Role and regulation of yeast Hrq1 and its human homolog, RECQL4, during DNA crosslink repair

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Human RECQL4 is a member of the RecQ family of DNA helicases and functions during DNA replication and repair. *RECQL4* mutations are associated with developmental defects and cancer. Although *RECQL4* mutations lead to disease, *RECQL4* overexpression is also observed in cancer, including breast and prostate. Thus, tight regulation of RECQL4 protein levels is crucial for genome stability. Because mammalian *RECQL4* is essential, how cells regulate RECQL4 protein levels is largely unknown. Utilizing budding yeast, we investigated the *RECQL4* homolog, *HRQ1*, during DNA crosslink repair. We find that Hrq1 functions to mediate bypass of intrastrand crosslinks. Although Hrq1 is involved in the processing of cisplatin-induced lesions, it is paradoxically degraded by the proteasome following cisplatin treatment. By identifying the targeted lysine residues, we show that preventing Hrq1 degradation results in increased recombination and mutagenesis following cisplatin exposure. Like yeast, human RECQL4 is similarly degraded upon exposure to crosslinking agents and over-expression of *RECQL4* results in increased RAD51 foci and mutagenesis. Using bioinformatic approaches, we demonstrate a link between *RECQL4* overexpression and good clinical response in cisplatin-treated triple negative breast cancers. Overall, our study uncovers a role for Hrq1/RECQL4 in DNA intrastrand crosslink processing and its protein regulation through proteasomal degradation.

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Glossary

5-Fluoroorotic Acid (5-FOA)

AP- Apurinic

ATM- Ataxia-telangiectasia mutated

BER- Base excision repair

BGS- Baller-Gerold syndrome

CldU - 5-chloro-2'-deoxyuridine

CPD- Cyclobutane pyrimidine dimers

CMG- CDC45-MCM2-7-GINS

DPC – DNA protein crosslink

DSB- Double-strand break

DSBR- Double-strand break repair

EdU- 5-ethynyl-2'-deoxyuridine

ER- Estrogen receptor

HPRT- Hypoxanthine-guanine phosphoribosyltransferase

IdU- 5-iodo-2'-deoxyuridine

IP- Immunoprecipitation

IR- Ionizing radiation

METABRIC- Molecular Taxonomy of Breast Cancer International Consortium

MLS- Mitochondrial localization sequence

mtDNA- Mitochondria DNA

NER- Nucleotide excision repair

NHEJ- Non-Homologous End Joining

NLS- Nuclear localization sequence

PARP- poly (ADP-ribose) polymerase

RTS- Rothmund-Thomson syndrome

xRTS- Xenopus RECQL4

SCE- Sister chromatid exchange

TCGA – The Cancer Genome Atlas

TNBC- Triple negative breast cancer

TIFs- Telomere dysfunction-induced foci

UV- Ultraviolet

VCP- Valosin-containing protein

Preface

To all who provided guidance and support:

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To my mom and cousin, I tried my best, this is for you.

Thank you.

1.0 Introduction

1.1 RECQL4

Maintaining genomic stability is crucial for preserving genetic information and preventing disease. The stability of our genome is constantly threated by exogenous and endogenous sources, such as ultraviolet radiation and reactive aldehydes, respectively. Our cells have developed an intricate system of proteins and pathways to counteract these insults. One such family of proteins is the evolutionarily conserved RecQ helicases, consisting of RECQL1, RECQL4, RECQL5, Bloom syndrome protein (BLM), and Werner syndrome ATP-dependent helicase (WRN) (**Figure 1a**). This family of 3' to 5' DNA helicases are referred to as the "Guardians of the Genome" through crucial roles in DNA recombination, replication, and repair [1–6].

One critical member of this helicase family is encoded by *RECQL4* gene, which is located on chromosome 8q24.3. The resulting 1208 amino acid protein has a conserved core helicase domain, however RECQL4 is quite different from the other RecQ helicases as it does not contain the conserved helicase and RNAse D C-terminal (HRDC) domain, which is needed for putative DNA binding (**Figure 1a**). Furthermore, RECQL4 has a strong DNA annealing activity, so much so that it was thought RECQL4 did not have a helicase activity. However, RECQL4 ATP-dependent helicase activity was later shown, when *in vitro* helicase assays were performed in the presence of excess complementary oligonucleotide to prevent re-annealing of the unwound strand [7]. The RecQ helicases, RECQL1, BLM, and WRN are nuclear and RECQL5 is both nuclear and cytoplasmic. RECQL4 is unique in that it can be localized to the cytoplasm, nucleus, and mitochondria [6]. RECQL4 cytoplasmic localization is regulated by acetylation at K376, K380,

K382, K385, K386 by p300 [8]. Moreover, RECQL4 is the only RecQ helicase with a mitochondrial localization signal (MLS) and is critical for upkeeping the mitochondrial genome (discussed below; **Figure 1b**).

Underscoring its many cellular functions, mutations in RECQL4 are linked to three hereditary diseases: Rothmund-Thomson syndrome (RTS), Baller-Gerold syndrome (BGS) and RAPADILINO [9,10].

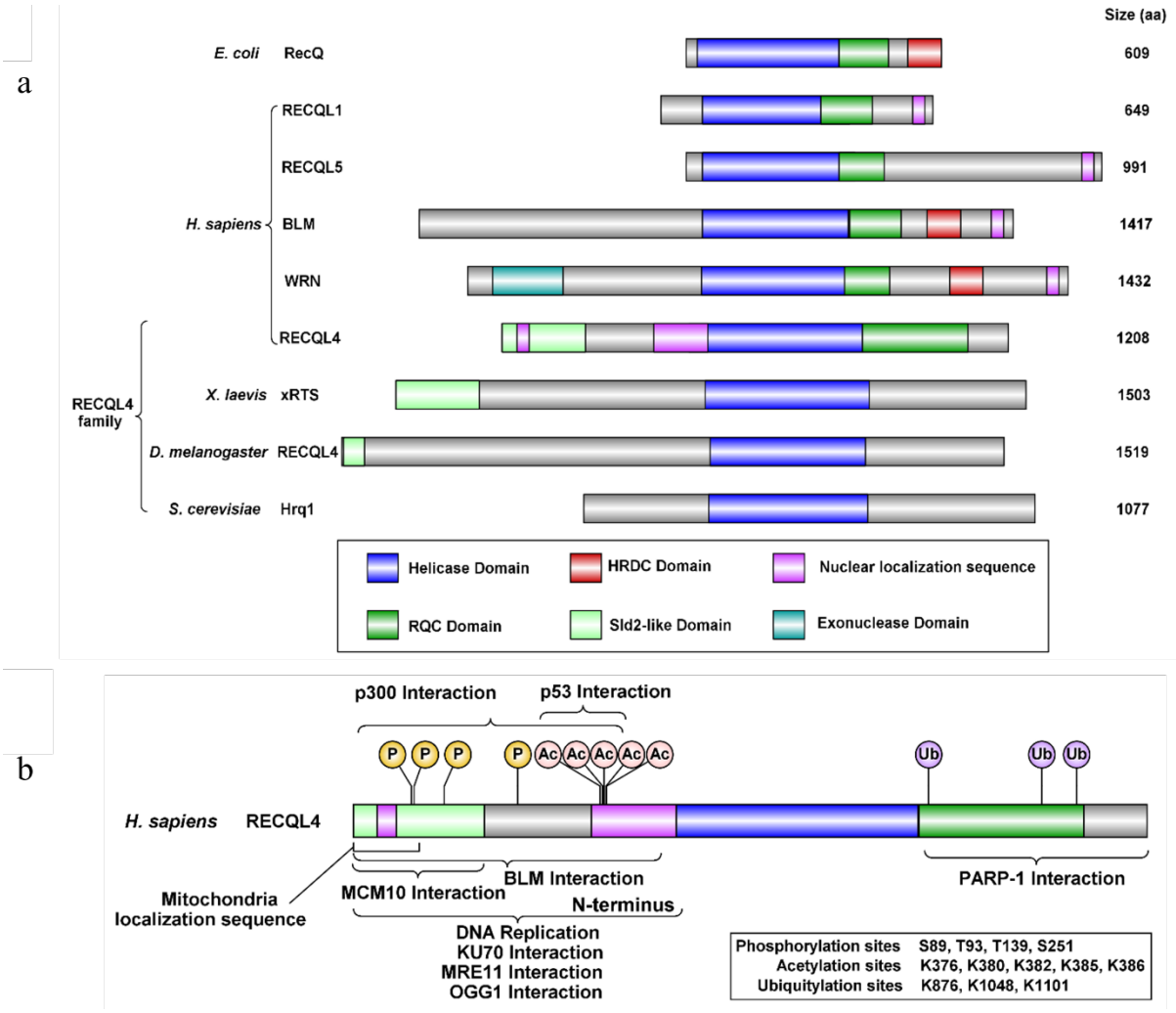

Figure 1. Structural features of RecQ helicases

a) The RecQ proteins have domains that are conserved from bacteria through humans. The core helicase domain (dark blue box) is found throughout the protein family. However, unlike the other members the RECQ family, RECQL4 does not contain the HRDC (red box) domain. With the exception of yeast, the RECQL4 family (including *H. sapiens, X. laevis, D. melanogaster*) have a conserved Sld2 (synthetic lethal to Dbp11-1)-like domain, which is crucial replication initiation (light green box). In yeast, Sld2 is encoded as a separate protein. The nuclear localization sequence is indicated by a purple box and the exonuclease domain of WRN with a light blue box. b) Interaction domains and post-translational modified regions of RECQL4. The mitochondrial localization sequence is located in the first 100 amino acid of RECQL4. Protein interactions with RECQL4 with BLM, MCM10, KU70, MRE11, OGG1, p300, p53, and PARP-1 are indicated by brackets. Phosphorylation sites (S89, T93, T139, S251) are indicated by a

yellow circled P, acetylation sites (K376, K380, K382, K385, K386) by pink circled Ac, and ubiquitylation sites (K876, K1048, K1101) by purple circled Ub. Created by using Illustrator for Biological Sequences (IBS) [11].

1.2 Relevance of RECQL4 to human health

RECQL4 is part of the evolutionarily conserved RecQ family of DNA helicases. This family has been referred to as the "Guardians of the Genomes" due to their roles in mediating DNA recombination, replication, and repair [1–6]. Consistent with a role in maintaining genomic stability RECQL4 dysfunction is associated with multiple diseases which are described below. It is still not understood how RECQL4 dysfunction can result in three different diseases, however when possible, we discussed potential mechanisms underlying the disease etiology.

1.2.1 Rothmund-Thomson syndrome

Rothmund-Thomson syndrome (RTS) is an autosomal recessive disorder that is extremely rare with approximately 500 reported cases [12]. RTS is characterized by poikiloderma, developmental abnormalities, cataracts, premature aging, and increased cancer predisposition, particularly osteosarcomas. Symptoms of RTS were first described in 1868 by Rothmund and then later in 1936 by Thomson [13,14]. In 1957, Taylor decided to group these two findings and classified the disease as RTS [15]. One of the main features of RTS is chromosomal aberrations including chromosomal rearrangements and aneuploidy [16–18]. Despite evidence for a genetic cause for RTS, the gene responsible for RTS remained elusive until the late 1990s. In 1998, the Shimamoto group cloned RECQL4 in a study to find new human RecQ helicases [19]. A year later

in 1999 the Furuichi group identified that a subset of RTS patients had mutations in *RECQL4* [20]. RECQL4 mutations range from frameshift, splicing and nonsense, which commonly leads to a truncated helicase domain [9,10]. Two-thirds of RTS patients are associated with mutations in RECQL4 and are referred to as RTS type II. The other third of RTS patients, referred to as RTS type I, are associated with mutations in another gene, anaphase promoting complex 1 (ANAPC1) or other yet to be identified gene(s) [21,22].

1.2.2 Baller-Gerold syndrome

Like RTS, Baller-Gerold syndrome (BGS) is a rare autosomal recessive disease. It was originally described by Baller in 1950 and later by Gerold in 1959 [23,24]. The prevalence is currently unknown and fewer than forty cases have been reported [25–28]. The most common RECQL4 mutations observed in BGS patients is a C-terminal missense mutation, R1021W, and a frame shift mutation in exon 9 which deletes nucleotide number 2886 (2886 delta T frameshift mutation). BGS symptoms share similarities to RTS including poikiloderma, developmental and radial ray defects. However, unlike RTS patients, BGS is associated with coronal craniosynostosis. Alongside RECQL4, mutations in fibroblast growth factor receptor 2, FGFR2, and the transcription factor, TWIST, have also been implicated in the development of BGS [29–31].

1.2.3 RAPADILINO

RAPADILINO was first described in 1989 by Kaariainen et al, when they coined the name RAPADILINO after an acronym related to the patients' syndromes. RA stands for radial ray malformations, PA for absent/hypoplastic patellae and cleft/high arched palate, DI for diarrhea and

dislocated joints, LI for limb abnormalities and little size, and NO for slender nose and normal intelligence [32]. RAPADILINO is an autosomal recessive disease which share similarities with RTS as its characterized by short stature, radial ray defects, predisposition to osteosarcoma and lymphoma. However, unlike RTS patients, RAPADILINO is also associated with palatal abnormalities as well as joint dislocation. Unlike RTS and BGS, RECQL4 in the only gene affiliated with RAPADILINO. Whereas RTS patients often have a truncated RECQL4 helicase domain (exon 8-14), the most common mutation in RAPADILINO leads to deletion of exon 7, which leaves the helicase domain intact but disrupts the second NLS of RECQL4 (Figure 1) [33]. Although the overall prevalence of RAPADILINO is unknown, it is most frequently observed in Finnish population [31]. The similarities and differences between these diseases are summarized below (**Figure 2)**.

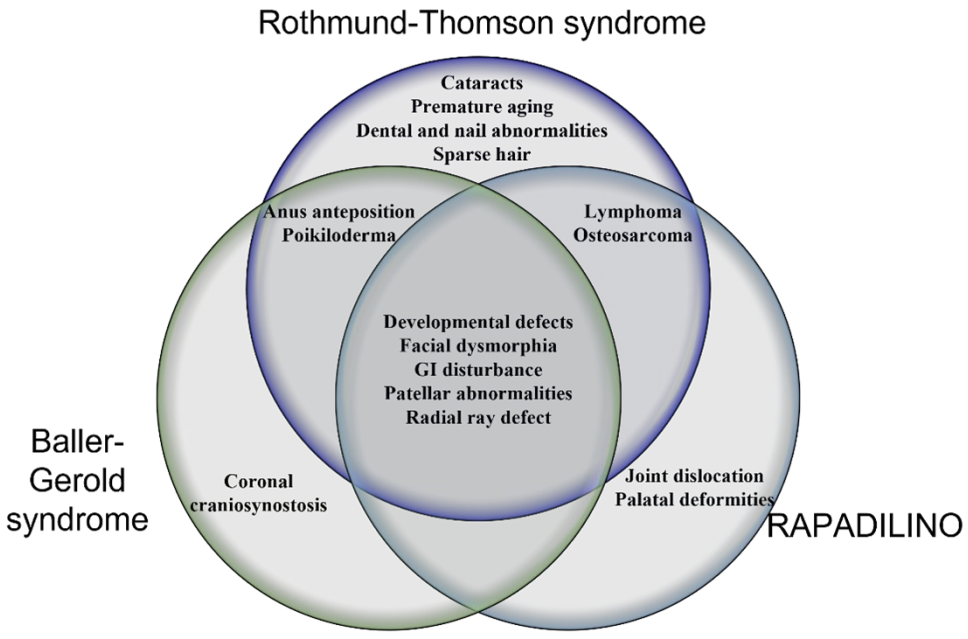

Figure 2. Venn diagram illustrating the similarities and differences between the three RECQL4 related diseases

1.2.4 RECQL4 in cancer

Loss of RECQL4 function is associated with chromosomal instability, which is a driver of cancer [34,35]. A recent case study of five families determined that pathogenic mutation in the helicase domain of RECQL4 is highly associated with osteosarcoma [36]. It has been suggested that RECQL4 is needed for normal osteoblast expansion and differentiation. Thus disruption to RECQL4 helicase activity, can lead to disruption of RECQL4 function in DNA repair in these cells which would promote genomic instability and can ultimately result in cancer [37]. In contrast, RTS patients with mutations outside that the helicase domain develop mild symptoms and do not develop cancer [36]. This suggests that the helicase function of RECQL4 is critical for maintenance of genomic stability and subsequent cancer prevention. However, there was a rare case in which a RAPADILINO patient had a mutation outside the helicase domain but still developed lymphoma [33]. Although patients with RECQL4 loss of function are predisposed to osteosarcomas, RECQL4 overexpression is also observed in several cancers including breast, cervical, gastric, oral, osteosarcoma, and prostate [38–44]. RECQL4 is associated with mediating different survival factors to promote cancer growth such as the apoptosis inhibitor, SURVIVIN, and the tumor suppressor, p53. SURVIVIN interacts with RECQL4 and RECQL4 knockdown results in reduced SURVIVIN expression [40]. RECQL4 also interacts with p53 and can sequester its transcriptional activity, ([45]; see the mitochondria section, Table 1). Consistent with promotion of tumor cell growth, RECQL4 over-expression is correlated with tumor aggressiveness in breast and prostate cancer and its suppression results in reduced proliferation [39,43]. Strikingly, in a prostate cancer focused study, suppressing RECQL4 to levels observed in non-malignant human epithelial cells is sufficient to reduce invasive growth and tumorigenic potential [39]. Another strategy that cancer cells use to survive is through the upregulation of drug efflux pumps, which

reduce the intracellular concentration of toxic chemotherapeutics [46]. Strikingly, RECQL4 transcriptionally activates the efflux pump, MDR1, through RECQL4 interaction with transcription factor, YB1. Transcriptional activation of MDR1 by YB1 resulted in cisplatin resistance [44]. Besides its role in modulating proteins critical for cellular survival and drug resistance, RECQL4 can also support cancer progression and survival by its replicative and DNA repair roles as discussed below. Intriguingly, RECQL4 is located on chromosome 8q24, which is a particular hotspot for overexpression in cancer as it is near the oncogene, c-Myc [37,47]. It is unclear whether RECQL4 upregulation is simply a byproduct of being near c-Myc or purely coincidental.

1.3 Multifaceted role in genomic stability

As RECQL4 is associated with a multitude of diseases, RECQL4 has diverse roles in maintaining genomic stability and cellular homeostasis. Below we discuss the known roles of RECQL4 before leading into our studies deciphering role and regulation of Hrq1 and RECQL4 in mediating DNA crosslink repair. **Figure 3** summarizes the diverse functions of RECQL4 discussed.

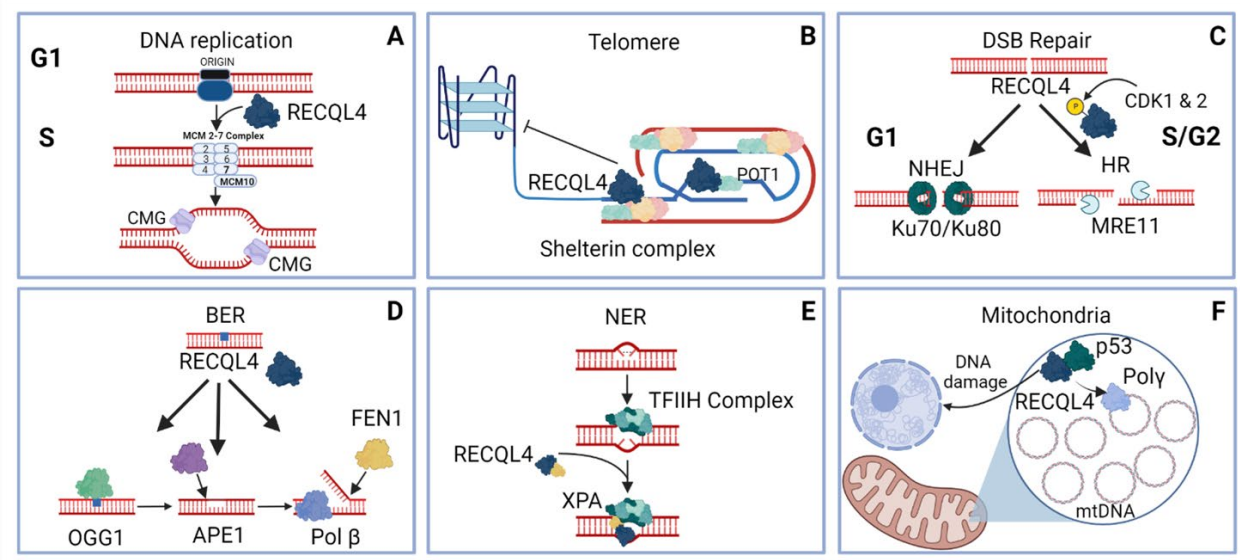

Figure 3. RECQL4 roles in genomic maintenance

a) RECQL4 (dark blue) is critical for replication initiation, RECQL4/xRTS is loaded following the pre-RC to mediate loading of DNA polymerase α and the CMG complex (light purple). The origin is indicated by a black and dark blue boxes and the MCM complex, consisting of MCM2-7, is shown with light blue boxes and MCM10 in a light blue box as well. b) RECQL4 interacts with the Shelterin complex [POT1-TRF1-TRF2 (teal-beige-pink proteins, respectively)] to resolve D-loops (blue and red DNA structure on right) and G-quadruplexes (light blue structured DNA on left). c) RECQL4 participates in DSB repair in a cell cycle dependent manner. RECQL4 functions with the KU complex (green circle) during G1 to mediate DNA end joining (NHEJ). During S/G2, RECQL4 cooperates with MRE11 (light blue pacman) to promote DNA end resection during homologous recombination (HR). d) RECQL4 stimulates the function of multiple BER proteins at different stages. At the initial stage of damage recognition, RECQL4 stimulates OGG1 (light green protein) lyase activity, subsequently RECQL4 stimulates APE1 (purple protein) endonuclease activity, and finally RECQL4 stimulates FEN1 (yellow protein) incision and Pol β (light blue protein) DNA synthesis activities. e.) RECQL4 mediates repair of UV lesions through its interaction with XPA (yellow). XPA works to verify the damage following damage recognition and DNA unwinding by the TFIIH complex (complex of teal proteins). f.) RECQL4 interacts with p53 (green protein) to mediate mtDNA synthesis through Polγ (light blue) and to sequester a portion of p53 in the mitochondria. However, following DNA damage, the interaction between RECQL4 and p53 is lost and both proteins re-localize to the nucleus to mediate the DNA damage response. Created using biorender.com

1.3.1 Replication initiation

The first clues that RECQL4 had a replication function was shown by Sangrithi et al who observed that both the N-terminus of human and frog RECQL4 have sequence similarity to the essential yeast replication initiation factor, Sld2 [48]. They demonstrated that immune-depletion of Xenopus RECQL4 (called xRTS) impairs replication initiation and nascent DNA synthesis in the Xenopus egg extract system. xRTS is loaded on chromatin following recruitment of the pre-replication complex and is needed for loading of the ssDNA binding protein, RPA, as well as the assembly of DNA polymerase α (**Figure 3a**). Interestingly, the authors show that expression of only the N-terminal Sld2-like domain led to 20% rescue whereas the full-length protein is needed for complementation of depleted xRTS. Importantly, this replication defect in Xenopus extracts is complemented with recombinant human RECQL4. RECQL4 replication function is dependent on its helicase activity as mutating the conserved Walker B motif (RECQL4-D605A), needed for ATP hydrolysis, does not rescue a xRTS immuno-depleted extract. Whether this is true in human cells is still not known and further investigations are needed. These results suggest that there are multiple regions of RECQL4 needed for replication initiation. Similar findings were also observed by Matsuno et al who demonstrated that N-terminal domain of xRTS physically interacts with Xenopus Cut5 (TOPBP1 in humans), an essential replication initiation factor (**Table 1**) [49]. The interaction between Cut5 and xRTS is independent of the phosphorylation status of xRTS on its n-terminal Sld2-like domain. Unlike Xenopus, the human ortholog of Cut5, TOPBP1, does not directly interact with RECQL4 [50]. Therefore, there are likely key differences between Xenopus and human RECQL4 family members.

Table 1 RECQL4 protein interactions

Process	Protein	Detection Methods*	Interaction region	Function	References
Localization	p300	Co-IP, Co-Loc, Pull Down	1-408 aa	RECQL4 cellular localization	Dietschy
Replication	Cut5	Co-IP	N-terminus	DNA Replication, *Xenopus* Cut5 with xRTS	Matsuno
	MCM7	Co-IP, MS	ND	DNA Replication	Xu
	MCM10	Co-IP, MS, Pull Down	1-200 aa	DNA Replication, inhibition of RECQL4 helicase activity	Xu
	SLD5	Co-IP, MS	ND	DNA Replication	Xu
Telomere	TRF1	Co-Loc	ND	Telomere maintenance, Stimulates RECQL4 helicase activity	Ghosh
	TRF2	Co-IP	ND	Telomere maintenance, Stimulates RECQL4 helicase activity	Ghosh
	WRN	Co-IP	ND	RECQL4 stimulates WRN telomeric D-loop resolution	Ghosh
DSB Repair	BLM	Co-IP, Y2H	1-471 aa	Increase RECQL4 retention time at DSB sites, BLM stimulation	Singh
	KU70	Co-IP	N-terminus	NHEJ, RECQL4 enhances KU complex DNA binding. KU inhibits RECQL4 helicase activity	Shamanna
	KU80	Co-IP	ND	NHEJ, RECQL4 enhances KU complex DNA binding. KU inhibits RECQL4 helicase activity	Shamanna
	MRE11	Co-Loc, IP, Pull Down	N-terminus	HR, DNA end resection	Lu
	RAD51	Co-Loc, Co-IP	ND	DSB Repair	Petkovic
BER	APE1	Co-Loc	ND	APE1 endonuclease stimulation	Schurman
	FEN1	Co-Loc	ND	FEN1 incision stimulation	Schurman
	OGG1	Co-IP	N-terminus	Stimulates OGG1 AP lyase activity	Duan
	PARP1	Co-IP, PDS	833-1208 aa	Base excision repair	Woo
	POL β	ND	ND	Stimulates POLβ DNA synthesis activity	Schurman
NER	XPA	Co-IP, Co-Loc, Fractionation, Pull Down	ND	Nucleotide excision repair	Fan
Mitochondria	p53	Co-IP, Co-Loc, Fractination	270-400 aa	Sequestering p53 from nucleus, mtDNA synthesis	De
	TOM20	Pull Down	13-18 aa	Mitochondrial import	De

* Abbreviations: Co-IP, Co-Immunoprecipitation; Co-Loc, Co-Localization; IP, immunoprecipitation; MS, mass spectrometry; ND, not determined; PDS, phage display.

Like *Xenopus*, *D. melanogaster* RECQL4 is also critical for DNA replication and RECQL4 knockout results in embryonic lethality and failure in cellular proliferation [51]. Although knockout of RECQL4 leads to embryonic lethality in flies, due to the powerful genetic tool of mosaicism researchers can study how RECQL4 knockout affects individual cells [51,52]. Importantly, similar to the Xenopus study, Drosophila RECQL4 helicase function is critical for replication as helicase dead Drosophila RECQL4 is not able to rescue the proliferation defect seen

in null RECQL4 [53]. These results suggest that the RECQL4 family has a conserved replication function in replication initiation.

Like frogs and flies, subsequent studies demonstrate that human RECQL4 has a critical role during replication initiation through loading the replicative helicase, CMG complex (CDC45-MCM2-7-GINS) (**Figure 3a**; [50,54]). Using bimolecular fluorescence complementation assays as well as chromatin fractionation, Im et al showed that knockdown of RECQL4 leads to decreased interaction between CMG complex members resulting in its failure to load on chromatin [54]. RECQL4 was subsequently found by Xu et al to interact with CMG complex members, MCM7 and SLD5, and another important replication initiation protein, MCM10 (**Figure 1, Table 1**) [50]. RECQL4 interaction with MCM7 and SLD5 is MCM10 dependent. Consistent with a role during replication initiation, RECQL4 interaction with the MCM7, SLD5, and MCM10 is enriched during G1/S. Furthermore, *siRECQL4* knockdown leads to decreased DNA synthesis [43,50]. Lastly, they demonstrated that MCM10 plays a critical role in regulating RECQL4 function by inhibiting its helicase activity *in vitro*. Interaction with MCM10 and subsequent inhibition of RECQL4, depends on RECQL4 phosphorylation state, as phospho-mimetic RECQL4 mutant (S89E, T93E, T139E) fails to interact with MCM10 [50]. The authors postulated that cyclin dependent kinase, CDK, mediated phosphorylation may regulate RECQL4 function in a cell cycle dependent manner. Later studies demonstrated that CDK-mediated phosphorylation can indeed modulate RECQL4 activity, albeit during DSB repair [55]. It remains to be determined whether CDK-mediates RECQL4 replicative functions.

1.3.2 Multifaceted role during DNA repair

1.3.2.1 Telomeric maintenance

There are many clues that suggested that RECQL4 has an important role in telomere maintenance. First, RTS is a progeroid syndrome, which is often associated with telomere dysfunction. Similarly, RTS patients exhibit symptoms analogous to dyskeratosis congenita, another disease caused by telomere dysfunction [56,57]. Like RTS patients, mutations in another RecQ helicase, WRN, result in Werner syndrome. Werner patients share clinical features with RTS patients such as developmental abnormalities and premature aging [58–60]. It is thought that Werner syndrome symptoms are largely due to WRN's crucial function in telomere maintenance [5,61]. Therefore, it was hypothesized that RECQL4 has an important role during telomere maintenance.

In 2012, Ghosh et al demonstrated that RTS cells have more fragile telomeres compared to age/sex-matched normal fibroblasts by telomeric fluorescent in situ hybridization (FISH) [62]. Like RTS cells, RECQL4 knockdown also increases telomere fragility, telomere sister chromatid exchanges, and telomere dysfunction-induced foci (TIFs). TIFs are indicative of a DNA damage response occurring at the telomere [63]. TIFs are characterized by 53BP1 colocalization with the telomere binding and Shelterin complex component, TRF1. These findings demonstrate that RECQL4 protects telomeres from DNA damage.

RECQL4 localizes to telomeres, where it interacts with multiple members of the Shelterin complex (**Figure 3b**, **Table 1**). The strongest interaction is between RECQL4 and TRF2. RECQL4 interaction with TRF1 and TRF2 stimulates RECQL4-dependent D-loops resolution at both undamaged and oxidized telomeres in vitro. Since both RECQ helicases, RECQL4 and WRN, have critical telomere functions, it was hypothesized that WRN and RECQL4 cooperatively

function. Indeed, RECQL4 physically and functionally interacts with WRN (**Table 1**). RECQL4 stimulates WRN-dependent D-loop unwinding at both undamaged and oxidized telomeric DNA *in vitro*. Further validating their roles in protecting telomeres from damage, both RECQL4 and WRN resolve oxidized telomeric D-loops better than undamaged D-loops [62]. The Bohr group also later demonstrated that RECQL4 can also unwind telomeric D-loops which contain thymine glycol, a potent replication fork stalling lesion, with even a greater affinity than telomeric D-loop containing 8-oxoG. Interesting in contrast with telomeric D-loops containing 8-oxoG, they observed that RECQL4 does not stimulate WRN ability to resolve telomeric D-loops with thymine glycol [64]. Together, RECQL4 has crucial roles in the repair of damage at telomere and telomeric DNA replication.

1.3.2.2 Double-strand break repair

DNA double-strand breaks (DSB) are considered the most lethal type of DNA damage, as single, unrepaired DSB results in cell death [65,66]. Early studies suggested that RECQL4 may function during DSB repair since a subset of RTS patients are sensitive to the DSB inducing agent, γ-irradiation [58,67]. RECQL4's role in DSBR was further collaborated by the Stagljar group who demonstrated that upon treatment with the topoisomerase II inhibitor, etoposide, RECQL4 colocalizes with ssDNA and RAD51 foci [68]. Furthermore, RECQL4 interacts with RAD51 by co-immunoprecipitation (**Table 1**). These early studies demonstrated that RECQL4 functions with RAD51 to repair DSBs.

RECQL4 function in DSB repair is likely conserved as the Enomoto group demonstrated that its *Xenopus* homolog, xRTS, localizes to chromatin upon DSB induction [69]. Furthermore, xRTS loading depends on the DNA damage checkpoint proteins, ataxia-telangiectasia mutated (ATM) and DNA-dependent protein kinase, DNA-PK, but not RAD51. These findings suggest

that xRTS may function upstream of RAD51 and perhaps to facilitate its loading. Consistent with xRTS role in DSB repair, the authors demonstrated that xRTS depletion led to prolonged γ-H2AX foci compared to mock-treated egg extracts. These early results from mammalian cells and *Xenopus* extracts were later corroborated by the Bohr group. In a 2010 study, Singh et al found that RECQL4 was recruited to laser-induced damage sites, where it colocalized with 53BP1 and γ-H2AX, by time-lapse microscopy [70]. Similar to *Xenopus*, *RECQL4* deficient human cells delay DSB repair as prolonged 53BP1 foci are observed. However, unlike the *Xenopus*, human RECQL4 is still recruited to damage sites, even in the absence of ATM. RECQL4 recruitment to DSB sites depends on its nuclear localization. Interestingly, it was shown that RECQL4 retention at DSB sites is diminished in the absence of another RecQ helicase, BLM, suggesting cooperativity between these two proteins (**Figure 1b**, **Table 1**) [71]. Although these studies suggested that RECQL4 functions during DSB repair, its precise role has remained enigmatic until recently.

RECQL4 has roles during the two major DSB repair pathways, non-homologous end joining (NHEJ) and homologous recombination (HR) (**Figure 3c;** [55,72–74]). In 2014, Shamanna et al demonstrated that RECQL4 knockdown leads to decreased end-joining both *in vitro* and *in vivo* [72]. Cellular extracts from *RECQL4* knockdown cells have decreased capacity to join DNA ends *in vitro*. This was recapitulated *in vivo* as *RECQL4* knockdown leads to decreased NHEJ using a GFP reporter system and similar results were later confirmed by others [55,72,74]. Shamanna et al also demonstrated that RECQL4 physically and functionally interacts with the key NHEJ protein, KU70, in the N-terminus of RECQL4. Furthermore, the addition of RECQL4 increases KU complex DNA binding *in vitro* [72]. KU complex interaction with RECQL4 was later confirmed *in vivo* (**Table 1**) [55,74]. Lu et al demonstrated that RECQL4 interacts with KU70 in a cell-cycle dependent manner with increased interaction during the G1 stage when NHEJ is the

predominant DSB repair pathway (**Figure 3c**) [55,75]. Tan et al later demonstrated that *RECQL4* knockdown leads to decreased recruitment of KU80 to DSB sites [74]. These results suggest that RECQL4 works cooperatively with the KU complex to mediate NHEJ.

RECQL4's role during HR was first shown by Lu et al where it was found to promote DNA end resection, a critical step in early HR (**Figure 3c**) [73]. RECQL4 physically and genetically interacts with the key end resection proteins, MRE11 and CtIP. RECQL4 functions before CtIP but after MRE11. A later study demonstrated that the interaction between MRE11 and RECQL4 is mediated by RECQL4 phosphorylation (S89/S251) by CDK1 and CDK2 during S/G2 cell cycle phase [55]. Phosphorylation of RECQL4 stimulates its helicase activity *in vitro*. RECQL4 helicase activity is critical for its function as a catalytically inactive mutant impairs end resection and HR [73]. Together, these findings demonstrate that RECQL4 functions with MRE11 to promote DNA end resection during HR.

1.3.2.3 Base excision repair

Base excision repair (BER) mediates repair of base damage that results from DNA methylation and oxidization as well as other types of base damage. The Hock group first hypothesized a potential role for RECQL4 during repair of base damage by postulating that cataracts observed in RTS patients could be due to the misrepair of oxidative damage in the eye lens. The authors showed that RECQL4 forms nuclear foci following exposure to the oxidative damaging agent, hydrogen peroxide (H_2O_2) [76]. Consistent with a function for RECQL4 in BER, RTS cells increase the amount of 8-oxo-guanine following H_2O_2 treatment compared to control cells. This increase in 8-oxo-guanine in RTS cells also correlates with decreased cell viability as well as more senescent cells.

Further evidence supporting a function for RECQL4 during BER was found by the Frank group who analyzed RECQL4 localization following exposure to a variety of DNA damaging agents. Utilizing a plasmid encoding GFP tagged RECQL4 and live-cell imaging, they found that RECQL4 is primarily nuclear and identified its nuclear localization sequence (NLS) (**Figure 1b**) [77]. Consistent with a function for RECQL4 in BER, RECQL4 re-localizes into distinct nuclear foci upon exposure to the oxidative DNA damaging agents, H_2O_2 and streptonigrin. Furthermore, RECQL4 foci colocalize with key BER proteins such as apurinic (AP) endonuclease 1, APE1, and the flap endonuclease, FEN1 (**Table 1**) [78]. Suggesting that the co-localization between RECQL4 and BER proteins is functionally important, RECQL4 stimulates APE1 endonuclease (~4 fold stimulation) as well as FEN1 incision activities (~4.3-6 fold stimulation) *in vitro* (**Figure 3d**). Although no colocalization was observed between RECQL4 and the key BER polymerase, Pol β, RECQL4 can also stimulate DNA synthesis by Pol β *in vitro* (**Figure 3d**) [78].

Suggesting a direct interaction between RECQL4 and BER proteins, RECQL4 immuno-precipitated with the 8-oxoguanine-glycosylase, OGG1, as well as a core member of the BER pathway, PARP1, through its C-terminus (**Figure 1b**; [77,79]). Like APE1, FEN1, and Pol β, RECQL4 stimulates the AP lyase activity of OGG1 *in vitro* (**Figure 3d**). RECQL4 interaction and subsequent stimulation of OGG1, upon oxidative stress, is induced by RECQL4 acetylation. RECQL4 acetylation is mediated by CREB-binding protein, CBP, which acetylates not only the five acetylation sites mentioned above (K376, K380, K382, K385, K386) but other undetermined sites as well as mutations of these sites did not abolish RECQL4 acetylation. Deacetylation of RECQL4 is mediated by deacetylase SIRT1. Suggesting that SIRT1 plays a key role in mediating RECQL4 activity during BER, RECQL4 deacetylation results in a decreased interaction between RECQL4 and OGG1 [79]. Therefore, RECQL4 acetylation plays a key role in the regulation of its

function during BER. Loss of RECQL4 results in misregulation of BER genes, as RTS cells fail to upregulate BER genes in response to oxidative damage by microarray analysis [78]. Together, these findings demonstrate that RECQL4 has a critical role during BER by stimulating the enzymatic activities of critical BER proteins, and that in its absence cells cannot properly activate an oxidative damage response.

