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Review

DNA repair mechanisms in eukaryotes: Special focus in *Entamoeba histolytica* and related protozoan parasites

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ABSTRACT

Eukaryotic cell viability highly relies on genome stability and DNA integrity maintenance. The cellular response to DNA damage mainly consists of six biological conserved pathways known as homologous recombination repair (HRR), non-homologous end-joining (NHEJ), base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), and methyltransferase repair that operate in a concerted way to minimize genetic information loss due to a DNA lesion. Particularly, protozoan parasites survival depends on DNA repair mechanisms that constantly supervise chromosomes to correct damaged nucleotides generated by cytotoxic agents, host immune pressure or cellular processes. Here we reviewed the current knowledge about DNA repair mechanisms in the most relevant human protozoan pathogens. Additionally, we described the recent advances to understand DNA repair mechanisms in *Entamoeba histolytica* with special emphasis in the use of genomic approaches based on bioinformatic analysis of parasite genome sequence and microarrays technology.

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1. Introduction

The maintenance of DNA integrity is vital for genomic stability and cell viability. The genome is under constant attack from endogenous metabolic processes and exogenous environmental factors that can alter its chemical structure. DNA lesions consist of single strand breaks (SSB), double strand breaks (DSB), inter- and intra-strand crosslinks and base modifications, as well as oxidation and alkylation of bases, formation of bulky chemical adducts and crosslinking of adjacent nucleotides. DNA damage can lead to multiple lesions including mutations, deletions, insertions, translocations, and loss of chromosomes and essential genetic information. This genome instability can consequently induce apoptosis and fatal diseases. It is therefore of vital importance that cells repair these lesions accurately and faithfully. The cellular response to DNA damage includes processes that require damage detection, activation of checkpoint pathways, cell cycle arrest and DNA repair mechanisms initiation. Several biological pathways operate in a concerted manner to minimize genetic information loss each time a DNA lesion occurs. Eukaryotic DNA repair can be divided into six highly conserved pathways: homologous recombination repair (HRR), non-homologous end-joining (NHEJ), base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and methyltransferase repair. Finally, recently described pathways known as transcription-coupled BER, break-induced replication, and nucleotide incision repair, are not discussed in this review.

Protozoan parasites are continuously exposed to drug action and host immune pressure, which can affect the stability of their genome. Their survival depends on DNA repair mechanisms that constantly supervise chromosomes to correct damaged nucleotides. Here we performed an exhaustive review on what is known about DNA repair mechanisms in protozoan that are important human pathogens, such as *Plasmodium falciparum*, the parasite responsible for malaria, *Trypanosoma brucei* the causative agent of sleeping sickness, *Leishmania*, the parasite responsible for the different forms of leishmaniasis, *Giardia* that causes diarrheal diseases, and *Entamoeba histolytica*, the etiological agent of amoebiasis. Taking advantage of the recent publication of the *E. histolytica* genome, we also compiled a widespread record of genes whose predicted products are homologous to members of the six major pathways of DNA repair previously described in yeast and human. Our study revealed that many of these fundamental DNA repair pathways have been conserved throughout eukaryotes evolution. However, the knowledge about DNA repair mechanisms in protozoa is still poor.

2. DNA damage and repair pathways

DSB, the most detrimental lesions of DNA, arises from endogenous sources including reactive oxygen species generated during cellular metabolism, collapsed replication forks, and nucleases action. DSB can also be directly or indirectly caused by exogenous sources such as ionizing radiation and chemical agents. This kind of injury can be repaired by HRR and NHEJ pathways (Fleck and Nielsen, 2004). HRR is also an accurate mechanism to generate genetic diversity within a given cell population. Other kinds of DNA damage include non-bulky lesions produced by alkylation, oxidation or deamination of bases, photoproducts induced by ultraviolet light (UV) and other bulky lesions, such as inter- and intra-strand crosslinks, as well as base mismatches and small insertion/deletion loops (IDL) introduced during replication. These aberrant nucleotides in ssDNA can be successfully restored by BER, NER and MMR, respectively, using the complementary strand as template for DNA synthesis (Krokan et al., 1997; Prakash and Prakash, 2000; Marti et al., 2002). In contrast, DNA alkylation at the O^6 position of guanine, which is regarded as one of the most critical events leading to induction of mutations and cancers, is repaired through the methyltransferase pathway without synthesis of new DNA strand (Sassanfar et al., 1991).

