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The roles of DNA polymerase ζ and the Y family DNA polymerases in promoting or preventing genome instability

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Abstract

Cancer cells display numerous abnormal characteristics which are initiated and maintained by elevated mutation rates and genome instability. Chromosomal DNA is continuously surveyed for the presence of damage or blocked replication forks by the DNA Damage Response (DDR) network. The DDR is complex and includes activation of cell cycle checkpoints, DNA repair, gene transcription, and induction of apoptosis. Duplicating a damaged genome is associated with elevated risks to fork collapse and genome instability. Therefore, the DNA Damage Tolerance (DDT) pathway is also employed to enhance survival and involves the recruitment of translesion DNA synthesis (TLS) polymerases to sites of replication fork blockade or single stranded DNA gaps left after the completion of replication in order to restore DNA to its double stranded form before mitosis. TLS polymerases are specialized for inserting nucleotides opposite DNA adducts, abasic sites, or DNA crosslinks. By definition, the DDT pathway is not involved in the actual repair of damaged DNA, but provides a mechanism to tolerate DNA lesions during replication thereby increasing survival and lessening the chance for genome instability. However this may be associated with increased mutagenesis. In this review, we will describe the specialized functions of Y family polymerases (Rev1, Pol η , Pol ι and Pol κ) and DNA polymerase ζ in lesion bypass, mutagenesis, and prevention of genome instability, the latter due to newly appreciated roles in DNA repair. The recently described role of the Fanconi anemia pathway in regulating Rev1 and Pol ζ -dependent TLS is also discussed in terms of their involvement in TLS, interstrand crosslink repair, and homologous recombination.

Keywords

POLH; POLI; POLK; REV1; REV3; REV7; postreplication repair; Fanconi anemia; Translesion DNA Synthesis; chemoresistance

1. Introduction

Cancer cells display numerous abnormal characteristics including loss of differentiated phenotypes, uncontrolled cell proliferation, and escape from apoptosis, all of which are initiated and maintained by increased mutation rates and genome instability [1]. Mutations in DNA arise from multiple mechanisms including replication errors, spontaneous decay of DNA, or damage created by endogenous reactive metabolites or environmental sources. The

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continuous accumulation of mutated genes, which leads to turning ‘ON’ or ‘OFF’ of crucial regulatory genes, creates pools of heterogeneous cancer cells possessing selective advantages for survival, duplication, and metastasis [2]. As a means to counteract carcinogenesis, a complex and interactive cellular response to damaged DNA, the DNA Damage Response (DDR), detects DNA lesions and initiates signal transduction cascades that lead to ‘protective’ cell cycle arrests at various positions in the cell cycle along with accurate DNA repair [3–6]. The importance of the DDR in genome maintenance is underscored by the elevated risk of cancer in patients harboring inactivating mutations in genes belonging to these pathways. Hereditary forms of cancer originating in the colon, breast, ovary, and skin are definitively linked to mutations in mismatch repair (MLH1), homologous recombination (BRCA1 and BRCA2), and nucleotide excision repair (Xeroderma Pigmentosum proteins) [7, 8]. Thus, a deficiency in a single gene can establish the conditions for carcinogenesis, by negatively impacting the fidelity of DNA repair.

The ability to duplicate the entire genome in an error-free manner is vital for limiting the risk for cancer. High fidelity nature of genome duplication is largely accredited to the proofreading capabilities of the DNA polymerase δ and ϵ , which ensure that a correct nucleotide is incorporated at each step. However, the properties of high fidelity and processivity can become limiting when a DNA polymerase encounters an altered template due to covalent adducts and/or distortions in the secondary structure of DNA. DNA polymerase δ and ϵ stall cannot accommodate bulky lesions or deformed structures within the template and will stall, ultimately leading to replication fork regression and collapse posing a risk for chromosomal translocations and aberrations. Thus, a second response to damaged DNA, termed DNA damage tolerance (DDT), has evolved to promote replication through and beyond an altered template. This highly conserved process, known as translesion DNA synthesis (TLS), invokes the activities of specialized DNA polymerases (TLS polymerases) that can accommodate lesions within template DNA, but in a potentially error-prone manner due to the fact that incorporating nucleotides opposite non-coding bases in the template is inherently mutagenic [9–12]. For these reasons, the DDT pathway is tightly regulated to prevent erroneous TLS during genome duplication. Despite the error-prone nature of the DDT pathway, the ability to replicate through polymerase blocking lesions is important for maintaining genome stability by lowering the overall risk replication fork stalling and degradation. In this review, we will describe the specialized functions of Y family TLS polymerases and DNA polymerase ζ in both mutagenesis and promotion of genome stability, the latter due to recently described roles in DNA repair.

2. TLS polymerases – tolerant enzymes that can use a damaged template to copy DNA

Exposure to ultraviolet (UV) light creates cyclobutane pyrimidine dimers and pyrimidine(6–4)pyrimidone photoproducts in DNA, both of which impose a substantial block to normal replicative DNA polymerases. Early studies in UV-treated prokaryotes brought forth the idea that mechanisms exist that fill in daughter strand gaps left behind after incomplete replication of UV-damaged DNA [13]. In other words, cells possess a unique ability to ‘repair’ gaps left behind where replication could not be completed due to the presence of polymerase blocking lesions (hence the term ‘postreplication repair’). DNA damage tolerance became linked with the active process of mutagenesis after the identification of mutant bacteria and yeast exhibiting non-mutable or ‘reversionless’ phenotypes post-UV treatment [14, 15]. The *rev1*, *rev3*, and *rev7* (for “defective mutation reversion”) genes were discovered using genetic screens that searched for mutants that are hypomutable in response to UV [15, 16]. Elimination of either *Rev* gene resulted in the same hypomutagenic phenotype suggesting they worked together to influence the rate of mutation. Even background mutation rates were reduced in the absence of *rev1*, *rev3*, or *rev7* gene function.

