

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

**8-Oxoguanine DNA glycosylase-1 links DNA repair to cellular responses via
the activation of the small GTPases, Ras and Rac1**

by

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DEBRECEN, 2015

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SUPPLEMENTARY 1.

Hajas G, Bacsı A, Aguilera-Aguirre L, Hegde ML, Tapas KH, Sur S, Radak Z, Ba X, Boldogh I. 8-Oxoguanine DNA glycosylase-1 links DNA repair to cellular signaling via the activation of the small GTPase Rac1.

Free Radic Biol Med. 2013 Aug;61:384-94

SUPPLEMENTARY 2.

German P, Szaniszló P, **Hajas G**, Radak Z, Bacsı A, Hazra TK, Hegde ML, Ba X, Boldogh I. Activation of cellular signaling by 8-oxoguanine DNA glycosylase-1-initiated DNA base excision repair.

DNA Repair. (Amst). 2013 Oct;12(10):856-63.

SUPPLEMENTARY 3.

Boldogh I, **Hajas G**, Aguilera-Aguirre L, Hegde ML, Radak Z, Bacsı A, Sur S, Hazra TK, Mitra S. Activation of ras signaling pathway by 8-oxoguanine DNA glycosylase bound to its excision product, 8-oxoguanine.

J Biol Chem. 2012 Jun 15;287(25):20769-73.

SUPPLEMENTARY 4.

Hajas G, Zsiros E, László T, Hajdú P, Somodi S, Réthi B, Gogolák P, Ludányi K, Panyi G, Rajnavölgyi E. New phenotypic, functional and electrophysiological characteristics of KG-1 cells.

Immunol Lett. 2004 Mar 29;92(1-2):97-106.

Abbreviations

8-OH-Ade	8-oxo-7,8-dihydroadenine
8-oxodG	8-oxo-7,8-dihydro-2 -deoxyguanosine
8-oxoG	8-oxo-7,8-dihydroguanine
APC	antigen presenting cell
APE1	apurinic/aprimidinic endonuclease 1
BER	base excision repair
BSA	bovine serum albumin
CCR7	CC type chemokine receptor 7
ChTX	charybdotoxin;
DC	dendritic cell
DNA-PKcs	DNA-dependent protein kinase
DSBs	double-strand breaks
ELISA	enzyme linked immuno-sorbent assay
ExoI	exonuclease 1
FapyG	2,6-diamino-4-hydroxy-5-formamidopyrimidine
FcγR	Fcγ gamma receptor
FU	fluorescence unit
GAP	GTPase activating protein
GC/MS	gas chromatography/mass spectrometry
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GITR	glucocorticoid-induced TNFR-related protein (TNFRSF18)
GG-NER	global genome repair
GM-CSF	granulocyte–monocyte-colony stimulating factor
GST	glutathione-S-transferase
GTP	guanosine triphosphate
GTPγS	guanosine 5-3-O-(thio)triphosphate
H/K/N-Ras	Harvey/Kirsten/neuroblastoma sarcoma virus oncogene homolog
H ₂ DCF-DA	2 -7 -dihydrodichlorofluorescein diacetate
HR	homologous recombination
HSC	hematopoietic stem cell
IPA	Ingenuity Pathways Analysis

IFN- γ	interferon- γ
IL-4/13	interleukin 4/13
LC	Langerhans cell
LC/IDMS	liquid chromatography and isotope dilution mass spectrometry
^{Mant} GDP	GDP (2 -(or-3)-O-(N-methylanthraniloyl)guanosine 5 -diphosphate
^{Mant} GTP	GTP (2 -(or-3)-O-(N-methylanthraniloyl)guanosine 5 -triphosphate
MDR	multidrog resistance
MHC	major histocompability complex
MIP-3	macrophage inflammatory protein
MMR	mismatch repair
MR	mannose receptor
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NAC	N-acetyl-L-cysteine
NOX1/2/3/4	NADPH oxidase type 1/2/3/4
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
Nrf2	NF-E2 related factor 2
OGG1	8-oxoguanine DNA glycosylase-1
OGG1/8-oxoG	OGG1 with bound 8oxoG
p22phox	regulatory subunit of NOXs
PBS	phosphate buffer solution;
PGE2	prostaglandin E2
Pol $\beta/\delta/\epsilon/\iota/\lambda/\mu$	DNA polymerase $\beta/\delta/\epsilon/\iota/\lambda/\mu$
PMA	phorbol-myristilacetate
PCNA	proliferating cell nuclear antigen
PARP1	poly (ADP-ribose) polymerase 1
Rac1	Ras-related C3 botulinum toxin substrate 1
Ras	small GTPase of the Ras superfamily (Rat sarcoma)
RPA	replication protein A
RT-PCR	reverse transcription followed by polymerase chain reaction
SLC	secondary lymphoid-tissue cytokine
SOD	superoxide dismutase
TNF-	tumor necrosis factor-
TC-NER	transcription-coupled repair

This dissertation describes new findings about the DNA glycosylase (OGG1) as an initiator of cellular responses in complex with its substrate the oxidized base 8-oxo-7,8-dihydroguanine (8-oxoG).

1. Introduction

Oxidative stress is an evolutionary driving force that often defined as an imbalance between the prooxidative and the antioxidative sources. It has been connected to serious health disorders like Parkinson's disease (Valko, Leibfritz et al. 2007), Alzheimer's disease (Pohanka 2013), cancer (Halliwell 2007), myocardial infarction (Ramond, Godin-Ribuot et al. 2013), chronic fatigue syndrome (Kennedy, Spence et al. 2005). On the other hand, organisms wouldn't be able to survive without the controlled production of reactive oxidative molecules. Redox sensitive amino acids like methionine play an important role in cellular signaling. The reversible oxidation of methionine can inhibit the phosphorylation of adjacent Tyr/Ser/Thr site influencing main signaling pathways (Hardin, Larue et al. 2009).

1.1 Oxidative stress

The environment is becoming richer and richer source of prooxidants because of the increasing amount of pollutants, chemicals, ionizing and ultraviolet radiation. These sources act directly or via activation of oxido-reductases and/or induction of mitochondrial dysfunction. When the antioxidant system of the cells cannot balance out the increased concentration of reactive oxygen species (ROS), these molecules indiscriminately modify proteins, lipids, and DNA (D'Autreaux and Toledano 2007) and disrupt normal cellular signaling processes.

As a part of their normal physiological activity cells produce ROS molecules. Notable cellular sources of ROS are: mitochondrial leakage during oxidative phosphorylation, xanthine oxidase, cytochromes P450 and NADPH oxidases (NOX1-5, DUOX1-2). These enzymes have the ability to transport electrons across the plasma membrane and to generate superoxide and other downstream reactive oxygen radicals. Phagocytic cells use NADPH oxidase 2 (gp91phox) to produce ROS after engulfing bacteria or viruses (Nathan and Shiloh 2000). In case of frustrated phagocytosis these toxic agents are released and damage surrounding tissues (Cannon and Swanson 1992).

The most common ROS molecules are: superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (OH^{\bullet}), alkoxy-radicals (RO^{\bullet}), peroxy-radicals (ROO^{\bullet}), hydrogen peroxide (H_2O_2), organic

hydroperoxides (ROOH), hypochlorous acid (HOCl), and peroxynitrite (ONOO⁻). The free-radicals can damage lipids via oxidation, which is often referred to as lipid peroxidation. During the reaction the free radicals "steal" electrons from the lipids in membranes. They mostly affect polyunsaturated fatty acids, because of their multiple double bonds. Lipid peroxides can participate in chain reactions that further increase damage to biomolecules like proteins (Negre-Salvayre, Coatrieux et al. 2008). Not only the lipid peroxides, but their degradation products (hydroxy-alkenals) can generate a variety of intra- and intermolecular covalent adducts that have influence on cell signaling, transcription factors and gene expression (Catala 2009).

As a major consequence of ROS formation, proteins are frequently damaged either at specific side chains of amino acids (i.e. by hydrogen peroxide) or non-specifically throughout the backbone (i.e. by hydroxyl radicals). Hydroperoxides can induce further oxidation, chain reactions and stable products that can be used as biomarkers. Most protein damage results in loss of function (enzyme activity, signaling), modified structure (unfolding, aggregation) and altered interactions with other molecules. Most oxidized proteins undergo selective proteolysis by proteasomal and lysosomal pathways, but in some cases, they may contribute to multiple human pathologies (Davies 2012).

One of the most common reactive oxygen species, the hydroxyl radical reacts with DNA by addition to double bonds of heterocyclic DNA bases and by abstraction of an H atom from the methyl group of thymine and each of the C-H bonds of 2-deoxyribose (Teoule 1987). In case of purines, hydroxyl radical can be added to the C4, C5, and C8 positions, generating OH adduct radicals. Depending on their redox properties, the redox environment and the reaction partners, radicals are reduced or oxidized. Product types and yields depend on absence and presence of oxygen and on other conditions (Dizdaroglu 1992). So far more than 20 base lesions have been identified (Fig. 1.) The consequences of these DNA lesions are diverse: they can cause mutations, conformational changes in DNA, deletions, epigenetic changes among others /reviewed by Cooke et al. in (Cooke, Evans et al. 2003)/.

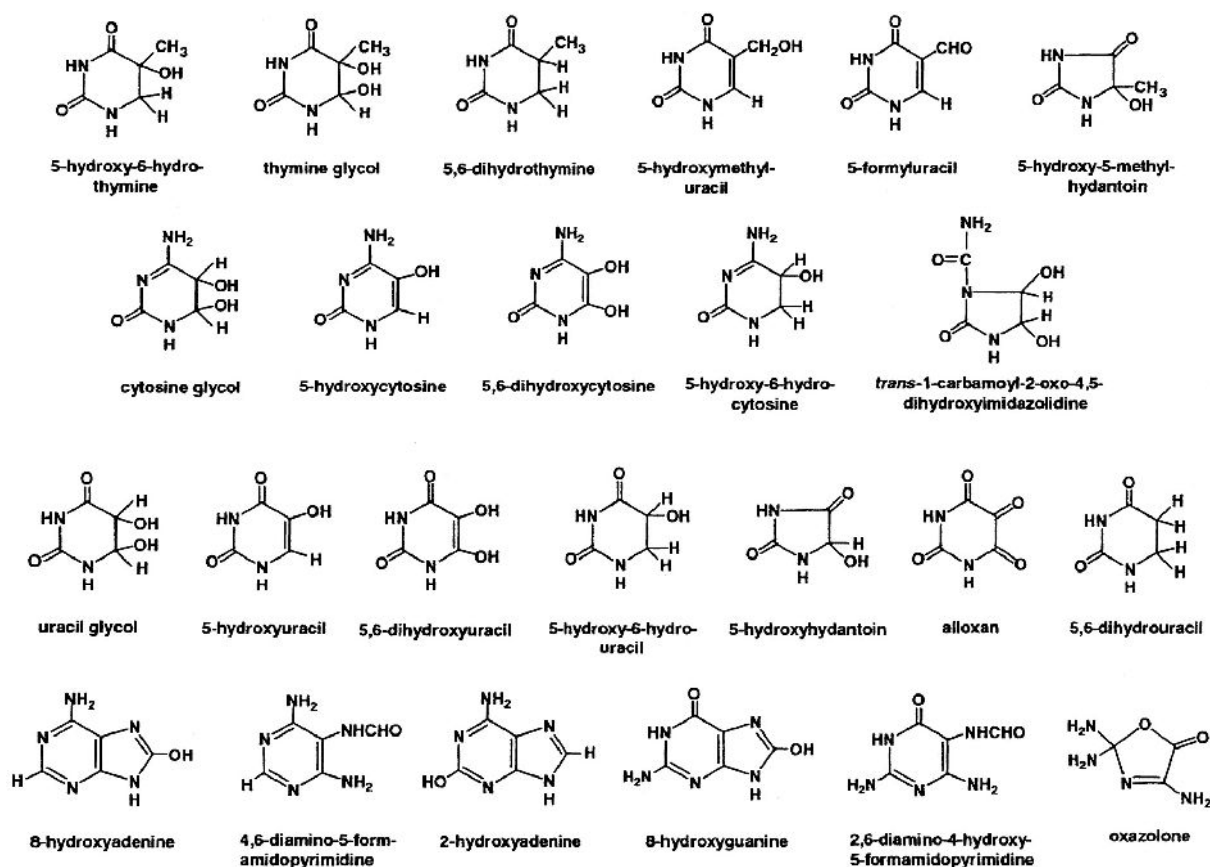


Figure 1. Oxidized DNA bases

DNA base products of interaction with reactive oxygen and free radical species (Cooke, Evans et al. 2003)

1.1.1 Formation of 8-oxo-7,8-dihydroguanine (8-oxoG)

The most susceptible base among the DNA and RNA bases to oxidative modification is guanine, due to its lowest reduction potential (midpoint potential is -1.29 mV vs. nickel-hydrogen electrode) (Jovanovic and Simic 1986). *In vivo*, guanine in DNA and RNA can be modified not only by $\bullet\text{OH}$ but also by other reactive species, including reactive oxygen (superoxide anion: $\text{O}_2^{\bullet-}$), nonradical (ozone: O_3 ; singlet oxygen: $^1\text{O}_2$; hydrogen peroxide: H_2O_2), and nitrogen species (nitric oxide: $\text{NO}\bullet$; peroxynitrite: ONOO^-), as well as nitrosoperoxycarbonate (ONOOCO_2^-), carbonate anions (CO_3^-) and the UVA component of solar light (Dizdaroglu, Jaruga et al. 2002; Cadet, Douki et al. 2006). The reaction of $\bullet\text{OH}$ with guanine can result in guanine C8-OH-adduct formation (Fig. 2). One-electron oxidation of guanine C8-OH-adduct results in 7,8-dihydro-8-oxoguanine (8-oxoG), while one-electron reduction of the guanine C8-OH-adduct radical leads to a ring opening, resulting in 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) or its isomer 2,5-diamino-4-hydroxy-6-formamidopyrimidine (Dizdaroglu, Kirkali et al. 2008; Jaruga, Kirkali et al. 2008). The free

8-oxoG base exists in both neutral (N9-H) and anionic (N9:⁻) forms at physiologic pH. Its presence as a free base in extracellular fluids is one of the most reliable gauges of the oxidative stress load of an organism (Fraga, Shigenaga et al. 1990; Svoboda, Maekawa et al. 2006). Due to its low redox potential, 8-oxoG is more reactive than guanine and serves as a primary target of reactive oxygen species and considered as a protective element in DNA (Sheu and Foote 1995). These observations were supported by findings showing that oligodeoxynucleotide damage and plasmid cleavage by reactive oxygen species (ROS) were inhibited in the presence of 8-oxodG (Kim, Choi et al. 2004).

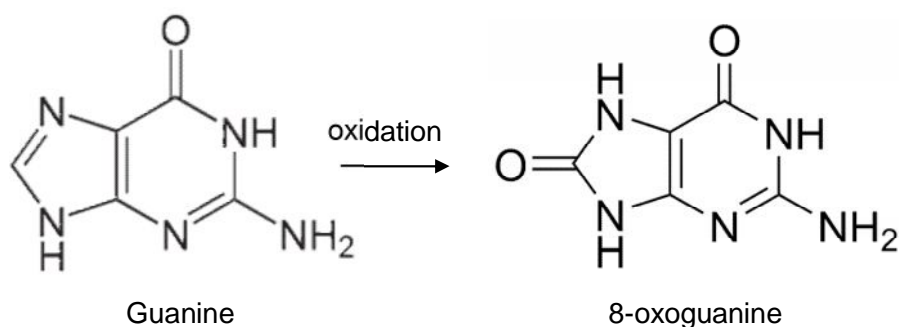


Figure 2. Guanine and 8-oxoguanine

Estimates show that under physiological conditions, several hundred 8-oxoG lesions could be formed in DNA per eukaryotic cell daily (Lindahl and Barnes 2000). It has been determined that 8-oxoG is one of the most abundant DNA lesions formed in oxidative stress conditions, such as those that exist in diseased and aged cells/tissues (Dizdaroglu 1985; Dizdaroglu, Jaruga et al. 2001). In mammals, the intra-helical 8-oxoG is recognized by its unique electronic properties (Markus, Daube et al. 2008) and excised by the *E. coli* Fpg homolog 8-oxoguanine DNA glycosylase 1 (OGG1) from nuclear and mitochondrial genomes during base excision repair (BER) processes (Mitra, Izumi et al. 2002; Dizdaroglu 2005). Unrepaired 8-oxoG may be paired with adenine during DNA replication, resulting in transversion mutations (Nishimura 2002). During mRNA synthesis, it may serve as a template to transcriptional mutagenesis (Saxowsky, Meadows et al. 2008). Kaneko and his co-workers showed a non-linear accumulation of 8-oxoG in nuclear DNA isolated from brain, heart, liver, and kidneys of rats, detecting a 2-fold increase in 30 month-old tissues compared to 2-24 month-old ones (Kaneko, Tahara et al. 1996). It is believed that this accumulation is caused by higher levels of ROS and/or decreased inactivity of OGG1 during the aging process (Chen, Hsieh et al. 2003).

As RNA molecules are present mostly in single stranded forms without protecting proteins, they are even more prone to oxidative damage (Li, Wu et al. 2006; Kong and Lin 2010). It is estimated that 30-70% of messenger RNA contains 8-oxoG because of the low

redox potential of guanine and the lack of repair systems (Thorp 2000; Hayakawa, Kuwano et al. 2001; Hayakawa, Uchiyama et al. 2002). Thus, the 8-oxoG level in RNA is estimated ten times higher than in DNA (Shen, Wu et al. 2000; Hofer, Badouard et al. 2005; Hofer, Seo et al. 2006). As the amount of RNA is approximately four times higher than DNA, and both guanine and 8-oxoG are susceptible to further oxidation, an antioxidant protective role has been hypothesized for the RNA pool (Martinet, De Meyer et al. 2005; Kong and Lin 2010).

1.1.2 Defense mechanisms against ROS

Cells have enzymatic and non-enzymatic antioxidants as protection against ROS. Non-enzymatic antioxidants are often reducing agents such as glutathione, ubiquinone, tocopherols (vitamin E), thiols (cysteine), ascorbic acid (vitamin C), beta carotene (precursor to vitamin A) or polyphenols. Hydrophilic antioxidants react with oxidants in the cytosol, while lipophilic antioxidants protect cell membranes from lipid peroxidation (Sies 1997). Many of the non-enzymatic agents are synthesized in the cells, others must be acquired from outer sources (Vertuani, Angusti et al. 2004). Cells also have interacting network of antioxidant enzymes such as glutathione enzymes (glutathione reductase, glutathione peroxidase and glutathione S-transferase), catalases, superoxide dismutases (SOD) and various peroxidases that protect against oxidative stress by metabolizing oxidative intermediates.

Oxidative stress activates the expression of a battery of defensive genes in order to eliminate ROS and to prevent free radical generation and further damage (Dhakshinamoorthy, Long et al. 2000; Jaiswal 2004). The Nrf2 (NF-E2 related factor 2) pathway is regarded as the most important one in the cells to protect against oxidative stress (Nguyen, Huang et al. 2000; Jaiswal 2004). Nrf2 binds to antioxidant responsive elements (ARE) that regulates the basal and inducible expression of antioxidant genes in response to UV light, xenobiotics, oxidants, heavy metals (Venugopal and Jaiswal 1996; Alam, Stewart et al. 1999; Wild, Moinova et al. 1999; Maher, Dieter et al. 2007; Copple, Goldring et al. 2008). Some of these genes encode enzymes such as γ -glutamylcysteine synthetase, glutathione S-transferases, heme oxygenase 1, quinone oxidoreductases, and ubiquitination enzymes (Dhakshinamoorthy, Long et al. 2000; Kwak, Cho et al. 2007). Other genes encode regulatory proteins with wide variety of cellular activities including signal transduction, proliferation, and immunologic defense reactions. Other factors associated with oxidative stress-induced cellular responses are: NF- κ B, heat shock response activator protein 1, p38 kinase, c-jun N-terminal kinases (JNKs) and TP53 (Halliwell and Gutteridge 2007; Wakabayashi, Slocum et al. 2010; Marinho, Real et al. 2014).

ROS molecules can cause DNA damage and start DNA damage response networks. These DNA damage sensing and signaling pathways enable the cell either to eliminate or cope with the damage or to activate a programmed cell death process, presumably to remove cells with potentially catastrophic mutations (Sancar, Lindsey-Boltz et al. 2004). The DNA damage response networks try to preserve genome integrity and prevent tumor growth while DNA repair mechanisms help to restore the damaged DNA to its original form.

1.2 DNA repair mechanisms

Elevated levels of ROS can generate over a hundred oxidative DNA adducts such as single/double-strand breaks, deoxyribose oxidation, DNA-protein cross-links and base modifications (Cadet, Berger et al. 1997). Majority of DNA damage has endogenous origin (De Bont and van Larebeke 2004) and one of the most common among them is spontaneous hydrolysis of the *N*-glycosidic bond between the DNA base and the deoxyribose (Lindahl and Nyberg 1972). The nucleobase loss generates an apurinic/apirimidic site (AP site), which is estimated to occur at a rate of ten thousand per cell per day (Lindahl 1993). Another example of spontaneous hydrolysis is the deamination of DNA bases containing exocyclic amino groups. Uracil from cytosine occurs most frequently (Sugiyama, Fujiwara et al. 1994), but guanine or adenine can also deaminate to form xanthine and hypoxanthine, respectively at a much lower rate (Kow 2002).

Among the ROS generated DNA adducts 8-oxoG is the most extensively studied and generally used as an indicator of DNA damage (Fraga, Shigenaga et al. 1990; Svoboda, Maekawa et al. 2006). Endogenous nitric oxide (NO•) and its derivatives can produce oxidative adducts too (Burney, Caulfield et al. 1999). Lipid peroxides can generate reactive alkylating agents such as methyl radicals, S-adenosylmethionine and nitrosated amines (De Bont and van Larebeke 2004). Nucleobases are being alkylated on the O- and N-atoms primarily. DNA polymerases also can cause endogenous errors by misincorporation of bases or chemically altered nucleotide precursors, such as 8-oxo-dGTP and dUTP (Shimizu, Gruz et al. 2003; McCulloch and Kunkel 2008). Even DNA repair mechanisms may be sources of DNA damage (Bridges 2005).

The environment serves with numerous forms of damaging agents. UV light may induce atypical covalent bond between adjacent pyrimidine bases (Ravanat, Douki et al. 2001). Ionization radiation is another external damaging source, which can be both artificial (X-rays) and natural (gamma radiation). The most harmful damages they induce either indirectly or through generating ROS are double-strand breaks and other DNA lesions (Ward

1988). Chemical agents are very potent at damaging DNA. Topoisomerase I or II inhibitors are used for treating cancer (camptothecin, etoposide, respectively) by inducing single or double strand breaks (Sinha 1995). Others can be originated from food such as heterocyclic amines (Sugimura 1997) or N-nitrosoamines (Jakszyn and Gonzalez 2006) from which the latter also can be found in tobacco (Schaal and Chellappan 2014). These type of chemicals induce bulky DNA adducts by covalently bond to DNA bases similarly to aflatoxins produced by *Aspergillus flavus* and *parasiticus* (Bedard and Massey 2006) found in various crops.

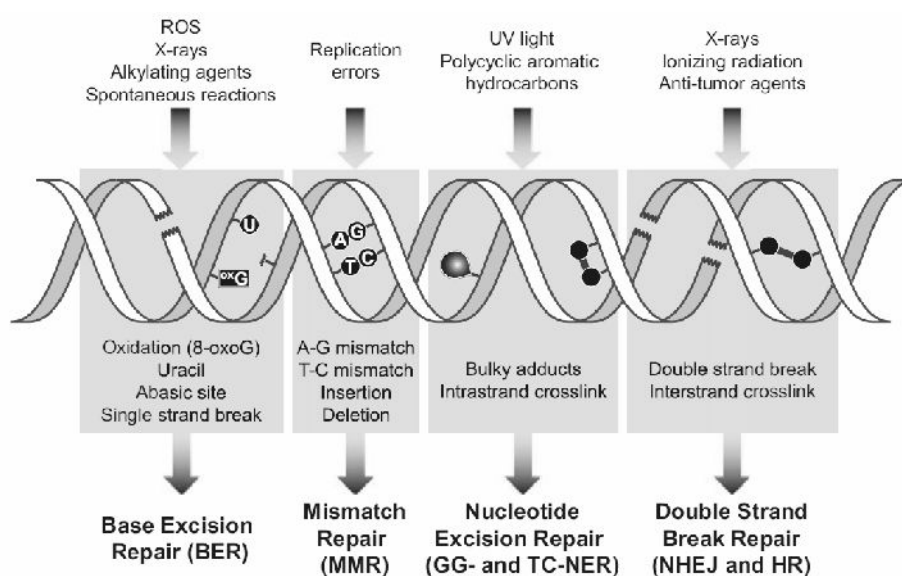


Figure 3. DNA damage and repair mechanisms

The diagram illustrates common DNA damaging agents, examples of DNA lesions caused by these agents, and the relevant DNA repair mechanism responsible for their removal. (DNA Repair of Cancer Stem Cells, page 21, Springer Link, 2013)

Depending on the type of damage, organisms developed multiple pathways to correct DNA lesions. There are five major DNA repair mechanism (Fig. 3) that mammalian cells can utilize: mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), homologous recombination (HR) and non-homologous end joining repair (NHEJ) (Christmann, Tomicic et al. 2003).

