

# New Insight on Entangled DNA Repair Pathways: Stable Silenced Human Cells for Unraveling the DDR Jigsaw

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## 1. Introduction

### 1.1 Need for outstanding cell models for studying interwoven DNA repair pathways

During normal human cell growth, each cell is exposed to numerous DNA-damaging events. DNA lesions are mainly inflicted by endogenous insults, such as normal biochemical activities, by-product synthesis and the *in situ* production of reactive oxygen species (ROS). DNA is also subject to genotoxic injuries resulting from diverse exogenous sources. It is not surprising that living organisms have evolved numerous intricate strategies to counteract these environmental pressures and to allow living cells to thrive in aerobic conditions. Through evolution various highly sophisticated pathways for protecting the genetic information have been retained. The first lines of defense include detoxification metabolisms and defense against oxidative stress. When these caretaker processes fail to insure a correct protection of biological molecules, such as genomic and mitochondrial DNA, DNA repair pathways become the ultimate bulwark against DNA damage. However, when DNA damage is not dealt with properly, it can adversely threaten the fidelity of the genetic information and ultimately lead to hereditary diseases or neoplastic processes.

Amongst DNA lesions, DNA double-strand breaks (DSBs) are likely to be the ultimate lethal ones because unrepaired they can lead to chromosomal rearrangements, malignant transformation or apoptosis (Roos & Kaina, 2006). Endogenous DSBs mainly arise from the processing of single-strand breaks (SSBs) when they are converted to DSBs by DNA replication and/or transcription mechanisms (Mladenov & Iliakis, 2011). Given the chemical variety of DNA lesions encountered, evolution has retained a **large diversity** of DNA repair pathways and a tight interplay between DNA replication and DNA repair. While numerous DNA repair mechanisms exist, the major pathways include mismatch, excision and recombinational repair (Hoeijmakers, 2001) and some factors can participate in divergent processes. This is the case of the structure-specific endonuclease ERCC1 / XPF, which is required in two distinct mechanisms: excision (nucleotide excision repair or NER) and recombinational (single-strand annealing or SSA) repair pathways. ERCC1 / XPF endonuclease plays a critical role in NER by being recruited at the site of damaged DNA in order to cleave one strand of the damaged DNA. It is also involved in SSA, which appears to

be an alternative pathway to homologous recombination (HR) or nonhomologous end joining (NHEJ) (Al-Minawi et al., 2008). Moreover, albeit belonging to the same complex, it is hypothesized that ERCC1 and XPF have distinct functions *in vivo* because their deficiency can lead to different phenotypes in humans. For instance, the only patient carrying a mutated *ERCC1* gene exhibits a cerebro-oculo-facio-skeletal syndrome with severe neurological defects but a moderate sensitivity to UV light and mitomycin C, the hallmark of XPF patients (Jaspers et al., 2007). Furthermore, there is a large body of evidence that raises the notion that the failure of one DNA repair pathway could modify the efficiency and/or fidelity of another one. An interesting example is the **cross-talk** between the mismatch repair (MMR) and recombinational pathways. MMR appears to be an essential mechanism for guaranteeing the fidelity of DNA replication because misincorporated nucleotides have to be excised immediately after DNA synthesis. Inherited defects in the MMR trigger a spontaneous mutation rate 50- to 1000-fold higher than that observed in MMR proficient cells, with a tremendous increase of spontaneous base substitution and frameshift mutations (for review (Iyer et al., 2006)). These mutational events could facilitate illegitimate recombination between nearly-homologous sequences, contributing to the onset of hereditary nonpolyposis colon cancer (HNPCC) (for review (Iyer et al., 2006)). Human MutS $\alpha$  ((Msh2-Msh6 heterodimer), and MutL $\alpha$  (Mlh1-Pms2 heterodimer) participate in the fidelity of genetic recombination and the suppression of gene amplification (Chen et al., 2001).

Another recurring theme in the DNA repair of complex genomes, such as the mammalian genome, is the existence of proteins with partly overlapping activities. This **genetic redundancy** appears essential for maintaining the stability of a complex genome but this represents a major drawback for experimental approaches designed to unravel the specific functions of a particular DNA repair protein. A classic example arises from the repair of uracil by BER. BER includes a recognition step which is performed by specific uracil-DNA glycosylases, following by a synthesis step conducted by the DNA polymerase beta (Pol  $\beta$ ). Different DNA glycosylases travel down the DNA molecule scanning for potential lesions (Sartori et al., 2002), and gene redundancy might make it difficult to generate uracil glycosylase-deficient cells because there are several genes in the mammalian genome that encode enzymes able to excise uracil from DNA (Pearl, 2000). Another example is the PARP family where PARP1 and PARP2 possess partially redundant functions as well as divergent activities (Menissier de Murcia et al., 2003, Schreiber et al., 2002). This functional partial overlap explains the survival of human cells when either the *PARP1* or *PARP2* gene is silenced (see below); in contrast double *PARP1* and *PARP2* knockdown leads to cell death (unpublished data). Fortunately, the genetic redundancy and the overlap between DNA repair pathways support the notion that **compensating repair activities** can take place over time. This is essential to understanding of DNA damage response (DDR)-deficient human cells, but also cells handled *in vitro*, such as knockdown cells.

An additional point in the complex study of DNA repair factors is that several of them are involved in **other physiological pathways**, even in the absence of DNA damage. This is the case for certain of the NER factors and their tight relationship with the transcriptional machinery (Le May et al., 2010a, Le May et al., 2010b). This is also observed when different DNA repair pathways are key building blocks in the primary and secondary antibody diversification processes taking place in B lymphocytes (for review (Durandy, 2009)).

Actually, BER, D-NHEJ (classic DNA PKcs-dependent NHEJ), b-NHEJ (backup NHEJ), MMR and DNA damage signaling factors actively contribute to immunoglobulin diversification.

These compelling data explain why a mutation of one DNA repair gene could trigger fetal or embryonic death or lead to a dramatic hereditary disorder. Human syndromes where one DNA repair gene is mutated have been collectively classified as “*DDR-defective syndromes*”. The range of clinical features associated with these disorders attests to the complexity of the DDR, its redundancy and its connection with other essential processes. That explains the diversity of the phenotypes observed in patients with DNA repair disorders (**Table 1**). It turned out that growing evidence demonstrates that ubiquitylation of key proteins is critically involved in the emergence of DDR-defective syndromes as observed for NER (DDB2), HR (FancD2) or TLS (PCNA). (for review (Huang & D'Andrea, 2006)). The pivotal role of DNA repair pathways during normal human development explains one hallmark of numerous DDR-defective syndromes. Actually, the main clinical features observed in numerous DDR-defective syndromes are hematopoietic defects (e.g. anemia or immunodeficiency) and neurological deficits (e.g. microcephaly), in parallel with genomic instability and specific DNA damage-induced sensitivities. This highlights crosslinks between DNA repair mechanisms and either neuronal development (O'Driscoll & Jeggo, 2008) or immunoglobulin diversification processes (for review (Durandy, 2009)). Hence, numerous DDR-defective disorders exhibit microcephaly, such as LIG4 syndrome (*DNA ligase IV* gene) but also XLF-Cernunnos-SCID (*XLF-Cernunnos* gene), Seckel syndrome (*ATR* gene), Nijmegen breakage syndrome (*NBS1* gene), Fanconi anemia (*FancD1/BRCA2* gene), Bloom syndrome (*BLM* gene), Cockayne syndrome (*CSA, CSB, XPB, XPD* and *XPG* genes), Xeroderma pigmentosum (*XPA* to *XPG* genes), and cerebro-oculo-facio-skeletal syndrome (*ERCC1* gene) ((Jaspers et al., 2007); for review (McKinnon, 2009, O'Driscoll & Jeggo, 2008)). LIG4 syndrome and XLF-Cernunnos-SCID also exhibit multiple immune abnormalities because both LigIV and XLF/Cernunnos are involved in the V(D)J and NHEJ pathways which are required during the primary repertoire of antibodies and the secondary diversification processes (Yan et al., 2007).