1.3.2.4 Nucleotide excision repair

Nucleotide excision repair (NER) mediates repair of bulky DNA adducts such as 6-4 photoproducts and cyclobutane pyrimidine dimers (CPD), which result from UV exposure [80,81]. Patients with defects in the canonical NER genes suffer from an inherited disorder called Xeroderma pigmentosum (XP). XP symptoms include profound sensitivity to the sun and an increased incidence of skin lesions and cancers. Multiple studies have implicated RECQL4 to have an important role during NER. For example, early studies reported that a subset of RTS patients were photosensitive, alongside developing erythema and other skin abnormalities [58,82]. Later reports demonstrated that cells from RTS patients have increased UV sensitivity, which is consistent with a role for RECQL4 during NER [83–85]. In a 2008 study by the Luo group, RECQL4 was found to re-localize into nuclear foci following UV irradiation [86]. Importantly, they showed that complementing RTS fibroblast cells with RECQL4 led to faster resolution of CPDs and increased survival following UV compared to the un-complemented RTS cells. To further validate RECQL4 role in CPD resolution, the Luo group observed that normal fibroblast cells resolved CPDs more slowly when RECQL4 is knocked down by siRNA. Suggesting a direct role for RECQL4 in NER, RECQL4 interacts and colocalizes with XPA, a critical NER protein, following UV treatment (**Figure 3e**, **Table 1**). XPA functions to verify the nucleotide damage and following which the DNA is unwound to facilitate DNA excision [80,81]. RECQL4 functions

downstream of XPA, as RECQL4 recruitment into UV-induced foci is diminished in XPA-null cells. Despite these studies, the exact function of RECQL4 during NER remains ambiguous.

1.3.3 Mitochondrial maintenance

Unlike WRN and BLM which are primarily nuclear, RECQL4 is localized to the cytoplasm as well as the nucleus. This is particularly intriguing because RECQL4 is the only RecQ helicase to have a mitochondrial localization sequence (**Figure 1b**, [45,87]). Furthermore, RECQL4 dysfunction is associated with progeroid syndrome. Alongside telomere shortening, another major contributor to aging is mitochondrial dysfunction [88–92]. Therefore, RECQL4 was hypothesized to have a role in mitochondrial maintenance. The Bohr group demonstrated that human RECQL4 localizes to the mitochondria both by immunofluorescence and cellular fractionation [93]. Furthermore, they showed RECQL4 depletion leads to increased mitochondrial DNA damage and decreased oxygen consumption rates. Together, these findings suggest that RECQL4 has a critical role in protecting the mitochondrial genome.

Later studies by the Sengupta and Zhao groups confirmed RECQL4 mitochondrial localization [45,87,94] and RECQL4 interaction with the mitochondria importer, TOM20, was later described by De et al [45]. Intriguingly though, RECQL4 protein is undetectable in the mitochondria during S phase, likely due to its role in DNA replication [45]. RECQL4 mitochondrial localization is important not only for RECQL4 function but also to recruit the tumor suppressor gene, p53, into the mitochondria under unperturbed conditions (**Figures 1b and 3f, Table 1**). RECQL4 and p53 colocalization to the mitochondria enables mitochondria polymerase, Polγ, to mediate mtDNA synthesis [45,95]. Another important function for the colocalization between RECQL4 and p53 in the mitochondria is to sequester a portion of p53 away from the

nucleus, thus inhibiting its transcriptional activity [45]. The authors postulated that p53 and RECQL4 interaction results in masking their respective NLS, keeping them in the mitochondria. However, upon DNA damage, p53 is recruited to the nucleus and the interaction between with the two proteins are lost and both proteins re-localize to the nucleus to mediate the DNA damage response (**Figure 3f**).

Later studies corroborated the importance of RECQL4 for mitochondrial maintenance, as disruption of RECQL4 MLS results in bioenergetics dysfunction indicated by increased aerobic glycolysis [87]. The shift to utilizing aerobic glycolysis is called the Warburg effect and is commonly observed in cancer cells [96]. It was hypothesized that the increased aerobic glycolysis caused by loss of RECQL4 mitochondrial localization could drive tumorigenesis. Consistent with this notion, cells with defective RECQL4 mitochondria localization have increased invasive capability [87]. RECQL4 is critical for mitochondrial maintenance but its exact role remains elusive. Unlike other cellular compartments, mitochondria are more susceptible to oxidative DNA damage as oxygen radicals are constantly released during ATP production [97,98]. BER plays a key role in the repair of oxidative damage in mitochondrial DNA [99,100]. Therefore, one possibility is that RECQL4 may function to enhance the BER polymerase, Pol β activity, in the mitochondria as has been shown in the nucleus [78]. Future studies are needed to pinpoint the exact function of RECQL4 in mitochondrial genome maintenance.

1.3.4 Does RECQL4 truly function in all of these pathways?

As discussed above RECQL4 has been implicated to have roles in a multitude of pathways ranging from replication to NER to DSBR. But the cognizant question is does RECQL4 truly function in all of these pathways? The likely answer is no. RECQL4 has been implicated in these

pathways mainly through RTS cells sensitivity to damaging agents and protein-protein interactions studies. However, there are caveats to both approaches. First as mentioned, RTS can arise from mutations in other genes besides *RECQL4* [21,22]. Thus, it is possible that the sensitivity that was observed in these cells were due to dysfunction in another gene. Secondly, there are technical limitations with some of the interaction studies that were performed. For example, in the Fan, Pekotvic, and Woo studies, the authors did not perform the co-immunoprecipitation experiments in the presence of ethidium bromide or benzonase [68,77,86]. Thus, it cannot be ruled out that these interactions are artifacts, mainly due to the DNA binding abilities of these proteins. Furthermore, in the Schurman study, they rationalized that since RECQL4 co-localize with BER proteins, APE1 and FEN1, that it may work together with them. This is not necessarily true as co-localization does not indicate that they are interacting or necessarily that they are working together. It should be noted though that the authors did demonstrate that RECQL4 stimulates APE1 and FEN1 enzymatic activity suggesting that RECQL4 may have a role during BER [78]. So far, the strongest evidence points to RECQL4 roles during replication initiation and DSBR, however RECQL4 functions in these other pathways cannot be ruled out yet in particular its potential role during BER.

1.4 Use of the budding yeast model system

Unlike the other two diseases-associated RecQ helicases, BLM and WRN, RECQL4 has not been as well characterized. Due to RECQL4 roles during replication initiation, previous studies of mammalian *RECQL4* were stymied due to technical difficulties, including the embryonic lethality of mouse knockout models thus another model system to study its function would be

valuable [51,56,101,102]. Sgs1 was thought to be the only RecQ helicase in yeast as it has conserved functions with other human RECQ protein family members, such as WRN and BLM [103,104]. However, in 2008, Hrq1 was discovered through a computational screen by Barea et al. In this study, hydrophobic cluster analysis was used to find yeast genes that have similar hydrophobic regions to RECQL4, which could indicate similar structure [105]. They revealed Hrq1 as a potential a RECQL4 homolog. Further supporting this notion, AlphaFold structures of Hrq1 and RECQL4 are well-aligned (**Figure 4a**), particularly the helicase domains of Hrq1 and RECQL4 (**Figure 4b**) [106]. Please note that the alignment was performed with the helicase and C-terminal domains of Hrq1 and RECQL4, as AlphaFold was not confident in its prediction of the N-terminal domain of RECQL4. Thus, so far two computational analyses has revealed that Hrq1 and RECQL4 may be homologs. Complementing these computational analyses, recent experiments were also performed to validate that Hrq1 and RECQL4 has some conserved function in genome integrity. For example, in *vitro* biochemical studies demonstrate that Hrq1 and RECQL4 bind and unwind similar DNA substrates including bubbles, D-loops, and poly(dT) forks (**Table 2**) [107]. The primary difference between yeast Hrq1 and human RECQL4 is that yeast encodes a separate Sld2 (synthetically lethal with Dpb11-1) protein whereas human RECQL4 is a fusion between Sld2-like domain at the N-terminus of the helicase (**Figure 1**). Because of this Hrq1 is non-essential, thus providing us with a potential model system to the role of RECQL4 during genomic maintenance.

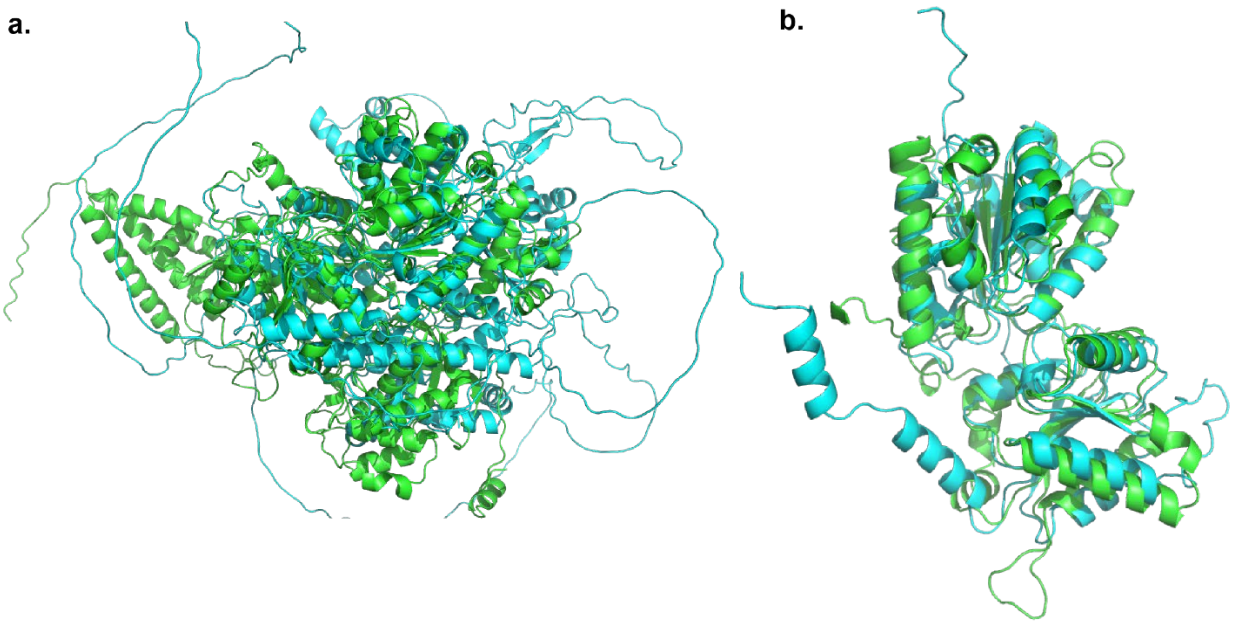

Figure 4. Alignment of AlphaFold structures of Hrq1 and RECQL4

a) Alignment of Hrq1 (green) and RECQL4 (cyan) helicase and C-terminal domains. **b)** Alignment of Hrq1 (green) and RECQL4 (cyan) helicase domains. Structure alignment was performed using PyMOL [108].

Table 2 Equilibrium dissociation constants (*Kd*) and Michaelis constants *(Km*) for Hrq1 and RECQL4

	Hrq1		RECQL4	
Substrate	Binding Kd(nM)	Unwinding Km (nM)	Binding Kd (nM)	Unwinding Km (nM)
Poly (dT) ssDNA	3.45 ± 0.19	N/A	2.38 ± 0.15	N/A
Random Fork	N/D	113.9 ± 19.5	107.7 ± 32.5	14.7 ± 1.1
3' tail	140.7 ± 59.4	11.4 ± 43.4	21.8 ± 4.2	98.1 ± 17.8
5' tail	154.5 ± 44.6	-	101.6 ± 29.4	-
Blunt dsDNA	-	-	-	-
Bubble	36.1 ± 4.9	23.6 ± 2.2	16.8 ± 2.3	143 ± 56.1
HJ	79.2 ± 22.7	-	90.1 ± 23.4	-
D-loop	9.9 ± 0.7	1.6 ± 0.1	12.6 ± 1.8	5.7 ± 0.8
G4	17.6 ± 2.2	14.1 ± 0.9	12.3 ± 2.5	38.4 ± 8.7
Poly (dT) Fork	6.5 ± 0.5	12.6 ± 1.7	50.9 ± 10.4	21.7 ± 1.1
Yeast Tel Fork	3.5 ± 0.4	2.0 ± 0.2	18.5 ± 3.1	9.2 ± 1.6
Human Tel Fork	15.7 ± 1.7	23.0 ± 1.5	39.7 ± 8.3	35.4 ± 5.4

Modified from Rogers et al 2017; N/D, not determined; N/A, not applicable

1.5 Limitations of yeast as a model organism

Although Hrq1 has been predicted to have a similar structure to RECQL4 (**Figure 4a,b**) [105,106], it is missing some distinguishing features that RECQL4 possess such as the Sld2-domain/MLS and the RQC domain (**Figure 1**). However, it should be noted that RECQL4 was long thought not to contain an RQC domain, and the RQC domain of RECQL4 was just recently identified [5,6,109,110]. Thus, it is possible that Hrq1 may also possess an unidentified RQC domain, nevertheless the structural differences between Hrq1 and RECQL4 suggest that Hrq1 may not fully recapitulate all of RECQL4 functions. Indeed, unlike RECQL4, Hrq1 does not seem to have roles during DSBR as *hrq1Δ* cells are not sensitive to DSB inducing agents, bleomycin and camptothecin [111]. Furthermore, so far Hrq1 has not been implicated to have any roles in the mitochondria as it does not contained a mitochondrial localization sequence and we observed Hrq1-YFP to be mainly in the nucleus (data not shown). All of this suggests that Hrq1 may not be a direct homolog for RECQL4. However, both Hrq1 and RECQL4 seems to have roles during repair of cisplatin-induced lesions as disruptions of these genes results in increased cisplatin sensitivity [43,44,111,112]. This suggests that Hrq1 may be a functional homolog for RECQL4 during repair of cisplatin induced lesions. However, the other RecQ helicases such as BLM and WRN needs to be tested to determine whether they function and are regulated similarly to Hrq1 following cisplatin exposure. This would provide insight into whether Hrq1 is truly a functional homolog to RECQL4 in mediating repair of cisplatin induced lesions. Another experiment that would support the notion that they are functional homologs would be to see if RECQL4 expression can rescue *hrq1Δ* cells cisplatin sensitivity.

1.6 Major hypotheses

An early study examining the sensitivity of RTS cells to different genotoxic agents, found that RTS cells exhibit increased sensitivity to cisplatin [85]. Years later studies in budding yeast found knockout of *HRQ1* results in increased cisplatin and MMC sensitivity [111,112]. Furthermore, Hrq1 helicase function is necessary for crosslink repair as catalytically inactive helicase mutant Hrq1-K318A, is more sensitive to both cisplatin and MMC [111]. Recent studies with mammalian cell lines demonstrated that *RECQL4* knockdown results in increased cisplatin sensitivity [43,44]. This suggests that the RECQL4 family has a conserved role during DNA crosslink repair. DNA crosslinks are extremely deleterious lesions as they can stall transcription and replication and can hinder the recruitment of proteins involved in genome maintenance [113–115]. Unrepaired DNA cross-links result in mitotic catastrophe and, ultimately, cell death [116]. DNA crosslinking agents, such as mitomycin C and cisplatin, are used as chemotherapeutics. Besides these exogenous sources, there are also endogenous sources of DNA crosslinking agents such as reactive aldehydes [117–120]. Reactive aldehydes are formed through normal metabolism and are extremely reactive and can interact with DNA to form crosslinks. Although Hrq1 and RECQL4 are needed for resistance of DNA crosslinking agents, the exact function and how these proteins are regulated during this process is still unknown [43,44,111,112].

Since Hrq1 and RECQL4 are proposed to be homologs and are both needed for resistance of cisplatin-induced lesions. I hypothesized that Hrq1 and RECQL4 functions and is regulated similarly to each during processing of cisplatin-induced lesions. In this thesis, I expanded on the role of Hrq1 during DNA crosslink processing by mediating damage bypass through recombination and uncovered a role by the proteasome to regulate Hrq1's levels. Utilizing a

combination of genetic and molecular biology, I uncovered the phenotypic consequences of its stabilization following cisplatin exposure; our results are summarized in Chapter II and III.

If Hrq1 is indeed a functional homolog of RECQL4 during repair of cisplatin-induced lesion, then we should observe similar function and regulation of RECQL4 during this process. Indeed, we observed that RECQL4 is regulated similarly to Hrq1 following treatment of DNA crosslinking agents and that overexpression of RECQL4 leads to similar phenotypes to Hrq1. Importantly, our bioinformatic analysis reveals that high levels of *RECQL4* are associated with increased tumor mutation burden and enrichment of genes involved in the homologous recombination pathway. These results are summarized in Chapter IV and V.

1.7 Acknowledgement

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2.0 Regulation of Hrq1 by the ubiquitin proteasome system

2.1 Introduction

Although *RECQL4* dysfunction is associated with hereditable diseases, recent studies have shown that overexpression of *RECQL4* is linked to multiple cancer types such as breast, hepatic, gastric, and prostate [39,43,44,121]. Since both inactivation or overexpression of *RECQL4* cause human disease and genetic instability, *RECQL4* protein levels must be tightly regulated. However not much is known about how RECQL4 is regulated. Using budding yeast as a model system, we find that although Hrq1 is needed for cisplatin resistance it is paradoxically degraded by the proteasome following cisplatin exposure. We find that this degradation is lesion specific as treatment with other DNA damaging agents such as MMS, IR, and HU lead to no decrease of Hrq1 protein level. Finally, we find that the E3 ubiquitin ligase, Rad16, mediates its degradation following cisplatin exposure. All together we uncovered a previously unidentified regulatory mechanism of Hrq1 during repair of cisplatin-induced lesions.

2.2 Material and methods

2.2.1 Yeast strains

The yeast strains used are listed in **Supplemental Table 1**. All strains are isogenic to W303 [122,123]. Yeast media and plates were prepared as previously described [124]. Strain construction

for knockouts and epitope tagging was performed as described in [125]. Experiments were performed to ensure that epitope tagging did not disrupt the function of the protein of interest. After transformation and selection, knockouts were verified by sequencing using primers that flank each gene; western blot or fluorescent microscopy was used to confirmed epitope tagging.

2.2.2 Cycloheximide chase

Cycloheximide chases in yeast were adapted from [126]. Yeast cultures were grown in 3 ml SC medium (pH 5.8) or YPD overnight at 30˚C, and subsequently diluted to 0.2 OD_{600} in 35 ml SC/YPD medium and grown until the cultures reached logarithmic phase. The translation inhibitor, cycloheximide (Sigma-Aldrich), was then added to a final concentration of 0.5 mg/ml. In addition to cycloheximide, DMSO (0.1%) or cisplatin (100 µg/ml) or MMC (100 µg/ml) diluted in DMSO was added to the culture and grown at 30˚C for the indicated time points. Cisplatin dosage was optimized by a previous post-doc in the lab. MMC dosage was chosen following a trial utilizing a range of MMC concentrations and there was no observable difference between the doses, thus the dose of MMC was chosen to match the dose of cisplatin. Where indicated in the text, cells were pretreated with proteasome inhibitor MG-132 (50 µM, SelleckChem S2619) for one hour prior to CHX addition. Equal amounts of cells (0.75 OD_{600}) were taken at each time-point, pelleted, supernatant was removed, and washed once with $_{dd}$H2O. The pellets were flash frozen on dry ice. Protein was extracted from whole cell lysates by TCA precipitation as described and resuspended in 51 µl of loading buffer [127]. Thirteen µl of protein was run on a 10% SDS-PAGE gel and transferred to a PVDF membrane by semidry transfer (Bio-Rad) at 13V for 2 hours 15 min. Western blot using anti-Myc antibodies were used detect Hrq1-9Myc (α-Myc, mouse

monoclonal, Santa Cruz Biotechnology, 1:500) or Kar2 (α-Kar2, rabbit polyclonal, Santa Cruz biotechnology, 1:5000) as a loading control.

2.2.3 Ubiquitin pulldown

Ubiquitin pulldown protocol was modified from the Tansey Lab [128,129]. Briefly, *pdr5Δ* Hrq1-9myc and *pdr5Δ* Hrq1-7KR-6xHA strains were transformed with YEpHisUbStu, which expresses 6x-His-Ubiquitin under the *CUP1* promoter. Cells were grown in SC-TRP medium to select for the YEpHisUbStu and grown to logarithmic phase. Expression of 6x-His-Ubiquitin expression was induced by addition of 500 μM $CuSO_4$. After 2.5 hours of growth, MG-132 (50 μM) was added then grown for another 45 minutes before addition of DMSO (0.1%) or cisplatin (100 μg/ml) and further grown for 45 minutes before being pelleted and frozen. The cell pellets were lysed using glass beads and ubiquitylated proteins were pulldown by affinity purification using Ni-NTA beads (ThermoFisher #88221). The Ni-NTA beads were then washed 3 times with 10 mM, followed by 3 times with 17.5 mM, then finally 1 time with 20 mM imidazole. The protein bound to the beads was eluted in 50 ul of 2x Laemmli buffer with 0.8 M imidazole. The entire sample was loaded on 1.5 mm 8% SDS-PAGE gel and ran at 85 volts for 2 hours and transferred to PVDF membrane via semidry transfer (Bio-Rad) at 19 V for 2 hours and 30 minutes. We probed using α-Myc (1:500 Santa Cruz) or α-HA (1:5000 Abcam) to detect ubiquitylation status of Hrq1-9xMyc or Hr1-7KR-6xHA. For each condition, we utilized densitometry (ImageJ) to quantify ubiquitylation amount by normalizing ubiquitylated band to the input. Photoshop was used to crop and grayscale the image.

2.2.4 Protein immunoprecipitation

Hrq1-9myc *pdr5Δ* or Hrq1-7KR-6xHA *pdr5Δ* strains were grown overnight in 3 mL of YPD. Subsequently, the cultures were diluted to 0.2 OD_{600} in 100 ml of YPD. After 2 hours, MG-132 (50 µM) was added for 45 minutes before addition of DMSO (0.1%) or cisplatin (100 µg/ml) for another 45 minutes before the cells were pelleted and frozen. The cell pellets were resuspended in lysis buffer [50 mM Tris, 100 mM NaCl, 2 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 1x Pierce Protease Inhibitor (Thermo), 1x Phosphatase Inhibitor (Thermo), 2 mM PMSF]. Glass beads were added, and the mixture was vortexed for 5 minutes at 4ºC. Next, 31.5 µl of 10% NP-40 was added and the mixture incubated on ice for 5 minutes. Afterwards, it was spun down at 5,000xg for 5 minutes at 4ºC. 15 µl of the supernatants were set aside for the input and 15 µl of loading buffer was added. The remaining 250 µl of the sample was incubated with 20 µl of washed magnetic anti-Myc or anti-HA beads at 4ºC for 3 hours on a rotator. The beads were washed four times with lysis buffer with 5 minutes incubations on ice between washes. Following the final wash, all the supernatants were removed and 25 µl of loading buffer was added to the beads. The samples were incubated at 65ºC for 10 minutes and then loaded on 10% SDS-PAGE gel and ran at 90 volts for 2 hours and transferred to a PVDF membrane via semidry transfer (Bio-Rad) at 13 V for 3 hours. The blots were probed using α-Myc (1:500 Santa Cruz) or α-HA (1:5000 Abcam) antibodies to detect Hrq1 and α-ubiquitin (1:1000 Santa Cruz) antibodies to detect ubiquitylated Hrq1-9xMyc or Hr1-7KR-6xHA. For each condition, we utilized densitometry (ImageJ) to quantify ubiquitylation amount by normalizing ubiquitylated band to the amount of Hrq1 pulled down. Photoshop was used to crop and grayscale the image.

2.3 Results

2.3.1 Degradation of Hrq1 is lesion specific

Previous reports demonstrate that Hrq1 is important during DNA crosslink repair [107,111]. Consistent with prior studies [107,111], we observe that *hrq1Δ* cells are cisplatin sensitive compared to wild-type (WT) cells (**Figure 5a**). Since Hrq1 is needed for resistance to DNA crosslinking agents, we reasoned that Hrq1 protein levels may increase upon cisplatin exposure. To address this, we 9xMyc tagged Hrq1 on its C-terminus at its endogenous locus and promoter. We verified that 9xMyc-Hrq1 was functional upon exposure of the *HRQ1-9myc* yeast strain to cisplatin by comparing its growth to the parental WT strain (**Appendix A: Supplemental Figure 1**). Although Hrq1 is needed for cisplatin resistance, we paradoxically observe that Hrq1 steady state levels significantly decrease upon cisplatin exposure (**Figure 5b**). Importantly, Hrq1 degradation is specific to cisplatin since other types of DNA damage, such as methyl methanesulfonate (MMS; an alkylating agent), ionizing radiation (IR; which induces double-strand breaks), or hydroxyurea (HU; which depletes dNTP pools), have no detectable effect on Hrq1 steady state levels (**Figure 5b**). These results suggest that the reduced Hrq1 protein levels observed are specific to cisplatin.

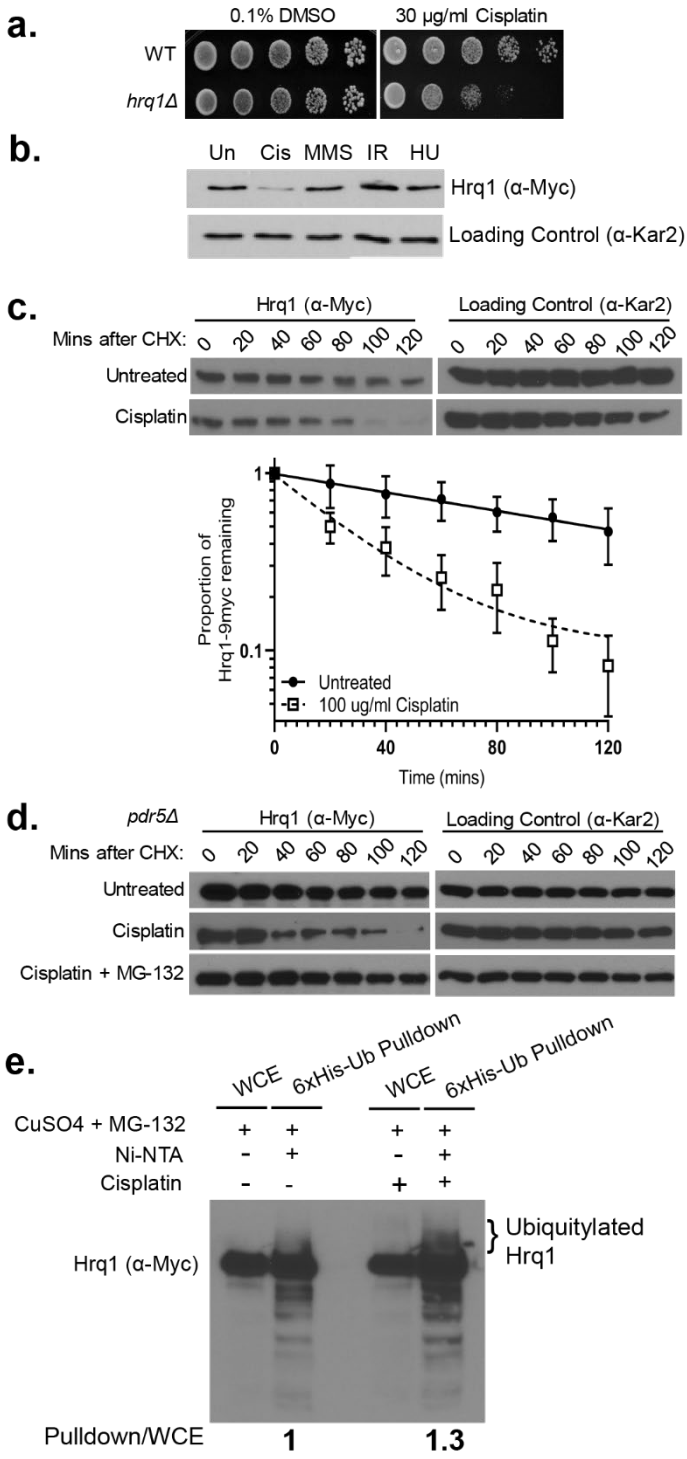


Figure 5. Although Hrq1 is needed for cisplatin resistance, it is degraded by the proteasome upon cisplatin exposure.

a) *HRQ1*-null cells are sensitive to cisplatin. Wild-type (WT) or *hrq1*Δ disrupted cells were five-fold serially diluted on medium containing 30 µg/ml cisplatin and/or 0.1% DMSO, grown for 48 hours at 30˚C, and photographed. **b)**

Hrq1 level is stable following treatment with other DNA damaging agents. Exponentially growing cells with Hrq1-9xMYC were treated with the indicated drugs (0.3% MMS, 100 µg/ml cisplatin, 100 Gy IR, 100 mM HU) for 2 hours before being harvested for western. **c)** Hrq1 protein levels are decreased upon cisplatin treatment. Exponentially growing cells with Hrq1-9xMYC were incubated with cycloheximide in the presence or absence of 100 µg/ml cisplatin and/or 0.1% DMSO. Quantification of the proportion of Hrq1 remaining relative to time 0 (before CHX addition) and the loading control, Kar2. The experiment was performed five times with mean and standard error plotted. **d)** The proteasome degrades Hrq1 following cisplatin exposure. *PDR5* disrupted cells were untreated (0.1% DMSO), cisplatin treated, or pretreated for one hour with 50 µM MG-132 before cisplatin addition with 0.1% DMSO. Cycloheximide chases were performed the same as b). **e)** Hrq1 is ubiquitylated *in vivo*. Lanes 1 (whole cell extract, WCE without pulldown, 10% input) serves as the control whereas lane 2 is the pulldown showing ubiquitylated Hrq1 as indicated. Lanes 3 and 4 is after treatment with cisplatin, where lane 3 is WCE and lane 4 is pulldown showing increased ubiquitylated Hrq1.

2.3.2 Hrq1 is ubiquitylated and marked for degradation following cisplatin treatment

Next, we sought to determine if the reduced Hrq1 protein levels observed in cisplatin-treated cells are due to protein degradation. To measure Hrq1 protein stability, we performed cycloheximide chase experiments and analyzed cells for Hrq1 protein every 20 minutes for 120 minutes following cycloheximide addition (**Figure 5c**). Consistent with our previous observations, cisplatin treatment led to a decrease in Hrq1 protein levels, where approximately 50% of Hrq1 remains 30 minutes following cycloheximide addition, whereas in DMSO treatment more than 50% of Hrq1 still remains at 120-minutes mark (**Figure 5c**).

Proteasomal degradation is an important regulatory mechanism to ensure proper and timely DNA repair for many DNA repair proteins [130]. Therefore, one possibility is that Hrq1 protein levels are regulated by the 26S ubiquitin proteasome system (UPS) following DNA damage. To

determine whether Hrq1 is degraded by the UPS following cisplatin treatment, we performed cycloheximide chase experiments in the presence of the proteasome inhibitor, MG-132 (**Figure 5d**). Hrq1 protein levels were stabilized following cisplatin exposure when the UPS is inhibited (**Figure 5d**). Note that in order to keep the intracellular concentration of MG-132 high, the drug efflux pump, *PDR5*, is also disrupted [131]. We next determined whether Hrq1 is ubiquitylated by performing a ubiquitin pulldown experiment. We observe that Hrq1 is indeed ubiquitylated as observed as a smear and that Hrq1 ubiquitylation increases 30% following cisplatin exposure (**Figure 5e;** compare 6xHis-Ub pulldown lanes 2 and 4). These findings are strengthened by performing the reciprocal experiment where Hrq1 was pulled down and then blotted for ubiquitin (**Appendix A: Supplemental Figure 1**). It is interesting that even in the absence of damage Hrq1 is seemingly ubiquitylated. This ubiquitylation could be a result of its cell cycle regulation, discussed further below, however it cannot be rule out that there could be some technical artifact from this experiment such as Hrq1 being pull-downed non-specifically by Ni-NTA. We are currently repeating these experiments with IgG magnetic beads to address this potential issue. However together, these results suggest that Hrq1 is degraded by the proteasome following cisplatin treatment.

2.3.3 Degradation of Hrq1 is trigged by intrastrand crosslinks

As cisplatin can cause both inter- and intra-strand crosslinks, we sought to determine which of these lesion(s) are triggering the degradation of Hrq1. To this, we repeated the cycloheximide chase experiment with MMC, which as stated creates predominantly interstrand crosslinks and UV-C which induces 6-4 photoproducts and cyclobutane pyrimidine dimers (CPDs), both of which cause similar types of DNA distortions to intrastrand crosslinks. We observed that MMC treatment

does not lead to decreased Hrq1 protein levels (**Appendix Figure A: Supplemental Figure 2a**) while UV-C treatment led to a reduction of Hrq1 protein levels (**Appendix Figure A: Supplemental Figure 2b**). These data suggest that Hrq1 degradation is induced in response to DNA intrastrand crosslinks.

2.3.4 Hrq1 is regulated by the E3 ubiquitin ligase, Rad16

Since Hrq1 protein levels are regulated by the proteasome, we sought to identify the E3 ubiquitin ligase that targets Hrq1. In *S. cerevisiae*, there are 60-100 E3 enzymes, so we prioritized E3 enzymes known to regulate DNA damage response proteins. For example, the NER gene, Rad4 (mammalian XPC), functions upstream of Hrq1 during crosslink repair, and its protein levels are regulated by the UPS following DNA damage [111,132]. Therefore, one possibility is that Rad4 and Hrq1 are targeted by the same E3 enzyme, Rad16 [133,134]. Thus, we examined whether Rad16 regulates Hrq1 protein levels. Indeed, we found that Hrq1 protein levels are largely stabilized in *rad16*Δ cells in both untreated and cisplatin treated conditions (**Figure 6a**). These results show that loss of *RAD16* E3 ubiquitin ligase results in Hrq1 protein stabilization following cisplatin exposure.

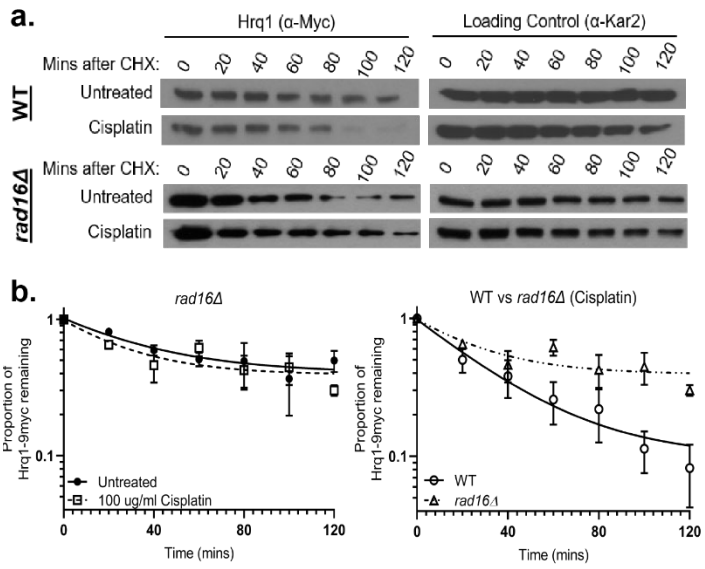


Figure 6. Hrq1 protein levels are stabilized in the absence of the E3 Ub-ligase, *RAD16*

a) Deletion of Rad16 stabilizes Hrq1 following cisplatin exposure. Hrq1-9xMYC expressing wild-type (WT) or *rad16*Δ, cells were incubated with cycloheximide in the presence or absence of 100 µg/ml cisplatin and/or 0.1% DMSO. Note blot from **Figure 5c** was reshown for comparison. **b)** Quantification of the proportion of Hrq1 remaining relative to time 0 (before CHX addition) and the loading control, Kar2, are plotted on the graph in log scale from WT and *rad16*Δ cells. Each experiment was performed three to 5 times with standard error plotted. Note that the WT cisplatin treated time course is replotted from **Figure 5c**, for direct comparison to *rad16*Δ cisplatin treated cells.

We also examined potential E2 enzymes that regulate Hrq1. In yeast, there are only thirteen known E2s. Of the E2 enzymes only two, Ubc13 and Rad6, are associated with the DNA damage response. To test whether either of these two genes may regulate Hrq1, we knocked out either *RAD6* or *UBC13* in a Hrq1-9myc tagged strain and performed cycloheximide chase experiments. Disruption of either *UBC13* or *RAD6* only led to a mild stabilization of Hrq1 (**Appendix A: Supplemental Figure 3**). These results suggest there are likely additional E2s that regulate Hrq1 protein levels. Or perhaps Rad6 and Ubc13 can compensate for each other, if this is the case then a double knockout of *RAD6* and *UBC13* may lead to increased Hrq1 stabilization.

2.4 Discussion

It is intriguing that degradation of Hrq1 is triggered by intrastrand adducts induction. Rad4, whose degradation is triggered by similar types of DNA lesions, is one of the classic examples of a DNA repair protein being degraded following repair. In the case of Rad4, it has been hypothesized that its degradation is a way to negatively regulate Rad4 activity, perhaps to prevent excessive DNA repair intermediates from accumulating. Studies in mammalian cells demonstrated that recruitment of downstream factor, XPG, is perturbed in cells where XPC is stabilized [135]. This is consistent with the notion that Rad4/XPC degradation is needed to prevent DNA repair intermediates from accumulating and for completion of the repair process itself. Future studies will be needed to determine whether stabilization of Hrq1/RECQL4 leads to more DNA repair intermediates or perturbed recruitment of any downstream repair factors.

Besides Rad4/XPC, there are many other DNA repair proteins that are regulated by the ubiquitin-proteasome system, including DNA helicases such as BLM and F-box DNA helicase (FBH1) [136]. Degradation of these key helicases is critical for dictating which pathway is used as well as for preventing the accumulation of toxic DNA repair intermediates. For example, BLM is marked for degradation by the ubiquitin ligase MlB1 during G1 to limit its end-resection activity thus promoting non-homologous end joining (NHEJ) [137]. While in the case of FBH1, it was shown that it needed to be degraded following UV damage to remove it from DNA and allow for the subsequent recruitment of Pol η to mediate lesion bypass [138]. It is still unknown whether degradation of Hrq1 is needed for pathway choice or recruitment of downstream factors. In the next chapter we discuss some of the consequences of Hrq1 stabilization.

