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DNA Repair in Pathogenic Eukaryotic Cells: Insights from Comparative Genomics of Parasitic Protozoan

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1. Introduction

Numerous external and internal DNA damaging agents can affect genetic material to produce single-strand breaks (SSB), double strand breaks (DSB), inter- and intra-strand cross-links in the form of cyclobutane pyrimidine dimers and (6-4)-photoproducts, oxidation and alkylation of bases, or formation of bulky chemical adducts. Cells possess several biological processes that act in a coordinated way to supervise DNA molecules and properly repair DNA lesions to minimize genetic information loss. This DNA repair system, which has been conserved throughout eukaryotes and prokaryotes evolution, includes various pathways that can be classified according to the type of DNA lesion they can restore: i) DSB, the most detrimental lesions of DNA, can be repaired by homologous recombination (HRR) and non-homologous end joining (NHEJ) pathways [Fleck & Nielsen, 2004]; ii) aberrant bases or nucleotides from a single strand DNA can be repaired by base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) pathways using the complementary strand as template for DNA synthesis. BER mainly restores non-bulky lesions that result from bases alkylation, oxidation or deamination [Krokan et al., 1997]. The main task of NER pathway, which consists in two subpathways: global genome repair (GGR) to remove damage in the overall genome and transcription-coupled repair (TCR) to specifically repair the transcribed strand of active genes, is to eliminate photoproducts produced by ultraviolet (UV) light and other bulky lesions, such as inter- and intra-strand crosslinks [Prakash &Prakash, 2000]. MMR allows the removal of base mismatches and small insertion/deletion loops (IDL) that are formed during the replication process [Marti et al., 2002].

The genome of protozoan parasites is continuously subjected to the effects of antiparasitic drugs and host immune system attacks, which can affect its stability and therefore parasite survival. Thus, efficient DNA maintenance mechanisms are necessary to detect and accurately repair damaged nucleotides. The fully sequenced genome of the four major human pathogens described here provides new insights into parasite biology, including molecular features of

DNA repair mechanisms, and genome evolution. *Entamoeba histolytica* and *Giardia lamblia* (syn. *G. intestinalis, G. duodenalis*) are intestinal parasites that cause diarrheal diseases. *E. histolytica* is responsible for fulminating dysentery, bloody diarrhea, weight loss, fatigue, abdominal pain, which affect 50 million people and provoke 100,000 deaths in developing countries each year. In some cases, *E. histolytica* trophozoites can cross the intestinal wall and use the blood stream to reach different vital organs of the human body, usually the liver (but also the lungs, brain or spleen) to provoke liver abscesses, which can be fatal if untreated [Guo et al., 2007]. *G. lamblia* is another contributor to the enormous burden of diarrheal diseases with over 250 million symptomatic human infections per year worldwide. This anaerobic flagellated protozoa colonises and reproduces in the small intestine of several vertebrates, including human, causing giardiasis, commonly known as Beaver fever, which is characterized by diarrhea, excess gas, stomach or abdominal cramps, upset stomach, and nausea. Additionally, *Giardia* infection has an adverse impact on child linear growth and psychomotor development since the parasite causes iron-deficiency anemia, micronutrient deficiencies and growth retardation associated with diarrhea and malabsorption syndrome [Ankarklev et al., 2010]. Individuals become infected by *E. histolytica* and *G. lamblia* through ingesting or coming into contact with food, soil, or water that have been contaminated by the feces of an infected human or animal. *Plasmodium falciparum* is the protozoan parasite responsible for human malaria, which is one of the most severe infectious diseases with 240 million cases in 2009 and more than 1 million deaths in children each year in Africa alone. The presence of the parasite in red blood cells lead them to stick to blood vessels through a process called cytoadherence, which produce the obstruction of the microcirculation and dysfunction of multiple organs, typically the brain in cerebral malaria. Symptoms usually include fever and headache, in severe cases progressing to coma, and death (Kokwaro, 2009). Trichomoniasis caused by *Trichomonas vaginalis* is the most common nonviral sexually transmitted disease (STD) in the world [WHO, 1995]. It has long been recognized as a frequent cause of vaginitis in women and urethritis in men, but data now link it to cervical cancer and bad pregnancy outcomes [Cotch et al., 1997], as well as to an enhanced risk for human immunodeficiency virus transmission [Sorvillo & Kerndt , 1998]. Here, we combined the use of genomic approaches based on bioinformatic analysis of parasite genome sequence with the review of published reports to perform a comparative description of DNA repair machineries from *E. histolytica*, *G. lamblia*, *P. falciparum* and *T. vaginalis*, which cause high morbidity and mortality in many developed and developing countries.

2. DNA repair machineries in pathogenic eukaryotic cells

2.1 Identification of DNA repair machineries

In order to identify amino acids sequences of *E. histolytica*, *G. lamblia*, *P. falciparum* and *T. vaginalis* proteins related to DNA repair factors, we performed similarity searches in the Eupath database (http://eupathdb.org/eupathdb/) using the *Saccharomyces cerevisiae* DNA repair proteins from HRR, NHEJ, BER, NER and MMR machineries as probes [reviewed in Lopez-Camarillo et al., 2009]. Putative gene products were selected from BLAST analysis against each parasite database using the Blosum 62 scoring matrix and the following criteria: (i) at least 20% identity and 35% homology to the query sequence and (ii) e-value lower than 0.002, unless a portion of the protein showed a very strong similarity. All sequences, as well as the *E. histolytica* sequences obtained from previous work [López-Camarillo et al., 2009], were then verified by BLAST against *S. cerevisiae* and *Homo sapiens* databases to confirm their identity. Additionally, we also retrieved data from published reports about *G. lamblia*,

P. falciparum and *T. vaginalis* (**Table 1**). The absence of a given sequence in the table indicates that the corresponding gene was not identified in the parasite genome or that the sequence was too divergent to be detected by our *in silico* strategy.