## 1.2 Long term silenced human cells

A better understanding of these hereditary disorders requires detailed insight into each DNA repair pathway that can operate on the damaged genome. Furthermore, the importance of the DDR during the multistage process leading to tumorigenesis emphasizes the need for outstanding biological tools to study DNA repair genes. Altogether this compelling evidence points to the need of outstanding cell models for unraveling the DDR jigsaw both for fundamental research and for the development of novel therapeutic strategies.

Over the last seven years we have developed a rational strategy to silence the main DNA repair factors so as to unveil their functions. Since the emergence of the RNA interference technology, many studies have developed transient or middle-term gene silencing experiments targeting DDR genes, but few of them have characterized stable clones. Our project is based on the exceptional efficiency of pEBVsiRNA vectors in ensuring stable gene silencing. Our approach has been extensively described previously (Biard, 2007, Biard & Angulo, 2007).

Pathways	Diseases or syndromes	Mutated genes (targeted genes*)	Main symptoms and/or remarks
Cell cycle control	Li-Fraumeni syndrome	<b>p53</b>	Sarcoma, breast, brain, leukemia
	familial retinoblastoma	<b>Rb</b>	Retinoblastoma, osteosarcoma
	familia melanoma	<b>p16</b>	Melanoma, pancreas cancers
	Sporadic cancers	<b>Chk1</b>	Colorectal, stomach, lung, endometrial, melanoma, mesothelioma cancers (for review (Solyom et al., 2010))
	Li-Fraumeni syndrome	<b>Chk2</b>	Breast, lung, colon, urinary, bladder, testis cancers, melanoma
Signaling pathways	Ataxia telangiectasia	<b>ATM</b>	Neurodegeneration, sterility, telangiectasia, dysarthria, immunological defects, sensitivity to IR, lymphomas
	ATR-Seckel syndrome	<b>ATR</b>	Microcephaly and mental retardation, growth defects
	Ataxia telangiectasia-like disorder	<b>MRE11</b>	Ataxia, neurodegeneration, dysarthria and oculomotor apraxia, mild immunological defects, lymphomas
	Familial breast cancer 1 & 2	<b>BRCA1, BRCA2</b>	Chromosome instability, sensitivity to DNA damage, HR deficiency, cancer
	Nijmegen breakage syndrome	<b>NBS1</b>	Microcephaly, immunological defects and lymphoid malignancy, lymphomas
	NBS-like disorder (NBSID)	<b>Rad50</b>	
DNA DSB repair	LIG4 syndrome	<b>LIG4</b>	Microcephaly, developmental/ growth delay, immunodeficiency and lymphomas
	Human immunodeficiency with microcephaly glioblastoma (M059J cells)	<b>XLFCernunnos</b>	Microcephaly, immunodeficiency
		<b>DNAPKcs</b>	(Allalunis-Turner et al., 1993)
	Fanconi anaemia	<b>BCRA2</b>	Microcephaly and medulloblastoma, Bone marrow and congenital defects
DNA SSB repair	Spinocerebellar ataxia with axonal neuropathy	<b>TDP1</b>	Ataxia, neurodegeneration, peripheral axonal motor, and sensory neuropathy, and muscle weakness...
	Ataxia with oculomotor apraxia 1	<b>APTX</b>	Ataxia, neurodegeneration, oculomotor apraxia and peripheral neuropathy, hypercholesterolaemia and hypoalbuminaemia
	cerebro-oculo-facio-skeletal syndrome	<b>ERCC1</b>	Microcephaly, moderate sensitivity to UV and mitomycin C (Jaspers et al., 2007)
NER	Xeroderma pigmentosum (XP)	<b>XPA to XPG</b>	Neurodegeneration and microcephaly, UV sensitivity and skin cancer
	Cockayne syndrome (CS)	<b>CSA, CSB, XPB, XPD, XPG</b>	Microcephaly and dysmyelination, TCR-specific disorder. Segmental progeria, no increase in cancer incidence
	Trichothiodystrophy (TDD)	<b>XPD, XPB, TTD-A</b>	Neurodevelopmental defects and dysmyelination, brittle hair, nails and scaly skin. Segmental progeria without an increase in cancer incidence (Giglia-Mari et al., 2004)

Pathways	Diseases or syndromes	Mutated genes (targeted genes*)	Main symptoms and/or remarks
TLS	XP variant (XP-V)	<b>XPV (pol iota)</b>	UV-induced skin cancer
DNA cross link repair & Ubiquitin ligase	Fanconi anaemia	<b>FancA, B, C, D1 (BRCA2), D2, E, F, G, I, J, L, M, N</b>	Microcephaly and medulloblastoma, cervical cancer, brain tumours i(FANCD2 and FANCN), anaemia, developmental defects, ovarian carcinomas, head and neck squamous cell carcinomas, bone marrow failure, and myeloid leukemias.
BER	Multiple colorectal adenoma and carcinoma	<b>MutYH</b>	DNA glycosylase involved in the repair of oxidative damage (Al-Tassan et al., 2002)
	Ligase I	<b>Ligase I</b>	Immunodeficiencies and cellular hypersensitivity to DNA-damaging agents (Barnes et al., 1992)
MMR	HNPCC	<b>MLH1, MLH3, MSH2, MSH6, PMS2</b>	Hereditary nonpolyposis colon cancer, rectum, gastric, endometrium, ovarian, urinary organ cancers (Peltomaki, 2003)
Helicase	Werner syndrome	<b>WRN</b>	Severe progeria, various cancers
	Rothmund Thomson syndrome	<b>RTS</b>	Osteosarcomas
	Bloom syndrome	<b>BLM</b>	Proportional dwarfism, leukemias, lymphomas and others cancer
	Ataxia with oculomotor apraxia 2	<b>SETX</b>	Ataxia, neurodegeneration and oculomotor apraxia

(\*: genes targeted with pEBVsiRNA plasmids)

Table 1. Main DDR defective Syndromes. (Adaptated from (Hoeijmakers, 2001) and (McKinnon, 2009)).