3. Homologous recombination repair (HRR)

3.1. HRR in yeast and human

In yeast and human, DSB are detected by MRE11-RAD50-NBS1 (XRS2 in yeast) complex and converted to 3′ ssDNA tails, which are subsequently bound by RPA. Then, RAD52 protein interacts with RPA and promotes RAD51 binding to ssDNA, which may be stabilized by RAD51 paralogues (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 in human, RAD55 and RAD57 in yeast). Subsequently, the RAD51 bound to ssDNA invades a homologous molecule in a reaction stimulated by RAD54. After DNA synthesis and ligation, two Holliday junctions are formed and branch migration can occur. The Holliday junctions are finally resolved by other proteins including Bloom and Werner DNA helicases and ligases (Aylon and Kupiec, 2004). Defects in HRR proteins cause ataxia telangiectasia, Nijmegen breakage, Bloom and Werner human syndromes, as well as hereditary breast and ovarian cancer associated with mutations in BRCA1 or BRCA2 genes.

3.2. HRR in protozoan parasites

HRR is the major pathway of DSB repair in lower eukaryotes including protozoan parasites (Bhattacharyya et al., 2004). *P. falciparum* has homologs for RAD51, MRE11, RAD54 and RPA (lacking the RAD52 interacting domain) which are probably involved in the extensive HRR-mediated DNA rearrangements exhibited by this parasite (Gardner et al., 2002; Voss et al., 2002). The *PfRad51* gene is overexpressed in the mitotically active schizont stage and in response to methyl methane sulfonate, indicating its participation in DNA repair (Bhattacharyya and Kumar, 2003). DMC1, RAD51 and related factors (McCulloch and Barry, 1999; Proudfoot and McCulloch, 2005, 2006), MRE11 (Tan et al., 2002), and sirtuin (Alsford et al., 2007) proteins have been identified in *T. brucei*. RAD51 has a role in the process of antigenic variation, enabling trypanosomes to escape host immune response (McCulloch and Barry, 1999). *Leishmania*, which is known to use HRR pathway to amplify drug resistance genes, possesses a RAD51 homolog that is induced by DNA damaging agent and exhibits DNA-binding and DNA-stimulated ATPase activities (McKean et al., 2001). The survey of the genome of *Giardia*, allowed the detection of putative meiotic genes, including *Rad50*/*Mre11*, *Rad52* and

Dmc1, that could be involved in meiotic recombination machinery by HRR although their functional relevance has not been determined yet (Ramesh et al., 2005).

E. histolytica genome contains genes homologous to yeast and human RAD52 epistasis group involved in HRR (Lopez-Casamichana et al., 2008); particularly, this parasite has *Ehmre11*, *Ehrad50* and *Ehnbs1* (*xrs2* in yeast) genes, which could encode the parasite putative MRN complex (Lopez-Casamichana et al., 2007) that represents the key sensor of DNA DSB in other organisms (Longhese et al., 2006). *E. histolytica* also contains genes encoding the EhRAD51 recombinase and its paralog EhRAD51C, EhRAD52, EhRAD54, EhRAD54B, EhRAD59 (EhRAD52/22 in *E. histolytica* Pathema database), as well as SCRPA1 and SCRPA2, which correspond to yeast RPA subunits, a highly conserved ssDNA binding protein involved in both HRR and NER. Furthermore, *E. histolytica* genome contains genes for yeast EXO1 nuclease and SGS1 helicase, the homolog of human BLM and WRN helicases, in addition to a gene for the checkpoint/DNA end-processing RAD24 protein. In contrast, this parasite does not seem to have homologs for the other *rad51* paralogs (*rad51b*, *rad51d*), HPR5 helicase nor 9-1-1 complex (RAD17-MEC3-DDC1) (Supplementary data 1).

4. Non-homologous end-joining (NHEJ)

4.1. NHEJ in yeast and human

DNA repair by NHEJ initiates when KU70-KU80 dimers (KU complex) bind both DSB ends. In higher eukaryotes the DNA protein kinase catalytic subunit (DNA-PKcs) is subsequently recruited. Once DSB are recognized and the ssDNA filaments of the lesion are processed, a DNA polymerase synthesizes short DNA strands and DNA ends are linked together in the presence of the XRCC4/LIF1-DNA ligase IV/DNL4 complex. DSB that are not suitable for ligation may be processed by MRE11-RAD50-NBS1 and FEN1/RAD27 nuclease. Given that DNA is repaired by synthesis and ligation without using a homologous sequence, NHEJ is often associated to nucleotide loss (Daley et al., 2005).