None of the protozoan parasites studied here has the complete DNA repair pathways reported in yeast. HRR is the most conserved pathway suggesting that it is the mayor DSB repair pathway in these protozoan parasites. *E. histolytica*, *G. lamblia*, *P. falciparum* and *T. vaginalis* genomes contain most of the RAD52 epistasis group genes, although their functional relevance remains to be determined. Homologs for RAD50, RAD51, MRE11, RAD54 and RPA (lacking the RAD52 interacting domain) have been previously reported in *P. falciparum* [Voss et al., 2002; Malik et al., 2008]. In agreement with its participation in DNA repair, the *PfRad51* gene is overexpressed in the mitotically active schizont stage and in response to methyl methane sulfonate [Bhattacharyya & Kumar, 2003]. In. *T. vaginalis*, RAD50 y MRE11 were previously published as components of the meiotic recombination machinery, although meiosis has not been observed in this organism [Malik et al., 2008]. Ramesh et al. [2005] and Malik et al. [2008] identified the *Rad50/Mre11*, *Rad52* and *Dmc1 genes* involved in meiotic recombination machinery by HRR in *Giardia*. Intriguingly, *G. lamblia* and *P. falciparum* lack the *nsb1* homologue (*xrs2* in Yeast) that is a component of the MRN complex involved in DSB detection and 3´ ssDNA tails conversion. Recently, we published the *E. histolytica* RAD52 epistasis group involved in HRR [Lopez-Casamichana et al., 2007, 2008]. Interestingly, RT-PCR assays evidenced that some genes were down-regulated, whereas others were up-regulated when DSB were induced by UV-C irradiation, which revealed an intricate transcriptional modulation of *E. histolytica* RAD52 epistasis group related genes in response to DNA damage. Particularly, *Ehrad51* mRNA expression was 16-, 11- and 4-fold increased at 30 min, 3 h and 12 h, respectively. DNA microarrays assays confirmed the activation of *EhMre11, EhRad50*, and *EhRad54* genes at 5 min after DSB induction, suggesting that they represent early sensors of damage in HRR pathway [Weber et al., 2009]. Additionally, the molecular characterization of EhRAD51 showed that the presence of all the functional domains reported in yeast and human homologues. EhRAD51 was upregulated and redistributed from cytoplasm to the nucleus of trophozoites at 3 h after DNA damage and it was able to catalyze specific single-strand DNA (ssDNA) transfer to homologous double strand DNA (dsDNA) forming the three-stranded pairing molecule called D-loop structure, confirming that it is a *bonafide* recombinase in *E. histolytica* [Lopez-Casamichana et al., 2008].

G. lamblia and P. *falciparum* only have three of the eight factors of the NHEJ pathway (including the MNR complex also involved in HRR), which strongly suggest that they preferably use HRR to repair DSB. In contrast, almost all NEHJ pathway factors have been identified in *E. histolytica* and *T. vaginalis*, including the LIF1 ligase, RAD27 nuclease and MRE11/RAD50/NSB1 proteins. However, *E. histolytica* genome does not contain a homologous gene for KU80 subunit [López-Camarillo et al., 2009] and *T. vaginalis* lacks both *ku70* and *ku80* genes [Carlton et al., 2007]. As these proteins form a single KU complex that recognizes DSB sites and recruits other DNA repair factors, our findings could appear contradictory. The absence of conserved KU proteins has also been reported in *Encephalitozoon cunili* [Gill & Fast, 2007] and yeast [Hefferin & Tomkinson, 2005], thus it is possible that these organisms use highly divergent KU proteins to perform the NHEJ pathway.

The other key DNA repair mechanisms represented by BER, NER and MMR pathways operate to repair aberrant bases or nucleotides from a ssDNA using the complementary strand as template for DNA synthesis. As in *E. histolytica* [Lopez-Camarillo et al., 2009], the *G. lamblia* BER pathway appears to be largely incomplete, lacking *apn1*, *mag1, ogg1*, *rad10*, *mus81* and *mms4* genes. Both parasites live under oxygen-limiting conditions and have a

highly reduced form of mitocondria called mitosomes [Tovar et al., 1999, 2003]. Then the absence of OGG1 could indicate that they do not suffer oxidative damage to mitochondrial DNA. In contrast, *Plasmodium* Flap endonuclease-1 (PfFEN-1) and Pf DNA Ligase I (PfLigI) have enzymatic activities similar to other species [Gardner et al., 2002; Casta et al., 2008], indicating that BER pathway should be functional in this parasite although several components are lacking.

Most genes involved in NER pathway are represented in *E. histolytica* [Lopez-Camarillo et al., 2009], *G. lamblia, P. falciparum and T. vaginalis* genomes suggesting that this mechanism could be potentially active in these eukaryotic parasites. PfXPB/RAD25, PfXPG/RAD2 and PfXPD/RAD3 have been previously reported in *P. falciparum* [Gardner et al., 2002; Bethke et al., 2007; Casta et al., 2008]. Additionally, the overexpression of *EhDdb1, EhRad23* and *EhRad54* genes after UV-induced DNA damage in *E. histolytica* [Weber et al., 2009] suggested that these genes could be involved in chromatin remodeling complexes as their homologues in human and yeast. *E. histolytica*, *G. lamblia* and *T. vaginalis* have various *rad3* genes to form the NEF3 complex (RAD2, RAD3, RAD25) of the BER pathway. Particularly, we identified six *rad3* genes and an additional truncated gene in *T. vaginalis*. On the other hand, all the parasites studied here lack almost one of the components of the TFIIH complex subunits (TFB1, TFB2 or TFB3).

As in bacteria, *Drosophila melanogaster*, *H. sapiens* and many other organisms [Lisby & Rothstein, 2005], *E. histolytica*, *G. lamblia* [Ramesh et al., 2005], *P. falciparum* [Bethke et al., 2007] and *T. vaginalis* [Malik et al., 2008] have almost all *S. cerevisiae* MMR genes, including the components of the MUTSα (MSH2/MSH6) heterodimer, which strongly suggest that MMR could be an active DNA repair pathway in these parasites. Notably, *E. histolytica* and *P. falciparum* have two *msh2* genes. However, neither *E. histolytica* nor *P. falciparum* present the *msh3* gene that is required for the formation of the MUTSβ (MSH2/MSH3) heterodimer. PfMSH2-1, PfMSH2-2, PfMSH6, PfMLH1 and PfPMS1 proteins potentially participating in MMR have been previously reported in *P. falciparum*. Inhibition of *PfMSH2-2* gene increased mutation rate and microsatellite polymorphism, indirectly demonstrating its relevance in MMR and microsatellite slippage prevention. Moreover, antimalarial drug resistance has been recently related to a defective DNA mismatch repair, mainly in PfMutLα content [Castellini et al., 2011], which demonstrated the relevance of this mechanism for the parasite biology.

