TLS Polymerases are involved in processing of EXO1-dependent lesions after UV-induced damage

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UV light mainly damages DNA by generating CPDs and 6-4PP photoproducts, which are responsible for the pathological effects of sunlight. In a healthy organism, such DNA helix distorting lesions are removed by Nucleotide Excision Repair (NER), a multistep process**.** Mutations in NER genes cause the onset of severe pathologies. The principal symptom common to all diseases is the strong sensitivity to UV. A high predisposition to tumors development arises in xeroderma pigmentosum (XP) patients, while neurological dysfunctions have been observed in both XP and Cockayne syndrome patients.
Upon DNA damage sensing, checkpoints are activated allowing a block or delay of cell cycle progression to ensure repair of the DNA lesions. Intriguingly, while in normal cells UV irradiation activates DNA damage checkpoints in all phases of the cell cycle NER yeast mutant strains and human fibroblasts derived from XP patients fail activate the checkpoint in G1 and G2.
Recently, we demonstrated that the checkpoint response to UV light in cells that are not actively replicating their genome requires prior processing of the UV lesions. This involves NER factors but also the Exo1 nuclease. In particular, acting on NER intermediates, Exo1 generates structures containing long tracts of ssDNA in response to UV irradiation. This role of Exo1 is only observed at a subset of problematic lesions that cannot properly repaired by canonic NER. It is these Exo1-induced structures that provide the signal for checkpoint activation both in yeast and human non-replicating cells. The essential role of Exo1 in UV-induced checkpoint activation in vivo has been recently supported by in vitro reconstitution of the activation pathway.
What are the problematic lesions that require EXO1 activity is still unknown. We hypothesized that Closely Opposing UV Lesions (COLs) on the two DNA strands could exist and may be a likely candidate. This scenario would require TLS polymerases bypass during repair synthesis step. Therefore, we are investigating Y-family polymerase recruitment at EXO1-positive local UV damage sites (LUDs). We found that Pol h is recruited at both EXO1-positive and EXO1-negative LUDs, while Pol ι and Pol κ always co-localize with the nuclease.
Using the CRISPR-Cas9 system, we generated EXO1 knock out cell lines that demonstrated a requirement for EXO1 in Pol ι and Pol κ recruitment, consistently with our working model.
Finally, when we silenced TLS polymerases we observed a hyper-activation of UV-induced DNA damage checkpoint, suggesting that EXO1 continues to process UV damaged DNA enlarging the gap and eventually producing DSBs. TLS polymerases, thus are crucial to prevent dangerous situations in non-replicating UV irradiated cells.

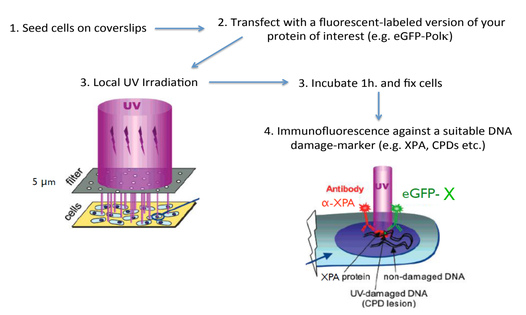

Fig. 1 Local UV Damaged sites (LUD).
Schematic view of LUD detection. We applied this technique to study protein recruitment at UV-damaged sites.
Cells were seeded on coverslip and UV irradiated through an Isopore filter with pores of 5 µm diameter.
One example is showed in this cartoon.

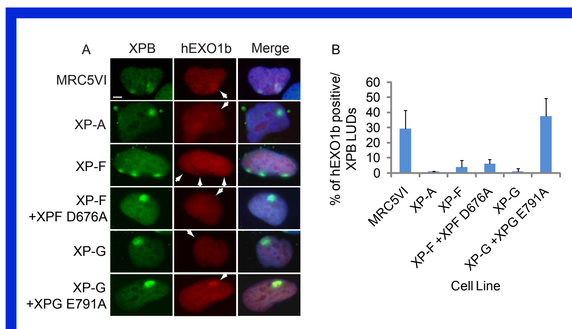

Fig. 2 NER preincision complex and 5′ incision are required for EXO1 accumulation at LUDs.
A) Cells were transfected with EXO1-mCherry, seeded on coverslips, and locally UV-irradiated (40 J/m2). After 1 h of incubation, cells were fixed and processed for immunofluorescence against the indicated protein. White arrows indicate the position of LUDs. (Scale bar=5 µm.) *B*) Quantification of immunofluorescence shown in *A)*.
Modified from *Sertic et al., 2011 PNAS*

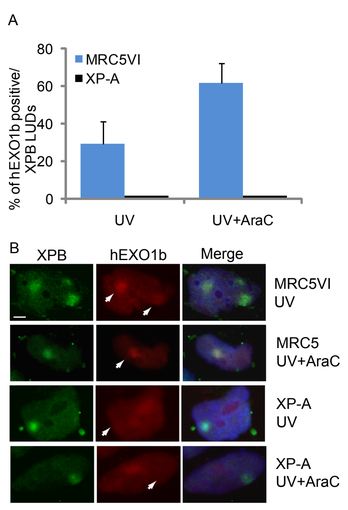

Fig. 3 Accumulation of EXO1 at LUDs is enhanced when repair synthesis is blocked.
MRC5VI and XP-A cells transfected with EXO1-mCherry were exposed to local UV irradiation (40 J/ m2) and incubated for 1 h in the presence or absence of AraC. *A*) Histograms indicating the percentage of XPB-positive LUDs that also contained EXO1, with or without AraC treatment.