4.2. NHEJ in protozoan parasites

T. brucei has KU70 and KU80 from NHEJ machinery, although characterization of null mutants showed that they are involved in telomere maintenance, but not in DNA repair (Burton et al., 2007).

Genes encoding putative DNL4/LIF1 ligase complex, RAD27 nuclease and MRE11/RAD50/NSB1 proteins are represented in the *E. histolytica* genome (Supplementary data 1 and 2), which strongly suggests that NHEJ pathway could be functional in this parasite. Intriguingly, *E. histolytica* genome contains a sequence for KU70 but not for KU80 subunit (Supplementary data 2). As both proteins operate as a single unit (KU complex) to recognize DSB sites and recruit other DNA repair factors, this finding could appear contradictory. However, the absence of a KU subunit has been reported for other eukaryotic parasites like *Trichomonas vaginalis* (Carlton et al., 2007) and *Encephalitozoon cuniculi* (Gill and Fast, 2007). Additionally, the yeast KU70/KU80 core is homologous to a smaller bacterial protein that performs the same function, suggesting that some NHEJ subunits may be dispensable (Hefferin and Tomkinson, 2005). Thus, it is possible that *E. histolytica* uses a NHEJ pathway mediated by a highly divergent KU80 protein.

5. Base excision repair (BER)

5.1. BER in yeast and human

BER mainly repairs non-bulky lesions produced by alkylation, oxidation or deamination of bases. During BER, damaged bases are recognized by a specific DNA glycosylase, which cleaves the N-glycosydic bond between the base and the deoxyribose to remove the base (Krokan et al., 1997). After cleavage, the damaged base is released and an apurinic/apyrimidinic (AP) site is created. An AP site can also occur spontaneously and represents damage by itself. Bifunctional glycosylases have an intrinsic AP lyase activity, which cleaves the sugar phosphate backbone 3′ to the AP site. After base was removed from DNA, a non-specific endonuclease (APN1 or APN2) releases the deoxyribose phosphate to produce a gap, which is filled by DNA polymerase β, whereas the FEN1/RAD27 endonuclease eliminates the displaced DNA and the CDC9 ligase closes up the nick. After strand displacement by Pol β, and Pol δ or Pol ε, a flap structure is formed, which is cleaved by FEN1/RAD27. The RAD1-RAD10 and MUS81-MMS4 endonucleases are also believed to play minor roles in BER by processing the DNA 3′ end (Boiteux and Guillet, 2004). No human disease is currently known to be associated with defects in BER.

5.2. BER in protozoan parasites

Several BER components, including uracyl DNA glycosylases, AP endonucleases, DNA polymerase, FEN-1, DNA ligase I and ERCC1 factors, have been identified in *P. falciparum* (Gardner et al., 2002). Particularly, PfFEN-1 protein, which has the classical DNA structure-specific flap endonuclease and 5′–3′ exonuclease activities, was able to generate a nicked DNA substrate that was repaired by recombinant Pf DNA ligase I *in vitro* (Casta et al., 2008). *T. brucei* also has an AP endonuclease that confers resistance to oxidizing agents in DNA repair-deficient *Escherichia coli* (Perez et al., 1999). Similarly, *Leishmania* possesses an AP endonuclease, which is active in AP sites repair in *E. coli*; moreover, its expression in AP endonuclease-deficient *E. coli* conferred resistance to alkylating and oxidizing agents (Perez et al., 1999). Additionally, AP endonuclease overexpression in *Leishmania* prevented DNA fragmentation and increased H_2O_2 and methotrexate resistance (Gallego et al., 2005). The AP protein–DNA complex shares structural characteristics with previously characterized homologs and the purified enzyme possesses the classical AP endonuclease and 3′-phosphodiesterase activities (Vidal et al., 2007). Additionally, *L. infantum* has a DNA polymerase β-like with 5′-deoxyribose-5-phosphate lyase activity (Alonso et al., 2006). Both BER enzymes are thought to play an important role for *Leishmania* survival in the highly oxidative environment within the host macrophage.