1.2.1 Mismatch Repair (MMR)

The MMR system recognizes and corrects misincorporated bases, erroneous insertion and deletion made by DNA replication polymerases. Cells lacking MMR have increased number of mutations, organisms with defective MMR genes are characterized by variety of cancers

including Lynch syndrome or also known as hereditary non-polyposis colon cancer (Peltomaki 2001).

The MMR is a strand specific pathway that remained quite conservative from bacteria to primates. The process consists of three main steps: recognition, excision and repair. In the first step mispaired bases are recognized, in the second one the error containing strand is partially degraded, leaving a gap, and in the third one DNA is synthesized to fill the gap (Fukui 2010). The initiation of the mismatch repair is carried out by two protein complexes: MutS (MutS α and β in humans) and MutL (MutL α , β and γ in humans). MutS is responsible for the mismatch recognition and MutL couples the recognition with downstream events leading to the removal of the error containing strand. Both MutS α and MutS β are heterodimers (homodimers in *E. coli*) consisting of a common MSH2 subunit and one MSH6 in MutS α and one MSH3 in MutS β (Modrich 2006). The MSH2-MSH6 heterodimer represents the 80-90% of the cellular MSH2 and recognizes insertion/deletion (ID) mispairs and base-base mismatches (Drummond, Li et al. 1995; Palombo, Gallinari et al. 1995). MutS β recognizes larger (2-10 IDs), but no base-base mismatches (Genschel, Littman et al. 1998). After the MutS-DNA complex is formed, a MutL homologue (MLH) heterodimers are recruited. The MutL α (MLH1-PMS2 heterodimer) carries out 90 % of the MutL activities, and supports the repair initiated by either MutS α or MutS β . The other two MutL homologues MutL β (MLH2-PMS2) and MutL γ (MLH1-MLH3) may have not known or minor roles in MMR (Raschle, Marra et al. 1999; Cannavo, Marra et al. 2005).

The assemblage of ATP-driven MutS-MutL-DNA ternary complex activates the exonuclease 1 (ExoI) and degrades the error containing DNA strand (Galio, Bouquet et al. 1999). ExoI has a 5' \rightarrow 3' exonucleotic activity and required for repair the base-base and single nucleotide ID mismatches (Tran, Erdeniz et al. 2004). The incision needed for ExoI is made by PCNA/replication factor C (proliferating cell nuclear antigen/RFC)-dependent activity of MutL α (Kadyrov, Dzantiev et al. 2006). DNA polymerase δ accompanied by PCNA and replication protein A (RPA) fills the gap left by ExoI and the repair is completed by DNA ligase I sealing the nick.

1.2.2 Nucleotide Excision Repair (NER)

NER machinery recognizes the bulky distortions of the double helix. Such DNA distorting lesions are cisplatin-DNA intrastrand crosslinks, pyrimidine dimers and 6-4 photoproducts caused by UV light. The process consists of the same biochemical steps both in prokaryotes

and in eukaryotes: damage recognition, verification, dual incisions, excision, repair synthesis and ligation (Costa, Chigancas et al. 2003; Gillet and Scharer 2006). While the NER in prokaryotes takes only six proteins, in eukaryotes more than thirty proteins are involved. The process mediated by the sequential assembly of repair proteins and the correct positioning at the site of the DNA lesion. Defects in NER lead to severe diseases including xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy, caused by genetic mutations of NER proteins. All of them characterized by extreme sun sensitivity and predisposition to cancer, neurodegeneration, immunological defects and premature aging (Nospikel 2008; Cleaver, Lam et al. 2009). The NER system contains two subpathways: global genome repair (GG-NER) and transcription-coupled repair (TC-NER). The two differ in the damage recognition step and while GG-NER eliminates lesions from the whole genome, TC-NER is initiated by the stalling of the RNA polymerase on the coding strand of DNA being transcribed. In GG-NER the damage recognition is carried out by XPC/HR23B/CEN2 (XP complementation group C/Rad23 homolog B/Centrin-2) protein complex (Sugasawa, Ng et al. 1998) and in some cases by the UV-damaged DNA binding complex (UV-DDB1, 2) (Sugasawa 2006). The UV-DDB binding to the damaged DNA increases the distortion of the helix and helps the recruitment of the XPC complex to the lesion site (Sugasawa 2010).

TC-NER damage recognition is initiated when RNA polymerase II (RNAPII) stalls at the site of the DNA damage (Fousteri and Mullenders 2008). Cockayne syndrome A (CSA) and B (CSB) are recruited to the site displacing RNAPII and allow NER proteins to continue with the repair process (Tornaletti 2009). Following initial damage recognition the two subpathways proceed through the same NER reactions recruiting the ten subunit transcription factor TFIIH to the site of damage. With the help of two ATP-dependent helicases (XPB and XPD) TFIIH unwinds the DNA helix to form a ~30 nucleotide bubble exposing the lesion. The unwinding allows another protein, XPA, to access the damaged region and perform a second level of damage recognition (Schaeffer, Roy et al. 1993; Evans, Moggs et al. 1997). The binding of XPA recruits replication protein A (RPA), which helps to stabilize the pre-incision complex. The lesion is excised by the endonucleases ERCC1-XPF and XPG at positions 3' and 5' relative to the damage, respectively (O'Donovan, Davies et al. 1994). Finally DNA polymerase δ or ϵ resynthesize the gap using the undamaged strand as a template. The nick is sealed by DNA ligase I or XRCC1-DNA ligase III α , completing the NER process (Moser, Kool et al. 2007).

1.2.3 Double-Strand Break Repair

DNA double strand breaks (DSBs) are amongst the threats that endanger genome stability and cell viability. They can be generated naturally during programmed genome rearrangement by nucleases (Paques and Haber 1999), V(D)J recombination (Franco, Alt et al. 2006) and from damaging agents, including ionizing radiation (Khanna and Jackson 2001), UV lights (Limoli, Giedzinski et al. 2002) and chemicals (Bosco, Mayhew et al. 2004). Failure to repair them can cause chromosomal aberrations, deletions leading to genomic instability or development of cancer (Khanna and Jackson 2001). Organisms use two pathways to repair DSBs: homologous recombination (HR) and non-homologous end-joining (NHEJ).

1.2.3.1 Homologous recombination (HR)

HR pathway utilizes the undamaged sister chromatid as template (Li and Heyer 2008) and is restricted to the late S and G2 phases of the cell cycle (Morrison, Sonoda et al. 2000). The process starts with generation of 3'-single-stranded tails by the MRN complex (Mre11-Rad50-Nbs1) together with CtIP (RBBP8) at the DNA ends of the DSB (Sartori, Lukas et al. 2007). Next, BLM helicase (Bloom syndrome, RecQ helicase-like) and Exo1 exonuclease continue the 5' to 3' resection (Nimonkar, Ozsoy et al. 2008), which enables RPA to bind to the single stranded tails and prepare the environment for Rad51 recombinase and several other mediator proteins such as Rad52, BRCA2 and Rad51 paralogs (Rad51B,C,D, XRCC2,3) (Forget and Kowalczykowski 2010). Then the single-stranded DNA tail coated Rad51 searches for the homologue DNA sequence and once it has been identified Rad51 starts the DNA strand invasion. During this process, the damaged DNA strand invades the template DNA (sister chromatids) and DNA polymerase η starts synthesizing DNA from the 3'-end of the invading strand followed by DNA ligase I, creating a four-way junction intermediate structure (McIlwraith, Vaisman et al. 2005). This so called "Holliday junction" is cleaved by either Gen1/Yen1 (symmetrically) or Slx1/Slx4 (asymmetrically) or dissolved by the BLM-TopII α complex (Seki, Nakagawa et al. 2006; Ip, Rass et al. 2008) finishing the correction of DSB.

1.2.3.2 Non-Homologous End-Joining (NHEJ)

This DSB repair pathway was named "non-homologous" because the break ends are directly ligated without the need for a homologous template. NHEJ is considered to be an error-prone repair, which operates in all phases of the cell cycle (Sonoda, Hohegger et al. 2006). The repair process starts with the recognition and binding of Ku70/80 heterodimer (Ku) to the DSB (Mari, Florea et al. 2006). Ku produces a ring-shaped structure that encircles the DNA helix by binding to the sugar backbone allowing the heterodimer to be sequence independent (Walker, Corpina et al. 2001). Once Ku is bound to the DNA, the heterodimer-DNA complex recruits the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) to create the DNA-PK holoenzyme. The binding of the DNA-PKcs on opposing ends of DSBs makes a synapsis of the two DNA molecules and results in an autophosphorylation of the DNA-PKcs promoting an accessible DNA termini (DeFazio, Stansel et al. 2002). In case the DNA termini have single-stranded overhangs, DNA polymerase μ or λ can resynthesize the missing strand or Artemis, a NHEJ-specific nuclease can excise the overhangs (Jeggo and O'Neill 2002; Lieber, Lu et al. 2008). Other options for making the overhangs ligatable are the lesion-specific base excision repair (BER) enzymes, such as Tdp1, PNKP and APE1 (Chappell, Hanakahi et al. 2002), or exonucleases ExoI and WRN (Bahmed, Seth et al. 2011). The final step is the ligation of the DNA ends by DNA ligase IV/XRCC4 complex with the help of an additional factor called XLF (XRCC4-like factor) (Ahnesorg, Smith et al. 2006).

1.2.4 Base Excision Repair (BER)

Base excision repair pathway removes small, non-helix distorting lesions from the DNA. The process is initiated by DNA glycosylases which excise mismatched (uracil) or damaged bases derived from alkylation (3-methyladenine), deamination (hypoxanthine) or oxidation (8-oxoguanine) (David, O'Shea et al. 2007; Zharkov 2008). There are at least twelve DNA glycosylases with very narrow substrate specificity (Jacobs and Schar 2012). They all use a common "flipping" mechanism by which the damaged base is flipped to an extrahelical position for excision (Hitomi, Iwai et al. 2007). DNA glycosylases cleave the N-glycosidic bond between the base and its deoxyribose leaving an abasic (AP) site. These AP sites are then processed by apurinic/apyrimidic endonuclease 1 (APE1) which hydrolyzes the phosphodiester backbone 5' to the AP site, generating a single-strand break bordered by 3'-OH and 5'-deoxyribose phosphate (5'-dRP) termini (Abbotts and Madhusudan 2010). The resulting single-strand break can be further processed by either short patch repair with a single nucleotide replacement or long patch repair where 2-10 nucleotides are replaced (Matsumoto,

Kim et al. 1999; Pascucci, Stucki et al. 1999). Some of the DNA glycosylases (i.e. NEIL1 and 2) have AP endonuclease activity too, and can cleave the AP site via β elimination reaction resulting s 3'-phospho- α , β -unsaturated aldehyde and 5'-phosphate at the ends of the break. This break contains 3'- and 5'- blocking lesions, which first must be changed to 3'-OH and 5'-phosphate in order to be processed by the subsequent DNA polymerase, then DNA ligase reactions. APE1 also has an intrinsic 3'-phosphodiesterase activity which enables it to restore 3'-OH from 3'-phospho- α , β -unsaturated aldehyde. The 3'-phosphate products generated by the bifunctional glycosylases, are converted to 3'-OH by the 3'-phosphatase activity of PNKP (polynucleotide kinase 3'-phosphatase). The 5'-dRP removal is primarily executed by DNA polymerase β (Pol β) which has an intrinsic dRP lyase activity (Loeb and Monnat 2008). Besides Pol β , DNA polymerase λ (Pol λ) and DNA polymerase ι (Pol ι) are also capable of removing 5'-dRP terminal groups (Bebenek, Tissier et al. 2001; Garcia-Diaz, Bebenek et al. 2001). Because of the many types of termini, DNA end-processing is a very diverse enzymatic step of BER. Besides the classic end-processing enzymes mentioned before, there are specific ones for the removal of "non-scheduled" single-strand 3'- and/or 5'- blocking lesions, such as tyrosyl-DNA phosphodiesterase 1 (Tdp1) (Interthal, Chen et al. 2005) and aprataxin (APTX) (Ahel, Rass et al. 2006).

After the hydroxyl group at the 3'-, and the phosphate group at the 5' ends are restored, the Pol β synthesizes the missing base for the short patch, while Pol λ and Pol ϵ in conjunction with proliferating cell nuclear antigen (PCNA) are believed to be responsible for the DNA synthesis of the long patch pathway (Robertson, Klungland et al. 2009). The Pol λ and Pol ϵ perform a strand displacement synthesis where the downstream 5' DNA end is displaced to create a flap intermediate. The displaced DNA strand is removed primarily by flap endonuclease 1 (FEN1) leaving a ligatable site (Storici, Henneke et al. 2002) for DNA ligase I to seal the nick.

There are additional proteins facilitating the BER process. For example X-ray repair cross-complementing protein 1 (XRCC1) functions as a scaffold that coordinates the assembly of BER protein including DNA glycosylases, DNA polymerase , APE1, APTX, PNKP, Tdp1, and ligase III (Caldecott 2003). Another example is poly (ADP-ribose) polymerase 1 (PARP1), which functions as a sensor of DNA breaks and catalyzes the ADP-rybosition of itself and other proteins enabling the recruitment of repair proteins (Malanga and Althaus 2005).

1.2.5 OGG1, a versatile DNA repair enzyme

OGG1 is the dedicated enzyme to excise the 8-oxoG during the DNA base excision repair process. OGG1 is a bifunctional glycosylase, it is able to both cleave the glycosidic bond of the mutagenic lesion and the phosphodiester bonds (3' and 5') causing a strand break in the DNA backbone (Chung, Kasai et al. 1991; Chung, Kim et al. 1991). OGG1-initiated BER encompasses four key steps (Fig. 4), including damaged base recognition and excision, 3'deoxyribose phosphate end-processing by AP endonuclease 1 (APE1), filling in the nucleotide gap by DNA polymerase β , and nick-sealing by DNA ligase (Mitra 2001). OGG1's repair activity is modulated by post-translational modifications, including phosphorylation (Dantzer, Luna et al. 2002), acetylation (Bhakat, Mokkapati et al. 2006), and by interactions with canonical repair and non-repair proteins (Hegde, Hegde et al. 2011). Studies have also unveiled a redox-dependent mechanism for the regulation of OGG1 activity (Bravard, Vacher et al. 2006; David, O'Shea et al. 2007).

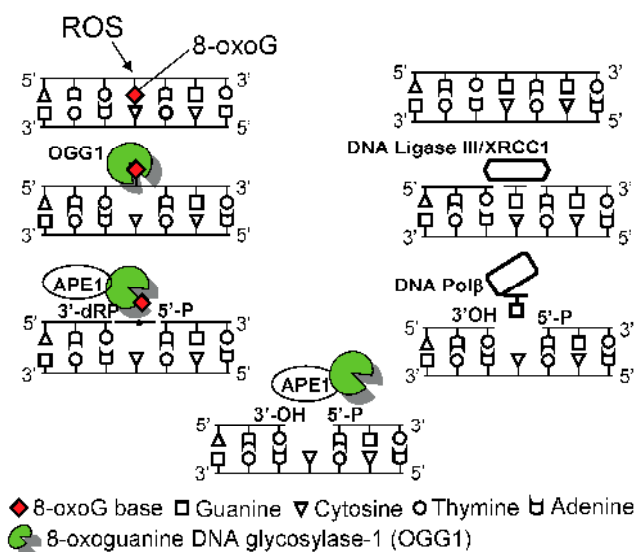


Figure 4. Graphical illustration of 8-oxoguanine DNA glycosylase-1 (OGG1)-initiated genome damage repair (Ba, Aguilera-Aguirre et al. 2014)

Depending on the last exon sequence of the C-terminal region of the OGG gene there are two major splice variants of OGG: nuclear (type 1 with 3 isoforms) and mitochondrial (type 2 with 5 isoforms) (Aburatani, Hippo et al. 1997; Nishioka, Ohtsubo et al. 1999). All variants have the N-terminal region in common. In eukaryotes, the N-terminus of this gene contains a mitochondrial targeting signal, essential for mitochondrial localization (Nishioka,

Ohtsubo et al. 1999). A conserved N-terminal domain contributes residues to the 8-oxoguanine binding pocket (van der Kemp, Charbonnier et al. 2004).

Accumulation of 8-oxoG in DNA has conventionally been associated with various diseases, accelerated telomere shortening, inflammatory and aging processes (Markesbery and Lovell 2006; Radak, Bori et al. 2011). In addition, unrepaired 8-oxoG lesion is potentially one of the most mutagenic lesions among oxidatively modified DNA bases, because its pairing with A will cause a GC → AT transition. Unexpectedly, OGG1 knock out (OGG1^{-/-}) mice have an unaltered lifespan, and show only moderate increases in tumor formation. In these animals, no organ failure can be observed despite the supraphysiological levels of 8-oxoG in their DNA (Klungland, Rosewell et al. 1999; Minowa, Arai et al. 2000). Furthermore, OGG1^{-/-} mice showed an increased tolerance to chronic oxidative stress (induced by KBrO₃ treatment), while 8-oxoG levels in the DNA increased by 250- to 500-fold compared to the wild type. Interestingly, lack of OGG1 activity protected mice from the trinucleotide repeat expansions underlying Huntington's disease (Kovtun, Liu et al. 2007).

Mabley and co-workers studied the role of OGG1 in inflammatory processes. They used three models of inflammation: endotoxic shock, diabetes, and contact hypersensitivity. According their results the OGG1 knockout mice are extremely resistant to most of the lipopolysaccharide-induced effects: LPS-induced organ dysfunction, neutrophil infiltration and oxidative stress, when compared to wild-type (OGG1^{+/+}) controls. Furthermore, OGG1^{-/-} mice had decreased serum cytokine and chemokine levels and prolonged survival after LPS treatment. In case of multiple low-dose streptozotocin-induced type I diabetes, OGG1^{-/-} mice were found to have significantly lower blood glucose and higher insulin levels followed by fewer incidence of diabetes as compared with wild type mice. These knockout mice also have higher levels of protective Th2 cytokines (IL-4, IL10) while lower levels of chemokine MIP-1 and Th1 cytokines (IL-12, TNF- α) compared to the levels measured in OGG1^{+/+} controls. In a model of oxazolone-induced contact hypersensitivity, results showed reduced neutrophil accumulation, chemokine (MIP-1, MIP-2), Th1 (IL-1, TNF- α) and Th2 cytokine levels (IL-4) in the ear tissue of OGG1^{-/-} mice. Their results suggest that OGG1 may primarily regulate Th1 cytokine levels rather than Th2 (Mabley, Pacher et al. 2005). On the other hand, mice lacking OGG1 have been shown to be susceptible to high-fat diet induced insulin resistance and obesity as well (Sampath, Vartanian et al. 2012). Others demonstrated that under chronic inflammatory conditions, cytokine-induced nitric oxide inhibits the activity DNA repair enzymes, including OGG1 (Jaiswal, LaRusso et al. 2000; Jaiswal, LaRusso et al. 2001). It has been hypothesized that DNA-dependent kinases recognize the single strand gaps made by

OGG1 and trigger inflammation. In this point of view, it looks more advantageous to down-regulate OGG1 and leave the 8-oxoG in the DNA (Radak and Boldogh 2010). This hypothesis also can explain why OGG1^{-/-} mice with significantly fewer DNA nicks are less prone to inflammation. In support, OGG1 expression was increased in islet cells of type 2 diabetes patients compared to healthy controls (Tyrberg, Anachkov et al. 2002). Although oxidative stress increases 8-oxoG in the DNA, but observations showed a decreased OGG1 activity until normal redox status returned (Bravard, Vacher et al. 2006).

Besides redox modulation, OGG1 activity can also be altered by acetylation/deacetylation. OGG1 activity can be increased by acetylation by transcriptional coactivator p300 in the presence of APE1 (Bhakat, Mokkaapati et al. 2006). Recent publications suggest that sirtuins, a group of regulatory proteins with NAD⁺-dependent deacetylase (or mono-ADP-ribosyltransferase) activity, have important role modifying OGG1's glycosylase activity. Cheng and colleagues showed a regulatory role of Sirt3 in the maintenance of mitochondrial DNA and turnover of OGG1. They found that by deacetylation, Sirt3 modified OGG1 incision activity and prevented its degradation leading to cell survival under oxidative stress. (Cheng, Ren et al. 2013). Another paper revealed an inverse correlation between Sirt1 and OGG1 in animal studies. They found that rats with higher aerobic capacity had increased Sirt1 and lower acetylated OGG1 levels (lower repair capacity), when compared to low aerobic capacity rats (Sarga, Hart et al. 2013).

OGG1 has been associated with aging because of the accumulation of 8-oxoG in DNA (Dherin, Radicella et al. 1999; Cortopassi 2002). Szczesny and co-workers showed a difference in efficiency of import of OGG1 (and APE1) into nuclear and mitochondrial compartments of young and aged cells. They hypothesized this phenomenon as reason for age-related DNA damage (Szczesny, Hazra et al. 2003). Physical exercise can upregulate OGG1 activity in the liver of old (21 month) rats and reduce the 8-oxoG content of both nuclear and mitochondrial DNA to the level of young rats (11 month) after 2 months regular exercise (Nakamoto, Kaneko et al. 2007).

1.3 Small GTPases

Small GTPases are a type of G-proteins found in the cytosol that are homologous to the alpha subunit of heterotrimeric G-proteins (large GTPases). They can hydrolyze guanosine triphosphate (GTP) to form guanosine diphosphate (GDP) (Bourne, Sanders et al. 1990). The GDP-bound form is their inactive state while the GTP-bound form is their active form (Fig. 5) in which they can activate downstream pathways by binding to effectors. Small GTPases

work like molecular switches with the help of guanine nucleotide exchange factors (GEFs) that facilitate GDP dissociation and of GTPase activating proteins (GAPs) that stimulate GTP hydrolysis (Bos, Rehmann et al. 2007).

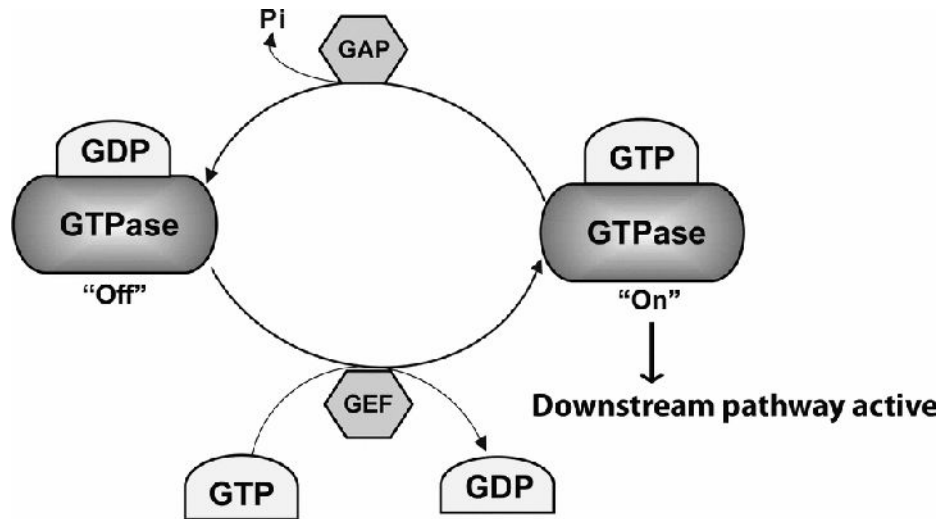


Figure 5. The GTPase activity cycle

GTPases cycle between the inactive 'off' GDP-bound state and the active 'on' GTP-bound state. The inactive state occurs by stimulation of intrinsic GTPase hydrolysis activity by GAPs. Activation is facilitated by GEFs to load GTP and dissociate GDP, allowing interaction with downstream effectors and in turn activation of downstream signaling pathways. (Original picture from Biochemical Society Transactions).