During the same period, human cell lines derived from patients with the variant form of Xeroderma Pigmentosum (XP-V), a hereditary disorder associated with sensitivity to sunlight and high incidence of skin cancer, were characterized as being deficient in filling in gaps in DNA left behind after replication of DNA damaged by UV light [17]. These findings implied that similar pathways exist in human cells and can influence the process of carcinogenesis, in addition to the development of human disease.

2.1 Rev1 and DNA polymerase ζ

Cloning and characterization of Rev3 revealed that mutagenesis is due to the activities of a DNA polymerase sharing sequence similarities within the catalytic domain within Pol δ , Pol ϵ and Pol α , all members of the B family of DNA polymerases [18–22]. Rev7 and Rev3 were later shown to form a DNA polymerase complex named Pol ζ [23]. Pol ζ possesses lower processivity and is devoid of the 3' $\rightarrow$ 5' proofreading exonuclease activity present in most B-family DNA polymerases [23]. Although, Rev3 alone is capable of polymerization, association of Rev3 with Rev7 has been shown to stabilize and significantly enhance the polymerase activity of Rev3 by 20–30 fold, suggesting that Rev7 functions as a processivity factor for Pol ζ [23]. Recently, the two accessory subunits (Pol31 and Pol32) which associate with the catalytic subunit (Pol3) of yeast Pol δ , were also identified in a complex with Pol ζ and the presence of Pol31 and Pol32 is needed for efficient TLS in yeast [24, 25][CC1]. Thus, Pol ζ is now considered to be a four subunit polymerase. These recent findings suggest that accessory subunit 'swapping' between Pol δ and ζ may facilitate the exchange of DNA polymerases during the transition from normal replication to TLS [26].

Compared to other TLS polymerases, Pol ζ is not very efficient in replicating through most DNA lesions, the exception being certain adducts like thymine glycol, where it has been reported to perform both the insertion and extension steps opposite the thymine glycol lesion in an error-free manner [27]. Rather than inserting nucleotides opposite DNA adducts, Pol ζ appears to be particularly specialized to extend distorted base pairs, such as mismatches that might result from an inaccurate base insertion by a TLS polymerase or a base pair involving a bulky DNA lesion [28, 29]. Therefore, the primary role of Pol ζ in TLS is performing extension beyond nucleotides inserted opposite the lesion by another TLS polymerase [30]. In addition, Pol ζ has recently been shown to be essential for bypassing ribonucleotides misincorporated into DNA and promotes tolerance to the presence of these toxic lesions [31]. The overall proficiency in extending insertions opposite mismatches and DNA adducts with a relatively high error rate for base substitutions is associated with the mutagenic properties of Pol ζ [32, 33]. Mammalian Rev3 (encoded by the *Rev3l* gene) is twice as large as the yeast protein, expressed at extremely low levels, and has yet to be characterized at the biochemical level [19, 20, 22]. Yeast can grow and thrive without Rev3 activity and suffer some survival disadvantage when cells are exposed to genotoxic stress. This is in direct contrast to mice where knocking out *Rev3l* leads to embryonic lethality as well as genome instability [34–39]. Embryonic cells, lacking Rev3 show high levels of apoptosis and display elevated numbers of spontaneous chromosomal aberrations and translocations. The embryonic lethality observed in *Rev3*^{-/-} mice strongly suggests that at least during early development, loss of Pol ζ activity cannot be compensated for by other TLS polymerases. The structural domains of mammalian Rev1, Rev3, and Rev7 are detailed in Figure 1.

Yeast and mammalian Rev1 (*Rev1l*) are related to the *E. coli* UmuC protein and not considered processive DNA polymerases. Instead Rev1 is a deoxycytidyl (dCMP) transferase, whose activity appears to be restricted to inserting dCMPs opposite guanines and abasic sites in template DNA [40–46]. Additional insights made from the crystal structure of the polymerase domain of *S. cerevisiae* Rev1 bound to a primer template and an incoming dCTP revealed that Rev1 uses a novel means of catalysis whereby the incoming dCTP pairs with an arginine within the active site and the template base is flipped out of the

catalytic site [47]. This limited dCMP transferase activity does not seem to be important for most functions of Rev1 since catalytic inactive Rev1 can still promote Rev3-dependent mutagenesis in yeast [48, 49]. Instead, Rev1 appears to play a structural role in regulating Pol ζ -dependent TLS.