E. histolytica BER pathway appears to be largely incomplete, lacking MAG1, OGG1, MUS81, MMS4, as well as APN1 and APN2 endonucleases (Supplementary data 3). The absence of OGG1 is compatible with the lack of a mitochondrial compartment, where repair of oxidative damage to mitochondrial DNA takes place. In contrast, *E. histolytica* genome has genes for DNA glycosylase AP lyase NTG1 and the uracil DNA glycosylase UNG1, which could be sufficient to perform the cleavage of the glycosydic bond between the base and the deoxyribose. Additionally, amoeba genome also encodes RAD1, RAD10, RAD27, CDC9, and PCNA factors, which also play roles in other DNA repair pathways and cell signaling.

6. Nucleotide excision repair (NER)

6.1. NER in yeast and human

The main function of NER pathway is to remove photoproducts induced by ultraviolet light (UV) and other bulky lesions, such as inter- and intra-strand crosslinks. NER consists of two subpathways: global genome repair (GGR), which removes damage in the overall genome, and transcription-coupled repair (TCR), which specifically repairs the transcribed strand of active genes. UV-DDB, consisting of DDB1 and DDB2, and XPC-hHR23B are involved in the

recognition step of GGR. Then, NEF4 complex, which is composed of RAD7 and RAD16, is recruited to the damaged site Finally, RAD7 binds the NF2 complex (RAD4/RAD23), stabilizing and increasing DNA binding recognition. In contrast, TCR initiation involves RNA polymerase arrest and the participation of CSA, CSB, RAD26 and RAD28 specific proteins. The protein complexes acting in further steps of both NER pathways are likely to be identical; they include NEF1 (RAD1–RAD10–RAD14) and NEF3, which contains RAD2, RAD3, RAD25 and SSL1, as well as the transcription elongation factor IIH (TFIIH), a complex consisting of nine subunits (Prakash and Prakash, 2000). Two subunits of TFIIH, XPB and XPD, exhibit helicase activity of opposite polarity, and unwind DNA around the lesion. The next factors that bind to the damaged site are XPG and XPA–RPA. XPA–RPA verifies whether the NER complex is correctly assembled and ensures proper incision of the damaged strand. After binding of XPF–ERCC1, dual incision occurs by XPG and XPF-ERCC1, which cut 3′ and 5′ ends, respectively. Repair is completed by DNA synthesis and ligation. Defects in NER proteins cause xeroderma pigmentosum, whereas cockayne syndrome and trichothiodystrophy are due to impaired TCR.

6.2. NER in protozoan parasites

P. falciparum XPB/RAD25, XPG/RAD2 and XPD/RAD3 are the only NER pathway members that have been reported in protozoan parasites (Gardner et al., 2002). In contrast, most genes involved in the two NER subpathways, GGR and TCR, are represented in *E. histolytica* genome (Supplementary data 4), suggesting that NER mechanism could be potentially active in this eukaryotic parasite. Particularly, *E. histolytica* has genes for DDB1 which could initiate the recognizing step of GGR, RAD7 and RAD16 (NF4 complex), as well as RAD4 and RAD23 (NF2 complex). It also has *rad26* and *rad28* genes potentially involved in TCR. In addition, *E. histolytica* genome encodes proteins acting further downstream in GGR and TCR, such as NEF1 complex (RAD1, RAD10, RAD14), NEF3 complex (RAD2, RAD3, RAD25), and TFIIH complex subunits (SSL1, TFB1, TFB3, TFB4), but it does not contain the *rad14* gene.

7. Mismatch repair (MMR)

7.1. MMR in yeast and human

The main task of MMR is to remove base mismatches and small insertion/deletion loops (IDL) introduced during replication. In *yeast*, single base mismatches are recognized by MUTSα (MSH2/MSH6) and IDL are sensed by MUTSβ (MSH2/MSH3). PCNA protein is also engaged in MMR, maybe supporting the damage detection and strand discrimination steps. Another complex named MUTLα, composed by MSH1 and PMS1 proteins, binds both MUTSα and MUTSβ to promote their efficient binding to mismatches. Finally, EXO1 removes these regions and gaps filling and closing are completed by DNA polymerase and DNA ligase, respectively (Marti et al., 2002). The inactivation of human MMR homologous proteins is cause of hereditary non-polyposis colorectal cancer.