Briefly, for each gene, three pEBVsiRNA vectors are constructed and validated through both short-term (several days) and long-term (several weeks) experiments. Afterwards, we used only one “validated” vector to establish stable clones (Fig. 1). Four years ago we adopted the DSIR program developed by Vandenbrouck and collaborators (Vert et al., 2006) to design shRNA sequences. This program includes an exact similarity search algorithm for potential off-target detection. In a recent comparison of methods for a rational siRNA design, DSIR is among the three best predictive programs (Matveeva et al., 2007). Our siRNA sequences mainly target the open reading frame of the targeted genes, but when necessary we also use siRNA sequences stretching to the 3'-UTR (e.g. for rescue experiments). Among the targeted genes and in using our approach we have always obtained at least one vector able to impose long-term shut down greater than 80% as compared with control cells (as evidenced by real-time RT-PCR).

Using this technology, more than 160 human genes in different human cell models such as HeLa (Ame et al., 2009, Amine et al., 2009, Aressy et al., 2008, Betous et al., 2009, Biard, 2007, Biard et al., 2005, Biard & Angulo, 2007, Boehler et al., 2011, Bouley et al., 2010, Britton et al., 2009a, Despras et al., 2007, Godon et al., 2008, Le May et al., 2010b, Ousset et al., 2010, Pennarun et al., 2008, Pennarun et al., 2010, Wu et al., 2007), U2OS (Betous et al., 2009, Rey et al., 2009) and MRC5-V1 (Bouquet et al., 2011, Britton et al., 2009b, Schmutz et al., 2010) cells have been silenced. Our approach has also been successfully tested in other human tumor-derived cell lines, such as RKO (Biard & Angulo, 2007), HCT-116 (Aressy et al., 2008), Caco2 (Coant et al., 2010), SH-SY5Y cells (Schulte et al., 2008), MCF7, MDA-MB 231, K562, UT7

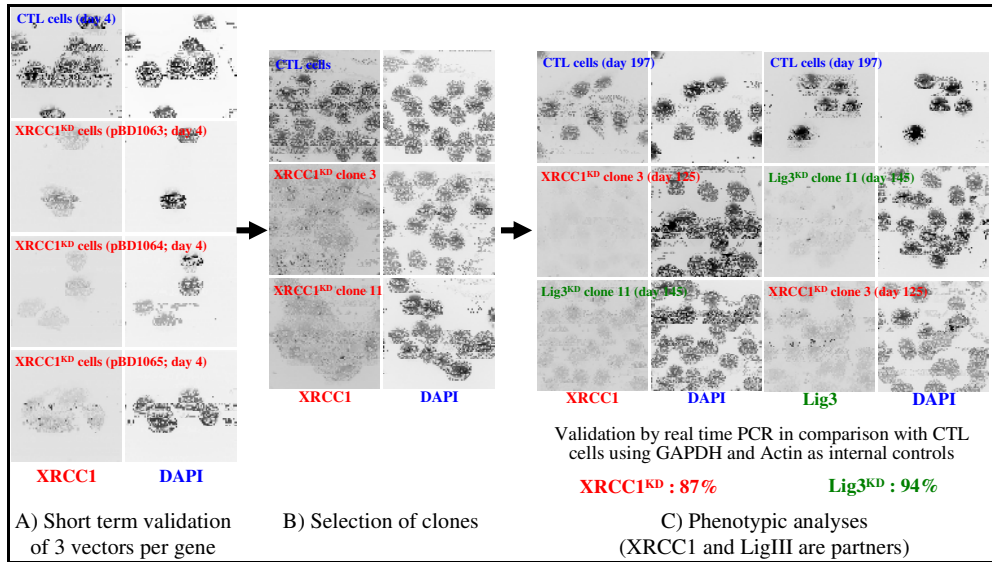


Fig. 1. Establishment of stable clones.

(papers in preparation), and even in mouse NIH-3T3 cells (Meulle et al., 2008). Some authors have previously suggested the importance of “position-specific” criteria for efficient gene silencing. With the benefit of hindsight, we have never observed such a positioning effect in either short-term (*few days*) or long term (*several months*) experiments. In **Figure 2** we show the position of different siRNA sequences able to impose a very efficient long-term gene silencing along a representative mRNA and we demonstrate no positioning effect.

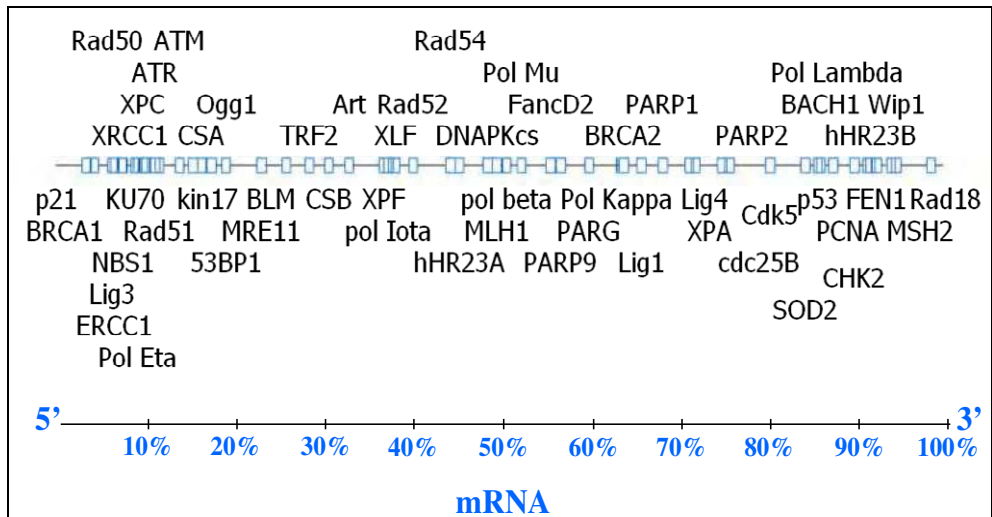


Fig. 2. Position of validated siRNA sequences along a representative mRNA.

The maintenance of stable gene silencing for several months affords the opportunity to validate different siRNA sequences for an unfailling and specific gene silencing. Importantly, transient assays may mask the real effects of gene silencing, due to the saturation of the RNAi (and miRNA) machinery and by side-effects resulting from the high siRNA concentrations currently used. In the long-term experiments, we do not exclude the possibility of skews, and the suppression of gene expression over a long period may provoke compensatory cellular responses during an “adaptive period”. During this period, cellular metabolism may compensate for the decrease in protein concentration, particularly if the protein plays an important role in the cell. These compensating activities are also observed during the multistage process leading to tumorigenesis, where a normal cell undergoes serial genetic changes, including initiation, clonal expansion, pre-malignant lesions, and malignant progression, before acquiring a tumor phenotype. These properties acquired by cells to escape DDR defects are essential to our understanding of tumor cell behavior following chemo- or radiotherapy. We can now assess the usability of the numerous stable clones affecting all branches of the DDR that have been created. This unique cell model appears relevant for studying DNA repair, DNA replication, DNA recombination and cross-talk between them.

To date, we have established numerous clones, creating a library of stable isogenic cells which no longer express a specific DNA repair gene. This approach has helped us to untangle the interwoven DNA repair pathways and represents a powerful tool for research, drug screening and for preclinical testing of new therapies. This review will concentrate on two fields of research investigated using these knockdown clones.