Gene name	*E. histolytica*	*G. lamblia*	*P. falciparum*	*T. vaginalis*	*S. cerevisiae*
Homologous recombination repair (HRR) pathway					
rad50	C4M2L7	Q6WD96	C6KSQ6	A2FAD3	P12753
mre11	Q86C23 C4LVX7 C4M8N7*	Q86CI9 A8BR27*	PFA0390w**	A2ECB0	P32829
nbs1	C4M874	-	-	A2DHF7	P33301
rad51	C4M4K4	Q86C21	Q8IIS8 Pf11_0087**	A2FXT7	P25454
rad52	C4M197	Q6WD95	-	-	P06778
rad54 *rad54b*	C4LVM6 C4M7S7	-	Q8IAN4	A2FNE0	P32863
rad51c *rad57*	C4M5L7	-	-	A2GIB8	P38953 P25301

Gene name	*E. histolytica*	*G. lamblia*	*P. falciparum*	*T. vaginalis*	*S. cerevisiae*
rad59	-	-	-	-	Q12223
exo1	C4MBM5	A8BQ11	Q8IBK1	A2E2N7	P39975
rpa1	C4M8G6	-	Q9U0J0 Q8I3A1	A2G5D0	P22336
rpa2	C4LT79	-	-	-	P26754
sgs1	C4M4V5	A8BAJ1 A8B9Y0	Q8I2W7 Q8ILG5	A2DYY2	P35187
rad24	C4M5T7	-	-	A2D9F4	P32641
hpr5	-	-	Q8I3W6	A2F783	P12954
rad17	-	-	-	-	P48581
ddc1	-	-	-	A2F0Q2	Q08949
mec3	-	-	-	-	Q02574
Non homologous end joining (NHEJ) pathway					
ku80	C4MBG9	-	-	-	P32807
ku70	-	-	-	-	Q04437
lif1	-	-	-	-	P53150
dnl4	C4M5H3	-	-	A2DFX6	Q08387
rad27	C4M6G8	A8B672 D3KG58	Q7K734 Q8IJW1	A2GNP0	P26793
Base excision repair (BER) pathway					
apn1	-	-	Q9BMG7	-	P22936
apn21	-	A8BGE2	O97240	-	P38207
mag1	-	-	-	-	P22134
ogg1	-	-	Q8I2Y2	-	P53397
ntg1	C4M764 C4LYM7	-	Q8II68	A2DS55	P31378
ung1	C4LUV5	A8B632	Q8ILU6	A2GFQ7	P12887
pcna	C4M9R9	A8BIU1	P61074 Q7KQJ9	A2DQV2	P15873
rad1	C4LT01	D3KH96	Q8ID22	A2DS24	P06777
rad10	C4LW01	-	O96136	A2DBF5	P06838
cdc9	C4M5H3	A8BWV4	Q8IES4	A2DFX6	P04819
mus81	-	-	-	A2FKU9	Q04149
mms4	-	-	-	A2DHF7	P38257
Nucleotide excision repair (NER) pathway					
rad2	C4M0V9	-	O96154	A2GNP0	P07276
rad3	C4M8K7 C4M8Q4 C4M6T8	A8BYS3 A8B495	Q8I2H7	A2G2G8 A2E4I6 A2F1W2 A2DDD4 A2E1B9 A2ELX1 A2G2G9*	P06839

Gene name	*E. histolytica*	*G. lamblia*	*P. falciparum*	*T. vaginalis*	*S. cerevisiae*
rad4	-	-	-	-	P14736
rad7	-	-	-	-	P06779
rad14	-	-	-	-	P28519
rad16	-	A8BL62	Q8I4S6	A2D9P9	P31244
rad23	C4MAR5	D3KF29*	Q8IJS8	A2FM19	P32628
rad25	C4MA19	A8BMI7	Q8IJ31	A2DEA8	Q00578
rad26	C4MAR8	A8BK31	-	A2EXQ4	P40352
rad28	-	-	Q8IJ73	A2DZ24	Q12021
ssl1	C4LV67	A8BA50	Q8IEG6	A2ENQ3	Q04673
tfb1	C4LWV8	-	-	-	P32776
tfb2	C4MIG0	-	Q8I4Y8	A2E2N2	Q02939
tfb3	-	D3KH94	Q8I3Y3	-	Q03290
tfb4	C4M9E2	A8B6C2	Q8IDG5	A2EYI3	Q12004
Mismatch repair (MMR) pathway					
mlh1	C4M5R1	-	Q8IIJ0	A2EGR5	P38920
msh2	C4M9J9 B1N4L6	Q6WD97	Q8ILI9 C0H4L8	A2EP54	P25847
msh3	-	-	-	-	P25336
msh6	C4M4T8	A8BC61	Q8I447	A2EA54	Q03834
pms1	C4LW71	A8B4I6	Q8IBJ3	A2G2B4	P14242

Table 1. Comparison of DNA repair machineries from *E. histolytica*, *G. lamblia*, *P. falciparum*, *T. vaginalis* and *S. cerevisiae*. * fragment, ** PlasmoDB database.

2.2 Conservation of DNA repair pathways

To investigate the degree of conservation of DNA repair pathways in protozoan parasites, we next determined the values of Smith-Waterman identity scores between *E. histolytica* proteins and their corresponding orthologues in *G. lamblia*, *P. falciparum* and *T. vaginalis* by BLAST analysis based in pairwaise sequence alignments and calculated the mean value for each DNA repair machinery (**Fig. 1**). Data of the MNR complex which participate in HRR and NHEJ pathways were included in both mechanisms. DSB repair pathways were generally more conserved than Excision Repair mechanisms. Considering amino acids identity, mean values for HRR and NHEJ pathways were higher in *E. histolytica*/*P. falciparum* comparison, suggesting that *E. histolytica* machinery was closer to *P. falciparum* than to *G. lamblia* and *T. vaginalis* machineries. The comparison *E. histolytica*/*G. lamblia* evidenced that HRR is highly conserved between both parasites, whereas components of the other pathways were more divergent. In the case of *E. histolytica*/*P. falciparum* comparison, NHEJ appeared to be more conserved that HRR, while the identity of HRR and NHEJ factors was very similar in *E. histolytica*/*T. vaginalis*. In all the parasites, the RAD51 recombinase is the most conserved protein (51%, 58% and 64% when *E. histolytica* protein sequence was compared with *G. lamblia*, *P. faciparum* and *T. vaginalis* orthologues, respectively), which is consistent with its relevant role in HRR mechanism.