B) Representative images of cells with EXO1-positive LUDs (with or without AraC treatment) LUDs are indicated by white arrows. (Scale bar=5 µm.)

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WORKING MODEL

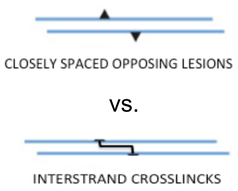

Fig. 4 ICLs and COLs similarities.
We propose that ”difficult” lesions processed by EXO1 exonuclease could be two lesions on the opposite DNA strand in close proximity one to each other, as shown in this schematic view. They structurally resemble ICLs. When repair synthesis starts after removal of the first lesion, it will stop when it encounters lesion of the template strand.
Are TLS polymerases required to proceed?

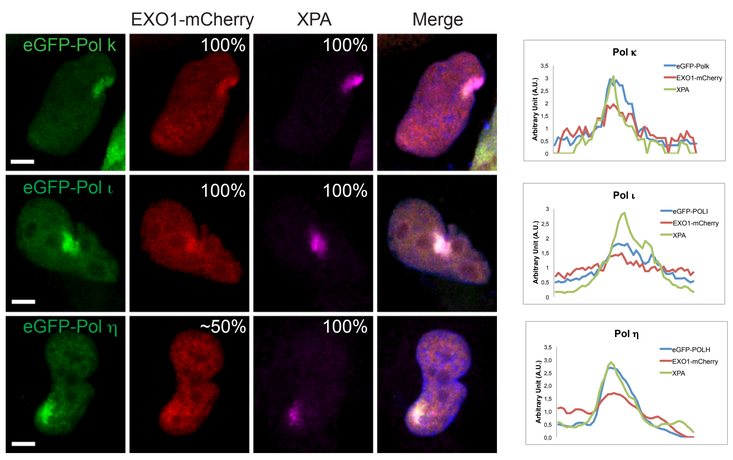

Fig. 5 Y-family TLS polymerases are recruited at the EXO1-dependent lesions.
MRC5VI cells were seeded and transfected with the indicated tagged protein. We started analyzing Pol k recruitment and we observed that all EXO1 positive LUDs were also positive for Pol k in the presence or absence of DNA synthesis inhibitor AraC.
We performed the same experiments transfecting tagged version of Pol ι and η. We observed that ι also localized at EXO1-positive LUD, while η is recruited in both EXO1-poisitive and –negative LUDs.

Therefore, ι, κ and η polymerases work together with EXO1, but with different mechanisms. Iota and kappa seem to act in concert with EXO1, while eta has also other roles.

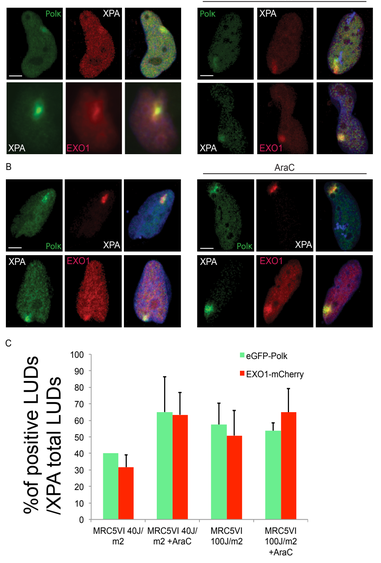

Fig. 6 Pol κ behaves like EXO1 in its recruitment at UV-induced “difficult” lesions.
MRC5VI cells were transiently transfected with the indicated proteins. Cells were locally UV irradiated with 40J/m^2 (A) or 100J/m^2 (B) and treated with AraC where indicated. Immunofluorescence was performed against XPA as a damage marker. Scale bar= 5 µm
C) Quantification of EXO1 and Pol κ positive LUDs at the different UV doses and with or without synthesis inhibitor AraC.

Pol k and EXO1 recruitment is dose dependent and when we inhibit DNA repair synthesis (mimicking “difficult” lesions) their presence at LUDs is increased.

CRISPR-Cas9 Knock Out for EXO1 nuclease!
gRNAs targeting exon 1 of the NG_029100(1q43) gene were selected using the Cas9 Design tool (http://cas9.cbi.pku.edu.cn/index.jsp. gRNA sequences were cloned into lentiCRISPRv2. MRC5VI cells were infected with lentiCRISPRv2, human codon-optimized Cas9-EXO1(+), -EXO1(-) or vector containing gRNA against Luciferase as control. Genomic DNA from clones was further analyzed by GeneArt Genomic Cleavage Detection kit. Targeted regions of positive clones were sub-cloned, extracted from *E.coli* and sequenced to identify biallelic InsDels.