1.3.1 Ras

The name "Ras" is an abbreviation of "Rat sarcoma" referring the way the first members of the protein family were discovered. Ras protein family members belong to small GTPases (Wennerberg, Rossman et al. 2005), as they can bind and hydrolyze guanosine triphosphate. Ras is encoded by the *ras* gene and the prototypical member of the Ras superfamily of proteins, which are all related in 3D structure. They regulate diverse cell behaviors and are involved in transmitting signals (Fig. 6) within cells (Bourne, Sanders et al. 1990). The family members share a set of conserved G box GDP/GTP-binding motif elements beginning at the N-terminus (Bourne, Sanders et al. 1991). Together, these elements make up a ~20 kDa G domain (Ras residues 5-166) that has a conserved structure and biochemistry shared by all Ras superfamily proteins. Ras (p21) protein contains a six-stranded beta sheet and 5 alpha helices (Vetter and Wittinghofer 2001).

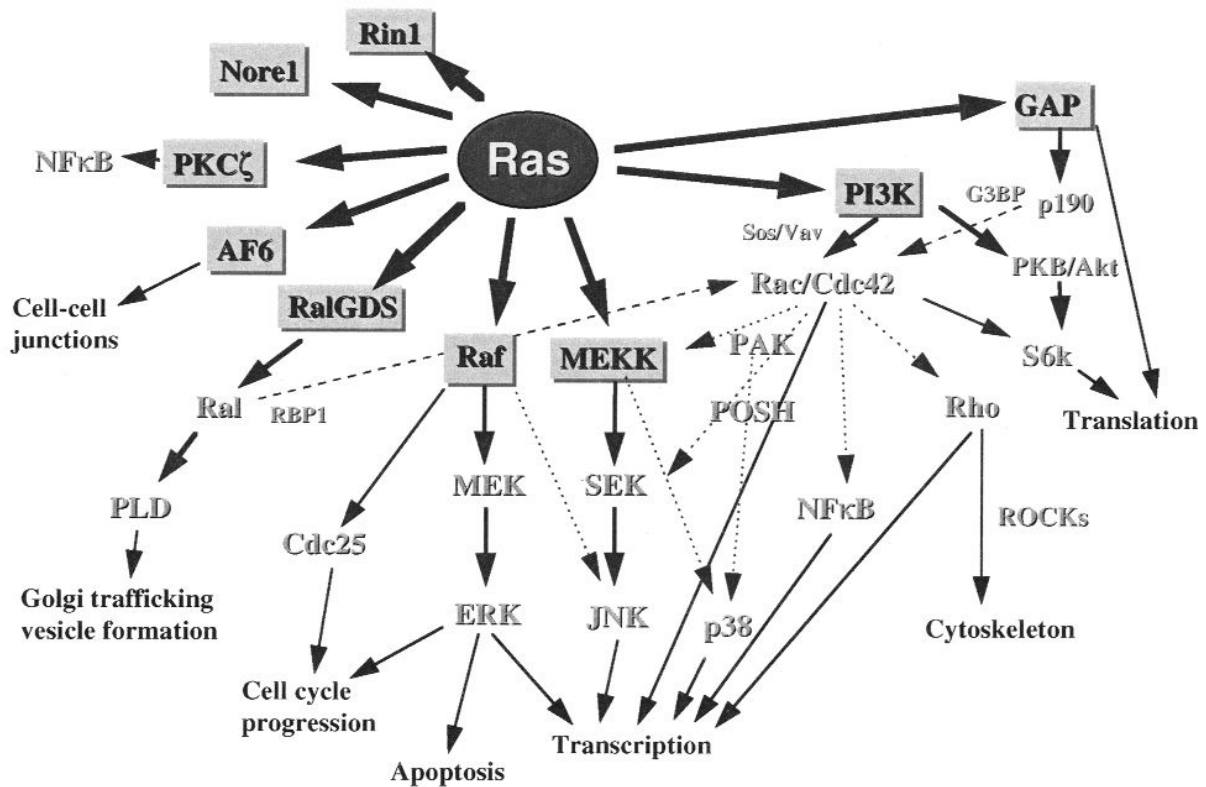


Figure 6. Ras effectors and downstream pathways

Activation of Ras GTPases involves the displacement of GDP with GTP, a process mediated by GEFs (Bourne, Sanders et al. 1990). GEFs first interact with GTPase and dissociate GDP at an increased rate, and then the bound GTP promotes the release of exchange factor leaving the GTPase in an active form (Bourne, Sanders et al. 1990; Boriack-Sjodin, Margarit et al. 1998). Well known GEFs are Son of Sevenless (Sos) (Rogge, Karlovich et al. 1991) and cdc25 (Sadhu, Reed et al. 1990). Ras-GTP binds to the RBD domain of the Raf1 serine/threonine kinase (Block, Janknecht et al. 1996), and its subsequent phosphorylation is essential, but not sufficient, for mediating Raf1's mitogen-activated protein kinase (MAPK) activity, as phosphorylated Raf1 requires additional protein-protein and membrane-lipid interactions (Kyriakis, App et al. 1992). The MAPK cascade transmits signals downstream and results in the transcription of genes involved in cell growth and division. While RasGEFs catalyze a "push and pull" reaction which releases GDP from Ras, RasGAPs enhance the catalytic machinery of Ras, since the protein intrinsic GTPase activity is very slow. Thus, GAPs accelerate Ras inactivation. An inorganic phosphate is released and the Ras molecule is now bound to a GDP which turns "off" further signaling (McCormick 1989). Because intracellular concentration of GTP is approximately 10-fold higher than that of GDP, GTP predominantly re-enters the nucleotide binding pocket of Ras. The balance between GEF and GAP activity determines the guanine nucleotide status of Ras, thereby regulating Ras

activity. Another protein may augment the activity of Ras is GDI (GDP Dissociation Inhibitor). This protein functions by slowing the exchange of GDP for GTP and thus, prolonging the inactive state of Ras (Boguski and McCormick 1993).

Ras-regulated signal pathways control cell growth, migration (Lee, Feig et al. 1996), differentiation (Crespo and Leon 2000), actin cytoskeletal integrity, cell adhesion (Chambers, Hota et al. 1993), apoptosis (Kauffmann-Zeh, Rodriguez-Viciano et al. 1997), proliferation and survival (Bonni, Brunet et al. 1999). The clinically most notable members of the Ras subfamily are H-RAS, K-RAS and N-RAS, mainly for being implicated in many types of cancer (Bos 1989). Ras proteins are attached to the cell membrane because of their prenylation and palmitoylation (H-RAS and N-RAS) or prenylation and a polybasic sequence adjacent to the prenylation site (K-RAS). Depalmitoylation releases the proteins from the membrane, and allows them to enter another cycle of palmitoylation and depalmitoylation. It is assumed that this cycle prevents the N-RAS and H-RAS to attach to other membranes over time and to maintain their localization along the Golgi apparatus, secretory pathway, plasma membrane and inter-linked endocytosis pathway (Rocks, Peyker et al. 2006). Ras inhibitor trans-farnesylthiosalicylic acid (FTS) disrupts the membrane attachment of Ras, thus works as an anti-oncogenic drug in many cancer cell lines (Blum, Jacob-Hirsch et al. 2005; Rotblat, Ehrlich et al. 2008). Ras and Ras-related proteins are often deregulated in cancers, leading to increased invasion and metastasis, and decreased apoptosis. Mutations in *ras* genes can result the production of continuously active Ras proteins. As a result, the signaling pathway(s) remain “switched-on” and the overactive signaling can lead to uncontrolled cell growth and cancer (Goodsell 1999). Ras mutations are found in 20-25% of all human tumors and up to 90% in certain types of cancer (e.g. pancreatic cancer) (Downward 2003).

H-Ras is involved in regulating cell division in response to growth factor stimulation. Growth factors act by binding cell surface receptors that span the cell's plasma membrane. Once activated, receptor stimulate signal transduction events in the cytoplasm, a process by which proteins and second messengers relay signals from outside the cell to the cell nucleus and instructs the cell to grow or divide. Once it is turned on, K-Ras recruits and activates proteins necessary for the propagation of growth factor and other receptors' signal, such c-Raf and PI 3-kinase (Castellano and Downward 2011).

1.3.2 Rac

Rac1, also known as Ras-related C3 botulinum toxin substrate 1 is a protein ubiquitously expressed and involved in signal pathways (Fig. 7) that regulate mobility and other processes

related to membrane trafficking and cell morphology (Ridley 2001; Vega and Ridley 2008). It is encoded by the *rac1* gene (Didsbury, Weber et al. 1989; Jordan, Brazao et al. 1999). The Rac protein belongs to the Rho GTPase family. The classical members of the four subfamilies of Rho are: Rac, Cdc42, Rho and Rif. Similarly to other GTPases, the classical Rho GTPases cycle between active GTP-bound forms and inactive GDP-bound forms (Symons and Settleman 2000). Their cycle is also controlled by three types of regulatory proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) (Boguski and McCormick 1993). Rac subfamily has three Rac isoforms: Rac1, Rac2 and Rac3. Rac1 protein is widely expressed in different tissues, whereas as Rac2 expression is restricted to cells of hematopoietic origin. Rac3 is predominantly found in the central nervous system (Bedard and Krause 2007).

Rac can regulate cell survival and proliferation via cell cycle progression by activating p42/p44 and p38 MAPK, JNK, and Akt kinases (Yang, Atkinson et al. 2001; Gu, Filippi et al. 2003; Cancelas, Lee et al. 2005; Carstanjen, Yamauchi et al. 2005). Rac1 is a well characterized member of the Rac subfamily. Previous studies found that Rac1 regulates a diverse array of cellular events, including the formation of lamellipodia and membrane ruffles, cell cycle, cell adhesion and mobility (Ridley 2006). In addition, Rac1 regulates endocytic and exocytic trafficking pathways. Rac1 has a characterized role in clathrin-dependent endocytosis (Lamaze, Chuang et al. 1996). Other physiological functions of the Rac1 GTPase includes: modulation of the cellular redox state, regulation of cell movements, cellular signaling, gene expression, and cell differentiation (Bedard and Krause 2007). In most types of cells, Rac is connected to the production of low levels of ROS which have an implicated role in growth, differentiation, migration, and angiogenesis, as well as in inflammation (Sulciner, Irani et al. 1996; Sundaresan, Yu et al. 1996; Irani, Xia et al. 1997; van Wetering, van Buul et al. 2002). Rac1 is ubiquitously expressed in nonphagocytic cells, including lung epithelial cells and fibroblasts. Rac1 is one of the three Rac family molecules that control NADPH oxidase (NOX) activity (NOX1, NOX2, and NOX4 as well as NOX3) both in phagocytes and in nonphagocytic cells (Hordijk 2006; Lambeth, Kawahara et al. 2007).

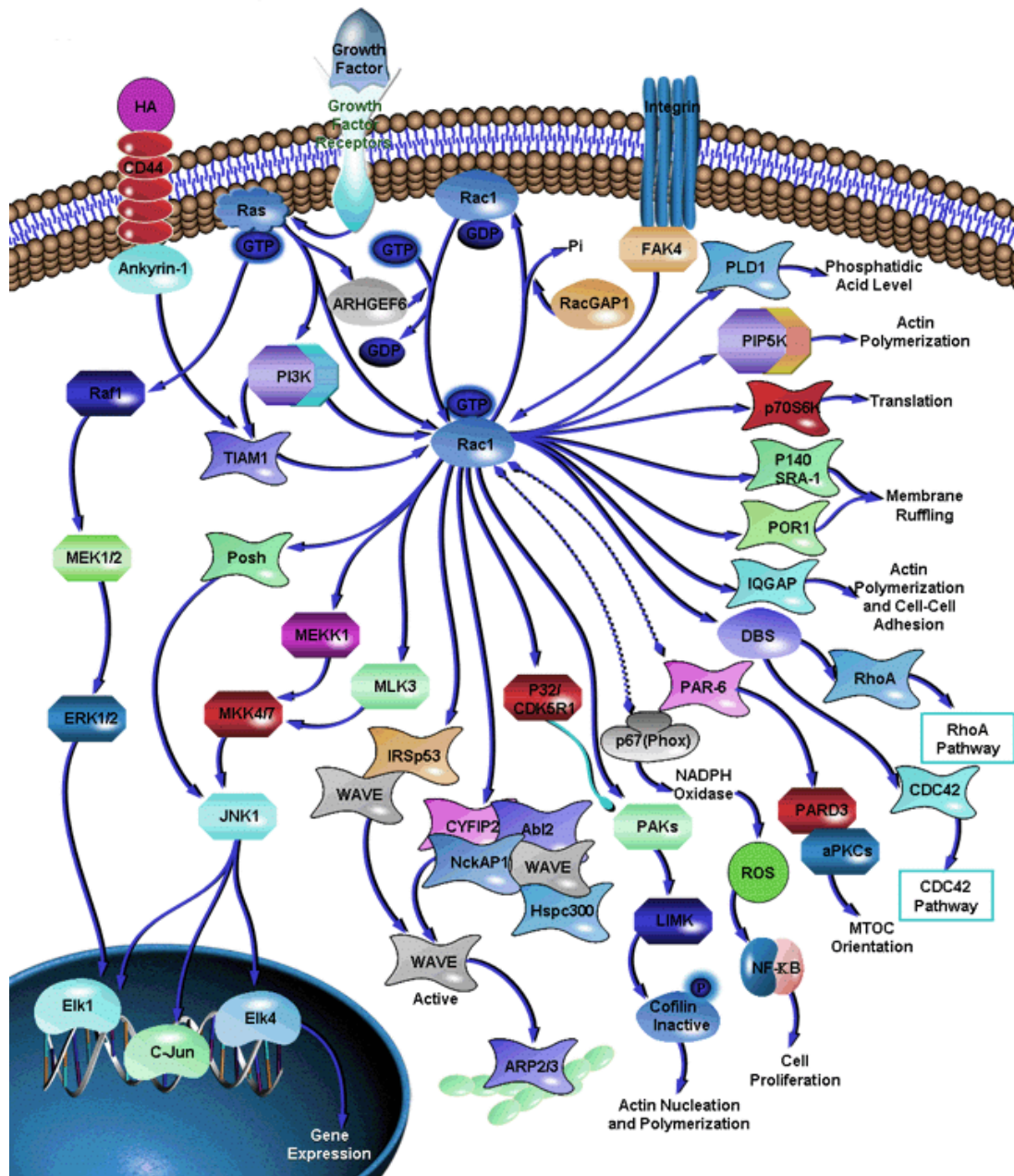


Figure 7. Rac1 related pathways
(Original picture from SABiosciences)

Rac2 is expressed primarily in myeloid cells (e.g. human neutrophils) (Hordijk 2006). Rac2 has a crucial role generating high levels of ROS in neutrophils upon cell stimulation and phagocytosis, and these ROS aid in the killing of ingested pathogens. To assemble a fully active oxidase complex, Rac2 with three other cytosolic proteins - p40phox, p47phox, p67phox - translocate to the plasma membrane and the active complex uses electrons from NADPH to reduce oxygen to form superoxide (Babior, Lambeth et al. 2002). Abnormal activation of Rac has been shown in a number of acute and chronic leukemias, including chronic myelogenous leukemia, chronic lymphocytic leukemias and acute myeloid leukemias

(Wertheimer, Gutierrez-Uzquiza et al. 2012). Rac1 is thought to play a significant role in the development of various cancers, including melanoma (Bauer, Chen et al. 2007; Krauthammer, Kong et al. 2012) and non-small cell lung cancer (McAllister 2012; Stallings-Mann, Waldmann et al. 2012). Another example that Rac1 overexpression may play a role in tumors was shown in pancreatic cancer progression (Wertheimer and Kazanietz 2011). Rac2 has been linked to leukemias (Muller, Schore et al. 2008) and mutations in the Rac2 gene have been found in human brain tumors (Hwang, Lieu et al. 2005).

Rac3 was originally identified from a chronic myelogenous leukemia cell line and has been implicated in human breast cancer (Mira, Benard et al. 2000; Morris, Haataja et al. 2000; Baugher, Krishnamoorthy et al. 2005), ovarian cancer, cellular transformation (Keller, Gable et al. 2005) and tumor invasion (Chan, Coniglio et al. 2005).

1.4. DNA repair-independent functions of OGG1

Previous studies have implied roles for OGG1 in multiple cellular processes in addition to that of being a canonical DNA BER protein (Mitra 2001; Dizdaroglu, Kirkali et al. 2008). For example, it has been shown that OGG1 colocalizes with centrioles (microtubule organizing center), microtubule networks, and mitotic chromosomes (Dantzer, Luna et al. 2002; Szczesny, Hazra et al. 2003; Conlon, Zharkov et al. 2004). These data show the implication of OGG1 in chromatin remodeling and transcriptional initiation. In mitochondria, aconitase enzyme has been suggested as a redox sensor because of its vulnerability to oxidative stress leading to inactivation or disassembly (Bulteau, Ikeda-Saito et al. 2003). OGG1 was reported to act as a chaperon for aconitase and to prevent mitochondrial dysfunction and apoptosis by interacting with it. They found that OGG1 silencing augmented oxidant induced caspase-9 activation (Panduri, Liu et al. 2009; Kim, Cheresch et al. 2014).

As described above, OGG1^{-/-} mice have increased resistance to inflammation (Mabley, Pacher et al. 2005). This observation raises the possibility that it may not be the genomic level of 8-oxoG but the free 8-oxoG generated by BER that provides the linkage to disease/aging processes. While several aspects of the involvement of OGG1 in DNA repair-independent cellular functions have already been revealed, the role of OGG1 and free 8-oxoG in the activation of canonical small GTPase-mediated pathways has not been investigated so far.

2. Objectives of the study

Previous studies on OGG1^{-/-} mice suggest that a lack of OGG1 activity is accompanied by dysfunction of signaling pathway(s) linking oxidative stress to cellular responses. These observations raise the possibility that the 8-oxoG base released from the genome by OGG1 could have physiological/pathophysiological relevance. The aim of this study was to reveal whether 8-oxoG and/or OGG1 are able to activate small GTPase-related signaling pathways.

Our hypotheses were the following:

- 8-oxoG has a biological role after being excised and not just a neutral byproduct of DNA repair,
- both exogenously added and endogenously excised 8-oxoG induces cellular responses,
- free 8-oxoG base can bind to OGG1,
- 8-oxoG/OGG1 complex can activate small GTPases, Ras and Rac1,
- 8-oxoG/OGG1 complex can induce ROS production via NADPH oxidases by activating Rac1.

3. Materials and methods

3.1 Reagents

8-OxoG was from Cayman Chemicals (Ann Arbor, MI); 7,8-dihydro-8-oxoadenine (8-oxoA) was from Axxora, LLC/BioLog Life Science Institute (San Diego, CA); 8-aminoguanine was purchased from Carbosynth Inc, (Berkshire, UK). Guanine, 8-oxo-deoxyguanosine (8-oxodG), maleic acid diethyl ester (DEM), N-acetyl-L-cysteine (NAC), L-glutathione reduced (GSH); β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (β -NADPH); 2'-deoxyguanosine, adenine, guanosine, were from Sigma-Aldrich (St. Louis, MO). 8-Oxo-7,8-dihydroadenine (8-OH-Ade); Biolog Life Science Institute, Bremen, Germany); 2,6-diamino-4-hydroxy-5-formo-midopyrimidine (FapyG) was a kind gift of Dr. Miral Dizdaroglu (National Institute of Standards and Technology, Gaithersburg, MD). Rac1 antibody (Thermo Fisher Scientific, Rockford, IL); Rac2 and Rac3, NADPH oxidase subunit Abs (Epitomics, Burlingame, CA); recombinant human Rac1 protein (Cytoskeleton, Denver, CO); recombinant human OGG1, H-Ras, N-Ras, and K-Ras proteins (Novus Biological, Littleton, CO); OGG1 Ab (Abcam, Cambridge, MA). GTP, GDP, and GTP γ S were from Cytoskeleton (Denver, CO); Pan-Ras antibody was from Millipore (Darmstadt, Germany); nickel-nitrilotriacetic acid-agarose beads were from Qiagen (Valencia, CA); K-Ras and N-Ras antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies to ERK1/2, MEK1/2, phospho- ERK1/2, -MEK1/2 were from Cell Signaling (Danvers, MA); and FITC- and Alexa Fluor 488-conjugated antibodies were from Invitrogen (Carlsbad, CA). HRP-conjugated anti-rabbit Ab (Southern Biotech, Birmingham, AL), anti-mouse IgG, GE Healthcare UK Ltd, (Pittsburgh, PA). Active Ras and Rac pull-down assay kit was from Pierce Biotechnology (Thermo Fisher Scientific, Waltham, MA); and siRNAs for Ras, Rac1, NOX4, and OGG1 depletion were from Dharmacon (Thermo Fisher Scientific Inc. Waltham, MA). Diphenyleneiodonium chloride (DPI); 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (eBioscience, San Diego, CA); H₂SO₄ (Fisher Scientific, Fair Lawn, NJ); (Mant)-GTP (2-(or-3)-O-(N-methylanthraniloyl)guanosine 5'-triphosphate, trisodium salt, ^{Mant}GTP) and (Mant)-GDP (2-(or-3)-O-(N-methylanthraniloyl)guanosine 5'-diphosphate, disodium salt, and ^{Mant}GDP) (Invitrogen, Carlsbad, CA). CD11a, CD11c, CD16, CD34, CD58, CD64, HLA-DR purchased from Immunotech (Commerce, CA), CD11b, CD32, CD36, CD45RA, CD45RO, CD95 (Fas), IL-3R α , β , γ , CD206 obtained from BD Pharmingen (San Diego, CA), CCR5, CCR6, CCR7, GPCR, E-cadherin purchased from R&D Systems (Minneapolis, MN), α 5 (Chemicon, Temecula, CA). The MDR-specific monoclonal antibody was a generous gift from Gabor Szabo, (Department of Biophysics and Cell Biology, University of Debrecen).

Isotype-matched antibodies labeled with the same fluorochrome (all from BD Pharmingen). pHyPer-Cyto, pHyPer-dMito and pHyPer-Nuc were acquired from Evrogen (Moscow, Russia).

3.2 Cell cultures

MRC-5, a human diploid lung fibroblast (ATCC# CCL-171) and human cervix carcinoma (HeLaS, ATCC# CCL-2.2) cells were maintained in Earle's minimum essential and Dulbecco's modified Eagle's low glucose medium, respectively. A549 type II alveolar epithelial cells (ATCC # CCL-185) were cultured in Ham's F12 (GIBCO-BRL), U937, a human monocytic cell line (ATCC# CRL-1593.2), were grown in and RPMI-1640. The human myelomonocytic KG-1 (ATCC# CCL-246) cells were grown in Iscove's Modified Dulbecco's Medium. All media were supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin; cells were grown at 37 °C in a 5% CO₂. KG1 cells were stimulated with 10 ng/ml PMA (Sigma-Aldrich, Steinheim, Germany) and 100 ng/ml ionomycin (Sigma-Aldrich) for 4 days as described previously (St Louis, Woodcock et al. 1999).

Monocyte-derived DCs were developed as described previously (Turner, Roder et al. 1999). Briefly, mononuclear cells were isolated from Buffy Coat by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) gradient centrifugation and monocytes were isolated by magnetic cell separation using positive selection with anti-CD14-coated beads (Miltenyi Biotech, Bergish Gladbach, Germany). Purified monocytes were plated at 2×10^6 cells/ml concentration and cultured in serum-free AIMV medium (Gibco) in the presence of 100 ng/ml IL-4 and 75 ng/ml GM-CSF (Peprotech EC, London, UK) given on days 0 and 2. Cells were differentiated for 5 days and immature DC were characterized by flow cytometry using anti-CD1a fluorescent antibody (Immunotech, Marseille, France). Activation of immature DC was induced by an inflammatory cocktail containing 10 ng/ml TNF- α , 5 ng/ml IL-1 β , 20 ng/ml IL-6, 75 ng/ml GMCSF and 1 μ g/ml PGE2 (Sigma-Aldrich). Mature DC were identified by anti-CD83 mAb (Immunotech).

3.3 Animals and treatments

Animal experiments were performed according to the National Institutes of Health Guidelines for Use of Experimental Animals and approved by the University of Texas Animal Care and Use Committee (Protocol number: 0807044A). Eight-week-old female BALB/c mice (The Jackson Laboratory) were challenged intranasally with 60 μ l of 8-oxoG (1 μ M) in saline (or

with control saline) under mild anesthesia (Boldogh, Bacsi et al. 2005). The animals were sacrificed after 15 min, their lungs homogenized in lysis buffer (detailed in “Assessment of GTP-bound Ras and Rac levels”) then extracts were prepared for measuring the Ras-GTP levels. For Rac1 measurement, mice were sacrificed and their lungs homogenized in a lysis buffer and GTP bound levels of Rac determined as described in “Assessment of GTP-bound Ras and Rac levels.”