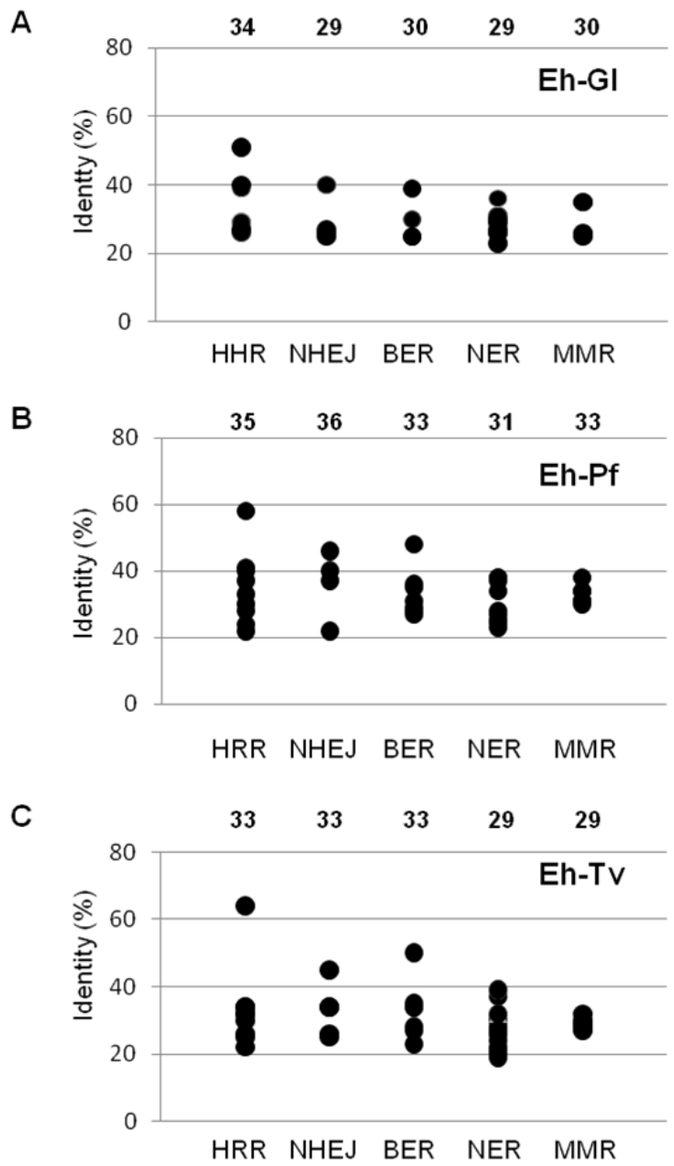

Fig. 1. **Conservation of DNA repair pathways between** *E. histolytica* **and** *G. lamblia* **(A),** *P. falciparium* **(B) and** *T. vaginalis* **(C).** Amino acids sequences from orthologous proteins were compared by Blast and the percentage of identity was determined through pair wise alignment of the most conserved region. Average identity of all pathways is indicated above each graph.

2.3 DNA repair activity in cell free lysates evidences the functionality of DNA repair proteins

Although insights about the activity of DNA repair proteins in protozoa have been mainly obtained from experimental evidence based in heterologous expression and characterization of recombinant proteins, some reports showed that DNA repair activity could be detected in whole cell extracts, supporting the notion that DNA repair pathways already operates *in vivo*. For instance, Haltiwanger et al., (2000) reported the characterization of an AP

endonuclease activity in a *P. falciparum* cell free lysate. Authors provide evidence for the presence of class II, Mg^{2+}–dependent and independent AP endonucleases in the extracts. Moreover, they detected that *Plasmodium* AP endonuclease(s) possessed a 3´-phosphodiesterase activity similar to those described in other class II AP endonucleases [Demple et al., 1986]. In a related study, it was reported that a *P. falciparum* lysate contained uracil DNA glycosylase, AP endonuclease, DNA polymerase, flap endonuclease, and DNA ligase activities [Haltiwanger et al., 2000]. In contrast, DNA repair activities in cell lysates have not been detected in *Entamoeba, Giardia* and *Trichomonas* parasites. These data remark the utility of cell free lysates to understand DNA repair pathways, and pointed out to the urgency to investigate endogenous DNA repair activities using whole cell extracts in parasites where no data is available.

3. Functional categorization of *Entamoeba histolytica* DNA repair genes

To define the putative functions of *E. histolytica* DNA repair genes in unrelated DNA repair processes, we investigated the functional diversity of genomic maintenance pathways using Gene Ontology (GO) annotations. Functional related gene groups were predicted by the David bioinformatic resources (http://david.abcc.ncifcrf.gov/gene2gene.jsp), using a functional classification tool which generates a gene-to-gene similarity matrix based in shared functional annotation using over 75,000 terms from 14 functional annotation sources, allowing the classification of highly related genes in functionally related groups. Results from this analysis revealed that a large number of DNA repair genes were miss-annotated in parasites genome databases (43%). However, our analysis clearly showed that the majority of these genes seems to participate in DNA repair related processes. Besides, 57% of genes were predicted to function in DNA repair related process. 11% of genes participates in DNA damage repair, and 18% and 8% have helicase and endonuclease functions, respectively (**Fig. 2**).