## 2. Example of stable DNA repair gene silencing studies

### 2.1 Dual roles of some NER factors

NER is one of the more versatile DNA repair processes and removes diverse bulky lesions located on one DNA strand, including UV-induced photoproducts. In mammals, more than 30 proteins are required for this process, which comprises first a DNA damage recognition and structure distortion step involving XPC-hHR23B-centrin 2 and XPE in the global genome (GG)-NER or RNA polymerase II in the transcription-coupled (TC)-NER. NER also includes the verification of lesions (XPA-RPA), strand-separating helicases (TFIIH containing XPB and XPD DNA helicases), structure-specific endonucleases (ERCC1-XPF and XPG), and the enzymes needed for gap filling (DNA polymerase  $\delta/\epsilon$ , PCNA, RFC, and RPA). For example, ERCC1<sup>KD</sup> and XPF<sup>KD</sup> cells exhibited a tremendous and stable decrease of both targeted mRNA and protein, as evidenced by real time PCR and immunofluorescence staining (**fig. 3**). Beside, as documented in the literature, the loss of one of these proteins induces the disappearance of the other partner.

In GG-NER, the XPC-hHR23B-centrin 2 complex is responsible for the detection of damaged DNA. In TC-NER the displacement of stalled RNA polymerase complexes with the CSA and CSB proteins allows coordination of transcription and DNA repair. In order to unravel new roles for some of these gene products in this wide DNA repair network, we have established stable XPA<sup>KD</sup>, XPC<sup>KD</sup>, hHR23A<sup>KD</sup>, hHR23B<sup>KD</sup>, ERCC1<sup>KD</sup> and XPF<sup>KD</sup> HeLa cells. In the **figure 4** established clones are represented for the NER and SSBR pathways.

Several clones displaying undetectable protein levels of XPA or XPC were established and grown for more than 300 days in culture with a tremendous stability of the gene-silenced

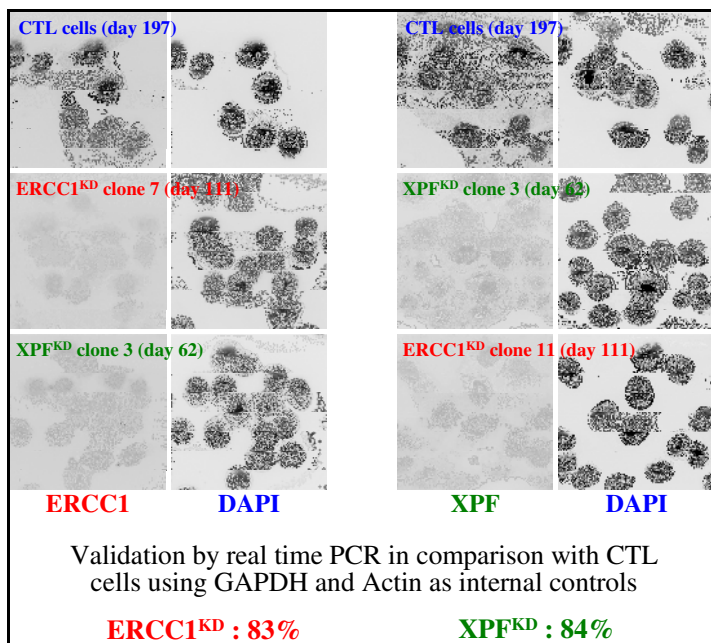


Fig. 3. Analysis of ERCC1<sup>KD</sup> and XPF<sup>KD</sup> cells by immunofluorescence.

and expected phenotypes (Biard et al., 2005). As expected, XPA<sup>KD</sup> and XPC<sup>KD</sup> HeLa cells were highly UVC sensitive and exhibited cell cycle arrest in early and middle S phase after UVC irradiation, showing that the persistence of UVC lesions blocks DNA replication. Both clones also show an impaired unscheduled DNA synthesis (UDS) after UVC irradiation. However, unlike XPA, the silencing of the XPC gene dramatically impeded HeLa cell growth. Furthermore, XPC<sup>KD</sup> HeLa clones were more sensitive to UVC than their XPA<sup>KD</sup> counterparts. In parallel we have analyzed the behavior of our hHR23B<sup>KD</sup> and hHR23A<sup>KD</sup> cells. hHR23B<sup>KD</sup> cells displayed a significant sensitivity to UVC, in contrast to their hHR23A<sup>KD</sup> counterparts which strongly tolerated UVC irradiation (Biard, 2007). While hHR23A<sup>KD</sup> cells were not blocked in S phase after UVC irradiation, the exit from the S-phase of hHR23B<sup>KD</sup> cells was hindered, suggesting the presence of unrepaired (or unreparable) UVC-induced DNA damage. These data clearly demonstrate that hHR23A and hHR23B have diverse biological functions in human cells and that hHR23B<sup>KD</sup> cells have a phenotype closely resembling that of XPC<sup>KD</sup> cells. To understand why the silencing of the XPC gene can trigger major changes in cell behavior, we have performed hygromycin B withdrawal experiments.

After about 200 days of culture, hygromycin B was removed from the culture medium in order to reverse the gene-silencing phenotype by the slow and progressive disappearance of pEBV episomes. Under these experimental conditions, XPA or XPC protein levels returned to “control” levels after 15 to 20 days in culture. Unexpectedly, reverted XPC<sup>KD</sup> cells (*XPC re-expressing cells*) did not recover a normal resistance to UVC, unlike XPA<sup>KD</sup> cells. This striking result suggests that irreversible genetic changes have been fixed in the genome during the long-term XPC gene silencing and that, beside their canonical roles, some NER





suggest that beside its canonical function in the early steps of the NER, the XPC protein could be essential in the coordination of other recovery pathways, such as those involved in the repair of IR- and etoposide-induced DNA damage.

In mock treated cells, the persistence of XPC on chromatin structures was shown by experiments in which the XPC protein remained tightly anchored to detergent-insoluble nuclear structures (Despras et al., 2007). Interestingly, XPC was released from these structures after induction of DSBs by calicheamicin or neocarzinostatin, two potent specific DSB inducers. The reduction of chromatin-fixed XPC correlated with the increase of H2AX phosphorylation and presumably with the recruitment of DNA repair factors at sites of damaged DNA. This sequence of events was partly confirmed by the subsequent recruitment of phosphorylated-XRCC4 and LigIV into the less extractable nuclear fraction after DSB induction, as previously described (Drouet et al., 2005). Therefore, XPC should be considered as a genome caretaker protein, which is (i) recruited for initiating the GG-NER in the presence of bulky DNA damage, but which (ii) also displays other functions in the presence of DSBs.