2.2 DNA polymerase eta

The gene responsible for XP-V (also referred to as *RAD30A* or *POLN*) turned out to be a DNA polymerase that replicates DNA templates containing thymine dimers efficiently and with high fidelity and has been named DNA polymerase eta (Pol η) [50–53]. In fact, Pol η appears to be specifically designed for accurate replication of these lesions [54, 55]. In the absence of Pol η , cells replicate DNA poorly following UV irradiation and are UV hypermutable emanating from the loss of error-free Pol η activity past UV photoadducts [17, 50, 56–61]. In this scenario, TLS is presumably carried out by more error prone polymerases, as has been observed in yeast where deletion of *rad30* reveals the well-characterized mutator phenotype dependent upon *rev1*, *rev3*, and *rev7* [48]. The increase in error-prone TLS in XP-V patients most likely contributes to carcinogenesis of the skin. Consistent with these observations, REV1, REV3, and REV7 have all been associated with UV-induced mutagenesis in human cells [41, 62–65].

2.3 DNA polymerase iota

It is unclear whether REV1 and REV3 are solely responsible for UV-induced hypermutability observed in XP-V patient cells since vertebrates express two additional Y-family TLS polymerases, Pol ι and Pol κ . Pol ι is a paralog of Pol η , and thus named RAD30B, and has been characterized as the most error-prone polymerase within the Y family, incorporating dGMP opposite thymine 10 times more frequently than dAMP, but accurately incorporating dTMP opposite adenine [29, 66, 67]. This unique property is explained by the fact that Pol ι utilizes Hoogsteen base pairing for efficient and correct dNMP incorporation opposite altered purines, such as 8-oxoguanine, which would otherwise be complementary for dA or dC [68, 69]. The unique structure of Pol ι restricts the template 8-oxoG in the syn conformation thereby promoting the correct insertion of dCMP [70]. On this note, Pol ι may play a specialized role in the tolerance of oxidative damage for which 8-oxoG is a major product of oxidative DNA damage by chemicals such as hydrogen peroxide [71]. Unlike Pol η , Pol ι is capable of inserting the correct nucleotide opposite highly distorting (6–4) photoproducts, but does not have the capacity to extend beyond the initial insertion [28, 29]. Other TLS polymerases are required to extend beyond the first insertion opposite this adduct (e.g. Rev3 and Pol κ). In the absence of Pol η , a significant amount of UV-induced mutagenesis has been attributed to Pol ι activity and may therefore also contribute to carcinogenesis in XP-V patients [72–76].

2.4 DNA polymerase kappa

Pol κ is the orthologue of the *E. coli dinB* (DNA Pol IV) in higher eukaryotes [77–79]. Pol κ is specialized in performing error-free bypass of bulky minor groove *N*²-deoxyguanine adducts among other lesions, including mispaired primers, and plays a critical role in limiting mutagenesis from specific bulky lesions such as benzo(a)pyrene adducts, an environmental carcinogen present in tobacco smoke [80–88]. In the absence of Pol κ , mice exhibit elevated mutation frequencies suggesting that Pol κ is needed to execute error-free lesion bypass of DNA damaged by reactive metabolites that form bulky adducts [89]. On the other hand, Pol κ is highly error-prone when replicating a normal template and therefore can be a notable source for mutagenesis in mammalian cells [77, 86, 90, 91]. The crystal structure of the ternary complex of the Pol κ catalytic core with DNA and an incoming nucleotide reveals that the DNA is completely encircled by the polymerase, with an N-clasp augmenting the thermodynamic stability of the catalytic complex. This locked structure

leads to increased polymerase residence on the template, thus allowing the efficient extension of distorted primer termini [92, 93]. Pol κ is also involved in nucleotide excision repair (NER) after UV irradiation, especially under conditions of deoxynucleotide imbalances, highlighting recent observations that TLS polymerases can be recruited to facilitate actual DNA repair processes [94, 95].

3. Regulation of TLS – ‘polymerase switching’

3.1 PCNA ubiquitination

In *S. cerevisiae*, genetic studies have shown that genes belonging to the *rad6* epistasis group are involved in the replication of damaged DNA, a process termed postreplication repair [96–98]. Of these, *rad6* and *rad18* are required for both error-free and error-prone replicative bypass of UV-induced DNA lesions, while *rad5*, *mms2*, and *ubc13* are important for regulating error-free lesion bypass using a TLS polymerase-independent mechanism that involves template switching [99]. Rad6 and the Mms2-Ubc13 complex function as E2 ubiquitin-conjugating enzymes in association with the Rad18 and Rad5 E3 ubiquitin ligases respectively, and regulate the ubiquitination state of the homotrimeric sliding clamp, proliferating cell nuclear antigen (PCNA) [96, 97, 100]. Replication fork stalling uncouples the coordinated efforts of DNA polymerase and DNA helicase resulting in the generation of long stretches of single-stranded DNA (ssDNA) [101, 102]. Replication factor A (RPA) rapidly binds to ssDNA and serves as a recruitment factor for Rad18 and other DNA damage response proteins such as ATR and ATRIP [103, 104]. Current models suggest that Rad6 and Rad18 are recruited to these regions via RPA to monoubiquitinate PCNA on lysine-164 (K164) triggering TLS (Figure 2). The first ubiquitin moiety conjugated to PCNA on K164 can then be further extended by Mms2–Ubc13 and Rad5 forming a poly-K63-linked ubiquitin chain which signals the error-free template switching mechanism [100, 105–107]. However, this view has recently been challenged with new data suggesting that the human homologue of Rad5, HTPF, transfers polyubiquitin chains to RAD6, permitting RAD18 to attach polyubiquitin K-63-linked ubiquitin chains to PCNA [108]. Thus, RAD18 may directly control both states of PCNA ubiquitination. Regardless of the precise mechanism of ubiquitin chain elongation on PCNA, it is generally believed that monoubiquitination of PCNA mediates the switch to TLS, whereas polyubiquitination of PCNA channels the postreplication repair pathway into an uncharacterized error-free damage avoidance pathway that involves template switching [9].