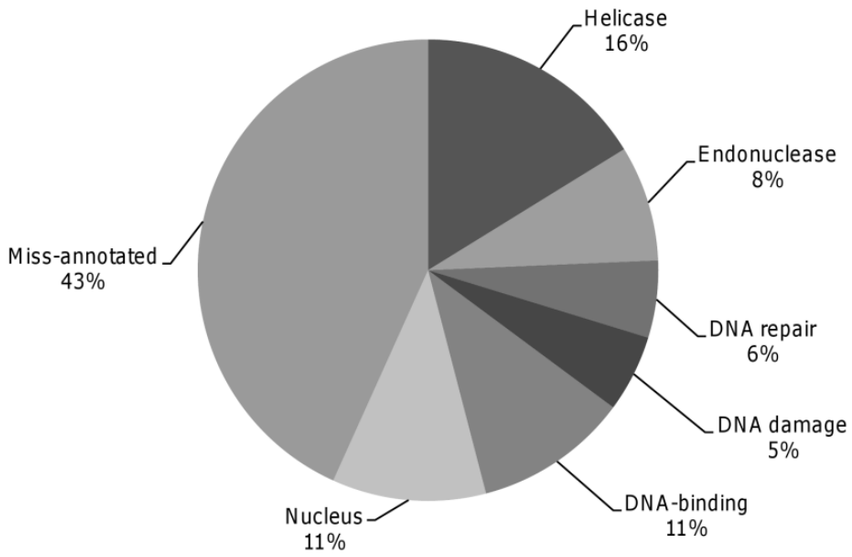

Fig. 2. **Functional categorizations of** *E. histolytica* **DNA repair genes.** Biological processes and molecular functions were determined using David software (http://david.abcc.ncifcrf.gov/gene2gene.jsp). Percentage of genes included in individual categories is given.

4. Duplicated genes: The case of *rad3*

Gene duplicates represent for 8-20% of the genes in eukaryotic cells, and the rates of gene duplication are estimated at between 0.2% and 2% per gene per million years. Gene duplications are one of the major motors in the evolution of genetic systems and may occur in homologous recombination, retrotransposition event, or duplication of an entire chromosome [Zhang, 2003]. Duplicated genes are believed to be a main system for the establishment of new gene functions generating evolutionary novelty [Long & Langley, 1993; Gilbert et al., 1997].

A detailed examination of **Table 1** revealed that several DNA repair genes are duplicated in protozoan parasites, while there is only one gene in yeast. For example, the HRR machinery includes two *rad51* genes in *P. falciparum*, two *rad54* and *mre11* genes in *E. histolytica* [Lopez-Casamichana et al., 2008], two *rpa1* genes in *T. vaginalis*, and two *sgs1* genes in *G. lamblia* and *P. falciparum*. We also identified two *rad27* genes in *P. falciparum* and *G. lamblia* NHEJ pathway, two *E. histolylica ntg1* and *P. falciparum pcna* genes in the BER pathway, as well as two *msh2* genes for the MMR pathway in *E. histolytica* and *P. falciparum*. But the most duplicated gene was the *rad3* gene from the NER mechanism, since there are three genes in *E. histolytica*, two in *G. lamblia* and six in *T. vaginalis*, whereas *P. falciparum* has only one *rad3* gene, alike yeast. Remarkably, gene duplication is evident for many other genes in *T. vaginalis* and reflexes the massive gene expansion inside the large genome of this pathogen [Hartl & Wirth, 2006]. In yeast, the RAD3 protein is involved in mitotic recombination and spontaneous mutagenesis, becoming essential for cell viability in the absence of DNA injury. Furthermore, this protein participates in the repair of UV-irradiated DNA via NER, and constitutes a subunit of RNA polII initiation factor TFIIH [Moriel-Carretero & Aguilera, 2010]. *S. cerevisiae* RAD3 is related to the *H. sapiens* XPD, also known as ERCC2. Defects in human XPD result in a wide range of diseases, including Xeroderma pigmentosum (XP), Cockayne's syndrome, and Trichothiodystrophy characterized by a wide spectrum of symptoms ranging from cancer susceptibility to neurological and developmental defects [Liu et al., 2008].

In order to describe the inferred evolutionary relationships among the most abundant duplicated gene found through the analysis of DNA repair machineries from the human pathogens studied here, we have undertaken a phylogenetic analysis of RAD3 helicase orthologues in *S. cerevisiae*, *E. histolytica*, *T. vaginalis*, *G. lamblia* and *P. falciparum*. We evaluated the minimum evolution of RAD3 proteins through the construction of Neighbor-Joining phylogenetic tree using the *MEGA* version 5.05 [Tamura et al., 2011]. The robustness was established by bootstrapping test, involving 500 replications of the data based on the criteria of 50% majority-rule consensus (**Fig. 3**). Two main branches that came from a common ancestor can be observed. On one branch, *T. vaginalis* RAD3 parologues are clustered into two sister proteins pairs (A2E1B9 and A2ELX1, A2E4I6 and A2DDD4), that have each evolved from the same ancestor. Besides, *E. histolytica* C4M6T8 is closer to *T. vaginalis* A2E4I6 and A2DDD4, than to its own paralogues. The other branch supports *T. vaginalis* A2G2G8 that is closely related to yeast and *P. falciparum* RAD3 proteins that came off the same node. Interestingly, these two organisms only have one *rad3* gene. This branch also includes *E. histolytica* C4M8K7 and C4M8Q4 sister proteins pair. Intriguingly, the two *Giardia* RAD3 proteins have emerged from different nodes and appeared to be more related to orthologues from other species than to each other; particularly, the branch supporting *Giardia* A8B495 also includes *Trichomonas* A2E1B9 and A2ELX1, while *Giardia* A8BYS3 is on the other branch, isolated from the other proteins, such as *Trichomonas* A2F1W2, which suggested that these proteins have evolved early.