Using the HeLa isogenic KD model we have also focused our attention on the efficiency of NER-deficient cells in performing NHEJ, using an *in vitro* assay making use of DNA PKcs<sup>KD</sup> and XRCC4<sup>KD</sup> cells. The DNA PKcs<sup>KD</sup> cells used displayed an undetectable protein level and a nearly total loss of the endogenous kinase activity (Despras et al., 2007), and the isolated XRCC4<sup>KD</sup> clones all displayed a residual XRCC4 protein level corresponding to about 15% of the control (CTL); this residual level might reflect the essential role played by XRCC4 in cell survival. These XRCC4<sup>KD</sup> cells are particularly interesting experimentally too as there are no human cell lines lacking the XRCC4 protein. In ligase IV<sup>KD</sup>, DNA PKcs<sup>KD</sup> and XRCC4<sup>KD</sup> cells, NHEJ efficiencies dropped to 50, 30 and 20%, respectively, as compared with control (personal data and (Despras et al., 2007)). This also correlated with a markedly increased sensitivity towards IR. Our results also argue for XRCC4 being a limiting factor in the NHEJ process, at least *in vitro*. Strikingly, while the expression of NHEJ factors was not altered in XPC<sup>KD</sup> cells, XPC deficiency led to a decrease of *in vitro* NHEJ efficiency. In both XPC<sup>KD</sup> and DNA PKcs<sup>KD</sup> cells, XRCC4 and ligase IV proteins were mobilized to damaged nuclear structures at lower doses of chemical DSB inducer in comparison with proficient cells. In contrast, XPA gene silencing did not modify HeLa cell response to DSBs. Our results reinforce the notion that XPC<sup>KD</sup> cells display an unexpected behavior towards DSBs, presumably due to an intrinsic characteristic of XPC, rather than being a consequence of NHEJ deficiency. We can also rule out a direct role of XPC in the NHEJ process *per se*. Presumably XPC deficiency could locally change the chromatin structure and interfere with other pathways.

It is notable that in our experiments we have always observed that XPA gene silencing could lead to an enhanced cell growth several weeks after transfection of HeLa cells and in the absence of genotoxic injuries. In contrast, knocking down of XPC triggered major growth defects and tremendous cellular stress as well as elevated sensitivity to genotoxic agents. Presumably XPA and XPC can participate in major pathways required for normal growth, but with opposite effects. Because relationships between some NER factors and transcription have been extensively related in the literature (for review (Le May et al., 2010a)), we have questioned whether XPA and XPC factors could be involved in the regulation of transcription in the absence of exogenous DNA damage. The transcription / repair factor TFIIH is organized into a core complex (XPB, XPD, p62, p52, p44, p34, and p8/TTDA) that associates with the Cdk-activating kinase (CAK) complex (Cdk7, cyclin H,

and MAT1). In response to DNA damage, XPA catalyzes the detachment of the CAK from the core TFIID, changing this transcription factor into a repair factor (Coin et al., 2008). Thereafter, new NER proteins are recruited around the TFIID factor such as XPC / hHR23B. After repair, resumption of CAK activity is required for continuation of transcription.

By using our XPA<sup>KD</sup>, XPC<sup>KD</sup> and ERCC1<sup>KD</sup> clones, we have determined the role of these NER proteins during the transcriptional regulation of active promoters. Interestingly, we observed that the recruitment of NER factors at promoters of inducible nuclear receptor genes (including the retinoic acid receptors  $\alpha$  and  $\gamma$ ) occurred in a sequential order and required XPC, CSB, XPA / RPA, the two endonucleases, XPG and ERCC1 / XPF and XPE with the RNA pol II machinery (Le May et al., 2010b). This transcriptional complex containing NER factors is formed in the absence of any genotoxic injury, at the site of the promoter. Contrary to the coordinated recruitment observed in control cells, none of the NER factors were recruited to the promoter in XPC<sup>KD</sup> HeLa cells. XPC association is thus a pre-requisite step and abnormal XPC protein levels could affect normal transcription. This XPC-dependent transcriptional complex is distinct from a repair complex. In contrast, in XPA<sup>KD</sup> cells, only XPC and CSB were detected at the promoter, and in ERCC1<sup>KD</sup> cells we detected XPC, XPA, and XPG together with RAR, RXR, RNA pol II, and TFIID. Furthermore, during the transcriptional initiation step, XPC is required to achieve optimal DNA demethylation and histone posttranscriptional modifications. In control cells, transcription initiation and recruitment of NER factors are accompanied by a global DNA demethylation. A local DNA demethylation at sites of 5'-CpG-3' islands was also detected around the proximal RAR $\beta$ 2 promoter region. In contrast, in XPC<sup>KD</sup>, XPA<sup>KD</sup>, and ERCC1<sup>KD</sup> HeLa cells the global methylation levels were lowered as compared with control cells. More importantly, XPC<sup>KD</sup> and XPA<sup>KD</sup> cells, but not ERCC1<sup>KD</sup> cells, failed to demethylate the RAR $\beta$ 2 promoter. Afterwards, during the transcription elongation in distal regions of the gene, NER factors escort the RNA-Pol and form a complex which now excludes XPC but needs CSB. This latter complex could appear as a pre-TC-NER complex. In all of these studies, the phenotype of the knockdown HeLa cells was compared with that of deficient XP and CS fibroblasts from patients.

Altogether these data demonstrate that NER factors could actively contribute to transcription of particular promoters in the absence of DNA damage and then interfere with cellular homeostasis. These results help us to explain the striking phenotype observed in our XPC<sup>KD</sup> and hHR23B<sup>KD</sup> cells in comparison with control cells or their XPA<sup>KD</sup> counterparts. Recently, in an effort to silence other genes belonging to the NER, we have observed that *DDB1* gene silencing strongly disrupts HeLa cell growth a few weeks after transfection (unpublished data). This raises the question whether XPE (DDB2-DDB1 heterodimer) also participates in transcription regulation in the absence of exogenous DNA damage, as has been seen for XPC.

## 2.2 Parp1, between inhibition and gene silencing

We have also employed our cell model to shed light on the poly(ADP-ribose) polymerase (PARP) family. New developments of mono- and combined therapeutic approaches based on PARP inhibitors reinforce the crucial role played by these proteins in the DDR. The PARP family contains 17 members and its founding member, PARP1, carries out the majority of poly(ADP-ribose) (PAR) synthesis in mammalian cells (Ame et al., 2004, D'Amours et al., 2001). Poly(ADP-ribosyl)ation is an immediate DNA damage-dependent posttranslational modification of numerous nuclear proteins indispensable for an accurate

DDR. In contrast to what is frequently stated in the literature, PARP1 is not a DNA repair protein in *stricto sensu* but rather a signaling and scaffold protein which binds to DNA nicks and breaks in order to facilitate DNA repair by attracting other factors to damaged sites (e.g. XRCC1). Hence, PARP1 participates in numerous DNA repair pathways. It is a key building block in the SSBR, more precisely in the SPR (short patch repair) pathway, but probably in the first steps of the LPR (long patch repair) pathway, but not after SSB generating agents (see below; **fig. 4**). In addition, PARP1 is also involved in NER, b-NHEJ (**fig. 5**), transcription, cellular bioenergetics, telomere cohesion and mitotic segregation, centromere and/or kinetochore function and energy metabolism (Schreiber et al., 2006). A recent study shows that loss of PARP1 leads to spontaneous hyper-recombinogenic phenotype in mice, suggesting a balance between SSBR and HR (Claybon et al., 2010). Moreover, Patel et al. have observed that transient chemical inhibition of PARP1 and gene silencing interfered with NHEJ activities, emphasizing an interplay between the error-prone NHEJ and the error-free HR (Patel et al., 2011).