3.4 Assessment of GTP-bound Ras and Rac levels

Ras-GTP levels were quantified with the Active Ras pull-down assay kits. Briefly, the cells were lysed in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 60 mM MgCl₂, 1% Nonidet P-40, and 5% glycerol, and Ras-GTP in 250 µg extracts was captured by the Ras-binding domain of Raf1 immobilized to glutathione resin (Block, Janknecht et al. 1996; Taylor, Resnick et al. 2001). After washing with binding buffer, the activated Ras was eluted with Laemmli buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, pH 6.8) and quantified by Western blotting and densitometry.

Changes in the levels of GTP-bound Rac1 were analyzed using the Active Rac pull-down and detection kit (Pierce, Thermo Scientific Inc. Waltham, MA) per the manufacturer's instructions with slight modifications. Briefly, cells were washed once with ice-cold TBS and lysed with 1x Lysis/Binding/Washing buffer (25 mM Tris-HCl /pH 7.5/, 150 mM NaCl, 1% NP-40, 1 mM DTT, 5% glycerol, 20 mM NaF, 1 mM sodium orthovanadate, 1 µg/ml leupeptin and 1 µg/ml aprotinin). Cell/tissue extracts were cleared by centrifugation, and GTP-bound Rac1 was captured by the Rac-binding domain of p21/Cdc42/Rac1-activated kinase 1 bound to GST beads (Benard and Bokoch 2002). GST beads were washed with lysis buffer and bound proteins were fractionated on a 4 to 20% PAGE. Changes in Rac1 levels were determined by Western immunoblot analysis.

3.5 Assessment of 8-oxoG's cellular uptake

MRC-5 cells at 80% confluence were washed, and PBS containing 10 µM of 8-oxoG (final concentration) was added. Aliquots were removed at 0, 1, 30 min, then immersed into liquid nitrogen, then transferred to a freeze-dryer (Dura-Dry MP, Stone Ridge, NY) and lyophilized at -80°C. The lyophilized materials were reconstituted in 10 mM NaOH and the 8-oxoG levels were measured by liquid chromatography and isotope dilution mass spectrometry (LC/IDMS). As an internal standard, a stable isotope-labeled analog of 8-oxoG (8-oxoG-¹³C₃, ¹⁵N) was used as previously described (Dizdaroglu, Jaruga et al. 2001).

3.6 Protein interaction assays

The interaction of OGG1 with H-, N-, or K-Ras was analyzed as described previously (Lai, Boguski et al. 1993; Chataway and Barritt 1995). Briefly, individual His-Ras proteins were immobilized on nickel-nitrilotriacetic acid (Ni-NTA)-agarose beads in interaction buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 7.5) and incubated for 30 min at 4 °C. After three washes in interaction buffer, untagged OGG1 ± 8-oxoG was added in the presence or absence of GTP or GDP. The samples were incubated for 30 min at 4 °C and washed twice with interaction buffer, and the proteins eluted with Laemmli buffer were analyzed by Western blotting.

To determine interactions between OGG1 and Rac1, we used enzyme-linked immunosorbent assays (ELISA). Briefly, Rac1 antibody-coated wells were washed with PBS-T (2.68 mM KCl, 1.47 mM KH₂PO₄, 136.8 mM NaCl, 9.58 mM NaH₂PO₄, 0.05% Tween-20), and then guanine nucleotide free (empty) Rac1 (5.3 pmol), GDP-, or GTP-loaded Rac1 protein (5.3 pmol) was added to parallel wells in PBS-T alone or together with OGG1 (5.3 pmol) and 8-oxoG (5.3 pmol) for 1 h at room temperature. Unbound proteins were removed by washing before incubation with anti-OGG1 Ab (1 h). HRP-conjugated secondary Ab was added for 45 min, and color was developed using 3,3',5,5'-Tetramethylbenzidine (TMB) substrate. Absorbance was determined on a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA). To confirm OGG1-Rac1 interactions by His-affinity pull-down assays, Ni-NTA-agarose beads (Qiagen Inc., Valencia, Ca) were mixed with His-Rac1 protein (6 pmol) (Cytoskeleton, Denver, CO) in 300 µl interaction buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 7.5) (Qin, Xie et al. 2009). After a 30 min incubation at 4 °C, His-Rac1-bound beads were washed 3 times, and equimolar, non-tagged OGG1 alone or OGG1 (6 pmol) plus 8-oxoG (6 pmol) was added to the interaction buffer. Samples were incubated for 30 min at 4 °C then washed twice with interaction buffer, and proteins eluted with Laemmli buffer at 100 °C for 5 min. The eluants were analyzed on Western immunoblots as described below.

3.7 Western blot analysis

Extracts from lung or cell lysates were clarified by centrifugation, and the supernatants were collected. Protein (25 µg Ras, 10 µg Rac per lane) samples were mixed with sample loading buffer, heated for 5 min at 95 °C, and separated by 5-20% SDS-PAGE. Proteins were transferred to Hybond-ECL nitrocellulose (GE Healthcare Biosciences, Pittsburgh, PA)

membrane by electroblotting. The membranes were then blocked with 3% BSA in TBS containing 0.1% Tween (TBS-T) for 3 h and incubated overnight at 4 °C with the primary antibody diluted in 3% BSA in TBS-T. The blots were then washed 4x with TBS-T and incubated for 1 h with HRP-conjugated secondary Ab in 5% non-fat dry milk in TBS-T. After washing, immunoreactive bands on membranes were visualized by chemiluminescence using an ECL substrate (GE Healthcare Biosciences, Pittsburgh, PA).

3.8 Preparation of 8-oxoG solution

8-OxoG is provided as a hydroacetate salt, and was dissolved in 12 mM NaOH (4 mM final concentration). An 8-oxoG stock solution was prepared freshly, diluted in PBS (w/o $\text{Ca}^{2+}/\text{Mg}^{2+}$, pH: 7.4), and used within 1 h for experiments. All nucleotide bases and nucleosides (2'-deoxyguanosine, guanine, adenine, guanosine, 7,8-dihydro-8-oxo-2'-deoxyguanosine, 8-hydroxyadenine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine) were solubilized in the same manner.

3.9 Fluorescence spectroscopy

The binding of 8-oxoG to OGG1 was assessed by monitoring the decrease in intrinsic tryptophan fluorescence (Hegde, Theriot et al. 2008). Briefly, 0.5 μM OGG1 (100 μl) was incubated with increasing concentrations of 8-oxoG base (or 8-oxodG and FapyG as controls; 0, 10, 50, 100, 200, 400, 800, 1200, 1600, or 2000 nM) for 10 min at 24 °C in 25 mM Tris-HCl (pH 7.6) containing 1 mM DTT. The tryptophan fluorescence at $\lambda_{\text{em}} = 290\text{--}400$ nm ($\lambda_{\text{ex}} = 280$ nm) was analyzed in a SPEX FluoroMax spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ). The binding constant K_d was calculated by plotting ΔF (change in fluorescence emission maximum, 336 nm) *versus* ligand concentration according to the equation $\Delta F = \Delta F_{\text{max}} [\text{ligand}]/K_d + [\text{ligand}]$ (Hegde, Theriot et al. 2008).

3.10 Guanine nucleotide exchange assay

Nucleotide-free H-Ras (6 pmol) was loaded with an equimolar amount of GDP or GTP in a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 3 μM MgCl_2 , 1mM dithiothreitol, and 50 μg of bovine serum albumin at 24 °C (Field, Broek et al. 1987; Lai, Boguski et al. 1993). Guanine nucleotide exchange assays were initiated by the addition of OGG1±8-oxoG in the presence of a 10-fold excess of GTP γ S or GDP. The molecular ratio of Ras and OGG1±8-oxoG was 1:1 or 10:1. After 0, 0.5, 1, 2, 4, 8, 16, or 32 min, nucleotide exchange reactions were terminated by adding 60 mM MgCl_2 . Ras-GTP levels were determined using Active Ras

pull-down assays. Changes in Ras levels were analyzed by Western blotting. The GDP-GTP and GTP-GDP exchange on Rac1 were determined by real-time fluorescence spectroscopic analysis (Qin, Xie et al. 2009). Rac1 (6 pmol) was loaded with the nucleotide analog (2-(or-3)-*O*-(*N*-methylantraniloyl)guanosine 5-triphosphate (Mant)-GTP (^{Mant}GTP) or GDP (^{Mant}GDP) in exchange buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 50 µg of bovine serum albumin for 30 min. In the case of GDP-GTP exchange, Rac1-^{Mant}GDP and OGG1 protein (6 pmol) + 8-oxoG base (6 pmol) were mixed with untagged GTP. A similar strategy was used to monitor GTP-GDP exchange. Kinetic changes in the fluorescence of Rac1-^{Mant}GDP or Rac1-^{Mant}GTP were determined using a POLARstar Omega reader (BMG: Bio Medical Gurrat; LABTECH). Curves were fitted using MS Excel. The half-life of Rac1-^{Mant}GDP was determined using POLARstar Omega software.

3.11 Gene expression and molecular network analysis

MRC-5 cells were treated with 10 µM 8-oxoG or mock solution and harvested at various time points. RNA was isolated, and after synthesis of double-stranded cDNA and biotin-labeled cRNA, the cRNA was hybridized to Affymetrix GeneChip® Human Genome Focus Arrays. The initial data were produced by Affymetrix Microarray Suite software and further processed for network, pathway, and functional analyses using the Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems) in accordance with IPA guidelines. A 3.25×10^{-5} normalized gene expression level (corresponding to ~200 units of fluorescent intensity level in the raw data file) was established as the background threshold. Genes with altered expression (1.5-fold) were overlaid onto a global molecular network developed from information contained in the IPA Knowledge Base; networks of these genes were then algorithmically generated based on their connectivity. These data were deposited (NCBI, Gene expression Omnibus# GSE26813). Canonical pathway analysis identified and ranked the signaling pathways from the IPA library that were most significant to the dataset, based on IPA statistical analysis.

3.12 Flow cytometry

Cells were harvested by pipetting, washed twice with saline and resuspended in the staining buffer /PBS supplemented with 0.5% BSA and 0.05% sodium azide (Sigma-Aldrich). 3×10^5 cells were labeled with fluorescent monoclonal antibody conjugates. Control samples were labeled with isotype-matched antibodies labeled with the same fluorochrome (all from BD Pharmingen). Expression level of cell surface markers was measured after direct or indirect immunofluorescence labeling using FACSCalibur flow cytometer (BD Biosciences, Franklin

Lakes, NJ, USA). Ten-thousand cells were counted and analyzed by the CellQuest program. Subcellular particles were gated out on the basis of forward and side scatter, the list-mode data were analysed by the WinMDI software.

3.13 Latex bead uptake

Unstimulated and activated cells (5×10^5 /ml) were incubated with 5×10^6 /ml carboxylate modified latex beads of 1 μ m diameter (Sigma-Aldrich) for 48 h at 37 °C, washed three times with staining buffer and analyzed by flow cytometry. Control cells were incubated at 4 °C under the same conditions.

3.14 Lucifer Yellow uptake

Unstimulated and control cells (1×10^6 /ml) were incubated with 250 μ g/ml Lucifer Yellow (Sigma-Aldrich) at 37 °C for 1 h, washed three times with the staining buffer and analyzed by flow cytometry. Control cells were incubated at 4 °C under the same conditions.

3.15 FITC-dextran uptake

FITC-dextran uptake was measured as described previously (Sallusto, Cella et al. 1995). Briefly, 1 mg/ml FITC-dextran (Sigma-Aldrich) was added to 1×10^6 unstimulated or activated cells and incubated for 1 h. Cells were washed three times with the staining buffer and analyzed by flow cytometry. Control cells were incubated at 4 °C under the same conditions.

3.16 Chemotaxis assay

Cell migration was assessed in a multiwell microchemotaxis chamber (Neuroprobe, Gaithersburg, MD, USA). 1×10^6 cells were resuspended in 1 ml RPMI 1640 medium (Sigma-Aldrich) supplemented with 0.5% BSA (Sigma-Aldrich). 3.5×10^5 cells in 350 μ l RPMI medium were placed into the upper well chambers, which were separated by a filter of 5 μ m pore size from the lower wells containing 430 μ l medium containing various concentrations of the recombinant chemokine MIP-3 (Peprotech) or medium alone. Cells were allowed to migrate for 3 h, 4 h and 5 h at 37 °C in a CO₂ incubator. Non-migrating cells were removed by washing the filter with PBS containing 2 mM EDTA. Cells, which migrated to the lower wells were centrifuged and resuspended in 50 μ l medium. The amount of migratory cells was assessed by MTT assay.

3.17 Down-regulation of gene expression

Cells were transfected with control siRNA (siGENOME nontargeting siRNA) or target-specific siRNA: Harvey (H)-Ras, Kirsten (K)-Ras, neuroblastoma Ras viral oncogene homolog (N)-Ras, OGG1, Rac1 and NOX4 siRNAs (siGENOME SMARTpool, Dharmacon, Thermo Scientific) using INTERFERin™ transfection reagent (Polyplus Transfection Inc.) per the manufacturer's instructions. Briefly, siRNAs (20 nM final concentrations, as determined in preliminary studies) were mixed with INTERFERin™ transfection reagent and added to cells. After 3 h incubation in serum-free medium, growth medium was added for 72 h. p22phox siRNA and a second control were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). OGG1 was depleted via a simultaneous siRNA transfection and plating method (Boldogh, Hajas et al. 2012). Depletion of the target genes' mRNA levels was determined by qRT-PCR and Western blot analysis.

3.18 Quantitative real-time PCR

qRT-PCR was done by the SYBRGreen method using an ABI 7000 System equipment and software (Applied Biosystems, Foster City, CA) per the manufacturer's recommended protocol. The thermal profile was: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 15 sec, followed by 60 °C for 1 min. A dissociation stage was added at the end of the run to verify the primers' specificity (95 °C for 15 sec, 60 °C for 20 sec and 90 °C for 15 sec). Expression levels (fold change) were determined by the delta-delta Ct method (ΔCt) (Livak and Schmittgen 2001; Aguilera-Aguirre, Bacsı et al. 2009). Primers: p22phox: F: 5'-AACGAGCAGGCGCTGGCGTCCG-3' R: 5'-GCTTGGGCTCGATG GGCGTCCACT-3'; Rac1: F: 5'-CTGATGCAGGCCATCAAGT-3' R: 5'-CAGGAAATGCATTGGTTG TG-3'; GAPDH: F: 5'-GAAGGTGAAGGTCGGAGT-3'; R: 5'-GAAGATGGTGATGGGATTTC-3'

3.19 Oligonucleotide excision assay

OGG1' base excision repair activity in nuclear lysates was determined by using a ³²P-labeled 31-mer oligonucleotide (5'-GAA GAG AGA AAG AGA *GAA GGA AAG AGA GAA G-3'; Midland Certified Reagent Co., Midland, TX) substrate containing one 8-oxoG as previously described (Bhakat, Mokkaṭṭi et al. 2006). The cleaved product was separated from the intact substrate in a 20% polyacrylamide gel containing 8 M urea in Trisborate-EDTA buffer, pH 8.4. Radioactivity in the separated DNA bands was visualized by using a Storm 860 PhosphorImager (Molecular Dynamics) and quantified by densitometry using ImageQuant software.

3.20 Assessment of cellular ROS levels

Changes in intracellular ROS levels were determined by using the fluorogenic probe 2'-7'-dihydro-dichlorofluorescein diacetate (H₂DCF-DA; Molecular Probes, Eugene, OR) (Das, Hazra et al. 2005; Bacsi, Chodaczek et al. 2007). Briefly, cells were grown to 70% confluence and loaded with 50 μM H₂DCF-DA at 37 °C for 30 minutes. Cells were then washed twice with PBS and exposed to nucleic acid bases, nucleosides, or solvent. Changes in DCF fluorescence were recorded in an FLx800 (Bio-Tek Instruments Inc., Winooski, VT) microplate reader at 485 nm excitation and 528 nm emission. Alternatively, in parallel experiments, changes in cellular ROS levels were determined by flow cytometry (BD FACSCanto flow cytometer; BD Biosciences, Franklin Lakes, NJ).

3.21 Microscopic imaging

Cells were transfected with pHyPer-Cyto, pHyPer-dMito, or pHyPer-Nuc and 72 h later challenged with 8-oxoG (10 μM) or H₂O₂ (10 μM). At times indicated, cells were washed in PBS, fixed with formalin (3.7%), dried, and mounted on microscope slides. Images were taken by a NIKON Eclipse Ti System (magnification: x125).

To visualize colocalization, cells were cultured on microscope coverslips then mock-treated or pulsed for 30 min with 10 μM 8-oxoG base and fixed in 4% paraformaldehyde at 4 °C and then permeabilized with Triton X100 for 30 min at 37 °C. The cells were then incubated for overnight at 4 °C with primary antibody to OGG1 (1:200), Rac1 (1:400), NADPH oxidase subunit 4 antibody (1:300). After washing with PBS-Tween 20 (PBS-T) cells were incubated for 1 h at room temperature with Alexa 488-, Alexa-594 and/or Texas Red-conjugated secondary antibodies. Nuclei of cells were stained for 15 min with DAPI (4'-6-diamidino-2-phenylindole dihydrochloride; 10 ng/ml). Cells were then mounted in anti-fade medium (Dako Inc. Carpinteria, CA) on a microscope slide. Microscopy was performed on a NIKON Eclipse Ti System (magnification: x125). Colocalization was visualized by superimposition of green and red images using Nikon NIS Elements Version 3.5 (NIKON Instruments, Tokyo, Japan).

Colocalizations (overlap coefficient) of proteins were calculated according to Manders (Manders, Verbeek et al. 1993). $R = S1 \times S2 / ((S1)^2 \times (S2)^2)$ where $S1$ represents the signal intensity of pixels in channel 1 and $S2$ represents signal intensity of pixels in channel 2. This coefficient is not sensitive to the limitations of typical fluorescence imaging, such as efficiency of hybridization, sample photo bleaching, and camera quantum efficiency

(Zinchuk, Zinchuk et al. 2007). The overlap coefficients k_1 and k_2 split the value of colocalization into a pair of separate parameters: $k_1 = S1 \times S2 / (S1)^2$; $k_2 = S1 \times S2 / (S2)^2$, where $S1$ represents the signal intensity of pixels in channel 1 and $S2$ represents signal intensity of pixels in channel 2 (Manders, Verbeek et al. 1993).

3.22 Statistical analysis

Data are expressed as the mean \pm S.D. Results were analyzed for significant differences using ANOVA procedures and Student's t-tests (Sigma Plot 11.0). Differences were considered significant at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

4. Results and Discussion

4.1 Signaling pathways induced at the transcriptomal level by 8-oxoG

The free 8-oxoG base is generated exclusively during the repair of 8-oxoG in DNA, initiated by OGG1 (Mitra, Hazra et al. 1997). To mimic a transient increase in its intracellular level, we added 8-oxoG base to OGG1-proficient cells (MRC-5) and analyzed the impact on the transcriptome using Affymetrix GeneChip. Ingenuity Pathways Analysis (IPA) of microarray gene expression data showed that 8 of the top 10 pathways that responded to 8-oxoG involved the small G protein Ras (Fig. 8.). These data were deposited to National Center for Biotechnology Information (NCBI), GEO accession number: GSE26813.

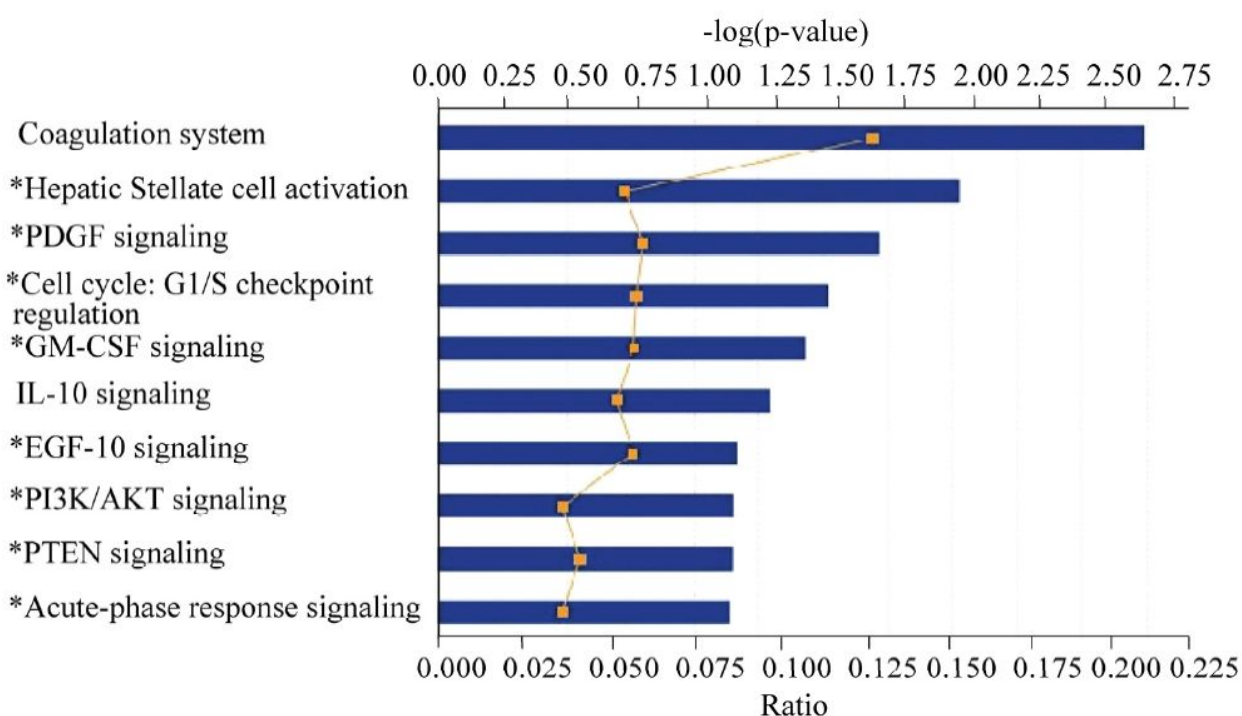


Figure 8. Signaling pathways induced at the transcriptomal level in human diploid fibroblasts after 8-oxoG exposure

MRC-5 cells were exposed to 8-oxoG base (10 μM) or mock-treated and harvested at various time points thereafter. Data for the 6-h time point are shown. Bars correspond to $-\log(p\text{-value})$. *Ras-regulated pathways.

- -, Ratio. The significance of the association between the dataset and a canonical IPA pathway was determined as a ratio of the number of genes from the dataset that map to the pathway divided by the total number of genes that map to the canonical pathway. Fisher's exact test was used to calculate a $p\text{-value}$ determining the probability that the association between the genes in the dataset and the canonical pathway is not explained by chance alone. A cutoff $p\text{-value}$ of $p = 0.05$ was used to determine significantly altered networks.

4.2 Ras activation in cell culture and lungs

To confirm Ras activation, we showed that the addition of 8-oxoG increased GTP-bound Ras levels in a time- (Fig. 9A) and dose-dependent (Fig. 9B) manner. The time course of Ras activation was in line with rapid cellular uptake of 8-oxoG base (Fig. 9C). For example, at 1 min after the addition, ~70% of 8-oxoG was taken up by the cells, as shown by LC/IDMS analysis (Fig. 9C).

