS sensitive in our genome-wide screen for genes involved in damage resistance (BEGLEY *et al.* 2004). Strains mutated in genes that are necessary for the Mec1-mediated DNA damage checkpoint (i.e. *MEC1*, *RAD53, RAD9, RAD17, RAD24*) are more sensitive to killing by MMS than wildtype strains (PAULOVICH and HARTWELL 1995; PAULOVICH *et al.* 1997). Given the sensitivity of the *anc1* strain to MMS and 4NQO damage, it seemed plausible that their sensitivity may be due their failure to arrest in response to DNA damage (DAHAN and KUPIEC 2004). To assess the effect of Anc1 on the Mec1-mediated DNA damage checkpoint, we analyzed cell cycle progression in wild-type and *anc1* yeast cultures in the presence of MMS (PAULOVICH and HARTWELL 1995) (Figure 2-2).

As previously shown, a moderately toxic dose of MMS (0.015%) induced a Mec1-dependent S-phase arrest in wildtype *S. cerevisiae* (PAULOVICH and HARTWELL 1995). The 0.015% dose of MMS used in this experiment causes minimal killing in wildtype and only moderate killing in *anc1* strains (Figures 2-3 and 2-4). Although *anc1* strains grow more slowly than wildtype (VINH *et al.* 1993), the MMS-induced S-phase arrest is clearly observed in both the wildtype and *anc1* strains (Figure 2-2); it is important to note that no such arrest is observed in *mec1-1*, *rad53*, *rad9*, *rad17* and *rad24* (PAULOVICH and HARTWELL 1995; PAULOVICH *et al.* 1997). However, *anc1* cells take longer than wildtype to reach an arrested state, and also take longer to move through S phase (Figure 2-2). This lag may be a result of the following: (i) *anc1*'s slow growth rate; (ii) a slower release

Figure 2-2

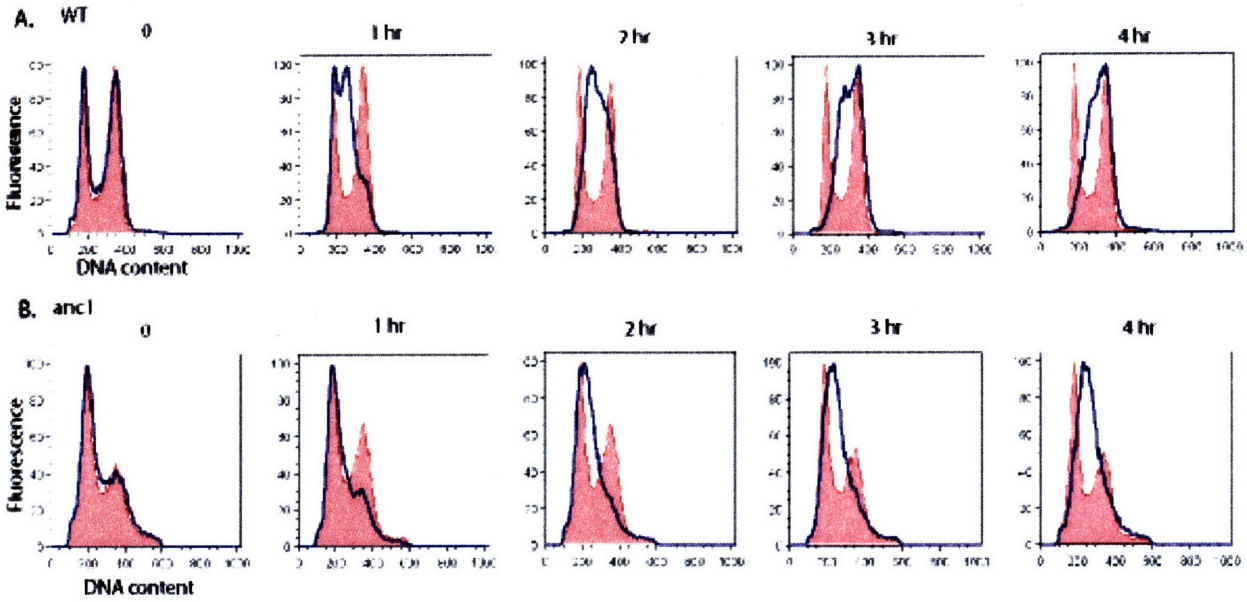

Figure 2: Cell cycle distribution of wildtype and *anc1* asynchronous cells before and after MMS exposure. A. WT cells, B. *anc1* cells. When log-phase growing cells in YPD reached an OD(600) of 0.2, MMS was added to half of the cells at a concentration of 0.015%, and aliquots were removed at the indicated times to monitor cell cycle distribution by flow cytometry. Profiles of untreated cells are shown in red shading, and profiles of treated cells are shown with a blue trace. The experiment was repeated twice to ensure reproducibility.

from the checkpoint; or (iii) a more strongly induced cell cycle arrest (Figure 2-2). Comparing the untreated cell cycle profiles of *anc1* and wildtype, we observed that *anc1* cells spend much longer in G1 than do wildtype cells, presumably contributing to their slow growth phenotype (Figure 2-2). The reason for a prolonged G1 in *anc1* cells is unclear, but may be linked to a role for Anc1 either in leaving G1 or in starting S phase. What does seem clear is that the sensitivity of *anc1* cells is not due to a complete failure to arrest at the Mec1-mediated DNA damage checkpoint, although there does seem to be a delay in triggering this S-phase checkpoint (PAULOVICH *et al.* 1997).

Epistasis of ANC1 with established DNA Repair pathways

To determine if *ANC1* functions within a canonical DNA repair pathway, we examined whether *anc1* could be assigned to established DNA repair epistasis groups, namely nucleotide excision repair (here represented by *rad2*), base excision repair (*apn1*), homologous recombination (*rad51, rad54*), transcription coupled repair (*rad26*) or postreplicative repair (PRR) (*rad5* and *rad6*) (Figure 2-3, Supplemental Figure 2-1). The MMS sensitivity of double mutant strains was compared to each of the single mutants as well as wildtype yeast. With the exception of *rad5anc1* and *rad6anc1*, the double mutants all showed additive or synergistic effects compared to the single mutants (Figure 2-3, Supplemental Figure 2-1). The sensitivity of the *rad5anc1* double mutant matches that of the *rad5* single mutant, indicating that *ANC1* shares a genetic pathway with *RAD5*, a member of the postreplicative repair pathway (Figure 2-3F). *rad6* strains are extremely sensitive to MMS, roughly 100X more sensitive than *anc1*, so we used two sets of MMS doses to observe sensitivity in this epistasis test (Figures 2-3G

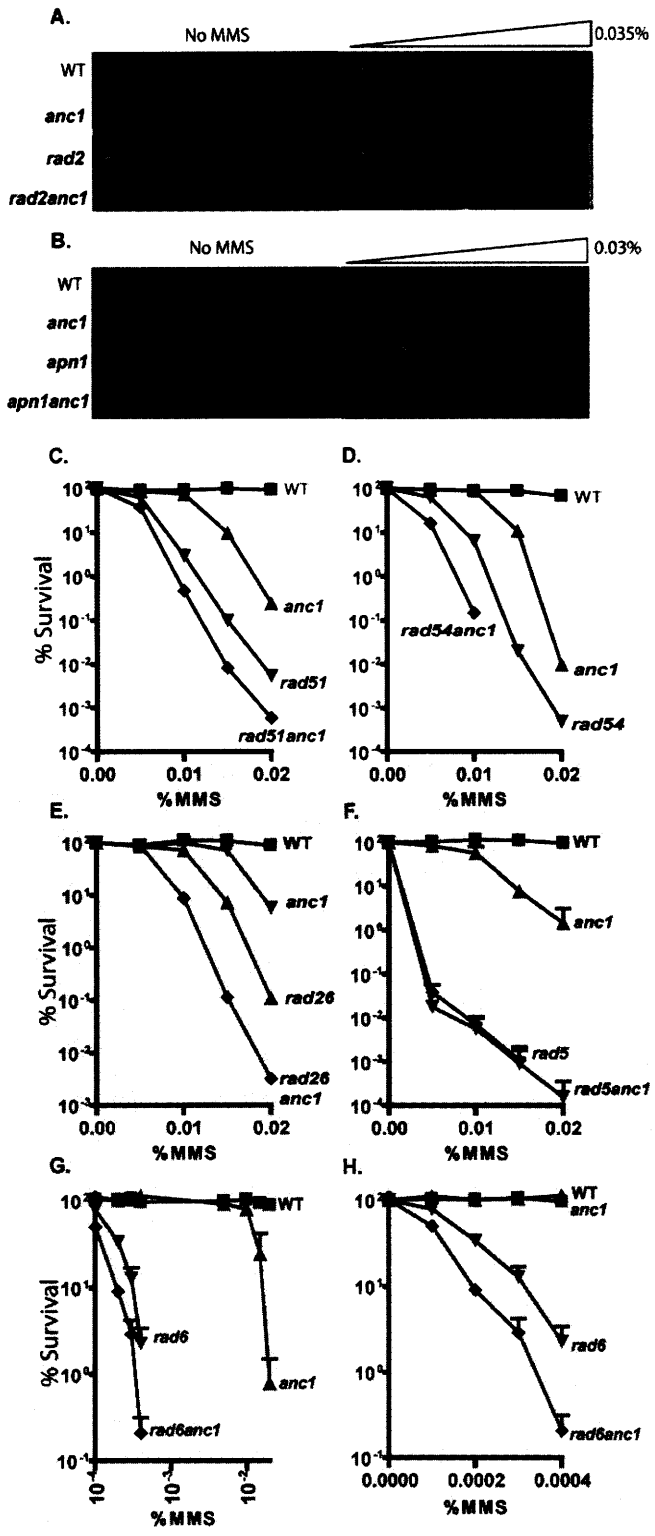

Figure 2-3: Epistasis analysis of *ANC1* in known DNA repair pathways. Survival after chronic MMS treatment for: A. YPD gradient plate with maximum dose 0.03% MMS, B. YPD gradient plate with maximum dose 0.035% MMS, C. WT (■), *anc1* (▲), *rad51* (▼), *rad51anc1* (♦). D. WT (■), *anc1* (▲), *rad54* (▼), *rad54anc1* (♦), E. WT (■), *anc1* (▲), *rad26* (▼), *rad26anc1* (♦), F. WT (■), *anc1* (▲), *rad5* (▼), *rad5anc1* (♦). Serial dilutions and gradient plates available in Supplemental Figure 2-1A-C.

and H). At the MMS doses to which *anc1* cells begin to show sensitivity, the sensitivity of the *rad6* and the *rad6anc1* strains was so great that survival could not be measured. But, looking at MMS doses on a log scale, we observed an apparently epistatic relationship between *ANC1* and *RAD6* (Figure 2-3G). A closer examination of the extremely low MMS dose range where the survival of *rad6* and *rad6anc1* strains can be accurately measured may, however, reveal a slightly synergistic relationship between these two genes (Figure 2-3H).

RAD5 is known to belong to the error-free branch of PRR, although studies have shown an additional role for Rad5 in the error-prone branch of the pathway (Schiestl et al. 1990; Minesinger and Jinks-Robertson 2005; Gangavarapu et al. 2006). *RAD5* has a complex relationship with other members of the error-free branch of PRR: the *rad5mms2* double mutant has an additive effect for UV- or MMS-induced cytotoxicity compared to the single mutants (XIAO *et al.* 2000), and Mms2/Ubc13-dependent and -independent roles for Rad5 have recently been described (GANGAVARAPU *et al.* 2006). *RAD6*, on the other hand, operates upstream of the branching between the error-prone and error-free pathways (Figure 2-4A).

After establishing the epistatic relationships between *ANC1*, *RAD5* and (possibly) *RAD6*, we assayed the genetic relationship between *ANC1* and other members of the PRR pathway, namely *SRS2*, *MMS2*, *UBC13*, *REV3*, and *RAD30* (Figure 2-4). The genetic relationships between the genes in the PRR pathway and the number of branches therein are a subject of some disagreement, but the pathway is generally divided into error-prone and error-free branches

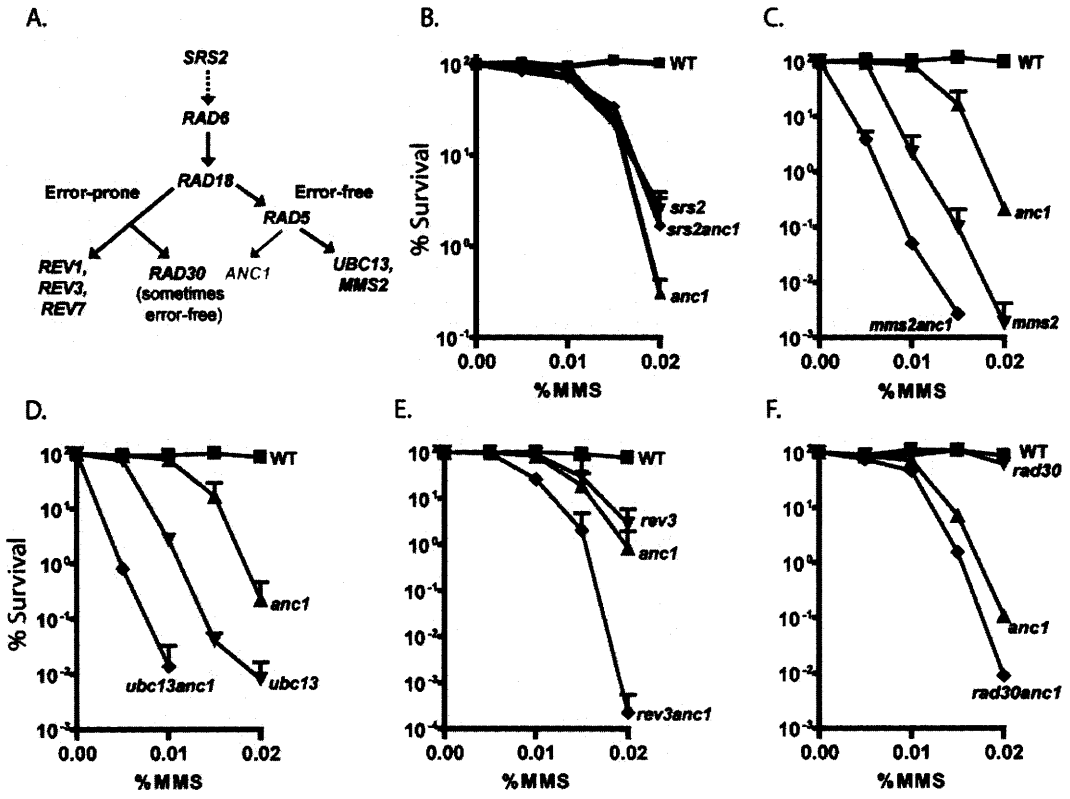

Figure 2-4: Epistasis analysis of *ANC1* with PRR pathway members. A. Genetic relationships within the PRR pathway in yeast. Epistasis was determined by sensitivity of mutants to damaging agents. *srs2* only suppresses the damage sensitivity of *rad5*, *ubc13* and *mms2* mutants (modified from Ulrich, 2006). Survival after chronic MMS treatment for: B. WT (■), *anc1* (▲), *srs2* (▼), *srs2anc1* (♦), C. WT (■), *anc1* (▲), *mms2* (▼), *mms2anc1* (♦) D. WT (■), *anc1* (▲), *ubc13* (▼), *ubc13anc1* (♦), E. WT (■), *anc1* (▲), *rev3* (▼), *rev3anc1* (♦) F. WT (■), *anc1* (▲), *rad30* (▼), *rad30anc1* (♦). Results are average of at least 2 replicates, error bars = SD, except in F.; gradient plate replica for F. in Supplemental Figure 1.