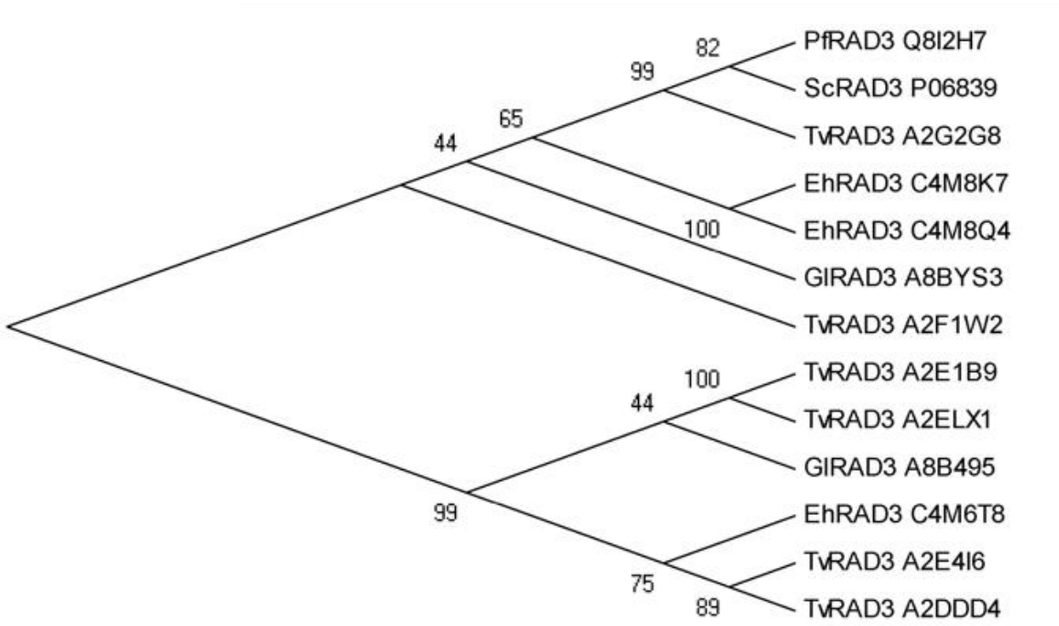

Fig. 3. **Phylogenetic relationships between RAD3 from** *S. cerevisiae*, *E. histolytica*, *T. vaginalis*, *G. lamblia* **and** *P. falciparum*. The unrooted tree was created with the MEGA 5.05 program using the Neighbor Joining algorithm based on ClustalW. Numbers above the tree nodes indicate the percentage of times that the branch was recovered in 500 replications.

5. Molecular organization of the MNR complex

The MRE11–RAD50–NBS1 (MRN) complex is considered to have an imperative function in DSB repair. This protein complex operates as DSB sensor, co-activator of DSB-induced cell cycle checkpoint signaling, and as a DSB repairs effector in both the HRR and NHEJ pathways [Taylor et al., 2010; Rass et al., 2009]. Additionally, it has also been found to associate with telomeres maintenance at the ends of linear chromosomes. MRE11 and RAD50 orthologues have been reported in all taxonomic Kingdoms. MRE11, RAD50, and XRS2 (the *S. cerevisiae* homologue of vertebrate-specific NBS1) were initially recognized through yeast resistance to DNA damage induced by UV light and X-rays and meiotic recombination studies [Ogawa et al., 1995]. To efficiently perform these functions, this complex has shown particular enzymatic roles. Biochemical experiments have revealed that the phosphoesterase domain of MRE11 works as both a single-and double-stranded DNA endonuclease, besides as 3´–5´ dsDNA exonuclease [D'Amours & Jackson, 2002]. Furthermore, RAD50 and NBS1/Xrs2 are able to promote the activity of MRE11, in an ATP dependent manner [Paul & Gellert, 1998]. ATP binding by RAD50 stimulates the binding of the MR complex to 3´ overhangs and, also, ATP hydrolysis is required to arouse the cleavage of DNA hairpins, inducing modification of endonuclease specificity via DNA relaxing [Paull & Gellert, 1998; de Jager et al., 2002].

In this chapter, we have identified the presence of *Mre11* and *Rad50* genes in the genome of *E. histolytica*, *T. vaginalis*, *G. lamblia* and *P. falciparum.* However, all analyzed pathogenic eukaryotic cells, with the exception *of E. histolytica,* lack the *Xrs2* homologue. The absence of a NBS1/Xrs2 homologous sequence in the other parasites might seem antagonistic to the idea of the existence of an active MRN complex. However we cannot discard the possibility that these microorganisms use a very divergent NBS1 protein, or even that this third component could be unessential. In order to initiate the characterization of components of MRN complex in these parasites, we studied the structural and evolutionary relationships between MRE11, RAD50 and NBS1 through PSI-BLAST analysis in comparison to human and yeast orthologues. This program generates a weighted profile from the sequences detected in the first pass of a gapped-BLAST search and iteratively searches the database using this profile as the query, allowing the inclusion of sequences with e-value cut off higher than 0.01 [Alschult et al., 1997]. Using the e-value threshold as a similarity measure, we evidenced a close relation between putative EhMRE11, HsMRE11, ScMRE11, TvMRE11 and PfMRE11. Conversely, GlMRE11 turned out to be less similar to the others, being closer to *E. histolytica* and *T. vaginalis* proteins (**Fig. 4**). On the other hand, analysis of RAD50 orthologues exposed a great conservation of these proteins, since all e-value threshold were <0.0001. As we have previously reported, EhNBS1 is closer to its human homologue than yeast [Lopez-Casamichana et al., 2007].

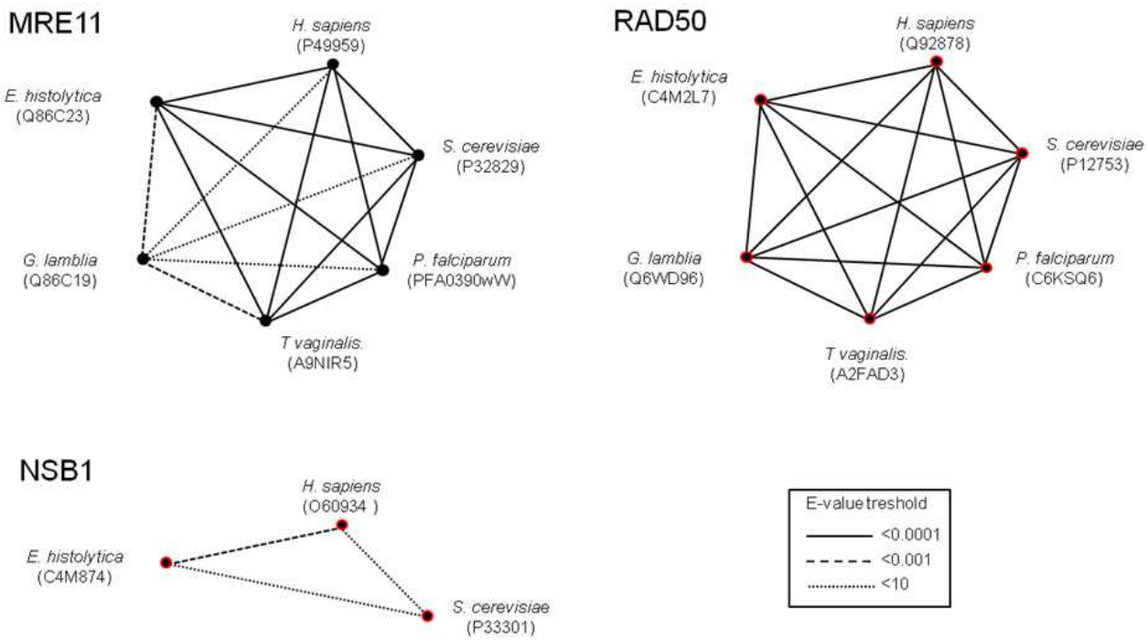

Fig. 4. **Individual protein relationships of MRN complex in pathogenic eukaryotic cells.** Similarity was evaluated through PSI-BLAST analysis. The width of connecting lines indicates similarity level.