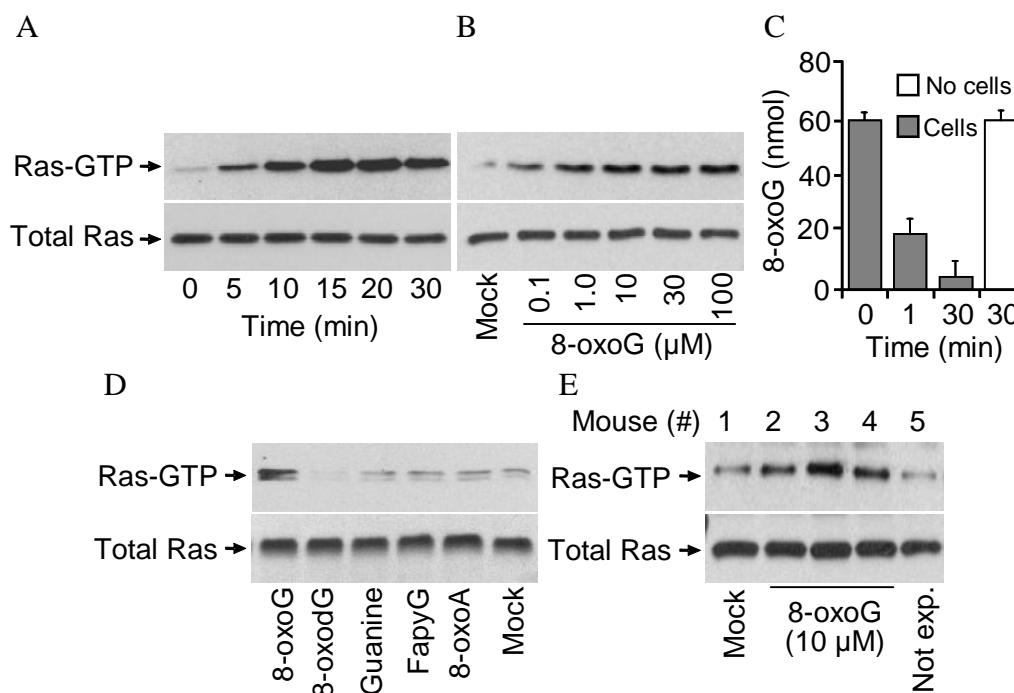


Figure 9. 8-oxoG activates Ras GTPases

Ras activation in time- (A) and dose-dependent (B) manner in MRC-5 cells. (C) 8-oxoG uptake by MRC-5 cells. (D) Only 8-oxoG but not other bases induces Ras activation. Parallel cell cultures were exposed to 1 μ M (A, D) or increasing concentration of 8-oxoG (B). Cell extracts were made at the times points indicated (A) or 15 min after treatment (B, D). (E) Increase in Ras-GTP levels in murine lungs. BALB/c mice were challenged intranasally with 60 μ l of 1 μ M 8-oxoG, and the lungs were excised after 15 min. Ras-GTP levels were determined by GST pull-down assays.

The lowest dose of 8-oxoG base that increased Ras-GTP to a detectable level was 100 nM in MRC-5 cells (Fig. 9B). There was no concentration-dependent increase in Ras-GTP levels above 10 μ M of 8-oxoG (e.g., 30 μ M, Fig. 9B), due to its low solubility at physiological pH (Qi, Spong et al. 2009). Free 8-oxoG was unique in increasing Ras-GTP levels as neither 8-oxodG nor other oxidized bases (FapyG, 8-oxoA) nor the original guanine base displayed this activity (Fig. 9D). To test the *in vivo* relevance of these findings we challenged mice intranasally with 8-oxoG and investigated the activated Ras levels in the

lungs excised after 15 min. We found that exposure of mice to 8-oxoG markedly increased Ras-GTP levels in the lungs (Fig. 9E).

4.3 Ras is not activated when OGG1 silenced

As 8-oxoG is recognized and excised by OGG1 in the genome, we hypothesized that 8-oxoG bound to OGG1 mediates Ras activation. To prove our hypothesis we down-regulated OGG1 in MRC-5 (Fig. 10A) and HeLaS (Fig. 10B) cells and measured the Ras-GTPase levels after 8-oxoG treatment (Fig. 10C, D). OGG1-depleted MRC-5 (Fig. 10A, C) or HeLaS cells (Fig. 10B, D) showed reduced OGG1 RNA levels (20% compared to control cells) and adding 8-oxoG did not cause an increase in Ras-GTP levels.

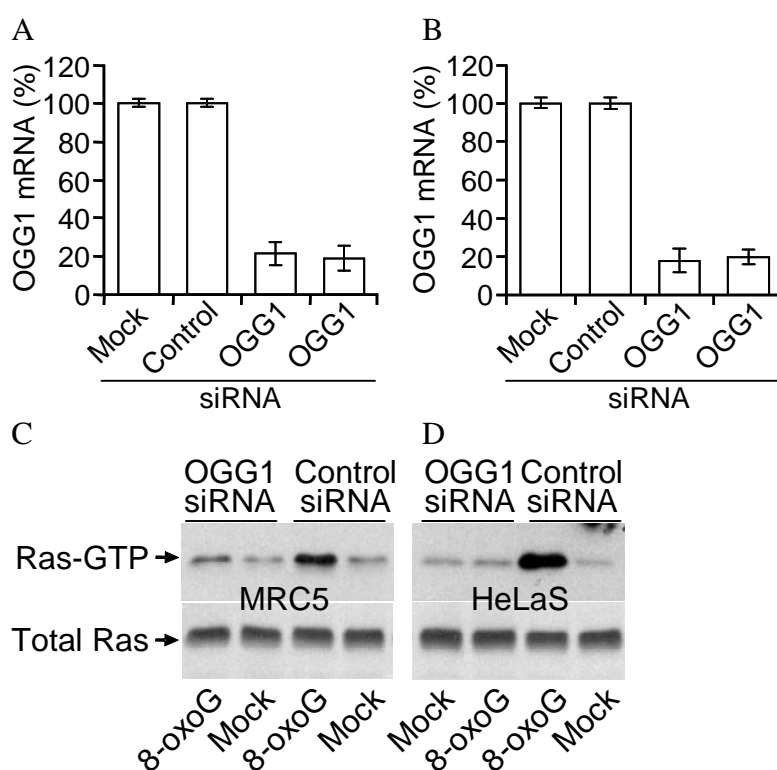


Figure 10. OGG1 down-regulation prevents Ras activation in 8-oxoG exposed cells

Silencing RNA-mediated OGG1 depletion at the RNA levels in MRC-5 (A) and HeLaS (B) cells. Cells were transfected with silencing (si)RNA to OGG1 or control siRNA using two cycles of transfection and sub-culturing. RNA levels were determined by qRT-PCR. Ras activation in OGG1 depleted MRC-5 (C) and HeLaS (D). Ras-GTP levels were determined from control siRNA- and OGG1-specific siRNA-transfected cells by GST pull-down assays.

In the experiments described above we have observed that externally added 8-oxoG is able to increase GTP-bound Ras levels both in cultured cells and in the lungs of experimental animals. Our further question was if Ras can be activated by 8-oxoG excised from DNA?

4.4 Ras activation by excised 8-oxoG: the KG-1 model

To investigate the effects of intracellularly generated 8-oxoG on Ras activation we used KG-1 cell line. The KG-1 is a human acute myelogenous leukemia cell line that represents an early stage of hematopoietic differentiation. KG-1 can spontaneously differentiate to granulocyte and macrophage like cells and they show a good response to colony stimulating factor (CSF) (Koeffler and Golde 1978). KG-1 cells have several features that make them a good model to study various cell functions. Hulette and his co-workers induced DC morphology and phenotype by using a defined cytokine cocktail. The cells grew hair-like cytoplasmic projections and expressed MHCII, CD80, CD86 and CD83 molecules. Functionally, KG-1-derived DCs were able to induce allogenic T-cell response and phagocytose latex beads. Furthermore, KG-1 cells also respond to inflammatory cytokines like IL-18 and TNF- α (Nakamura, Otani et al. 2000).

We previously described some important functional features of KG-1 cells compared to immature dendritic cells (DCs) of monocytic origin. We found that the efficiency of Lucifer Yellow uptake by pinocytosis is similar in monocyte-derived DCs and in KG-1 cells (Fig. 11A). Phagocytosis of latex beads, however, was less efficient by KG-1 cells than by immature DCs (Fig. 11B). Both immature DCs and KG-1 cells internalized FITC-dextran and expressed the mannose receptor (MR) as shown in Fig. 11D and C, respectively. The difference in the efficacy of receptor-mediated internalization correlated with MR expression (CD206), which was higher in monocyte-derived immature DCs than in KG-1 cells (Fig. 11C).

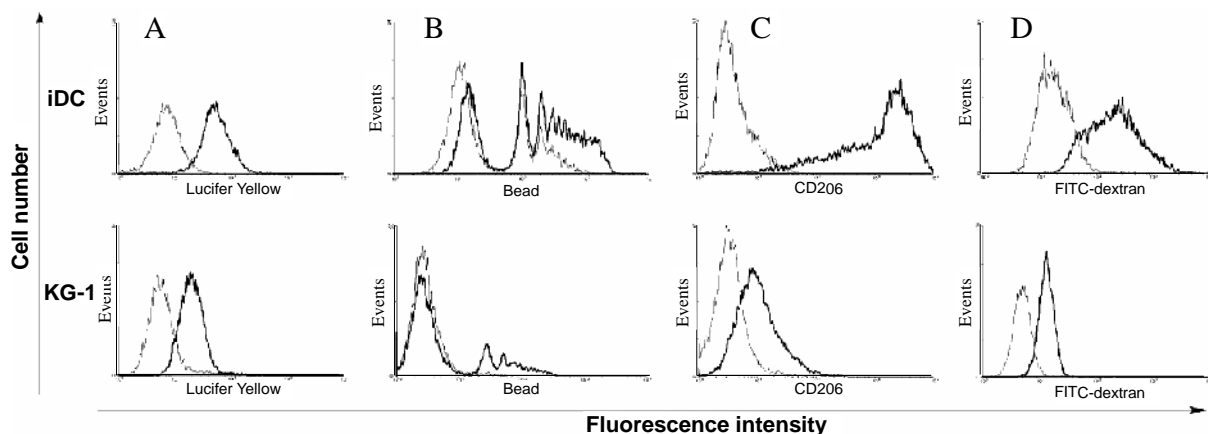


Figure 11. The internalizing capacity of immature monocyte-derived DCs and KG-1 cells

The uptake of Lucifer Yellow (A), latex beads (B) and FITC-dextran (D) was measured as described in Materials and Methods. Bold lines represent cells incubated at 37 °C, hairpin lines correspond to control cells incubated at 4 °C. Panel C shows cell surface expression of mannose receptor on immature monocyte-derived DC and KG-1 cells. Bold line represents mannose receptor expression as compared to isotype-matched control antibody (hairpin line).

We also investigated the migratory capacity of monocyte-derived DCs and KG-1 cells. The expression of the CCR7 chemokine receptor on mature DCs, naive and central memory T-cells is essential for their migration to the T-cell areas of draining lymph nodes (Scandella, Men et al. 2002). We found that immature monocyte-derived DCs express the CCR7 receptor at low levels (Fig. 12A), but significant up-regulation was detected in a small population of mature monocyte-derived DCs followed by their activation with inflammatory cytokines (Fig. 12A). Our results show that unstimulated as well as activated KG-1 cells express high levels of the chemokine receptor CCR7 (Fig. 12B).

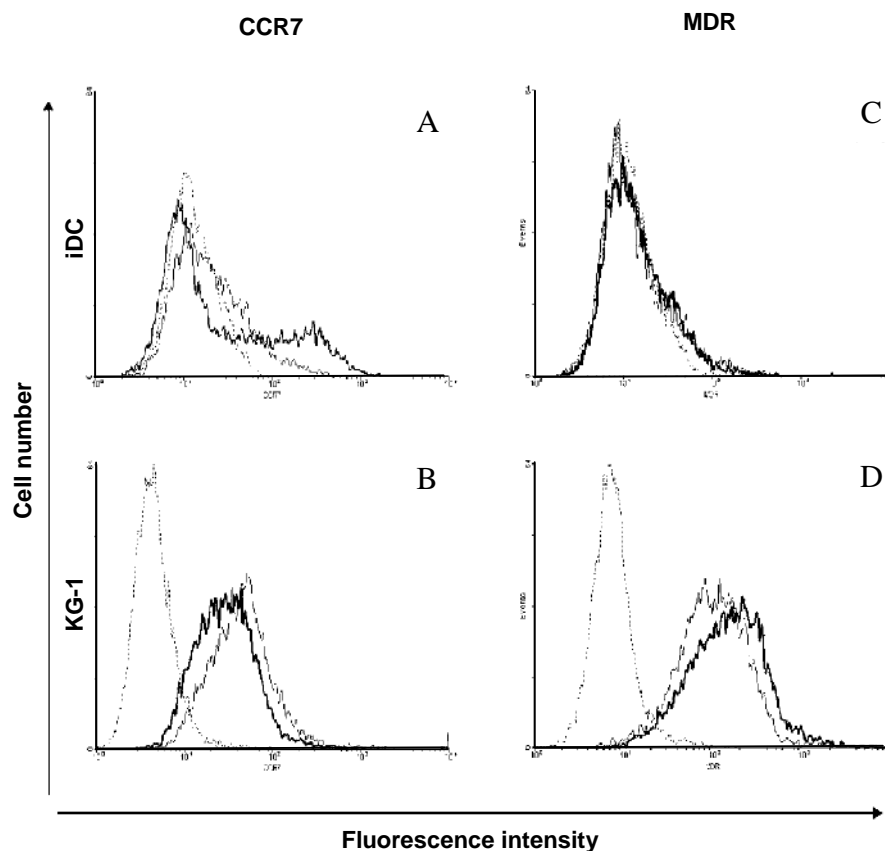


Figure 12. Cell surface expression of CCR7 chemokine and MDR transporter on monocyte-derived DCs (A, C) and KG-1 cells (B, D)

Monocyte-derived DCs were activated by an inflammatory cocktail for 24 h, KG-1 cells were activated by PMA and ionomycin for 4 days. Dotted line: isotype matched control.

Next, we compared the migratory capacity of monocyte-derived mature DCs and KG-1 cells towards a chemokine gradient. In contrast to monocyte-derived DCs (Fig. 13A) KG-1 cells were not able to migrate to the direction of chemokine attraction (Fig. 13B). These results show that high cell surface expression of CCR7 is not sufficient for inducing migration of KG-1 cells and its lower expression on DCs also allows mobilization.

It has been proposed that the ABC transporters P-glycoprotein/MDR-1 and Mrp-1 are involved in the mobilization of DC cells from tissues to draining lymph nodes (Robbiani, Finch et al. 2000). We could not detect MDR-1 in the surface of monocyte-derived DC (Fig. 12C) but KG-1 cells expressed high level of this transporter (Fig. 12D). These results suggest that the defect in chemokine driven migration of KG-1 cells is conferred by factors independent of the expression of CCR7 or MDR-1.

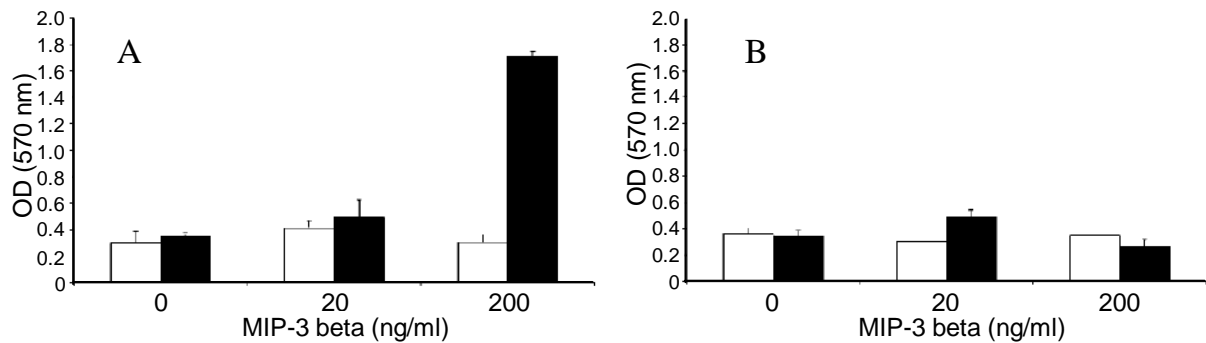


Figure 13. Chemokine-mediated migration of monocyte-derived DCs and KG-1 cells

The migration of cells was measured in the presence of 20 and 200 ng/ml MIP-3 (CCL19) in a 4-h *in vitro* chemotactic assay. Empty columns correspond to immature monocyte-derived DCs (A) and unstimulated KG-1 cells (B), respectively. Dark columns show activated monocyte-derived DCs (A) and activated KG-1 cells (B). Monocyte-derived DCs were activated by an inflammatory cocktail for 24 h, KG-1 cells were activated by PMA and ionomycin for 4 days.

Our observations altogether suggest that KG-1 cells resemble professional myeloid antigen presenting cells but also possess special phenotypic and functional characteristics.

The unique feature that makes KG-1 cell line a suitable model for study the 8-oxoG-mediated Ras activation is the expression of a thermolabile OGG1 mutant (OGG1^{R229Q}). At physiological temperature (37 °C), these cells accumulate 8-oxoG in their genome due to the lack of OGG1^{R229Q}'s enzymatic activity (Hill and Evans 2007). Shifting KG-1 cell cultures from 37 °C to a lower temperature (e.g., 25 °C) restores the 8-oxoG excision activity of OGG1^{R229Q}. Since KG-1 cells cultured at 37 °C have supraphysiological 8-oxoG levels in their genome they mimic normal cells in tissues exposed to oxidative stress generated by endogenous and exogenous mechanisms. Intriguingly, the viability of KG-1 cells is similar to that of cells expressing wild-type OGG1. Importantly, the KG-1 cell model allowed us to examine the consequences of 8-oxoG repair without exposing cells to ROS.

To confirm that the catalytic activity of OGG1 in KG-1 cells is defective under our culture conditions, nuclear extracts were prepared and 8-oxoG excision assays were carried out. Figure 14 shows that there was no detectable 8-oxoG excision activity at 37 °C. The activity of OGG1^{R229Q} was re-established at 25 °C, in line with data published previously (Hyun, Choi et al. 2000; Hill and Evans 2007). In control experiments, nuclear extracts of U937 cells expressing wild-type OGG1 showed nearly identical 8-oxoG excision activity at both 25 °C and 37 °C (Fig. 14). To determine if the release of 8-oxoG from the genome is associated with an increased level of Ras-GTP, cultures of KG-1 cells were transferred from 37 °C to 25 °C for 45 min or 90 min, cell extracts were prepared, and GTP-bound Ras levels were determined using active Ras pull-down assays. Control cells were kept at 37 °C (Fig. 15).

The results summarized in Figure 15 show that at 25 °C, GTP-bound Ras levels were elevated at 45 min and further increased at 90 min. In contrast, U937 cells (expressing wild-type OGG1) showed no change in active Ras levels at 25 °C. Incubation of KG-1 and U937 cells at 25 °C resulted in no alteration in total Ras levels (Fig. 15). These data support the idea that OGG1-initiated BER is associated with activation of Ras GTPase as we proposed previously.

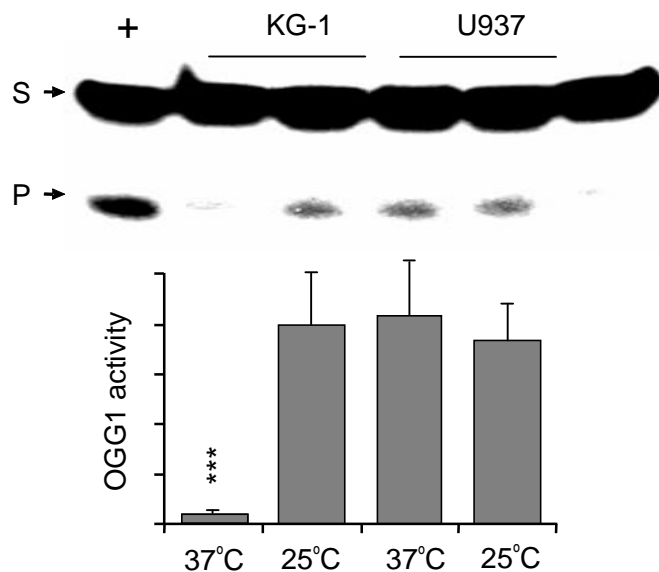


Figure 14. OGG1 activity in KG-1 and U937 cells at 25 °C vs. 37 °C

Nuclear extracts were prepared from KG-1 and U937 cells and DNA repair assays were carried out at 37 °C and 25 °C as we previously described. Upper panel: Band intensities were quantified by densitometry by using ImageQuant software and graphically illustrated (lower panel). S: substrate; P: product; (+): 25 fmol recombinant OGG1; (-): ³²P-labeled substrate only.

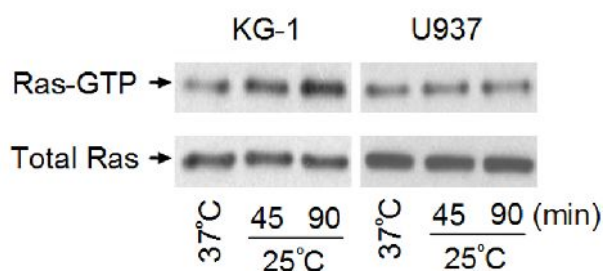


Figure 15. Levels of activated Ras in KG-1 and U937 cells at 25 °C vs. 37 °C

Cultures of KG-1 and U937 cells were incubated at 37 °C or transferred to 25 °C, and Ras-GTP levels were assessed at 45 min and 90 min. Upper panels: Ras-GTP levels in KG-1 and U937 cells. Lower panels: Total Ras levels in KG-1 and U937 cells.

It has been reported that GEFs' and GAPs' activities were affected by temperature change (Chan, Stang et al. 1999), so they could influence the relative levels of Ras-GTP and Ras-GDP in KG-1 cells. For example, Ras GAP could be inhibited at 25 °C, resulting in the accumulation of Ras-GTP. To exclude this possibility, OGG1 expression in KG-1 cells was downregulated using siRNA, and the cells then incubated at 25 °C for 90 min (Fig. 16).

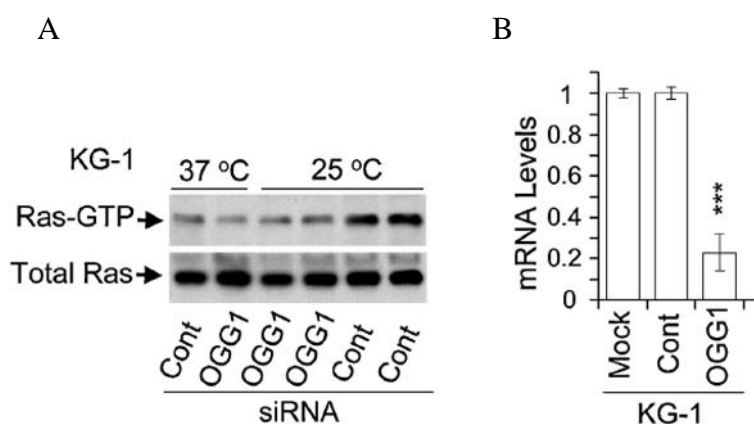


Figure 16. Lack of Ras activation in OGG1-depleted KG-1 cells

(A) OGG1-depleted and control (control siRNA-transfected) cells were transferred to 25 °C for 90 min and Ras-GTP levels were determined in cell extracts. (B) Changes in OGG1 RNA levels after transfection of cells with control and OGG1-specific siRNA. *** $p < 0.001$.

OGG1-depleted cells showed no increase in Ras-GTP levels (Fig. 16A). In contrast, Ras-GTP levels were increased in cells transfected with control siRNA (Fig. 16A). The extent of OGG1 downregulation is shown in Figure 16B. These results confirmed that in KG-1 cells, activation of OGG1^{R229Q} and generation of 8-oxoG base is the primary cause of increased Ras-GTP levels, rather than an inhibitory effect of low temperature on Ras GAP.

4.5 OGG1 binds free 8-oxoG at an independent site and not in the DNA lesion-recognition site

Next, we investigated the interaction between OGG1 and free 8-oxoG. In many cases, the binding of the ligand to the protein changes its intrinsic tryptophan (Trp) (or tyrosine) fluorescence. If the ligand is close enough to the tryptophan(s) or if a conformational change is associated with ligand binding and this brings a quenching group into the vicinity of the fluorophore, then the intensity decreases and the K_d value of protein-ligand binding can be determined (Royer and Scarlata 2008). Based on these previous observations the binding of free 8-oxoG base to OGG1 was analyzed by changes in the intrinsic Trp fluorescence of OGG1 (Hegde, Theriot et al. 2008). A concentration-dependent decrease in Trp fluorescence (Fig. 17A) indicated the OGG1 conformational change as a result of the interaction.

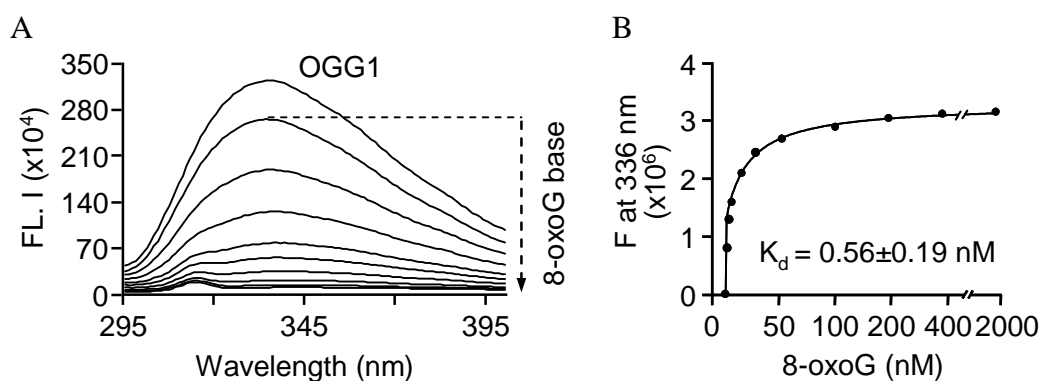


Figure 17. Fluorescent spectroscopic analysis of 8-oxoG binding to OGG1

(A) Changes in the intrinsic Trp fluorescence of OGG1 after addition of increasing concentrations of 8-oxoG base were assessed by fluorescence spectrometry. FL indicates fluorescence. (B) K_d was calculated from binding isotherm.