(Xiao et al. 2000; Cejka et al. 2001; Gangavarapu et al. 2006; Ulrich 2006) (Figure 2-4A). Like *RAD5*, *SRS2* was also found to be in the same genetic pathway as *ANC1*, with the *srs2* mutation suppressing the MMS sensitivity of *anc1* (Figure 2-4B). The suppression of *anc1* sensitivity in the *srs2anc1* double mutant is consistent with earlier observations that the *srs2* deletion suppresses the MMS sensitivity of several mutants in the error-free branch of the postreplicative repair pathway, including *rad5, ubc13* and *mms2* (FRIEDL *et al.* 2001; ULRICH 2001) (Figure 2-4B) These data support the hypothesis that Anc1 functions in the error-free branch of postreplicative repair, downstream of Srs2.

Epistasis analysis of *ANC1* with *RAD18* was not carried out because we were unable to create a *rad18anc1* double mutant by either mating or transformation (*anc1* transformation into *rad18* or vice versa). The defective alpha-factor response and sporulation of *anc1* have been previously noted (VINH *et al.* 1993). Mutants for two other error-free pathway components, *MMS2* and *UBC13*, showed a synergistic pattern of sensitivity to MMS when combined with the *ANC1* mutation (Figure 2-4C, D). From this we infer that Anc1 might act on the same type of damage as Mms2/Ubc13, but through a different pathway. *ANC1*'s epistasis with *RAD5* and (possibly) *RAD6* does not help us determine to which branch of PRR it belongs, as each gene has a role in both the error-prone as well as the error-free pathway. *SRS2, MMS2* and *UBC13* are all characterized members of the error-free branch, and given *ANC1*'s epistasis with *SRS2*, but synergistic relationship with *MMS2* and *UBC13*, *ANC1*'s role in PRR does not fit into the canonical error-free branch.

Because *ANC1* is synergistic rather than epistatic with the *MMS2* and *UBC13* members of the error-free branch of the PRR pathway, we determined whether *ANC1* lies in the error-prone pathway (BROOMFIELD *et al.* 1998). We analyzed the alkylation sensitivity of *anc1* in combination with *rev3* or *rad30*, mutants in two translesion DNA polymerases involved in PRR: *REV3* (with *REV7*) encodes DNA polymerase ζ, an error-prone polymerase, and *RAD30* encodes polymerase η, a polymerase that is sometimes characterized as error-prone, and sometimes as error-free depending on the type of lesion being bypassed (PRAKASH *et al.* 2005). The *rev3anc1* double mutant showed an additive MMS-sensitivity phenotype relative to the single mutants, indicating that Anc1 probably lies in a non-overlapping pathway with Rev3 (Figure 2-4E). The *rad30anc1* double mutant, however, appeared to have synergistic sensitivity when compared to the sensitivities of the single mutants, possibly indicating a partially overlapping function between Rad30 (Pol η) and Anc1 (Figure 2-4F). Thus, with respect to its genetic pathway, *ANC1* appears to be independent from both the error-prone and error-free branches of postreplicative repair. Taken together, from the lack of epistasis between *ANC1* and error-free branch members *UBC13* and *MMS2*, and from the lack of epistasis with error-prone branch members *REV3* and *RAD30*, we infer that *ANC1* functions in a genetic pathway that is independent from the two established branches, and thus defines a member of a new branch of PRR (Figure 2-4A).

Induced and spontaneous mutagenesis in anc1 cells

As discussed, PRR has been divided into "error-prone" and "error-free" branches. When the error-prone pathway is impaired, cells become refractory to spontaneous and

damage-induced mutagenesis; when the error-free pathway is impaired, cells become, if anything, more susceptible to damage-induced mutagenesis. Given that *ANC1* is not epistatic with all members of the error-free branch of postreplicative repair, it was important to determine whether *ANC1* acts in an error-free or error-prone pathway with respect to mutagenesis.

Yeast lacking Anc1 were assayed for both frameshift and point mutations as previously described (Tran et al. 1997; Glassner et al. 1998; Stelter and Ulrich 2003; Hanna et al. 2004). Point mutations were monitored in the *CAN1* gene by canavanine resistance, and frameshift mutations were monitored by reversion of the *lys2* A_{12} and A_{14} alleles containing mononucleotide runs of adenines (Tran et al. 1997; Hanna et al. 2004). Functional *LYS2* is only expressed after a -1 or +1 frameshift mutation in *lys2* A_{12} and *lys2* A_{14}, respectively (TRAN *et al.* 1997). Rev3 is a well-characterized member of the error-prone branch of PRR, and is used here as a positive control for monitoring the phenotype associated with a deficiency in an error-prone pathway (Figure 2-5).

ANC1 deleted cells were slightly more sensitive than wildtype yeast to UV-induced point mutation and -1 framshift mutations; in contrast, UV-induced +1 frameshift mutations were similar between *anc1* and wildtype. (Figure 2-5A, B, C). At the *CAN1* locus *rad5* has been observed to result in a slight increase in UV-induced point mutagenesis compared to wildtype, although the induced mutagenesis in *rad5* strains has been previously characterized as being very dependent on the mutational target being assayed (Johnson et al. 1992; Broomfield and Xiao 2002; Gangavarapu et al. 2006). This is consistent with the slight increase in induced mutagenesis observed in our *anc1* strain at the *CAN1* locus (Figure 2-5A). In contrast, the *rev3* deleted strain is

Figure 2-5

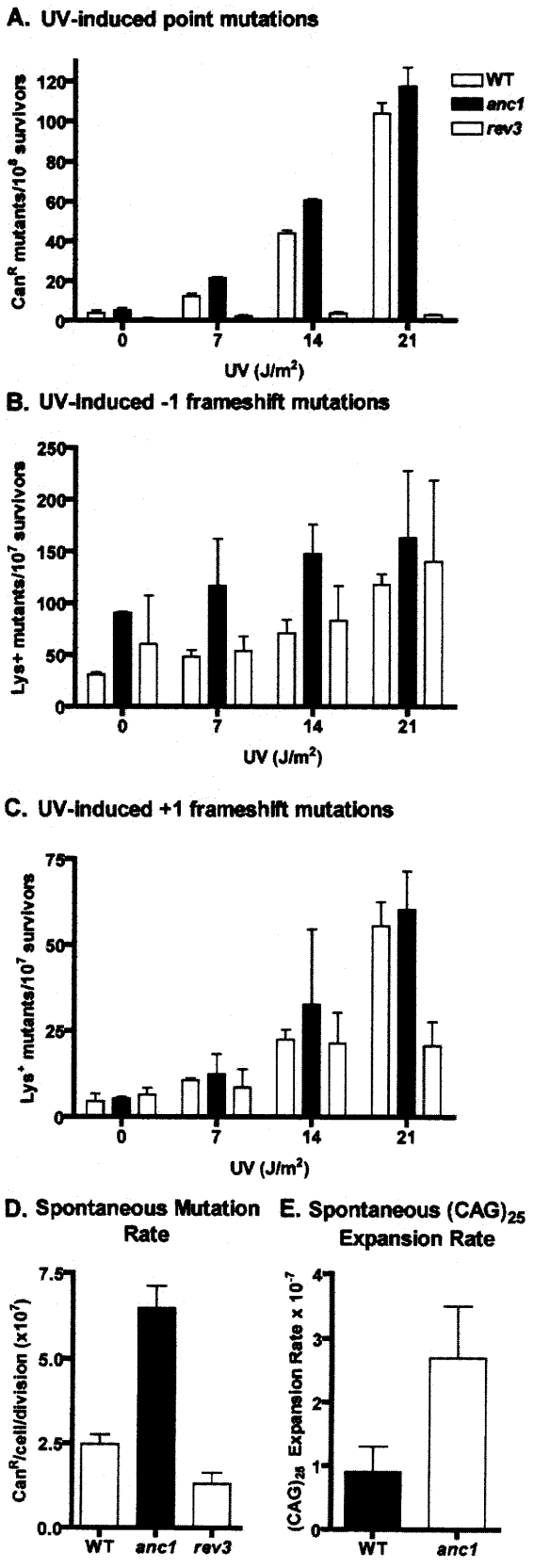

Figure 2-5: UV-induced point and frameshift mutagenesis and spontaneous mutagenesis. A. Induced point mutagenesis in BY4741 background: wildtype, *anc1* and *rev3* cells were exposed to UV doses as indicated. Results are mean of two replicates, +/- SD. B. and C. Induced frameshift mutagenesis in CG379-A12 and CG379-A14 backgrounds: WT and *anc1* frameshift reversions to a functional Lys2 gene. Cells were exposed to UV doses as indicated. Results are mean of two replicates, +/- SD. D. Spontaneous point mutagenesis, +/- SD, measured as described in Materials and Methods. E. Spontaneous expansion in (CAG)25 repeats, +/- SD, measured as described in Materials and Methods.

refractory to UV-induced point mutations compared to both wildtype and *anc1* strains (Figure 2-5A). Thus, *anc1*'s sensitivity to damage-induced mutagenesis is consistent with Anc1 acting in an error-free rather than an error-prone pathway.

Previous studies have shown an increase in spontaneous mutation rates among mutants in the error-free branch of PRR, and a decrease in the spontaneous mutation rate among mutants in the error-prone branch (BROOMFIELD *et al.* 1998; BRUSKY *et al.* 2000; CEJKA *et al.* 2001). Here we show that, the deletion of *ANC1* results in an increased frequency of spontaneous -1 frameshift mutations (Figure 2-5B), and also in an increased spontaneous base pair substitution mutation rate compared to wildtype (Figure 2-5D). Note that the *rev3* control displays a decreased spontaneous base pair substitution mutation rate compared to wildtype. Thus, in terms of both induced and spontaneous mutation, the newly defined Anc1 branch of PRR is clearly error-free, protecting *S. cerevisiae* from both cytotoxicity and mutagenesis.

Anc1 protects against trinucleotide repeat expansions

It was recently reported that gene deletion for several members of the error-free branch of the PRR pathway, including *RAD5* and *SRS2*, results in an expansion of CAG and CTG trinucleotide repeats (TNRs); expansion of such repeats have been associated with Huntington's disease and myotonic dystrophy (Timchenko and Caskey 1996; Bhattacharyya and Lahue 2004; Daee et al. 2007). In those studies, it was observed that the disease-associated TNRs, but not dinucleotide repeats or non-

disease associated TNRs, are prone to expansion, but not contraction, in cells deficient in the error-free branch of the PRR pathway (DAEE *et al.* 2007).

To determine whether Anc1 plays a role in limiting CAG expansions like other members of the error-free PRR pathway, an *anc1* deletion was introduced into a strain containing a $(CAG)_{25}$ construct, and assayed for CAG expansions as described (DAEE *et al.* 2007). Like other PRR members, *anc1* displays a statistically significant (three-fold) increase in CAG expansions compared to wildtype (Figure 2-5E). This expansion is statistically indistinguishable from those in *rad5* and *mms2* strains, although it is considerably lower than the expansion observed for several other PRR mutants (DAEE *et al.* 2007). These data indicate that Anc1, like other members of the error-free PRR pathway, plays a role in preventing the expansion of CAG trinucletide repeat sequences.

DISCUSSION

Anc1 is known to directly interact with the catalytic protein subunits for six of the seven Anc1-containing multi-protein complexes, including TFIID, TFIIF, RSC, Ino80, SWI/SNF and NuA3 (Treich et al. 1995; Sanders et al. 2002; Kabani et al. 2005). Of the six subunits with which Anc1 directly interacts, Sth1, Ino80 and Snf2 are DNA-dependent ATPases/helicases with sequence similarity to the SNF2 family of DNA-dependent ATPases (Laurent et al. 1992; Ebbert et al. 1999; Kabani et al. 2005). The other three catalytic subunits are Tsm1 and Tgf1 that are both involved in general transcription initiation, and Sas3, the catalytic subunit of NuA3 that acetylates histone H3 (KABANI *et al.* 2005). Given the interaction between Anc1 and the catalytic domains

of nearly all of its component complexes, plus the putative interaction between histones and Anc1's YEATS domain, it seems likely that Anc1 acts as a regulatory adapter between chromatin and the complexes that act upon it (KABANI *et al.* 2005; ZEISIG *et al.* 2005). The damage sensitivity of cells mutant in individual components of so many of these complexes suggests that Anc1 is involved in regulating transcription, chromatin remodeling, and as reported here, PRR, upon exposure to DNA damaging agents.

The *ANC1* transcript belongs to a minority of yeast transcripts that contain a splice site. It was recently reported that *ANC1* mRNA splicing is regulated by Cdc40, a protein involved in controlling cell cycle progression (DAHAN and KUPIEC 2004). In the absence of *CDC40*, cells arrest in G2/M, and the addition of intronless *ANC1* cDNA only partially mitigates this arrest (Vaisman et al. 1995; Dahan and Kupiec 2004) indicating that Cdc40 may have other splicing targets in addition to the *ANC1* mRNA, or may have yet another function. The slow transition out of G1 that we observed in *anc1* cells is also observed in *cdc40* cells (Vaisman et al. 1995; Kaplan and Kupiec 2007), and like *anc1*, *cdc40* mutants are sensitive to a variety of DNA damaging agents, including hydroxyurea, MMS, 4NQO and UV (BEGLEY *et al.* 2004; KAPLAN and KUPIEC 2007). However, the sensitivity of *cdc40* cells to MMS or HU is not suppressed when intron-less *ANC1* cDNA is expressed (KAPLAN and KUPIEC 2007). Of relevance to this study, a temperature sensitive allele of *cdc40* was shown to be epistatic to an allele of *rad6* in terms of MMS sensitivity during log phase growth, although neither allele was characterized as being null (KUPIEC and SIMCHEN 1986). Since a correctly spliced *ANC1* transcript does not suppress the MMS or HU sensitivity of *cdc40* cells, we must conclude that Cdc40 has another function in allowing cells to survive after DNA damage

that is independent from *ANC1* transcript splicing. Like *ANC1*, *UBC13* and *MMS2* are intron-containing genes in the PRR pathway (DAVIS *et al.* 2000). Given the observed epistasis between alleles of *cdc40* and *rad6* after MMS treatment (KUPIEC and SIMCHEN 1986), and the failure of the correctly spliced *ANC1* transcript to complement a *cdc40* mutant's damage sensitivity (KAPLAN and KUPIEC 2007), it is worth exploring whether Cdc40 mediates the splicing of the *MMS2* and/or *UBC13* transcripts as well.