3.0 Hrq1 has a role during post-replicative repair/damage tolerance

3.1 Introduction

To determine why Hrq1 needs to be degraded following cisplatin exposure, we need to further identify its function during DNA crosslink repair. Knowing Hrq1 function during this process could provide insights into the potential consequences if Hrq1 is not degraded following cisplatin exposure. DNA crosslinking agents, such as cisplatin and MMC, can induce two types of damage including, interstrand crosslinks (ICL) and intrastrand crosslinks. In ICLs, the Watson and Crick strands are covalently linked. However, the same DNA strand is covalently linked to itself in an intrastrand crosslink [116,139–141]. Due to the different nature of the crosslinks, the mechanism of how these two adducts are repaired is also unique. While there are some key differences in how ICLs are repaired from yeast to man, during replication-coupled ICL repair, the steps are largely conserved [113–115,142]. Following damage recognition, endonucleases nick either side of the damaged DNA, which then mediates exonuclease to come in and degrade the damaged DNA. This "unhooking" step results in a ssDNA gap. This gap may be filled in by the post-replicative repair (PRR) pathway [142–146]. The PRR pathway consists of an error-prone and error-free branch. Utilization of the different branches is mediated by PCNA (Pol30 in yeast) ubiquitylation. For example, in both yeast and humans mono-ubiquitylation of PCNA at lysine 164 (K164) recruits error-prone translesion synthesis polymerases to fill the gap [147]. On the other hand, polyubiquitylation of the same K164 residue on PCNA results in error-free homology-directed repair [148]. In contrast to ICLs, intrastrand crosslinks are primarily repaired by the nucleotide excision repair (NER) pathway [149–152]. However, if the replication fork encounters

the intrastrand crosslink and stalls, then the PRR and DNA damage tolerance pathways can mediate bypass of this adduct using the mechanism described above [147,148,153,154]. Subsequently NER, will excise and degrade the damaged DNA and the gap with be filled using DNA polymerases and ligases. We find that like RECQL4, Hrq1 is cell cycle regulated with protein levels peaking is S/G_2 suggesting that it may have a function during DNA replication. Consistent with this we find that Hrq1 mediates recombination during replication to bypass intrastrand crosslinks. By mutating conserved and predicted ubiquitylation residues in Hrq1, we were able to stabilize its levels following cisplatin exposure. We find that by stabilizing or overexpressing Hrq1, we observed increased recombination and mutation following cisplatin exposure. Overall, we find that Hrq1 has function during replication to bypass intrastrand crosslinks, however it is degraded by the proteasome to prevent excess recombination and mutation.

3.2 Material and methods

3.2.1 Cell cycle analysis

Cells expressing Hrq1-9myc were grown overnight in 4 ml YPD at RT, and subsequently diluted to 0.2 OD_{600} in 30 ml YPD and grown to logarithmic phase at RT. Alpha factor (Genescript) diluted in DMSO was added to a final concentration of 10 μM, and cells were arrested in G1 for 3 hours. The asynchronous control sample was removed directly prior to alpha-factor addition. To release the cells from alpha-factor, the yeast was pelleted, washed twice in an equal volume of water, and transferred into 30 mL YPD medium. Samples of equal amounts of cells were taken

every 20-minutes for FACS and protein analysis. Protein samples were prepared as described above, with the exception that Clb2 (G2/M cyclin) was also analyzed (rabbit polyclonal, Santa Cruz Biotechnology, 1:2000). FACS samples were pelleted and washed once with water before being fixed with 70% EtOH and stored in the dark at 4ºC. Subsequently, the samples were treated with protease and RNase and DNA was stained with propidium iodide and analyzed by FACS as described [127].

3.2.2 Serial dilutions, cisplatin and mitomycin C plates

The indicated cultures were grown in 4 ml SC medium (pH 5.8) for cisplatin exposure or YPD for MMC exposure overnight at 30˚C, and subsequently diluted to 0.2 OD_{600} and grown for 2.5 hours until the cultures reached logarithm phase. The cultures were five-fold serially diluted starting at 0.2 OD_{600} and 5 µl were spotted on the indicated plates and grown for 2 days at 30˚C before being photographed. Photoshop was used to crop and enhance the contrast of the images, and subsequently converted to black and white images in the Figures.

Cisplatin (SelleckChem S1166) was dissolved to 100 mg/ml stock solution in dimethyl sulfoxide (DMSO). Synthetic complete (SC) plates containing the indicated doses of cisplatin, were made by diluting the stock solution. Cisplatin was handled in the dark and plates were stored in the dark and used within 24 hours.

MMC (SelleckChem S8146) was dissolved to 50 mg/ml stock solution in dimethyl sulfoxide (DMSO). YPD plates containing the indicated doses of MMC, were made by diluting the stock solution. MMC was handled in the dark and plates were stored in the dark and used within 24 hours.

3.2.3 Direct repeat recombination assay

Direct repeat recombination assays were performed as described [155] except that the cultures were grown overnight in SC with 2% galactose either with or without cisplatin. In this assay, two *leu2* heteroalleles are disrupted with EcoRI and BsteII, respectively, with an intervening *URA3 gene.* Total recombination is measure by restoration of a function *LEU2* gene, which allows yeast to grow on plates that lack leucine. The experiment was done in with nine individual colonies in each condition and repeated. Recombination rate was calculated and graphed alongside the median from the two experiments. Mann-Whitney test was used to determined significance.

3.2.4 Canavanine mutagenesis assay

Mutagenesis assay protocol was adapted from Godin *et al.* 2016 [156]. Two individual colonies of wild-type (WT), Hrq1-7KR, Hrq1-OE were grown in 3 ml of SC with 2% galactose, to induce Hrq1-overexpression, either with or without cisplatin overnight at 30ºC to saturation. Subsequently, the culture was diluted to 2 OD600 and 200 µl was plated onto SC-ARG+CAN plate or further diluted 100,000-fold and 200 µl was plated onto SC plate (these plates were made with galactose). The plates were incubated for 3 days at 30ºC. Plates were scanned and colonies were counted using OpenCFU software [157]. Mutation rate was calculated by counting colonies that grew on SC-ARG+CAN vs total colonies that grew on SC. For each trial, a mean rate was calculated and graphed alongside the median from multiple experiments. Mann-Whitney test was used to determined significance.

3.3 Results

3.3.1 Hrq1 is cell cycle regulated

As stated above, DNA crosslinks are repaired using different pathways depending upon the cell cycle stage. For example, during replication, DNA intrastrand crosslinks are bypassed by the PRR pathway and then subsequently repaired by NER. Therefore, to determine whether Hrq1 functions during a specific cell cycle stage, we asked whether its protein levels are cell cycle regulated. We synchronized Hrq1-9xMYC expressing cells in G1 with alpha factor and released them into fresh medium to enable cell cycle progression. Protein extracts were made from equal cell numbers every 20 minutes and Hrq1 protein levels analyzed and compared to Kar2, endoplasmic reticulum protein, as a loading control and Clb2 as a G2/M regulated cyclin. Consistent with a role during replication, Hrq1 protein levels begin to peak in S/G2 at 40 min after alpha factor release whereas Clb2 protein peaks starting at 60 min (**Figure 7a)**. Cell cycle progression was confirmed by FACS analysis (**Figure 7a**). These results demonstrate that Hrq1 is enriched during S/G2, the cell cycle stage when PRR functions.

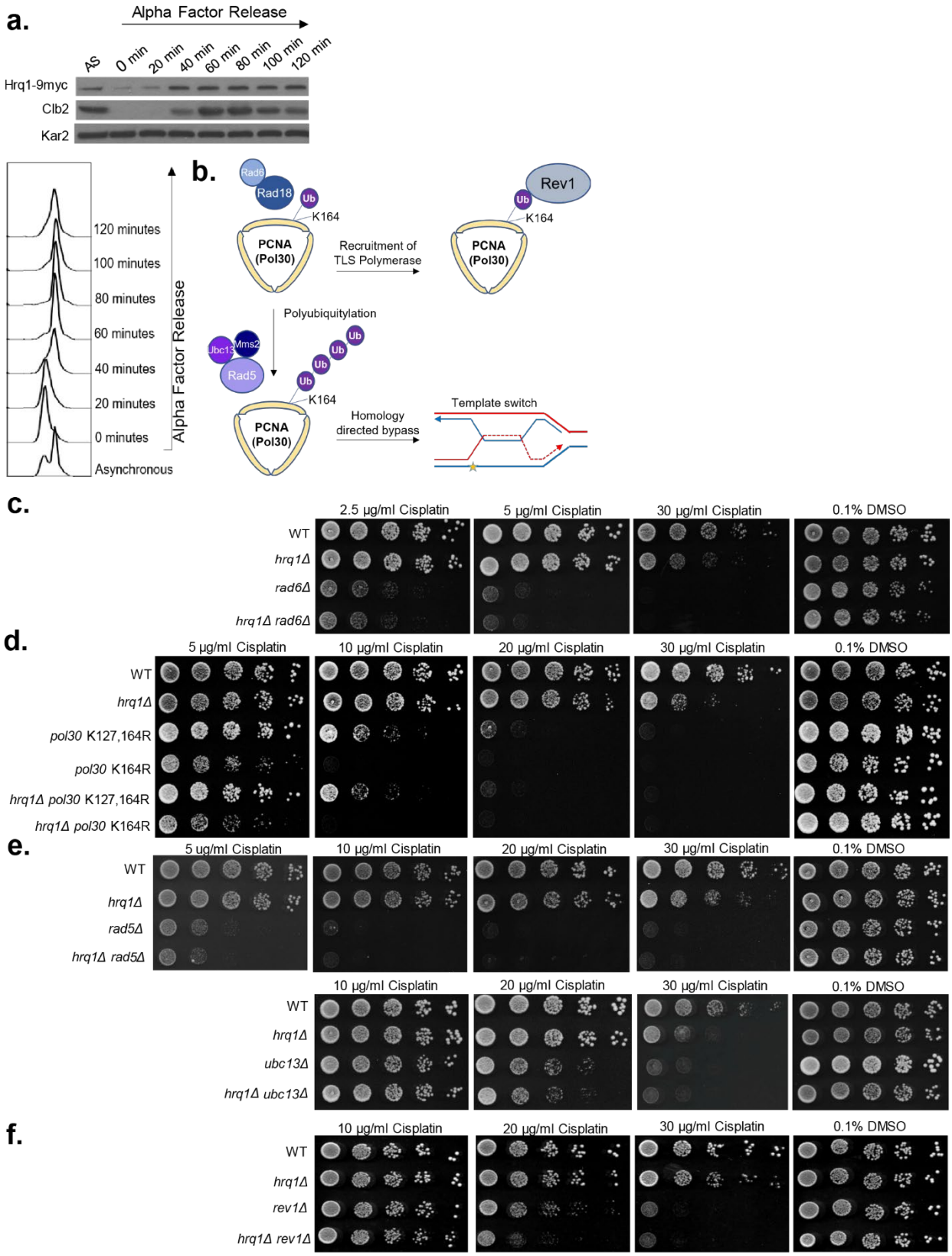


Figure 7. Hrq1 functions during error-free post-replicative repair

a) Hrq1 expression peaks during S/G2. Hrq1-9xMYC expressing cells were either untreated (asynchronous, AS) or cell cycle arrested in G1 with α-factor. The α-factor arrested cells were subsequently released into fresh YPD medium (0 min) and grown for 120 min. Protein samples from the indicated time points were analyzed by western blot for Hrq1 (anti-MYC), the G2/M cyclin, Clb2, (anti-Clb2), or a loading control, Kar2 (anti-Kar2). The cell cycle stage was analyzed FACS. **b)** Schematic of post-replicative repair pathways. When the replication fork stalls, Pol30 (yellow trimer) is initially monoubiquitylated (Ub, purple circle) via Rad6-Rad18 (light and dark blue circles) on lysine 164 (K164). Monoubiquitylation of PCNA recruits the error-prone translesion synthesis polymerases, i.e. Rev1 (gray circle) to bypass the lesion. Alternatively, PCNA is further poly-ubiquitylated by Rad5-Ubc13-Mms2 (light, medium, and dark purple complex). Polyubiquitylation of PCNA mediates error-free repair through template switching, which is a homolog directed process. A bypass intermediate is shown with the newly synthesized DNA in red and the lesion as a yellow star. **c)** Hrq1 functions in the same pathway as Pol30. The indicated yeast strains were five-fold serial diluted onto SC medium containing DMSO and/or SC medium containing the indicated amount of cisplatin. The plates were photographed after 2 days of incubation at 30˚C in the dark. **d**) Hrq1 functions in the same pathway as Rad5 and Ubc13. Serial dilutions of the indicated yeast strains were performed as described in **c)**. **e)** Hrq1 functions in a different pathway than Rev1. Serial dilutions of the indicated yeast strains were performed as described in **c).**

3.3.2 Epistatic relationship between *hrq1Δ* and the early responders of PRR

Previous studies in plants, fission yeast, and budding yeast have confounding results as to whether Hrq1 functions during PRR [112,158,159]. Therefore, it remains controversial as to the role of Hrq1 during crosslink repair. To determine if Hrq1 has a role in PRR, we carefully and systematically examined the genetic relationship between *HRQ1* and multiple members of the PRR pathway. We combined *HRQ1* mutants with disruption of PRR genes and tested these mutants for increased cisplatin sensitivity. Since Rad6/Rad18 ubiquitylates Pol30 during early PRR steps (**Figure 7b**), we began our analysis with *rad6*Δ cells. When compared to wild-type, *rad6*Δ cells are sensitive to 2.5 μg/ml cisplatin whereas *hrq1*Δ cells are sensitive to 30 μg/ml cisplatin. Yeast

disrupted with both *HRQ1* and *RAD6*, exhibit sensitivity comparable to *rad6*Δ alone (**Figure 7c**). These results suggest that *HRQ1* likely functions in the PRR pathway downstream of *RAD6*.

After Rad6/Rad18 mono-ubiquitinates PCNA, PCNA becomes poly-ubiquitylated on lysine 164 during error-free PRR (**Figure 7b**). At the same time, PCNA can be independently SUMOylated on lysine 127, which recruits the DNA helicase Srs2 (not drawn). To address whether *HRQ1* functions downstream of *PCNA* ubiquitylation, we examined the genetic relationship between *HRQ1* and *PCNA* ubiquitylation/sumo mutants, *POL30-K164R* and *POL30-K127R/K164R*. Disruption of *HRQ1* in combination with *POL30-K164R or POL30-K127R/K164R*, results in cisplatin sensitivity comparable to either *POL30* single mutants (**Figure 7d**). These results are consistent with *HRQ1* functioning downstream of *POL30* ubiquitylation/sumoylation during PRR.

3.3.3 Epistatic relationship between *hrq1Δ* and mediators of template switch branch

We next asked whether Hrq1 functions in the "error-free" template switching or "error-prone" translesion synthesis branches of PRR. To do this, we first examined the genetic relationship between *HRQ1* and members of the "error-free" branch of PRR, *RAD5-UBC13-MMS2*, which polyubiquitylates PCNA (**Figure 7b**). Cells with both *HRQ1* and *RAD5* disrupted exhibit cisplatin sensitivity comparable to a *RAD5* single mutant (**Figure 7e**). These findings are consistent with a plant study demonstrating that *RAD5* functions in the same pathway as *HRQ1*, and contrast with a budding yeast study, where *HRQ1* was found to function independently of *RAD5* [112,158,159]. Potential reasons behind these discrepancies are discussed below. Suggesting that *HRQ1* does indeed function during TS, we observe similar genetic results with

another PRR member, *UBC13*, where *hrq1*Δ *ubc13*Δ double mutants exhibit cisplatin sensitivity to *ubc13*Δ (**Figure 7e**). Together, these results suggest that *HRQ1* functions in the "error-free" branch of PRR.

3.3.4 Synthetic lethality between *hrq1Δ* and *rev1Δ*

Next, we determined if *HRQ1* also functions in the "error-prone" translesion synthesis branch of PRR by examining the genetic relationship between *HRQ1* and *REV1*, a translesion synthesis polymerase. We observe that *hrq1*Δ *rev1*Δ double mutant cells exhibit increased cisplatin sensitivity in comparison to either a *hrq1*Δ or a *rev1*Δ single mutant (**Figure 7f;** 20 μg/ml cisplatin). These results suggest that Hrq1 functions primarily in the "error-free" branch of PRR.

3.3.5 Hrq1 function in ICL repair is distinct from intrastrand crosslink repair

Loss of *HRQ1* results in sensitivity to different types of DNA crosslinking agents including cisplatin and MMC. While both cisplatin and MMC cause ICLs and intrastrand crosslinks, cisplatin damage results in 90-95% intrastrand crosslinks whereas mitomycin C results in 90-95% ICLs. Therefore, by using MMC, we asked if Hrq1 has a similar function during ICL repair when ICLs are the predominant lesion. To address this, we examined *hrq1*Δ cells for sensitivity to MMC either alone or in combination with a *rev1*Δ or *ubc13*Δ mutant (**Appendix A: Supplemental Figure 4a**). As reported in a prior study [111], *hrq1*Δ cells are MMC sensitive (**Appendix A: Supplemental Figure 4a**). Like cisplatin induced DNA damage, the *hrq1*Δ *rev1*Δ double mutant exhibits increased MMC sensitivity compared to the single mutants (**Appendix A: Supplemental Figure 4a**). These results suggest that *HRQ1* and *REV1* are functioning in different pathways upon

MMC exposure. However, in contrast to what we observed with cisplatin damage, a *hrq1*Δ *ubc13*Δ double mutant exhibits increased MMC sensitivity compared to the single *hrq1*Δ or *ubc13*Δ mutants (**Appendix A: Supplemental Figure 4a**). These results suggest that Hrq1 function in ICL repair may be functionally distinct from intrastrand crosslink repair.

To validate Hrq1 role in bypass of DNA intrastrand adducts, we performed genetic analysis using UV-C, which induces primarily 6-4 photoproducts and cyclobutane pyrimidine dimers. Suggesting a role in template switch, we find that a *hrq1Δ rad5Δ* double mutant has similar sensitivity to UV-C compared to *rad5Δ* (**Appendix A: Supplemental Figure 4b**). We observe similar results with *UBC13,* as a *hrq1Δ ubc13Δ* double mutant has similar sensitivity to UV-C as a *ubc13Δ* single mutant (**Appendix A: Supplemental Figure 4b**). Suggesting *HRQ1* functions in a different pathway to TLS, a *hrq1*Δ *rev1*Δ double mutant exhibits increased UV-C sensitive compared to the single mutants (**Appendix A: Supplemental Figure 4c**). Together these results strengthen the conclusion that Hrq1 functions during intrastrand crosslink repair via PRR.

3.3.6 Overexpression or stabilization of Hrq1 leads to increased recombination and mutation

Since we find that Hrq1 functions during "error-free" PRR during intrastrand crosslink repair and is regulated by the 26S proteasome, we sought to determine if misregulation of Hrq1 protein levels alter PRR. To do this, we determined the lysine residues that catalyze Hrq1 degradation upon cisplatin exposure (**Figure 8a**). Hrq1 is 1077 amino acids and contains 77 lysines. To identify potential ubiquitylation sites, we analyzed Hrq1 protein using UbPred, which predicts five ubiquitinated lysines (K164, K219, K221, K366, K872) [160]. UbPred utilized a random forest approach, where the classification algorithm was trained using positive and negative

ubiquitylation sites. In addition, we included two lysines that are conserved between Hrq1 and its mammalian ortholog, RECQL4 (K839, K938), as well as one lysine (K366), which is both predicted to be ubiquitylated by UbPred and conserved with RECQL4 [160,161]. We mutated these seven lysine residues to arginine at the endogenous HRQ1 locus and herein refer to this mutant as Hrq1-7KR. We next determined whether mutating these Hrq1 lysine residues results in Hrq1 protein stabilization. To test this, we performed cycloheximide chases on Hrq1-3xHA and Hrq1-7KR-6xHA. HA tagged was chosen in this case was because the plasmid used to place Hrq1 under GAL promoter had an HA tag. To keep it consistent with the OE strain, the 7KR strain was also tagged with HA, as we would use the OE and 7KR strains in the same experiments. Initial efforts to tag WT Hrq1 with 6xHA failed, thus we performed the experiments with Hrq1-3xHA. However, we later made a Hrq1-6xHA strain and confirm that it behaved similarly to Hrq1-3xHA. As previously observed, Hrq1 protein levels are reduced upon cisplatin treatment (**Figure 8b**). In contrast, Hrq1-7KR protein expression remains similar in both cisplatin treated and untreated conditions (**Figure 8b**). It is interesting to note that the Hrq1-7KR mutant is not fully stabilized, suggesting that additional lysine residues may contribute to Hrq1 degradation independently of its DNA damage response function. These results suggest that the Hrq1-7KR mutant protein levels are misregulated in response to cisplatin.

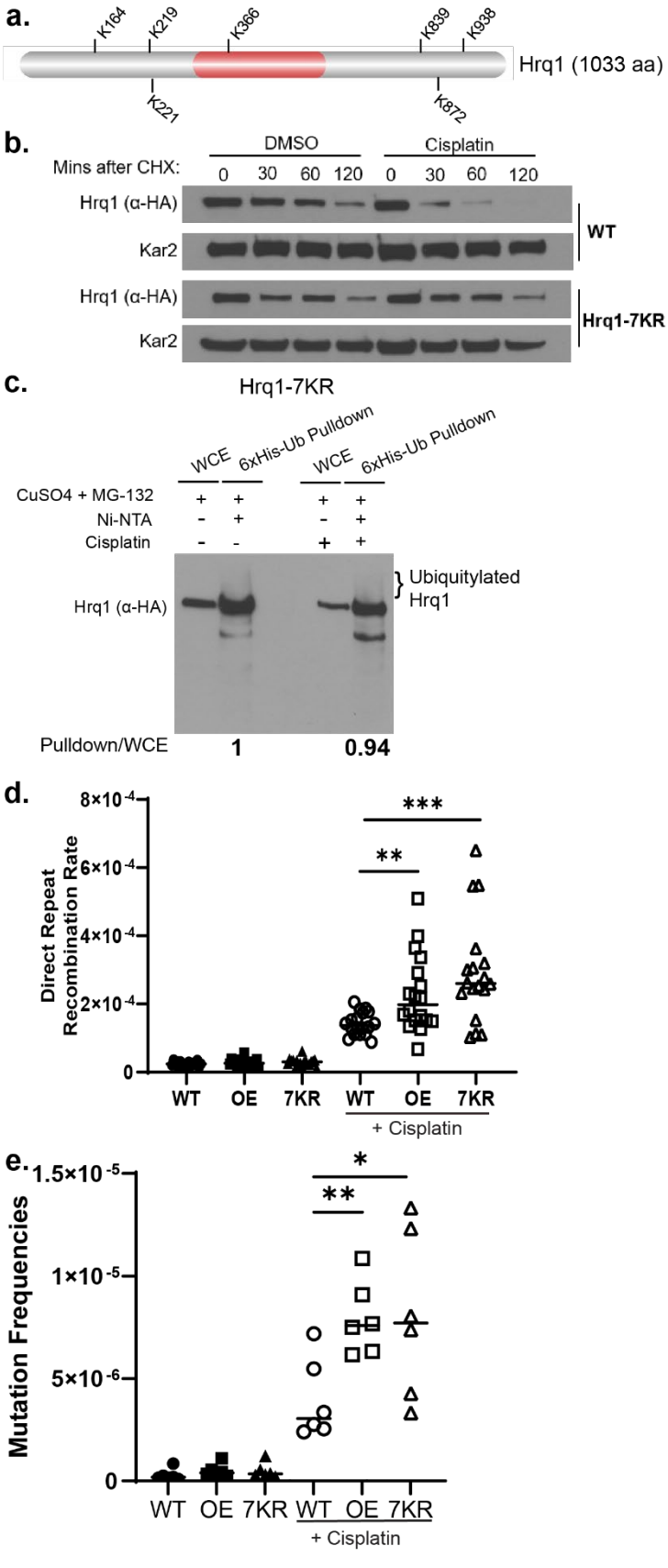

a.
K164
K219
K366
K839
K938
Hrq1 (1033 aa)
K221
K872
b.
DMSO
Cisplatin
Mins after CHX:
0 30 60 120 0 30 60 120
Hrq1 (α-HA)
Kar2
WT
Hrq1 (α-HA)
Kar2
Hrq1-7KR
c.
Hrq1-7KR
WCE
6xHis-Ub Pulldown
WCE
6xHis-Ub Pulldown
CuSO4 + MG-132
Ni-NTA
Cisplatin
Hrq1 (α-HA)
Ubiquitylated Hrq1
Pulldown/WCE
1
0.94
d.
Direct Repeat Recombination Rate
8×10-4
6×10-4
4×10-4
2×10-4
0
***
**
WT
OE
7KR
WT
OE
7KR
+ Cisplatin
e.
Mutation Frequencies
1.5×10-5
1×10-5
5×10-6
0
*
**
WT
OE
7KR
WT
OE
7KR
+ Cisplatin

Figure 8. Hrq1 protein levels are stabilized following cisplatin exposure by mutating the predicted Hrq1 ubiquitylated lysine residues to arginine

a) Schematic of Hrq1 with lysines residues that are predicted to be ubiquitylated (K164, K219, K221, K872) or conserved between Hrq1 and RecQL4 (K366, K839, K938) is shown. The helicase domain is indicated in red (287-496 aa, InterPro). **b)** Hrq1 protein levels are stabilized in Hrq1-7KR compared to WT. Cycloheximide chase experiments were performed in Hrq1-3xHA or Hrq1-7KR-6xHA expressing cells. Equal number of cells were collected every 30 minutes for 120 minutes. Experiment was performed in duplicate and a representative image (image was processed using Photoshop as described above) is shown. **c)** Hrq1-7KR shows similar ubiquitylation in treated versus untreated conditions. Lanes 1 (whole cell extract, WCE without pulldown, 10% input) serves as the control whereas lane 2 is the pulldown showing ubiquitylated Hrq1 as indicated. Lanes 3 and 4 is after treatment with cisplatin, where lane 3 is WCE and lane 4 is pulldown showing similar levels of ubiquitylated Hrq1 as untreated condition. **d)** Overexpression or stabilization of Hrq1 leads to increased recombination following cisplatin treatment. Each measurement (dots) and the median value from three experiments (horizontal bar) were plotted. ** represents $p<0.01$, *** represents $p<0.001$ **e)** Overexpression or stabilization of Hrq1 leads to increased mutagenesis following cisplatin treatment. Each measurement (dots) and the median value from two experiments (horizontal bar) were plotted. * represents $p<0.05$, ** represents $p<0.01$.

In **Figure 5e**, we show that Hrq1 is ubiquitylated and that its ubiquitylation increases 30% upon cisplatin exposure. Since Hrq1-7KR protein levels are stabilized upon cisplatin, we examined whether Hrq1-7KR ubiquitylation is altered. To address this, we performed ubiquitin pulldown experiments in the presence or absence of cisplatin as described in **Figure 5e**. Unlike wild-type Hrq1, we do not detect increased ubiquitylation of the Hrq1-7KR mutant upon cisplatin treatment (**Figure 8c**). To confirm these findings, we performed the reciprocal experiment where Hrq1-7KR was pulled down and then subsequently blotted for ubiquitin (**Appendix A: Supplemental Figure 1c**). However, we are unable to reliably detect ubiquitylated Hrq1-7KR either in the presence or absence of cisplatin (**Appendix A: Supplemental Figure 1c**). Together, these experiments

suggest that mutation of these seven lysine residues results in deregulation of Hrq1 ubiquitylation upon cisplatin exposure resulting in its protein stabilization (**Figure 8b**).

We have thus far identified a function for Hrq1 in error-free PRR to mediate intrastrand crosslink repair and found that Hrq1 is ubiquitylated and targeted for degradation upon cisplatin treatment. Therefore, we hypothesized that Hrq1 may need to be degraded for completion of PRR. Since Hrq1 functions during template switching, which is a recombination-based pathway, we asked whether stabilization of Hrq1 protein levels leads to increased recombination. To test this, we utilized the Hrq1-7KR mutant strain. At the same time, we created a strain where we induce Hrq1 over-expression (Hrq1 OE) by replacing HRQ1's endogenous promoter with a galactose-inducible/dextrose-repressible GAL1 promoter. GAL-3xHA-Hrq1 results in an approximately five-fold increase in Hrq1 expression compared to Hrq1-3xHA (**Appendix A: Supplemental Figure 5a**). Therefore, we used a Hrq1-7KR strain characterized above for our studies but treated the cells in the same manner as the GAL-3xHA-Hrq1 cells to enable direct comparisons between the two strains.

Using the Hrq1-7KR-6xHA mutant and the GAL-3xHA-Hrq1 strains, we performed a direct repeat recombination assay in the presence of galactose containing rich medium. We find that over-expression or stabilization of Hrq1 results in a 1.5 to 2-fold increase in total recombination compared to WT cells following cisplatin treatment (**Figure 8d**). We tried to perform this assay utilizing *hrq1Δ* cells, but they were too sensitive, and all the cells die. These results suggest high levels of Hrq1 following cisplatin exposure results in increased recombination. Yeast that grew on -Leu plates were replica plated onto 5-fluorooritic acid (5-FOA) plates, to

counter-select for yeast that has a functional URA3 gene. However, due to the high background the software could not reliably differentiate between the colonies. We plan to repeat these experiments but instead of replica-plating on 5-FOA plates, we will plate on SC-Leu-Ura alongside Sc-Leu plates.

Although recombination during template switching is considered "error-free", aberrant template switching can result in increased DNA mutations [162,163]. Therefore, we examined whether Hrq1 over-expression or stabilization increases mutagenesis. To measure mutation rates, we performed a canavanine mutagenesis assay. This assay measures mutations in the CAN1 permease gene, which enables cell viability upon exposure to the toxic arginine analog, canavanine. We observe over two-fold increase in mutation rates in Hrq1 over-expressing or 7KR cells in the presence cisplatin in comparison to WT cells (**Figure 8e**). In untreated conditions, the mutation rates in Hrq1 over-expressing or 7KR cells were similar to WT cells (**Figure 8e**). These results suggest that Hrq1 must be tightly regulated following cisplatin exposure to prevent excess recombination and mutagenesis.

Lastly, it is possible that preventing the degradation of Hrq1 results in accumulation of toxic DNA repair intermediates. To test this hypothesis, we examined the sensitivity of Hrq1-7KR cells upon cisplatin exposure (**Appendix A: Supplemental Figure 5b**). Surprisingly, we do not observe decreased cell viability of Hrq1-7KR cells compared to WT (**Appendix A: Supplemental Figure 5b**). This suggests that stabilization of Hrq1 during PRR may result in DNA repair intermediates that are ultimately resolved using an alternative pathway. Although we do not observe cisplatin sensitivity in the Hrq1-KR mutant, overexpression of the HRQ1-7KR (by using

a galactose inducible promoter) results in cell lethality even in the absence of DNA damage (**Appendix A: Supplemental Figure 5c**).

3.4 Discussion

Previous studies presented conflicting results as to whether Hrq1 functions during PRR [112,158,159]. There are multiple explanations for this discrepancy. For example, in *S. pombe* polyubiquitylation of PCNA (Pol30) triggers both TS and TLS. Therefore, the synthetic lethality observed between fission yeast *HRQ1* and factors that mediate PCNA polyubiquitylation may be due to function in TLS rather than an independent function in TS [158]. Separation-of-function alleles are needed to differentiate between these two functions. Our study also contradicts another in *S. cerevisiae,* which found that Hrq1 functions independently of PRR [112]. It is possible that there are background strain differences since we used W303, and the other group used the YPH499 genetic background (derived from YNN216). It is important to note that our study here performed extensive genetic analysis at each step of the PRR pathway whereas the other study only analyzed *RAD5* and *REV3*, another TLS polymerase, mutants [112]. It is also important to note that *HRQ1* has been proposed to have a function in NER as well to mediate repair of cisplatin induced lesions [112,158]. Thus, the mild synthetic sickness they observed between *HRQ1* and *RAD5/REV1* may be due to *HRQ1* function during NER. Furthermore, we find that our results are consistent with UV-C damage as well (**Appendix A: Supplemental Figure 4**). Altogether, we provide strong evidence that Hrq1 promotes recombination during TS to mediate repair of intrastrand crosslinks.

Recent studies *S. pombe* and *A. thaliana* showed that there is synthetic sickness between *HRQ1* and *MUS81* in repair of cisplatin-induced lesions. Consistently, we observe the same phenomenon in *S. cerevisiae* where the double knockout of *HRQ1* and *MUS81* has increased sensitivity compared to the single knockouts (**Appendix A: Supplemental Figure 6a**). It is postulated that Mus81 is needed to resolve recombination intermediates that may arise in the absence of *HRQ1*. Mus81 is canonically thought to act as a Holliday junction resolvase; however, it also has a role in cleaving stalled replication forks to mediated replication restart [154,164]. Thus, it is possible that in the absence of *HRQ1,* there might be an increased amount of stalled replication forks, which would need to be cleaved by Mus81 to mediate replication restart. We observed similar synthetic sickness between *HRQ1* and *SLX4* (**Appendix A: Supplemental Figure 6a**). Slx4 is another endonuclease that functions as a Holliday junction resolvase and has roles during replication. It is important to note that in the absence of *HRQ1,* there are increased gross chromosomal rearrangements (GCRs) following MMC treatment, which could be indicated of more double-strand breaks that could result from replication fork collapse [111]. This assay needs to be repeated with cisplatin to determine whether intrastrand crosslinks can also result in increased GCRs in the absence of *HRQ1*.

Consistent with a role for Hrq1 in recombination during replication, we observed a synthetic sickness between *HRQ1* and *RAD27* (**Appendix A: Supplemental Figure 6b**). *RAD27* is the homolog to *FEN1* and is critical in Okazaki fragment maturation. Early studies indicated that in the absence of *RAD27,* HR genes are critical for cellular survival [165]. This is likely because HR genes can mediate recombination to fill in the gaps that form in *rad27Δ* cells. If replication gaps are not filled, this can lead to a DSB where HR genes would then be required for

their repair. *HRQ1* is not needed for DSB repair directly, as *hrq1Δ* cells are not sensitive to DSB inducing agents such as camptothecin and bleomycin [111]. Therefore, the synthetic sickness observed between *hrq1Δ* and *rad27Δ* could be due Hrq1 mediating recombination during replication. Lastly, we found that *HRQ1* is epistatic to *RAD51*, since the double *hrq1*Δ *rad51*Δ mutant exhibits similar cisplatin sensitivity to a *rad51Δ* single mutant (**Appendix A: Supplemental Figure 6c**). This result suggests that Hrq1 works with Rad51, which is also consistent with a role for Hrq1 during recombination.

Although epistasis and synthetic lethality/sickness are powerful genetic tools, they are not without their limitations. For example, *rad6Δ* cells are exquisitely sensitive to cisplatin (**Figure 7c**) thus almost any double knockout in combination with *rad6Δ* would look like a *rad6Δ*. This could lead to misinterpretation where genes that are not involved in the *RAD6* pathway look like they are. Thus, genetic relationship needs to be determined with multiple genes along the pathway to get a better sense to whether the gene of interest belongs in the pathway of interest. Furthermore, in epistatic relationships, sensitivity is used to gauge whether a gene is "upstream" or "downstream" of another gene, while this could be useful it also has its limitations. This is because sensitivity is broad phenotype which could result from multiple sources. For example, although *RAD6* is famous for its role during damage tolerance/post-replicative repair, it can also function with *BRE1* to regulate cell cycle progression through transcriptional regulations [166]. Because of its multiple roles during cellular homeostasis, *RAD6*-null cells are slow growing thus it may look more "sensitive" and thus "upstream" of another gene which may not have as many cellular functions.

A synthetic lethality or sickness relationship is defined when $\varepsilon < 0$ in the formula $\varepsilon = W_{AB} - W_A * W_B$. Where the fitness of the double mutant is less than the fitness of the multiplication of the single mutants [167]. Based on that calculation, we observed a rather mild synthetic sickness between *hrq1Δ* and *rev1Δ* (**Figure 7f**). A potential explanation for this mild phenotype could be due to the fact that there are multiple TLS polymerases and there are redundancies between the TLS polymerases. For example, Rad30, POL η homolog, mediates bypass of UV induced lesions, thus it is possible that it could also mediate bypass of cisplatin-induced intrastrand crosslinks [168]. If Rad30 and Rev1 could compensate for each other, then *hrq1Δ rad30Δ rev1Δ* cells should be more sensitive than *hrq1Δ rad30Δ* or *hrq1Δ rev1Δ*. Overall, although epistasis and synthetic lethality/sickness have technical limitations it is still a critical genetic tool, and when combined with functional assays it becomes a powerful discovery tool.

Overexpression/stabilization of Hrq1 alone seems to be well tolerated, as there are no noticeable growth defects and no increased sensitivity to genotoxic agents. However, it is still unclear what are the long-term effects of Hrq1 overexpression/stabilization and whether it could lead to increased genomic instability. It is interesting that overexpression of Hrq1-7KR resulted in decreased viability even in the absence of treatment. In this scenario, it is possible that the decreased viability is due to accumulation of Hrq1 on the DNA which could stalled DNA replication and transcription. Stalled DNA replication and transcription could lead to replication fork collapse which would result in a DSB, which is lethal to the cell if left unrepaired.

Lastly, it is possible that the increased mutations that arise from Hrq1 overexpression or stabilization could be due to increased utilization of TLS polymerases, such as Rev1. However, overexpression of *HRQ1* in a *rev1Δ* background did not increase cisplatin sensitivity compared to

a *rev1Δ* single mutant (**Appendix A: Supplemental Figure 6d**). These results suggest that there is no increased dependency on *REV1* when *HRQ1* is overexpressed. It is still possible that one of the other TLS polymerases, such as Rad30, are causing this increase in mutations. However, it is important to note that Rev1 is thought to be the main TLS polymerase in bypassing cisplatin-induced DNA lesions [168]. Furthermore, there are other error-prone DNA repair pathways that may be involved to repair cisplatin-induced DNA damage such as break-induced replication (BIR) and single-strand annealing (SSA) [154,169]. Future studies can test whether knocking out BIR factor, *POL32*, or SSA factor, *RAD52*, in an Hrq1 overexpressed or stabilized strain will reduce mutation rates following cisplatin exposure.

4.0 RECQL4 has a conserved role during cisplatin resistance and is similarly regulated

4.1 Introduction

The RecQ family of DNA helicases is highly conserved. In fact most of what is known about Bloom syndrome patients was first shown in yeast, as *sgs1Δ* cells phenocopied what was seen in these patients including increased sister-chromatid exchange (SCEs), premature aging, and meiotic and telomeric defects [103,170,171]. So naturally, we wanted to determine how much was conserved from Hrq1 to RECQL4. We find that, like Hrq1, RECQL4 levels decrease following cisplatin exposure. Furthermore, we find that RECQL4 functions in a different pathway than the TLS polymerase, REV1, suggesting that it may have a role during TS. Suggesting that RECQL4 may mediate recombination, overexpression of RECQL4 leads to increased RAD51 foci formation and that this is partly due to its helicase function. Lastly, we observed that this increase in RAD51 foci formation was not due to increased DSBs or ssDNA from *RECQL4* overexpression. Overall, we observed a conserved regulatory mechanism between Hrq1 and RECQL4, that similar to Hrq1, RECQL4 functions in a separate pathway from REV1 and suggesting a role in recombination, high levels of *RECQL4* is associated with increased RAD51 foci formation.