The binding constant (K_d) 0.56 ± 0.19 nM calculated from the binding isotherms (Fig. 17B) indicates its high affinity for 8-oxoG, which was unexpected. In controls, OGG1 did not bind either 8-oxodG or free FapyG base (data not shown), the latter one with abundance similar to that of 8-oxoG in oxidatively damaged DNA and an equally good OGG1 substrate (Hu, de Souza-Pinto et al. 2005). Additionally, the 8-oxoG base did not affect the intrinsic fluorescence of Ras protein (data not shown). Thus, the 8-oxoG base is unique in its strong affinity for OGG1, which functions not only in repairing oxidized purines, but together with 8-oxoG, has an independent function in cellular signaling.

We observed that 8-oxoG base stimulated OGG1's activity in a concentration-dependent fashion, which indicates that the 8-oxoG base serves as a cofactor to OGG1 (Fig. 18). This observation also implies that the 8-oxoG base binds tightly at an independent site of

OGG1 and not in the DNA lesion-recognition site which was previously suggested by Fromme and coworkers (Fromme, Bruner et al. 2003).

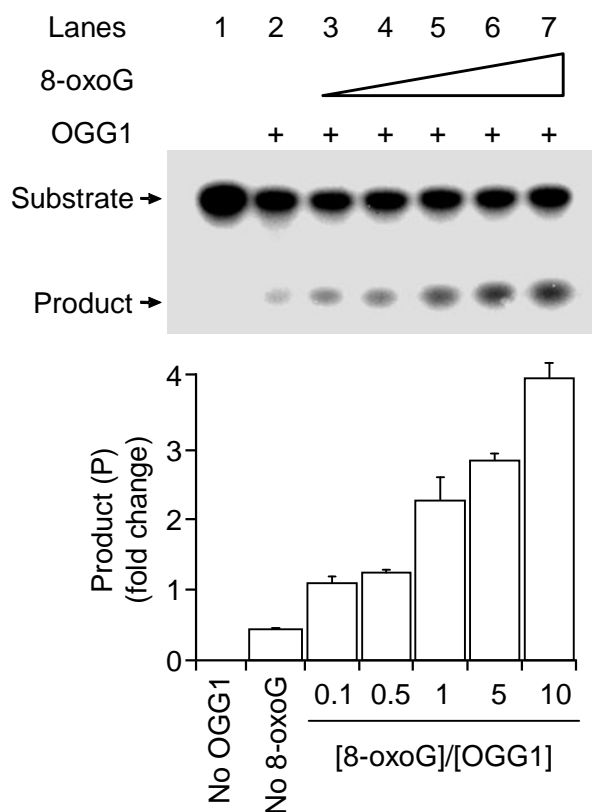


Figure 18. OGG1 binds 8-oxoG base at an independent site and not in the DNA lesion recognition site
 OGG1 was incubated with 0, 0.1, 0.5, 1.0, 5, and 10-fold molar excess of 8-oxoG, and OGG1's repair activity was assayed using a ^{32}P -labeled 31-mer oligonucleotide (5'-GAA GAG AGAAAG AGA *GAA GGA AAG AGA GAA G-3'; substrate containing one 8-oxoG (at the *). The excision reactions were carried out as described in the Materials and methods section. The cleaved product was separated from the intact substrate in a polyacrylamide gel. Radioactivity in the separated DNA bands was visualized with a PhosphorImager and quantified by densitometry by using ImageQuant software. Upper panel: a representative image showing cleavage of ^{32}P -labeled 31-mer oligonucleotide. Lower panel: graphical depiction of radioactivity in the product.

4.6 OGG1/8-oxoG complex functions as a GEF on Ras

Activation of GTPases involves displacement of GDP by GTP, a process mediated by GEFs (Bourne, Sanders et al. 1990). GEFs accelerate the exchange of GDP for GTP in Ras-GTPase, and active GTP-bound Ras releases GEF (Boriack-Sjodin, Margarit et al. 1998; Meller, Irani-Tehrani et al. 2002). Next, we explored a possible interaction between OGG1 and Ras (Lai, Boguski et al. 1993; Mosteller, Han et al. 1994; Chataway and Barritt 1995) and observed that in the presence of 8-oxoG, OGG1 was able to bind to H-Ras (Fig. 19A, lane 2).

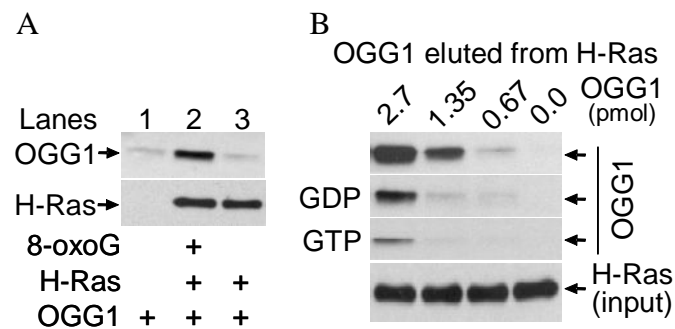


Figure 19. Physical interactions of OGG1 with Ras protein

(A) Interaction of OGG1 with Ras protein requires 8-oxoG base. H-Ras (2.7 pmol) was bound to nickel-agarose beads and OGG1±8-oxoG was added. After washing three times, the amount of OGG1 bound was analyzed by immunoblotting. (B) Guanine nucleotides decrease the OGG1 interaction with Ras. His-H-Ras (2.7 pmol) bound to agarose beads was incubated with untagged OGG1 (2.7, 1.35, 0.67, and 0 pmol) and equimolar 8-oxoG for 30 min, and a 10-fold molar excess of GTP or GDP was then added for 30 min at 24 °C. Levels of eluted proteins were determined by Western blotting.

However, OGG1 alone did not interact with H-Ras under identical conditions (Fig. 19A, lane 1), suggesting that an 8-oxoG-induced conformational change in OGG1 allows its binding to Ras. Furthermore, GTP was more effective than GDP in inhibiting the interaction between Ras and OGG1/8-oxoG (Fig. 19B). These data strongly suggest that the conformation of nucleotide-free Ras allows the most stable interaction with OGG1/8-oxoG, which is weakened in the presence of guanine nucleotides. Similar interactions of K-Ras and N-Ras with OGG1/8-oxoG were also observed, and guanine nucleotides, especially GTP, decreased these interactions (data not shown). Our observations are consistent with those showing high affinity binding between nucleotide-free Ras and GEF (*e.g.* CDC25), which is decreased to an undetectable level by guanine nucleotides, especially GTP, due to nucleotide-induced conformational changes in the Ras protein (Kyriakis, App et al. 1992; Lai, Boguski et al. 1993). In controls, other oxidized base-specific DNA glycosylases (NEIL1 and NEIL2) did not interact with H-Ras (data not shown).

Increases in the Ras-GTP level upon exposure of cells to 8-oxoG (Fig. 9) and physical interaction between Ras and OGG1 (Fig. 19A and B) could cause guanine nucleotide exchange. Indeed, in the presence of 8-oxoG, OGG1 caused replacement of GDP-bound to Ras with GTP (Fig. 20A) at equimolar or higher molar ratios of H-Ras:OGG1. We subsequently showed that OGG1 also catalyzed the H-Ras-bound GTP replacement with GDP (Fig. 20B).

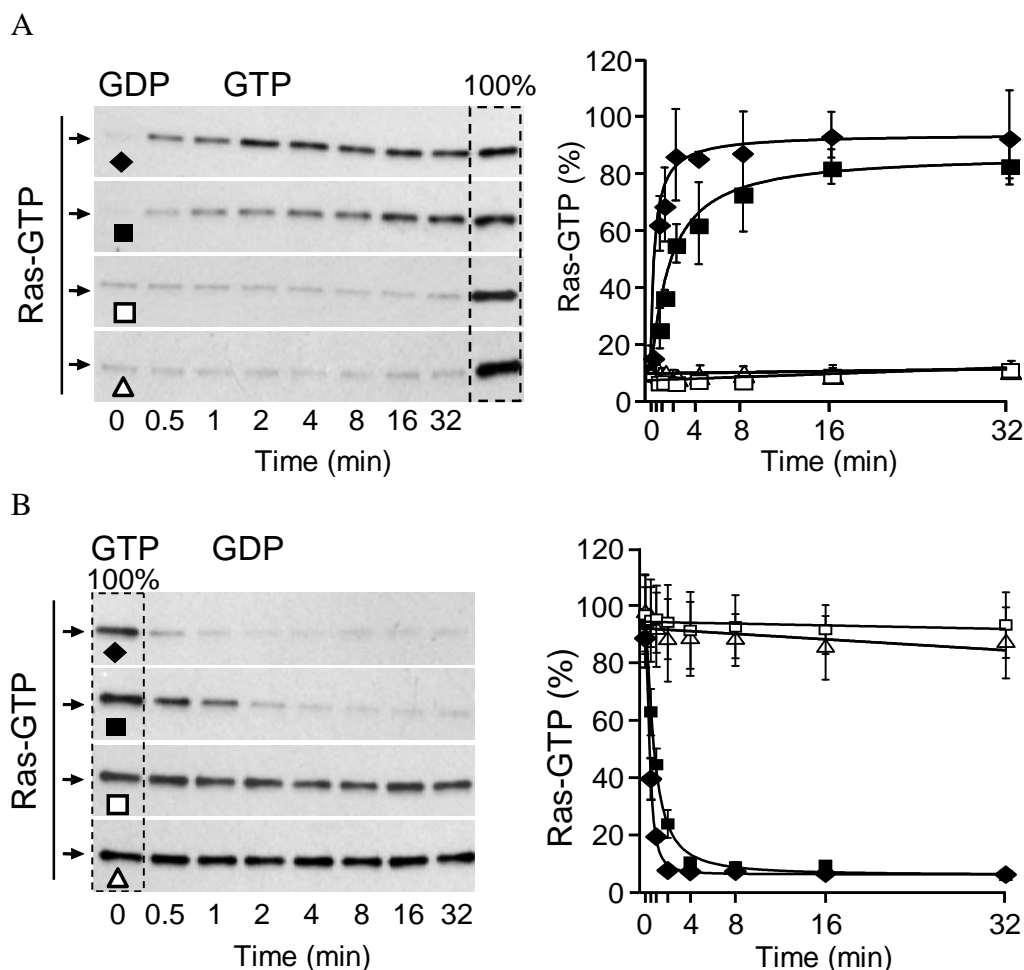


Figure 20. OGG1/8-oxoG complex can exchange both Ras-bound GDP and GTP for free GTP/GDP (A) H-Ras protein (6 pmol) was loaded with GDP (6 pmol), and nucleotide exchange was initiated by adding OGG1 (6 pmol) plus 8-oxoG (◆) or 0.6 pmol of OGG1 plus 8-oxoG (■), OGG1 (□) or 8-oxoG (△) alone, together with GTP γ S (60 pmol). (B) GTP-GDP exchange by OGG1. H-Ras protein (6 pmol) was loaded with GTP, and guanine nucleotide exchange was initiated by adding 6 pmol of OGG1 plus 8-oxoG (◆) or 0.6 pmol of OGG1 plus 8-oxoG (■), OGG1 (□), or 8-oxoG (△) alone, together with 10-fold excess GDP. Ras-GTP was quantified using pulldown immunoblot assays. Right panels: bands in left panels were quantitated by densitometry, and the time course of nucleotide exchange is depicted graphically.

OGG1 or 8-oxoG alone did not induce guanine nucleotide exchange (Fig. 20. A and B). Densitometric analysis of the bands in Fig. 20A and B (left panels) shows striking similarities between the kinetics of GDP → GTP and GTP → GDP exchange on Ras (Fig. 20A and B, right panels), suggesting that OGG1/8-oxoG indiscriminately releases the nucleotide *in vitro* and allows rebinding; thus its activity is similar to that of other Ras-GEFs (Kyriakis, App et al. 1992; Lai, Boguski et al. 1993). In the intracellular environment, due to the high GTP:GDP ratio (~10-fold higher GTP than GDP; (Meller, Irani-Tehrani et al. 2002)/, the released GDP is exchanged for GTP in the Ras protein. OGG1/8-oxoG induced similar guanine nucleotide exchange with N-Ras and K-Ras (data not shown).

4.7 Ras activation induced by 8-oxoG treatment leads to phosphorylation of MAPKs

Ras-GTP binds to the Ras-binding domain (RBD) of the Raf1 serine/threonine kinase (Block, Janknecht et al. 1996), and its subsequent phosphorylation is necessary, but not sufficient, for mediating the mitogen-activated protein kinase (MAPK) activity of Raf1 as phosphorylated Raf1 requires additional protein-protein and membrane-lipid interactions (Kyriakis, App et al. 1992). An increase in the cellular 8-oxoG level in MRC-5 cells induced rapid phosphorylation of the MAPK kinase (MEK1/2) and extracellular signal-regulated kinase (ERK1/2) (Fig. 21A) and the nuclear translocation of the latter (Fig. 21C). The MEK1/2 inhibitor PD98059 decreased phosphorylation and the nuclear translocation of ERK1/2 after treatment of cells either with 8-oxoG base or PDGF (Fig. 21B and C).

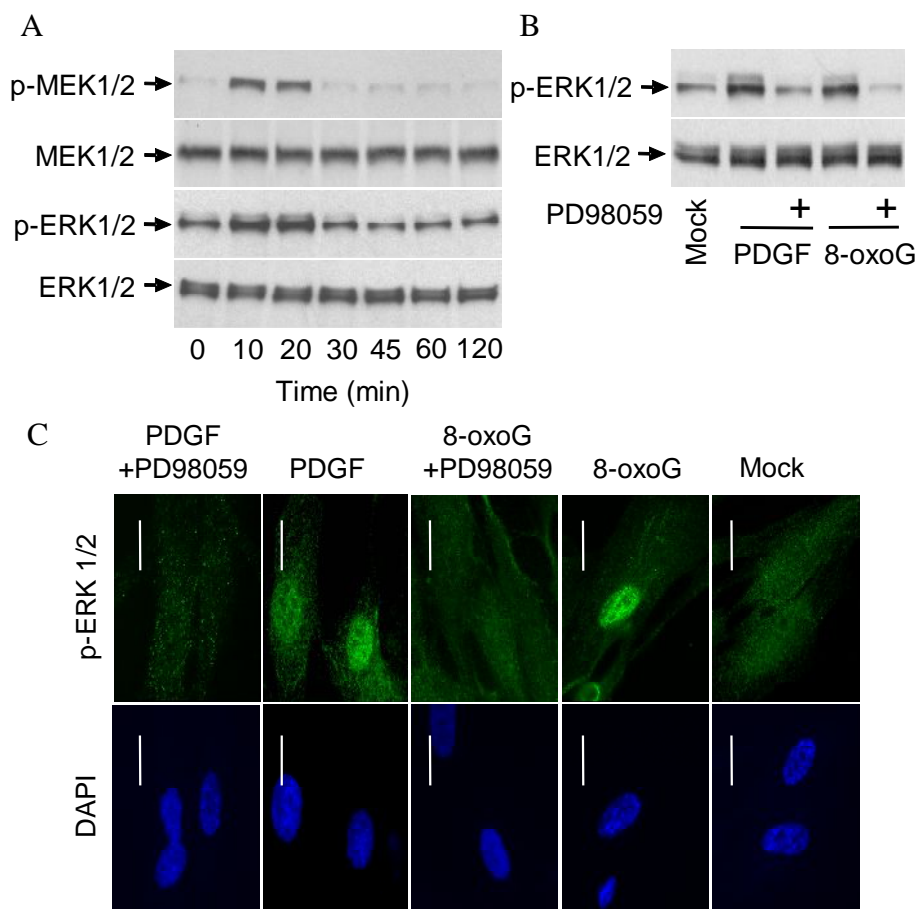


Figure 21. Activated Ras-mediated phosphorylation of MAPKs in 8-oxoG treated cells

(A) MRC-5 cells were exposed to 1 μ M 8-oxoG and lysed at the times indicated, and phosphorylated MEK 1/2 (p-MEK1/2) and -ERK1/2 (p-ERK1/2) levels were determined (upper panels) by Western blotting. Total MEK1/2 and ERK1/2 levels served as loading controls (lower panels). (B) 8-oxoG or PDGF induced ERK1/2 phosphorylation was prevented by PD98059 a MEK1/2 inhibitor. (C) 8-oxoG induces ERK1/2 translocation into the nucleus. The 8-oxoG base exposure, similar to PDGF treatment, induces nuclear accumulation of p-ERK1/2. ERK1/2 translocation was inhibited by PD98059. Green: indirect labeled ERK1/2 (Alexa 488). Blue: DAPI (4,6-diamidino-2-phenylindole dihydrochloride)-stained nuclei (scale bars: 10 μ m).

To confirm that ERK1/2 phosphorylation is indeed Ras-dependent in 8-oxoG-exposed cells, we depleted the canonical Ras family members H-, K- and N-Ras with siRNA (Fig. 22A, upper panel). The results showed a decrease in the phosphorylation of ERK1/2 after 8-oxoG addition, primarily in N-Ras-ablated MRC-5 cells (Fig. 22A, middle panel, last lane). To test which Ras isoform mediated MEK1,2/ERK1,2 phosphorylation in MRC-5 cells, first we demonstrated that the majority of Ras consisted of N-Ras (>60%) and K-Ras (36%) proteins, while H-Ras was nearly undetectable (Fig. 22B middle panel). After 8-oxoG exposure of MRC-5 cells, ~14% of total N-Ras and ~1.7% of K-Ras was in GTP-bound form

(Fig. 22B upper panel). These data are in line with those showing that siRNA to N-Ras significantly decreased ERK1/2 phosphorylation, while a marginal effect of K-Ras-siRNA was observed (Fig. 22A middle panel).

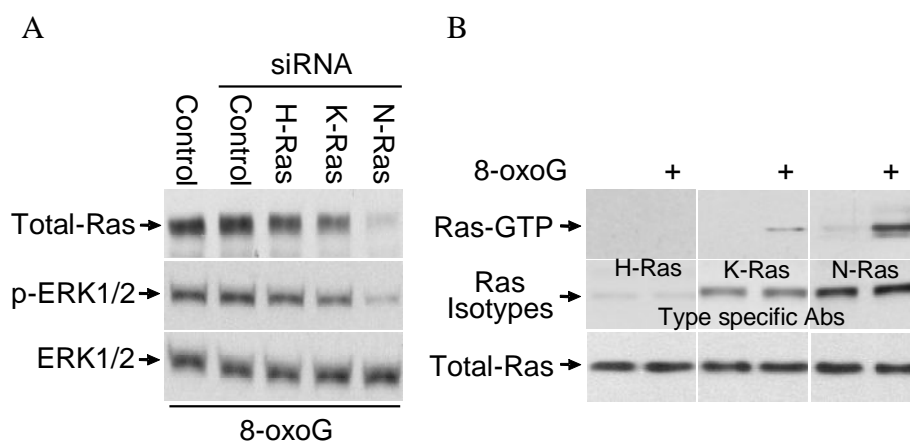


Figure 22. Isotype-specific ERK1/2 phosphorylation and Ras activation in MRC-5 cells

(A) Upper panel, levels of total Ras after depletion by siRNA to H-, K-, and N-Ras, shown by Western blotting. SiRNA-transfected cells were treated with 1 μ M 8-oxoG for 15 min, and phosphorylated ERK1/2 and ERK1/2 levels were assessed by Western blot analysis (middle and lower panels). (B) Isotype-specific activation of Ras in 8-oxoG-exposed MRC-5 cells. Upper panel: activation of Ras isoforms after 8-oxoG addition (15 min). Ras-GTP levels were determined in 250 μ g of cell extracts by pull-down assay. Middle panel: H-, K-, and N-Ras levels in mock- and 8-oxoG-treated cells. Lower panel: total Ras in cell extracts (25 μ g/lane) is shown by pan-Ras antibody.

These results so far suggest a complex signaling network activated by OGG1, and triggered by the generation and repair of 8-oxoG in the genome.

4.8 Treatment with 8-oxoG also increases cellular ROS levels

Li and co-workers have recently showed that downregulation of OGG1 decreases the house dust mite (HDM) extract induced ROS in MLE-12 cells. They also proposed that expression of OGG1 affects the levels of oxidative stress during asthmatic conditions (Li, Yuan et al. 2012). Based on these previous observations we also tested whether upon addition of free 8-oxoG base to the cells it increases cellular ROS levels via an OGG1 dependent mechanism. We found that the treatment of MRC-5 or A549 cells with 8-oxoG elevated the intracellular ROS levels (Fig. 23A–D). Pretreating the cells with N-acetyl-L-cysteine (NAC; 10 mM, for 3 h), a precursor of glutathione biosynthesis and a scavenger of oxygen radicals (Das, Bacsi et al. 2006), prevented the 8-oxoG-mediated increase in intracellular ROS levels (Fig. 23B). Furthermore, 8-oxoG induced significantly lower levels of ROS in OGG1-depleted cells (Fig.

23C). The 8-oxoG base is unique in increasing ROS levels, because the guanine base, FapyG, 8-OH-Ade, adenine, and guanine do not have this activity (Fig. 23D).

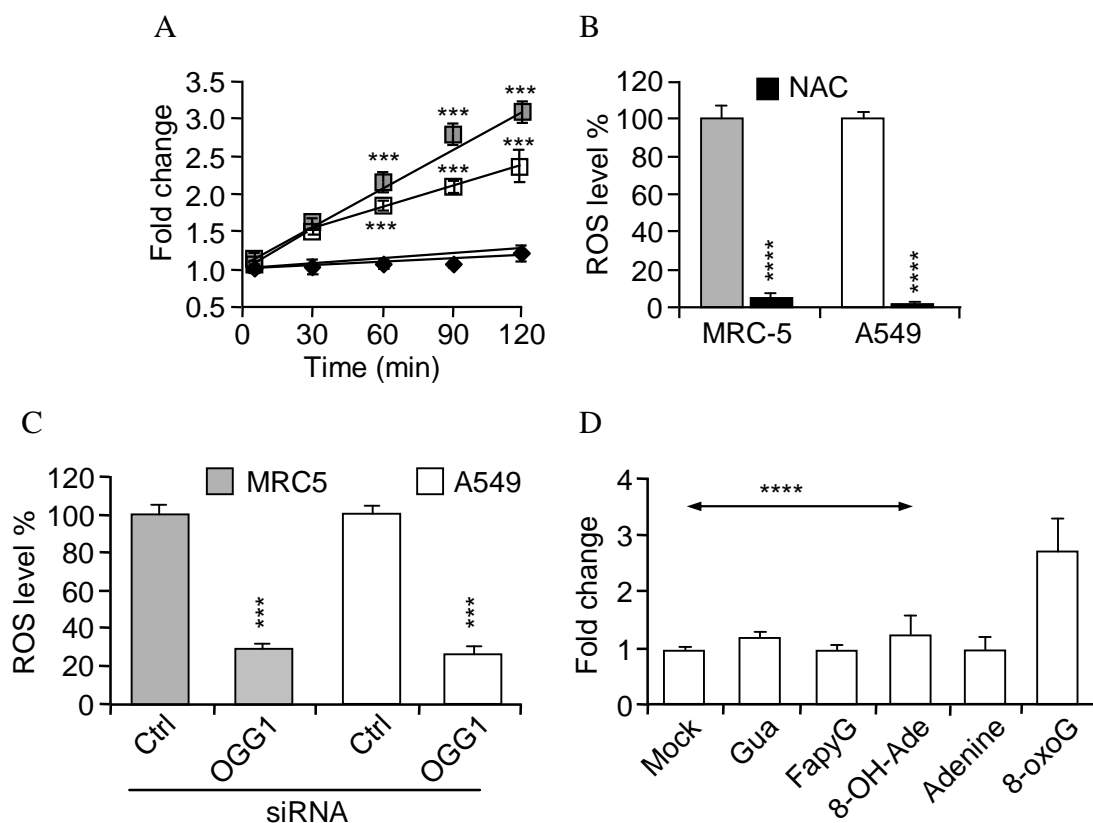


Figure 23. Changes in ROS levels in OGG1-expressing cells after exposure to 8-oxoG base (A) Kinetic changes in ROS levels in MRC-5 and A549 cells after 8-oxoG treatment. □, A549; ●, MRC-5; ○, mock-treated A549; ▲, mock-treated MRC-5. (B) 8-OxoG base exposure failed to increase ROS levels in N-acetyl-L-cysteine (NAC)-pretreated cells. (C) Depletion of OGG1 prevented the 8-oxoG-mediated increase in ROS levels. Cells were transfected with OGG1 siRNA and exposed to 8-oxoG. (D) Exposure of cells to FapyG, 8-oxo-Ade, adenine, guanine, or 8-OH-adenine failed to change intracellular ROS levels. In panels A, B, C, and D, cells at 80% confluence were kept in 0.1% serum-containing media for 4 h, H₂DCF-DA-loaded, and then exposed for 30 min before DCF fluorescence was assessed.