To better understand the functionality of MRN complex in these parasites, predicted amino acid sequences of RAD50 and MRE11 were compared through multiple alignment using ClustalW software (http://www.ebi.ac.uk/ clustalw/). Reported functional and structural domains were surveyed using Prosite (http://www.expasy.org/tools/scanprosite/), Pfam (http://www.sanger.ac.uk /Software/Pfam/), SMART (http://smart.emblheidelberg.de/) and Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motifscan) programs. For all studied parasites, our search revealed that the MRE11 orthologues contain the N-terminal Mn2+/Mg2+-dependent nuclease domain including the five conserved phosphoesterase motifs described in yeast protein [Hopkins & Paull, 2008]. Moreover, C-terminal DNA binding domains were also identified [Williams et al., 2007; D'Amours & Jackson, 2002] (**Fig. 5A**).

RAD50 proteins displayed sequence and organizational homology to structural maintenance of chromosome (SMC) family members that control the higher-order structure and dynamics of chromatin. The N-terminal Walker A and C-terminal Walker B nucleotide binding motifs, which associate one with another to form a bipartite ATP-binding cassette (ABC)-type ATPase domain, were predicted [Hopfner et al., 2000; Hopfner et al, 2001]. Furthermore, amino acids flanking Walker motifs form coiled-coil configurations that converge with the cysteine zinc hook (CysXXCys) motif [Hopfner et al., 2002] (**Fig. 5B**). In the interphase of Walker domains, there are two MRE11 binding sites. Formation of the stable MRE11-RAD50 complex is reached by each unit of the MRE11 dimer binding a RAD50 molecule at the intersection of its globular and coiled-coil domains [de Jager et al., 2001a]. Scanning force microscopy experiments have demonstrated that whereas the globular head of the Mre112Rad502 complex links with the ends of linear dsDNA, the two coiled-coil regions of RAD50 are stretchy ''arms", and project outward away from the DNA [Hopfner et al., 2002].

The third member of the MRN complex is NBS1 protein that was only detected in *E. histolytica*, but not in *G. lamblia*, *P. falciarum* neither *T. vaginalis*. We have previously reported that EhNBS1 consists of an FHA domain and adjacent BRCT domains at its N-terminus [Lopez-Casamichana et al., 2007]. In *Homo sapiens*, the FHA domain binds phosphorylated threonine residues in Ser-X-Thr motifs present in DNA damage proteins, including CTP1 and MDC1. The BRCT domains in human NBS1 fix Ser-X-Thr motifs when the serine residue is phosphorylated. These phospho-dependent interactions are significant for recruiting repair machineries and checkpoint proteins to DNA DSBs [Lloyd et al., 2009; Williams et al., 2009]. In reconstitution studies, the affinity of MRE11-RAD50 for DNA and its nuclease activity is further enhanced by the addition of NBS1 [Paull & Gellert, 1999].

6. Molecular organization of the RAD51 recombinase

RAD51 recombinase is an essential protein in HRR pathway that catalyzes strand transfer between a broken DNA and its undamaged homologous strand, allowing damaged region to be repaired [Thacker, 2005] Strand exchange reaction is initiated by RAD51-coating of ssDNA released from DSBs, to generate a nucleoprotein filament. This active thread binds the intact dsDNA substrate, searching and locating homologous sequences, and promoting DNA strand exchange in an ATP-dependent manner, forming a heteroduplex structure [Paques & Haber, 1999]. After DNA damage, RAD51 protein has been observed in nuclear complexes forming discrete foci, which are considered as the recombinational DNA repair