4.2 Material and methods

4.2.1 Mammalian cell culture

U2OS cells (ATCC) were cultured in DMEM w/ 10% FBS and 1x penicillin-streptomycin (Life Technologies: 100 U/mL penicillin, 100 µg/mL streptomycin) at 5% CO_2.

4.2.2 Mammalian cycloheximide chase

Mammalian CHX chases were adapted from the yeast protocol and performed using U2OS cells. Cells were seeded into 6-well plates at 75,000 cells per well and were grown for three days in 10% FBS-DMEM. The cells were then treated with CHX (50 µg/ml) and 0.39% DMSO, 50 µM cisplatin (diluted in DMSO), or 75 µM acetaldehyde (Sigma-Aldrich). One well of each 6-well dish per time-point was collected and lysed in RIPA buffer [150 mM NaCl, 50 mM Tris-HCL pH 7.2, 1% Triton X-100, 0.5 Na-deoxycholate, 0.1% SDS, 1mM EDTA, 1x Pierce Protease Inhibitor (Thermo), 1x Phosphatase Inhibitor (Thermo)]. Protein concentration was measured via Bradford assay, equal amount of protein was run on a 10% SDS-PAGE gel, transferred to PVDF membrane, and blotted for endogenous RECQL4 (rabbit polyclonal, Cell Signaling, 1:500, WB) and γ-tubulin ((mouse monoclonal, Sigma Aldrich, 1:5000) or GAPDH (mouse monoclonal, UBP Bio, 1:1000) as a loading control. The blots were scanned, the images were cropped using Photoshop. ImageJ was used to determine relative protein quantity by mean gray value measurement. It was then normalized to the loading control and to timepoint zero.

4.2.3 Cell proliferation (MTS) assay

Cells were seeded at 50,000/well in a 6 well dish. The next day 30 nM of siRNAs were transfected using INTERFERin (VWR #89129-930) per manufacturer's instructions. After 48 hours, the cells were then seeded at 5,000 cells/well in a 96-well plate, the remaining cells were used for western to confirm protein knockdown levels. The next morning, cells were treated with the indicated concentration of cisplatin. After 72 hours of treatment, 20 µl of MTS reagent was added to the media and allowed to incubate in the dark for two hours. Growth was measured by absorbance at 490 nm.

4.2.4 Clonogenic survival assay

Initial seeding and siRNAs transfection were performed as described for the MTS assays, except that after the 48 hours of siRNA treatment, the cells were then seeded 250 cells/well in a 6-well plate. The next morning, cells were treated with the indicated concentration of cisplatin. After 24 hours of treatment, cells were washed with PBS and fresh medium was added. Colonies were grown for 17 days. For staining, cells were washed twice with PBS before being fixed with methanol for 20 minutes and stained with crystal violet solution (0.5% crystal violet, 20% methanol) for 30 minutes. Colonies were counted using ImageJ plugin, ColonyArea [172]. Each condition was plated in triplicate and the experiment was independently performed three times. Results are plotted as a normalized area, where in the treated conditions were normalized to the untreated conditions.

4.2.5 Annexin V/PI staining

Initial seeding and siRNAs transfection were performed as described for the MTS assays, except that after the 48 hours of siRNA treatment, cisplatin was added at 1 μM and the cells were treated for 24 hours. Cells were then harvest for staining according to manufacturer protocol (ab14085). Accuri C6 was used to quantify the number of apoptotic/dead cells.

4.2.6 Immunostaining and imaging

U2OS cells were seeded at 50,000 cells/well in a 6-well dish that contained a coverslip in each well. The next day, the cells were transfected with an empty plasmid or plasmid expressing RECQL4 under a CMV promoter. 72 hours following transfection, the cells were treated with or without cisplatin for one hour in the dark. Afterwards, the cells were washed with PBS and fresh medium was added. The treated cells were allowed to recover for 2 hours. The coverslip was then removed and fixed with 4% PFA for 20 minutes in the dark at 4˚C. After fixation, the sample was treated with extraction buffer and then washed and incubated overnight with α-RAD51 (1:2000). Subsequently, the samples were washed and then incubated with Goat anti-Rabbit IgG Alexa Fluor 488 (1:2000) for an hour at RT. Following secondary antibody incubation, the samples were washed then mounted onto a slide using Prolong Gold solution with DAPI. The slides were allowed to dry overnight before imaging. Foci were image with Nikon-TiE, RAD51 foci was quantified using ImageJ plug-in, Fiji.

4.2.7 Neutral comet assay to detect DSBs and ssDNA

Initial seeding and transfection and cisplatin treatment are the same as described for the immunofluorescence experiments with the exception that a coverslip was not added. Following the 2 hours recovery, the cells were trypsinized and washed before being resuspended in PBS at 100,000 cells per milliliter. Subsequently, the cells were combined with molten LMAgarose (Cat# 4250-050-02) at 1:10 (cells: agarose) ratio, and immediately 30 µl was pipetted onto two 20-well CometSlideTM (Cat# 4252-02K-01). The mixture solidified at 4ºC before being immersed in chilled lysis solution (Cat# 4250-050-01) overnight. Subsequently, the slides were washed three times with 1x S1 nuclease buffer (50 mM NaCl, 30 mM sodium acetate, pH 4.6 and 5% glycerol). S1 nuclease (ThermoFisher #18001016) at 20 U/ml in S1 nuclease buffer and 50 mM NaCl was added to one of the two slides and incubated for 30 min at 37 ºC. As a control, the other slide was incubated with the same solution, but without S1 addition. The slides were then washed three times in chilled neutral electrophoresis buffer (100 mM Tris base, 300 mM sodium acetate). Afterwards, the slides equilibrated in the neutral electrophoresis buffer for 30 minutes at 4ºC. The slides were then placed in a chilled electrophoresis unit (Cat# 4250-05-ES) and submerged with chilled electrophoresis buffer. Electrophoresis was then run at 21 volts for 45 minutes at room temperature (RT). After electrophoresis, the slides were transferred to DNA precipitation buffer (6.7 ml of 7.5 M ammonium acetate and 43.3 ml 95% EtOH) and incubated at RT for 30 minutes. Following DNA precipitation, the slides were immersed in 70% EtOH and incubated for 30 minutes at RT. The slides were then allowed to dry overnight at RT and subsequently stained using SYBR Gold solution (1 ul of SYBR in 30 ml Tris-EDTA buffer, pH 7.5) for 30 minutes at RT. Afterwards, excess SYBR solution was removed, and the slides were dried overnight at RT. Comets were imaged with Nikon-TiE and quantified using Comet Assay IV software by Instem.

4.3 Results

4.3.1 RECQL4 protein level decreases following cisplatin or acetaldehyde exposure

We next sought to determine whether there is a conserved regulatory role for human RECQL4 following cisplatin exposure. We used U2OS cells since they are commonly used in DNA repair studies and Rothmund-Thompson patients with *RECQL4* mutations are predisposed to osteosarcomas. Since RECQL4 is needed for cisplatin resistance, we hypothesized that similar to Hrq1 its protein levels may also decrease follow cisplatin exposure. To test this hypothesis, we repeated our cycloheximide chase experiments in the U2OS cell line. Similar to yeast Hrq1, RECQL4 protein levels decrease following cisplatin exposure in comparison to the untreated control (**Figure 9a**).

We next determined whether this phenomenon holds true with an endogenous crosslinking agent, acetaldehyde. Acetaldehyde is known as the toxic byproduct of alcohol metabolism, but it is also naturally produced from the breakdown of various foods including yogurt, apples, etc. [118,119]. Acetaldehyde is highly reactive and can produce intrastrand crosslinks [117,120]. Like cisplatin, RECQL4 levels decrease following acetaldehyde exposure (**Figure 9b**). Together, these results suggest that there is a conserved regulatory mechanism between yeast Hrq1 and human RECQL4 during intrastrand crosslinks repair.

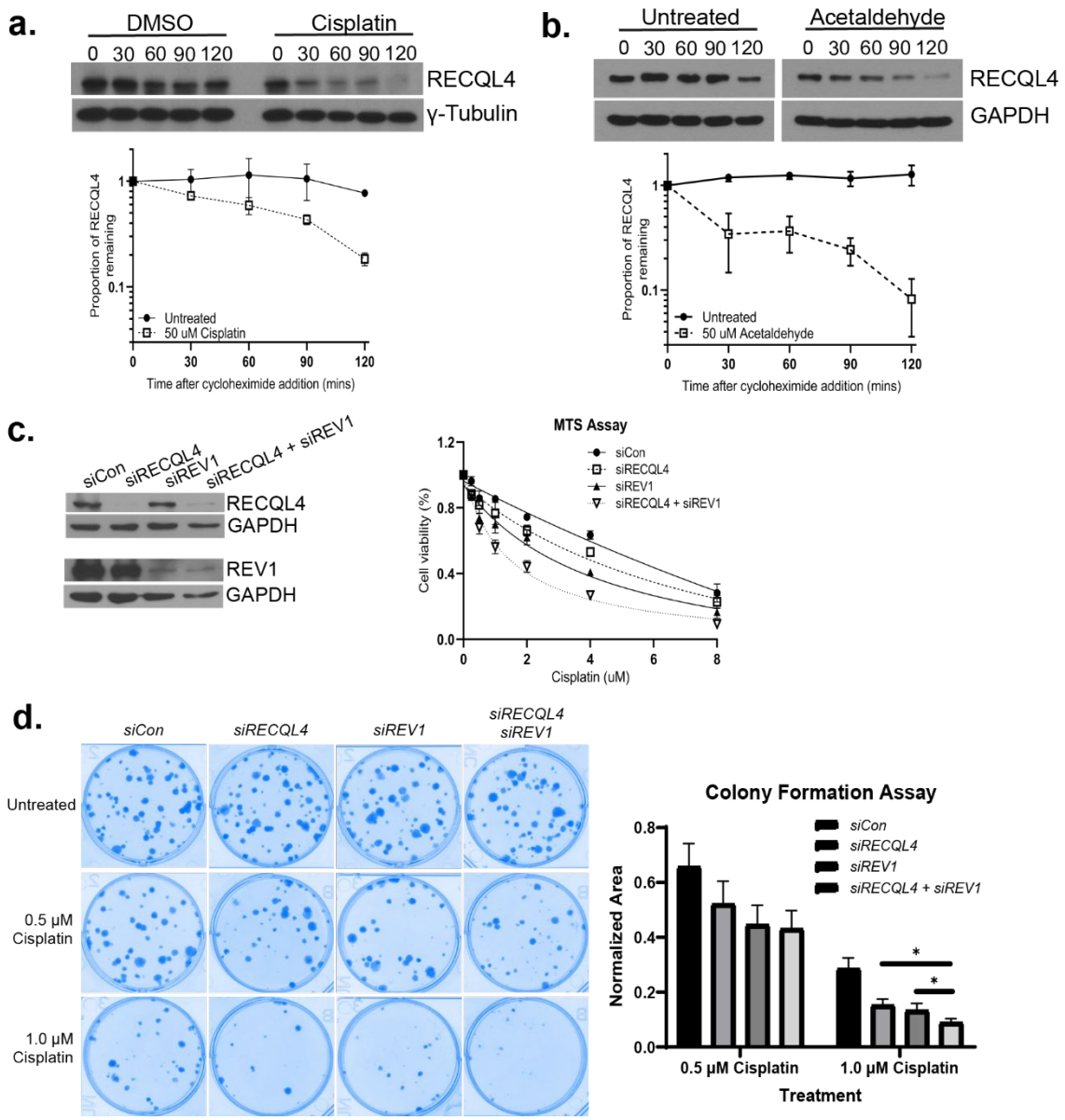


Figure 9. RECQL4 functions in a separate pathway from REV1 and is degraded following DNA crosslinks

a) RECQL4 levels decrease following cisplatin treatment. U2OS cells were incubated with 50 μg/ml cycloheximide in the presence or absence of 50 μg/ml cisplatin. Protein extracts were analyzed by western blot for endogenous RECQL4 protein levels (α-RECQL4) or a loading control, Tubulin (γ-Tubulin), at the indicated time points. Quantification of the proportion of RECQL4 remaining relative to time 0 (before CHX addition) and the loading control, Tubulin, are plotted on the graph in log scale. Both untreated and treated conditions were normalized to time 0 in their respective conditions. Each experiment was performed in triplicate with standard error plotted. **b)** RECQL4 protein levels decrease following acetaldehyde treatment. U2OS cells were incubated with 50 μg/ml cycloheximide

in the presence or absence of 50 μg/ml acetaldehyde. Protein extracts were analyzed by western blot for endogenous RECQL4 protein levels (α-RECQL4) or a loading control, GAPDH (α-GAPDH), at the indicated time points. Quantification of the proportion of RECQL4 remaining relative to time 0 (before CHX addition) and the loading control, GAPDH, are plotted on the graph in log scale. Both untreated and treated conditions were normalized to time 0 in their respective conditions. Each experiment was performed in triplicate with standard error plotted. **c)** Cisplatin exposed U2OS cells exhibit decreased viability when both RECQL4 and REV1 are knocked down by siRNA. MTS assay was performed on after 72 hr exposure to the indicated dose of cisplatin. Western is shown to confirmed knockdown of proteins, note since RECQL4 and REV1 are of similar size, the same samples were loaded twice to detect RECQL4 and REV1. Each experiment was performed three times with mean and standard errors plotted. **d)** Decreased cell viability following cisplatin exposure when both RECQL4 and REV1 are knocked down. Clonogenic survival assay with siControl (scrambled siCon), siRECQL4, siREV1, and siRECQL4 siREV1 treated cells with or without cisplatin. Each condition was plated in triplicate for each experiment and the experiment was performed three times with mean and standard errors plotted.

4.3.2 Knockdown of *REV1* and *RECQL4* results in increased cisplatin sensitivity

Since we find that Hrq1 functions in the error-free branch of PRR, we hypothesized that RECQL4 functions similarly. To test this, we determined the cisplatin sensitivity of U2OS cells with siRNA knockdown of *RECQL4* alone or in combination with the error-prone PRR polymerase, *REV1*. Using an MTS assay, we find that both *siRECQL4* and *siREV1* individually treated cells are cisplatin sensitive (**Figure 9c**). If *RECQL4* and *REV1* function in the same PRR branch, we expect that siRNA knockdown of both *RECQL4* and *REV1* will result in similar cisplatin sensitivity as one of the single knockdown. However, if *RECQL4* and *REV1* function in different pathways, we expect increased cisplatin sensitivity when both *RECQL4* and *REV1* are knocked down. Consistent with *RECQL4* and *REV1* functioning in different pathways, we observe increased cisplatin sensitivity in the double treated *siRECQL4 siREV1* cells using both MTS and

clonogenic survival assays (**Figure 9c,d,** respectively). These results suggest that, like *HRQ1*, *RECQL4* may function in the error-free PRR pathway. However, functional studies are needed to validate these genetic results. One such experiment could be to determine whether mutation rates increase after cisplatin treatment in *RECQL4* knock-down cells, which would suggest an increased utilization of TLS polymerases when RECQL4 levels are low.

4.3.3 Knockdown of *RECQL4* does not increase apoptosis following cisplatin exposure

Consistent with other studies, we found that knockdown of *RECQL4* in U2OS cells resulted in increased sensitivity to cisplatin (**Figure 9c,d**). We wanted to determine whether this decrease in cell number following cisplatin treatment in *RECQL4* knockdown cells was due to increased number of cells undergoing apoptosis. To test this, we performed Annexin V/PI staining following 24 hours of cisplatin treatment. We observed that *RECQL4* knockdown did not lead to a noticeable increase in apoptosis, indicative of cell death (**Figure 10a,b**). As this was the case, we next wanted to test whether there was an increase in senescence. To do this, we performed western blot analysis to examine p21 levels following *RECQL4* knockdown and observed increased p21 levels compared to the control conditions (**Figure 10c**). These results suggest that senescence may be increased upon RECQL4 knockdown. However other senescence markers, such beta-galactosidase and γ-H2AX staining and loss of Lamin B1, are needed to validate the senescence phenotype observed in *RECQL4* knockdown U2OS cells [173,174]. It should be noted that the p21 experiment was only performed once and is currently being repeated. However, in support of our preliminary findings, another study utilizing primary fibroblasts showed that RECQL4 dysfunction either by knockdown or helicase mutations leads to increased senescence, which might result from increased/persistence DNA damage [175]. Together, these results suggest that the

decrease cell number/colony observed may be due to senescence however more studies are needed to validate this conclusion.

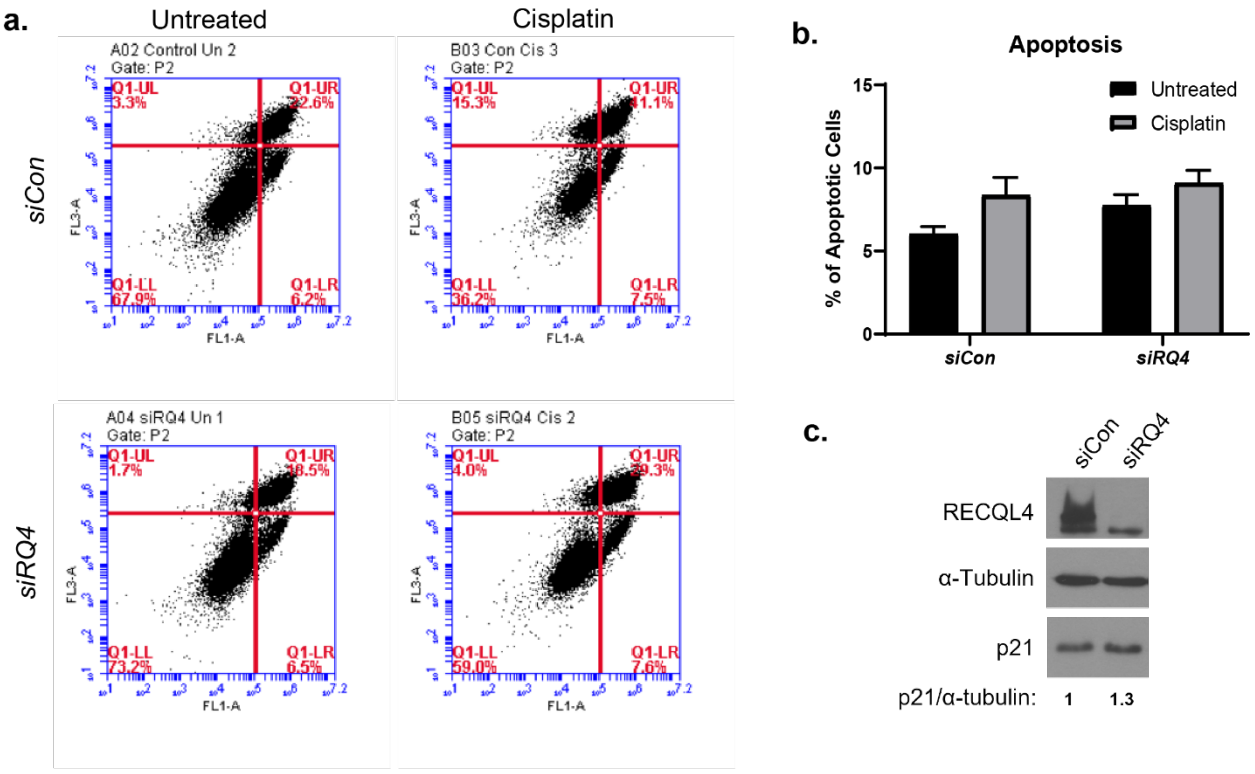


Figure 10. Knockdown of *RECQL4* does not lead to increased apoptosis but instead increased senescence

a) Annexin V/PI staining following *RECQL4* knockdown with or without cisplatin. **b)** Quantification of apoptotic cells from two experiments. **c)** Knockdown of *RECQL4* leads to increased p21 protein levels. p21 was normalized to tubulin, siRQ4 was normalized to siCon.

4.3.4 Overexpression of *RECQL4* results in increased RAD51 foci formation and decreased tail moment

Since yeast Hrq1 over-expression led to increased recombination, we examined whether over-expression of human RECQL4 also leads to increased recombination. To do this, we created a plasmid that over-expressed RECQL4 (**Appendix A: Supplemental Figure 7a**) and measured these cells for RAD51 focus formation in the presence or absence of cisplatin. RAD51 is a central

homologous recombination protein that is relocated to DNA repair sites following DNA damage. Suggesting that there is increased recombination, we find that RECQL4 over-expression results in a significant increase in the number of cells with RAD51 foci in both untreated and cisplatin treated cells (**Figure 11a;** $p < 0.0001$ for both). The number of RAD51 foci per cell were determined utilizing an ImageJ plug-in, Fiji. To be counted as a focus, it must be at least a certain size, that size was optimized from the cisplatin treated condition in each experiment. RAD51 foci from all conditions in an individual experiment were counted utilizing the same parameter. Since RECQL4 is a DNA helicase, we asked whether its helicase activity would be crucial for RAD51 focus formation upon cisplatin exposure. Suggesting that RECQL4 DNA unwinding activity likely contributes to RAD51 focus formation, we observe that that RECQL4 helicase mutant, RECQL4-K508A, exhibits reduced RAD51 foci compared to RECQL4 over-expressing cells (**Figure 11a**; $p < 0.01$). All of this suggests RECQL4 may have a role in mediating recombination. However RAD51 has other roles besides recombination, thus it should be tested whether *RECQL4* overexpression can lead to increased SCEs to determine whether *RECQL4* overexpression results in increased recombination.

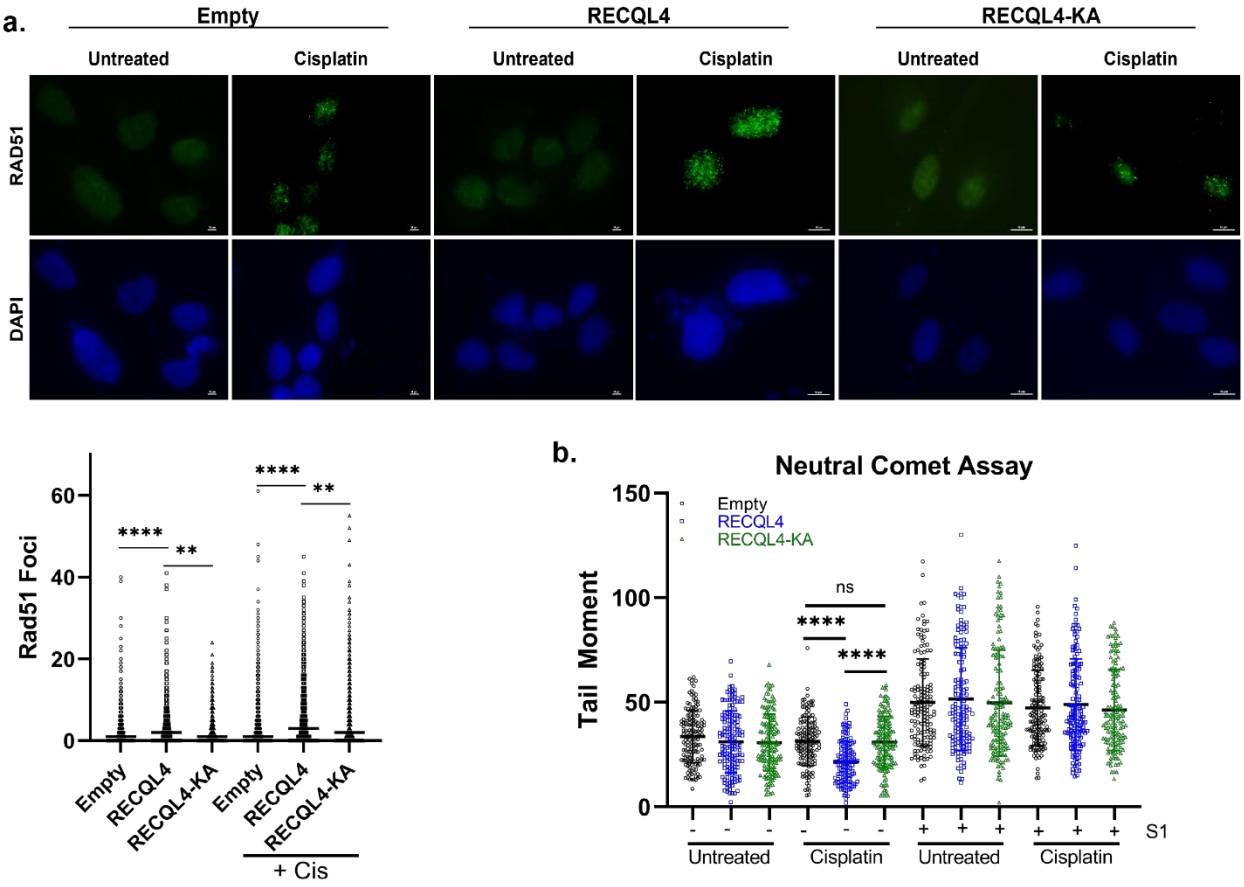


Figure 11. Overexpression of RECQL4 results in increased RAD51 foci and decreased tail moment

a) Overexpression of *RECQL4* results in increased RAD51 foci, which is dependent on its helicase activity. U2OS cells were transfected with an empty plasmid or a plasmid expressing RECQL4 or RECQL4-K508A under a CMV promoter. The cells were either mock or cisplatin treated for one hour and after a two-hour recovery, imaged for RAD51 foci or DAPI by immunofluorescence. RAD51 foci were quantified from 200 cells per condition for each experiment. The experiment was performed three to five times and the median was graphed. Representative images are shown. **b)** Overexpression of *RECQL4* results decreased tail moment following cisplatin exposure, which is dependent on its helicase activity. U2OS cells were treated similarly to the immunofluorescence experiment, before being harvested for neutral comet assay. At least 40 comets were counted per condition for each experiment. The experiment was performed four times and the mean and standard deviation was graphed.

The RAD51 foci observed upon RECQL4 over-expression could be indicative of increased DSB formation, recombination, or even ssDNA from excess RECQL4-mediated DNA unwinding. To examine whether RECQL4 overexpression leads to increased DSB formation, we performed neutral comet assays. We find that RECQL4 over-expression in untreated or cisplatin-treated cells do not increase tail moments compared to the empty control U2OS cell line (**Figure 11b**). Intriguingly, RECQL4 over-expression decreases tail moments upon cisplatin exposure.

Suggesting that RECQL4 helicase activity is critical to prevent DSB formation following cisplatin, the RECQL4 helicase mutant, RECQL4-K508A, has similar tail moments to the empty control (**Figure 11b**). These finding are consistent with a role for RECQL4 in preventing DSB formation and fork collapse, perhaps, by enabling lesion bypass through TS.

It is possible that the increased RAD51 foci observed may be due to more ssDNA caused by RECQL4 DNA unwinding. To examine whether RECQL4 over-expression results in more ssDNA, we quantitated the tail moments following S1 nuclease treatment. If more ssDNA is present, the S1 nuclease will degrade the ssDNA resulting in more DSBs. We find that treatment with S1 nuclease did not lead to increased neutral tail moments in *RECQL4* overexpressed cells suggesting that *RECQL4* overexpression does not result in increased ssDNA (**Figure 11b**). Overall, our studies suggests that RECQL4 over-expression does not significantly increase either DSBs or ssDNA. Therefore, the increased RAD51 foci observed upon *RECQL4* overexpression could be due to increased recombination happening at the replication fork.

4.4 Discussion

Unlike some of the other DNA helicases, RECQL4 has a critical role in DNA replication. So, besides the reasons discussed above why DNA helicases might need to be degraded following damage, since RECQL4 has a critical role in DNA replication initiation, it is easy to hypothesize that it needs to be degraded to ensure proper cell cycle progression. Our preliminary data suggests that this might be one of the reasons RECQL4 is degraded, as *RECQL4* overexpression results in an increased level of cyclin B1 (G_2/M) compared to the empty control (**Appendix A: Supplemental Figure 8a**). Studies suggest that cells transitioning from G2 to M phase are particularly sensitive to DNA damaging agents [176,177]. Thus, it possible that RECQL4 needs to be degraded to prevent transitioning to M-phase, where DNA damaging agents are particularly lethal. As we performed transient transfections to over-express *RECQL4*, there is a possibility that would we see an even more pronounced cell cycle arrest if we sorted out cells where *RECQL4* is overexpressed. Or perhaps another way around this is to create a stable cell line where we can induce *RECQL4* overexpression. That way we can gauge the effect of *RECQL4* overexpression on cell cycle progression and at the same time test whether these cells have increased sensitivity to cisplatin. Besides overexpression, it would be critical to determine which residues lead to RECQL4 stabilization. There are three conserved lysine residues in RECQL4: K568, K991, and K1101. Future studies should test whether mutation of these residues stabilizes RECQL4 following cisplatin exposure. Lastly, it should be noted that we did not observe cell cycle defects when we overexpressed/stabilized Hrq1 in the presence or absence of cisplatin (**Appendix A: Supplemental Figure 8b**). These results suggest that there are some differences between the two homologs, and that Hrq1 degradation is mainly due to a response to DNA damage.

It is interesting that even in the absence of damage *RECQL4* knockdown leads to increased cellular senescence [175]. RECQL4 as mentioned has roles in replication, DNA damage response, and telomere maintenance. Dysfunction in any of these processes could lead to cellular senescence. However, it is important to note that *RECQL4* knockdown leads to persistent DNA damage as observed by more 53BP1 and γH2AX foci, indicating that low levels of *RECQL4* leads to an increased in endogenous DNA damage [175]. As aldehydes are a potential endogenous source of DNA crosslinks, it would be interesting whether treatment with aldehyde dehydrogenase inhibitors leads to increased cellular senescence in *RECQL4* knockdown cells.

Since *RECQL4* overexpression leads to increased RAD51 foci and decrease neutral comet tails following cisplatin exposure, it is tempting to speculate that RECQL4 mediates recombination at stalled replication forks to prevent replication fork collapse. This could be tested by knocking down *RECQL4* and performing a neutral comet assay with and without cisplatin. It is important to note that besides its role in recombination, RAD51 has roles during DNA fork regression and fork protection also discussed further below.

4.5 Acknowledgement

Phoebe Parker helped quantify the number of RAD51 foci as well as tail moments from the neutral comet assay.

5.0 Bioinformatic analysis reveals elevated RECQL4 results in similar phenotypes

5.1 Introduction

Our results from yeast cells demonstrated that high levels of Hrq1 are associated with increased recombination and mutation (**Figure 8d,e**). Our preliminary data with mammalian cells indicates that high levels of RECQL4 may also be associated with increased recombination, as *RECQL4* overexpression is associated with increased RAD51 foci formation. Thus, we then wondered how much of this is seen in patients. Utilizing a bioinformatic approach, we find that high levels of *RECQL4* are seen more in tumors than normal tissue matched samples. Like our experimental results, we observed that high levels of *RECQL4* are associated with increased tumor mutation burden and *RAD51* enrichment.

5.2 Material and methods

5.2.1 Bioinformatic analysis

R version 3.6.1. was used for all the bioinformatic analysis. *RECQL4* expression in different samples was determined by first transcriptomic data from The Cancer Genome Atlas (TCGA) and Molecular Taxonomy of Breast Cancer International Consortium (METABRIC). TCGA data were downloaded from GSE62944 and Log2 (TPM+1) values were used for downstream analysis. For the METABRIC data set, normalized probe intensity values were

obtained from Synapse (Syn1688369). For genes with multiple probes, probes with the highest inter-quartile range (IQR) were selected to represent the gene.

Tumor mutation burden (TMB) calculation was performed as previous described [178]. Briefly, TCGA mutation annotation files from 982 patients were downloaded from FireBrowse and mutation subtypes were summarized using "maftools" package. Mutations subtypes were classified into truncated (nonsense, frame-shift deletion, frame-shift insertion, splice-site) and non-truncated mutations (missense, in-frame deletion, in-frame insertion, nonstop). TMB was calculated as 2X Truncating mutation numbers + non-truncating mutation numbers.

Pathway enrichment was performed by gene set variation analysis (GSVA). Using the GSVA program, we inputted the TNBC transcriptomic data from TCGA and METABRIC, and we used the defined 50 Hallmark gene sets from molecular signature database (MSigDB) as our gene sets. The program then provided which pathways and genes were enriched, we then stratified the results based on *RECQL4* expression. Clinical and pathological outcome was based on analysis from a (Silver et al. 2010) study where cisplatin was given as a neoadjuvant therapy in 24 TNBC tumors. Transcriptomic data were downloaded from GSE18864. Clinical response to cisplatin was measured as progressive disease (PD), stable disease (SD), partial response (cPR), complete response (cCR). For clinical response, good and poor responders were defined as patients with cPR/cCR status and PD/SD status respectively. Pathological response to cisplatin was monitored by Miller Payne metric score (grade 1 is no significant tumor reduction and grade 5 is compete tumor reduction) [179]. Good and poor responders were defined as patients with a Miller Payne Score of 4-5 or 0-3 respectively.

5.3 Results

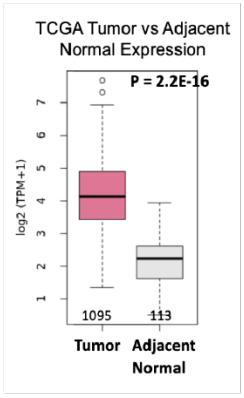
5.3.1 Overexpression of *RECQL4* leads to increased tumor mutation burden and *RAD51* enrichment

We find that Hrq1/RECQL4 over-expression results in genomic instability, which is a cancer hallmark. Therefore, we asked whether RECQL4 is over-expressed in breast tumors relative to normal tissue. By examining mRNA levels of *RECQL4* in TCGA, we observe that *RECQL4* expression is increased in tumors versus normal tissues (**Figure 12a**). Several tumor types where *RECQL4* is overexpressed are treated with cisplatin, including breast cancer. Therefore, we focused on breast cancer where we can delineate between breast cancers that are classically treated with cisplatin versus those that are not. Estrogen receptor negative (ER-) breast cancers are generally more aggressive and typically treated with cisplatin including TNBC, an ER- breast cancer subtype [180]. In contrast, ER+ breast cancers are classically treated with hormone therapy [180]. When compared to ER+ breast cancers, ER- breast cancers have elevated levels of *RECQL4* expression (**Figure 12b**). Our result is consistent with another study where *RECQL4* expression was elevated in more aggressive cancers [43].

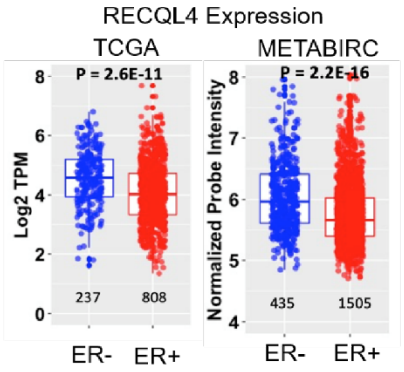
As elevated Hrq1 protein levels in yeast result in increased mutations, we postulated that increased human *RECQL4* expression correlates with increased tumor mutation burden. Indeed, when examining ER- cancers, high levels of *RECQL4* correlates with increased mutational burden (**Figure 12c**).

When yeast Hrq1 is over-expressed, we find that both mutations and recombination are increased (**Figure 8d,e**). Therefore, we asked whether over-expression of RECQL4 results in increased expression of recombination genes. Using the METABRIC and TCGA mRNA expression datasets, we examined which cellular pathways are enriched in RECQL4 over-expressing ER- tumors. This analysis revealed that cell cycle progression, transcriptional regulation, and DNA repair are enriched in RECQL4 high subset of TNBC (**Figure 12d**). Upon further analysis, we find that high levels of *RECQL4* significantly correlates with genes in the HR pathway and specifically with RAD51 (**Figure 12e**). These results are consistent with our yeast and mammalian studies here suggesting that high levels of *RECQL4* correlate with increased mutations and recombination.

a.



b.



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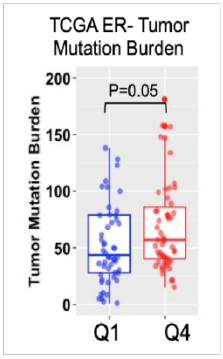

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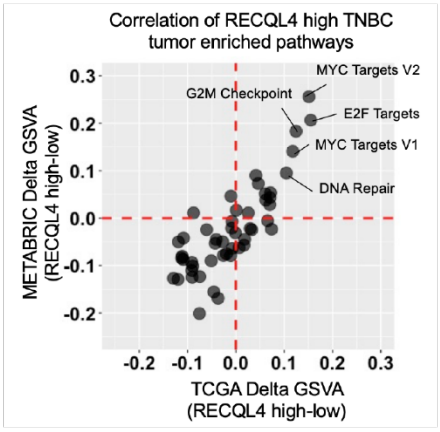
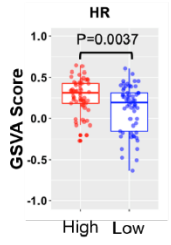


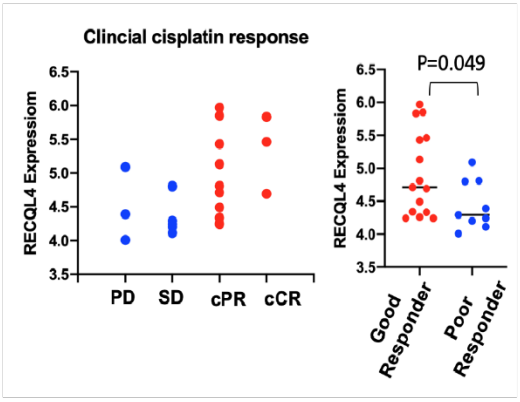
e.

Top20 Enriched DNA Repair Pathway genes

	Log2FC	padj
RAD51	0.907	6.60E-06
ZWINT	0.876	5.80E-46
ZNF707	0.773	1.56E-41
TYMS	0.767	4.64E-54
VPS28	0.657	3.44E-31
ALYREF	0.632	1.05E-46
POLD1	0.622	2.74E-41
FEN1	0.613	1.61E-43
ADRM1	0.612	2.25E-33
LIG1	0.595	1.76E-46
SAC3D1	0.577	4.74E-45
NME1	0.558	3.50E-30
PCNA	0.522	9.06E-21
NME3	0.510	2.77E-27
RFC4	0.474	1.19E-15
POLR2K	0.457	1.46E-27
BRF2	0.438	2.80E-22
NT5C	0.425	9.43E-08
POLA2	0.388	2.47E-16
APRT	0.385	1.04E-21



f.



g.