Several pieces of evidence support Anc1's role in the PRR pathway. Based on the suppression of *anc1*'s sensitivity by *srs2, ANC1* can be placed genetically downstream of *SRS2*, as was previously observed for other members of the error-free PRR pathway (Schiestl et al. 1990; Friedl et al. 2001; Ulrich 2001). *ANC1* also shares a genetic pathway with *RAD5*, a downstream member of the error-free pathway and possibly with *RAD6*, which lies between *SRS2* and *RAD5* in the genetic model of the PRR pathway (Figure 4A). If the slight synergism observed at low MMS doses between *rad6* and *anc1* is genuine, it may imply a role for Anc1 that is partially parallel to that of Rad6. The lack of epistasis between *ANC1* and other error-free branch members *MMS2* and *UBC13* provides evidence for a new, Mms2/Ubc13 independent branch of the PRR pathway. Given that we were unable to create a *rad18anc1* double mutant by mating or transformation, even in the presence of a covering plasmid bearing an intact *RAD18*, we do not yet know whether Rad18 also plays a role in the new pathway defined by Anc1; however, since no Rad6-independent role for Rad18 has been described, it seems likely that Rad18 also plays a role in the Anc1-branch of PRR.

Two types of mutagenesis data indicated that the Anc1-containing branch of the PRR pathway deals with DNA damage in an error-free manner. First, the *ANC1*

deletion, similar to deletions for most members of the error-free PRR pathway (Ulrich 2001; Broomfield and Xiao 2002), causes an increase in both induced and spontaneous point mutation compared to wildtype. Second, *anc1* mutants display a significant increase in the expansion of CAG tri-nucleotide repeats compared with wildtype, a trait that was recently identified in all of the tested members of the error-free branch of the PRR pathway, including *srs2* and *rad5* (DAEE *et al.* 2007). These mutagenesis data are consistent with a role for Anc1 in error-free PRR.

Anc1's role in PRR may be crucial for understanding the interaction of key players in the cellular response to DNA damage. Anc1 interacts physically with Mus81, a structure-specific endonuclease in the XPF family involved in cleaving stalled replication forks (HO *et al.* 2002; OSMAN and WHITBY 2007). Mus81 forms a heterodimer with Mms4 for its endonuclease activity, and deletions of either partner results in sensitivity to MMS and 4NQO (BEGLEY *et al.* 2002; BEGLEY *et al.* 2004). Mus81 is speculated to be involved with the PRR pathway (in addition to its better characterized role in homologous recombination) by means of its cleavage of the stalled replication forks that the PRR pathway acts upon (OSMAN and WHITBY 2007). Furthermore, there is genetic evidence in *S. pombe* that *srs2* and *mus81* are epistatic with respect to their MMS, UV and HU sensitivities (DOE and WHITBY 2004), although in *S. cerevisiae* the *mms4srs2* double mutant displays a synergistic effect compared with either of the single mutants after MMS or UV treatment, suggesting that their pathways may partially overlap (ODAGIRI *et al.* 2003) Having demonstrated *ANC1*'s membership in the error-free branch of PRR, it seems likely that the physical interaction between Mus81 and Anc1 relates to Mus81's cleavage function in PRR. The method by which Mus81

recognizes its substrates is not well understood, but it seems possible that Anc1, through its presumed interaction with histones (ZEISIG *et al.* 2005), allows the Mus81 endonuclease access to sites where its cleavage will initiate the sister-strand recombination that drives error-free PRR.

Given the direct interaction between the YEATS domain of ENL with histones H1 and H3, and Anc1's interaction with the catalytic subunits of so many transcriptionally-important complexes (KABANI *et al.* 2005; ZEISIG *et al.* 2005), it may be hypothesized that Anc1 acts as a DNA-damage mediated adapter between chromatin, transcription and PRR repair at or near sites of DNA damage. Since transcription generally continues through S-phase, while DNA is being replicated, the collision of the transcriptional machinery and stalled replication forks is thought to be a common event (AGUILERA 2002). In recent years there has been considerable interest in the phenomena of transcription-associated mutation (TAM) and transcription-associated recombination (TAR), which characterize the mutagenesis and recombination that occur when the transcription and replication machineries collide (AGUILERA 2002). Mediation of the interaction between these machineries by a common member (Anc1) of the transcription complexes is a possibility worthy of further exploration. It is possible that the new branch of postreplicative repair represented by Anc1 is responsible for mediating the repair of replication forks that have stalled as a result of the collision between transcription and replication machineries (Figure 2-6). Furthermore, the role of the human YEATS containing leukemia-associated proteins, ENL, AF9 and GAS41, in both the human post-replication repair pathway, and in polyglutamine expansions such as those associated with Huntington's disease is certainly worthy of further exploration,

and may provide insight into the molecular basis of such disparate diseases as leukemia and Huntington's disease.

MATERIALS AND METHODS

Yeast Strains and Media: Yeast strains used in this study are listed in Supplementary Table 2-1. Yeast strains were grown in standard media, including YPD and synthetic complete (SC) medium. All strains are congenic with the BY4741 background (MAT*a* *his*3Δ*1 leu2Δ0 met15Δ0 ura3Δ0*), except for the spontaneous mutagenesis and the trinucleotide repeat assays as specified below in Induced and Spontaneous Mutagenesis Assays and Supplemental Table 1. Double mutants were created by transformation of an *anc1*::URA3 linear cassette into G418 resistant strains from the genome-wide deletion collection (Invitrogen-ResGen) (WACH *et al.* 1994). Homologous ends allowed the cassette to recombine into the ANC1's endogenous location (WACH *et al.* 1994). Constructs were confirmed by PCR and/or DNA sequencing.

Flow Cytometry: Log phase cells were washed twice in 50mM Tris pH 7.8, resuspended in 50mM Tris pH 7.8 containing RNase A (1mg/ml) and incubated at 37°C overnight. Cells were pelleted and resuspended in 55mM HCl containing 5 mg/ml Proteinase K, incubated at 37°C for 30 min, washed once with 200mM Tris pH 7.5, 211mM NaCl, 78mM $MgCl_2$, then resuspended in the same buffer with 1mg/ml of propidium iodide before assaying by flow cytometry using a FACScan cytometer (Becton Dickinson) and CellQuest Pro software. Two independent assays were performed to confirm reproducibility, and analysis was performed using FlowJo software

Figure 2-6

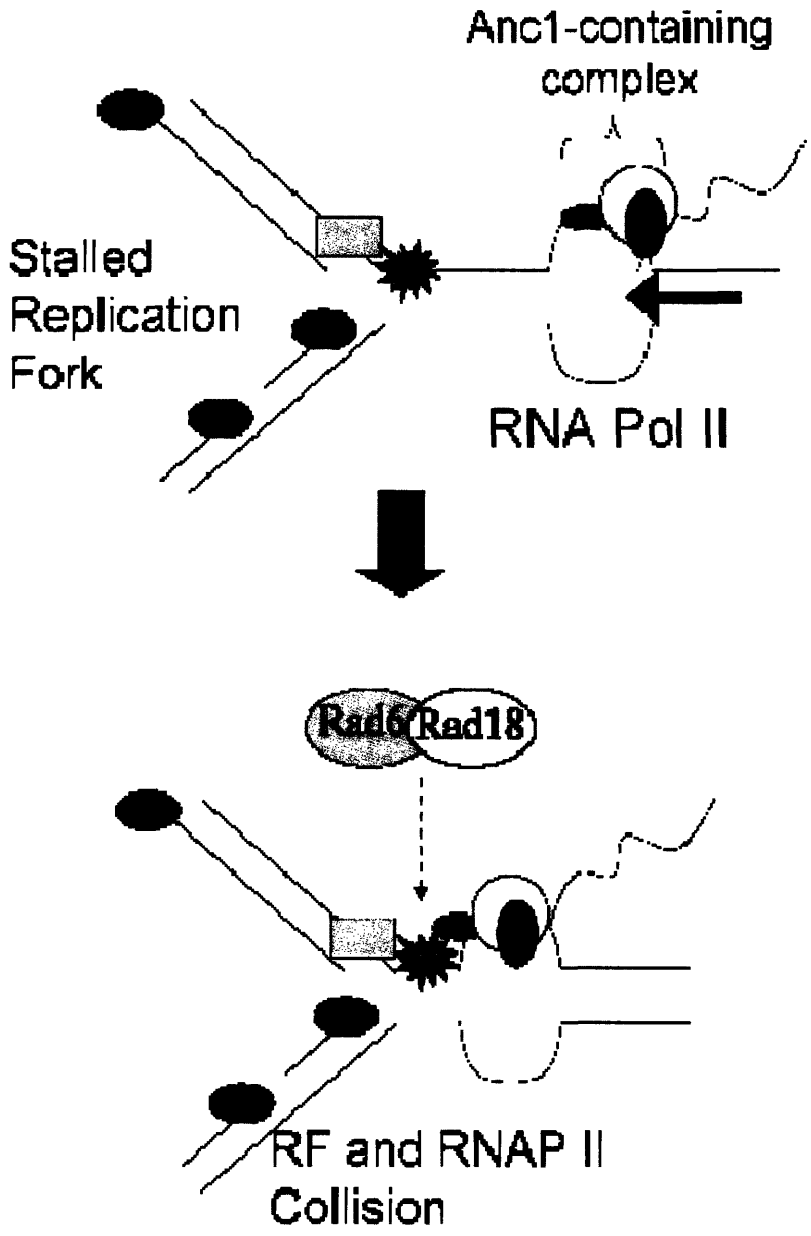

Figure 2-6 Model of Transcription-Coupled Postreplication Repair. Model depicts an Anc1-containing transcription complex colliding with a stalled replication fork (RF), resulting in the recruitment of the PRR pathway.

Version 6.4.7.

Sensitivity of deletion strains to DNA damaging agents in the genome-wide screen

The sensitivity of every non-essential gene deletant in *S. cerevisiae* was previously determined (BEGLEY *et al.* 2002; BEGLEY *et al.* 2004). Relative sensitivity values were generated using a scoring scheme that allocated values of 4, 3, 2, or 1 depending on the concentration of agent where strain sensitivity was identified; 4 is allocated to the lowest, and 1 is allocated to the highest concentration of damaging agent in the plate. These values were allowed to accumulate in each replicate, and then they were summed across all replicates. For example, in replicate 1, strains sensitive to all concentrations of agents received a score of 10 (4 + 3 + 2 +1), and this was summed over all 3 replicates for a final score of 30 (10 + 10 + 10). Damage-sensitive strains had scores that ranged from 30 (most sensitive) to 2 (least sensitive) (BEGLEY *et al.* 2004). All data is available at http://genomicphenotyping.mit.edu/newpages/source2.html.

Survival Curves/Epistasis Assays:

Log-phase cells were diluted and plated on MMS-containing YPD-agar plates or onto control plates with no MMS. Colonies were allowed to grow at 30°C for 2-5 days, depending on rate of growth for each strain, and survival was calculated by dividing the number of surviving colonies at a given MMS dose by the number of colonies that grew in the untreated sample. At least two replicates were counted per trial.

Induced and Spontaneous Mutagenesis Assays:

Yeast strains CG379-A_{12} and CG379-A_{14} from (TRAN *et al.* 1997) revert by -1 and +1 frameshifts in *LYS2::InsE-A_{12} and LYS2::InsE-A_{14}*, respectively, were used to measure frameshift mutation frequencies. These strains are isogenic with CG379 (*MATα ade5-1 his7-2 leu2-3, 112 trp1-289 ura3-52*) (TRAN *et al.* 1997). Frameshifts were calculated by comparing the number of Lys^+ revertants growing on Lys^- media to the number of colonies on a YPD control. Point mutation frequencies were measured in a BY4741 background. Canavanine-resistant mutations were measured on synthetic complete medium containing 0.004% (or 30 mg/liter) canavanine (HANNA *et al.* 2004). In the induced mutagenesis assay, UV doses of 0, 7, 14 and 21 J/m^2 were administered using a UV Stratalinker 2400 (Stratagene). Cells were grown into log phase, then serially diluted and plated onto YPD or Canavanine containing plates before exposure to UV. Colony formation on YPD was used to calculate the total number of cells plated on canavanine-containing plates, for a final calculation of mutants per 10^7 survivors.

In the spontaneous mutagenesis assay, forward mutations at *CAN1* were determined based on the protocol previously described in Glassner et al. (GLASSNER *et al.* 1998). Briefly, an overnight culture of each strain was diluted to 4000 cells/ml in 5mL of YPD in 10 cultures. The cultures were allowed to grow at 30°C for 5 days, then a small amount diluted 10^5-fold on YPD to assay for viable cells, and the remainder concentrated to 1 mL, and 100ul plated on 0.04% Canavanine-containing synthetic complete medium to assay for Can^R mutants. Mutagenesis rates were calculated using the Drake Formula (ROSCHE and FOSTER 2000).

Trinucleotide Repeat Assay

Expansion rates were measured by fluctuation analysis as described previously (Miret et al. 1998; Rolfsmeier et al. 2001; Dixon et al. 2004; Daee et al. 2007). All experiments were conducted in BL035, a *leu2* version of the wild type strain MW3317-21A (*MATα Δtrp1 ura3-52 ade2Δ ade8 hom3-10 his3-kpn1 met4 met13*) (KRAMER *et al.* 1989). $(CAG)_{25}$ tracts were cloned into a yeast promoter-reporter construct that allows spacing-sensitive expression of the downstream *URA3* reporter. Yeast cells harboring an expansion of four or more repeats do not express *URA3* and are identified by their resistance to 5-fluoroorotic acid. Mutation rates are calculated by the method of the median (LEA and COULSON 1948). Six independent clones were tested to ensure reproducibility. Single colony PCR analysis of expansions were done as previously described and rates were corrected by multiplying the percent *bona fide* expansions by the apparent mutation rates obtained by fluctuation analysis (DIXON *et al.* 2004). All statistical analyses were performed using the T-test (two-tailed distribution and two-sample equal variance) in Microsoft Excel and P-values less than 0.05 were considered statistically significant.