We previously showed that in a solution the 8-oxoG molecule slowly transforms into a hydroperoxy form, which in turn serves a substrate for peroxidases (i.e. glutathione peroxidase) and catalase (Hajas, Bacsi et al. 2012) and can increase cellular ROS levels. The kinetics of intracellular ROS increase induced by freshly dissolved 8-oxoG indicate that an indirect mechanism is responsible for the observed phenomenon and the involvement of regulator molecules.

4.9 8-OxoG induces ROS via activating NADPH oxidase

We hypothesized that the increase in cellular ROS levels by 8-oxoG base is due to the activation of cellular oxido-reductases, which may require small GTPase(s). One particular protein that has a direct role in ROS production is Rac1 a multifunctional GTPase, e.g., that is clearly involved in the modulation of cellular redox state via regulating NADPH oxidase(s)' activities (Bedard and Krause 2007). To prove our hypothesis we pretreated cells with DPI, a widely used inhibitor of NADPH oxidases, prior exposure to 8-oxoG (Fig. 24A). Pretreating the cells with DPI (15 μ M, the sufficient concentration was determined in preliminary studies) before 8-oxoG addition decreased cellular ROS levels by 62 ± 3 and $70\pm 7\%$ in MRC-5 and A549 cells, respectively, compared to cells exposed to 8-oxoG alone (Fig. 24A). Then we used siRNA-mediated depletion of p22phox, an essential regulatory subunit of NOX1-4 (Bedard and Krause 2007), Rac1, and NOX4, expressed in both MRC-5 and A549 cells (Bedard and Krause 2007; Lambeth, Kawahara et al. 2007) to get further evidence of the association of NADPH oxidase activity with cellular ROS increase after 8-oxoG treatment (Fig. 24B, C).

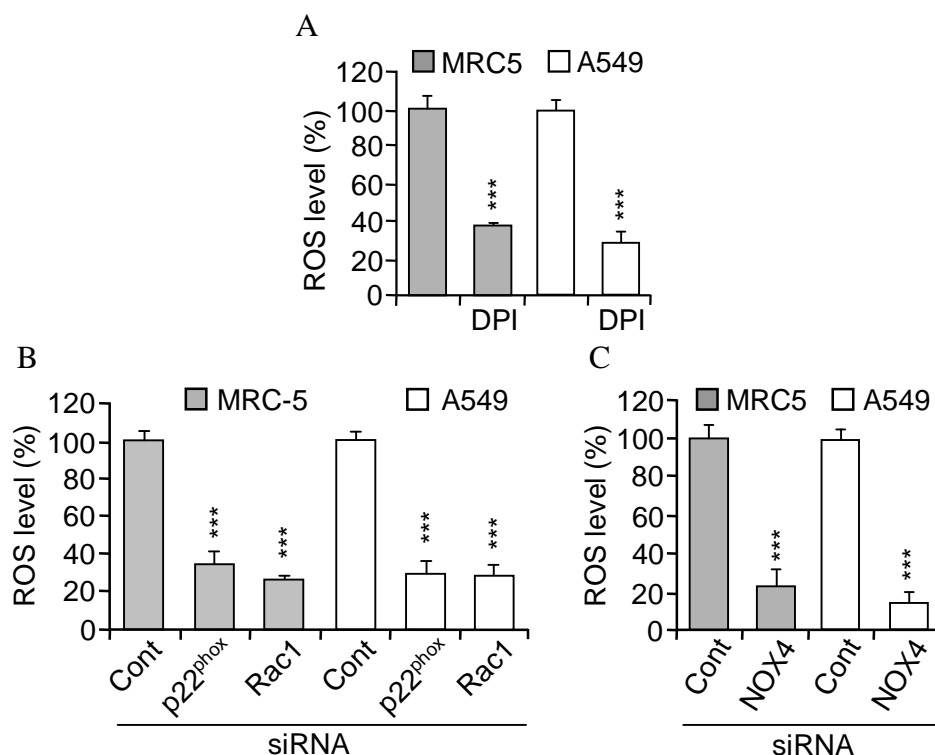


Figure 24. Inhibition of NOX4 or its regulatory subunits decrease cellular ROS levels in 8-oxoG-exposed cells (A) DPI pretreatment of cells lowered ROS levels in MRC-5 and A549 cells after exposure to 8-oxoG. Cells were given DPI (15 μ M) for 1 h and then loaded with H₂DCF-DA and exposed to 8-oxo-G. Changes in ROS levels were determined after 30 min. (B, C) Depletion of p22phox, NOX4 or Rac1 decreased ROS levels. Parallel cultures of cells (MRC-5, A549) were transfected with siRNA to p22phox, NOX4 or Rac1, and 48 h later cells were exposed to 8-oxoG base (10 μ M).

The siRNA mediated depletion of p22phox lowered ROS levels by $64\pm 8\%$ (MRC-5 cells) and $69\pm 6\%$ (A549 cells) in 8-oxoG-exposed cells, which further confirmed the involvement of NOXs in ROS generation (Fig. 24B). NOX4 specific siRNA significantly decreased ($67\pm 9.5\%$ for MRC-5; $76\pm 4\%$ for A549) ROS levels (Fig. 24C). Depletion of Rac1 decreased ROS levels by $72\pm 3\%$ in MRC-5 and $68\pm 7\%$ in A549 cells after 8-oxoG exposure (Fig. 24B). These data show the significance of Rac1 in the 8-oxoG mediated ROS production via NADPH oxidase so next we investigated the role of OGG1 in the activation of this small GTPase.

4.10 Rac1 activation in cultured cells

First we explored whether exposure of the cells to the 8-oxoG base changes cellular Rac1-GTP levels. MRC-5 and A549 cells were maintained in low serum (1%)-containing medium for 24 h and exposed to 8-oxoG base for increasing time intervals.

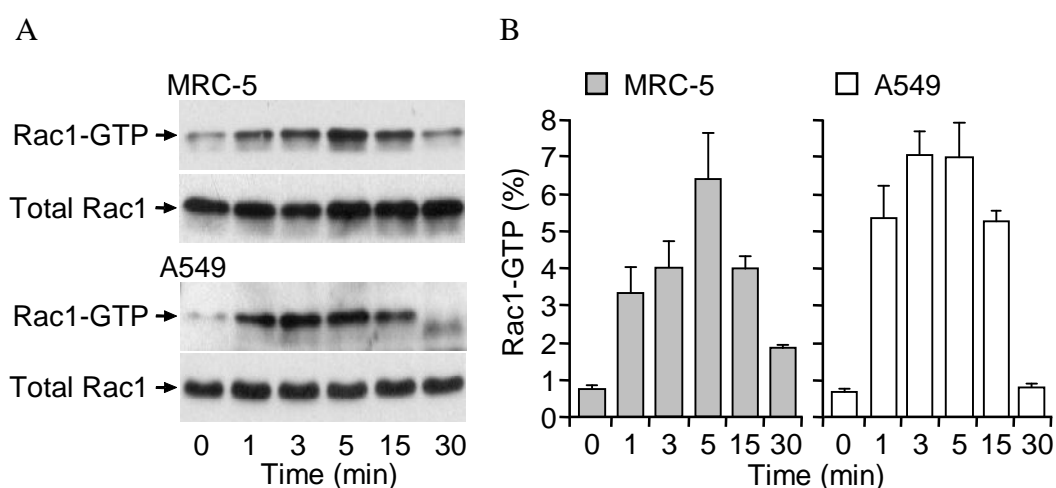


Figure 25. Changes in Rac1-GTP levels in MRC-5 and A549 cells after 8-oxoG exposure

(A) 8-oxoG base ($10\ \mu\text{M}$) was added to cells and extracts were made at indicated times points. Rac1-GTP levels were determined by GST pull-down assays. Upper panels: Rac1-GTP levels were determined in $100\ \mu\text{g}$ cell extract. Lower panel: total Rac1 protein levels in $10\ \mu\text{g}$ cell lysate. (B) Graphical depiction of percentage of Rac1-GTP levels. Rac1-GTP band intensities were determined by densitometry (Image J 1.44), and the percentage changes were calculated with MS Excel.

The results, summarized in Fig. 25A, showed a rapid increase in Rac1-GTP levels in MRC-5 cells between 1 and 5 min of treatment, which peaked at 5 min and then decreased nearly to basal levels by 30 min. Similar results were obtained by using A549 cells (Fig. 25A lower panel). Figure 25B shows the percentage changes in Rac1-GTP levels after 0, 1, 3, 5, 15, and 30 min of 8-oxoG exposure. At time 0, the percentage of GTP-bound Rac1 was $0.78 \pm$

0.2 and $0.74 \pm 0.1\%$ in MRC-5 and A459 cells, respectively, whereas after a 5-min exposure to 8-oxoG, the percentage of GTP-bound Rac1 levels increased to $6.5 \pm 2\%$ (MRC-5) and $7.05 \pm 1.7\%$ (A459) cells. These data indicate that the kinetics of Rac1 activation in these cells was faster than that we observed in case of Ras-GTPase. It also correlates with the rapid cellular uptake of 8-oxoG we showed earlier (Fig 9C) and the beginning of cellular ROS level increase after 8-oxoG exposure (Fig 23A).

To further confirm that the 8-oxoG-induced increase in Rac1-GTP levels required OGG1, we decreased its levels with siRNA.

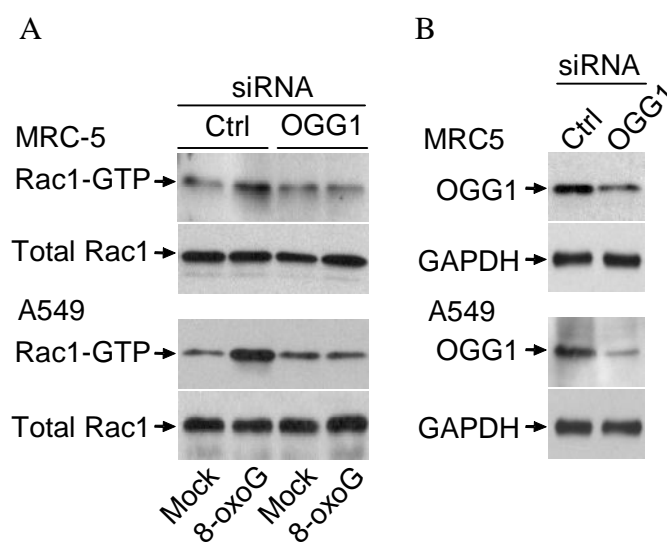


Figure 26. Depletion of OGG1 decreased 8-oxoG exposure-induced Rac1-GTP levels

(A) MRC-5 and A549 cells were transfected with OGG1-specific siRNA or control siRNA and then exposed to 8-oxoG ($10 \mu\text{M}$) for 10 min. Rac1-GTP levels were determined by pull-down assays. (B) siRNA-mediated decrease in OGG1 levels. GAPDH is shown for equal protein loading.

In OGG1-depleted cells (MRC-5, A549), there was no significant increase in Rac1-GTP levels after 8-oxoG addition (Fig. 26A, upper and lower panels) when compared to Rac1-GTP levels in the OGG1-expressing cells. The extent of OGG1 depletion was confirmed by Western blot analysis (Fig. 26B). These results indicate that similarly to Ras protein, Rac1 also requires the presence of OGG1 in order to get activated by free 8-oxoG.

4.11 Rac1 activation in lung

Next, we examined whether 8-oxoG base increases Rac1-GTP levels in a tissue environment. The 8-oxoG base was instilled into the lungs of mice, and Rac1-GTP levels were determined as described in Materials and methods section.

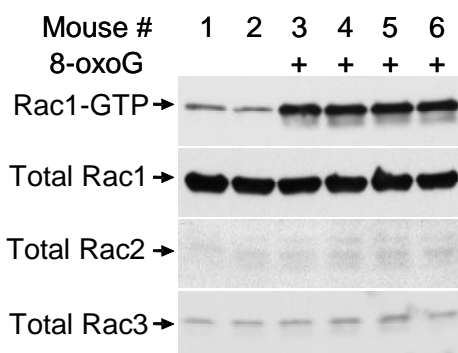


Figure 27. Activation Rac1 by 8-oxoG challenge in lungs

Mice were challenged intranasally with saline \pm 10 μ M 8-oxoG. At 15 min thereafter Rac1-GTP levels were determined by GST pull-down assays in 100 μ g lung extracts. Total Rac1, Rac2 and Rac3 levels were assessed by immunoblotting (25 μ g extract per lane).

Compared to saline-challenged lungs (Fig. 27, mice Nos. 1 and 2) there was a robust increase in Rac1-GTP levels at 15 min post-challenge (Fig. 27, mice Nos. 3, 4, 5, and 6), consistent with our cell culture studies. As shown by immunoblot analysis, Rac1 is abundantly expressed in lung tissues (Fig. 27, total Rac1), while Rac2 and Rac3 expression was nearly undetectable. Although the significance of Rac1 activation in lungs by the 8-oxoG base is yet to be defined, these results are in line with those observed in normal diploid lung fibroblasts (MRC-5) and A549 cells (type II alveolar lung epithelial cells). Rac1 has various roles in lung tissues. One of them is taking part in the reorganization of actin cytoskeleton, which leads to endothelial barrier recovery and elimination of interendothelial gaps (Vogel and Malik 2012). Lung endothelial cells (EC) have all components of NOXs and are major contributors of ROS related lung inflammation (Pendyala, Usatyuk et al. 2009).

4.12 Changes in ROS levels begin at perinuclear membrane

NOX type 4 (NOX4) have been reported to be localized to cytoplasmic compartments and nuclear membranes, and are involved in multiple cellular processes, including localized redox modulation, and cellular signaling (Bedard and Krause 2007; Gordillo, Fang et al. 2010). To determine the cellular localization of NOX4 that generate ROS in response to 8-oxoG exposure, cells were transfected with the biosensor pHy-Pers (Belousov, Fradkov et al. 2006; Malinouski, Zhou et al. 2011), expressed in cytoplasm (pHyPer-Cyto) or targeted to nucleus (pHyPer-Nuc), and mitochondria (pHyPer-Mito). Intracellular sites of fluorescence were recorded by microscopic imaging.

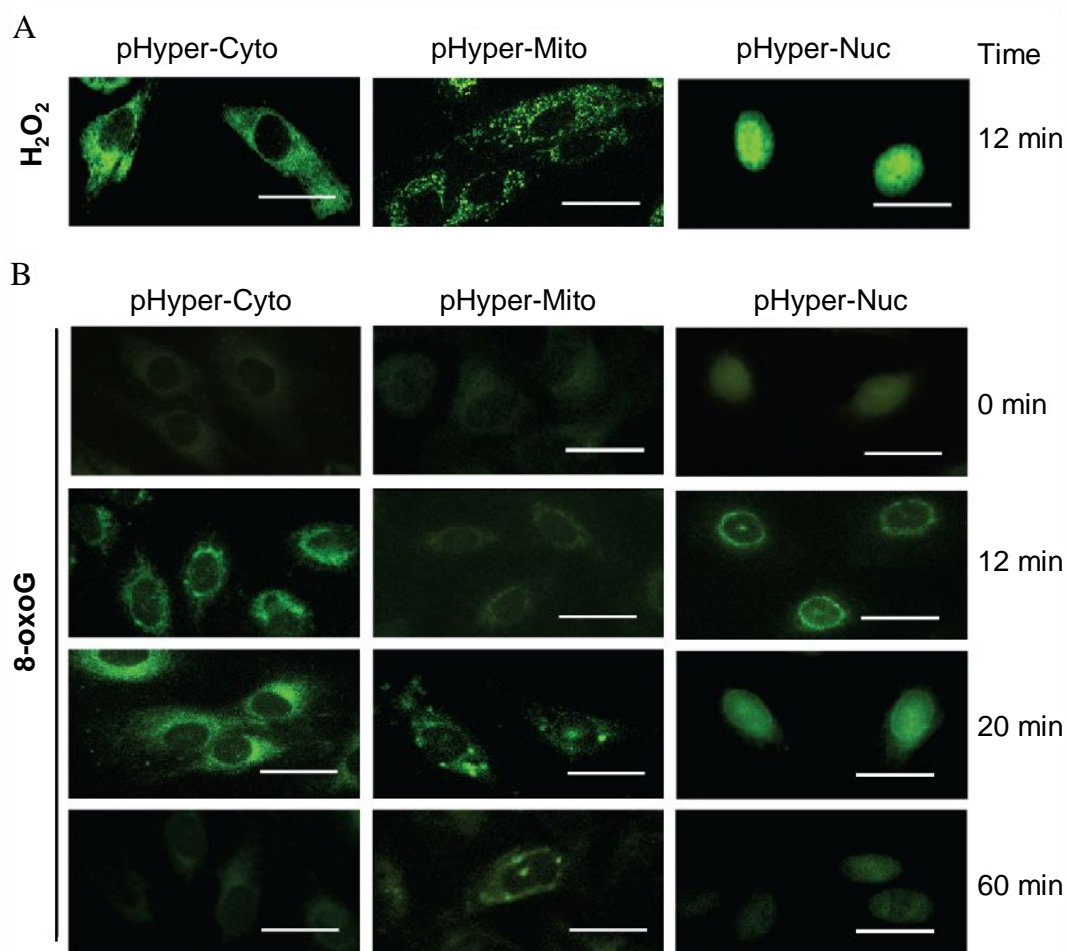


Figure 28. Transient and localized changes in cellular ROS levels in response to 8-oxoG exposure. Cells were transfected with pHyPer-Cyto, pHyPer-Mito, or pHyPer-Nuc and 72 h later challenged with 8-oxoG (10 μ M) or H_2O_2 (10 μ M). At times indicated, cells were washed in PBS, fixed with formalin (3.7%), dried, and mounted on microscope slides. Images were taken by a NIKON Eclipse Ti System (magnification: $\times 125$). (A) H_2O_2 exposure-induced changes in fluorescence in subcellular compartments of cells. (B) Localized ROS generation by 8-oxoG exposure of cells. Scale bars: 20 μ m.

As shown in Figure 28A, H₂O₂-induced pHyPer signal was localized to cytoplasm, mitochondria, and nuclei of cells. When cells were exposed to 8-oxoG pHyPer-Nuc fluorescence appeared first at the perinuclear region (10 to 12 min after exposure) and then a nuclear fluorescence was observed (~20 min). pHyPer-Nuc signal decreased to the basal level by 60 min. pHyPer-Cyto-mediated fluorescence was initiated around the nuclei of cells (10 to 12 min after post-exposure) and then spread to the cytoplasm (20 min; Fig. 28B). The pHyPer-Mito fluorescence appeared at 20 min after 8-oxoG addition. Interestingly, only few mitochondria showed fluorescence, suggesting that pHyPer-Mit oxidation is a secondary event and possibly due to ROS generated by NOX4. There were no increased fluorescence of biosensors observed at 60 min post-exposure with 8-oxoG. Together these data suggest that NOX4 generating ROS is localized to the nuclear membrane and 8-oxoG exposure-induced ROS generation is transient.

Redox sensors (pHyPer-Nuc and pHyPer-Cyto) showed changes in ROS levels first at the perinuclear region of cells, which seems suggestive of localized ROS generation. These results were supported by our data showing that Rac1, NOX4, and OGG1 are in close proximity in the nuclear membrane of A549 cells.

4.13 Rac1 and OGG1 colocalize with NOX4 in nuclear membrane

To obtain an insight on the close proximity of OGG1, Rac1 and NOX4 at perinuclear regions, we immunostained A549 cells by using specific antibodies to these proteins. Figure 29A, B, and C show that OGG1 was primarily localized to the cell nuclei, but a fraction of it was associated with the nuclear membrane. Microscopic imaging showed that like NOX4, Rac1 is associated with the nuclear membrane. Lamin A/C antibody stained the nuclear membrane exclusively (Fig. 29C). We utilized digital imaging tools to examine the colocalization of OGG1 with Rac1 and NOX4 in images obtained by fluorescence microscopy. The fluorescence intensities emitted by OGG1, Rac1, and NOX4 were different, so we employed and calculated overlap coefficients according to Manders, a method which allows a reliable estimation of close proximity localization of proteins (Manders, Verbeek et al. 1993; Zinchuk, Zinchuk et al. 2007).

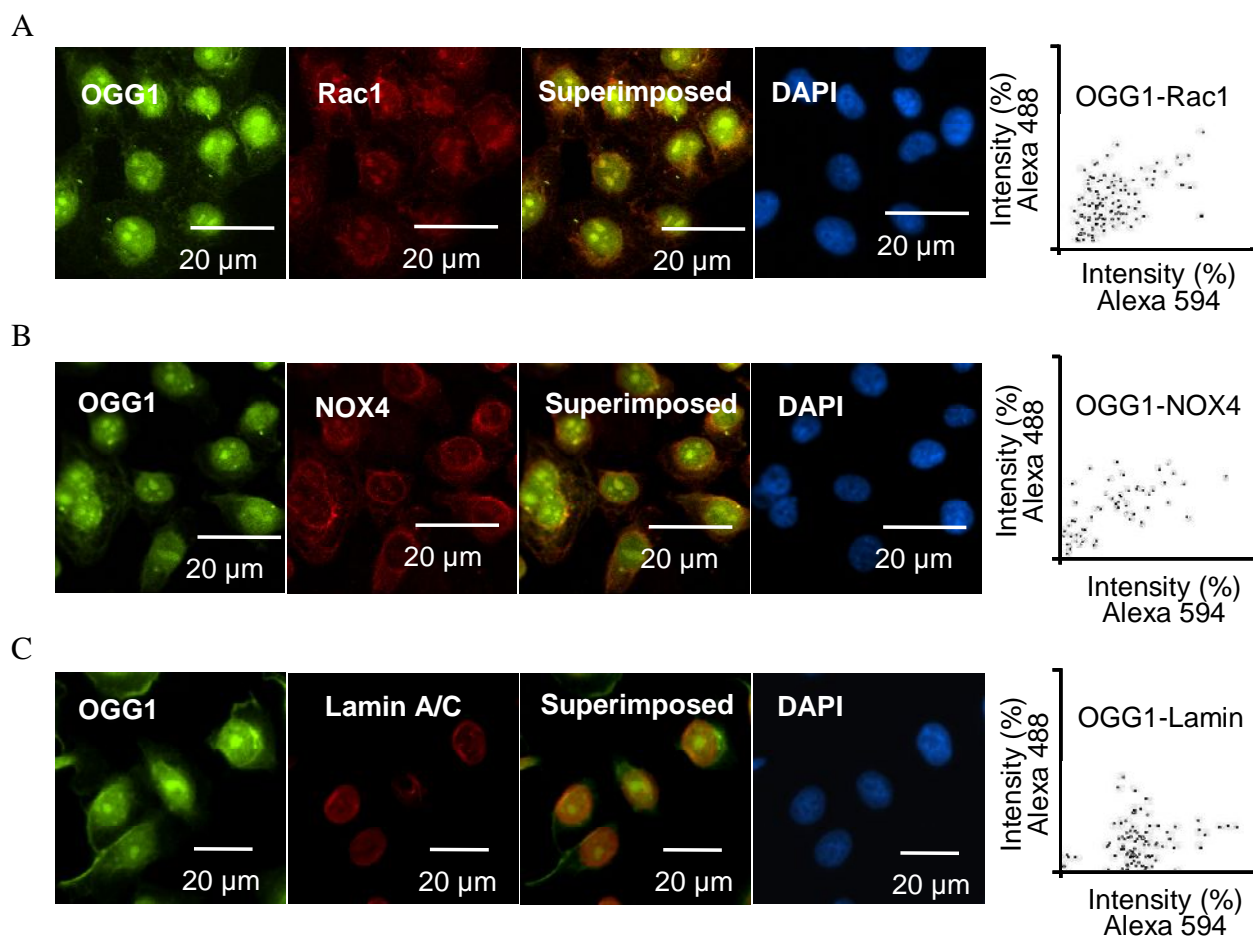


Figure 29. Microscopic imaging of OGG1 colocalization with Rac1 and NOX4

A549 cells were cultured on microscope coverslips, then incubated for 1h with Abs to OGG1, Rac1 (panel A) and/or NOX4 (panel B). Panel C shows OGG1's localization to the nuclear membrane based on lamin A/C. Binding of primary Abs was visualized using Alexa 488- and Alexa 594-conjugated secondary Abs. Staining of cells was carried out as described in Materials and Methods. Images were captured with a NIKON Eclipse Ti System (magnification: $\times 125$). Colocalization was visualized by superimposition of green and red images using Nikon NIS Elements Version 3.5 (NIKON Instruments, Tokyo, Japan). Mander's overlap coefficient is 0.935431 for OGG1 and Rac1 (upper right panel); 0.909245 for OGG1 and NOX4 (middle right panel). DAPI = 4'-diamidino-2-phenylindole dihydrochloride.