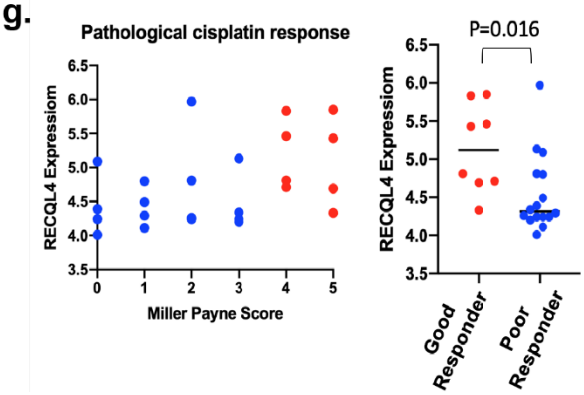


Figure 12. High RECQL4 expression coincides with increased recombination, mutations, and tumorigenesis

a) RECQL4 expression is elevated in tumors in comparison to adjacent normal tissue. RNAseq data from tumors and normal tissue matched samples was acquired from TCGA. The data was normalized by transcripts per million (TPM), then analyzed for *RECQL4* expression. Box and whisker plots graph the median of the data alongside the 25th and 75th percentile. The number of samples alongside p-value are shown using Mann-Whitney U test. **b)** RECQL4 expression is higher in ER- breast cancer. Transcriptome data from TCGA (RNA-seq) and METABRIC (microarray) was analyzed for *RECQL4* expression in ER- (blue) and ER+ (red) cancers. Log2 (TPM+1) and normalized probe intensity were used respectively. Box and whisker plots alongside each data point was graphed. The number of samples alongside p-value are shown using Mann-Whitney U test. **c)** Elevated levels of RECQL4 are associated with increased tumor mutation burden. The expression of RECQL4 in ER- breast tumors from TCGA was divided into quartiles based upon expression level. The mutation burden between the least expressing tumors (Quartile 1 – Q1, blue) and the highest expressing tumors (Quartile 4 – Q4, red) was analyzed. Box and whisker plots alongside each data point was graphed. The number of samples alongside p-value are shown. Annotated mutational data from ER- breast cancer was acquired from Firebrower. The mutational data was categorized using "maftools" in R. Tumor mutation burden was scored as described (Wang *et al* 2019). Significance was determined by Mann-Whitney U test. **d)** High expression of *RECQL4* correlates with enrichment of DNA repair, G2M checkpoint, MYC Targets V1, E2F Targets 1 and 2 in TNBC. Gene set variation analysis (GSVA) was performed on TNBC gene expression data from both TCGA and METABRIC using the 50 Hallmark gene sets from MSigDB. Correlation between TCGA and METABRIC datasets was plotted for enriched pathways. **e)** Homologous recombination and RAD51 is enriched in TNBC tumors expressing high levels of *RECQL4*. GVSA results of "KEGG Homologous Recombination" gene set (MSigDB M11675) was parsed for the top 20 DNA repair genes enriched in TNBC tumors expressing high levels of *RECQL4*. HR pathway was enriched by GSVA analysis in RECQL4 high expressing TNBC compared to loss expressing TBNC. Box and whisker plots alongside each data point was graphed. The number of samples alongside p-value are shown using Mann-Whitney U test. **f, g)** *RECQL4* expression serves as a predictor for clinical outcomes. High expression of *RECQL4* correlates with positive response to cisplatin and favorable clinical outcome (Good responder). Analysis from a (Silver *et al* 2010) study where cisplatin was given as a neoadjuvant therapy in 24 TNBC tumors. Lower expressing RECQL4 tumors are shown as blue dots and high expressing RECQL4 tumors are shown as red dots. Clinical response to cisplatin was measured as progressive disease (PD), stable disease (SD), partial response (cPR),

complete response (cCR). Good and poor responders were defined as patients with cPR/cCR status and PD/SD status respectively. Increased RECQL4 expression correlates with a good clinical response as determined by Mann-Whitney U test. Pathological response to cisplatin was monitored by Miller Payne metric score (grade 1 is no significant tumor reduction and grade 5 is compete tumor reduction) and graphed based on RECQL4 expression. Good and poor responders were defined as patients with a Miller Payne Score of 4-5 or 0-3 respectively. Increased RECQL4 expression correlates with a good pathological response as determined by Mann-Whitney U test.

5.3.2 High levels of *RECQL4* correlates with positive therapeutic response to cisplatin

In tumor cells, DNA repair defects can result in therapeutic sensitivity. Since *RECQL4* overexpression leads to increased genomic instability, we asked whether *RECQL4* expression may predict therapeutic response to cisplatin. In cisplatin-treated TNBCs, tumors with elevated *RECQL4* expression have a better clinical response compared to tumors with lower *RECQL4* expression (**Figure 12f,g**). This suggests that increased *RECQL4* levels may provide a prognostic marker for therapeutic response to cisplatin.

5.4 Discussion

Although high levels of RECQL4 are associated with tumorigenesis (**Figure 12a**), the potential underlying mechanisms of how RECQL4 could promote tumorigenesis are still being elucidated. First, RECQL4 is associated with replication initiation, which could be beneficial for cancer cells which are rapidly dividing and proliferating. Secondly, RECQL4 is critical in DNA repair, whereas chemotherapeutic agents are classically DNA damaging agents, such ionizing radiation and cisplatin, thus high levels of RECQL4 can mediate repair allowing cancer cells to

survive and progress even in the presence of excess DNA damage. Lastly, RECQL4 has a role in telomere maintenance, which could be advantageous for cancers as they need to maintain telomere length to survive. Here, we defined another manner of how RECQL4 dysregulation could promote tumor progression by potentially mediating recombination, which could lead to genomic instability, a hallmark of cancer.

Besides increased recombination, RECQL4 dysregulation could also lead to increased mutations as observed in our TNBC analysis. We also repeated this analysis in ovarian cancer where we saw positive correlation with tumor mutation burden and *RECQL4* expression levels (**Appendix A: Supplemental Figure 7b**). Increased mutations could be advantageous or deleterious for cancer cells. For example, mutations in certain oncogenes can lead to constitutive activation thus promoting tumorigenesis, one classic example being the BRAF V600E mutation [181]. Also, mutations could also lead to restoration of function allowing for cancer to combat chemotherapeutics, such as restoration of *RAD51C* and *RAD51D* in ovarian cancer to acquire resistance to PARP inhibitors [182]. However, mutations have also been implicated to have a positive impact in immunotherapy due to production of neoantigens [183,184]. For example, there are studies that have indicated that increased mutational load led to increased response to anti-PD1/PDL-1 therapy [185]. Thus, it would be interesting to determine whether increased mutations associated with *RECQL4* could serve as a predictive marker for therapeutic response to immunotherapy treatment. To our knowledge this has not been investigated. However there have been studies linking *RECQL4* expression levels to cisplatin response. For example, a recent study showed that gastric cancer cell lines with higher expression of *RECQL4* correlates with cisplatin resistance [44]. However, in our studies we saw that elevated levels of *RECQL4* correlated with a

better response to cisplatin (**Figure 12f,g**). The difference between these two results could simply be because one is in a cell system while the other is in patients, where the immune system may have a role. It would also be interesting to determine whether there would be a synergistic effect with cisplatin and immunotherapy treatment in patients with increased levels of *RECQL4*.

5.5 Acknowledgement

The bioinformatic work done in this section was performed by Zheqi Li and Nolan Priedigkeit from Adrian Lee's lab. I repeated the tumor mutation burden analysis with ovarian cancer data.

6.0 Discussion and future directions

Here we identified a role for Hrq1 in mediating bypass of intrastrand crosslinks during replication. Although Hrq1 is needed for bypassing intrastrand crosslinks, it is paradoxically degraded by the proteasome following damage. Overexpression or stabilization of Hrq1 leads to increased recombination and mutation. We extended this study to mammalian cells showing that there is likely a conserved regulatory mechanism shared between Hrq1 and RECQL4, as RECQL4 protein levels also decrease following cisplatin exposure. Overexpression of RECQL4 results in increased RAD51 foci following cisplatin exposure. Lastly, our bioinformatic analysis reveals that high levels of *RECQL4* are associated with increased RAD51 expression and increased tumor mutation burden.

Our current working model is after a DNA intrastrand crosslink occurs during DNA replication, the crosslink can stall the fork, which leads to mono-ubiquitylation of PCNA by Rad6/Rad18 (**Figure 13**). Subsequently, the lesion is bypassed by the translesion polymerase, Rev1, or alternatively, polyubiquitylation by Rad5-Ubc13-Mms2 promotes a template switching mechanism. Our model shows that Hrq1 mediates template switching and is subsequently ubiquitylated by Rad16 and degraded by the proteasome (**Figure 13**). After the replication fork bypasses the damage, then NER mediates removal of the intrastrand crosslink. In this chapter, we will discuss the outstanding questions and the broader implications of our work.

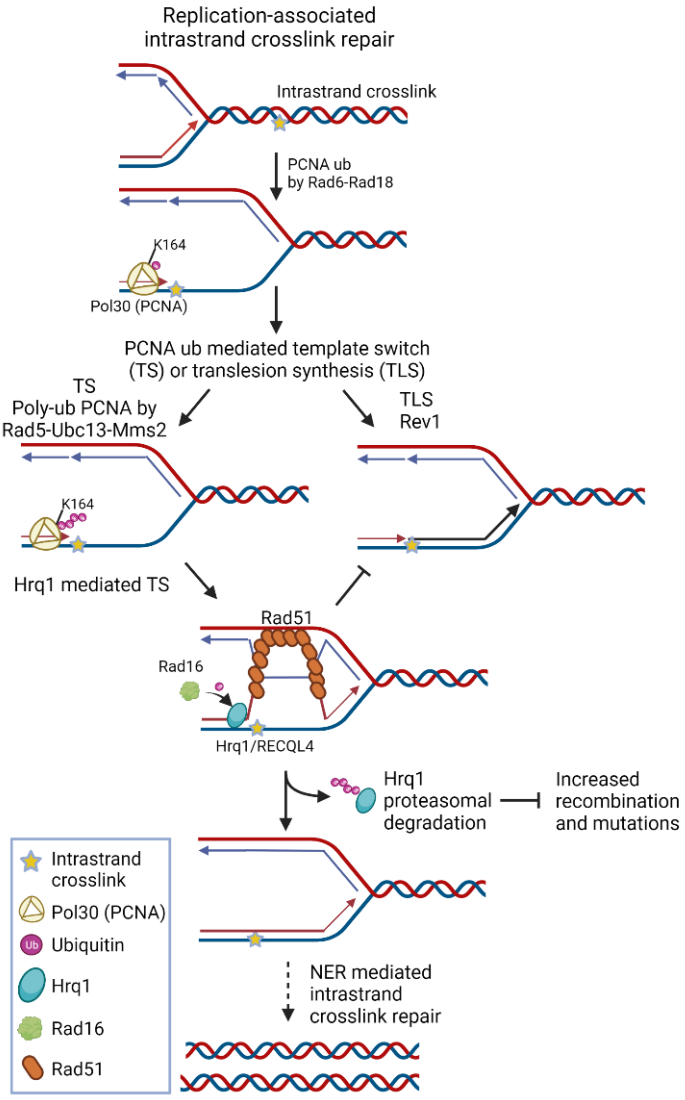


Figure 13. Model of Hrq1/RECQL4 function during replication-associated intrastrand crosslink repair

6.1 Does RECQL4 belong in error-free template switch pathway

The big question that remains is whether RECQL4 also has a function in the error-free template switching pathway during post-replicative repair. DNA damage repair or tolerance during replication is critical for our genomic integrity. In fact, work from Bert Vogelstein and Cristian

Tomasetti suggests that errors that arise during replication should be considered one of the major determinants of cancer development alongside genetics and environmental factors [186,187]. One of the major players during replication is the damage tolerance/PRR pathway. This pathway as discussed is composed of error-prone TLS branch and "error-free" template switch (TS) branch. Our results from budding yeast indicate that Hrq1 has a function during TS. Our initial results in mammalian cells suggest that RECQL4 may function similarly to Hrq1 as there is a synthetic sickness between *RECQL4* and *REV1* following cisplatin exposure. Moreover, RECQL4 is also cell-cycle regulated with protein levels peaking in S-phase and has a high affinity to DNA fork substrates with a *Kd* in the nanomolar range (**Table 2**, [107]).

To determine whether RECQL4 acts in the TS pathway, future studies should test whether RECQL4 is epistatic to the Rad5 homologs, HLTF and SHPRH, following cisplatin treatment [188–190]. Furthermore, TS can occur with or without fork regression [162,191]. As RECQL4 can potentially mediate DNA fork regression due to its DNA annealing and unwinding activities, it would be interesting to determine what RECQL4 function is during this process. *In vitro* replication fork regression assays could be performed to test RECQL4s ability to mediate fork regression[192,193]. Another approach would be to use electron microscopy to determine whether *RECQL4* overexpression leads to increased fork regression following cisplatin exposure. Determining RECQL4 function during DNA crosslink repair would provide insights into how *RECQL4* dysfunction contributes to diseases such as cancer.

6.1.1 How does Hrq1/RECQL4 get recruited to replication fork?

If RECQL4 does indeed have a role during TS, the question then becomes how does Hrq1/RECQL4 get recruited to the replication fork? Is this recruitment dependent on DNA

damage? It is well characterized how TLS polymerases are recruited to stalled replication forks, as they have a putative PCNA interacting motif (PIP boxes) alongside a ubiquitin interacting motif. It is because of these two motifs, TLS polymerases can interact with ubiquitylated PCNA. However, it is still unknown how poly-ubiquitylation of PCNA can trigger TS. Furthermore, whether this poly-ubiquitylation of PCNA lead to recruitment of proteins involved in this pathway is still unclear. To test whether PCNA poly-ubiquitylation leads to recruitment of Hrq1/RECQL4, we could perform chromatin fractionation experiments where factors, Rad5-Ubc13-Mms2 (HLTF-UBE2N-UBE2V2), that mediated poly-ubiquitylation of PCNA are knockdown [194]. Would knockdown of these factors lead to decreased Hrq1/RECQL4 at the chromatin?

When the fork stalls the replicative helicase and DNA polymerases become uncoupled, the replicative will continue ahead of the polymerase leading to excess ssDNA. RPA will bind to the ssDNA, not only to protect it but also serve as a platform for many proteins perhaps even RECQL4. Consistent with this idea a study from the Bohr's group identified that RECQL4 and RPA co-localized via immunofluorescence [195]. Furthermore, utilizing *in vitro* biochemical assays they identified that RPA stimulates RECQL4 helicase activity. Thus, it is possible that RECQL4 gets recruited to stalled replication forks via an interaction with RPA. However, the potential interaction between RPA and RECQL4 needs to be tested by other approaches such as co-IP with and without damage.

Lastly, if we do observe RECQL4 enriched at the chromatin following damage, this still does not necessarily mean it is being recruited to the replication fork. One technique that could be utilized to test whether RECQL4 is at fork is isolation of proteins on nascent DNA (iPOND). This technique allows for temporal analysis of proteins that are recruited to replication forks, this is done by labeling newly synthesized DNA with 5-ethynyl-2'-deoxyuridine (EdU), followed by

crosslinking proteins to the DNA. The EdU labeled DNA can be linked to biotin using click chemistry, this facilitates purification of by a streptavidin-biotin purification. The protein of interest can then be identified by western blot. To date iPOND has only been done with hydroxyurea and methyl methanesulfonate [196–199]. Performing iPOND and mass-spectrometry in the absence or presence of cisplatin could determine whether RECQL4 is present at the replication fork as well as other key factors.

6.1.2 How does Hrq1/RECQL4 mediate recombination

Overexpression of Hrq1 or RECQL4 correlates with increased recombination or increased RAD51 foci respectively. It is important to note that RAD51 has roles outside of recombination, discussed below, nevertheless this section will focus on its roles in recombination. The question now becomes how does Hrq1/RECQL4 mediate recombination? Although recombination is a complex process involving a multitude of proteins, which can either promote or antagonize the process, it can be broken down to a few core steps. The first step is substrate availability, which in this case is ssDNA, which can arise from various sources such DNA resection, CMG (replicative) helicase run-off at stalled replication forks, or DNA unwinding [200,201]. The second step is binding of ssDNA by RPA to prevent nucleolytic degradation and/or secondary structure formation [202–204]. Third is the displacement of RPA for RAD51, which will facilitate homolog search and recombination [205,206]. DNA helicases have the ability to be pro- and/or anti-recombinogenic [207–209]. One classic example is the RecQ helicase, Sgs1/BLM, where it can promote recombination by mediating DNA end resection, but it can also antagonize RAD51 loading thus suppressing recombination [171,208,210,211].

Unlike BLM, RECQL4 only has pro- recombinogenic activity, as observed in our studies and others, where it promotes DNA end resection during repair of DSBs [73]. We wanted to test whether *RECQL4* overexpression would lead to increased ssDNA. We saw that there was not an increase of ssDNA following *RECQL4* overexpression (**Figure 11b**), but this was examined at a fixed point in time, and therefore, we cannot rule out that *RECQL4* overexpression may lead to increased ssDNA. Moreover, a possible technical limitation of this assay is that ssDNA cleavage by the S1 endonuclease may be inhibited by RPA. Thus, it is possible that *RECQL4* overexpression may lead to increased ssDNA but was just not picked up by this assay. In fact, we noticed that overexpression of *RECQL4* leads to increased phospho-CHK1, which is indicative of increased ssDNA and/or replication stress (**Appendix A: Supplemental Figure 8c**). However, this phenotype may be simply due to RECQL4's role in replication initiation. Future studies should test the effects of Hrq1/RECQL4 overexpression on RPA foci formation as well as chromatin association with or without cisplatin exposure. This would give us insights on whether there is increased ssDNA as well as whether RPA is being displaced to mediated RAD51 loading which DNA helicases such as FBH1 have been shown to do. Of course, this should be correlated with RAD51 foci and chromatin loading and be analyzed over time, which would provide additional kinetic information.

6.2 RAD51 and fork regression/protection

We observed that *RECQL4* overexpression results in increased RAD51 foci. Although RAD51 is canonically known as a central recombinase, recent studies have indicated RAD51 has other roles including fork regression and fork protection. Following fork stalling, fork regression

is critical to limit ssDNA accumulation, provide time and space, place the lesion in the context of dsDNA for repair, and enable HR-mediated processes [154,212]. Although it is still unknown how RAD51 mediates fork regression, it has been suggested that its strand exchange activities is not required for this process [213]. Once the regressed fork is formed, RAD51 is also critical for protecting the fork from enzymatic degradation. If the regressed arm is left unprotected, it is susceptible to degradation from EXO1 or DNA2 [214–216]. Consistent with a role in fork protection, RAD51 destabilizing mutant, *RAD51-T131P*, has impaired fork protection activity [217]. Since RAD51 has roles during these processes, it will be critical to determine whether RECQL4 has any role in promoting these processes. Above we already discussed possible assays that could be used to determine whether RECQL4 can drive DNA regression. If RECQL4 has roles in mediating RAD51 during fork protection, then we should see less nascent DNA degradation following *RECQL4* overexpression. To look at fork protection, we can perform a modified fork protection assay. Following transient transfection with empty control or *RECQL4*, we can then pulse with 5-chloro-2'-deoxyuridine (CldU) followed by 5-iodo-2'-deoxyuridine (IdU) following by treatment with or without cisplatin. Nascent DNA degradation can be gauged by calculating the ratio of Idu/CldU, nascent DNA degradation following fork regression would lead to a lower IdU/CldU ratio. If *RECQL4* in mediate fork protection through RAD51, then *RECQL4* overexpression should result in higher IdU/CldU compared to the empty control.

6.3 Hrq1/RECQL4 and DNA protein crosslink repair

Besides intrastrand crosslinks; acetaldehyde, cisplatin, and UV could also induce DNA-protein crosslinks (DPCs), where proteins are covalently linked to the DNA. DPCs are highly toxic

lesions as they can interfered with normal DNA metabolism. Furthermore, DPCs can stalled DNA replication and transcription, which could lead to replication fork collapse and ultimately cell death [218]. Whether or not Hrq1 or RECQL4 has a role during DPC repair needs to be investigated. Human SPRTN and yeast Wss1 are proteases that mediated repair of DPCs by degrading proteins that are covalently linked to the DNA [218,219]. If Hrq1 has a role during DPC repair, one would expect that in the absence of Wss1, there will be increased dependency in Hrq1. This could be tested by determining the genetic relationship between Hrq1 and Wss1, if Hrq1 is needed for DPC repair, then deletion of *HRQ1* in a *wss1Δ* cells should result in increased sensitivity to DPC inducing agents. Similar experiments could be performed in mammalian cells to determine whether *RECQL4* knockdown in *SPRTN* knockdown cells will resulted in increased sensitivity to DPC inducing agents.

6.4 Role of ubiquitylation in regulating RECQL4 function and level

It is still unclear what mediates RECQL4 degradation and whether its degradation is lesion specific. Early on, RECQL4 was shown to interact with ubiquitin ligases, UBR1 and UBR2, of the N-end rule pathway [220]. However, in this study they were unable to detect ubiquitylated RECQL4 in HeLa cells and RECQL4 was relatively stable. It should be of note though that these assays were done without damage, so it is possible that ubiquitylation and subsequent degradation of RECQL4 is dependent on DNA damage.

Indeed, this seems to be the case, as recent studies demonstrated that RECQL4 is ubiquitylated during DSBR. However, these studies have conflicting results. Lu et al observed that RECQL4 is ubiquitylated by DDB1-CUL4A following CDK1/CDK2 phosphorylation at S89 and

S251. Ubiquitylation of RECQL4 results in increased RECQL4 recruitment to chromatin and sites of laser-induced DSBs [55]. However, a later study by Tan et al demonstrated that RECQL4 is ubiquitylated at K876, K1048, and K1101 by RNF8 with the help of WRAP53β. In this case, RECQL4 ubiquitylation led to dissociation of RECQL4 from DSBs sites [74]. The second study did not interrogate whether ubiquitylation of RECQL4 by RNF8 is facilitated by CDK1/CDK2 phosphorylation or not. Furthermore, the study by Lu et al did not determine where DDB1-CUL4A ubiquitylates RECQL4. Thus, it is possible that the ubiquitylation sites are different, where ubiquitylation by DDB1-CUL4A leads to accumulation of RECQL4 whereas RNF8-mediated ubiquitylation leads to its dissociation. It is also possible that DDB1-CUL4A and RNF8 compete to dynamically regulate RECQL4 at DSB sites. Additional studies are needed to differentiate between these possibilities. In these studies, they demonstrated that ubiquitylation of RECQL4 is critical in regulating its function during DSBR, but they did not test whether ubiquitylation of RECQL4 leads to its degradation. However, it is interesting to note that we did not observe decreased Hrq1 protein levels following IR treatment. These results are consistent with another study which also noted no change in RECQL4 protein levels following IR treatment as well [221].

However, there was a recent study that show RECQL4 is marked for degradation after treatment with bleomycin by the E2/E3 ubiquitin-conjugating enzyme, UBE2O, to negatively regulate RECQL4 function during DNA end resection [222]. It was subsequently noted that UBE2O overexpression induces RECQL4 degradation and thereby inhibits HR-mediated DSB repair as measured by DR-GFP [222]. The discrepancies between this study and the Shamanna study could arise from the fact that they used different cell lines, HEK293T and U2OS respectively.

To date the only DNA damaging agent that has been shown to induce RECQL4 degradation is bleomycin as shown in the Huang study. We showed that RECQL4 is also degraded following cisplatin and acetaldehyde. It would be interesting to test whether other DNA damaging agents evokes a similar response as RECQL4 has been implicated in multiple DNA repair pathways. Due to the similarities of lesions that UV and cisplatin causes, the most pertinent source of DNA damage to test would be UV.

6.5 RECQL4's role and regulation during NER?

Besides its potential role in TS to bypass intrastrand crosslinks, one of the more interesting future directions would be to dissect Hrq1/RECQL4 potential role during NER. As discussed, a subset of RTS patients have increased UV sensitivity. Furthermore, studies in yeast and mammalian cells demonstrated that decreased levels of Hrq1/RECQL4 lead to increased UV sensitivity. Is this UV sensitivity simply due to its role during replication to bypass these lesions or is there something more? Supporting the notion that RECQL4 may function during canonical NER, RECQL4 forms foci and interacts with XPA following UV treatment, although this may be an artifact.[86] However in yeast, it has been shown that Hrq1 can interact with Rad14, yeast homolog of XPA, via Y2H [223], however other assays are needed to confirm this interaction. Nevertheless, if Hrq1/RECQL4 does have a role during this process, the question becomes what could RECQL4 be doing? Due to RECQL4 helicase activity as well as its high affinity to DNA bubble substrates (**Table 2**), could it perhaps aid in DNA unwinding following damage recognition? If this is the case, then one could expect recruitment of downstream factors such as endonuclease, XPF, to be diminished if *RECQL4* knockdown following UV irradiation.

Our preliminary results suggest that Hrq1 is degraded following UV exposure, induces 6-4 photoproducts and CPDs, (**Appendix A: Supplemental Figure 2**), it is intriguing to speculate that RECQL4 may also be degraded following UV irradiation. This should be tested by performing cycloheximide chases following UV irradiation. Ubiquitylation and subsequent degradation plays a key role in orchestrating lesion "hand-off" in NER [224]. For example, it has been suggested that DDB2 that persists at damaged DNA needs to be degraded to allow for efficient recruitment of downstream factors, such as the TFIIH complex [225]. It has been suggested that RECQL4 interacts with XPA following UV irradiation. Could there perhaps be a similar regulatory mechanism where if RECQL4 stays at the damaged site too long it would disrupt recruitment of downstream factors? A stabilization mutant of RECQL4 would help address these questions. But for now, simply overexpressing *RECQL4* and measuring recruitment of downstream factors following UV irradiation could be analyzed. Another way would be to determine which protein marks RECQL4 for degradation. Our studies demonstrated that in yeast, the E3 ubiquitin ligase, Rad16, helps regulate Hrq1 protein levels. Rad16 has no sequence homolog, but it functions similarly to UV-DDB [226]. As Rad16 binds to UV-damaged DNA and ubiquitylates yeast XPC homolog, Rad4 [133,227], it would be intriguing if UV-DBB could regulate RECQL4 protein levels during repair of UV-induced lesions.

Another approach would be to test if VCP/p97 could mediate RECQL4 degradation. Valosin-containing protein (VCP)/p97 is an AAA ATPase which has been implicated to be involved in protein turnover and degradation [228,229]. Moreover, VCP/p97 has been implicated to remove ubiquitylated DNA repair proteins from the DNA to mediate timely repair [228–230]. Recently there have developments of p97 inhibitors, so we can test the hypothesis that p97 could

mediate RECQL4 degradation [231]. If p97 has a role in mediating RECQL4 degradation, then treatment with p97 inhibitors should lead to stabilization of RECQL4 following damage.

6.6 Friend or foe: tumor suppressor or therapeutic target

The RecQ family of helicases has been labeled the "Guardians of the Genome", whose function is to maintain genomic stability and thus prevent disease [1,3–6]. However, there have been recent studies that have suggested that RecQ helicases have oncogenic potential [43,232]. In fact, high levels of RECQL4 are associated with multiple types of cancer, such as breast and gastric [43,44]. That leads to the question whether RECQL4 functions as a tumor suppressor or is it a viable target for cancer therapeutics, perhaps both. Of course, the idea of targeting DNA repair proteins for cancer treatment is not new. One of the most famous examples is the development of PARP inhibitors (PARPi) to treat HR-deficient tumors [233–236]. The idea being that inhibition of PARP will lead to unrepaired ssDNA breaks, which could eventually lead to a collapse replication fork where HR factors would have to repair the resultant DSB (alternative models discussed below). Besides PARPi there has been a recent interest to develop DNA ligase inhibitors as most DNA repair processes require DNA ligases for completion [237–240].

Like DNA ligases inhibitors, there has been recent interest in inhibiting RECQL4 as a therapeutic target as it is involved in multiple DNA repair pathways. RECQL4 is also an attractive therapeutic target because inhibiting it could block tumor cell proliferation due to its role in replication initiation [47]. However, given RECQL4's importance in replication initiation, targeting RECQL4 would have off target affects due to causing cellular death to both proliferating normal and cancer cells. Inhibitors that block RECQL4's helicase function may be a potential

therapeutic target. However, studies in *Xenopus* suggest that RECQL4 ATPase activity is critical for replication initiation. Another approach would be to create inhibitors that disrupt RECQL4 interaction with its protein partners (**Figure 1, Table 1**). However, to date, there is still not a full crystal structure of RECQL4. The closest is from the Kisker group where they have a crystal structure encompassing the ATPase domain and a large fraction of the C-terminus (427-1116 aa) [241]. With the advance of AlphaFold and other computational approaches, perhaps one day we will be able to accurately predict the full protein structure of RECQL4.

Since high levels of *RECQL4* are seen in multiple tumor types, it could potentially serve as an attractive biomarker for cancer progression. Our studies indicate that in TNBC patients, *RECQL4* expression could serve as a predictive biomarker for cisplatin response. However it should be noted that since RECQL4 has a role in processing of cisplatin induced lesions, there have been studies that implicated that high levels of *RECQL4* results in resistance to cisplatin in gastric cancer cell lines [44]. This is in direct contrast to what we just shown. It is possible that the increased mutations observed with RECQL4 over-expression elicits a tumor immune response that is not observed in cultured cells or perhaps the small sample size or cohort analyzed may be distinct. Thus, more studies are needed to explain the differences that was observed in cell lines versus our results.

6.7 Limitations of bioinformatic approaches

Although bioinformatic approaches are powerful tools, all results that come from these analyses need to be validate experimentally before drawing any major conclusion. For example, we observed that ER- tumors that had higher expression of *RECQL4* had increased tumor mutation

burden. However, this is correlation and does not necessarily mean that high levels of RECQL4 led to increased mutations in these tumors. To test whether high levels of RECQL4 could lead to increased mutations, we can perform a HPRT mutagenesis assay with or without cisplatin following overexpression of *RECQL4* [242]. If high levels of RECQL4 can lead to increased mutations, then we should see increased mutations in *RECQL4* overexpression cells versus the empty control. Our results indicates that high levels of *RECQL4* is correlated with enrichment of HR genes, which is consistent with our yeast data that high levels of *HRQ1* may lead to increased recombination. However, this also needs to be tested. If RECQL4 can promote recombination, then we should observe increased SCE in cells that overexpressed *RECQL4.* It would also be interesting to take tumor tissues and stain for RECQL4 and RAD51 to see whether they co-localized or their expressions correlate with each other. Overall although our bioinformatic studies demonstrate results that are consistent with our yeast data, these are still correlation studies and experimental studies are needed to validate these results.

6.8 Replication gaps suppression by RECQL4

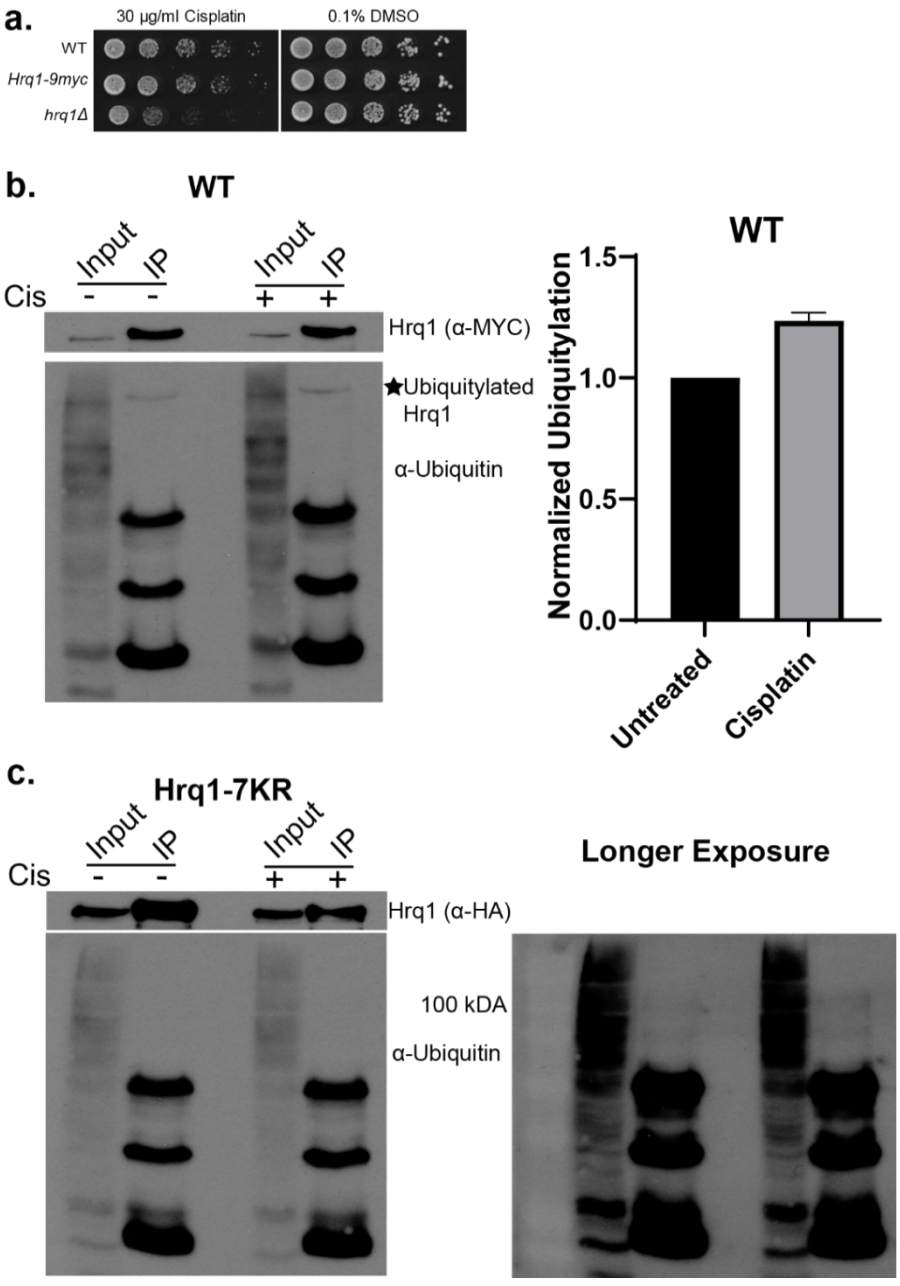
Loss or inhibition of Rad27/FEN1 results in increased dependency in HR factors [165,243,244]. It has been suggested that HR factors are needed to suppress replication gaps that arise from unprocessed Okazaki fragments when Rad27/FEN1 is inhibited. In yeast, we observed synthetic sickness between *rad27Δ* and *hrq1Δ,* as the double knockout strain is more sensitive to cisplatin than the single knockout strains (**Appendix A: Supplemental Figure 6b**). This suggests that Hrq1 may play a role in replication gap suppression. It would be interesting to determine if we would observe a similar genetic interaction between FEN1 and RECQL4. This is particularly

important given the recent work by the Cantor lab, that suggests replication gap accumulation is one of the key determinants in therapeutic response to PARPi in HR-deficient tumors [245,246]. If we do observe similar genetic interactions between FEN1 and RECQL4, perhaps we can target FEN1 in tumors that have low *RECQL4* expression.

6.9 Concluding remarks

In this work, we expanded the role of Hrq1 to mediate intrastrand crosslinks repair. Furthermore, we identified that Hrq1 is degraded by the proteasome following induction of intrastrand crosslinks. We find that stabilization or overexpression of Hrq1 results in increased recombination and mutations following damage. We then extended this work in U2OS cells and observed that human RECQL4 is regulated similarly to yeast Hrq1 and that *RECQL4* overexpression is associated with increased recombination. Importantly, we found that treatment with an endogenous aldehyde, acetaldehyde, also leads to a decrease in RECQL4 levels. Suggesting that dysregulation of RECQL4 could perhaps lead to genomic instability in tissues that are exposed to high levels of aldehydes. Lastly, we extended our work analyzing breast cancer tumor samples and observed that high levels of *RECQL4* is associated with increased RAD51 expression and tumor mutation burden. Our studies provide additional insights into how dysregulation of *RECQL4* could lead to genomic instability, which is a hallmark of cancer.