One potential outcome of attaching a single ubiquitin moiety to PCNA is mutagenic lesion bypass by Rev1 and Pol ζ . It is important to note here that Rev1 and Pol ζ -dependent TLS not only occurs during replication, but also after DNA replication has completed, being responsible for filling in gaps left behind after stalled replication forks resume DNA synthesis downstream of a replication-blocking lesion [109–113]. Hence, significant TLS activity occurs in G2 after replication has completed. Alternatively, Pol η can accurately insert nucleotides opposite thymine dimers and reduce UV-induced mutagenesis, and hence Pol η 's activity is often referred to as being responsible for error-free lesion bypass within the postreplication repair pathway, at least in the more simplified model described for yeast.

All eukaryotic Y-family polymerases possess ubiquitin binding motifs (UBM) or ubiquitin-binding zinc finger (UBZ) domains which increase their affinity for ubiquitinated PCNA [114–119]. Any DNA adduct that poses a block to replication induces the formation of TLS polymerase-containing foci, and this event is driven by PCNA monoubiquitination [120–128]. It is unclear why such a high concentration of TLS polymerases is needed within the region surrounding stalled replication forks such that they form visible foci via immunofluorescence microscopy. Polymerase foci colocalize with PCNA and γ H2AX, a

marker of DNA damage and replication stress, implying these are regions of chromatin undergoing postranslational modifications by both the DDR and DDT pathways [127, 129].

Although ubiquitination of PCNA is absolutely necessary for TLS in yeast, recent studies suggest that alternative pathways may regulate at least some polymerase switching in vertebrates. Analysis of the replication of damaged DNA in chicken DT40 cells demonstrated a predominant role for PCNA ubiquitination only in promoting the filling in of post-replication gaps rather than TLS occurring at a stalled replication fork [130]. This latter activity requires Rev1 and its ability to contact ubiquitin and other TLS polymerases (see section 3.2). In addition, Rev1-dependent TLS across a pyrimidine 6–4 photoproduct in DT40 cells carrying a PCNA K164 mutation appears to be normal as measured by a plasmid system [131]. Recent work from the Livneh group utilized mouse embryonic fibroblasts in which specific TLS genes associated with ubiquitination of PCNA were manipulated. These studies showed that eliminating expression of Rev3, Pol η or Rev1 in PCNA^{K164R/K164R} mouse embryo fibroblasts further increased their sensitivity to UV-radiation indicating the existence of a TLS pathway that is independent of PCNA ubiquitination [132]. At least in DT40 cells, this non-canonical TLS pathway appears to be largely dependent on Rev1 [130, 131]. Recent studies have identified the Fanconi anemia core complex as also being an important regulator of Rev1 localization to stalled replication forks resulting from UV or cisplatin treatment [127, 133, 134].

As with all posttranslational modifications, the presence of ubiquitinated PCNA is tightly regulated by both its addition and removal. The deubiquitinase (DUB), ubiquitin specific protease 1 (USP1), is a key regulator of this pathway by maintaining PCNA in a ubiquitin-free form [135]. USP1 protein levels are reduced following UV exposure thereby shifting the balance towards PCNA ubiquitination by Rad18 and Rad6. The mechanism for this decrease in response to UV involves an autocleavage event followed by proteasomal degradation of the cleaved products. However, this appears to be a unique response to UV damage and not other genotoxic agents. Studies conducted in cells lacking USP1 reveal that these cells exhibit increased TLS activity as well as increased mutation rates due to erroneous TLS [132, 135]. Two recent studies revealed that a major function of USP1 and its partner UAF1 is to suppress sporadic monoubiquitination of PCNA during normal DNA replication [136, 137]. Reduced replication fork speed and genomic instability observed in USP1-deficient cells has been attributed to increased levels of PCNA monoubiquitination and hyperaccumulation of Pol κ to replication forks [138]. *USP1*^{-/-} mice also display increases in PCNA monoubiquitylation and exhibit high rates of perinatal lethality, depletion of male germ cells, infertility, hypersensitivity to the crosslinking agent mitomycin C, and chromosome instability [139]. Thus, inappropriate recruitment of TLS polymerases can interfere with the maintenance of genome stability during DNA replication.

Other factors have been found to regulate the state of PCNA ubiquitination. Elg1 comprises an alternative Replication Factor C (RFC) complex when substituted for the RFC1 subunit. Human ELG1 (which is also called ATAD5) interacts with PCNA, colocalizes with the sliding clamp at stalled replication forks, and also associates with the USP1-UAF1 complex which removes ubiquitin moieties from PCNA [137]. Similar to USP1-depleted cells, cells with reduced ELG1 function display hyper-PCNA monoubiquitylation, elevated mutation rates, and chromosomal instability. [137, 140]. Furthermore, deletion of Elg1 in mice leads to chromosomal instability and a strong predisposition to cancer [141]. These results indicate that inappropriate recruitment of TLS polymerases to replication forks can have detrimental consequences to genome stability and therefore, their activities must be tightly regulated.