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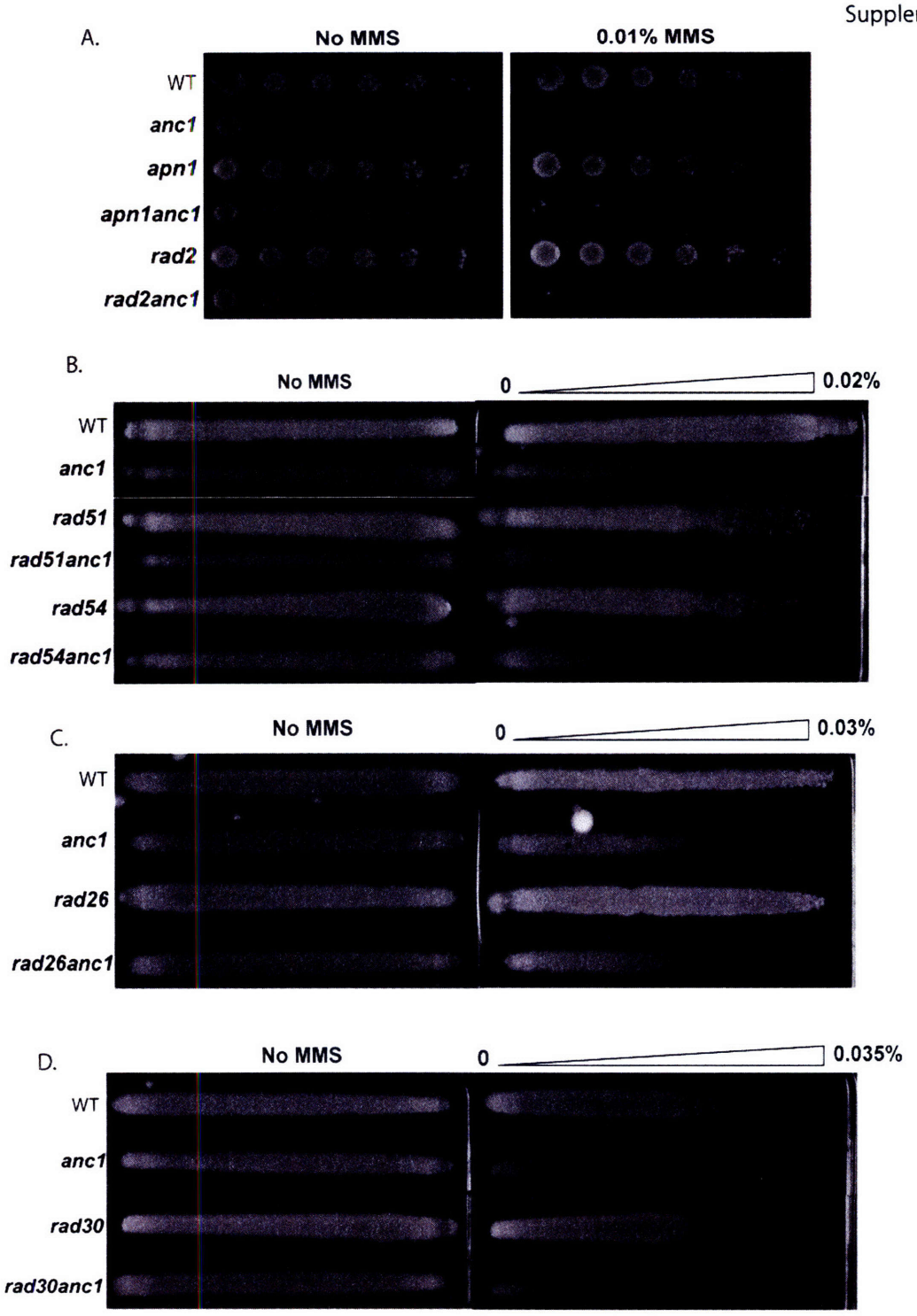

Supplementary Figure 1. Epistasis of *ANC1* with DNA repair pathway members A. Five-fold serial dilutions on YPD containing 0.01% MMS. Cell concentrations were normalized after overnight growth. B., C., D. Gradient plates on YPD containing the indicated concentrations of MMS. Cell concentrations were normalized after overnight growth.

Supplemental Table 2-1: Strains used in this study		
Strain	genotype	reference
S. cerevisiae		
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Wach et al, 1994
BY4741*anc1*	BY4741 *anc1::URA3*	this study
BY4741*rad2*	BY4741 *rad2::kanMX4*	Wach et al, 1994
BY4741*rad2anc1*	BY4741 *rad2::kanMX4 anc1::URA3*	this study
BY4741*apn1*	BY4741 *apn1::kanMX4*	Wach et al, 1994
BY4741*apn1anc1*	BY4741 *apn1::kanMX4 anc1::URA3*	this study
BY4741*rad51*	BY4741 *rad51::kanMX4*	Wach et al, 1994
BY4741*rad51anc1*	BY4741 *rad51::kanMX4 anc1::URA3*	this study
BY4741*rad54*	BY4741 *rad54::kanMX4*	Wach et al, 1994
BY4741*rad54anc1*	BY4741 *rad54::kanMX4 anc1::URA3*	this study
BY4741*rad26*	BY4741 *rad26::kanMX4*	Wach et al, 1994
BY4741*rad26anc1*	BY4741 *rad26::kanMX4 anc1::URA3*	this study
BY4741*rad5*	BY4741 *rad5::kanMX4*	Wach et al, 1994
BY4741*rad5anc1*	BY4741 *rad5::kanMX4 anc1::URA3*	this study
BY4741*srs2*	BY4741 *srs2::kanMX4*	Wach et al, 1994
BY4741*srs2anc1*	BY4741 *srs2::kanMX4 anc1::URA3*	this study
BY4741*rad6*	BY4741 *rad6::kanMX4*	this study
BY4741*rad6anc1*	BY4741 *rad6::kanMX4 anc1::URA3*	this study
BY4741*mms2*	BY4741 *mms2::kanMX4*	Wach et al, 1994
BY4741*mms2anc1*	BY4741 *mms2::kanMX4 anc1::URA3*	this study
BY4741*ubc13*	BY4741 *ubc13::kanMX4*	Wach et al, 1994
BY4741*ubc13anc1*	BY4741 *ubc13::kanMX4 anc1::URA3*	this study
BY4741*rev3*	BY4741 *rev3::kanMX4*	Wach et al, 1994
BY4741*rev3anc1*	BY4741 *rev3::kanMX4 anc1::URA3*	this study
BY4741*rad30*	BY4741 *rad30::kanMX4*	Wach et al, 1994
BY4741*rad30anc1*	BY4741 *rad30::kanMX4 anc1::URA3*	this study
CG379-A_{12}	*MATα ade5-1 his7-2 leu2-3, 112 trp1-289 ura3-52 lys2::InsE-A12*	Tran et al., 1997
CG379-A_{14}	*MATα ade5-1 his7-2 leu2-3, 112 trp1-289 ura3-52 lys2::InsE-A14*	Tran et al., 1997
CG379-A_{12}*anc1*	CG379-A_{12} *anc1::kanMX4*	this study
CG379-A_{14}*anc1*	CG379-A_{14} *anc1::kanMX4*	this study
CG379-A_{12}*rev3*	CG379-A_{12} *rev3::HIS3*	Rusyn et al. (in preparation)
CG379-A_{14}*rev3*	CG379-A_{14} *rev3::kanMX4*	Klapacz et al (in preparation)
BL035	*MATα trp1 ura3-52 ade2 ade8 hom3-10 his3-kpn1 met4 met13 leu2*	Daee et al. 2007
BL035*anc1*	BL035 *anc1::kanMX4*	this study

CHAPTER 3: ANC1 REGULATES THE ENVIRONMENTAL STRESS RESPONSE AND MAY MEDIATE A RESPONSE TO DAMAGE-INDUCED MEC1 SIGNALING

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ABSTRACT

The Anc1 protein has been shown to interact with 85 different proteins in yeast. The large majority of Anc1's interactors are involved directly in RNA polymerase II-mediated transcription, but a few interactors, involved in signaling and posttranslational modification, such as Dun1, hint at an additional function for Anc1. Here, we analyze the gene expression profiles of *anc1* cells in the presence and absence of DNA damage, and find that they indicate a role for Anc1 in modulating the expression of the roughly 900 genes involved in the transcriptional response to environmental stress. Specifically, in the absence of *ANC1,* the expected up- or down-regulation of transcripts after exposure to environmental stress is significantly reduced. The transcription of these genes is under the control of the Mec1 signaling pathway, such that mutants in *mec1* or *dun1* disrupt their transcriptional regulation similarly to the *anc1* strain. Sml1, a downstream effector of the Mec1-signaling pathway, was found to be inappropriately degraded in the absence of Anc1 after MMS treatment, strengthening the possibility of a connection between Anc1 and the Mec1 pathway, especially given that Sml1 is a direct target of Dun1, an Anc1 interactor.

INTRODUCTION

Eighty-five protein-protein interactions have been identified for Anc1 (Ho et al., 2002; Stark et al., 2006) (Figure 1-1). Of these, all but four interactions are involved, either directly or indirectly in RNA polymerase II (RNA pol II) transcription. Since Anc1's presence in transcriptional complexes has been well-characterized, the four protein interactors that are not implicated in transcription are of particular interest; these proteins may indicate a link between Anc1's known functions in transcription and DNA repair, and a hitherto undiscovered function. The four interacting proteins unrelated to transcription are: Mus81, a structure dependent endonuclease involved in homologous recombination; Sap185, which is necessary for Sit4 phosphatase function; Smt3, a protein in the SUMO signaling family; and the Dun1 kinase, a member of the Mec1/Tel1 damage signaling pathway (Ebbert et al., 1999; Gardner et al., 1999; Luke et al., 1996; Soustelle et al., 2004). The involvement of three of these proteins (Sap185, Smt3 and Dun1) in post-translational modification may reveal a role for Anc1 in this process, or as a target of this process. Of these three proteins, only Dun1 shares Anc1's pattern of DNA damage sensitivity to MMS, 4NQO and UV (Begley et al., 2004).

The Mec1/Tel1 signaling pathway in *S. cerevisiae* is homologous to the human ATM/ATR pathway. Both pathways are activated in response to DNA damage, and in the event of a DNA damage insult, a cascade of activation through phosphorylation propagates signals from upstream members (e.g. Mec1, Rad53, Dun1) to downstream members (e.g. Rad55, Sml1) (Bashkirov et al., 2000). The downstream members of the Mec1/Tel1 pathway are involved in a wide variety of cellular processes, ranging from

the regulation of cell cycle arrest (e.g.Chk1) to the regulation of nucleotide precursors for DNA repair (e.g. Sml1).

Several genes in the Mec1-mediated pathway have been shown to regulate the transcriptional response of yeast to a wide variety of stressors, in addition to DNA damage (Gasch et al., 2001). In these experiments, transcriptional changes in yeast cells that had been exposed to stresses such as high salt concentration, temperature shock or MMS treatment were assayed (Gasch et al., 2001; Gasch et al., 2000). A set of about 900 transcripts whose regulation was modulated similarly in response to a variety of different stressors was identified, and dubbed the Environmental Stress Response (ESR) (Gasch et al., 2000). Gasch et al. observed that the deletion of upstream members of the Mec1 pathway, like Mec1 or Dun1, results in a loss of the wildtype transcriptional response to stress, that is, the ESR was abrogated (Gasch et al., 2001).

Here, we show that Anc1 is necessary for the normal transcriptional response to the DNA damaging agent MMS, and that, in its absence, the transcription of ESR genes is significantly reduced. Given that *mec1* and *dun1* strains had previously expressed this same phenotype, we assayed the effect of Anc1 on this signaling pathway. We demonstrate that the regulation of Sml1, a downstream target of the Mec1 pathway, is disrupted after DNA damage when Anc1 is absent, and that this disruption takes place at the protein, not the transcript level.

RESULTS

Anc1 *is a Protein Hub that may have an Intrinsically Disordered C-terminus*

On average, each protein in *S. cerevisiae* interacts with approximately 5 other proteins (Grigoriev, 2003). Proteins that interact with a large number of other proteins are called "hub" proteins, and their characteristics have been much studied, given their disproportionate involvement in cellular contacts (Dosztanyi et al., 2006). Although there are differing opinions regarding the cutoff for how many interactions define a "hub" protein (usually about eight interactions), Anc1, with 85 protein-protein interactions, clearly surpasses the threshold for definition as a "hub" protein (Ho et al., 2002; Stark et al., 2006). Hubs are sometimes divided into static "party" hubs and dynamic "date" hubs (Ekman et al., 2006). Static hubs are proteins that interact with most of their protein partners at the same time (i.e. independent of time and location), and dynamic hubs are proteins that bind their partners at different times/locations (Ekman et al., 2006). Given Anc1's large number of protein interactors, and their variety of functions in cellular regulation, it seems most likely that Anc1 functions as a dynamic hub, interacting with its various protein partners at different times and places.

There are several traits that have been associated with hub proteins: highly connected proteins are more likely to be essential than those that are less connected, to be more highly conserved evolutionarily, and to have regions of intrinsic disorder (Ekman et al., 2006). Although Anc1 is not essential, it contains a YEATS domain that is highly conserved between yeast and humans, and its presence is characterized in many eukaryotic organisms (Gasteiger et al., 2003). To determine whether Anc1 contains any regions of intrinsic disorder, we utilized the PondR ("*P*redictor *o*f *N*aturally *D*isordered *R*egions) program (http://www.pondr.com/) that predicts protein disorder based on amino acid sequence. According to this algorithm, Anc1's N-terminal,

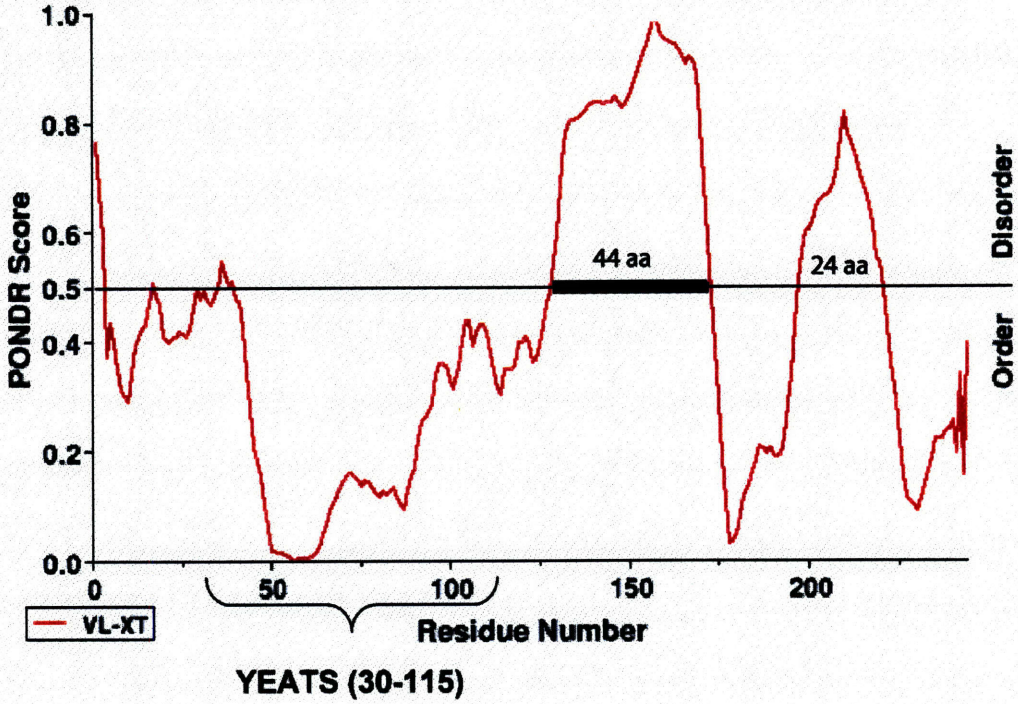
YEATS-containing region is highly ordered, whereas the middle and C-terminal regions are very likely to contain areas of disorder, including one stretch of 44 consecutive amino acids that are predicted to be disordered (Figure 3-1) (Li et al., 1999; Romero et al., 1997; Romero et al., 2001).

Intrinsically disordered (or "intrinsically unstructured") proteins have several qualities that are thought to be useful within hubs, including the ability to bend into many different conformations so as to accommodate many different partners, the ability to respond quickly and reversibly to changes in the cellular environment and the ability to be targeted for degradation quickly in their unfolded state, allowing for rapid regulation (Ekman et al., 2006). Proteins involved in transcription, cell cycle control and signaling are more likely than other proteins to contain long regions of disorder (>40 residues), and dynamic hubs are more likely than static hubs to contain regions of intrinsic disorder (Ekman et al., 2006). Anc1 is no exception; although it is a rather small protein (only 244 amino acids) it contains one predicted disordered region of 44 amino acids, and a second region of 24 amino acids (Figure 3-1).

anc1 mutants display massive transcriptional changes both in the presence and absence of alkylation damage

Given Anc1's participation in seven protein complexes involved in general transcription it seems possible that Anc1 is involved in genome-wide transcriptional regulation. A set of microarray experiments was performed to determine how the absence of *ANC1* affects transcription in the presence and absence of DNA damage.