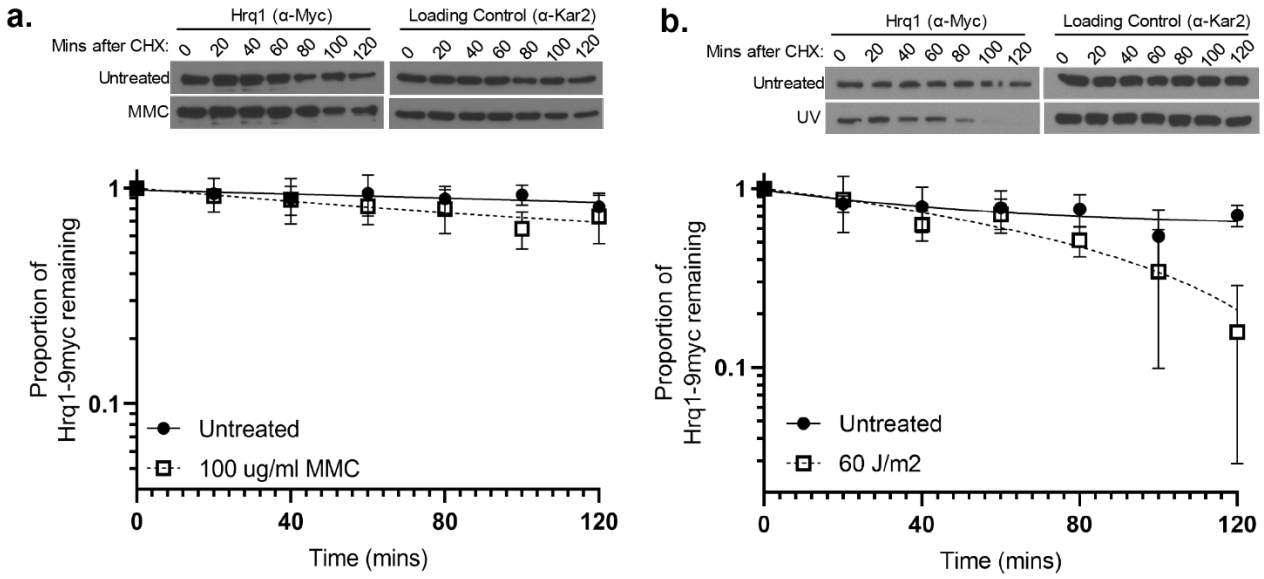
Appendix A Supplemental Figures



Appendix A.1 Supplemental Figure 1 9-Myc tagging Hrq1 does not result in cisplatin sensitivity

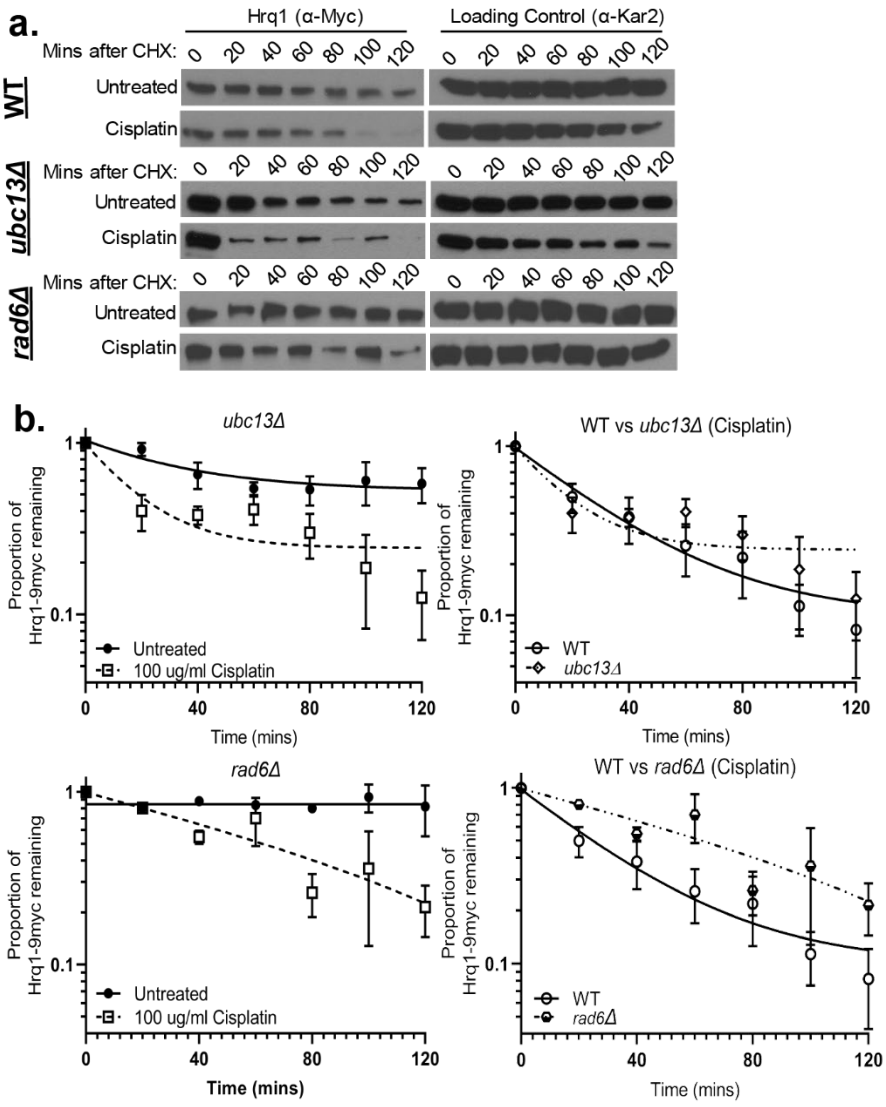
a) Hrq1-9myc cells displayed similar cisplatin sensitivity compared to WT cells. The indicated yeast strains were five-fold serial diluted onto SC medium containing DMSO and/or SC medium containing the indicated amount of cisplatin.

The plates were photographed after 2 days of incubation at 30˚C in the dark. **b)** Hrq1 ubiquitylation levels are increased upon cisplatin exposure. Hrq1 WT strain was treated with or without 100 µg/ml cisplatin. Subsequently, Hrq1 was pulldown using Myc beads then analyzed by western blot for Hrq1 (α-MYC) and ubiquitin levels (α-UB). The mean with standard error was graph from two experiments. **c)** Ubiquitylated Hrq1-7KR was not detected. Hrq1-7KR strain was treated with or without 100 µg/ml cisplatin. Subsequently, Hrq1 was pulldown using HA beads then analyzed by western blot for Hrq1 (α-MYC) and ubiquitin levels (α-UB).



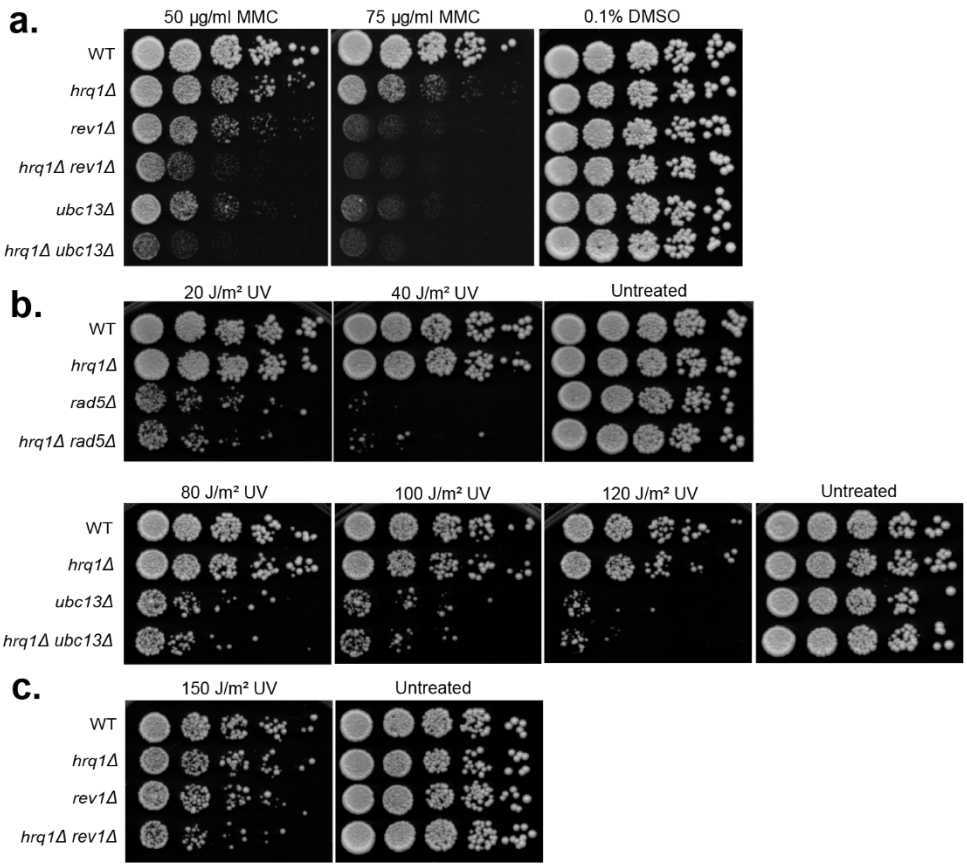
Appendix A.2 Supplemental Figure 2 Hrq1 levels are largely unchanged following MMC treatment, but decreases following UV treatment

a) Hrq1 protein levels are unchanged upon MMC treatment. Exponentially growing cells with Hrq1-9xMYC were incubated with cycloheximide in the presence or absence of 100 µg/ml MMC and/or 0.1% DMSO. Quantification of the proportion of Hrq1 remaining relative to time 0 (before CHX addition) and the loading control, Kar2. The experiment was performed three times with mean and standard error plotted. **b)** Hrq1 protein levels decreases upon UV-C treatment. Exponentially growing cells with Hrq1-9xMYC were incubated with cycloheximide in the presence or absence of 100 µg/ml MMC and/or 0.1% DMSO. Quantification of the proportion of Hrq1 remaining relative to time 0 (before CHX addition) and the loading control, Kar2. The experiment was performed three times with mean and standard error plotted.



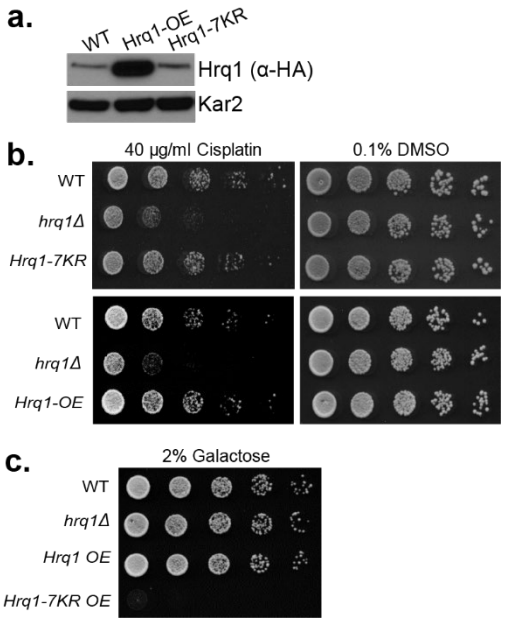
Appendix A.3 Supplemental Figure 3 Hrq1 protein levels are mildly stabilized in the absence the E2 Ub-conjugating enzyme, UBC13, or the E2 Ub-conjugating enzyme, RAD6

a) Hrq1-9xMYC expressing *ubc13*Δ or *rad6*Δ cells were incubated with cycloheximide in the presence or absence of 100 µg/ml cisplatin and/or 0.1% DMSO. Protein extracts were analyzed by western blot for Hrq1 protein levels (α-MYC) or a loading control, Kar2 (α-Kar2), at the indicated time points. **b)** Quantification of the proportion of Hrq1 remaining relative to time 0 (before CHX addition) and the loading control, Kar2, are plotted on the graph in log scale from *ubc13*Δ or *rad6*Δ cells. Each experiment was performed in triplicate with standard error plotted. Note that the WT cisplatin treated time course is replotted from Figure 1C, for direct comparison to *ubc13*Δ or *rad6*Δ cisplatin treated cells.



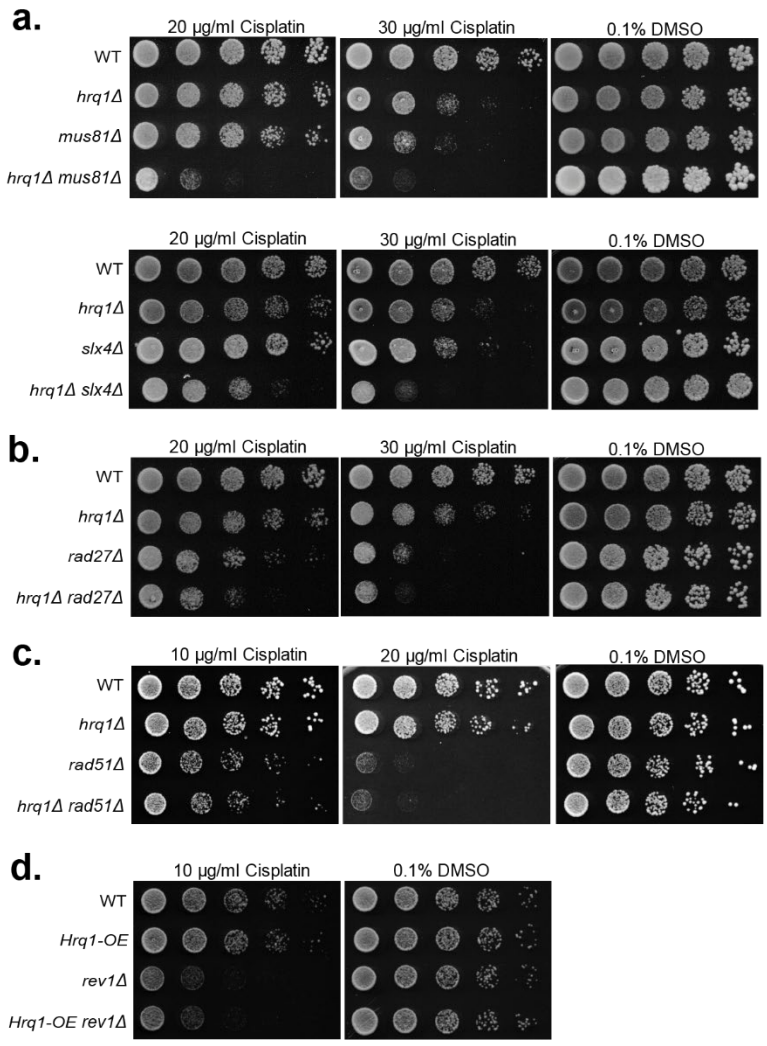
Appendix A.4 Supplemental Figure 4 Hrq1 function in ICL repair is distinct from intrastrand crosslink repair

a) Hrq1 functions in a different pathway as Rev1 and Ubc13 to repair ICL. The indicated yeast strains were five-fold serial diluted onto YPD medium containing DMSO and/or YPD medium containing the indicated amount of MMC. The plates were photographed after 2 days of incubation at 30˚C in the dark. **b)** Hrq1 functions in the same pathway as Rad5 and Ubc13 to repair intrastrand adducts. The indicated yeast strains were five-fold serial diluted onto YPD medium before being treated with the indicated dosages of UV-C. The plates were photographed after 2 days of incubation at 30˚C in the dark. **c)** Hrq1 functions in different pathway as Rev1 to repair intrastrand adducts. The indicated yeast strains were five-fold serial diluted onto YPD medium before being treated with the indicated dosage of UV-C. The plates were photographed after 2 days of incubation at 30˚C in the dark.



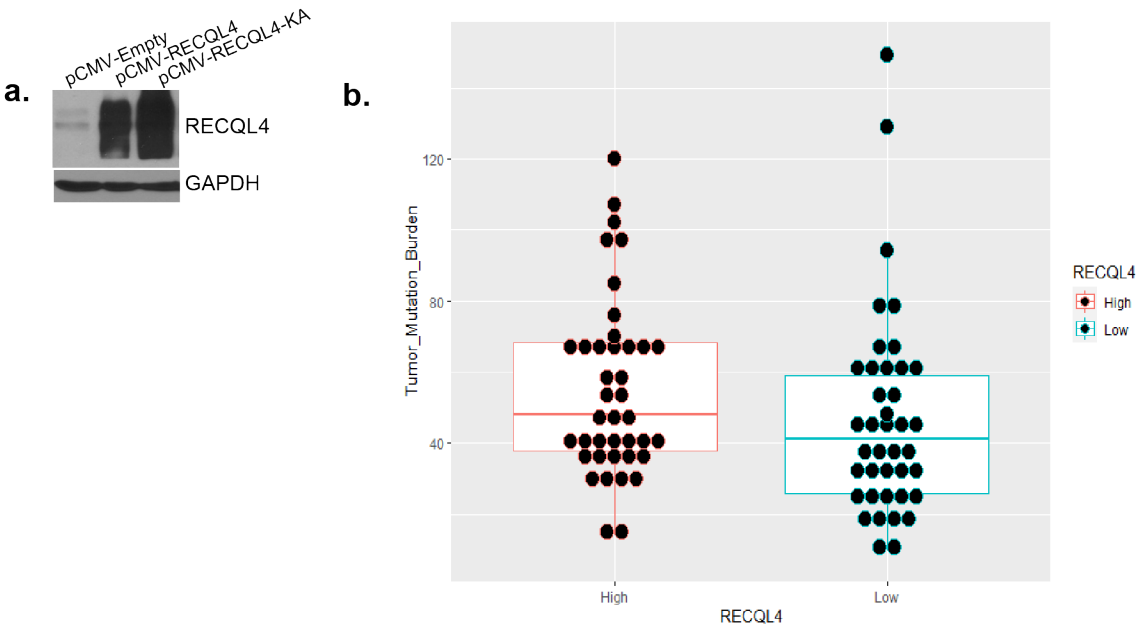
Appendix A.5 Supplemental Figure 5 High level of Hrq1-7KR results in cell lethality

a) Hrq1-7KR protein levels are similar to WT in basal conditions. The indicative strains were grown overnight in 2% galactose, subsequently TCA was performed and western was run to determined protein level. **b)** Stabilization or overexpression of Hrq1 does not lead to increased cisplatin sensitivity. The indicated yeast strains were five-fold serial diluted onto SC medium containing DMSO and/or SC medium containing the indicated amount of cisplatin. The plates were photographed after 2 days of incubation at 30˚C in the dark. Plates that were used with *Hrq1-OE* (GAL-HRQ1, galactose inducible/dextrose repressible promoter) strains were made with galactose instead of glucose medium. **c)** Overexpression of the Hrq1-7KR mutant results in cell lethality. The indicated yeast strains were grown overnight in SC medium containing raffinose. Subsequently they were five-fold serial diluted onto galactose SC plates. The plates were photographed after 2 days of incubation at 30˚C in the dark.



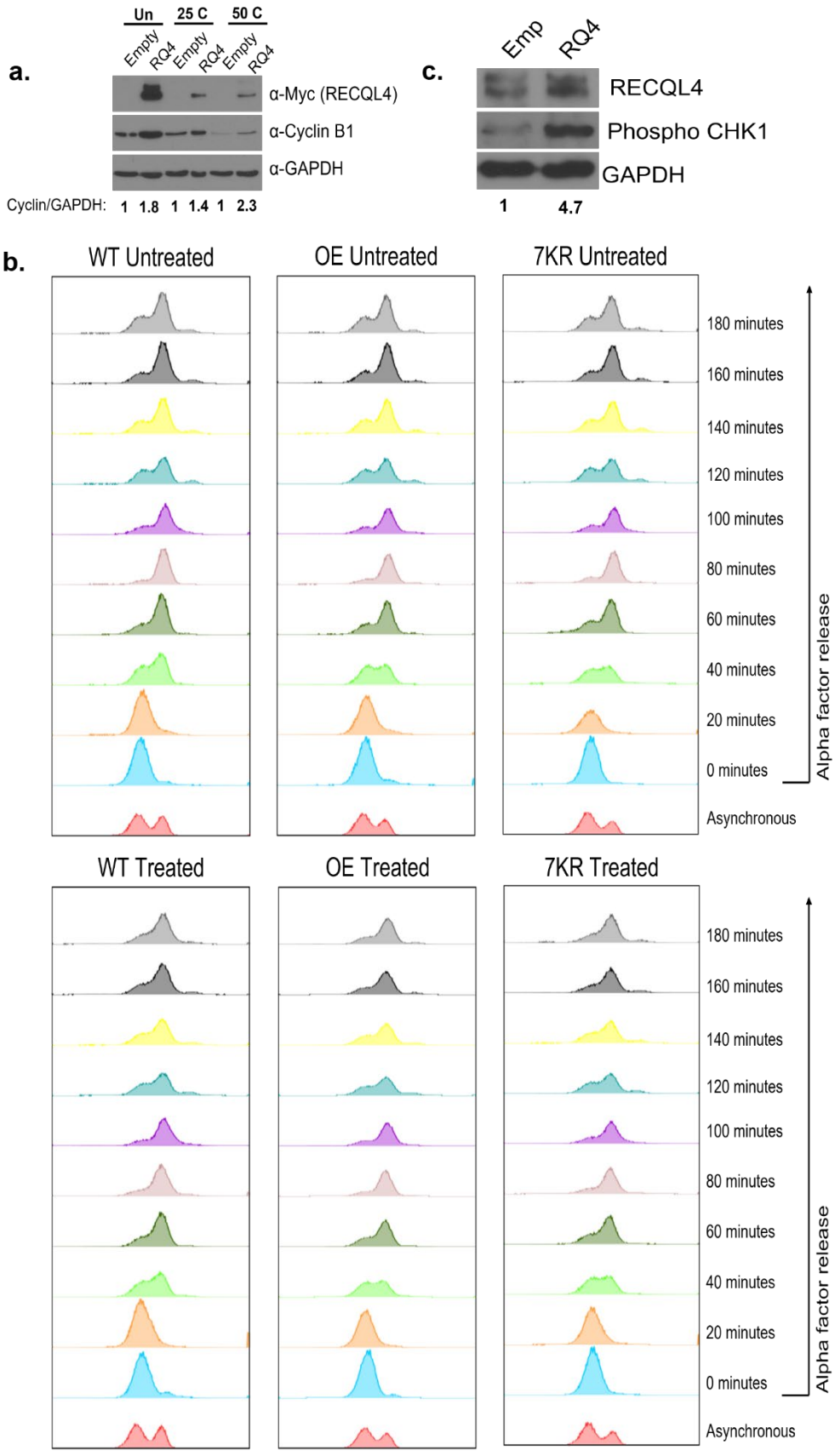
Appendix A.6 Supplemental Figure 6 Hrq1 functions with Rad51 to mediate recombination

a) b) Hrq1 functions in a different pathway than Mus81, Slx4, and Rad27 to repair cisplatin induced lesions. The indicated yeast strains were five-fold serial diluted onto SC medium containing DMSO and/or the indicated amount of cisplatin. The plates were photographed after 2 days of incubation at 30˚C in the dark. **c)** Hrq1 functions in the same pathway as Rad51 to repair cisplatin induced lesions. The indicated yeast strains were five-fold serial diluted onto SC medium containing DMSO and/or the indicated amount of cisplatin. The plates were photographed after 2 days of incubation at 30˚C in the dark. **d)** Overexpression of Hrq1 does not rescue *rev1Δ.* The indicated yeast strains were five-fold serial diluted onto SC medium with 2% galactose containing DMSO and/or indicated amount of cisplatin. The plates were photograph after 2 days of incubation at 30˚C in the dark.



Appendix A.7 Supplemental Figure 7 High RECQL4 levels is associated with increased tumor mutation burden in ovarian cancer.

a) pCMV-RECQL4-KA promotes similar overexpression compared to pCMV-RECQL4. Protein extracts from cells 3 days post-transfection. Endogenous RECQL4 was detected using α-RECQL4, GAPDH served as a control. **b)** Elevated levels of RECQL4 are associated with increased tumor mutation burden in ovarian cancer. The expression of RECQL4 in ovarian tumors from TCGA was divided into quartiles based upon expression level. The mutation burden between the least expressing tumors (Quartile 1 – Q1, blue) and the highest expressing tumors (Quartile 4 – Q4, red) was analyzed. Box and whisker plots alongside each data point was graphed. The number of samples alongside p-value are shown. Annotated mutational data from ovarian cancer was acquired from Firebrower. The mutational data was categorized using "maftools" in R. Tumor mutation burden was scored as described (Wang *et al* 2019).


a.
Un
25 C
50 C
Empty
RQ4
Empty
RQ4
Empty
RQ4
α-Myc (RECQL4)
α-Cyclin B1
α-GAPDH
Cyclin/GAPDH: 1 1.8 1 1.4 1 2.3
c.
Emp
RQ4
RECQL4
Phospho CHK1
GAPDH
1
4.7
b.
WT Untreated
OE Untreated
7KR Untreated
180 minutes
160 minutes
140 minutes
120 minutes
100 minutes
80 minutes
60 minutes
40 minutes
20 minutes
0 minutes
Asynchronous
Alpha factor release
WT Treated
OE Treated
7KR Treated
180 minutes
160 minutes
140 minutes
120 minutes
100 minutes
80 minutes
60 minutes
40 minutes
20 minutes
0 minutes
Asynchronous
Alpha factor release

Appendix A.8 Supplemental Figure 8 Unlike yeast, *RECQL4* overexpression is associated with cell cycle defects **a)** Three-day post transfection, the cells were treated with the indicated dosages of cisplatin (μM) for an hour, before protein was extracted. Transfected RECQL4-myc was detected using α-myc, cyclin B1 served as the G_2/M marker, GAPDH served as a control. **b)** Stabilization or overexpression of Hrq1 lead to no cell cycle defects. The indicated strains were either untreated (asynchronous, AS) or cell cycle arrested in G1 with α-factor. The α-factor arrested cells were subsequently released into fresh YPD medium or YPD medium containing cisplatin (100 μg/ml) and grown for 180 min. Samples were taken even 20 minutes and subsequently fix and stained with propidium iodide. The cell cycle stage was analyzed FACS. **c)** *RECQL4* overexpression leads to increased phosphor-CHK1. Protein extracts from cells 3 days post-transfection. Endogenous RECQL4 was detected using α-RECQL4, GAPDH served as a control, phosphor-CHK1 was normalized to total CHK1 levels. Quantification is the average from two separate experiments.

Appendix B Supplemental Tables

Appendix B.1 Supplemental Table 1: Yeast strains and plasmids

Yeast strains				
Appreviated Genotype	Strain ID	Genotype	Strain Background	Reference
WT	W9100-17D	MAT **a** *ADE2 leu2-3,112 his3-11, 15 ura3-1 TRP1 lys2Δ RAD5*	W303	This study
Hrq1-9myc	KBY642-1	MAT **a** *ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1 LYS2 RAD5 Hrq1-9myc:HISMX6*	W303	This study
*pdr5*Δ Hrq1-9myc	KBY872-4D	MAT **a** Hrq1-9myc::HIS3MX6 *pdr5::hphNT1 ADE2 his3-11,15 leu2-3,112 trp1-1 ura3-1 lys2Δ RAD5*	W303	This study
*pdr5*Δ Hrq1-9myc UB	KBY1346	MAT **a** Hrq1-9myc::HIS3MX6 *pdr5::hphNT1 ADE2 his3-11,15 leu2-3,112 trp1-1 ura3-1 lys2Δ RAD5* CUP-6His-UB	W303	This study
*pdr5*Δ Hrq1-7KR UB	KBY1347	MAT **a** Hrq1-7KR-6HA::KanMX *pdr5::hphNT1 ADE2 his3-11,15 leu2-3,112 trp1-1 ura3-1 lys2Δ RAD5* CUP-6His-UB	W303	This study
*rad16*Δ Hrq1-9myc	KBY1293-1	MAT **a** *ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1 LYS2 RAD5 Hrq1-9myc:HISMX6 rad16::hphNT1*	W303	This study
*rad6*Δ Hrq1-9myc	KBY1326	MAT **a** *ade2-1 can1-100 leu2-3,112 trp1-1 ura3-1 LYS2 RAD5 Hrq1-9myc:HISMX6 rad6::KanMX*	W303	This study
*ubc13*Δ Hrq1-9myc	KBY1291-1	MAT **a** *ade2-1 can1-100 his3-11,15 leu2-3,112 ubc13::TRP1 ura3-1 LYS2 RAD5 Hrq1-9myc:HIS3MX6*	W303	This study
Hrq1-OE	KBY1348	MAT **a** *ADE2 leu2-3,112 his3-11, 15 ura3-1 TRP1 lys2Δ RAD5* GAL1-3HA-HRQ1	W303	This study

Supplemental Table 1 continued

Hrq1-7KR	KBY1349	MAT **a** *ADE2 leu2-3,112 his3-11, 15 ura3-1 TRP1 lys2Δ RAD5* HRQ1-7KR-6HA::KanMX	W303	This study
WT CAN1	KBY 824-29A	MAT **alpha** *ADE2 TRP1 LYS2 CAN1*	W303	Gaines et al. 2015
Hrq1-OE CAN1	KBY1350	MAT **alpha** *ADE2 TRP1 LYS2 CAN1* GAL1-3xHA-HRQ1	W303	This study
Hrq1-7KR CAN1	KBY1351	MAT **alpha** *ADE2 TRP1 LYS2 CAN1* HRQ1-7KR-6HA::KanMX	W303	This study
WT DRR	KBY225-9C	MAT **alpha** *ADE2 his3-11 leu2ΔEcoRI::URA3-HO::leu2ΔBsteII lys2Δ trp1-1*	W303	This study
Hrq1-OE DRR	KBY1352	MAT **alpha** *ADE2 his3-11 leu2ΔEcoRI::URA3-HO::leu2ΔBsteII lys2Δ trp1-1* GAL1-3HA-HRQ1	W303	This study
Hrq1-7KR DRR	KBY1353	MAT **a** ADE2 *his3-11 leu2ΔEcoRI::URA3-HO::leu2ΔBsteII lys2Δ trp1-1* HRQ1-7KR-6HA::KanMX	W303	This study
rad6Δ	W9197-7A	MAT **a** *rad6::KanMX TRP1 ADE2 LYS2 ura3-1 leu2-3,112 his3-11,15*	W303	Boehm et al. 2016
hrq1Δ	KBY640-3A	MAT **alpha** *hrq1::hphNT1 ADE2 leu2-3,112 his3-11,15ura3-1 trp1-1 lys2Δ RAD5*	W303	This study
rad6Δ hrq1Δ	KBY1325	MAT **alpha** *hrq1::hphNT1 rad6::KanMX ADE2 leu2-3,112 his3-11,15ura3-1 trp1-1 lys2Δ RAD5*	W303	This study
Pol30-K127,164R	R1350	MAT **a** *ADE2 his3-11,15 bar1::LEU2 trp1-1 LYS2 ura3-1 RAD5* pol30-K127R,K164R	W303	This study
Pol30-K164R	R1351	MAT **a** *ADE2 his3-11,15 bar1::LEU2 trp1-1 LYS2 ura3-1 RAD5* pol30-K164R	W303	This study
Pol30-K127,164R *hrq1Δ*	KBY1313-2B	MAT **a** *ADE2 his3-11,15 bar1::LEU2 trp1-1 LYS2 ura3-1 RAD5 pol30-K127R,K164R hrq1::hphNT1*	W303	This study
Pol30-K164R *hrq1Δ*	KBY1314-6B	MAT **a** *ADE2 his3-11,15 bar1::LEU2 trp1-1 LYS2 ura3-1 RAD5 pol30-K164R hrq1::hphNT1*	W303	This study
rad5Δ	KBY851-5C	MAT **alpha** *ADE2 TRP1 lys2 rad5::natNT2*	W303	This study
rad5Δ hrq1Δ	KBY1305-2A	MAT **a** *ADE2 TRP1 hrq1::kanMX4 rad5::natNT2*	W303	This study

Supplemental Table 1 continued

ubc13Δ	KBY1145-2A	MAT **alpha** *ADE2 LYS2 TRP1 leu2-3,112 ura3-1 ubc13::HIS3 RAD5*	W303	This study
ubc13Δ hrq1Δ	KBY1297-1D	MAT **alpha** *ADE2 hrq1::kanMX4 ubc13::HIS3 TRP1 ura3-1 leu2-3,112 RAD5*	W303	This study
rev1Δ	KBY1322-1	MAT **a** *ADE2 leu2-3,112 his3-11,15 ura3-1 TRP1 lys2Δ RAD5 rev1::KanMX*	W303	This study
rev1Δ hrq1Δ	KBY1323-4D	MAT **a** *ADE2 leu2-3,112 his3-11,15 ura3-1 TRP1 lys2Δ RAD5 rev1::KanMX hrq1::hphNT1*	W303	This study

Plasmids				
Name	Background	Purose	Selection marker	Reference
pFA6a-HIS	pFA6a	Knockout yeast gene with HIS3 Marker	HIS, Amp	This study
pFA6a-hphNT1	pFA6a	Knockout yeast gene with hphNT1 Marker	HYG, Amp	This study
pFA6a-KanMX	pFA6a	Knockout yeast gene with KanMX Marker	G418, Amp	This study
pFA6a-natNT2	pFA6a	Knockout yeast gene with natNT2 Marker	NAT, Amp	This study
pFA6a-TRP1	pFA6a	Knockout yeast gene with HIS3 Marker	TRP1, Amp	This study
pYM14	PYMN	6HA C terminal tag	G418, Amp	This study
pYM19	pYM	9Myc C terminal tag	HIS, Amp	This study
pYM-N24	pYM	Place gene of interest under GAL1 promoter, N-terminal 3-HA tag	NAT, Amp	This study
pHrq2	YIplac211	Intergration vector which contains Hrq1-7KR to preform mutagenesis	Amp	This study
YEpHisUbStu	Yep	2μ plasmid for pCUP-6xHis-ubiquitin expression	TRP1, Amp	This study
pCMV-3B	pCMV	Empty control plasmid	Kan	This study

Supplemental Table 1 continued

pKB-240	pCMV	N terminal Myc tagged RECQL4 under CMV promoter, to overexpress RECQL4	Kan	This study
pKB-999	pCMV	N terminal Myc tagged RECQL4-K508A under CMV promoter, to overexpress RECQL4-K508A	Kan	This study

Supplemental Table 2: PCR Oligonucleotide and siRNAs

PCR Oligonucleotide		
Name	Sequence	Purpose
Hrq1-S1	CATATTGAGATGGTTAAGGTCGTAGAAAAG AAATGTTCATTTGAGAAGGAAAAATGCGTA CGCTGCAGGTCGAC	Knockout/Tagging of *HRQ1*
Hrq1-S2	GTAGTAGAATAGAGTATTTATATTCGGTTTA CAAACTACAAATAGCGTGCTCAATCGATGA ATTCGAGCTCG	Knockout/Tagging of *HRQ1*
Hrq1-S3	GAGCTACGAAAGACGATACTCATACAAATG AAATCATTAAAAAAGAGATATGACGTACGC TGCAGGTCGAC	Tagging of *HRQ1*
Hrq1-S4	CCACTCCCTTGACCTGCTGACTTCAGTTTCT TTTTGATAGGTCCTTCCTCCATCGATGAATT CTCTGTCG	Tagging of *HRQ1*
Hrq1-Seq_F	CAGAAGAGAAAGGCATACCGTC	Sequencing of *HRQ1*
Hrq1-Seq_R	CTGTGCATCAACAAGGTGACAG	
Hrq1-Seq1	AACGCTCATGGTCTGCAA	Internal primers for sequencing of *HRQ1* to confirm mutagenesis
Hrq1-Seq2	AGCCAGAGGAAAGAGCTT	
Hrq1-Seq3	CCCACTATCAATGGCGAA	
Hrq1-Seq4	AGGATCATCGATGCCATC	
Prd5-S1	AGACCCTTTTAAGTTTTCGTATCCGCTCGTT CGAAAGACTTTAGACAAAAATGCGTACGCT GCAGGTCGAC	Knockout of *PDR5*
Prd5-S2	AAAAAGTCCATCTTGGTAAGTTTCTTTTCTT AACCAAATTCAAAATTCTATTAATCGATGA ATTCGAGCTCG	
Pdr5-Seq_F	AACCTTATGGCTGTTCGC	Sequencing of *PDR5*
Prd5_Seq_R	CCGATGAGATAACCTAGG	
Pol30-Seq_F	CGTACTTTGCTTCCTCTG	Sequencing of *POL30*
Pol30-Seq_R	GAAACCCTGAATACCACG	
Rad16-S1	GTAATTTTAGATACTCTTGGCCGTATAACTG GTGTACCAACTGAAAAATCATGCGTACGCT GCAGGTCGAC	Knockout of *RAD16*
Rad16-S2	GAAGAAAATGCGTTGTATCCTTTGCTACAC ATGGTCTTAGACAACTTACCTGAATCGATG AATTCGAGCTCG	
Rad16-Seq_F	GCACAGATAGGACCTTAAGG	Sequencing of *RAD16*
Rad16-Seq_R	GAGTCGGCCATTCCAATA	

Supplemental Table 2 continued

Rev1-S1	ACAGATTTTCTCAAAATAAATCGATACTGC ATTTCTAGGCATATCCAGCGATGCGTACGCT GCAGGTCGAC	Knockout of *REV1*
Rev1-S2	TTCGCAAACTGCGTGTTTACTGTATGCTGAA ATGTTTTTTTTTTTTTAATTCAATCGATGAAT TCGAGCTCG	
Rev1-Seq_F	GGCAACCTTTAAGCACCA	Sequencing of *REV1*
Rev1-Seq_R	GAGTCGGCCATTCCAATA	
siRNAs		
Name	Sequence	Purpose
siCon	Dharmacon, ON-TARGETplus, Non-targeting siRNA #1, Catalog #D-001810-01	siRNA control
siRECQL4	CAAUACAGCUUACCGUACA, Dharmacon, Custom siRNA, ON-TARGETplus	RECQL4 knockdown
siREV1	Dharmacon, ON-TARGETPlus, REV1, Catalog# J-008234-05	REV1 knockdown

Appendix B.3 Supplemental Table 3: Antibodies

Antibodies				
Name	Catalog	Species	Purpose	Dilution
C-Myc	Santa Cruz Biotechnology #sc-40	Mouse monoclonal	Western	1:500
Clb2	Santa Cruz Biotechnology #sc-9071	Rabbit polyclonal	Western	1:2000
Kar2	Santa Cruz Biotechnology #sc-33630	Rabbit polyclonal	Western	1:5000
RECQL4	Cell Signaling #2814	Rabbit polyclonal	Western	1:500
γ- tubulin	Sigma Aldrich #T5326	Mouse monoclonal	Western	1:5000
GAPDH	UBP Bio #Y1040	Mouse monoclonal	Western	1:1000
REV1	Santa Cruz Biotechnology #sc-393022	Mouse monoclonal	Westwern	1:1000
Ubiquitin	Santa Cruz Biotechnology # sc-8017	Mouse monoclonal	Western	1:1000
RAD51	Abcam #ab63801	Rabbit polyclonal	Immunofluorescence	1:2000
Anti-Mouse HRP	Jackson ImmunoResearch Laboratories #115-035-003	Goat	Western	1:10000
Anti-Rabbit HRP	Jackson ImmunoResearch Laboratories #111-035-003	Goat	Western	1:10000
Anti-Rabbit IgG Alexa Fluor 488	ThermoFisher #A-11034	Goat	Immunofluorescence	1:2000

Bibliography

1. Hickson, I. D. RecQ helicases: caretakers of the genome. *Nat. Rev. Cancer* **2003**, *3*, 169–178, doi:10.1038/nrc1012.

2. Hanada, K.; Hickson, I. D. Molecular genetics of RecQ helicase disorders. *Cell Mol. Life Sci.* **2007**, *64*, 2306–2322, doi:10.1007/s00018-007-7121-z.

3. Bohr, V. A. Rising from the RecQ-age: the role of human RecQ helicases in genome maintenance. *Trends Biochem. Sci.* **2008**, *33*, 609–620, doi:10.1016/j.tibs.2008.09.003.

4. Chu, W. K.; Hickson, I. D. RecQ helicases: multifunctional genome caretakers. *Nat. Rev. Cancer* **2009**, *9*, 644–654, doi:10.1038/nrc2682.

5. Bernstein, K. A.; Gangloff, S.; Rothstein, R. The RecQ DNA helicases in DNA repair. *Annu. Rev. Genet.* **2010**, *44*, 393–417, doi:10.1146/annurev-genet-102209-163602.

6. Croteau, D. L.; Popuri, V.; Opresko, P. L.; Bohr, V. A. Human RecQ helicases in DNA repair, recombination, and replication. *Annu. Rev. Biochem.* **2014**, *83*, 519–552, doi:10.1146/annurev-biochem-060713-035428.