We have addressed this issue by creating PARP1<sup>KD</sup>, PARP2<sup>KD</sup>, PARP3<sup>KD</sup> and PARG<sup>KD</sup> silenced cells (**fig. 6**). Our aim was to analyze spontaneous and genotoxic-induced genetic instability (Ame et al., 2009, Boehler et al., 2011, Godon et al., 2008). In a preliminary approach, we focused our attention on the requirement of PARP1 in the two SSBR pathways (SPR *versus* LPR). This approach requires the establishment of additional clones such as XRCC1<sup>KD</sup>, ligase III<sup>KD</sup> and ligase I<sup>KD</sup> cells, together with other knockdown cells which are presently under evaluation (Fen1<sup>KD</sup>; PNK<sup>KD</sup>, APTX<sup>KD</sup>, polβ<sup>KD</sup>).

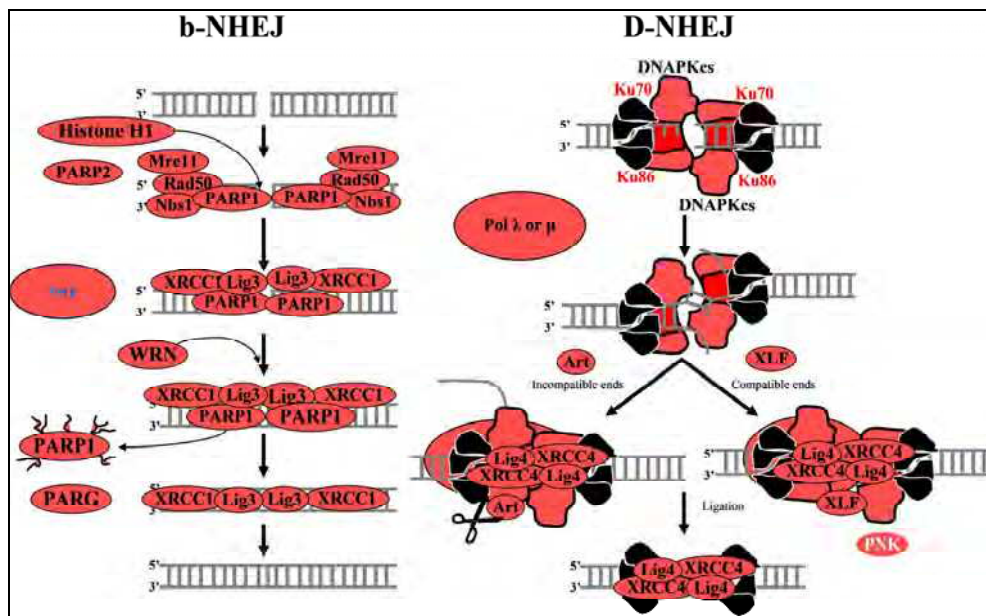


Fig. 5. b-NHEJ and D-NHEJ pathways. Stable knock down clones are identified as indicated in the legend of fig. 2.

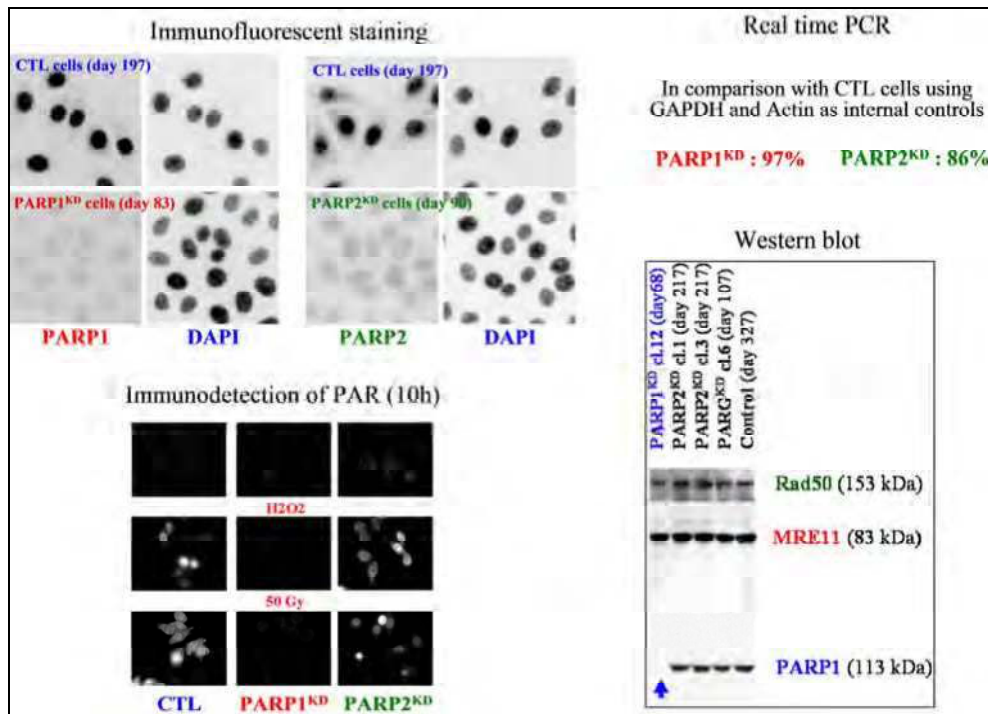


Fig. 6. Characterization of PARP1<sup>KD</sup> and PARP2<sup>KD</sup> stable clones.

This work has also been carried out to point pitfalls arising from conflicting data obtained after gene silencing *versus* chemical inhibition. Interestingly, PARP1 inhibition and gene silencing triggered different outcomes in terms of SSB and radiosensitivity. Our PARP1<sup>KD</sup> HeLa cells display a substantial reduction in both protein and mRNA levels, with undetectable poly(ADP-ribose) (PAR) synthesis following exposure to H<sub>2</sub>O<sub>2</sub> (1 mM, 10 min) or even after exposure to 50 Gy  $\gamma$  rays (Godon et al., 2008). PARP1<sup>KD</sup> cells are 2.5-fold more radiosensitive than both controls and XRCC1<sup>KD</sup> cells, and XRCC1<sup>KD</sup> cells are 5-fold more sensitive to methyl methane sulfonate than their PARP1<sup>KD</sup> counterparts. *PARP1* gene silencing prevents XRCC1-YFP recruitment at sites of local laser irradiation (405 nm), but does not affect the lifetime of PCNA-GFP foci, suggesting that impaired SPR (PARP1- and XRCC1-dependent) could be efficiently replaced by LPR (PCNA- and ligase I-dependent). However, we can not rule out the partial resolution of SSB by way of HR, as suggested elsewhere (Clayton et al., 2010). S phase-irradiated PARP1<sup>KD</sup> (and XRCC1<sup>KD</sup>) cells complete SSB as rapidly as controls, while SSB is slower in G1 cells but reaches completion. In contrast, PARP1 inhibition with 4-amino-1,8-naphthalimide (ANI) enhances radiosensitivity in highly proliferating cells (e.g. tumor cells), presumably due to the collision of unrepaired DNA lesions with replication forks (Noel et al., 2006). This also prevents XRCC1-YFP recruitment at sites of damaged DNA (laser micro-irradiation) and cells displayed a 10-fold slower SSB. We also observe accumulation of huge PARP1-GFP and PCNA-GFP foci. These results suggest that the chemically inhibited PARP1 protein remains tethered to nuclear structures and that this steric hindrance impedes the recruitment of further DNA

repair proteins. These data emphasize that the need for careful interpretation of results from the use of chemical inhibitors which could be riddled with pitfalls. Moreover, it is noteworthy that PARP inhibitors not only inhibit PARP1, but also PARP2 and PARP3 (Loseva et al., 2010).