Figure 3-1



==========PREDICTOR OUTPUT==========
"D" = Disordered
==========

```
1       MVATVKRTIR IKTQQHILPE VPPVENFPVR QWSIEIVLLD DEGKEIPATI
VLXT    DDD                D                       DDDD

51      FDKVIYHLHP TFANPNRTFT DPPFRIEEQG WGGFPLDISV FLLEKAGERK
VLXT

101     IPHDLNFLQE SYEVEHVIQI PLNKPLLTEE LAKSGSTEET TANTGTIGKR
VLXT                                  DD DDDDDDDDDD DDDDDDDDDD

151     RTTTNTTAEP KAKRAKTGSA STVKGSVDLE KLAFGLTKLN EDDLVGVVQM
VLXT    DDDDDDDDDD DDDDDDDDDD DD                        DDDD

201     VTDNKTPEMN VTNNVEEGEF IIDLYSLPEG LLKSLWDYVK KNTE
VLXT    DDDDDDDDDD DDDDDDDDDD
```

Predicted residues: 244
Number Disordered Regions: 5
Number residues disordered: 76
Longest Disordered Region: 44
Overall percent disordered: 31.15
Average Prediction Score: 0.4137

Modified from http://www.pondr.com/
Li et al, 1999
Romero et al, 2000
Romero et al, 1997

Figure 3-1: Anc1 contains a predicted disordered region. The Anc1 translated ORF sequence was analyzed using the VL-XT algorithm in the PondR ("*P*redictor *of* *N*aturally *D*isordered *R*egions) program (http://www.pondr.com/) (Romero et al. 1997; Li et al. 1999; Romero et al. 2001). Amino acids with a greater than 0.5 PONDR score are predicted to have diminished tertiary structure, and disordered regions of greater than 40 amino acids (marked with a thick black line) are thought to be highly predictive. The YEATS domain is labeled on the X-axis.

The genome-wide transcription of wildtype and *anc1* cells were assayed in the presence and absence of MMS treatment, yielding four transcriptional profiles, all performed in triplicate: wildtype untreated (WTU), *anc1* untreated (ancU), wildtype treated (WTT), and *anc1* treated (ancT) (Figure 3-2A). We used two metrics to determine changes in these transcriptional profiles; the difference between WTU and anc1U, hereafter called "basal" changes (or basal regulation), and the difference between the ratios of WTT/WTU and ancT/ancU, hereafter called "delta eta," or differential changes (Figure 3-2B, C). Delta eta describes a difference in transcriptional response to MMS depending on the presence/absence of *ANC1* (i.e. the difference in the extent of change in expression between the wildtype and *anc1* strains after MMS treatment) (Figure 3-2C).

The transcriptional response of wildtype cells to MMS has been assayed previously, and there is a good deal of overlap between the data from the arrays in this study and the data from the earlier publication; the correlation between those transcripts that are significantly changed after treatment is $r^2 = 0.81$ (Jelinsky et al., 2000) (Figure 3-3). Using FUNSPEC software, we determined which Gene Ontology (GO) categories were overrepresented in the set of genes that were up- and down-regulated after MMS treatment compared with the untreated wildtype control (Robinson et al., 2002) (Tables 3-1 and 3-2). In wildtype strains treated with MMS we observed increased expression of genes involved in carbohydrate metabolism, protein degradation, stress response and DNA repair, and decreased expression of genes involved in ribosome synthesis, ribosome assembly and nucleotide biosynthesis, similar to groups represented in the earlier study (Tables 3-1 and 3-2) (Jelinsky et al., 2000; Jelinsky and Samson, 1999).

Figure 3-2

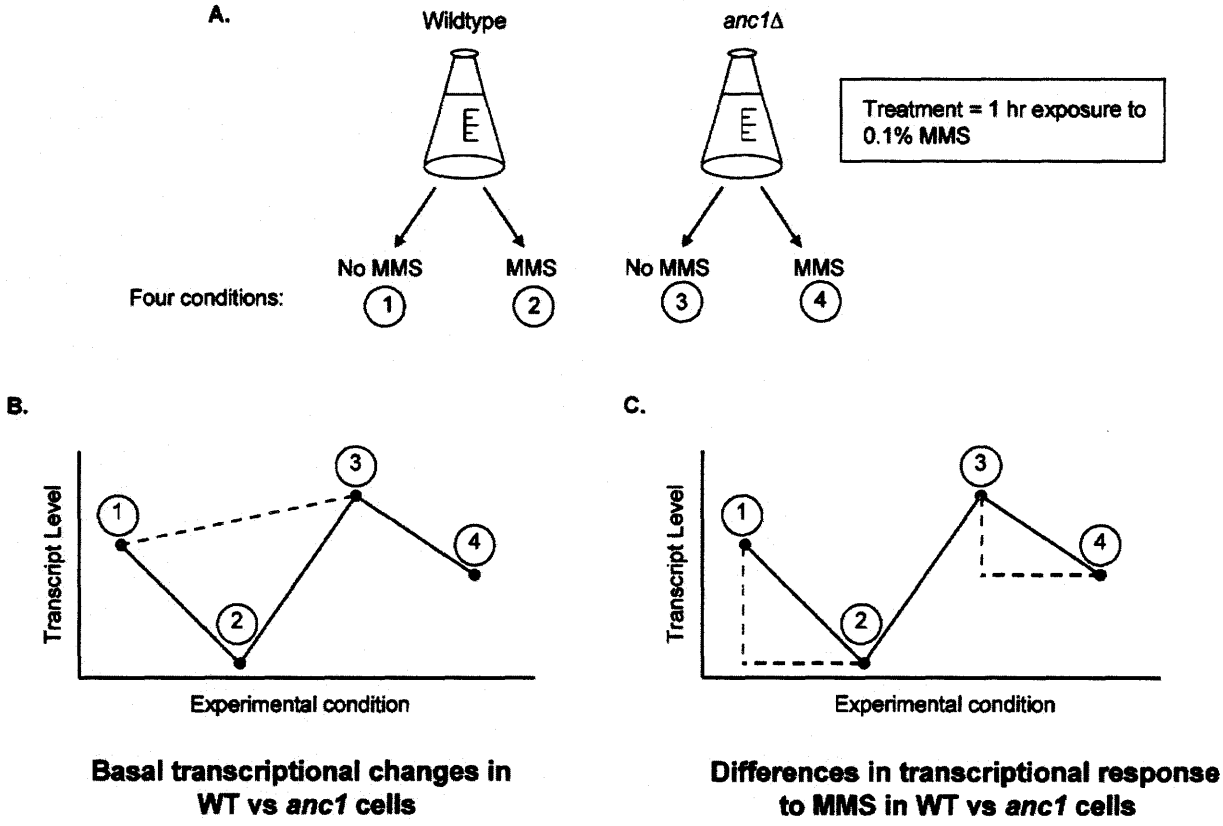


Figure 3-2: Experimental design for MMS-mediated transcriptional changes in *anc1* cells. A. Four cultures of cells were grown to mid-log phase: 1) wildtype untreated, 2) wildtype treated, 3) *anc1* untreated, 4) *anc1* treated. Treated cells were exposed to 0.1% MMS for one hour. Total RNA was extracted and hybridized to Affymetrix YG-S98 microarrays as noted in Materials and Methods. B. Example of a basal response to MMS; comparison of cultures 1 and 3. C. Example of a differential response to MMS; difference between the ratios 2/1 and 4/3.

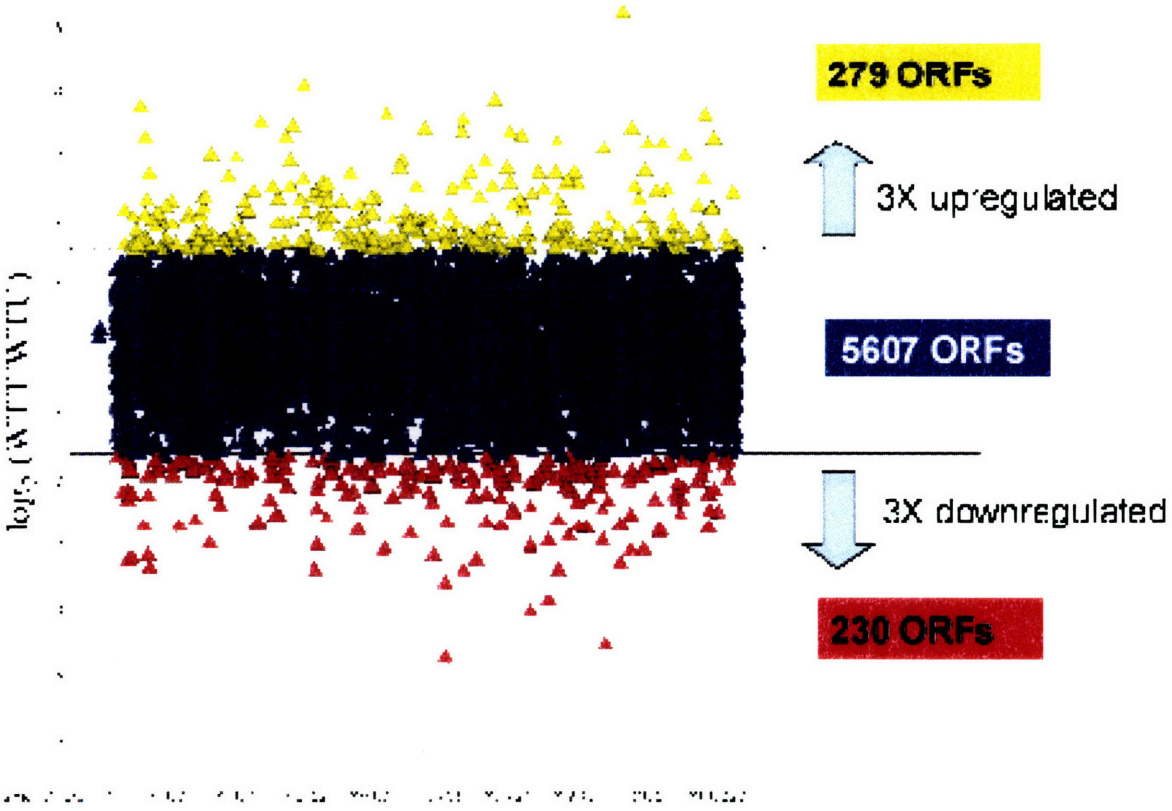


Figure 3-3. Wildtype transcriptional response to NMS. Upregulated ORFs are shown in yellow, downregulated ORFs are shown in red, and ORFs whose expression is unchanged after NMS treatment are shown in blue. A 3X cutoff was used to determine changes, as in (Jelinsky et al 2000). The corresponding gene ontology categorizations are described in Tables 1 and 2.

There are 1,386 transcripts whose basal expression is different between wildtype and *anc1* deleted cells, using a 1.5X fold change cutoff (Figure 3-4A). We are able to use a 1.5X fold-change cutoff (as opposed to the 3X fold change used above) because the high level of correlation between our three replicates makes the large majority of these changes statistically significant (data not shown). This corresponds to approximately 23% of the genome being transcriptionally-regulated by the presence/absence of *ANC1*. Gene ontology categories that are down-regulated in the absence of *ANC1* include genes involved in a variety of biosynthetic processes, DNA replication, lipid biosynthesis and the S-phase of the mitotic cell cycle (Figure 3-5B). We have observed that *anc1* cells spend a disproportionate amount of their cell cycle in G1 compared to wildtype cells (Figure 2-2). Presumably this accounts for the apparent down-regulation of transcripts involved in the S-phase of the cell cycle. Gene ontology categories up-regulated (i.e. whose expression has increased) in response to *ANC1* deletion include carbohydrate metabolism, energy pathways, response to stress and monosaccharide transport (Figure 3-5B). Despite these basal differences, there is a positive correlation between the transcriptional responses of wildtype and *anc1* strains to MMS ($r^2 = 0.64$) (Figure 3-4B).

Transcriptional changes in the Postreplication Repair Pathway

As demonstrated in Chapter 2, *ANC1* is a new member of the error-free branch of the postreplication repair pathway. Given this newly uncovered relationship between Anc1, a member of seven complexes involved in transcription, and postreplication repair, we sought to determine whether the transcriptional expression of genes involved

Figure 3-4

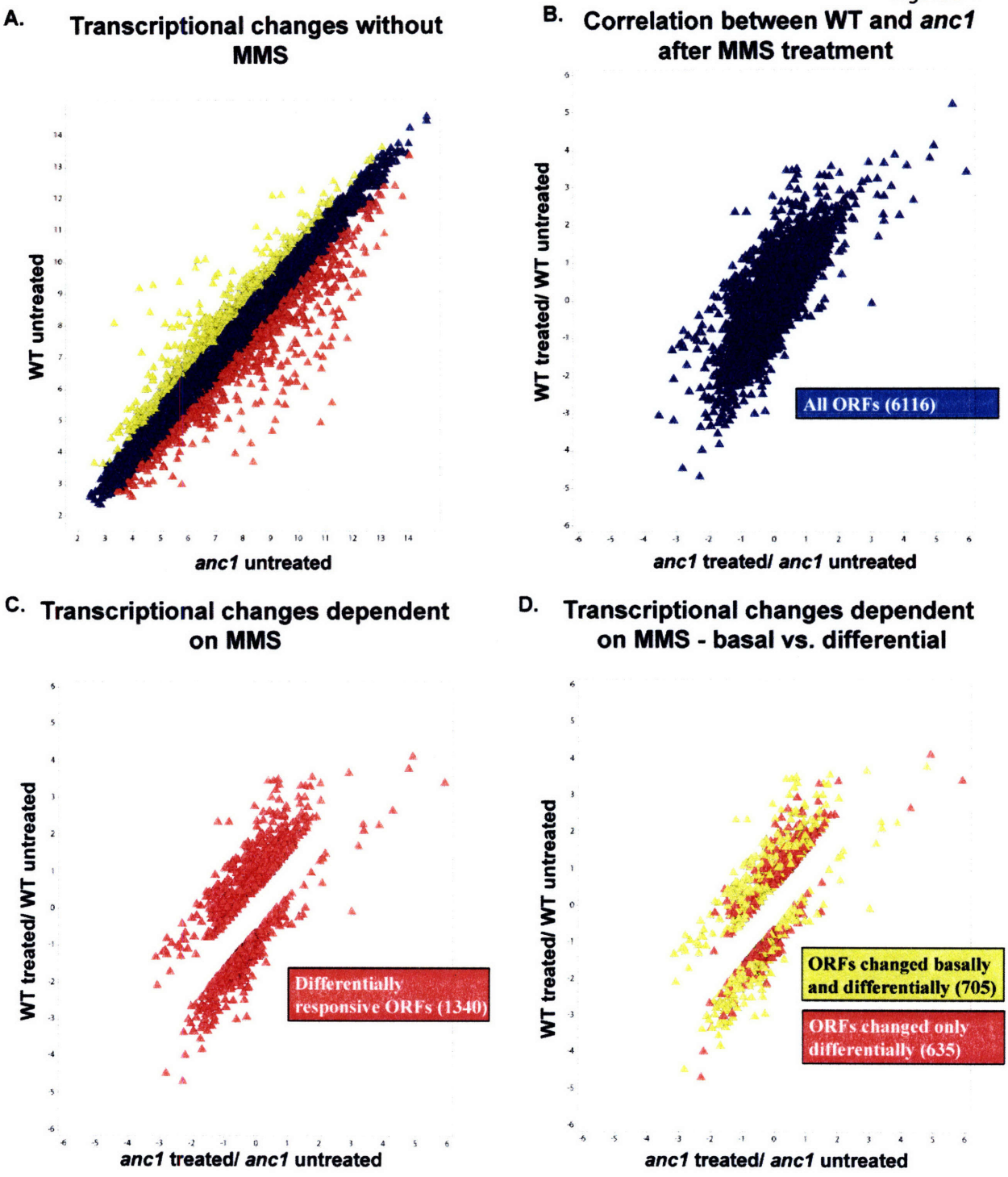
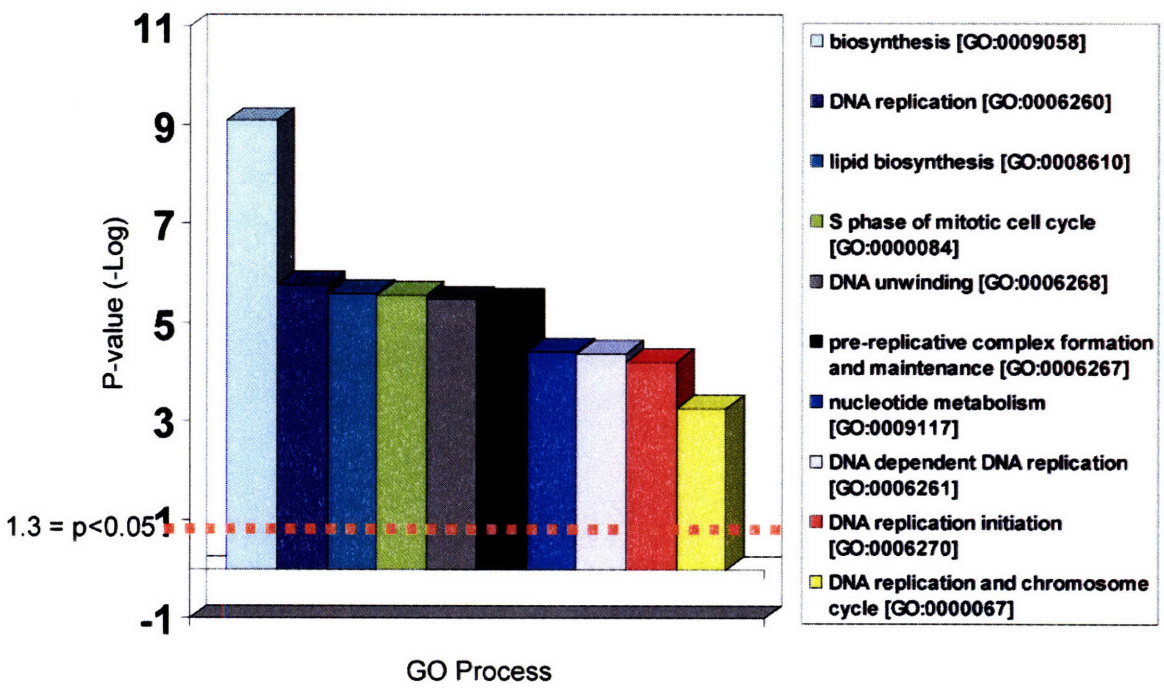