7. Xu, X.; Liu, Y. Dual DNA unwinding activities of the Rothmund-Thomson syndrome protein, RECQ4. *EMBO J.* **2009**, *28*, 568–577, doi:10.1038/emboj.2009.13.

8. Dietschy, T.; Shevelev, I.; Pena-Diaz, J.; Hühn, D.; Kuenzle, S.; Mak, R.; Miah, M. F.; Hess, D.; Fey, M.; Hottiger, M. O.; Janscak, P.; Stagljar, I. p300-mediated acetylation of the Rothmund-Thomson-syndrome gene product RECQL4 regulates its subcellular localization. *J. Cell Sci.* **2009**, *122*, 1701–1701, doi:10.1242/jcs.052944.

9. Larizza, L.; Magnani, I.; Roversi, G. Rothmund-Thomson syndrome and RECQL4 defect: splitting and lumping. *Cancer Lett.* **2006**, *232*, 107–120, doi:10.1016/j.canlet.2005.07.042.

10. Larizza, L.; Roversi, G.; Volpi, L. Rothmund-Thomson syndrome. *Orphanet J Rare Dis* **2010**, *5*, 2, doi:10.1186/1750-1172-5-2.

11. Liu, W.; Xie, Y.; Ma, J.; Luo, X.; Nie, P.; Zuo, Z.; Lahrmann, U.; Zhao, Q.; Zheng, Y.; Zhao, Y.; Xue, Y.; Ren, J. IBS: an illustrator for the presentation and visualization of biological sequences. *Bioinformatics* **2015**, *31*, 3359–3361, doi:10.1093/bioinformatics/btv362.

12. Wang, L. L.; Plon, S. E. Rothmund-Thomson Syndrome. In *GeneReviews(®)*; Pagon, R. A., Adam, M. P., Ardinger, H. H., Wallace, S. E., Amemiya, A., Bean, L. J., Bird, T. D.,

Fong, C.-T., Mefford, H. C., Smith, R. J., Stephens, K., Eds.; University of Washington, Seattle: Seattle (WA), 1993.

13. Rothmund Uber cataracten in Verbindung mit einer eigentumlichen Hautgeneration. *Arch Klein Exp Ophthalm* **1868**, 159–182.

14. Thomson, M. S. Poikiloderma congenitale. *Br. J. Dermatol.* **1936**, 221–234.

15. Taylor, W. B. Rothmund-Thomson's syndrome. *Arch. Dermatol.* **1957**, 236–244.

16. Ying, K. L.; Oizumi, J.; Curry, C. J. Rothmund-Thomson syndrome associated with trisomy 8 mosaicism. *J. Med. Genet.* **1990**, *27*, 258–260, doi:10.1136/jmg.27.4.258.

17. Orstavik, K. H.; McFadden, N.; Hagelsteen, J.; Ormerod, E.; van der Hagen, C. B. Instability of lymphocyte chromosomes in a girl with Rothmund-Thomson syndrome. *J. Med. Genet.* **1994**, *31*, 570–572, doi:10.1136/jmg.31.7.570.

18. Lindor, N. M.; Devries, E. M.; Michels, V. V.; Schad, C. R.; Jalal, S. M.; Donovan, K. M.; Smithson, W. A.; Kvols, L. K.; Thibodeau, S. N.; Dewald, G. W. Rothmund-Thomson syndrome in siblings: evidence for acquired in vivo mosaicism. *Clin. Genet.* **1996**, *49*, 124–129, doi:10.1111/j.1399-0004.1996.tb03270.x.

19. Kitao, S.; Ohsugi, I.; Ichikawa, K.; Goto, M.; Furuichi, Y.; Shimamoto, A. Cloning of two new human helicase genes of the RecQ family: biological significance of multiple species in higher eukaryotes. *Genomics* **1998**, *54*, 443–452, doi:10.1006/geno.1998.5595.

20. Kitao, S.; Shimamoto, A.; Goto, M.; Miller, R. W.; Smithson, W. A.; Lindor, N. M.; Furuichi, Y. Mutations in RECQL4 cause a subset of cases of Rothmund-Thomson syndrome. *Nat. Genet.* **1999**, *22*, 82–84, doi:10.1038/8788.

21. Ajeawung, N. F.; Nguyen, T. T. M.; Lu, L.; Kucharski, T. J.; Rousseau, J.; Molidperee, S.; Atienza, J.; Gamache, I.; Jin, W.; Plon, S. E.; Lee, B. H.; Teodoro, J. G.; Wang, L. L.; Campeau, P. M. Mutations in ANAPC1, Encoding a Scaffold Subunit of the Anaphase-Promoting Complex, Cause Rothmund-Thomson Syndrome Type 1. *Am. J. Hum. Genet.* **2019**, *105*, 625–630, doi:10.1016/j.ajhg.2019.06.011.

22. Zirn, B.; Bernbeck, U.; Alt, K.; Oeffner, F.; Gerhardinger, A.; Has, C. Rothmund–Thomson syndrome type 1 caused by biallelic ANAPC1 gene mutations. *Skin Health and Disease* **2021**, *1*, doi:10.1002/ski2.12.

23. Baller, F. Radiusaplasie und Inzucht. *Z Menschl Vererb Konstitutionsl* **1950**, *29*, 782–790.

24. Gerold, M. Frakturheilung bei einem seltenen Fall kongenitaler Anomalie der oberen Gliedmassen [Healing of a fracture in an unusual case of congenital anomaly of the upper extremities]. *Zentralbl Chir* **1959**, *84*, 831–834.

25. Mégarbané, A.; Melki, I.; Souraty, N.; Gerbaka, J.; Ghouzzi, V.; Bonaventure, J.; Mornand, A.; Loiselet, J. Overlap between Baller-Gerold and Rothmund-Thomson syndrome. *Clin Dysmorphol* **2000**, *9*, 303–305.

26. Van Maldergem, L.; Siitonen, H. A.; Jalkh, N.; Chouery, E.; De Roy, M.; Delague, V.; Muenke, M.; Jabs, E. W.; Cai, J.; Wang, L. L.; Plon, S. E.; Fourneau, C.; Kestilä, M.; Gillerot, Y.; Mégarbané, A.; Verloes, A. Revisiting the craniosynostosis-radial ray hypoplasia association: Baller-Gerold syndrome caused by mutations in the RECQL4 gene. *J. Med. Genet.* **2006**, *43*, 148–152, doi:10.1136/jmg.2005.031781.

27. Piard, J.; Aral, B.; Vabres, P.; Holder-Espinasse, M.; Mégarbané, A.; Gauthier, S.; Capra, V.; Pierquin, G.; Callier, P.; Baumann, C.; Pasquier, L.; Baujat, G.; Martorell, L.; Rodriguez, A.; Brady, A. F.; Boralevi, F.; González-Enseñat, M. A.; Rio, M.; Bodemer, C.; Philip, N.; Cordier, M. P.; Goldenberg, A.; Demeer, B.; Wright, M.; Blair, E.; Puzenat, E.; Parent, P.; Sznajer, Y.; Francannet, C.; DiDonato, N.; Boute, O.; Barlogis, V.; Moldovan, O.; Bessis, D.; Coubes, C.; Tardieu, M.; Cormier-Daire, V.; Sousa, A. B.; Franques, J.; Toutain, A.; Tajir, M.; Elalaoui, S. C.; Geneviève, D.; Thevenon, J.; Courcet, J. B.; Rivière, J. B.; Collet, C.; Gigot, N.; Faivre, L.; Thauvin-Robinet, C. Search for ReCQL4 mutations in 39 patients genotyped for suspected Rothmund-Thomson/Baller-Gerold syndromes. *Clin. Genet.* **2015**, *87*, 244–251, doi:10.1111/cge.12361.

28. Mo, D.; Zhao, Y.; Balajee, A. S. Human RecQL4 helicase plays multifaceted roles in the genomic stability of normal and cancer cells. *Cancer Lett.* **2018**, *413*, 1–10, doi:10.1016/j.canlet.2017.10.021.

29. Gripp, K. W.; Stolle, C. A.; Celle, L.; McDonald-McGinn, D. M.; Whitaker, L. A.; Zackai, E. H. TWIST gene mutation in a patient with radial aplasia and craniosynostosis: Further evidence for heterogeneity of Baller-Gerold syndrome. *Am. J. Med. Genet.* **1999**, *82*, 170–176.

30. Seto, M. L.; Lee, S. J.; Sze, R. W.; Cunningham, M. L. Another TWIST on Baller-Gerold syndrome. *Am. J. Med. Genet.* **2001**, *104*, 323–330, doi:10.1002/ajmg.10065.

31. Siitonen, H. A.; Sotkasiira, J.; Biervliet, M.; Benmansour, A.; Capri, Y.; Cormier-Daire, V.; Crandall, B.; Hannula-Jouppi, K.; Hennekam, R.; Herzog, D.; Keymolen, K.; Lipsanen-Nyman, M.; Miny, P.; Plon, S. E.; Riedl, S.; Sarkar, A.; Vargas, F. R.; Verloes, A.; Wang, L. L.; Kääriäinen, H.; Kestilä, M. The mutation spectrum in RECQL4 diseases. *Eur. J. Hum. Genet.* **2009**, *17*, 151–158, doi:10.1038/ejhg.2008.154.

32. Kääriäinen, H.; Ryöppy, S.; Norio, R. RAPADILINO syndrome with radial and patellar aplasia/hypoplasia as main manifestations. *Am. J. Med. Genet.* **1989**, *33*, 346–351, doi:10.1002/ajmg.1320330312.

33. Siitonen, H. A.; Kopra, O.; Kääriäinen, H.; Haravuori, H.; Winter, R. M.; Säämänen, A.-M.; Peltonen, L.; Kestilä, M. Molecular defect of RAPADILINO syndrome expands the

phenotype spectrum of RECQL diseases. *Hum. Mol. Genet.* **2003**, *12*, 2837–2844, doi:10.1093/hmg/ddg306.

34. Miozzo, M.; Castorina, P.; Riva, P.; Dalprà, L.; Fuhrman Conti, A. M.; Volpi, L.; Hoe, T. S.; Khoo, A.; Wiegant, J.; Rosenberg, C.; Larizza, L. Chromosomal instability in fibroblasts and mesenchymal tumors from 2 sibs with Rothmund-Thomson syndrome. *Int. J. Cancer* **1998**, *77*, 504–510, doi:10.1002/(sici)1097-0215(19980812)77:4<504::aid-ijc5>3.0.co;2-y.

35. Beghini, A.; Castorina, P.; Roversi, G.; Modiano, P.; Larizza, L. RNA processing defects of the helicase gene RECQL4 in a compound heterozygous Rothmund-Thomson patient. *Am. J. Med. Genet. A* **2003**, *120A*, 395–399, doi:10.1002/ajmg.a.20154.

36. Colombo, E. A.; Locatelli, A.; Cubells Sánchez, L.; Romeo, S.; Elcioglu, N. H.; Maystadt, I.; Esteve Martínez, A.; Sironi, A.; Fontana, L.; Finelli, P.; Gervasini, C.; Pecile, V.; Larizza, L. Rothmund-Thomson Syndrome: Insights from New Patients on the Genetic Variability Underpinning Clinical Presentation and Cancer Outcome. *Int. J. Mol. Sci.* **2018**, *19*, doi:10.3390/ijms19041103.

37. Ng, A. J. M.; Walia, M. K.; Smeets, M. F.; Mutsaers, A. J.; Sims, N. A.; Purton, L. E.; Walsh, N. C.; Martin, T. J.; Walkley, C. R. The DNA helicase recql4 is required for normal osteoblast expansion and osteosarcoma formation. *PLoS Genet.* **2015**, *11*, e1005160, doi:10.1371/journal.pgen.1005160.

38. Choi, Y. W.; Bae, S. M.; Kim, Y. W.; Lee, H. N.; Kim, Y. W.; Park, T. C.; Ro, D. Y.; Shin, J. C.; Shin, S. J.; Seo, J. S.; Ahn, W. S. Gene expression profiles in squamous cell cervical carcinoma using array-based comparative genomic hybridization analysis. *Int J Gynecol Cancer* **2007**, *17*, 687–696, doi:10.1111/j.1525-1438.2007.00834.x.

39. Su, Y.; Meador, J. A.; Calaf, G. M.; Proietti De-Santis, L.; Zhao, Y.; Bohr, V. A.; Balajee, A. S. Human RecQL4 helicase plays critical roles in prostate carcinogenesis. *Cancer Res.* **2010**, *70*, 9207–9217, doi:10.1158/0008-5472.CAN-10-1743.

40. Fang, H.; Nie, L.; Chi, Z.; Liu, J.; Guo, D.; Lu, X.; Hei, T. K.; Balajee, A. S.; Zhao, Y. RecQL4 helicase amplification is involved in human breast tumorigenesis. *PLoS One* **2013**, *8*, e69600, doi:10.1371/journal.pone.0069600.

41. Lu, L.; Jin, W.; Liu, H.; Wang, L. L. RECQ DNA helicases and osteosarcoma. *Adv. Exp. Med. Biol.* **2014**, *804*, 129–145, doi:10.1007/978-3-319-04843-7_7.

42. Yong, Z. W. E.; Zaini, Z. M.; Kallarakkal, T. G.; Karen-Ng, L. P.; Rahman, Z. A. A.; Ismail, S. M.; Sharifah, N. A.; Mustafa, W. M. W.; Abraham, M. T.; Tay, K. K.; Zain, R. B. Genetic alterations of chromosome 8 genes in oral cancer. *Sci. Rep.* **2014**, *4*, 6073, doi:10.1038/srep06073.

43. Arora, A.; Agarwal, D.; Abdel-Fatah, T. M.; Lu, H.; Croteau, D. L.; Moseley, P.; Aleskandarany, M. A.; Green, A. R.; Ball, G.; Rakha, E. A.; Chan, S. Y.; Ellis, I. O.; Wang, L. L.; Zhao, Y.; Balajee, A. S.; Bohr, V. A.; Madhusudan, S. RECQL4 helicase has oncogenic potential in sporadic breast cancers. *J. Pathol.* **2016**, *238*, 495–501, doi:10.1002/path.4681.

44. Mo, D.; Fang, H.; Niu, K.; Liu, J.; Wu, M.; Li, S.; Zhu, T.; Aleskandarany, M. A.; Arora, A.; Lobo, D. N.; Madhusudan, S.; Balajee, A. S.; Chi, Z.; Zhao, Y. Human Helicase RECQL4 Drives Cisplatin Resistance in Gastric Cancer by Activating an AKT-YB1-MDR1 Signaling Pathway. *Cancer Res.* **2016**, *76*, 3057–3066, doi:10.1158/0008-5472.CAN-15-2361.

45. De, S.; Kumari, J.; Mudgal, R.; Modi, P.; Gupta, S.; Futami, K.; Goto, H.; Lindor, N. M.; Furuichi, Y.; Mohanty, D.; Sengupta, S. RECQL4 is essential for the transport of p53 to mitochondria in normal human cells in the absence of exogenous stress. *J. Cell Sci.* **2012**, *125*, 2509–2522, doi:10.1242/jcs.101501.

46. Ughachukwu, P.; Unekwe, P. Efflux pump-mediated resistance in chemotherapy. *Ann. Med. Health Sci. Res.* **2012**, *2*, 191–198, doi:10.4103/2141-9248.105671.

47. Balajee, A. S. Human recql4 as a novel molecular target for cancer therapy. *Cytogenet Genome Res* **2021**, *161*, 305–327, doi:10.1159/000516568.

48. Sangrithi, M. N.; Bernal, J. A.; Madine, M.; Philpott, A.; Lee, J.; Dunphy, W. G.; Venkitaraman, A. R. Initiation of DNA replication requires the RECQL4 protein mutated in Rothmund-Thomson syndrome. *Cell* **2005**, *121*, 887–898, doi:10.1016/j.cell.2005.05.015.

49. Matsuno, K.; Kumano, M.; Kubota, Y.; Hashimoto, Y.; Takisawa, H. The N-terminal noncatalytic region of Xenopus RecQ4 is required for chromatin binding of DNA polymerase alpha in the initiation of DNA replication. *Mol. Cell. Biol.* **2006**, *26*, 4843–4852, doi:10.1128/MCB.02267-05.

50. Xu, X.; Rochette, P. J.; Feyissa, E. A.; Su, T. V.; Liu, Y. MCM10 mediates RECQ4 association with MCM2-7 helicase complex during DNA replication. *EMBO J.* **2009**, *28*, 3005–3014, doi:10.1038/emboj.2009.235.

51. Wu, J.; Capp, C.; Feng, L.; Hsieh, T. Drosophila homologue of the Rothmund-Thomson syndrome gene: essential function in DNA replication during development. *Dev. Biol.* **2008**, *323*, 130–142, doi:10.1016/j.ydbio.2008.08.006.

52. Germani, F.; Bergantinos, C.; Johnston, L. A. Mosaic analysis in drosophila. *Genetics* **2018**, *208*, 473–490, doi:10.1534/genetics.117.300256.

53. Capp, C.; Wu, J.; Hsieh, T. Drosophila RecQ4 has a 3'-5' DNA helicase activity that is essential for viability. *J. Biol. Chem.* **2009**, *284*, 30845–30852, doi:10.1074/jbc.M109.008052.

54. Im, J.-S.; Ki, S.-H.; Farina, A.; Jung, D.-S.; Hurwitz, J.; Lee, J.-K. Assembly of the Cdc45-Mcm2-7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 15628–15632, doi:10.1073/pnas.0908039106.

55. Lu, H.; Shamanna, R. A.; de Freitas, J. K.; Okur, M.; Khadka, P.; Kulikowicz, T.; Holland, P. P.; Tian, J.; Croteau, D. L.; Davis, A. J.; Bohr, V. A. Cell cycle-dependent phosphorylation regulates RECQL4 pathway choice and ubiquitination in DNA double-strand break repair. *Nat. Commun.* **2017**, *8*, 2039, doi:10.1038/s41467-017-02146-3.

56. Hoki, Y.; Araki, R.; Fujimori, A.; Ohhata, T.; Koseki, H.; Fukumura, R.; Nakamura, M.; Takahashi, H.; Noda, Y.; Kito, S.; Abe, M. Growth retardation and skin abnormalities of the Recql4-deficient mouse. *Hum. Mol. Genet.* **2003**, *12*, 2293–2299, doi:10.1093/hmg/ddg254.

57. Walne, A. J.; Vulliamy, T.; Beswick, R.; Kirwan, M.; Dokal, I. Mutations in C16orf57 and normal-length telomeres unify a subset of patients with dyskeratosis congenita, poikiloderma with neutropenia and Rothmund-Thomson syndrome. *Hum. Mol. Genet.* **2010**, *19*, 4453–4461, doi:10.1093/hmg/ddq371.

58. Vennos, E.; Collins, M.; James, W. Rothmund-Thomson syndrome: world literature Review of the. *J. Am. Acad. Dermatol.* **1992**.

59. Opresko, P. L.; Otterlei, M.; Graakjaer, J.; Bruheim, P.; Dawut, L.; Kølvraa, S.; May, A.; Seidman, M. M.; Bohr, V. A. The Werner syndrome helicase and exonuclease cooperate to resolve telomeric D loops in a manner regulated by TRF1 and TRF2. *Mol. Cell* **2004**, *14*, 763–774, doi:10.1016/j.molcel.2004.05.023.

60. Ghosh, A.; Rossi, M. L.; Aulds, J.; Croteau, D.; Bohr, V. A. Telomeric D-loops containing 8-oxo-2'-deoxyguanosine are preferred substrates for Werner and Bloom syndrome helicases and are bound by POT1. *J. Biol. Chem.* **2009**, *284*, 31074–31084, doi:10.1074/jbc.M109.027532.

61. Chang, S.; Multani, A. S.; Cabrera, N. G.; Naylor, M. L.; Laud, P.; Lombard, D.; Pathak, S.; Guarente, L.; DePinho, R. A. Essential role of limiting telomeres in the pathogenesis of Werner syndrome. *Nat. Genet.* **2004**, *36*, 877–882, doi:10.1038/ng1389.

62. Ghosh, A. K.; Rossi, M. L.; Singh, D. K.; Dunn, C.; Ramamoorthy, M.; Croteau, D. L.; Liu, Y.; Bohr, V. A. RECQL4, the protein mutated in Rothmund-Thomson syndrome, functions in telomere maintenance. *J. Biol. Chem.* **2012**, *287*, 196–209, doi:10.1074/jbc.M111.295063.

63. Takai, H.; Smogorzewska, A.; de Lange, T. DNA damage foci at dysfunctional telomeres. *Curr. Biol.* **2003**, *13*, 1549–1556, doi:10.1016/s0960-9822(03)00542-6.

64. Ferrarelli, L. K.; Popuri, V.; Ghosh, A. K.; Tadokoro, T.; Canugovi, C.; Hsu, J. K.; Croteau, D. L.; Bohr, V. A. The RECQL4 protein, deficient in Rothmund-Thomson syndrome is active on telomeric D-loops containing DNA metabolism blocking lesions. *DNA Repair (Amst.)* **2013**, *12*, 518–528, doi:10.1016/j.dnarep.2013.04.005.

65. Featherstone, C.; Jackson, S. P. DNA double-strand break repair. *Curr. Biol.* **1999**, *9*, R759–61, doi:10.1016/S0960-9822(00)80005-6.

66. Jeggo, P. A.; Löbrich, M. DNA double-strand breaks: their cellular and clinical impact? *Oncogene* **2007**, *26*, 7717–7719, doi:10.1038/sj.onc.1210868.

67. Smith, P. J.; Paterson, M. C. Enhanced radiosensitivity and defective DNA repair in cultured fibroblasts derived from Rothmund Thomson syndrome patients. *Mutat. Res.* **1982**, *94*, 213–228, doi:10.1016/0027-5107(82)90183-x.

68. Petkovic, M.; Dietschy, T.; Freire, R.; Jiao, R.; Stagljar, I. The human Rothmund-Thomson syndrome gene product, RECQL4, localizes to distinct nuclear foci that coincide with proteins involved in the maintenance of genome stability. *J. Cell Sci.* **2005**, *118*, 4261–4269, doi:10.1242/jcs.02556.

69. Kumata, Y.; Tada, S.; Yamanada, Y.; Tsuyama, T.; Kobayashi, T.; Dong, Y.-P.; Ikegami, K.; Murofushi, H.; Seki, M.; Enomoto, T. Possible involvement of RecQL4 in the repair of double-strand DNA breaks in Xenopus egg extracts. *Biochim. Biophys. Acta* **2007**, *1773*, 556–564, doi:10.1016/j.bbamcr.2007.01.005.

70. Singh, D. K.; Karmakar, P.; Aamann, M.; Schurman, S. H.; May, A.; Croteau, D. L.; Burks, L.; Plon, S. E.; Bohr, V. A. The involvement of human RECQL4 in DNA double-strand break repair. *Aging Cell* **2010**, *9*, 358–371, doi:10.1111/j.1474-9726.2010.00562.x.

71. Singh, D. K.; Popuri, V.; Kulikowicz, T.; Shevelev, I.; Ghosh, A. K.; Ramamoorthy, M.; Rossi, M. L.; Janscak, P.; Croteau, D. L.; Bohr, V. A. The human RecQ helicases BLM and RECQL4 cooperate to preserve genome stability. *Nucleic Acids Res.* **2012**, *40*, 6632–6648, doi:10.1093/nar/gks349.

72. Shamanna, R. A.; Singh, D. K.; Lu, H.; Mirey, G.; Keijzers, G.; Salles, B.; Croteau, D. L.; Bohr, V. A. RECQ helicase RECQL4 participates in non-homologous end joining and interacts with the Ku complex. *Carcinogenesis* **2014**, *35*, 2415–2424, doi:10.1093/carcin/bgu137.

73. Lu, H.; Shamanna, R. A.; Keijzers, G.; Anand, R.; Rasmussen, L. J.; Cejka, P.; Croteau, D. L.; Bohr, V. A. RECQL4 Promotes DNA End Resection in Repair of DNA Double-Strand Breaks. *Cell Rep.* **2016**, *16*, 161–173, doi:10.1016/j.celrep.2016.05.079.

74. Tan, Q.; Niu, K.; Zhu, Y.; Chen, Z.; Li, Y.; Li, M.; Wei, D.; Balajee, A. S.; Fang, H.; Zhao, Y. RNF8 ubiquitinates RecQL4 and promotes its dissociation from DNA double strand breaks. *Oncogenesis* **2021**, *10*, 24, doi:10.1038/s41389-021-00315-0.

75. Mao, Z.; Bozzella, M.; Seluanov, A.; Gorbunova, V. DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells. *Cell Cycle* **2008**, *7*, 2902–2906, doi:10.4161/cc.7.18.6679.

76. Werner, S. R.; Prahalad, A. K.; Yang, J.; Hock, J. M. RECQL4-deficient cells are hypersensitive to oxidative stress/damage: Insights for osteosarcoma prevalence and heterogeneity in Rothmund-Thomson syndrome. *Biochem. Biophys. Res. Commun.* **2006**, *345*, 403–409, doi:10.1016/j.bbrc.2006.04.093.

77. Woo, L. L.; Futami, K.; Shimamoto, A.; Furuichi, Y.; Frank, K. M. The Rothmund-Thomson gene product RECQL4 localizes to the nucleolus in response to oxidative stress. *Exp. Cell Res.* **2006**, *312*, 3443–3457, doi:10.1016/j.yexcr.2006.07.023.

78. Schurman, S. H.; Hedayati, M.; Wang, Z.; Singh, D. K.; Speina, E.; Zhang, Y.; Becker, K.; Macris, M.; Sung, P.; Wilson, D. M.; Croteau, D. L.; Bohr, V. A. Direct and indirect roles of RECQL4 in modulating base excision repair capacity. *Hum. Mol. Genet.* **2009**, *18*, 3470–3483, doi:10.1093/hmg/ddp291.

79. Duan, S.; Han, X.; Akbari, M.; Croteau, D. L.; Rasmussen, L. J.; Bohr, V. A. Interaction between RECQL4 and OGG1 promotes repair of oxidative base lesion 8-oxoG and is regulated by SIRT1 deacetylase. *Nucleic Acids Res.* **2020**, *48*, 6530–6546, doi:10.1093/nar/gkaa392.

80. Schärer, O. D. Nucleotide excision repair in eukaryotes. *Cold Spring Harb. Perspect. Biol.* **2013**, *5*, a012609, doi:10.1101/cshperspect.a012609.

81. Van Houten, B.; Kong, M. Eukaryotic Nucleotide Excision Repair. In *Encyclopedia of Cell Biology*; Elsevier, 2016; pp. 435–441.

82. Berg, E.; Chuang, T.-Y.; Cripps, D.; Madison A case report, phototesting, and literature review. *J. Am. Acad. Dermatol.* **1987**.

83. Shinya, A.; Nishigori, C.; Moriwaki, S.; Takebe, H.; Kubota, M.; Ogino, A.; Imamura, S. A Case of Rothmund-Thomson Syndrome With Reduced DNA Repair Capacity. *Arch Dermatolol* **1993**.

84. Vasseur, F.; Dalaporte, E.; Zabot, M. T.; Sturque, M. N.; Barrut, D.; Savary, J. B.; Thomas, L.; Thomas, P. Excision Repair Defect in Rothmund Thomson Syndrome.pdf. *Acta Derm. Venereol.* **1999**.

85. Jin, W.; Liu, H.; Zhang, Y.; Otta, S. K.; Plon, S. E.; Wang, L. L. Sensitivity of RECQL4-deficient fibroblasts from Rothmund-Thomson syndrome patients to genotoxic agents. *Hum. Genet.* **2008**, *123*, 643–653, doi:10.1007/s00439-008-0518-4.

86. Fan, W.; Luo, J. RecQ4 facilitates UV light-induced DNA damage repair through interaction with nucleotide excision repair factor xeroderma pigmentosum group A (XPA). *J. Biol. Chem.* **2008**, *283*, 29037–29044, doi:10.1074/jbc.M801928200.

87. Kumari, J.; Hussain, M.; De, S.; Chandra, S.; Modi, P.; Tikoo, S.; Singh, A.; Sagar, C.; Sepuri, N. B. V.; Sengupta, S. Mitochondrial functions of RECQL4 are required for the prevention of aerobic glycolysis-dependent cell invasion. *J. Cell Sci.* **2016**, *129*, 1312–1318, doi:10.1242/jcs.181297.

88. Trifunovic, A.; Larsson, N. G. Mitochondrial dysfunction as a cause of ageing. *J. Intern. Med.* **2008**, *263*, 167–178, doi:10.1111/j.1365-2796.2007.01905.x.

89. Cui, H.; Kong, Y.; Zhang, H. Oxidative stress, mitochondrial dysfunction, and aging. *J. Signal Transduct.* **2012**, *2012*, 646354, doi:10.1155/2012/646354.

90. Lane, R. K.; Hilsabeck, T.; Rea, S. L. The role of mitochondrial dysfunction in age-related diseases. *Biochim. Biophys. Acta* **2015**, *1847*, 1387–1400, doi:10.1016/j.bbabio.2015.05.021.

91. Payne, B. A. I.; Chinnery, P. F. Mitochondrial dysfunction in aging: Much progress but many unresolved questions. *Biochim. Biophys. Acta* **2015**, *1847*, 1347–1353, doi:10.1016/j.bbabio.2015.05.022.

92. Haas, R. H. Mitochondrial dysfunction in aging and diseases of aging. *Biology (Basel)* **2019**, *8*, doi:10.3390/biology8020048.

93. Croteau, D. L.; Rossi, M. L.; Canugovi, C.; Tian, J.; Sykora, P.; Ramamoorthy, M.; Wang, Z. M.; Singh, D. K.; Akbari, M.; Kasiviswanathan, R.; Copeland, W. C.; Bohr, V. A. RECQL4 localizes to mitochondria and preserves mitochondrial DNA integrity. *Aging Cell* **2012**, *11*, 456–466, doi:10.1111/j.1474-9726.2012.00803.x.

94. Chi, Z.; Nie, L.; Peng, Z.; Yang, Q.; Yang, K.; Tao, J.; Mi, Y.; Fang, X.; Balajee, A. S.; Zhao, Y. RecQL4 cytoplasmic localization: implications in mitochondrial DNA oxidative damage repair. *Int. J. Biochem. Cell Biol.* **2012**, *44*, 1942–1951, doi:10.1016/j.biocel.2012.07.016.

95. Gupta, S.; De, S.; Srivastava, V.; Hussain, M.; Kumari, J.; Muniyappa, K.; Sengupta, S. RECQL4 and p53 potentiate the activity of polymerase γ and maintain the integrity of the human mitochondrial genome. *Carcinogenesis* **2014**, *35*, 34–45, doi:10.1093/carcin/bgt315.

96. Warburg, O. On the origin of cancer cells. *Science* **1956**, *123*, 309–314.

97. Turrens, J. F. Mitochondrial formation of reactive oxygen species. *J. Physiol. (Lond.)* **2003**, *552*, 335–344, doi:10.1113/jphysiol.2003.049478.

98. Martínez-Reyes, I.; Cuezva, J. M. The H(+)-ATP synthase: a gate to ROS-mediated cell death or cell survival. *Biochim. Biophys. Acta* **2014**, *1837*, 1099–1112, doi:10.1016/j.bbabio.2014.03.010.

99. Prasad, R.; Çağlayan, M.; Dai, D.-P.; Nadalutti, C. A.; Zhao, M.-L.; Gassman, N. R.; Janoshazi, A. K.; Stefanick, D. F.; Horton, J. K.; Krasich, R.; Longley, M. J.; Copeland, W. C.; Griffith, J. D.; Wilson, S. H. DNA polymerase β: A missing link of the base excision repair machinery in mammalian mitochondria. *DNA Repair (Amst.)* **2017**, *60*, 77–88, doi:10.1016/j.dnarep.2017.10.011.

100. Sykora, P.; Kanno, S.; Akbari, M.; Kulikowicz, T.; Baptiste, B. A.; Leandro, G. S.; Lu, H.; Tian, J.; May, A.; Becker, K. A.; Croteau, D. L.; Wilson, D. M.; Sobol, R. W.; Yasui, A.; Bohr, V. A. DNA polymerase beta participates in mitochondrial DNA repair. *Mol. Cell. Biol.* **2017**, *37*, doi:10.1128/MCB.00237-17.

101. Ichikawa, K.; Noda, T.; Furuichi, Y. [Preparation of the gene targeted knockout mice for human premature aging diseases, Werner syndrome, and Rothmund-Thomson syndrome caused by the mutation of DNA helicases]. *Nihon Shinkei Seishin Yakurigaku Zasshi* **2002**.

102. Mann, M. B.; Hodges, C. A.; Barnes, E.; Vogel, H.; Hassold, T. J.; Luo, G. Defective sister-chromatid cohesion, aneuploidy and cancer predisposition in a mouse model of type II Rothmund-Thomson syndrome. *Hum. Mol. Genet.* **2005**, *14*, 813–825, doi:10.1093/hmg/ddi075.

103. Gangloff, S.; McDonald, J. P.; Bendixen, C.; Arthur, L.; Rothstein, R. The yeast type I topoisomerase Top3 interacts with Sgs1, a DNA helicase homolog: a potential eukaryotic reverse gyrase. *Mol. Cell. Biol.* **1994**, *14*, 8391–8398, doi:10.1128/mcb.14.12.8391.

104. Watt, P. M.; Louis, E. J.; Borts, R. H.; Hickson, I. D. Sgs1: A eukaryotic homolog of E. coil RecQ that interacts with topoisomerase II in vivo and is required for faithful chromosome segregation. *Cell* **1995**, *81*, 253–260, doi:10.1016/0092-8674(95)90335-6.

105. Barea, F.; Tessaro, S.; Bonatto, D. In silico analyses of a new group of fungal and plant RecQ4-homologous proteins. *Comput Biol Chem* **2008**, *32*, 349–358, doi:10.1016/j.compbiolchem.2008.07.005.

106. Jumper, J.; Evans, R.; Pritzel, A.; Green, T.; Figurnov, M.; Ronneberger, O.; Tunyasuvunakool, K.; Bates, R.; Žídek, A.; Potapenko, A.; Bridgland, A.; Meyer, C.; Kohl, S. A. A.; Ballard, A. J.; Cowie, A.; Romera-Paredes, B.; Nikolov, S.; Jain, R.; Adler, J.; Back, T.; Petersen, S.; Reiman, D.; Clancy, E.; Zielinski, M.; Steinegger, M.; Pacholska, M.; Berghammer, T.; Bodenstein, S.; Silver, D.; Vinyals, O.; Senior, A. W.; Kavukcuoglu,

K.; Kohli, P.; Hassabis, D. Highly accurate protein structure prediction with AlphaFold. *Nature* **2021**, *596*, 583–589, doi:10.1038/s41586-021-03819-2.

107. Rogers, C. M.; Wang, J. C.-Y.; Noguchi, H.; Imasaki, T.; Takagi, Y.; Bochman, M. L. Yeast Hrq1 shares structural and functional homology with the disease-linked human RecQ4 helicase. *Nucleic Acids Res.* **2017**, *45*, 5217–5230, doi:10.1093/nar/gkx151.

108. Schrödinger, L.; DeLano, W. *Available at : http://www.pymol.org/pymol*; PyMOL, 2020.

109. Marino, F.; Vindigni, A.; Onesti, S. Bioinformatic analysis of RecQ4 helicases reveals the presence of a RQC domain and a Zn knuckle. *Biophys Chem* **2013**, *177-178*, 34–39, doi:10.1016/j.bpc.2013.02.009.

110. Mojumdar, A.; De March, M.; Marino, F.; Onesti, S. The Human RecQ4 Helicase Contains a Functional RecQ C-terminal Region (RQC) That Is Essential for Activity. *J. Biol. Chem.* **2017**, *292*, 4176–4184, doi:10.1074/jbc.M116.767954.

111. Bochman, M. L.; Paeschke, K.; Chan, A.; Zakian, V. A. Hrq1, a homolog of the human RecQ4 helicase, acts catalytically and structurally to promote genome integrity. *Cell Rep.* **2014**, *6*, 346–356, doi:10.1016/j.celrep.2013.12.037.

112. Choi, D.-H.; Min, M.-H.; Kim, M.-J.; Lee, R.; Kwon, S.-H.; Bae, S.-H. Hrq1 facilitates nucleotide excision repair of DNA damage induced by 4-nitroquinoline-1-oxide and cisplatin in Saccharomyces cerevisiae. *J. Microbiol.* **2014**, *52*, 292–298, doi:10.1007/s12275-014-4018-z.

113. Moldovan, G.-L.; D'Andrea, A. D. How the fanconi anemia pathway guards the genome. *Annu. Rev. Genet.* **2009**, *43*, 223–249, doi:10.1146/annurev-genet-102108-134222.

114. Kottemann, M. C.; Smogorzewska, A. Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. *Nature* **2013**, *493*, 356–363, doi:10.1038/nature11863.

115. Ceccaldi, R.; Sarangi, P.; D'Andrea, A. D. The Fanconi anaemia pathway: new players and new functions. *Nat. Rev. Mol. Cell Biol.* **2016**, *17*, 337–349, doi:10.1038/nrm.2016.48.

116. Deans, A. J.; West, S. C. DNA interstrand crosslink repair and cancer. *Nat. Rev. Cancer* **2011**, *11*, 467–480, doi:10.1038/nrc3088.

117. Schärer, O. D. DNA interstrand crosslinks: natural and drug-induced DNA adducts that induce unique cellular responses. *Chembiochem* **2005**, *6*, 27–32, doi:10.1002/cbic.200400287.

118. Langevin, F.; Crossan, G. P.; Rosado, I. V.; Arends, M. J.; Patel, K. J. Fancd2 counteracts the toxic effects of naturally produced aldehydes in mice. *Nature* **2011**, *475*, 53–58, doi:10.1038/nature10192.

119. Uebelacker, M.; Lachenmeier, D. W. Quantitative determination of acetaldehyde in foods using automated digestion with simulated gastric fluid followed by headspace gas chromatography. *J. Autom. Methods Manag. Chem.* **2011**, *2011*, 907317, doi:10.1155/2011/907317.

120. Sonohara, Y.; Yamamoto, J.; Tohashi, K.; Takatsuka, R.; Matsuda, T.; Iwai, S.; Kuraoka, I. Acetaldehyde forms covalent GG intrastrand crosslinks in DNA. *Sci. Rep.* **2019**, *9*, 660, doi:10.1038/s41598-018-37239-6.

121. Li, J.; Jin, J.; Liao, M.; Dang, W.; Chen, X.; Wu, Y.; Liao, W. Upregulation of RECQL4 expression predicts poor prognosis in hepatocellular carcinoma. *Oncol. Lett.* **2018**, *15*, 4248–4254, doi:10.3892/ol.2018.7860.