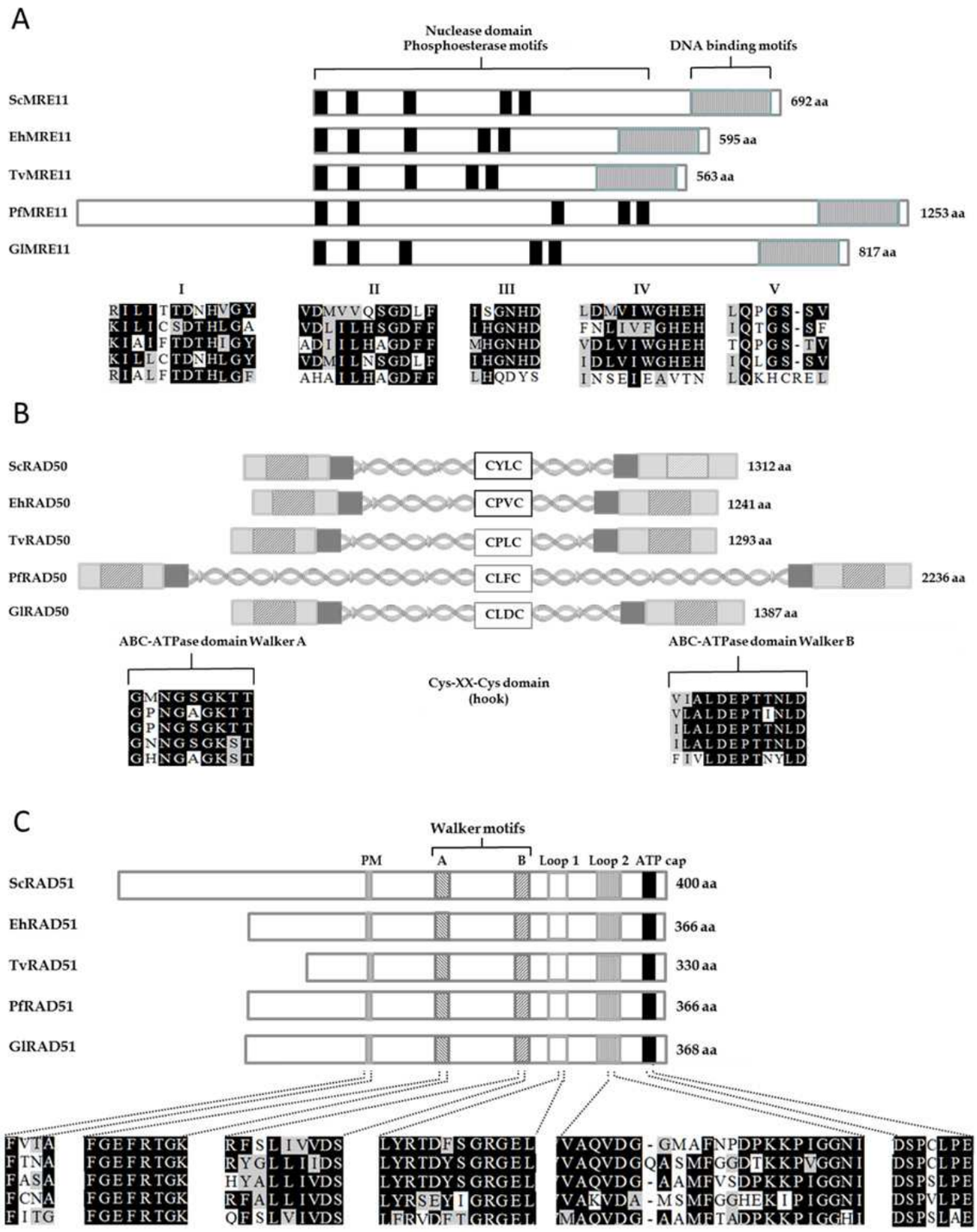

Fig. 5. **Comparison of the amino acids sequence of MRE11, RAD50 and RAD51 proteins of *S. cerevisae E. histolytica, T. vaginalis, P. falciparum* and *G. lamblia*. (A)**. Functional and structural domains of MRE11 proteins. MRE11 phosphoesterase motifs I-V (black

rectangles) and DNA-binding domains (gray rectangles). **(B)**. Functional and structural domains of RAD50 proteins. Walker A (N terminus) and Walker B (C terminus) are marked in the ABC-ATPase domains (gray rectangles), while MRE11-binding sites flank Walker motifs (dark gray rectangles) and the CysXXCys hook domain (white rectangles) are labeled in the coiled-coil central regions. (**C)**. Functional and structural domains of RAD51 proteins. Polymerization motif (PM), as well as Walker A and B motifs, L1 and L2 regions and ATP cap appear as colored rectangles. Boxshade panels: black boxes, identical aa; grey boxes, conserved substitutions.

sites [Tashiro et al., 2000]. RAD51 proteins have been identified in *Trypanosoma brucei* and *Plasmodium falciparum* parasites, which perform HRR to switch the expression of genes encoding surface membrane glycoproteins and generate antigenic variation [Conway et al., 2002; Freitas-Junior et al., 2000]. Furthermore, recombinational rearrangements are responsible for amplification of the multidrug resistance *pfmdr1* gene in *P. falciparum* [Triglia et al., 1991] demonstrating the relevance of HRR to generate genomic versatility and plasticity in protozoan parasites. Molecular analysis and functional assays confirmed that recombinant EhRAD51 is a *bonafide* recombinase that is able to catalyze specific ssDNA transfer to homologous dsDNA forming the three-stranded pairing molecule called D-loop structure. In addition, *E. histolytica RAD51* sequence conserves the typical architecture of RECA/RAD51 family members [Lopez-Casamichana et al., 2008]. Amino acid sequences multiple alignment of RAD51 orthologues from *E. histolytica, S. cerevisae, T. vaginalis*, *G. lamblia* and *P. falciparum* revealed that all these proteins share functional and structural conserved motifs (**Fig. 5C**). Each of them contains the putative polymerization motif (PM), which tethers individual subunits to form quaternary assemblies in human RAD51 protein [Bell, 2005]. We also identified the ATPase Walker A or phosphate binding loop (P-loop) and Walker B motifs residues, the ssDNA binding loops L1 and L2, as well as the ATP stacking motif or ATP cap at the C terminus, which are essential for nucleofilament assembling and ATP hydrolysis in RAD51/RECA-like recombinases [Shin et al., 2003; Conway et al., 2004].

7. Conclusions

Protozoan parasites are continuously subjected to the effects of antiparasitic drugs and host immune system attacks, which can affect their genome stability and therefore, their survival. In order to maintain the integrity of their DNA molecules, parasites have developed several mechanisms that are efficient to detect and accurately repair damaged nucleotides. Bioinformatic analyses of fully sequenced genomes are useful to identify molecular machineries for DNA repair in protozoan parasites of clinical relevance such as *Entamoeba histolyica*, *Giardia lamblia*, *Plasmodium falciparum* and *Trichomonas vaginalis,* which have a world-wide distribution with a high prevalence in developing countries. The computational data presented here provide new information on the evolution of DNA repair proteins and their potential relevance for DNA damage response in these major human pathogens. Future directions would include functional assays, as well as protein expression and protein-protein interactions analysis for the most relevant proteins, in order to contribute to the further elucidation of mechanisms regulating genome integrity in these organisms.

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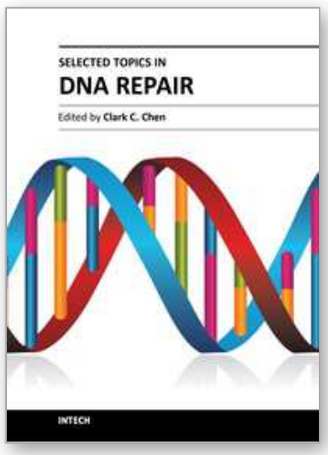

Selected Topics in DNA Repair

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This book is intended for students and scientists working in the field of DNA repair, focusing on a number of topics ranging from DNA damaging agents and mechanistic insights to methods in DNA repair and insights into therapeutic strategies. These topics demonstrate how scientific ideas are developed, tested, dialogued, and matured as it is meant to discuss key concepts in DNA repair. The book should serve as a supplementary text in courses and seminars as well as a general reference for biologists with an interest in DNA repair.

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