3.2 Rev1 and Polζ: completing TLS across many DNA lesions

Today, it is clear that for most DNA adducts, more than one TLS polymerase is needed to accomplish a single lesion bypass event [30, 127, 142]. Current models suggest a two step mechanism where a Y family polymerase inserts the first nucleotide opposite a damaged base and a second polymerase (notably Polζ) performs the extension step, even if the resulting primer/template pair is highly distorted (Figure 2). Rev1 is thought to be an essential regulator of TLS polymerase switching between the first TLS polymerase and Polζ [143, 144]. Rev1 has several protein-protein interactions domains that facilitate its function as a TLS polymerase scaffold protein. Rev1 is the only Y family polymerase that contains an N-terminal BRCA1 C-terminal homology (BRCT) domain which is capable of making direct contact with PCNA and DNA, the latter with a preference towards 5'-phosphorylated primer/template junctions that mimic a postreplication gap in DNA [145, 146]. Important to its overall function, Rev1 possesses a unique TLS polymerase interacting domain that makes direct contact with Rev7 of Polζ and a variety of TLS DNA polymerases including Polη and Rev3 [128, 143, 144, 147–150]. Disruption of the polymerase-interaction domain essentially eliminates Rev1-mediated mutagenesis [151, 152]. A Rev1-interacting region (RIR) has been identified in all Y family polymerases (Polη, Polι and Polκ) and is required for efficient binding to the c-terminus of Rev1. This motif is defined by short sequences in which the presence of two consecutive phenylalanine (F) residues is critical [153–155]. The ability to interact with Rev1 via the RIR motif is necessary for Polκ function in mouse embryonic fibroblasts [153].

We are now beginning to understand how mammalian Rev1 can interact with Polζ and other Y family polymerases simultaneously to regulate multi-polymerase TLS. The similarity of phenotypes in cells depleted of either Rev1 or Rev3 has strongly suggested direct interactions between Rev1 and Polζ to form a functional complex [127, 156–160]. At least in yeast, protein interactions with the c-terminal domain of Rev1 appear to be limited to Polζ via the Rev7 subunit [143, 150]. Recently, the crystal structure between human REV7 and the REV7 binding site on REV3 (amino acids 1847–1898) has been solved [161]. Based on this data, Hara *et al* suggest that REV7 binds both REV3 and REV1 simultaneously where binding of REV7 to REV3 alters the conformation of REV7 in a way that reveals an additional REV1 binding site [161]. This is an intriguing result since it has always been assumed that REV7 interactions with REV1 and REV3 were mutually exclusive. Over the past year, several structural and biochemical analyses of protein fragments belonging to REV1, REV7, plus the RIR from different Y family polymerases now indicate that the REV1 C-terminal domain can simultaneously contact both insertion and extension TLS polymerases [154, 155, 162, 163]. Future studies will be needed to fully disclose how REV1 performs its function as a TLS polymerase ‘switcher’ during complex lesion bypass events.

4. TLS polymerases directly implicated in DNA repair and genome maintenance

4.1 Rev1 and Polζ promote resistance to many forms of DNA damage

Deletion of *Rev7*, *Rev3* or *Rev1* in chicken DT40 cells or mammalian cells leads to remarkable hypersensitivity to a wide variety of genotoxic stresses including UV, methyl-methanesulfonate (MMS), DNA crosslinking agents, and DNA double-strand breaks (DSBs) [35, 127, 156, 157, 159, 164–170]. This has led to the idea that Rev1 and Polζ may have additional roles in promoting survival, genome stability, and DNA repair that is not compensated for by other TLS polymerases and beyond the conical postreplication repair pathway. An open question is whether lack of Polζ-dependent extension beyond most if not all DNA adducts explains this profound hypersensitivity to such a variety of DNA lesions, especially ionizing radiation (IR), where one would presume most damage is the result of

ssDNA breaks which are rapidly repaired. Increased radiosensitivity, chromosomal aberrations, and slow resolution of γ -H2AX foci after IR suggest insufficient or slow repair of DSBs, as has been observed in irradiated nasopharyngeal carcinoma cells expressing shRNA specific for REV7 (MAD2B) as well as other tumor cell lines cells depleted of REV1, REV3 or REV7 [156, 164, 171]. Recently, direct evidence has been obtained demonstrating that Pol ζ plays a direct role in executing homologous recombination repair, potentially explaining this extreme hypersensitivity to DSBs and replication stress induced by various genotoxic agents [156, 169].