Figure 3-4: Correlations between wildtype and *anc1* gene expression profiles. All scales are $\log_2$. A. Basally changed ORFS (1.5X cutoff). 735 ORFs whose expression is higher in wildtype are shown in yellow, 582 ORFs whose expression is significantly higher in *anc1* cells are shown in red. B. Correlation between the treated/untreated ratios of wildtype and *anc1* cells. C. ORFs that are differentially responsive to MMS (i.e. identified by the delta eta algorithm) are shown in red. D. ORFS that are basally responsive and differentially responsive are shown in yellow, ORFs that are only responsive in the presence of MMS are shown in red.

A.



B.

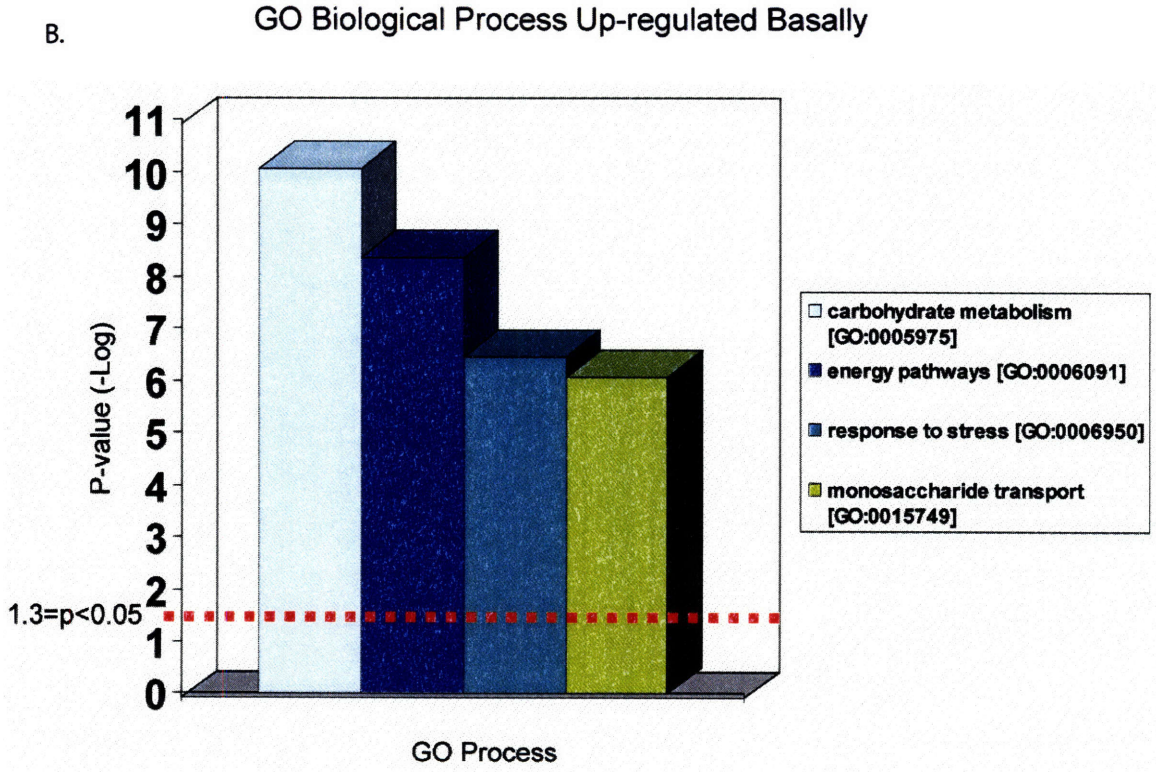


Figure 3-5: Biological pathways represented in the basal transcriptional differences between wildtype and *anc1* cells. A. Gene ontology biological processes for ORFs that were significantly downregulated basally. B. Gene ontology biological processes for ORFs that were significantly upregulated basally. A significance of $p<0.05$ was used for the determination of these categories.

in postreplication repair is altered in *anc1* cells. We analyzed both the basal and the differential responses of the genes involved in postreplication repair to the deletion of *ANC1*, and determined that none of the genes in this pathway (*RAD6, RAD18, RAD5, MMS2, UBC13, REV1, REV3 REV7 RAD30* and *SRS2*) show a statistically significant change in transcriptional expression (data not shown).

The Environmental Stress and DNA Damage Responses are Abrogated in anc1 mutants

The differential transcriptional response to MMS involves 1340 ORFS, 750 (56%) of which are not changed basally between wildtype and *anc1* cells (Figure 3-4C, D). Gene ontologies that are basally unchanged and differentially expressed (i.e. there is no transcriptional change between the WTU and ancU, but there is a difference between the ratios WTT/WTU and ancT/ancU) include ribosome biogenesis, metabolism, RNA processing and transcription from the Pol I promoter and amino acid biosynthesis. We observed that there was a very significant overlap between differentially responsive "delta eta" genes and genes involved in the response to environmental stress (Gasch et al., 2000). The set of about 900 ESR genes was identified by exposing wildtype yeast to a variety of environmental stressors ranging from high salt to MMS treatment (Gasch et al., 2000). A set of nine genes (*ALG14, DIN7, DUN1, RNR2, RNR4, PLM2, RAD54, FMP52, RAD51*) termed the "DNA Damage Signature" that is transcriptionally responsive only to DNA damaging agents was also identified in this study (Gasch et al., 2001). The overlap between our set of differentially responsive genes and genes involved in the ESR is extremely significant (Fisher's Exact Test, $p = 1x10^{-78}$), this

significance decreases, but is still maintained, when delta eta genes that are already changed basally are excluded (Fisher's Exact Test, $p=2x10^{-40}$). The overlap between our set of delta eta genes and the nine genes of the DNA Damage Signature is also significant (Fisher's Exact Test, p=0.04).

The delta eta metric identifies transcripts for which the difference in expression has changed between the wildtype and *anc1* strains after MMS treatment, but says nothing about the directionality of the change. To determine the directionality of the changes between the WTT/WTU ratio and the ancT/ancU we aligned heatmaps of the expression levels of ESR genes for these ratios (Figure 3-6). We observed that the regulation of genes involved in the ESR was abrogated in the *anc1* mutant strain, compared to the high level of regulation observed in the wildtype (Figure 3-6). That is, the WTT/WTU ratio shows an extensive increase/decrease in expression after MMS treatment, but these changes are muted in the *anc1* strain. The basal response (ancU/WTU) shows that expression of the large majority of ESR genes did not show changes basally between wildtype and *anc1* mutant strains (Figure 3-6). Similarly, none of the transcripts involved in the DNA damage signature show statistically significant basal expression changes (i.e. the expression of these transcripts does not change significantly between wildtype and *anc1* strains in the absence of MMS), and a majority show a significantly muted transcriptional response in the absence of *ANC1* (p=0.04) (data not shown).

Anc1 *modulates the Protein Expression of Sml1, a Downstream Member of the Mec1/Tel1 Signaling Pathway*

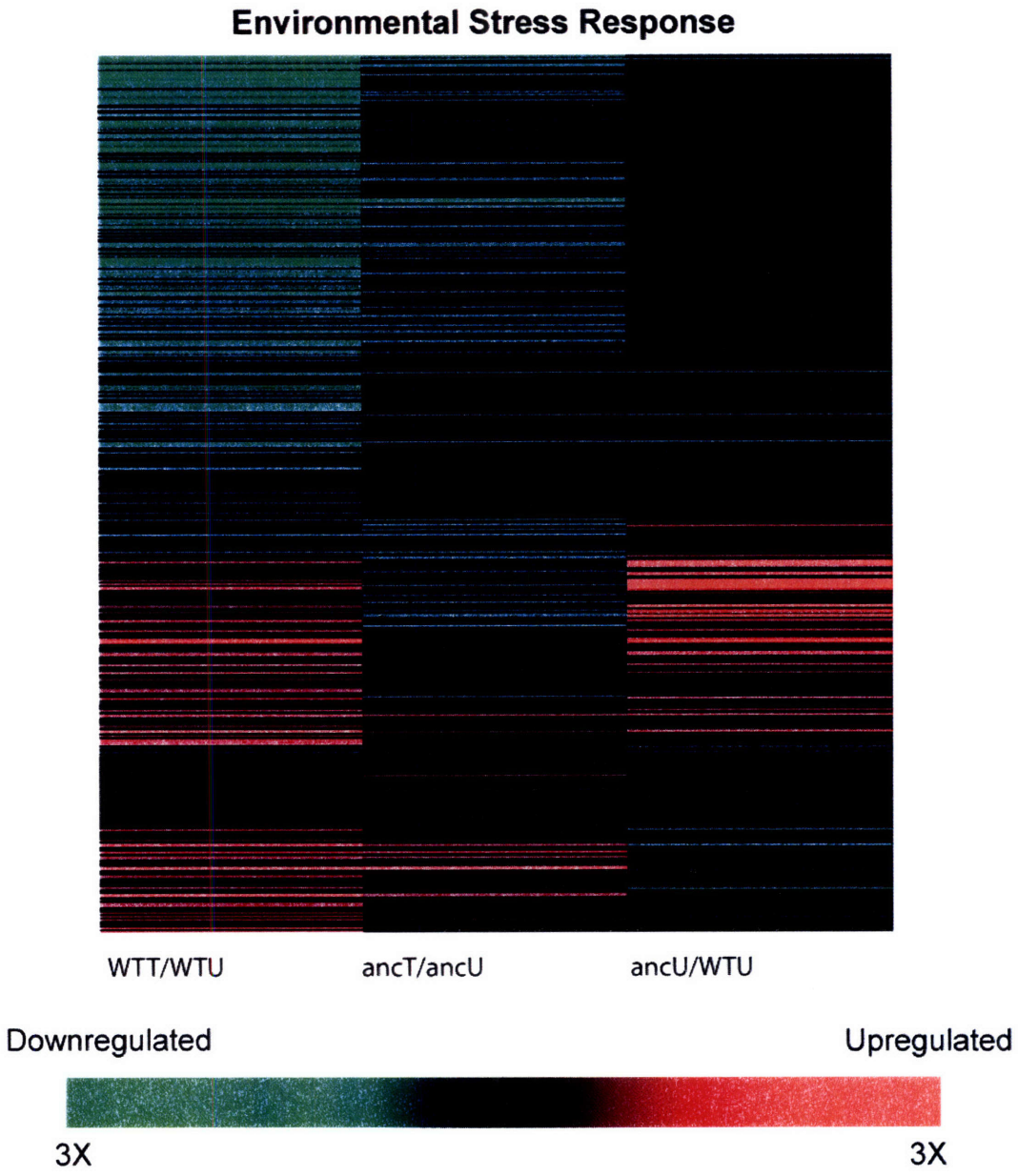


Figure 3-6: Transcriptional response of genes involved in the ESR. Transcriptional heatmap of the 904 genes involved in the ESR. Columns from left to right are: wildtype response to MMS, *anc1* response to MMS, and basal changes between WTU and ancU.

There are three lines of reasoning that lead us to explore the role of Anc1 in the Mec1-mediated DNA damage signaling pathway. First, Anc1 was identified in a screen for genes that, when overexpressed, allow for the survival of the usually lethal *mec1* and *rad53* mutations (Desany et al., 1998). Other deletion suppressors include proteins known to function downstream of Mec1 and Rad53 in the DNA damage signaling pathway, like Rnr1, as well as several transcriptional factors thought to function indirectly in the rescue (Desany et al., 1998). Secondly, Anc1 was observed to interact directly with the Dun1 kinase, a member of this signaling pathway (Ho et al., 2002). And finally, the abrogated ESR and DNA damage responses that we observe in *anc1* mutants treated with MMS is similar to the abrogation observed in *mec1* and *dun1* mutants (Gasch et al., 2001).