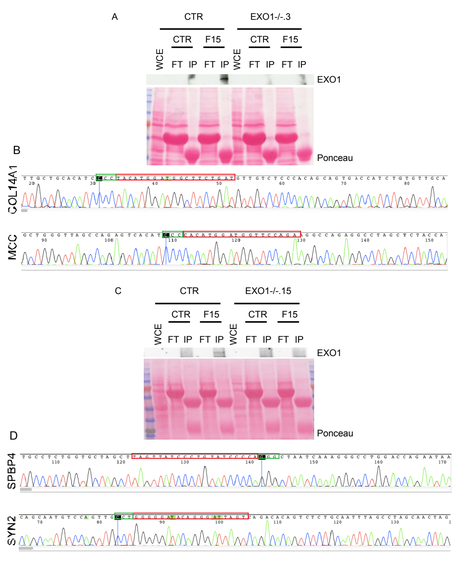

Fig. 7 CRISPR-Cas9 mediated EXO1 knock out.
MRC5VI cells were infected with LentiCRISPRv2 in which we cloned two different gRNAs sequences against EXO1 exon1.
A) and C) panels show immunoprecipitation to verify EXO1 protein absence in EXO1 KO clones.
B) and D) Putative off-target of gRNAs sequences were scored using the algorithm. Sequences were controlled amplifying the region of interest from genomic DNA of positive KO clones and sent for sequencing.

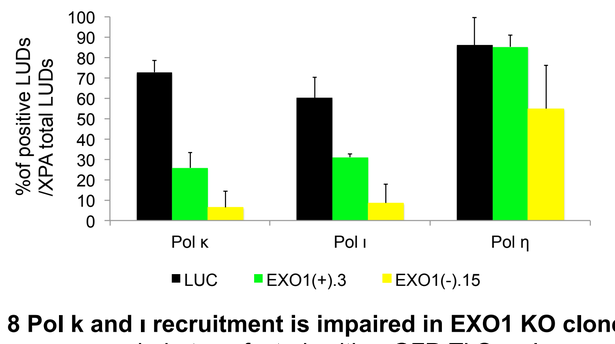

Fig. 8 Pol k and ι recruitment is impaired in EXO1 KO clones.
Cells were seeded, transfected with eGFP-TLS polymerases, Local UV irradiated (100 J/m^2) and fixed after 1 hour. Immunofluorescence was performed against XPA as a marker of NER recruitment and DNA damage. XPA-positive LUDs were analyzed in eGFP channel and scored for TLS polymerases recruitment.

Pol κ and ι, but not η recruitment depends upon EXO1-processing

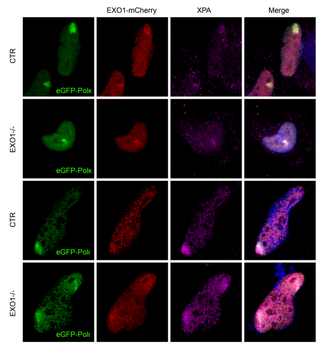

Fig. 9 Y-family Pol κ and ι recruitment is rescued by EXO1 expression.
EXO1 KO clones were seeded and transfected with EXO1-mCherry. The day after, cells were Local UV treated and processed for immunofluorescence.

TLS recruitment is rescued In all the cells transfected with EXO1-mCherry plasmid

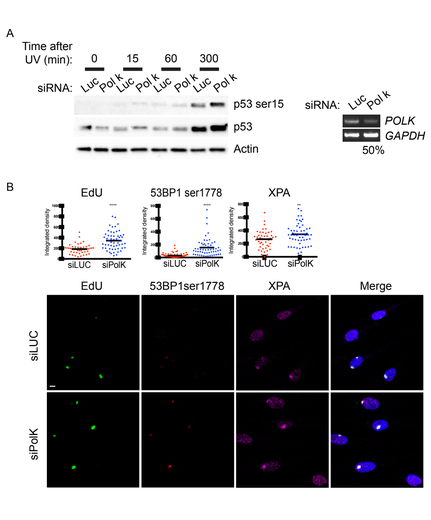

Fig. 10 Checkpoint activation is enhanced in the absence of Pol κ.
A) 48BR primary fibroblasts were silenced for Pol k and serum starved to let them reach a non-proliferating state, UV irradiated with 20 J/m^2 and harvested for western blot analysis. Checkpoint activation at different time points after UV damage was measured by looking at p53 phosphorylation state.
B) 48BR primary fibroblasts were silenced for Pol k and serum starved to let them reach a non-proliferating state. Local UV-irradiated with 100 J/m^2 and fixed after 1h, Immunofluorescence was performed against the indicated proteins.
Graphs represent fluorescence intensity measured for single LUD.

FUTURE PROSPECTIVES:

- **Are other Polymerases recruited at EXO1-positive lesions?**
- **Are Polymerases η and ι involved in quenching of UV-induced checkpoint activation?**
- **Are other polymerases involved in this?**
- **Is the lesion on the template strand UV-induced only, or is it from other origin?**
- **In the absence of TLS refilling of the gap, are some other damages generated?**