4.2 Are Rev1 and Pol ζ part of the fanconi anemia pathway?

Fanconi anemia (FA), first described in 1927 by the Swiss pediatrician Guido Fanconi, is a genome instability syndrome characterized by bone marrow failure, developmental abnormalities and an increased incidence of developing cancers [172, 173]. At the cellular level, FA cells display increases in chromosomal aberrations, particularly of the type referred to as radial chromosomes, and exhibit extreme hypersensitivity to DNA interstrand crosslink (ICL) inducing agents, such as mitomycin C, cisplatin and diepoxybutane [174–176]. At least 15 FA or FA-interacting genes have been identified and these ‘FA’ proteins have been shown to form several complexes that regulate or perform ICL repair [134, 172, 177]. Eight of these proteins (FANCA, -B, -C, -E, -F, -G, -L and -M) along with five FA-associated proteins (FAAP100, FAAP24, HES1, MHF1, MHF2 and the newly identified FAAP20) form the nuclear core complex [173]. The core complex functions as an E3 ubiquitin ligase by monoubiquitinating the FANCD2-FANCI heterodimer in response to DNA damage (Figure 3) [178–181]. Monoubiquitination takes place constitutively in S-phase and is dramatically increased upon DNA damage [181, 182]. In response to DNA damage, the monoubiquitinated FANCD2-I complex is recruited to chromatin where it interacts with downstream FA proteins (FANCD1/BRCA2, FANCI/BACH1, FANCN/PALB2, FANCO/RAD51C, FANCP/SLX4, and FA-associated nuclease 1 (FAN1)), all of which are involved in promoting homologous recombination repair [183–192]. Monoubiquitinated FANCD2 provides binding sites for SLX4, a protein scaffold that binds multiple structure-specific endonucleases including the FA-associated nuclease, FAN1. This complex is believed to unhook an ICL, participate in HR repair of the resulting replication associated break, and potentially facilitate TLS across the unhooked crosslink [191, 193–195]. Deficiencies in any of these proteins results in the characteristic deficiency in DSB resolution following exposure to DNA crosslinking agents, as well as reductions in gene conversion efficiencies, identical to what has been observed in REV1 or Pol ζ -depleted cells [127, 156].

Ketan Patel first demonstrated an epistatic relationship between the FANCC gene, which encodes one of the fanconi anemia core proteins, and Rev1 or Rev3 in chicken DT40 cells treated with cisplatin, thus directly implicating Rev1 and Rev3 in ICL repair [160]. The authors found little difference in the sensitivities of FANCC^{-/-} DT40 cells compared to FANCC^{-/-}/Rev1^{-/-} cells or FANCC^{-/-}/Rev3^{-/-} cells to loss in viability or the accumulation of chromosomal aberrations following cisplatin treatment, thus establishing an epistatic relationship between these genes. Sonoda’s laboratory confirmed these important observations [166]. Direct evidence implicating the involvement of the FA pathway and Pol ζ in ICL repair was obtained using the *Xenopus leavis* egg extract system, wherein it was shown FANCD2-FANCI ubiquitination promotes both the incision and TLS steps of ICL repair [196, 197]. This work led to the proposal that like PCNA ubiquitination, FANCD2-I ubiquitination plays a role in recruiting TLS polymerases to ICLs via their ubiquitin-binding domains. However to date, no evidence exists suggesting a direct interaction between FANCD2 and Rev1. Regardless, these studies brought forth the most current model for ICL

repair that involves the cooperation between the FA pathway, translesion DNA synthesis by Rev1 and Pol ζ , and homologous recombination (Figure 3).

Early studies into the molecular defects of FA patient cell lines discovered that these cells are hypomutagenic in response to UV, a phenotype very similar to Rev1 and Rev3-deficient cells [198]. Similarly, *FANCG*^{-/-} hamster CHO cells show reduced generation of viable HPRT mutations, thereby pointing towards a defect in TLS at the *HPRT* locus during DNA replication [199]. Also, *FANCC*-deleted DT40 cells have a lower frequency of spontaneous point mutations, suggesting that an intact Fanconi anemia pathway is required for Rev1-mediated error prone TLS [160]. Overall, these results suggest that Rev1 and Rev3 are regulated by the FA pathway in executing lesion bypass in two different scenarios: bypass of UV-generated photoproducts or cisplatin intrastrand crosslinks and repair of ICLs. Of importance, there appears to be specific differences in the requirement of FA proteins for TLS in response to replication blockade versus ICL repair. The recruitment of Rev1 into nuclear foci post UV and cisplatin treatment requires an intact Fanconi anemia core complex but is independent of FANCD2 monoubiquitination, thereby suggesting that FA core complex alone regulates TLS in response to replication fork stalling by UV or cisplatin [127, 133]. Accordingly, FA core-deficient patient cells are not as efficient in generating spontaneous and UV-induced point mutations as cDNA-corrected cells [133]. FAAP20 was recently identified by several studies to be an integral part of the FA core complex which plays a role in maintaining the integrity of the core complex, and promotes FANCD2 monoubiquitination [134, 200, 201]. Recent work from the D'Andrea group discovered that FAAP20 directly binds to FANCA and contains a c-terminal UBZ4 domain that associates with ubiquitinated Rev1, thereby providing a physical link between Rev1 and the FA pathways [134]. Together, these studies suggest that FAAP20 specifically promotes Rev1-dependent TLS across replication stalling lesions like thymine dimers and bulky cisplatin adducts and may also directly promote Rev1-dependent TLS during ICL repair.