To determine if Anc1 has a role in the Mec1 signaling pathway we looked at protein expression and modification of a known downstream member of the Mec1 pathway, namely Sml1. Sml1 is a ribonucleotide reductase inhibitor that regulates dNTP production after DNA damage; its degradation after DNA damage allows for the production of nucleotides needed for DNA repair (Zhao et al., 2001). We chose Sml1 for analysis because of its moderate to high level of protein expression in wildtype cells (Ghaemmaghami et al., 2003), its Mec1-pathway dependent phosphorylation after DNA damage (Zhao et al., 2001; Zhao and Rothstein, 2002), and its direct interaction with Dun1 (which also interacts physically with Anc1) (Ghaemmaghami et al., 2003; Ho et al., 2002) (Figure 3-7A). Specifically, in response to DNA damage (i.e. MMS, UV, γ-irradiation), Sml1 is phosphorylated by Dun1 and, subsequently, degraded; DNA

Figure 3-7

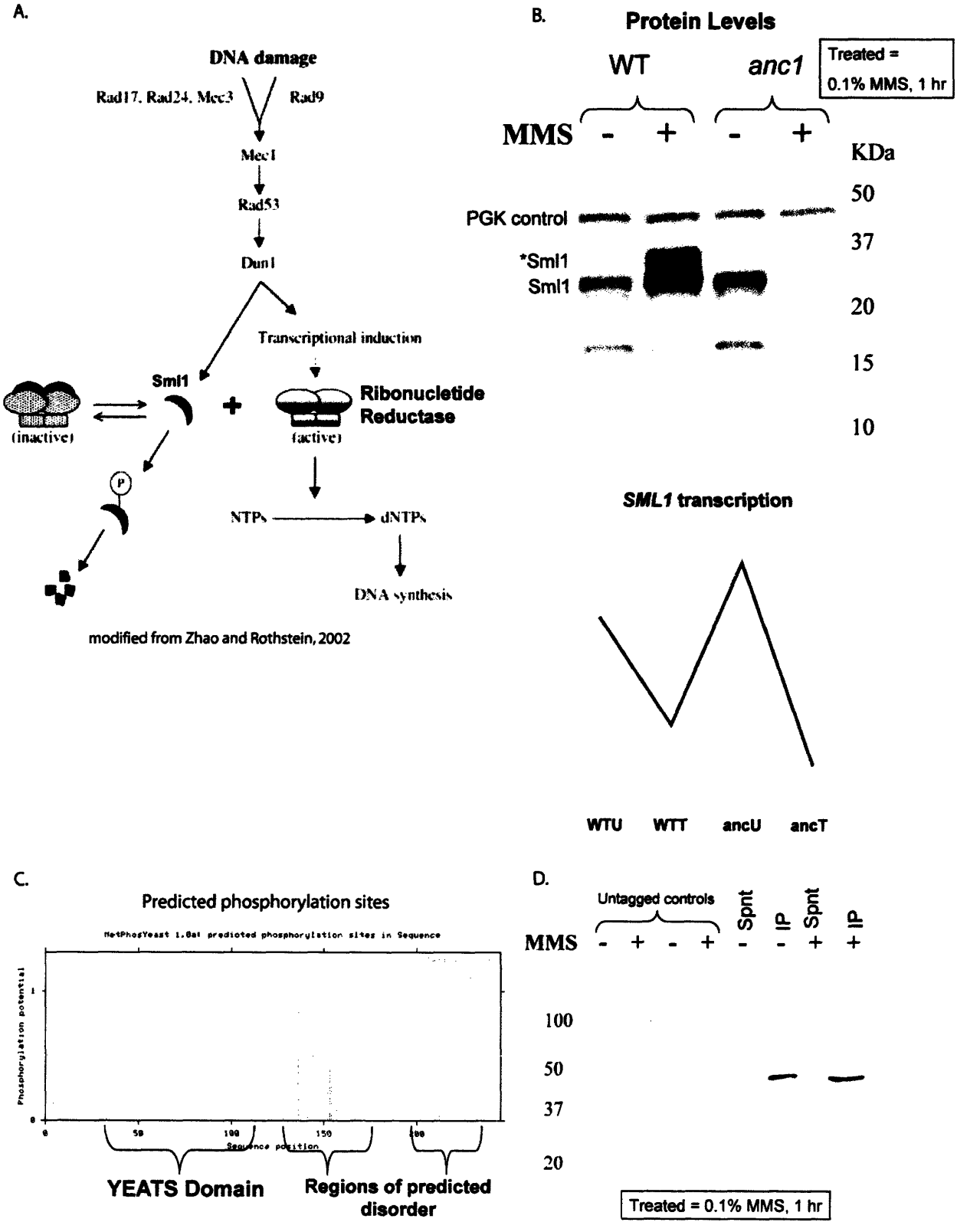

A.
DNA damage
Rad17, Rad24, Mec3
Rad9
Mec1
Rad53
Dun1
Transcriptional induction
Sml1
(inactive)
(active)
Ribonucleotide Reductase
P
NTPs
dNTPs
DNA synthesis
modified from Zhao and Rothstein, 2002
B.
Protein Levels
WT
anc1
Treated = 0.1% MMS, 1 hr
MMS
KDa
PGK control
*Sml1
Sml1
50
37
20
15
10
SML1 transcription
WTU
WTT
ancU
ancT
C.
Predicted phosphorylation sites
NetPhosYeast 1.0at predicted phosphorylation sites in Sequence
Phosphorylation potential
Sequence position
YEATS Domain
Regions of predicted disorder
D.
Untagged controls
Spnt
IP
Spnt
IP
MMS
100
50
37
20
Treated = 0.1% MMS, 1 hr

Figure 3-7: Posttranslational modification of Sml and Anc1 after MMS treatment. A. Model of Sml1 position within the Mec1-mediated damage response. Modified from Zhao and Rothstein, 2002. B. Western blot using antibody against Sml1-TAP, as described in Materials and Methods. Corresponding *SML1* transcription levels are shown below. C. Predicted phosphorylation sites for the translated *ANC1* ORF sequence. Predictions were determined using the NetPhosYeast algorithm at http://www.cbs.dtu.dk/services/NetPhosYeast/ (Ingrell et al., 2007). D. Western blot using antibody against immunoprecipitated Anc1-TAP, as described in Materials and Methods.

damage treated *dun1* strains show an accumulation of unphosphorylated Sml1 protein (Zhao and Rothstein, 2002) (diagrammed in Figure 3-7A).

Using an antibody against epitope-tagged Sml1 we monitored the expression of Sml1 protein in wildtype and *anc1* strains in the presence and absence of MMS treatment (Figure 3-7B). The expected phosphorylation of Sml1 was observed in the MMS treated wildtype cells, however the apparent increase in Sml1 protein levels in the wildtype treated sample is an artifact of this particular blot, and is not a consistent feature of this experiment (Figure 3-7B). Sml1 protein levels appear to be normal in untreated *anc1* cells, however in *anc1* cells that have been treated with MMS, the amount of Sml1 protein decreases, in contrast to wildtype cells, and its phosphorylation state cannot be observed due to the low protein levels (Figure 3-7B). Looking at transcript levels in MMS-treated and untreated cells, we observe that the gene expression of the *SML1* transcript decreases after MMS treatment in both wildtype and *anc1* backgrounds, but the extent of this change is not significantly different between the two strains (Figure 3-7B). We deduce, therefore, that the difference in protein expression observed in *anc1* cells is most likely regulated at the protein, not the transcript level. Two large-scale phosphoproteomic screens were recently completed in yeast, identifying roughly 900 protein phosphorylation substrates (Ficarro et al., 2002;

Gruhler et al., 2005); although Anc1 was not determined to be a phosphorylation substrate, an algorithm developed from these sets does predict several possible phosphorylation sites within the Anc1 protein (Ingrell et al., 2007) (Figure 3-7C). It is noteworthy that the majority of the predicted phosphorylation sites fall within the two predicted unstructured domains that are likely to interact with many protein partners (Figures 3-1 and 3-7C).

If Anc1 acts directly within the Mec1 signaling pathway, we might expect to see Anc1 act as a substrate for Mec1 or a downstream kinase. To determine if the Anc1 protein itself is modified in response to DNA damage, we immunoprecipitated epitope-tagged Anc1, and probed Anc1 with an antibody against the C-terminal epitope tag. We did not observe a shift in Anc1 that would have been indicative of posttranslational modification, although this does not rule out the possibility of a very small or faint shift not resolvable on a 2D gel (Figure 3-7D). We did observe a reproducible two-fold increase in Anc1 protein expression in the MMS-treated sample, but given the limitations of the immunoprecipitation assay, this may be a spurious result (Figure 3-7D).

ANC1 does not share an Epistasis Group with DUN1

Given the physical interaction between Dun1 and Anc1 (Ho et al., 2002), and the similar DNA damage sensitivity patterns between these two genes (Begley et al., 2002; Begley et al., 2004), we determined whether Anc1 and Dun1 operate in the same genetic pathway. To determine their genetic relationship we performed epistasis analysis using MMS sensitivity as the phenotype. We observed that *DUN1* and *ANC1*

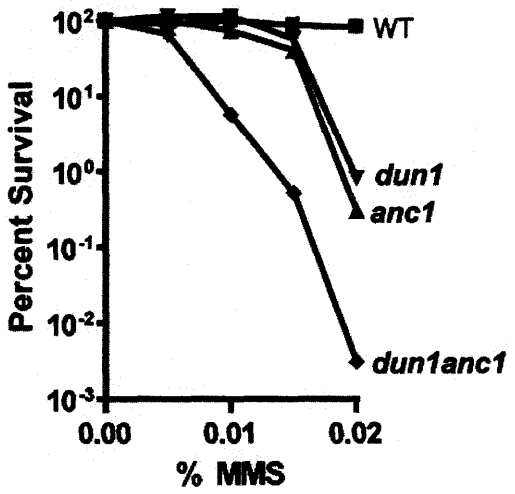


Figure 3-8: Epistasis analysis of *ANC1* and *DUN1*. Survival after chronic MMS treatment for: WT (■), *anc1* (▲), *dun1* (▼), *dun1anc1*(♦).

do not operate in the same epistasis group, and seem to display a synergistic interaction (Figure 3-8). This synergistic genetic interaction implies that Dun1 and Anc1 may operate in partially overlapping or parallel pathways, or possibly, they may have a more complex interaction.

Discussion

Mec1, the highly conserved homolog of the human ATR protein, has a critical role in signaling after DNA damage. It operates upstream of a variety of important responses to DNA damage, including cell cycle arrest, nucleotide production and DNA replication as well as the transcriptional regulation of the ESR and DNA Damage Signature (Gasch et al., 2000). Two pieces of evidence point to a role for Anc1 in modulating the Mec1-mediated signaling pathway's response to DNA damage. First, MMS-treated *anc1* cells, like *dun1* and *mec1* strains, lack some of the transcriptional changes normally observed after MMS treatment, although we known from our earlier cell cycle analysis (Figure 2-2), that the Mec1-mediated S-phase checkpoint is intact. Second, Sml1, a phosphorylation target of Dun1 (Zhao and Rothstein, 2002), is degraded prematurely in *anc1* cells after 1 hour of 0.1% MMS treatment.

If the absence of Anc1 were muting Dun1's phosphorylation of Sml1 in response to damage, we would expect to see an accumulation of unphosphorylated Sml1 (Zhao and Rothstein, 2002), however we see, instead, a premature degradation of Sml1. This may indicate that in the absence of Anc1, Dun1 constitutively phosphorylates Sml1 in the presence of DNA damage (at a higher rate than wildtype cells), leading to its abnormally fast degradation. Given Anc1's putative binding to histones through its

YEATS domain (Zeisig et al., 2005), and its direct interaction with Dun1, it also seems possible that the early degradation of Sml1 in *anc1* cells in response to MMS exposure may be due to destabilization of a protein complex containing Sml1, also a Dun1 interactor. Alternately, it is certainly possible that Anc1 is involved more directly in the signaling pathway. There is not, at this point, evidence that Anc1 itself is a member of the signaling pathway, despite its modulation of Sml1 regulation. Anc1 has several domains that are strongly predictive of phosphorylation (Ingrell et al., 2007), but, we observed no shift that would correspond to posttranslational modification in Anc1 after MMS treatment; it should be noted that these are preliminary results that need to be repeated. In addition, mass spectroscopy should be used to determine Anc1's posttranslational modifications with more precision.

We have noted that Anc1 is a protein hub, interacting with many more protein partners than the average yeast protein (Stark et al., 2006). The large majority of these proteins are involved in the process of RNA polymerase II transcription and the associated chromatin remodeling processes, but our data demonstrate that Anc1's physical interaction with Dun1, a protein outside of the transcriptional machinery, may also be highly relevant to its cellular function.

Furthermore, our epistasis data show that, despite their sensitivities to similar DNA damaging agents, Anc1 and Dun1 do not function in the same genetic pathway with respect to the cause for their MMS sensitivity, although they may function in partially overlapping pathways. Although the exact function of Anc1 with respect to the Mec1 signaling pathway has not yet been characterized, its function seems likely to be tied to its physical interaction with Dun1. Despite the lack of epistasis between *ANC1*

and *DUN1*, there are several pieces of evidence that tie the postreplicative repair pathway, of which Anc1 is a newly characterized member, and the Mec1 signaling pathway together functionally. It has been noted that the *MEC1* gene and the genes involved in the error-free postreplicative repair pathway (i.e. *SRS2, RAD5* and *RAD18*) act synergistically with respect to rates of chromosome loss and recombination, both markers of genetic stability (Smirnova and Klein, 2003). A putative role for Dun1 in the posttranscriptional regulation of Rad5 has also been described (Hammet et al., 2002). Specifically, it was found that Rad5 is upregulated in *dun1* mutant strains, and when *RAD5* is overexpressed, *dun1* strains have an increased sensitivity to the alkylating agent, hydroxyurea (Hammet et al., 2002). Although it seemed a likely possibility, the mechanism for the interaction between the Mec1 signaling pathway and postreplication repair is not a result of Anc1-mediated transcriptional regulation, as the members of this pathway showed neither basal nor differential regulation in the absence of Anc1.

Thus, our data demonstrate, using both transcriptional and protein assays, a role for Anc1 in modulating the Mec1 signaling pathway in its response to DNA damage. We have shown that in the absence of Anc1 the normal transcriptional response to treatment with DNA damage is absent, similar to strains lacking Mec1 and Dun1, and that Sml1, a downstream protein in the Mec1 pathway, is degraded improperly in the absence of Anc1. These data reveal an additional, novel role for Anc1 in the cellular response to DNA damage.

MATERIALS AND METHODS

Yeast Strains and Cultures

All strains were congenic with BY4741 (*MATa*, *his3*Δ1, *leu2*Δ0, *met15*Δ0, *ura3*Δ0) obtained from Invitrogen-ResGen. Cells were grown and maintained in YPD, under selection of Geneticin for strains containing $G418^R$, or in SC-URA- or SC-HIS- to select for the auxotrophic markers.

RNA Extraction

Three independent colonies of both wildtype and *anc1*::$G418^R$ were grown overnight, then diluted and grown into log phase for 4-5 hours in YPD. The cultures were then divided into treated and untreated samples. Treated samples were exposed to 0.1% MMS for 1 hour, then cells were pelleted and washed with water. RNA was extracted using Qiagen's RNeasy Mini Kit, checked for quality using an AgilentBioanalyzer and 20ug of total RNA were sent to Paradigm (now Cogenics) for hybridization on Affymetrix YG-898 microarrays.