7.2. MMR in protozoan parasites

P. falciparum possesses PfMSH2-1, PfMSH2-2, PfMSH6, PfMLH1 and PfPMS1 proteins. Inhibition of *PfMSH2-2* gene increased mutation rate and microsatellite polymorphism, indirectly demonstrating its relevance in MMR and microsatellite slippage prevention (Bethke et al., 2007). MLH1, PMS2, MSH2, MSH3 and MSH8, have also been reported in *T. brucei*. Particularly, genetic *knock-out* of *MSH2* and *MLH1* genes resulted in increased sequence variation at microsatellite loci and tolerance to alkylating agents (Augusto-Pinto et al., 2001; Bell et al., 2004). The survey of the genome of *Giardia* allowed the detection of *Msh2*, *Msh6*, *Msh1*, *Msh2* and *PMs1* genes involved in meiotic recombination machinery by MMR, although their functional relevance is still unknown (Ramesh et al., 2005).

E. histolytica genome survey showed the presence of almost all *S. cerevisiae* MMR genes in this parasite, including components of MUTSα (MSH2/MSH6) and MUTSβ (MSH2/MSH3) heterodimers, with the presence of two *msh2* genes. In addition, *E. histolytica* has *mlh1*, *pms1*, *exo1* and *pcna* genes (Supplementary data 5), which strongly suggested that MMR could be an active DNA repair pathway in this organism. Notably, both EXO1 and PCNA factors also play roles in HRR and BER, respectively.

8. Methyltransferase repair

Methyltransferases, including the canonical MGT1 protein found in eukaryotes, catalyze the irreversible transfer of methyl groups from DNA to their own cysteine residues (Sassanfar et al., 1991). To our knowledge, no methyltransferase has been reported in protozoa yet. Similarly, *E. histolytica* genome does not seem to contain a *mgt1* gene, suggesting that this pathway does not operate in this parasite. The same omission has been reported in *E. cuniculi*, a member of a distinctive group of unicellular parasitic eukaryotes called microsporida (Gill and Fast, 2007).

9. Recent insights in DNA repair in *E. histolytica*

9.1. Transcriptomic analysis of E. histolytica in response to DNA damage

The study of DNA repair mechanisms in *E. histolytica* was performed using a 254 nm UV-C light irradiation model to efficiently induce DNA damage in trophozoites (Lopez-Casamichana et al., 2008). Direct evidence for DSB was provided by TUNEL, FACS and single-cell gel electrophoresis assays. Moreover, cell survival after DNA damage suggested that efficient DNA repair mechanisms were activated. Microarray assays evidenced a weak transcriptional activation after DNA damage (Weber et al., 2009). Several genes correspond to cell cycle, signal transduction and DNA damage repair pathways, including *EhMre11*, *EhRad50* and *EhRad54* genes from HRR, and *EhRad23* and *EhDdb1* genes from NER pathways, as well as three ORFs codifying for iron–sulfur clusters-containing proteins, which are thought to act as cofactors in DNA repair (Lukianova and David, 2005). However, most genes were involved in unexpected pathways, such as RNA processing, protein synthesis and degradation, cell structure, in addition to numerous hypothetical genes.

As it has been described in human, it is possible that EhRAD23 interacts with EhRAD54, which is homologous to the human RAD54 protein that belongs to the Swi2/Snf2 family of DNA-stimulated ATPases and exhibits chromatin remodeling activity *in vivo* through interaction with the RAD51-DNA complex (Mazin et al., 2003; Alexiadis et al., 2004; Wolner and Peterson, 2006). The specific phosphorylation of EhH2AX histones after UV-C irradiation also suggested that some chromatin components could be a dynamic substrate for DNA repair in *E. histolytica* (Lopez-Casamichana et al., 2008).

E. histolytica has a large family of 20 EhDEAD and 13 EhDExH-box RNA helicases (Marchat et al., 2008). Particularly, EhDEAD1 protein, a conserved DEAD-box RNA helicase with ATPase and ATP-dependent RNA unwinding activities, seems to participate in S to G2/M phase transition during cycle progression (Lopez-Camarillo et al., 2008). Microarray data showed that four putative RNA helicases were specifically modulated in trophozoites in response to DNA damage (Weber et al., 2009), although their homologous proteins have been involved in cell cycle, rRNA biogenesis, and