The identification of BRCA2 and RAD51C mutations causing fanconi anemia-like syndromes provided a direct link between the FA pathway and regulation of homologous recombination repair [183, 190]. Other members of the FA pathway have also been implicated in regulating or participating in HR repair [160, 178, 202–206]. Deficiencies in the FA pathway are typically associated with partial defects in HR repair, the exception being BRCA2 deficiency since this protein is essential for RAD51 loading. We have also discovered that depletion of REV1, REV3, or REV7 in human cells is associated with a reduction in HR efficiency by approximately 50%, similar to FA cells, as opposed to 90–95% seen in cells deficient in the RAD51 protein [156]. This same reduction in HR repair is observed in cells depleted of another DNA polymerase implicated in ICL repair and HR, Pol ν , and no additive reductions in gene conversion efficiencies are observed when FANCD2 is co-depleted along with Pol ν , suggesting that Pol ν participates in the same FA pathway regulating HR repair [207]. Since these studies are measuring repair of a site specific DSB by HR, they imply that alternative DNA polymerases may be needed to perform a subset of these reactions and are not confined to preparing the sister chromatid for HR repair after ICL unhooking. This idea has been recently confirmed in genetic studies using *Drosophila melanogaster* as a model system, demonstrating a specific requirement for Pol ζ during HR repair [169]. Together, these observations suggest that Rev1 and Pol ζ (possibly in collaboration with Pol ν) may be required to synthesize DNA during a subset of HR reactions that involve extension of distorted or mispaired primer templates that would otherwise cause stalling of normal DNA polymerases. It is of interest to note that the RAD51D gene has been linked to mutation rates in mammalian cells thus HR repair may not always 'error-free' [208]. At least in mammalian cells, the Fanconi anemia pathway may be important for regulating TLS during HR repair and rescues problematic HR templates by recruiting the TLS pathway (Figure 3).

5. Deregulated TLS and cancer

5.1 Lack of TLS activity → genome instability

Pol η is the only known TLS polymerase that is directly linked to preventing cancer in humans. Inactivating mutations in Pol η have always been thought to result in the substitution of alternative error prone polymerases that leads to a constant state of hypermutability upon exposure to sunlight. It is interesting to note that one of the emerging hallmark phenotypes of cancer cells is increased hypermutability [209–216]. However, cancer progression in XP-V patients may be more complex than just more mutations resulting in more risk for carcinogenesis. At the cellular level, the absence of Pol η leads to enhanced genome instability, especially when p53 function is disrupted - a frequent occurrence during the progression of skin cancer [217–219]. The fact that increases in DNA double stranded breaks marked by DNA damage response proteins like MRE11 occur after exposure to UV irradiation, suggests that replication forks collapse more frequently in XP-V cells. The inability to rapidly engage in TLS following replication fork stalling at DNA adducts, fragile sites, repetitive sequences, or secondary structures formed in template DNA can lead to ‘replication stress’, ultimately leading to replication fork breakage (Figure 4) [220–223]. Incorrect repair of replication-associated DSBs is directly implicated in chromosomal translocations and chromosomal aberrations [205, 224–227].

Absence of Pol ζ has been shown to influence tumorigenesis in mouse models [228]. Both thymic lymphomas and mammary gland tumors form more rapidly in a p53 null background. These results indicate that even under conditions of ‘reduced mutagenesis’, absence of Pol ζ creates a state of increased genomic instability [39, 228]. One would have expected that absence of Pol ζ -dependent mutagenesis would reduce the rate of carcinogenesis. Instead, lack of the essential extender polymerase likely leads to greater instability by incapacitating the TLS pathway, thereby influencing the rate of replication fork collapse and DSB formation during S phase. A deficiency in HR repair could also be responsible for increased chromosome instability in cells lacking Pol ζ and the rapid onset of cancer observed in mice [156, 169]. Thus far it has been difficult to correlate a lack of REV1 and Pol ζ to the incidence of cancer in humans. However, recent studies indicate that both the levels of REV1 and REV3 can be modulated by specific miRNAs that may be dysregulated in cancer [229, 230].

5.2 Too much TLS activity → genome instability

The ‘overuse’ of TLS polymerases during DNA replication can clearly promote carcinogenesis due to their error prone nature on a non-damaged template (Figure 4). Multiple studies have searched for unusual DNA polymerase expression patterns in cancer cell lines and patient tumor samples, as well as, characterized the consequences of polymerase overexpression on genome stability [231–241]. Thus far, studies have focused on identifying differences in mRNA levels or the presence of variant alleles when comparing normal and cancer tissue. It is not surprising that there is little evidence thus far indicating that TLS polymerases are grossly up- or downregulated in cancer cells. Large changes in expression levels may be incompatible with cell survival and hence selected against [242, 243]. Therefore, subtle modifications to DDT signaling must also be considered when attempting to identify events that may alter TLS polymerase activity in cancer cells [137, 138, 244–246]. Any change in the steady state level of PCNA ubiquitination can significantly affect the fidelity of DNA replication and upset the balance between normal versus TLS polymerase activity. On the other hand, reduced levels of TLS activity would in turn lead to higher incidences of replication-associated DSBs or frank deficiencies in DNA repair, such as when lacking a specific polymerase like Pol ζ . The

damage tolerance pathway is in all likelihood much more delicately balanced than currently realized.

6. Conclusion

The past two decades have seen extraordinary advances in our understanding of how mutations may occur and potentiate carcinogenesis. Some of the same polymerases that have been characterized as facilitators of mutagenesis, are now implicated in suppressing tumorigenesis by maintaining genomic stability (e.g. Pol ζ). Recent evidence suggests that cancer cells may be particularly sensitive to inhibition of the DDR pathway that has evolved to promote replication fork stability [247]. Interfering with the ATR-Chk1 kinase pathway has been shown to be selectively toxic in cancer cells that have become dependent upon this pathway to cope with constant replication stress [248]. If it holds true, that cancer cells are more dependent upon Pol ζ for growth and survival (and perhaps HR repair) as suggested by Knobel et al [243], we anticipate that a potential new Achilles heel may have been identified. Suppressing the expression of TLS polymerases, such as REV1 and Pol ζ , has been shown to limit mutagenesis and sensitize tumor cells to cancer therapy suggesting that it may be fruitful to explore this avenue for drug development [167, 249–252]. Blocking the TLS pathway to sensitize tumor cells to conventional treatment is rational, and may be even more effective when combined with an inhibitor of Chk1 or ATR. Of course, understanding the implications for inhibiting TLS polymerases on overall genome maintenance in normal somatic cells is paramount before such an undertaking can be considered.