After a genotoxic injury, PARP1 activation leads to a tremendous but transient synthesis of PAR, in order to label DNA-damaged sites, open the chromatin structure and recruit repair factors, such as the scaffold protein XRCC1 (Dantzer et al., 2006). Because this reaction is transient, PAR polymers have to be rapidly degraded by PARG. PARP1 and PARG display opposite enzymatic activities which govern the balance between life and death after DNA injuries. Our knockdown clones clearly demonstrate that PARP1, PARP2, PARP3 and PARG activities contribute to this homeostasis, even in the absence of exogenous genotoxic attack (Ame et al., 2009, Boehler et al., 2011). PARG<sup>KD</sup> HeLa cells exhibit a stable loss of the three PARG isoforms (nuclear, cytoplasmic and mitochondrial) and a spectacular loss of function. Surprisingly, constitutive PARG depletion and subsequent PAR accumulation are rather beneficial in that they protect cells from spontaneous SSBs and telomeric abnormalities. In contrast, irradiation of PARG<sup>KD</sup> cells triggers PAR accumulation, delayed SSB and DSB repair, centrosome amplification and mitotic defects, all of which contribute to cell death by mitotic catastrophe (Ame et al., 2009).

The complexity and the redundancy of the PARP family members toward the DDR are reinforced by our recent data demonstrating that PARP3 is a newcomer in the cellular response to DNA damage and mitotic progression (Boehler et al., 2011). PARP3 is closely related to PARP1 and PARP2, but unlike these two counterparts PARP3 is a mono(ADP-ribose) polymerase. It has been proposed that PARP3 could be involved in transcriptional silencing in association with Polycomb group proteins. Moreover, PARP3 could also be a component of the DDR because it is found in complexes mainly containing Ku70 and Ku80, but also PARP1, DNA ligase III, DNA PKcs and DNA ligase IV (Rouleau et al., 2007). This raises the question whether PARP3 participates in SSBR (when PARP1 is deficient?), D-NHEJ (with DNA PKcs, DNA ligase IV, Ku70, and Ku80), b-NHEJ (with DNA ligase III) and telomere maintenance (with Ku70 and Ku80). This was partly confirmed by a recent study which shows that PARP3 might be a novel DSB sensor which functions in the same pathway as APLF (aprataxin- and PNK-like factor) in order to accelerate chromosomal DSB repair (Rulten et al., 2011). APLF is a poly(ADP-ribose)-binding protein which interacts directly with Ku80 and XRCC4 at sites of DSBs (Macrae et al., 2008). To gain further insight into PARP3 function in the DDR we have validated pEBVsiPARP3 plasmids targeting the two known PARP3 isoforms. Stable clones exhibiting an almost complete depletion of PARP3 were carefully characterized (Boehler et al., 2011). PARP3<sup>KD</sup> cells displayed spontaneous DSBs and genome instability, delayed repair after irradiation, but no significant radiosensitivity as compared with control cells. Our results reinforce recent data showing that PARP3-deficient cells were as sensitive to a topoisomerase I poison (camptothecin) as control cells (Loseva et al., 2010). These unexpected results could be explained by partly compensating activities between PARP3 and PARP1. These data strongly suggest a functional synergistic cross-talk between PARP1 and PARP3. Interestingly, PARP3 interacts directly and strongly with PARP1 and PARP3 is able to activate PARP1 in the absence of DNA (Loseva et al., 2010). Another significant event observed in PARP3<sup>KD</sup> cells is an elevated frequency of sister telomere fusions and sister telomere loss. This is explained by the functional association between PARP3, tankyrase I and NuMa (microtubule-associated protein involved in spindle dynamics). Altogether, these three proteins appear to be key

regulators of mitotic progression. This study will now continue by establishing new cell lines silenced for other members of the PARP family such as PARP9, PARP14, tankyrase 1 (PARP5a) and tankyrase 2 (PARP5b).

### 3. Conclusions

In the field of cancer research, numerous questions remain unanswered, such as how do different pathways cooperate to repair DNA damage in tumor cells? How can we explain the chemo- and radioresistance of tumor cells? Can we target DDR to enhance chemotherapy? How do genetic compensation events take place? How can we detect the combinations of genes leading to **synthetic lethality**? Are DNA repair factors involved in other processes? All of these questions have to be carefully analyzed in order to design specific and less toxic therapies for cancer. Currently, chemotherapeutic approaches are based on the fact that highly proliferating (*tumor and unfortunately hair, bone marrow and colon*) cells are more sensitive to DNA damage than their slowly proliferating (*normal*) counterparts. Alterations in DNA repair pathways in tumor cells can make some cancer cells dependent on a reduced set of DNA repair pathways for their survival. These adaptive but potentially error-prone bypasses could render DNA damage-based cancer therapies less efficient and allow tumor cells to escape specific treatments. Recently substantial progress has been made through studies of genes involved in the DDR in order to circumvent rescue pathways. A breakthrough has emerged with the concept of synthetic lethality, which is defined as a genetic interaction where the minimal combination of two nonlethal mutations leads to cell death. Because naturally occurring synthetic lethal mutants are unviable we have to develop outstanding cell models in order to unravel the DDR and subsequently to detect these combinations that give rise to synthetic lethality. In light of these concerns, an emerging strategy has been to use PARP inhibitors (e.g. iniparib, olaparib or veliparib) combined or not with DNA-damaging chemotherapeutic agents in the treatment of breast and ovarian cancers exhibiting germ-line mutations in *BRCA* genes (Bryant et al., 2005, Farmer et al., 2005, Mullan et al., 2006). Because of the partial **redundancy** between *BRCA* functions, PARP inhibitors have to be administered to patients displaying loss of copies of both the *BRCA1* and *BRCA2* (*FancD1*) genes. This approach is based on compelling evidence demonstrating why *BRCA1* and 2 act as molecular determinants in the response to chemotherapeutic agents (Quinn et al., 2003). Amongst prominent defects observed in *BRCA1/2*-deficient tumor cells, aberrant G2/M checkpoint control and impaired DNA repair (HR) modulate sensitivity to genotoxic agents (Hartman & Ford, 2002, Moynahan et al., 1999). Interestingly, *BRCA1* also participates in GG-NER (but not TC-NER) in a p53-independent manner by inducing the expression of XPC, DDB2 (XPE), and GADD45 (Hartman & Ford, 2003). In tumor cells, **compensating repair activities** taking place during clonal expansion could compensate HR (and GG-NER) deficiencies with other DNA repair pathways, such as those dependent on PARP1 (SSBR or b-NHEJ). In these conditions, PARP inhibition might lead to the persistence of DNA lesions normally repaired by HR and trigger tumor cell death without affecting normal cells (Farmer et al., 2005). Other genetic defects could lead to synthetic lethality associated with PARP inhibition, such as impaired PTEN (phosphatase and tensin homolog) (Mendes-Pereira et al., 2009), Fanconi anemia genes (D'Andrea, 2010) or ATM (Williamson et al., 2010) genes. Now, this approach has been enlarged to metastatic triple-negative breast cancers having inherent defects in DNA repair (O'Shaughnessy et al., 2011). Interestingly, a recent paper shows that PARP inhibition could