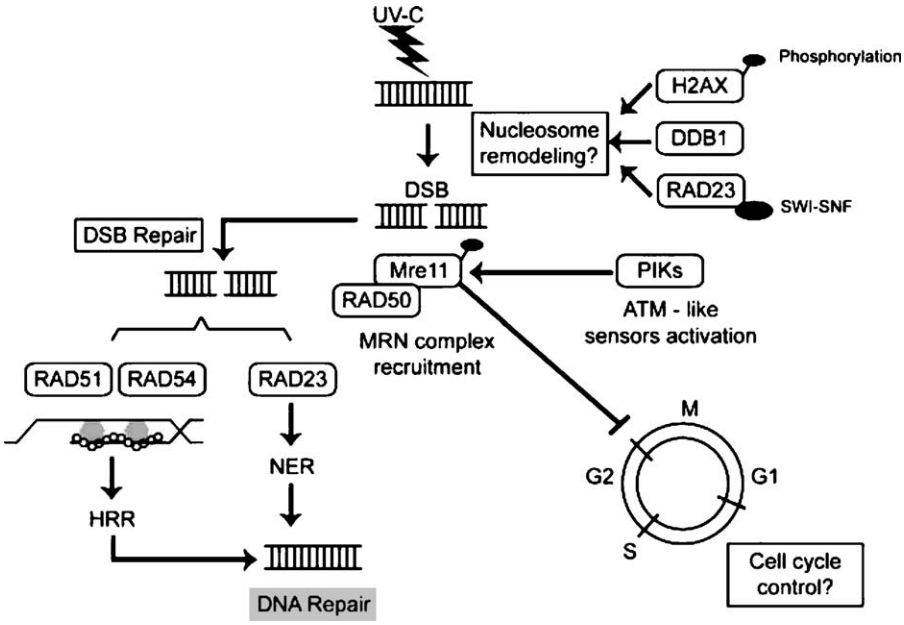

Fig. 1. Hypothetical working model for DNA damage response in *Entamoeba histolytica*.

mRNA non-sense mediated decay pathway in other organisms, but not in DNA damage response.

9.2. EhRAD52 epistasis group genes and DNA repair in E. histolytica

Both microarray and RT-PCR assays evidenced that the transcriptional response of RAD52 epistasis group-related genes was not coordinated after DNA damage (Lopez-Casamichana et al., 2008; Weber et al., 2009). Particularly, *Ehrad51* mRNA expression was upregulated at 30 min after UV-C irradiation. Immunolocalization assays confirmed the overexpression of EhRAD51 after UV-C treatment and evidenced its redistribution from cytoplasm to nucleus of trophozoites during the first 3 h after DNA damage. 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 (Lopez-Casamichana et al., 2008).

On the other hand, *Ehrad54* mRNA was upregulated at 5 min after DNA damage and mRNA levels were decreased at 30 min after UV-C irradiation (Lopez-Casamichana et al., 2008; Weber et al., 2009). Extensive *in silico* analysis revealed that EhRAD54 has all the molecular characteristic of RAD54 proteins. Western blot assays confirmed the coordinated expression of EhRAD51 and EhRAD54 factors in trophozoites nucleus at 5 min after UV-C irradiation, suggesting that both proteins could be participating in early steps of DNA repair by HRR in *E. histolytica* (our unpublished data).

9.3. Hypothetical working model for DNA damage response in E. histolytica

Based on our experimental observations, we propose a hypothetical working model for DNA damage response in *E. histolytica* trophozoites (Fig. 1). Three proteins could be early actors in DSB detection and processing. At 5 min after UV-C irradiation, the *E. histolytica* RAD23 a protein involved in NER pathway could interact with Swi2/Snf2 chromatin remodeling proteins to promote nucleosome modification and facilitate the recruitment of DNA repair proteins at DSB sites. DDB1, which initiates NER in other organisms, could also be participating in chromatin remodeling at DSB surrounding sites, as well as the phosphorylated H2AX histones. Three phosphatidylinositol 3-kinases (PIK) proteins could represent the sensors of DSB in *E. histolytica*. After DSB detection by chromatin remodeling proteins and PIKs, MRE11 and RAD50 proteins could be recruited to DSB sites to initiate HRR pathway. Notably, MRE11 could also act as a cell cycle modulator. Other proteins represented by RAD51 and RAD54, as well as RAD23, could be subsequently activated to perform DNA repair through HRR and NER pathways, respectively.

10. Concluding remarks

Here we showed that factors participating in the fundamental DNA repair pathways described in yeast and human appeared to be conserved in the most relevant protozoan parasite for human health. Particularly, DNA microarray, bioinformatic analyses and functional data provide new information on the evolution of DNA repair proteins and their potential relevance for DNA damage response in *E. histolytica*. Future directions will include functional assays of recombinant protein expression and protein–protein interaction studies in order to contribute to the further elucidation of mechanisms regulating genome integrity in the protozoan parasite responsible for human amoebiasis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2009.06.024.

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