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Figure 1. The structural domains of Pol ζ (comprised of REV3 and REV7) and the Y family of DNA polymerases

BRCT, BRCA1 C-terminus like domain; PIP, PCNA interacting peptide; PAD, polymerase-associated domain; UBZ, ubiquitin-binding Zinc finger domain; UBM, ubiquitin-binding motif; RIR, REV1-interacting region; PID, C-terminal polymerase-interacting domain; HORMA; HOP1, REV7 and MAD2.

Figure 2. A model for the regulation of the TLS pathway

Replication forks may inevitably encounter DNA adducts that are not repaired (such as a 6–4 photoproduct). These lesions block high fidelity polymerases, potentially leading to replication fork stalling, gaps in replicated DNA, and the generation of DNA double-strand breaks (DSBs). Current models suggest that replication fork stalling uncouples the replicative helicase from normal high fidelity DNA polymerases resulting in further unwinding of the double helix and the creation of abnormal large stretches of single stranded DNA (ssDNA). These single-stranded, unreplicated regions become coated with the ssDNA binding protein Replication protein A (RPA) which serves to initiate ATR signaling, as well as recruit the RAD18 E3 ubiquitin ligase to trigger the postreplication repair pathway which includes TLS. Together, the E3 ubiquitin ligase RAD18 and the RAD6 E2 ubiquitin conjugating enzyme monoubiquitinate PCNA on Lys-164, the latter a homotrimeric protein that functions as an auxiliary factor for DNA polymerases. This event is thought to operate as a molecular switch from normal DNA replication to the TLS. In this example of the two polymerase model for lesion bypass across a 6–4 photoproduct, Polymerase eta (Polη) inserts a nucleotide directly opposite the lesion and requires an additional TLS polymerase, such as DNA polymerase zeta (Polζ), to extend beyond the insertion. REV1 facilitates the exchange from Polη to Polζ which subsequently performs the extension step beyond the inserted nucleotide opposite the damaged base. Lesion bypass of DNA adducts may be executed with high fidelity or can lead to mutations that promote carcinogenesis. The Fanconi anemia (FA) core complex plays an important role localizing REV1 to stalled replication forks via interaction with the FA core complex-associated FAAP20 protein. The deubiquitinase protein USP1 maintains PCNA in a non-ubiquitinated state thereby minimizing TLS activity in the cell under normal conditions. USP1 proteins levels are reduced following UV exposure thereby shifting the balance towards PCNA ubiquitination by RAD18 and RAD6. ELG1 is an alternative component of the replication factor C complex (which loads PCNA onto DNA) and associates with the USP1-UAF1 complex to down regulate PCNA monoubiquitination and TLS. Potential outcomes of TLS activity or lack of TLS activity are illustrated.

Figure 3. Interactions of the Fanconi anemia (FA) pathway with TLS pathway

High fidelity DNA polymerases stall upon encounter of an interstrand crosslink (ICL) in DNA. This event triggers the Fanconi anemia (FA) pathway, whereby the FA core complex detects an ICL and functions as an E3 ubiquitin ligase to monoubiquitinate the FANCD2-FANCI heterodimer. The monoubiquitinated FANCD2-I complex is recruited to the chromatin where it promotes 'unhooking' of the ICL and further nucleolytic processing of DNA by structure specific endonucleases - MUS81-EME1, SNM1A, FAN1, XPF-ERCC1 and SLX1-SLX4. Replication-associated DSBs are generated during the repair process and become substrates for homologous recombination (HR) to ultimately complete ICL repair. FAAP20 interacts with the FA core complex and binds to monoubiquitinated REV1. FAAP20 may direct REV1 and Polζ to the unhooked crosslink where they perform lesion bypass across the adduct creating a substrate for HR repair. We speculate that REV1 and Polζ are not only involved in TLS across the 'unhooked ICL' in preparation HR repair, but also perform DNA synthesis during HR repair when the template is incompatible for normal replicative DNA polymerases and may still contain unhooked ICL lesions or other

polymerase blocking lesions. Both the FA pathway and REV1/Pol ζ have been implicated in facilitating HR repair of DSBs separate from the ICL repair pathway.

Figure 4. Consequences of TLS polymerase imbalance

A) Overactive or inappropriate Translesion DNA synthesis (TLS) polymerases activity (yellow circle) may lead to error-prone DNA replication, resulting in a hypermutable phenotype, carcinogenesis and chemoresistance. B) Balanced activity between TLS polymerases and high fidelity polymerases (blue circle) leads to normal replication of an undamaged template or appropriate TLS of a damaged template, both routes maintain genomic stability, the latter by preventing replication fork collapse. C) Cells with deficient TLS polymerase activity may not effectively deal with replication stress, leading to an increased frequency of DSBs and chromosome instability, that in turn can result in cell death or cancer.