also interfere with the NHEJ pathways in that PARP inactivation in HR-deficient cells enhances NHEJ activities (Patel et al., 2011). We have to keep in mind that, in mammalian cells, the high-speed 'classic' DNA-PKcs-dependent NHEJ (D-NHEJ) pathway repairs general DSBs. While some DNA ends may be rapidly joined through the D-NHEJ, other breaks are processed for homology searches. These ends may be substrates for the alternative low-speed *backup* NHEJ (b-NHEJ, also termed microhomology-based end-joining pathway) involving ligase III, XRCC1 and PARP1 (Audebert et al., 2006, Iliakis et al., 2004, Verkaik et al., 2002, Wang et al., 2006). Hence, this raises the question whether PARP1 inactivation induces NHEJ compensation due to impaired HR function or b-NHEJ function or both. To strengthen this notion we have observed that NHEJ activities were enhanced in ligase III<sup>KD</sup> HeLa cells when the b-NHEJ was expected to be hampered (as well as BER) (unpublished data). Altogether this striking example clearly highlights the requirement to study the interwoven DNA repair pathways in tumor cells using a relevant cell model. Now we are seeking to evaluate the compensating activities between different pathways, such as D-NHEJ *versus* b-NHEJ or HR *versus* D-NHEJ. We have also established TLS-deficient clones in order to determine the role of specialized (TLS) DNA polymerases in the absence of DNA injuries (fig. 7). Our published results show that these polymerases facilitate the progression of the replication fork through external replication barriers (e.g. bulky adducts) and also through naturally occurring DNA structures (G4 structures, H-DNA or Z-DNA). More precisely, Pol  $\eta$  and Pol  $\kappa$  help to prevent genomic instability occurring at such natural DNA sequences (Betous et al., 2009). Pol  $\eta$  also maintains chromosomal stability and prevents common fragile site breakage during unperturbed S phase (Rey et al., 2009).

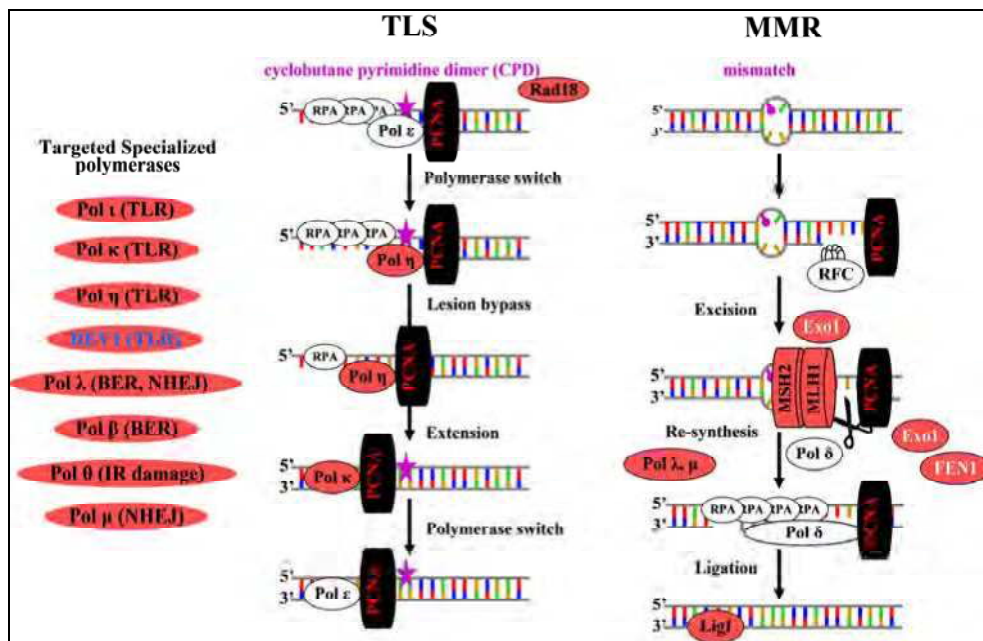


Fig. 7. TLS and MMR pathways. Stable knock down clones are identified as indicated in the legend of fig. 3.



To conclude, the major advantage of this strategy is the rapid establishment of new stable knockdown clones in various tumor-derived cells, which display stable gene silencing. A recent development has been to create dual pEBVsiRNA plasmids allowing efficient knockdown of two or more genes. For instance, double knockdown cells have been created where both DNA PKcs and ligase III were efficiently silenced with a single pEBVsiRNA vector. These cells, which grow normally, are expected to be deficient for both D-NHEJ and b-NHEJ. We have also developed plasmids targeting an endogenous gene and re-expressing an exogenous transcript carrying functional mutations. The latter approach allows mutant cells to be generated when the loss of the targeted gene is lethal. Hence, because we can easily and efficiently create DDR-deficient cells where one or more genes are silenced, we are now able to unravel the spectacular network of DNA repair pathways.

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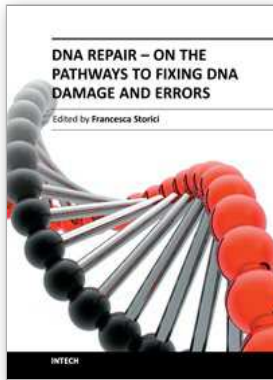
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## **DNA Repair - On the Pathways to Fixing DNA Damage and Errors**

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DNA repair is fundamental to all cell types to maintain genomic stability. A collection of cutting-edge reviews, DNA Repair - On the pathways to fixing DNA damage and errors covers major aspects of the DNA repair processes in a large variety of organisms, emphasizing foremost developments, questions to be solved and new directions in this rapidly evolving area of modern biology. Written by researchers at the vanguard of the DNA repair field, the chapters highlight the importance of the DNA repair mechanisms and their linkage to DNA replication, cell-cycle progression and DNA recombination. Major topics include: base excision repair, nucleotide excision repair, mismatch repair, double-strand break repair, with focus on specific inhibitors and key players of DNA repair such as nucleases, ubiquitin-proteasome enzymes, poly ADP-ribose polymerase and factors relevant for DNA repair in mitochondria and embryonic stem cells. This book is a journey into the cosmos of DNA repair and its frontiers.

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