122. Thomas, B. J.; Rothstein, R. Elevated recombination rates in transcriptionally active DNA. *Cell* **1989**, *56*, 619–630, doi:10.1016/0092-8674(89)90584-9.

123. Zhao, X.; Muller, E. G.; Rothstein, R. A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. *Mol. Cell* **1998**, *2*, 329–340, doi:10.1016/s1097-2765(00)80277-4.

124. Sherman, F. Getting started with yeast. *Meth. Enzymol.* **2002**, *350*, 3–41, doi:10.1016/S0076-6879(02)50954-X.

125. Janke, C.; Magiera, M. M.; Rathfelder, N.; Taxis, C.; Reber, S.; Maekawa, H.; Moreno-Borchart, A.; Doenges, G.; Schwob, E.; Schiebel, E.; Knop, M. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast* **2004**, *21*, 947–962, doi:10.1002/yea.1142.

126. Böhm, S.; Mihalevic, M. J.; Casal, M. A.; Bernstein, K. A. Disruption of SUMO-targeted ubiquitin ligases Slx5-Slx8/RNF4 alters RecQ-like helicase Sgs1/BLM localization in yeast and human cells. *DNA Repair (Amst.)* **2015**, *26*, 1–14, doi:10.1016/j.dnarep.2014.12.004.

127. Böhm, S.; Szakal, B.; Herken, B. W.; Sullivan, M. R.; Mihalevic, M. J.; Kabbinavar, F. F.; Branzei, D.; Clark, N. L.; Bernstein, K. A. The budding yeast ubiquitin protease ubp7 is a novel component involved in S phase progression. *J. Biol. Chem.* **2016**, *291*, 4442–4452, doi:10.1074/jbc.M115.671057.

128. Muratani, M.; Kung, C.; Shokat, K. M.; Tansey, W. P. The F box protein Dsg1/Mdm30 is a transcriptional coactivator that stimulates Gal4 turnover and cotranscriptional mRNA processing. *Cell* **2005**, *120*, 887–899, doi:10.1016/j.cell.2004.12.025.

129. Tansey, W. P. Detection of ubiquitylated proteins in yeast. *CSH Protoc* **2006**, *2006*, doi:10.1101/pdb.prot4615.

130. Vlachostergios, P. J.; Patrikidou, A.; Daliani, D. D.; Papandreou, C. N. The ubiquitin-proteasome system in cancer, a major player in DNA repair. Part 1: post-translational

regulation. *J. Cell Mol. Med.* **2009**, *13*, 3006–3018, doi:10.1111/j.1582-4934.2009.00824.x.

131. Tran, J. R.; Brodsky, J. L. Assays to measure ER-associated degradation in yeast. *Methods Mol. Biol.* **2012**, *832*, 505–518, doi:10.1007/978-1-61779-474-2_36.

132. Lommel, L.; Ortolan, T.; Chen, L.; Madura, K.; Sweder, K. S. Proteolysis of a nucleotide excision repair protein by the 26 S proteasome. *Curr. Genet.* **2002**, *42*, 9–20, doi:10.1007/s00294-002-0332-9.

133. Ramsey, K. L.; Smith, J. J.; Dasgupta, A.; Maqani, N.; Grant, P.; Auble, D. T. The NEF4 complex regulates Rad4 levels and utilizes Snf2/Swi2-related ATPase activity for nucleotide excision repair. *Mol. Cell. Biol.* **2004**, *24*, 6362–6378, doi:10.1128/MCB.24.14.6362-6378.2004.

134. Gillette, T. G.; Yu, S.; Zhou, Z.; Waters, R.; Johnston, S. A.; Reed, S. H. Distinct functions of the ubiquitin-proteasome pathway influence nucleotide excision repair. *EMBO J.* **2006**, *25*, 2529–2538, doi:10.1038/sj.emboj.7601120.

135. Wang, Q.-E.; Praetorius-Ibba, M.; Zhu, Q.; El-Mahdy, M. A.; Wani, G.; Zhao, Q.; Qin, S.; Patnaik, S.; Wani, A. A. Ubiquitylation-independent degradation of Xeroderma pigmentosum group C protein is required for efficient nucleotide excision repair. *Nucleic Acids Res.* **2007**, *35*, 5338–5350, doi:10.1093/nar/gkm550.

136. Sommers, J. A.; Suhasini, A. N.; Brosh, R. M. Protein degradation pathways regulate the functions of helicases in the DNA damage response and maintenance of genomic stability. *Biomolecules* **2015**, *5*, 590–616, doi:10.3390/biom5020590.

137. Wang, J.; Chen, J.; Gong, Z. TopBP1 controls BLM protein level to maintain genome stability. *Mol. Cell* **2013**, *52*, 667–678, doi:10.1016/j.molcel.2013.10.012.

138. Bacquin, A.; Pouvelle, C.; Siaud, N.; Perderiset, M.; Salomé-Desnoulez, S.; Tellier-Lebegue, C.; Lopez, B.; Charbonnier, J.-B.; Kannouche, P. L. The helicase FBH1 is tightly regulated by PCNA via CRL4(Cdt2)-mediated proteolysis in human cells. *Nucleic Acids Res.* **2013**, *41*, 6501–6513, doi:10.1093/nar/gkt397.

139. O'Donovan, A.; Davies, A. A.; Moggs, J. G.; West, S. C.; Wood, R. D. XPG endonuclease makes the 3' incision in human DNA nucleotide excision repair. *Nature* **1994**, *371*, 432–435, doi:10.1038/371432a0.

140. Bauer, G. B.; Povirk, L. F. Specificity and kinetics of interstrand and intrastrand bifunctional alkylation by nitrogen mustards at a G-G-C sequence. *Nucleic Acids Res.* **1997**, *25*, 1211–1218, doi:10.1093/nar/25.6.1211.

141. Lopez-Martinez, D.; Liang, C.-C.; Cohn, M. A. Cellular response to DNA interstrand crosslinks: the Fanconi anemia pathway. *Cell Mol. Life Sci.* **2016**, *73*, 3097–3114, doi:10.1007/s00018-016-2218-x.

142. McHugh, P. J.; Ward, T. A.; Chovanec, M. A prototypical Fanconi anemia pathway in lower eukaryotes? *Cell Cycle* **2012**, *11*, 3739–3744, doi:10.4161/cc.21727.

143. McHugh, P. J.; Sarkar, S. DNA interstrand cross-link repair in the cell cycle: a critical role for polymerase zeta in G1 phase. *Cell Cycle* **2006**, *5*, 1044–1047, doi:10.4161/cc.5.10.2763.

144. Noll, D. M.; Mason, T. M.; Miller, P. S. Formation and repair of interstrand cross-links in DNA. *Chem. Rev.* **2006**, *106*, 277–301, doi:10.1021/cr040478b.

145. Shen, X.; Li, L. Mutagenic repair of DNA interstrand crosslinks. *Environ Mol Mutagen* **2010**, *51*, 493–499, doi:10.1002/em.20558.

146. Daee, D. L.; Myung, K. Fanconi-like crosslink repair in yeast. *Genome Integr* **2012**, *3*, 7, doi:10.1186/2041-9414-3-7.

147. Budzowska, M.; Kanaar, R. Mechanisms of dealing with DNA damage-induced replication problems. *Cell Biochem. Biophys.* **2009**, *53*, 17–31, doi:10.1007/s12013-008-9039-y.

148. Ulrich, H. D.; Walden, H. Ubiquitin signalling in DNA replication and repair. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 479–489, doi:10.1038/nrm2921.

149. Köberle, B.; Masters, J. R.; Hartley, J. A.; Wood, R. D. Defective repair of cisplatin-induced DNA damage caused by reduced XPA protein in testicular germ cell tumours. *Curr. Biol.* **1999**, *9*, 273–276, doi:10.1016/s0960-9822(99)80118-3.

150. McKay, B. C.; Becerril, C.; Ljungman, M. P53 plays a protective role against UV- and cisplatin-induced apoptosis in transcription-coupled repair proficient fibroblasts. *Oncogene* **2001**, *20*, 6805–6808, doi:10.1038/sj.onc.1204901.

151. Welsh, C.; Day, R.; McGurk, C.; Masters, J. R. W.; Wood, R. D.; Köberle, B. Reduced levels of XPA, ERCC1 and XPF DNA repair proteins in testis tumor cell lines. *Int. J. Cancer* **2004**, *110*, 352–361, doi:10.1002/ijc.20134.

152. Basu, A.; Krishnamurthy, S. Cellular responses to Cisplatin-induced DNA damage. *J. Nucleic Acids* **2010**, *2010*, doi:10.4061/2010/201367.

153. Branzei, D.; Foiani, M. Maintaining genome stability at the replication fork. *Nat. Rev. Mol. Cell Biol.* **2010**, *11*, 208–219, doi:10.1038/nrm2852.

154. Berti, M.; Cortez, D.; Lopes, M. The plasticity of DNA replication forks in response to clinically relevant genotoxic stress. *Nat. Rev. Mol. Cell Biol.* **2020**, *21*, 633–651, doi:10.1038/s41580-020-0257-5.

155. Godin, S.; Wier, A.; Kabbinavar, F.; Bratton-Palmer, D. S.; Ghodke, H.; Van Houten, B.; VanDemark, A. P.; Bernstein, K. A. The Shu complex interacts with Rad51 through the Rad51 paralogues Rad55-Rad57 to mediate error-free recombination. *Nucleic Acids Res.* **2013**, *41*, 4525–4534, doi:10.1093/nar/gkt138.

156. Godin, S. K.; Zhang, Z.; Herken, B. W.; Westmoreland, J. W.; Lee, A. G.; Mihalevic, M. J.; Yu, Z.; Sobol, R. W.; Resnick, M. A.; Bernstein, K. A. The Shu complex promotes error-free tolerance of alkylation-induced base excision repair products. *Nucleic Acids Res.* **2016**, *44*, 8199–8215, doi:10.1093/nar/gkw535.

157. Geissmann, Q. OpenCFU, a new free and open-source software to count cell colonies and other circular objects. *PLoS One* **2013**, *8*, e54072, doi:10.1371/journal.pone.0054072.

158. Groocock, L. M.; Prudden, J.; Perry, J. J. P.; Boddy, M. N. The RecQ4 orthologue Hrq1 is critical for DNA interstrand cross-link repair and genome stability in fission yeast. *Mol. Cell. Biol.* **2012**, *32*, 276–287, doi:10.1128/MCB.06184-11.

159. Röhrig, S.; Dorn, A.; Enderle, J.; Schindele, A.; Herrmann, N. J.; Knoll, A.; Puchta, H. The RecQ-like helicase HRQ1 is involved in DNA crosslink repair in Arabidopsis in a common pathway with the Fanconi anemia-associated nuclease FAN1 and the postreplicative repair ATPase RAD5A. *New Phytol.* **2018**, *218*, 1478–1490, doi:10.1111/nph.15109.

160. Radivojac, P.; Vacic, V.; Haynes, C.; Cocklin, R. R.; Mohan, A.; Heyen, J. W.; Goebl, M. G.; Iakoucheva, L. M. Identification, analysis, and prediction of protein ubiquitination sites. *Proteins* **2010**, *78*, 365–380, doi:10.1002/prot.22555.

161. Madeira, F.; Park, Y. M.; Lee, J.; Buso, N.; Gur, T.; Madhusoodanan, N.; Basutkar, P.; Tivey, A. R. N.; Potter, S. C.; Finn, R. D.; Lopez, R. The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res.* **2019**, *47*, W636–W641, doi:10.1093/nar/gkz268.

162. Lovett, S. T. Template-switching during replication fork repair in bacteria. *DNA Repair (Amst.)* **2017**, *56*, 118–128, doi:10.1016/j.dnarep.2017.06.014.

163. Löytynoja, A.; Goldman, N. Short template switch events explain mutation clusters in the human genome. *Genome Res.* **2017**, *27*, 1039–1049, doi:10.1101/gr.214973.116.

164. Pepe, A.; West, S. C. MUS81-EME2 promotes replication fork restart. *Cell Rep.* **2014**, *7*, 1048–1055, doi:10.1016/j.celrep.2014.04.007.

165. Debrauwère, H.; Loeillet, S.; Lin, W.; Lopes, J.; Nicolas, A. Links between replication and recombination in Saccharomyces cerevisiae: a hypersensitive requirement for homologous recombination in the absence of Rad27 activity. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 8263–8269, doi:10.1073/pnas.121075598.

166. Enserink, J. M.; Kolodner, R. D. What makes the engine hum: Rad6, a cell cycle supercharger. *Cell Cycle* **2012**, *11*, 249–252, doi:10.4161/cc.11.2.19023.

167. Collins, S. R.; Schuldiner, M.; Krogan, N. J.; Weissman, J. S. A strategy for extracting and analyzing large-scale quantitative epistatic interaction data. *Genome Biol.* **2006**, *7*, R63, doi:10.1186/gb-2006-7-7-r63.

168. Kondratick, C. M.; Washington, M. T.; Spies, M. Making choices: DNA replication fork recovery mechanisms. *Semin. Cell Dev. Biol.* **2020**, doi:10.1016/j.semcdb.2020.10.001.

169. Alexander, J. L.; Orr-Weaver, T. L. Replication fork instability and the consequences of fork collisions from rereplication. *Genes Dev.* **2016**, *30*, 2241–2252, doi:10.1101/gad.288142.116.

170. Bernstein, K. A.; Shor, E.; Sunjevaric, I.; Fumasoni, M.; Burgess, R. C.; Foiani, M.; Branzei, D.; Rothstein, R. Sgs1 function in the repair of DNA replication intermediates is separable from its role in homologous recombinational repair. *EMBO J.* **2009**, *28*, 915–925, doi:10.1038/emboj.2009.28.

171. Bernstein, K. A.; Mimitou, E. P.; Mihalevic, M. J.; Chen, H.; Sunjaveric, I.; Symington, L. S.; Rothstein, R. Resection activity of the Sgs1 helicase alters the affinity of DNA ends for homologous recombination proteins in Saccharomyces cerevisiae. *Genetics* **2013**, *195*, 1241–1251, doi:10.1534/genetics.113.157370.

172. Guzmán, C.; Bagga, M.; Kaur, A.; Westermarck, J.; Abankwa, D. ColonyArea: an ImageJ plugin to automatically quantify colony formation in clonogenic assays. *PLoS One* **2014**, *9*, e92444, doi:10.1371/journal.pone.0092444.

173. Hernandez-Segura, A.; Nehme, J.; Demaria, M. Hallmarks of cellular senescence. *Trends Cell Biol.* **2018**, *28*, 436–453, doi:10.1016/j.tcb.2018.02.001.

174. González-Gualda, E.; Baker, A. G.; Fruk, L.; Muñoz-Espín, D. A guide to assessing cellular senescence in vitro and in vivo. *FEBS J.* **2021**, *288*, 56–80, doi:10.1111/febs.15570.

175. Lu, H.; Fang, E. F.; Sykora, P.; Kulikowicz, T.; Zhang, Y.; Becker, K. G.; Croteau, D. L.; Bohr, V. A. Senescence induced by RECQL4 dysfunction contributes to Rothmund-Thomson syndrome features in mice. *Cell Death Dis.* **2014**, *5*, e1226, doi:10.1038/cddis.2014.168.

176. Fang, H.; Niu, K.; Mo, D.; Zhu, Y.; Tan, Q.; Wei, D.; Li, Y.; Chen, Z.; Yang, S.; Balajee, A. S.; Zhao, Y. RecQL4-Aurora B kinase axis is essential for cellular proliferation, cell cycle progression, and mitotic integrity. *Oncogenesis* **2018**, *7*, 68, doi:10.1038/s41389-018-0080-4.

177. Patterson, J. C.; Joughin, B. A.; van de Kooij, B.; Lim, D. C.; Lauffenburger, D. A.; Yaffe, M. B. ROS and Oxidative Stress Are Elevated in Mitosis during Asynchronous Cell Cycle

Progression and Are Exacerbated by Mitotic Arrest. *Cell Syst.* **2019**, *8*, 163–167.e2, doi:10.1016/j.cels.2019.01.005.

178. Wang, X.; Li, M. Correlate tumor mutation burden with immune signatures in human cancers. *BMC Immunol.* **2019**, *20*, 4, doi:10.1186/s12865-018-0285-5.

179. Ogston, K. N.; Miller, I. D.; Payne, S.; Hutcheon, A. W.; Sarkar, T. K.; Smith, I.; Schofield, A.; Heys, S. D. A new histological grading system to assess response of breast cancers to primary chemotherapy: prognostic significance and survival. *Breast* **2003**, *12*, 320–327, doi:10.1016/S0960-9776(03)00106-1.

180. Sheikh, M. S.; Shao, Z. M.; Li, X. S.; Dawson, M.; Jetten, A. M.; Wu, S.; Conley, B. A.; Garcia, M.; Rochefort, H.; Fontana, J. A. Retinoid-resistant estrogen receptor-negative human breast carcinoma cells transfected with retinoic acid receptor-alpha acquire sensitivity to growth inhibition by retinoids. *J. Biol. Chem.* **1994**, *269*, 21440–21447, doi:10.1016/S0021-9258(17)31823-9.

181. Ascierto, P. A.; Kirkwood, J. M.; Grob, J.-J.; Simeone, E.; Grimaldi, A. M.; Maio, M.; Palmieri, G.; Testori, A.; Marincola, F. M.; Mozzillo, N. The role of BRAF V600 mutation in melanoma. *J. Transl. Med.* **2012**, *10*, 85, doi:10.1186/1479-5876-10-85.

182. Kondrashova, O.; Nguyen, M.; Shield-Artin, K.; Tinker, A. V.; Teng, N. N. H.; Harrell, M. I.; Kuiper, M. J.; Ho, G.-Y.; Barker, H.; Jasin, M.; Prakash, R.; Kass, E. M.; Sullivan, M. R.; Brunette, G. J.; Bernstein, K. A.; Coleman, R. L.; Floquet, A.; Friedlander, M.; Kichenadasse, G.; O'Malley, D. M.; Oza, A.; Sun, J.; Robillard, L.; Maloney, L.; Bowtell, D.; Giordano, H.; Wakefield, M. J.; Kaufmann, S. H.; Simmons, A. D.; Harding, T. C.; Raponi, M.; McNeish, I. A.; Swisher, E. M.; Lin, K. K.; Scott, C. L.; AOCS Study Group Secondary Somatic Mutations Restoring RAD51C and RAD51D Associated with Acquired Resistance to the PARP Inhibitor Rucaparib in High-Grade Ovarian Carcinoma. *Cancer Discov.* **2017**, *7*, 984–998, doi:10.1158/2159-8290.CD-17-0419.

183. Schumacher, T. N.; Schreiber, R. D. Neoantigens in cancer immunotherapy. *Science* **2015**, *348*, 69–74, doi:10.1126/science.aaa4971.

184. Wang, R.-F.; Wang, H. Y. Immune targets and neoantigens for cancer immunotherapy and precision medicine. *Cell Res.* **2017**, *27*, 11–37, doi:10.1038/cr.2016.155.

185. Johnson, D. B.; Frampton, G. M.; Rioth, M. J.; Yusko, E.; Xu, Y.; Guo, X.; Ennis, R. C.; Fabrizio, D.; Chalmers, Z. R.; Greenbowe, J.; Ali, S. M.; Balasubramanian, S.; Sun, J. X.; He, Y.; Frederick, D. T.; Puzanov, I.; Balko, J. M.; Cates, J. M.; Ross, J. S.; Sanders, C.; Robins, H.; Shyr, Y.; Miller, V. A.; Stephens, P. J.; Sullivan, R. J.; Sosman, J. A.; Lovly, C. M. Targeted Next Generation Sequencing Identifies Markers of Response to PD-1 Blockade. *Cancer Immunol Res* **2016**, *4*, 959–967, doi:10.1158/2326-6066.CIR-16-0143.

186. Tomasetti, C.; Vogelstein, B. Cancer etiology. Variation in cancer risk among tissues can be explained by the number of stem cell divisions. *Science* **2015**, *347*, 78–81, doi:10.1126/science.1260825.

187. Tomasetti, C.; Li, L.; Vogelstein, B. Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. *Science* **2017**, *355*, 1330–1334, doi:10.1126/science.aaf9011.

188. Motegi, A.; Liaw, H.-J.; Lee, K.-Y.; Roest, H. P.; Maas, A.; Wu, X.; Moinova, H.; Markowitz, S. D.; Ding, H.; Hoeijmakers, J. H. J.; Myung, K. Polyubiquitination of proliferating cell nuclear antigen by HLTF and SHPRH prevents genomic instability from stalled replication forks. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 12411–12416, doi:10.1073/pnas.0805685105.

189. Lin, J.-R.; Zeman, M. K.; Chen, J.-Y.; Yee, M.-C.; Cimprich, K. A. SHPRH and HLTF act in a damage-specific manner to coordinate different forms of postreplication repair and prevent mutagenesis. *Mol. Cell* **2011**, *42*, 237–249, doi:10.1016/j.molcel.2011.02.026.

190. Elserafy, M.; Abugable, A. A.; Atteya, R.; El-Khamisy, S. F. Rad5, HLTF, and SHPRH: A fresh view of an old story. *Trends Genet.* **2018**, *34*, 574–577, doi:10.1016/j.tig.2018.04.006.

191. Laranjo, L. T.; Klaric, J. A.; Pearlman, L. R.; Lovett, S. T. Stimulation of Replication Template-Switching by DNA-Protein Crosslinks. *Genes (Basel)* **2018**, *10*, doi:10.3390/genes10010014.

192. Bétous, R.; Mason, A. C.; Rambo, R. P.; Bansbach, C. E.; Badu-Nkansah, A.; Sirbu, B. M.; Eichman, B. F.; Cortez, D. SMARCAL1 catalyzes fork regression and Holliday junction migration to maintain genome stability during DNA replication. *Genes Dev.* **2012**, *26*, 151–162, doi:10.1101/gad.178459.111.

193. Blastyák, A.; Pintér, L.; Unk, I.; Prakash, L.; Prakash, S.; Haracska, L. Yeast Rad5 protein required for postreplication repair has a DNA helicase activity specific for replication fork regression. *Mol. Cell* **2007**, *28*, 167–175, doi:10.1016/j.molcel.2007.07.030.

194. Unk, I.; Hajdú, I.; Fátyol, K.; Hurwitz, J.; Yoon, J.-H.; Prakash, L.; Prakash, S.; Haracska, L. Human HLTF functions as a ubiquitin ligase for proliferating cell nuclear antigen polyubiquitination. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 3768–3773, doi:10.1073/pnas.0800563105.

195. Rossi, M. L.; Ghosh, A. K.; Kulikowicz, T.; Croteau, D. L.; Bohr, V. A. Conserved helicase domain of human RecQ4 is required for strand annealing-independent DNA unwinding. *DNA Repair (Amst.)* **2010**, *9*, 796–804, doi:10.1016/j.dnarep.2010.04.003.

196. Sirbu, B. M.; Couch, F. B.; Feigerle, J. T.; Bhaskara, S.; Hiebert, S. W.; Cortez, D. Analysis of protein dynamics at active, stalled, and collapsed replication forks. *Genes Dev.* **2011**, *25*, 1320–1327, doi:10.1101/gad.2053211.

197. Sirbu, B. M.; Couch, F. B.; Cortez, D. Monitoring the spatiotemporal dynamics of proteins at replication forks and in assembled chromatin using isolation of proteins on nascent DNA. *Nat. Protoc.* **2012**, *7*, 594–605, doi:10.1038/nprot.2012.010.

198. Sirbu, B. M.; McDonald, W. H.; Dungrawala, H.; Badu-Nkansah, A.; Kavanaugh, G. M.; Chen, Y.; Tabb, D. L.; Cortez, D. Identification of proteins at active, stalled, and collapsed replication forks using isolation of proteins on nascent DNA (iPOND) coupled with mass spectrometry. *J. Biol. Chem.* **2013**, *288*, 31458–31467, doi:10.1074/jbc.M113.511337.

199. Mohni, K. N.; Wessel, S. R.; Zhao, R.; Wojciechowski, A. C.; Luzwick, J. W.; Layden, H.; Eichman, B. F.; Thompson, P. S.; Mehta, K. P. M.; Cortez, D. HMCES Maintains Genome Integrity by Shielding Abasic Sites in Single-Strand DNA. *Cell* **2019**, *176*, 144–153.e13, doi:10.1016/j.cell.2018.10.055.

200. Li, X.; Heyer, W.-D. Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res.* **2008**, *18*, 99–113, doi:10.1038/cr.2008.1.

201. Byun, T. S.; Pacek, M.; Yee, M.; Walter, J. C.; Cimprich, K. A. Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. *Genes Dev.* **2005**, *19*, 1040–1052, doi:10.1101/gad.1301205.

202. Maréchal, A.; Zou, L. RPA-coated single-stranded DNA as a platform for post-translational modifications in the DNA damage response. *Cell Res.* **2015**, *25*, 9–23, doi:10.1038/cr.2014.147.

203. Yates, L. A.; Aramayo, R. J.; Pokhrel, N.; Caldwell, C. C.; Kaplan, J. A.; Perera, R. L.; Spies, M.; Antony, E.; Zhang, X. A structural and dynamic model for the assembly of Replication Protein A on single-stranded DNA. *Nat. Commun.* **2018**, *9*, 5447, doi:10.1038/s41467-018-07883-7.

204. Bhat, K. P.; Cortez, D. RPA and RAD51: fork reversal, fork protection, and genome stability. *Nat. Struct. Mol. Biol.* **2018**, *25*, 446–453, doi:10.1038/s41594-018-0075-z.

205. Rein, H. L.; Bernstein, K. A.; Baldock, R. A. RAD51 paralog function in replicative DNA damage and tolerance. *Curr. Opin. Genet. Dev.* **2021**, *71*, 86–91, doi:10.1016/j.gde.2021.06.010.

206. Sullivan, M. R.; Bernstein, K. A. RAD-ical New Insights into RAD51 Regulation. *Genes (Basel)* **2018**, *9*, doi:10.3390/genes9120629.

207. Fugger, K.; Mistrik, M.; Danielsen, J. R.; Dinant, C.; Falck, J.; Bartek, J.; Lukas, J.; Mailand, N. Human Fbh1 helicase contributes to genome maintenance via pro- and anti-recombinase activities. *J. Cell Biol.* **2009**, *186*, 655–663, doi:10.1083/jcb.200812138.

208. Patel, D. S.; Misenko, S. M.; Her, J.; Bunting, S. F. BLM helicase regulates DNA repair by counteracting RAD51 loading at DNA double-strand break sites. *J. Cell Biol.* **2017**, *216*, 3521–3534, doi:10.1083/jcb.201703144.

209. Zhang, H.; Schaub, J. M.; Finkelstein, I. J. RADX condenses single-stranded DNA to antagonize RAD51 loading. *Nucleic Acids Res.* **2020**, *48*, 7834–7843, doi:10.1093/nar/gkaa559.

210. Nimonkar, A. V.; Genschel, J.; Kinoshita, E.; Polaczek, P.; Campbell, J. L.; Wyman, C.; Modrich, P.; Kowalczykowski, S. C. BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA break repair. *Genes Dev.* **2011**, *25*, 350–362, doi:10.1101/gad.2003811.

211. Campos-Doerfler, L.; Syed, S.; Schmidt, K. H. Sgs1 Binding to Rad51 Stimulates Homology-Directed DNA Repair in Saccharomyces cerevisiae. *Genetics* **2017**, *208*, 125–138, doi:10.1534/genetics.117.300545.

212. Neelsen, K. J.; Lopes, M. Replication fork reversal in eukaryotes: from dead end to dynamic response. *Nat. Rev. Mol. Cell Biol.* **2015**, *16*, 207–220, doi:10.1038/nrm3935.

213. Mason, J. M.; Chan, Y.-L.; Weichselbaum, R. W.; Bishop, D. K. Non-enzymatic roles of human RAD51 at stalled replication forks. *Nat. Commun.* **2019**, *10*, 4410, doi:10.1038/s41467-019-12297-0.

214. Lemaçon, D.; Jackson, J.; Quinet, A.; Brickner, J. R.; Li, S.; Yazinski, S.; You, Z.; Ira, G.; Zou, L.; Mosammaparast, N.; Vindigni, A. MRE11 and EXO1 nucleases degrade reversed forks and elicit MUS81-dependent fork rescue in BRCA2-deficient cells. *Nat. Commun.* **2017**, *8*, 860, doi:10.1038/s41467-017-01180-5.

215. Kolinjivadi, A. M.; Sannino, V.; De Antoni, A.; Zadorozhny, K.; Kilkenny, M.; Técher, H.; Baldi, G.; Shen, R.; Ciccia, A.; Pellegrini, L.; Krejci, L.; Costanzo, V. Smarcal1-Mediated Fork Reversal Triggers Mre11-Dependent Degradation of Nascent DNA in the Absence of Brca2 and Stable Rad51 Nucleofilaments. *Mol. Cell* **2017**, *67*, 867–881.e7, doi:10.1016/j.molcel.2017.07.001.

216. Mijic, S.; Zellweger, R.; Chappidi, N.; Berti, M.; Jacobs, K.; Mutreja, K.; Ursich, S.; Ray Chaudhuri, A.; Nussenzweig, A.; Janscak, P.; Lopes, M. Replication fork reversal triggers fork degradation in BRCA2-defective cells. *Nat. Commun.* **2017**, *8*, 859, doi:10.1038/s41467-017-01164-5.

217. Zadorozhny, K.; Sannino, V.; Beláň, O.; Mlčoušková, J.; Špírek, M.; Costanzo, V.; Krejčí, L. Fanconi-Anemia-Associated Mutations Destabilize RAD51 Filaments and Impair Replication Fork Protection. *Cell Rep.* **2017**, *21*, 333–340, doi:10.1016/j.celrep.2017.09.062.

218. Stingele, J.; Habermann, B.; Jentsch, S. DNA-protein crosslink repair: proteases as DNA repair enzymes. *Trends Biochem. Sci.* **2015**, *40*, 67–71, doi:10.1016/j.tibs.2014.10.012.

219. Stingele, J.; Bellelli, R.; Boulton, S. J. Mechanisms of DNA-protein crosslink repair. *Nat. Rev. Mol. Cell Biol.* **2017**, *18*, 563–573, doi:10.1038/nrm.2017.56.

220. Yin, J.; Kwon, Y. T.; Varshavsky, A.; Wang, W. RECQL4, mutated in the Rothmund-Thomson and RAPADILINO syndromes, interacts with ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway. *Hum. Mol. Genet.* **2004**, *13*, 2421–2430, doi:10.1093/hmg/ddh269.

221. Shamanna, R. A.; Lu, H.; Croteau, D. L.; Arora, A.; Agarwal, D.; Ball, G.; Aleskandarany, M. A.; Ellis, I. O.; Pommier, Y.; Madhusudan, S.; Bohr, V. A. Camptothecin targets WRN protein: mechanism and relevance in clinical breast cancer. *Oncotarget* **2016**, *7*, 13269–13284, doi:10.18632/oncotarget.7906.

222. Huang, Q.; Qin, D.; Pei, D.; Vermeulen, M.; Zhang, X. UBE2O and USP7 co-regulate RECQL4 ubiquitinylation and homologous recombination-mediated DNA repair. *FASEB J.* **2022**, *36*, e22112, doi:10.1096/fj.202100974RRR.

223. Min, M.-H.; Kim, M.-J.; Choi, Y.-J.; You, M.-J.; Kim, U.-R.; An, H.-B.; Kim, C.-H.; Kwon, C.-Y.; Bae, S.-H. Characterization of Hrq1-Rad14 Interaction in Saccharomyces cerevisiae. *The Korean Journal of Microbiology* **2014**, *50*, 95–100, doi:10.7845/kjm.2014.4016.

224. Van Houten, B.; Schnable, B.; Kumar, N. Chaperones for dancing on chromatin: Role of post-translational modifications in dynamic damage detection hand-offs during nucleotide excision repair. *Bioessays* **2021**, *43*, e2100011, doi:10.1002/bies.202100011.

225. Ribeiro-Silva, C.; Sabatella, M.; Helfricht, A.; Marteijn, J. A.; Theil, A. F.; Vermeulen, W.; Lans, H. Ubiquitin and TFIIH-stimulated DDB2 dissociation drives DNA damage handover in nucleotide excision repair. *Nat. Commun.* **2020**, *11*, 4868, doi:10.1038/s41467-020-18705-0.

226. Mao, P.; Wyrick, J. J. Emerging roles for histone modifications in DNA excision repair. *FEMS Yeast Res* **2016**, *16*, doi:10.1093/femsyr/fow090.

227. Sugasawa, K.; Okuda, Y.; Saijo, M.; Nishi, R.; Matsuda, N.; Chu, G.; Mori, T.; Iwai, S.; Tanaka, K.; Tanaka, K.; Hanaoka, F. UV-induced ubiquitylation of XPC protein mediated by UV-DDB-ubiquitin ligase complex. *Cell* **2005**, *121*, 387–400, doi:10.1016/j.cell.2005.02.035.

228. Vaz, B.; Halder, S.; Ramadan, K. Role of p97/VCP (Cdc48) in genome stability. *Front. Genet.* **2013**, *4*, 60, doi:10.3389/fgene.2013.00060.

229. Meyer, H.; Weihl, C. C. The VCP/p97 system at a glance: connecting cellular function to disease pathogenesis. *J. Cell Sci.* **2014**, *127*, 3877–3883, doi:10.1242/jcs.093831.

230. Zhu, Q.; Wani, A. A. Nucleotide Excision Repair: Finely Tuned Molecular Orchestra of Early Pre-incision Events. *Photochem. Photobiol.* **2017**, *93*, 166–177, doi:10.1111/php.12647.

231. Vekaria, P. H.; Home, T.; Weir, S.; Schoenen, F. J.; Rao, R. Targeting p97 to disrupt protein homeostasis in cancer. *Front. Oncol.* **2016**, *6*, 181, doi:10.3389/fonc.2016.00181.

232. Kaur, E.; Agrawal, R.; Sengupta, S. Functions of BLM Helicase in Cells: Is It Acting Like a Double-Edged Sword? *Front. Genet.* **2021**, *12*, 634789, doi:10.3389/fgene.2021.634789.

233. Bryant, H. E.; Schultz, N.; Thomas, H. D.; Parker, K. M.; Flower, D.; Lopez, E.; Kyle, S.; Meuth, M.; Curtin, N. J.; Helleday, T. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* **2005**, *434*, 913–917, doi:10.1038/nature03443.

234. Fong, P. C.; Boss, D. S.; Yap, T. A.; Tutt, A.; Wu, P.; Mergui-Roelvink, M.; Mortimer, P.; Swaisland, H.; Lau, A.; O'Connor, M. J.; Ashworth, A.; Carmichael, J.; Kaye, S. B.; Schellens, J. H. M.; de Bono, J. S. Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. *N. Engl. J. Med.* **2009**, *361*, 123–134, doi:10.1056/NEJMoa0900212.

235. O'Connor, M. J. Targeting the DNA damage response in cancer. *Mol. Cell* **2015**, *60*, 547–560, doi:10.1016/j.molcel.2015.10.040.

236. Kristeleit, R. S.; Miller, R. E.; Kohn, E. C. Gynecologic cancers: emerging novel strategies for targeting DNA repair deficiency. *Am. Soc. Clin. Oncol. Educ. Book* **2016**, *35*, e259–68, doi:10.1200/EDBK_159086.

237. Ellenberger, T.; Tomkinson, A. E. Eukaryotic DNA ligases: structural and functional insights. *Annu. Rev. Biochem.* **2008**, *77*, 313–338, doi:10.1146/annurev.biochem.77.061306.123941.

238. Chen, X.; Zhong, S.; Zhu, X.; Dziegielewska, B.; Ellenberger, T.; Wilson, G. M.; MacKerell, A. D.; Tomkinson, A. E. Rational design of human DNA ligase inhibitors that target cellular DNA replication and repair. *Cancer Res.* **2008**, *68*, 3169–3177, doi:10.1158/0008-5472.CAN-07-6636.

239. Howes, T. R. L.; Tomkinson, A. E. DNA ligase I, the replicative DNA ligase. *Subcell. Biochem.* **2012**, *62*, 327–341, doi:10.1007/978-94-007-4572-8_17.

240. Sallmyr, A.; Matsumoto, Y.; Roginskaya, V.; Van Houten, B.; Tomkinson, A. E. Inhibiting Mitochondrial DNA Ligase IIIα Activates Caspase 1-Dependent Apoptosis in Cancer Cells. *Cancer Res.* **2016**, *76*, 5431–5441, doi:10.1158/0008-5472.CAN-15-3243.

241. Kaiser, S.; Sauer, F.; Kisker, C. The structural and functional characterization of human RecQ4 reveals insights into its helicase mechanism. *Nat. Commun.* **2017**, *8*, 15907, doi:10.1038/ncomms15907.

242. Johnson, G. E. Mammalian cell HPRT gene mutation assay: test methods. *Methods Mol. Biol.* **2012**, *817*, 55–67, doi:10.1007/978-1-61779-421-6_4.

243. Ward, T. A.; McHugh, P. J.; Durant, S. T. Small molecule inhibitors uncover synthetic genetic interactions of human flap endonuclease 1 (FEN1) with DNA damage response genes. *PLoS One* **2017**, *12*, e0179278, doi:10.1371/journal.pone.0179278.

244. Guo, E.; Ishii, Y.; Mueller, J.; Srivatsan, A.; Gahman, T.; Putnam, C. D.; Wang, J. Y. J.; Kolodner, R. D. FEN1 endonuclease as a therapeutic target for human cancers with defects in homologous recombination. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 19415–19424, doi:10.1073/pnas.2009237117.

245. Panzarino, N. J.; Krais, J. J.; Cong, K.; Peng, M.; Mosqueda, M.; Nayak, S. U.; Bond, S. M.; Calvo, J. A.; Doshi, M. B.; Bere, M.; Ou, J.; Deng, B.; Zhu, L. J.; Johnson, N.; Cantor, S. B. Replication gaps underlie BRCA deficiency and therapy response. *Cancer Res.* **2021**, *81*, 1388–1397, doi:10.1158/0008-5472.CAN-20-1602.

246. Cong, K.; Peng, M.; Kousholt, A. N.; Lee, W. T. C.; Lee, S.; Nayak, S.; Krais, J.; VanderVere-Carozza, P. S.; Pawelczak, K. S.; Calvo, J.; Panzarino, N. J.; Turchi, J. J.; Johnson, N.; Jonkers, J.; Rothenberg, E.; Cantor, S. B. Replication gaps are a key determinant of PARP inhibitor synthetic lethality with BRCA deficiency. *Mol. Cell* **2021**, *81*, 3128–3144.e7, doi:10.1016/j.molcel.2021.06.011.