Analysis of Microarray Data

Repair proficient and deficient strains were analyzed in triplicate on YG-S98 arrays. Normalization was carried out using the Robust Multichip Average (RMA) algorithm (Irizarry et al., 2003). Arrays were analyzed using Microarray Suite 5.0 to obtain Absent/Present calls and filtered for transcripts that were not expressed in any experiment. Differential gene expression was calculated using a dual filtering criteria; (1) an estimation of statistical significance through the Local Pooled Error test (LPE) (Jain et al., 2003) calculated using S-Plus Array Analyzer (ref) with an adjustment for false discovery rate calculation of p value of <0.05 (Benjamini Hochberg) and (2) a fold

change (FC) limit of 1.5. Differential responsiveness between strains was calculated using "delta eta" {$\log_2$ (WTT treated/WTU)- $\log_2$ (anc1T/anc1U)} with a FC greater than 1.5 identifying significance in magnitude of differential transcript modulation in response to treatment. Gene Ontologies were determined using online FUNSPEC algorithms at http://funspec.med.utoronto.ca/ (Robinson et al., 2002).

Western Blot

Log-phase wildtype and *anc1* cells were treated with 0.1% MMS for one hour (as in the microarray experiment), then proteins were isolated and diluted to a common concentration using BCA Protein Assay Kit (Pierce), and confirmed by Ponceau staining. Samples were loaded on Tris-HCl 12% gels (BioRad). The TAP-tagged Sml1 protein was probed with Peroxidase-anti-Peroxidase antibody (Sigma), and PGK was probed with anti 3-phophoglycerate kinase (yeast) mouse IgG, monoclonal antibody (Molecular Probes). Anc1-TAP was immunoprecipitated using IgG Sepharose 6 Fast Flow (Amersham Biosciences).

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Table 3-1: ORFs downregulated in response to MMS in wildtype (3X cutoff). (Robinson et al, 2002)

GO Biological Processes	p-value
biosynthesis [GO:0009058]	2.47E-12
cell growth and/or maintenance [GO:0008151]	4.83E-11
protein biosynthesis [GO:0006412]	1.42E-10
macromolecule biosynthesis [GO:0009059]	2.40E-10
ribosome biogenesis and assembly [GO:0042254]	3.94E-09
metabolism [GO:0008152]	2.30E-07
ribosomal subunit assembly [GO:0042257]	3.34E-07
ribosome assembly [GO:0042255]	5.20E-07
ribonucleotide metabolism [GO:0009259]	1.81E-06
purine ribonucleotide metabolism [GO:0009150]	1.81E-06
ribosome biogenesis [GO:0007046]	4.07E-06
transcription from Pol I promoter [GO:0006360]	6.13E-06
purine nucleotide metabolism [GO:0006163]	7.75E-06
nucleotide metabolism [GO:0009117]	1.20E-05
purine ribonucleotide biosynthesis [GO:0009152]	1.54E-05
ribonucleotide biosynthesis [GO:0009260]	1.54E-05
nucleotide biosynthesis [GO:0009165]	3.36E-05
GTP metabolism [GO:0046039]	3.66E-05
purine ribonucleoside triphosphate metabolism [GO:0009205]	3.66E-05
ribonucleoside triphosphate biosynthesis [GO:0009201]	3.66E-05
purine nucleoside triphosphate biosynthesis [GO:0009145]	3.66E-05
purine ribonucleoside triphosphate biosynthesis [GO:0009206]	3.66E-05
GTP biosynthesis [GO:0006183]	3.66E-05
ribonucleoside triphosphate metabolism [GO:0009199]	3.66E-05
purine nucleoside triphosphate metabolism [GO:0009144]	3.66E-05
purine nucleotide biosynthesis [GO:0006164]	6.16E-05
ribosomal large subunit assembly and maintenance [GO:0000027]	0.0001259
nucleoside triphosphate biosynthesis [GO:0009142]	0.0001427
steroid metabolism [GO:0008202]	0.0003029
sterol biosynthesis [GO:0016126]	0.0003197
nucleoside triphosphate metabolism [GO:0009141]	0.000348
lipid metabolism [GO:0006629]	0.0004477
rRNA processing [GO:0006364]	0.0004565
sterol metabolism [GO:0016125]	0.0005611
ribosomal small subunit assembly and maintenance [GO:0000028]	0.000678
organic acid biosynthesis [GO:0016053]	0.0009246
carboxylic acid biosynthesis [GO:0046394]	0.0009246
steroid biosynthesis [GO:0006694]	0.001079
ergosterol metabolism [GO:0008204]	0.001211
ergosterol biosynthesis [GO:0006696]	0.001211
regulation of CDK activity [GO:0000079]	0.001228

lipid biosynthesis [GO:0008610]	0.001482
nucleobase metabolism [GO:0009112]	0.002065
aerobic respiration [GO:0009060]	0.00216
fatty acid biosynthesis [GO:0006633]	0.002646
cellular respiration [GO:0045333]	0.00266
purine nucleoside monophosphate metabolism [GO:0009126]	0.003688
ribonucleoside monophosphate metabolism [GO:0009161]	0.003688
purine ribonucleoside monophosphate metabolism [GO:0009167]	0.003688
oxidative phosphorylation, ubiquinone to cytochrome c [GO:0006122]	0.003688
methionine metabolism [GO:0006555]	0.003824
electron transport [GO:0006118]	0.003824
nucleoside monophosphate metabolism [GO:0009123]	0.004948
fatty acid elongation [GO:0030497]	0.006353
purine base metabolism [GO:0006144]	0.008164

Table 3-2: ORFs upregulated in response to MMS in wildtype (3X cutoff). (Robinson et al, 2002)

GO Biological Processes	p-value
ubiquitin-dependent protein catabolism [GO:0006511]	2.89E-09
protein-ligand dependent protein catabolism [GO:0019941]	1.29E-08
protein catabolism [GO:0030163]	2.32E-08
proteolysis and peptidolysis [GO:0006508]	7.34E-08
nitrogen metabolism [GO:0006807]	4.87E-07
arginine biosynthesis [GO:0006526]	9.59E-07
urea cycle intermediate metabolism [GO:0000051]	1.49E-06
macromolecule catabolism [GO:0009057]	2.78E-06
response to stress [GO:0006950]	6.42E-06
catabolism [GO:0009056]	2.64E-05
siderochrome transport [GO:0015891]	5.30E-05
iron-siderochrome transport [GO:0015892]	5.30E-05
arginine metabolism [GO:0006525]	6.14E-05
protein folding [GO:0006457]	7.16E-05
amino acid biosynthesis [GO:0008652]	0.0001319
amine biosynthesis [GO:0009309]	0.0002579
glutamine family amino acid biosynthesis [GO:0009084]	0.000295
amino acid metabolism [GO:0006520]	0.0004321
aldehyde metabolism [GO:0006081]	0.0004834
nucleotide-excision repair, DNA damage recognition [GO:0000715]	0.000808
multidrug transport [GO:0006855]	0.000808
response to chemical substance [GO:0042221]	0.0008633
amino acid and derivative metabolism [GO:0006519]	0.0008638
amine metabolism [GO:0009308]	0.0009985
ornithine metabolism [GO:0006591]	0.001563
non-protein amino acid metabolism [GO:0019794]	0.001563
nucleotide-excision repair [GO:0006289]	0.001741
iron homeostasis [GO:0006879]	0.001876
trehalose metabolism [GO:0005991]	0.002646
drug transport [GO:0015893]	0.002646
response to drug [GO:0042493]	0.002884
glutamine family amino acid metabolism [GO:0009064]	0.003398
nitrogen utilization [GO:0019740]	0.004095
aspartate family amino acid biosynthesis [GO:0009067]	0.004517
response to abiotic stimulus [GO:0009628]	0.004813
trehalose catabolism [GO:0005993]	0.00571
energy reserve metabolism [GO:0006112]	0.009482

Chapter 4: Conclusions

At the outset of this study, the only characterized roles for Anc1 were as members of complexes involved in RNA pol II transcription, and as a potential mediator of actin function. A recent screen, however, showed that *anc1* cells have a strong and distinctive pattern of DNA damage sensitivity (Begley et al. 2002; Begley et al. 2004), indicating that the wildtype *ANC1* gene or its products somehow help cells to survive in the presence of DNA damage. In addition to its role in transcription, Anc1 was an object of particular interest because of its homology to three genes involved in human acute leukemias. Thus, we set out to learn more about Anc1's function.

To this end, we began by analyzing the Anc1-containing complexes (TFIID, TFIIF, Mediator, SWI/SNF, RSC, INO80 and NuA3) to see if mutants in any other members of these complexes share *anc1*'s pattern of DNA damage sensitivity. We reasoned that if the other components of a complex contributed to MMS, 4NQO and/or UV resistance, that the entire complex may be involved in the same role as Anc1 in survival after DNA damage. Since Anc1 is the only non-essential member of TFIID and TFIIF, we were only able to conduct this analysis for the other five complexes. We determined that the deleted non-essential members of Mediator, SWI/SNF, RSC and INO80 all had similar DNA damage sensitivities to *anc1*. This indicates, although not conclusively, that Anc1's role in survival after DNA damage may take place in the context of several of its transcription-associated complexes.

Our analysis of Anc1's function in DNA damage-induced cell cycle arrest allowed us to determine whether the Mec1-mediated S-phase arrest is intact in *anc1* cells. While we observed that *anc1* cells are not deficient in this cell cycle checkpoint in the

manner of *mec1* and *rad53* cells, the response of *anc1* cells to DNA damage was not entirely normal. Untreated *anc1* cells spend more time in G1 than wildtype cells, and after treatment, their entry into S-phase and passage into G2 is even more delayed than that of wildtype. This phenotype could be due to a variety of factors, ranging from a role for Anc1 in leaving G1 to a role for Anc1 in removing DNA lesions to an artifact of *anc1*'s slow growth.

We analyzed the membership of Anc1 in known DNA repair pathways (Base Excision Repair, Nucleotide Excision Repair, Homologous Recombination, Transcription Coupled Repair, and Postreplication Repair) using epistasis testing. In addition to epistasis with *RAD6*, *ANC1* showed epistasis with *RAD5*, a DNA dependent ATPase of the SWI/SNF family that is a member of the postreplication repair (PRR) pathway. Anc1 interacts with DNA-dependent ATPases in the context of several of its protein complexes (SWI/SNF, RSC and INO80); it is possible that the putative interaction between Anc1's YEATS domain and histones serves to localize these ATPases to their substrates. Epistasis testing also showed that, like members of the error-free branch of PRR, the deletion of *srs2* suppressed the MMS sensitivity *anc1* cells. These epistasis results, along with the synergistic relationship between *ANC1* and the other error-free pathway members, show that *ANC1* defines a new branch of error-free PRR that is *RAD5*-dependent, but *MMS2*- and *UBC13*-independent. Mutagenesis data backs up the placement of *ANC1* in the error-free pathway, as its absence increases point and frameshift mutagenesis.

Another piece of evidence that backs *ANC1*'s placement in the error-free branch of PRR is its role in suppressing the expansion of triplet repeats. It was recently shown

that in cells deleted for error-free branch members, there is an expansion of disease-associated triplet repeats (Daee et al. 2007), and the results for *anc1* cells are consistent with the earlier findings. The molecular basis for the expansion of CAG triplet-repeats, associated with Huntington's Disease, is still being worked out, and given this recent data, it seems that the error-free branch of PRR may play a role in this process, although its mechanism is not yet understood. The prospect of a role for DNA repair in maintaining the length of triplet repeats is not surprising, although how the recombination-based error-free branch is involved in this process is not immediately clear.

The deletion of *ANC1* is synthetically lethal with the deletions of two genes involved in DNA repair: *YAF9*, and *DST1* (Zhang et al. 2004; Fish et al. 2006). Yaf9 is another member of the YEATS family whose DNA repair function is linked to its membership in the NuA4 complex, and *DST1* encodes TFIIS, a general transcription factor that helps resolve stalled replication forks (Zhang et al. 2004; Fish et al. 2006). Given Anc1's newly established role in PRR, these synthetic lethalities can be better understood: both of these genes operate in pathways that may be at least partially redundant with Anc1's function in PRR for an essential cellular function, thus, when both pathways are absent, the cell cannot survive.

Anc1 has an uncommonly large number of protein interactors (85), most of which are associated with its role in transcription, though a few hint at a possible role in cell signaling. The YEATS domain, in Anc1's human homolog ENL, has been shown to interact directly with histones 1 and 3 (Zeisig et al. 2005), and, given the high degree of conservation in this domain, we expect that Anc1's YEATS domain has a similar

function. We analyzed the Anc1 protein for regions predicted to be unstructured, and for sites predicted to be phosphorylated (Romero et al. 1997; Li et al. 1999; Romero et al. 2001; Ingrell et al. 2007). There was considerable overlap between these predictions: there are two main regions predicted to be unstructured in Anc1, and these regions correspond to regions with a high density of predicted phosphorylation sites. It is thought that that unstructured protein domains encourage the quick and reversible binding of proteins to a broad range of interaction sites in response to changes in the cellular environment (Dunker et al. 2002). We suspect that these regions, which lie in the middle and C-terminal portion of the Anc1 protein, are the domains through which Anc1 makes the majority of its protein-protein interactions, and that the YEATS domain functions to bring these complexes in proximity to chromatin.

The transcriptional response of cells to MMS treatment has been characterized previously (Jelinsky and Samson 1999; Jelinsky et al. 2000), and we were eager to learn whether Anc1, with its many roles in transcription-related complexes, has a function in regulating the global transcriptional response to DNA damage. We discovered that the transcriptional response of *anc1* cells to MMS treatment did not exhibit the transcriptional changes that would be expected after MMS-treatment or other environmental stressors. The ~900 genes that are transcriptionally responsive to environmental stress have been previously identified, and were shown to be under the control of the Mec1 signaling pathway (i.e. *mec1* and *dun1* cells are transcriptionally unresponsive to environmental stress) (Gasch et al. 2000; Gasch et al. 2001). Thus, Anc1 plays an extensive role in mediating the transcriptional response to MMS treatment.

Given the similarity between *anc1*'s transcriptional response to MMS and those of *mec1* and *dun1*, the physical interaction between Anc1 and Dun1 (Ho et al. 2002), and earlier data showing that an *ANC1* overexpression strain can suppress the lethality of *mec1* cells (Desany et al. 1998), we postulated a role for Anc1 in mediating the Mec1 signaling pathway. We selected Sml1, a protein known to be phosphorylated by Dun1 after DNA damage (Zhao et al. 2001; Zhao and Rothstein 2002), for this purpose. Although Sml1 levels in untreated *anc1* cells were similar to those found in untreated wildtype cells, after MMS treatment, Sml1 was almost entirely absent in *anc1* cells, rather than present and phosphorylated, as it is in wildtype. We show that this regulation of Sml1 levels takes place at the protein, not the transcript levels, as the level of *SML1* transcript in *anc1* cells is not significantly different from that of wildtype. We suspect that this abnormally fast degradation of the Sml1 protein after DNA damage is linked to Anc1's interaction with Dun1, but the mechanism by which this interaction may affect the Mec1 signaling pathway is as yet unknown

In summary, we have examined a gene, *ANC1*, whose function in survival after DNA damage was previously uncharacterized, and have made considerable progress towards describing its contribution to the DNA damage response. We have shown that *ANC1* is a member of the postreplication repair pathway, and that within this pathway, it defines a previously undiscovered branch of error-free repair. Furthermore, we have described a role for Anc1 in the genome-wide transcriptional response to environmental stress, and shown that proper Anc1 function has consequences for protein-level changes as well. Given the homology of Anc1 to three human genes involved in

chemically-associated leukemias, we are hopeful that this new understanding of Anc1's role in repair may shed light on the function of its homologs in human disease.

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