Manders' formula (see Materials and methods) showed a close proximity localization of OGG1 and Rac1 (Manders' overlap=0.935431, overlap coefficients $k_1=0.886159$ and overlap coefficients $k_2=0.987443$) and NOX4 (Manders' overlap=0.909245, overlap coefficients $k_1=0.872817$, and overlap coefficients $k_2=0.980908$). In controls, OGG1 was localized to the nuclear membrane, like lamin A/C; however, the Manders' equation (overlap=0.898948, coefficients $k_1=1.341340$, coefficients $k_2=0.602463$) showed that OGG1 and lamin A/C are not in close proximity. The close proximity of OGG1 with Rac-1 (Fig. 29A) and NOX4 (Fig. 29B) is consistent with an increase in Rac1-GTP levels and the

observed nuclear membrane-associated ROS generation (Fig. 28). Colocalizations in the nuclear membrane of OGG1 with Rac1 and NOX4 (based on Manders overlap coefficients) are novel observations, while another report has already documented the nuclear membrane localization of NOX4 (and NOX1, 2) and their activators of Rac1, as well as p22phox (and p47phox), and their contribution to localized ROS generation (Spencer, Yan et al. 2011).

4.14 OGG1 physically interacts with small GTPase Rac1

Next, we investigated the molecular mechanism of OGG1-mediated Rac1 activation. We tested if physical interaction between OGG1 and Rac1 occurs similarly to that we experienced with Ras proteins.

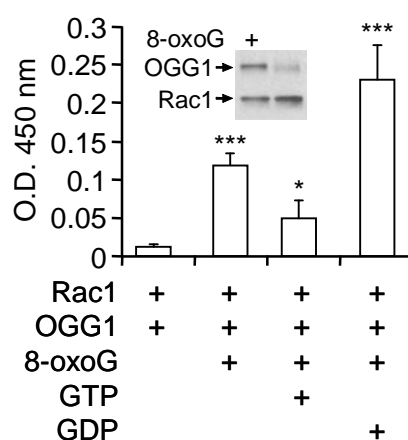


Figure 30. Rac1 binding to OGG1 measured with ELISA and pull-down assays

Rac1 protein was added to Rac1 Ab coated wells and incubated with OGG1±8-oxoG in the presence or absence of GDP or GTP. OGG1 binding was detected by HRP-conjugated Ab at 450 nm (Materials and methods). **Inset:** Interaction of OGG1 with Rac1 shown by pull-down assay. Rac1 was bound to Ni-NTA-agarose beads, and OGG1±8-oxoG was added. After washing, the amount of OGG1 bound to Rac1 was analyzed by Western blot (Materials and methods).

The results summarized in Figure 30 show that OGG1 protein alone interacted poorly with guanine nucleotide-free (empty) Rac1 protein. However, in the presence of 8-oxoG, physical interactions between OGG1 and empty Rac1 were significantly increased, as shown by ELISA and Ni-NTA pull-down assays (Fig. 30, inset). OGG1/8-oxoG bound most extensively to GDP-loaded Rac1 (Fig. 30), while binding to Rac1-GTP was significantly lower than to either empty Rac1 or GDP-Rac1 (Fig. 30). Together these data reveal a physical interaction of the OGG1/8-oxoG complex with Rac1, and also suggest that the conformation of GDP-bound Rac1 allows the most stable interaction with OGG1/8-oxoG.

4.15 OGG1/8-oxoG complex acts as a GEF on Rac1

To examine the possibility that OGG1/8-oxoG may serve as a GEF and increase Rac1-GTP levels, we performed guanine nucleotide exchange assays utilizing fluorescently labeled guanine nucleotides ($^{\text{Mant}}$ GDP and $^{\text{Mant}}$ GTP) (Zhang, Zhang et al. 2000).

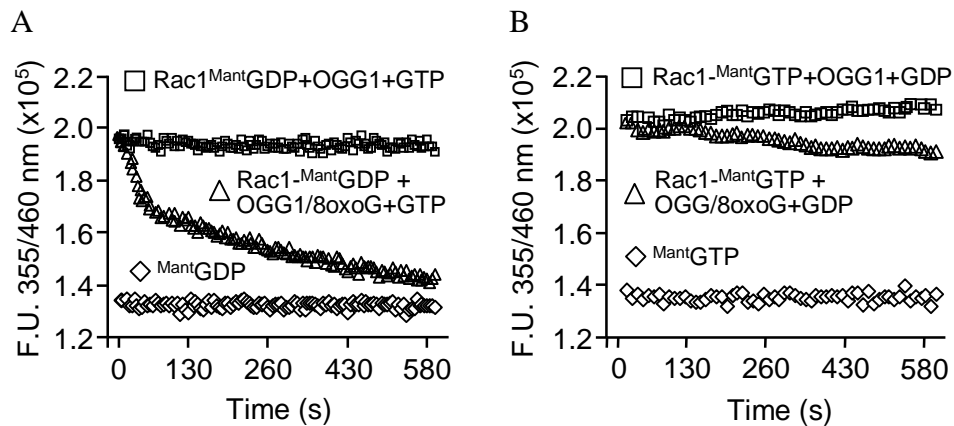


Figure 31. The OGG1/8-oxoG complex has guanine nucleotide exchange factor activity

(A) Exchange of Rac1-bound GDP for GTP by OGG1 in the presence 8-oxoG base. Rac1 protein was loaded with $^{\text{Mant}}$ GDP, and nucleotide exchange was initiated by adding OGG1 + 8-oxoG and GTP (6 pmol) (). In controls, GTP alone was added to Rac1- $^{\text{Mant}}$ GDP (). Fluorescence of $^{\text{Mant}}$ GDP alone (). (B) OGG1 does not catalyze Rac1-GTP- GDP exchange. Rac1 protein was loaded with $^{\text{Mant}}$ GTP and OGG1 + 8-oxoG and GDP () or GDP alone () was added. Fluorescence of $^{\text{Mant}}$ GTP alone (). In A and B, changes in fluorescence of Rac1- $^{\text{Mant}}$ GDP and Rac1- $^{\text{Mant}}$ GTP were determined by real-time measurements by using a POLARstar Omega (BMG Germany). Curves were fitted by using MS Excel.

When Rac1 protein was loaded with $^{\text{Mant}}$ GDP (1:1 molar ratio), the fluorescence intensity of $^{\text{Mant}}$ GDP increased from 1.36×10^5 fluorescence unit (FU) to 1.9×10^5 FU, as determined by spectroscopic analysis (Fig. 31A). On addition of OGG1 and 8-oxoG along with unlabeled GTP to Rac1- $^{\text{Mant}}$ GDP, the fluorescence rapidly decreased, indicating that Rac1-bound $^{\text{Mant}}$ GDP was replaced by nonfluorescent GTP (Fig. 31A). The release of $^{\text{Mant}}$ GDP and replacement with GTP were rapid, as during a 45 s time period more than 50% of $^{\text{Mant}}$ GDP was exchanged for GTP (Fig. 31A). In controls, OGG1 plus GTP did not change Rac1- $^{\text{Mant}}$ GDP fluorescence (Fig. 31A), and OGG1 alone also had no effect (data not shown). Next, we found that the fluorescence intensity of $^{\text{Mant}}$ GTP was increased (from 1.38×10^5 FU to 2.02×10^5 FU) (Fig. 31B) when bound to Rac1 in a manner similar to that observed for Rac1- $^{\text{Mant}}$ GDP (Fig. 31A). Addition of OGG1 plus 8-oxoG along with non-labeled GDP resulted in a slow guanine nucleotide exchange (Fig. 31B). OGG1 caused no change in Rac1- $^{\text{Mant}}$ GTP fluorescence in the presence of GDP, and OGG1 alone also had no effect (data not shown). These data are in line with the poor interaction of OGG1 with Rac1-GTP (Fig. 30). These

results thus show that OGG1 in complex with 8-oxoG catalyzes the release of GDP efficiently, and may function as a GEF in the intracellular environment.

OGG1 protein alone showed poor binding to guanine nucleotide-free (empty) Rac1 protein, but the 8-oxoG base increased this interaction. On the other hand, OGG1 showed poor interactions with Rac1-GTP in the presence of 8-oxoG however, less than with the empty protein. These observations resemble our findings that OGG1/8-oxoG binds to canonical Ras family proteins and those reporting interaction between Rac1 and GEFs such as T lymphoma invasion and metastasis (Tiam) protein (Haeusler, Blumenstein et al. 2003) or Vav2 (a homolog of the vav protooncogene) (Sauzeau, Sevilla et al. 2010). The interaction of OGG1 with GDP-loaded Rac1 suggested that 8-oxoG binding results in a conformational change which allows OGG1 to bind to Rac1. In support of this hypothesis, it has been shown that GEFs require either posttranslational modification(s) or binding to regulatory molecules for interaction and catalysis of guanine nucleotide exchange on Rac1 GTPases (Fan, Koch et al. 1998; Innocenti, Zippel et al. 1999). Tiam1's interaction with Rac1 and its GEF activity are increased by association with phosphoinositides in its N-terminal pleckstrin homology domain (Fleming, Gray et al. 2000). The GEF activity of Ras guanine nucleotide-releasing factor 2 requires Ca^{2+} /calmodulin binding to its IQ motif structure (Fan, Koch et al. 1998; Innocenti, Zippel et al. 1999). The activity of Ras guanine nucleotide release protein is regulated by Ca^{2+} - and diacylglycerol (Buday and Downward 2008). Together these data suggest that the OGG1 protein is functionally similar to other Rac1 GEFs, as it requires binding of a cofactor (8-oxoG) to gain the proper conformation necessary for its binding to Rac1-GDP.

4.16 The role of ROS-OGG1-Rac1/Ras triangle in cellular responses

Although the biological significance of our findings needs further investigation, at first glance it seems feasible that ROS could alter OGG1's repair activity as well as subcellular localization in response to the cell's need to repair oxidatively damaged DNA (Conlon, Zharkov et al. 2004; Szczesny, Bhakat et al. 2004). It has been shown that OGG1 is redistributed in response to locally generated oxidative stress within the nucleus, as shown by the accumulation of OGG1-GFP specifically at sites of laser-targeted ROS/DNA damage (Zielinska, Davies et al. 2011). On the other hand, ROS generated locally could produce oxidative modifications at cysteine residues, especially those at 253 and 255, in close proximity to OGG1's active site (Qi, Spong et al. 2009), which may thereby result in decreased OGG1's activity. This hypothesis is consistent with functional studies observing decreases in OGG1's activity by oxidative modifications at cysteine residues (Bravard, Vacher

et al. 2006). It has also been shown that NOX4-derived ROS cause DNA oxidation resulting in 8-oxoG and alterations in gene expression, especially those associated with inflammation (Gordillo, Fang et al. 2010; Weyemi and Dupuy 2012). OGG1 is considered to be a canonical DNA BER protein (Mitra, Izumi et al. 2002); however, our observations show that OGG1/8-oxoG complex functions both as a Ras and Rac1 GEF (Boldogh, Hajas et al. 2012; Hajas, Bacsi et al. 2012). We propose that the complex not only activates small GTPase dependent pathways, but initiates signal transduction for transcriptional activation of downstream genes. As a matter of fact, in a 2014 study, Ba and co-workers have reviewed the biological significance of the 8-oxoG base and the role of OGG1-BER in the activation of small GTPases and changes in gene expression, including those that regulate pro-inflammatory chemokines/cytokines and cause inflammation (Ba, Aguilera-Aguirre et al. 2014). Another report show an OGG1-BER induced K-Ras activation leading to innate inflammatory responses in mouse airways via MAP kinases, PI3 kinases, mitogen-stress-related kinase-1 and NF- κ B activation (Aguilera-Aguirre, Bacsi et al. 2014). OGG1 was found to play a role in innate immunity by enhancing Cxcl2 (a chemokine produced by monocytes and macrophages) expression after TNF- α treatment. The TNF- α induced ROS increased the 8-oxoG levels in the genome, including the promoter region(s) leading to the accumulation of inflammatory cells in the airways and a quick expression of chemokines/cytokines. OGG1 facilitated the recruitment of transcription initiation factor II-D, NF- κ B/RelA, Sp1 (specificity protein 1) and p-RNA pol II (p-RNA polymerase II) by binding to the Cxcl2 promoter region. The authors suggest that OGG1 binding to the 8-oxoG in the promoter region could be an epigenetic mechanism to prompt gene expression for innate immune response (Ba, Bacsi et al. 2014).

Although we only show that activated Rac1 increases ROS levels via NOX4, it could also be involved in other cellular processes such as regulation of the cytoskeletal network. Indeed, OGG1 was also observed to be associated with the microtubule organizing center and microtubule network (Dantzer, Luna et al. 2002; Conlon, Zharkov et al. 2004). Thus, by activating Rac1 OGG1 may participate in the organization of microtubules and the mitotic spindle assembly and centrosomes, and thereby could play a critical role in cell cycle progression, including mitosis as previously proposed (Conlon, Zharkov et al. 2004). Supporting this hypothesis, Luo and her colleagues have shown that similarly to Rac1, in the presence of 8-oxoG, OGG1 physically interacts with Rho GTPase and increases Rho-GTP levels in cultured cells and lungs, which mediates α -smooth muscle actin (α -SMA)

polymerization into stress fibers. This way OGG1 could be involved in the cytoskeletal changes and organ remodeling observed in various chronic diseases (Luo, Hosoki et al. 2014).

5. New scientific results

The new scientific results claimed by the author are the following:

- By binding 8-oxoG, OGG1 goes through sterical changes, which enable its physical interaction with small GTPases,
- These interactions result in GDP to GTP exchange activating both Ras and Rac1,
- Both externally added and intracellularly generated 8-oxoG bases are able to induce Ras activation,
- Administration of 8-oxoG increases both Ras-GTP and Rac1-GTP levels in the lungs of experimental mice
- OGG1/8-oxoG complex formation can lead to MEK/ERK phosphorylation via Ras,
- or a NOX4-mediated increase in cellular ROS levels via Rac1

Fig. 32 shows a summarized working model of the OGG1/8-oxoG complex leading to cellular signaling.

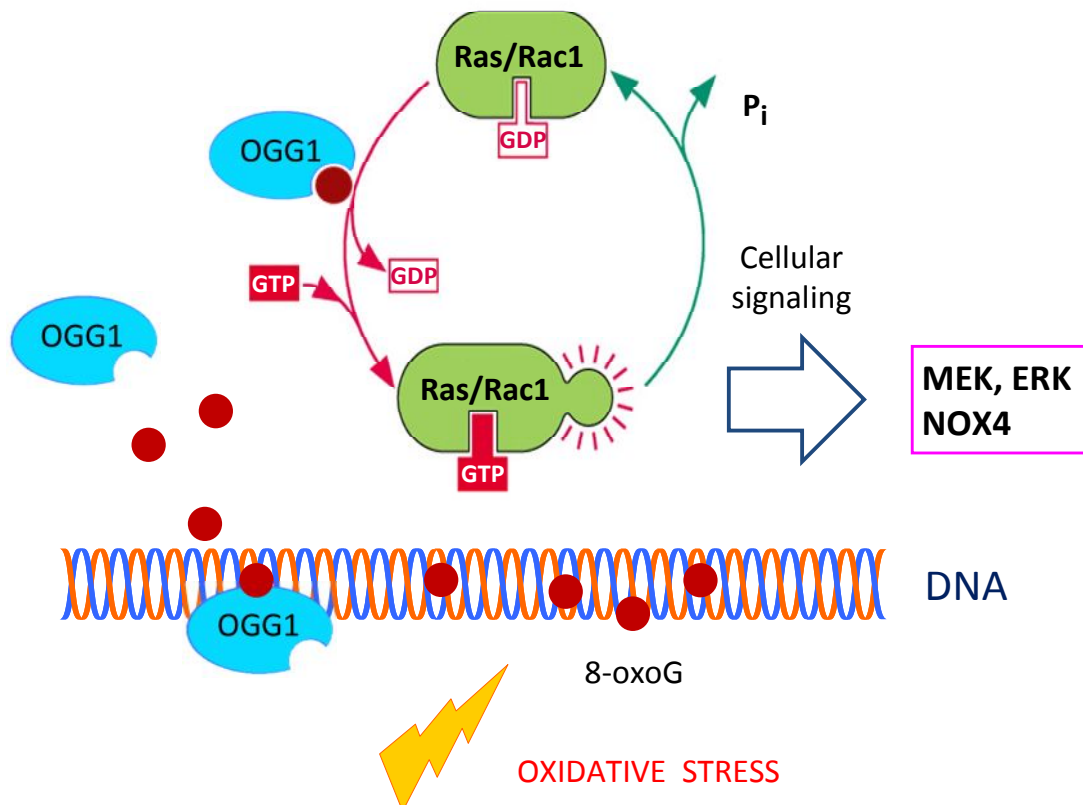


Figure 32. Schematic picture of OGG1/8-oxoG complex induced events

OGG1 excises 8-oxoG from DNA and bind the oxidized base at a non-catalytic site. The complex formation results in sterical changes which enables OGG1 to bind to Ras/Rac1 and work as a GEF. The activation of small GTPases followed by MEK, ERK activation and ROS production via NOX4.

6. Summary

DNA is a frequent target of oxidative agents from both inner and outer sources. One of the most studied DNA adduct among the oxidized bases is 8-oxoG and determination of its level can be used as a reliable gauge of the oxidative stress load of an organism. The 8-oxoguanine DNA glycosylase 1 is the dedicated enzyme to excise the 8-oxoG during the DNA base excision repair process from nuclear and mitochondrial genomes. Accumulation of 8-oxoG in the DNA connected to various disorders like cancer, aging, Alzheimer disease, although OGG1 knock-out mice have normal life span, show only moderate increases in tumor formation, have an increased tolerance to chronic oxidative stress and are less susceptible to inflammatory processes. In this work we describe that OGG1 can exert non-repair functions after binding its excised product, 8-oxoG. We demonstrated that OGG1 has a non-catalytic binding site for 8-oxoG. In a complex with its product, OGG1 not only becomes more efficient removing oxidized guanine, but also goes through sterical changes, which enable its physical interaction with small GTPases, Ras and Rac1 proteins. These interactions result in GDP to GTP exchange activating these small GTPases. We found that intranasal administration of 8-oxoG increases both Ras-GTP and Rac1-GTP levels in the lungs of experimental mice. Our results also show that both externally added and intracellularly generated 8-oxoG bases are able to form a complex with OGG1 leading to Ras activation. We revealed that OGG1/8-oxoG complex formation can lead to MEK/ERK phosphorylation via Ras or a NOX4-mediated increase in cellular ROS levels via Rac1.

While the biological significance of our findings has yet to be fully elucidated, it appears that activation of the small GTPase Rac1, leading to localized ROS generation could be part of a physiological DNA damage/repair response initiated by OGG1. As the OGG1/8-oxoG not only activates Rac1 but also the canonical Ras family GTPases, it could be considered that Rac1/NOX4/ROS and Ras-mediated signaling are involved in maintaining cellular homeostasis. We also speculate that a failure in the control of OGG1 activity may lead to excessive release of 8-oxoG from DNA, resulting in unscheduled activation of Rac and Ras family GTPases that could result in pathophysiological cellular responses, contributing to diseases, cellular senescence, and aging processes.

Összefoglalás

A DNS gyakori célpontja mind a külső, mind a belső eredetű oxidatív ágenseknek. Az egyik legtöbbet tanulmányozott oxidált DNS bázis az 8-oxoguanin (8-oxoG), aminek mért mennyisége a szervezetet ért oxidatív stressz mércéjeként használható. A 8-oxoG kivágásáért a 8-oxoguanin DNS glikoziláz-1 (OGG1) a felelős enzim, ami a bázis-eltávolító javítás során kivágja az oxidált guanint mind a nukleáris, mind a mitokondriális genomból. Annak ellenére, hogy a 8-oxoG akkumulálódása a DNS-ben összeköthető olyan rendellenességekkel, mint a rák, Alzheimer-kór, öregedés, az OGG1 knock-out egerek élettartama nem csökken, tumor képződési hajlamuk csak kevéssé emelkedik, valamint mind a krónikus oxidatív stresszrel, mind a gyulladásos folyamatokkal szembeni állóképességük megnövekszik. Ebben a tézisben az OGG1 egy olyan új működését írjuk le, amit a kivágott 8-oxoG megkötésével képes kifejteni. Bemutatjuk, hogy az OGG1 rendelkezik egy katalitikustól független 8-oxoG kötő helyvel és a kivágott termékével komplexben nemcsak sokkal hatékonyabban távolítja el az oxidált guanint, hanem olyan térszerkezeti változáson megy át, ami lehetővé teszi Ras és Rac1 szabályzó GTP-ázokhoz való kötődését. Ez a kapcsolódás elősegíti a GDP lecserélődését GTP-re, ami ezen a szabályzó GTP-ázok aktiválásához vezet. Kimutattuk, hogy 8-oxoG intranazális beadása után a tüdőben megnövekszik a Ras és a Rac1 fehérjék szintje. Az eredményeink szerint mind a hozzáadott, mind a sejten belül keletkezett 8-oxoG bázis képes az OGG1-el komplexet képezni és aktiválni a Ras fehérjét. Kimutattuk, hogy az OGG1/8-oxoG komplex képződése MEK/ERK aktivációhoz vezethet a Ras protein-, míg reaktív oxigén gyökök (ROS) képződéséhez a Rac1/NOX4 aktiválásán keresztül.

Konklúzióként levonhatjuk, hogy habár ezen eredmények jelentősége még nem teljesen ismert, úgy gondoljuk, hogy a szabályzó GTP-áz, Rac1 aktivációja és helyi ROS keletkezése egy DNS javító mechanizmus OGG1-en keresztüli fiziológiai válasz részét képezi. Mivel az OGG1/8-oxoG komplex nemcsak a Rac1-et, hanem a Ras család klasszikus GTP-ázait is aktiválja, lehetséges, hogy a Rac1/NOX4/ROS és Ras általi jelátvitel a sejt homeosztázisának fenntartásában is szerepet játszik. Szintén elképzelhetőnek tartjuk, hogy az OGG1 szabályozás feletti kontrol elvesztése a 8-oxoG molekula nagy mennyiségű eltávolításához, és ez által a Rac1-, és Ras GTP-ázok soron kívüli aktiválásához vezet, ami hozzájárulhat kóros sejtválaszok, valamint betegségek és öregedési folyamatok kialakulásához.

7. References

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7.2 Publication list prepared by the Kenézy Life Sciences Library



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Registry number: DEENK/67/2015 PI
Subject: Ph.D. List of Publications

Candidate: György Hajas
Neptun ID: M255ZY
Doctoral School: Doctoral School of Molecular Cell and Immune Biology

List of publications related to the dissertation

1. **Hajas, G.**, Bácsi, A., Aguilera-Aguirre, L., Hegde, M.L., Tapas, K.H., Sur, S., Radák, Z., Ba, X., Boldogh, I.: 8-Oxoguanine DNA glycosylase-1 links DNA repair to cellular signaling via the activation of the small GTPase Rac1.
Free Radic. Biol. Med. 61C, 384-394, 2013.
DOI: <http://dx.doi.org/10.1016/j.freeradbiomed.2013.04.011>
IF:5.71
2. Germán, P., Szaniszló, P., **Hajas, G.**, Radák, Z., Bácsi, A., Hazra, T.K., Hegde, M.L., Ba, X., Boldogh, I.: Activation of cellular signaling by 8-oxoguanine DNA glycosylase-1-initiated DNA base excision repair.
DNA Repair. 12 (10), 856-863, 2013.
DOI: <http://dx.doi.org/10.1016/j.dnarep.2013.06.006>
IF:3.362
3. Boldogh, I., **Hajas, G.**, Aguilera-Aguirre, L., Hegde, M.L., Radák, Z., Bácsi, A., Sur, S., Hazra, T.K., Mitra, S.: Activation of ras signaling pathway by 8-oxoguanine DNA glycosylase bound to its excision product, 8-oxoguanine.
J. Biol. Chem. 287 (25), 20769-20773, 2012.
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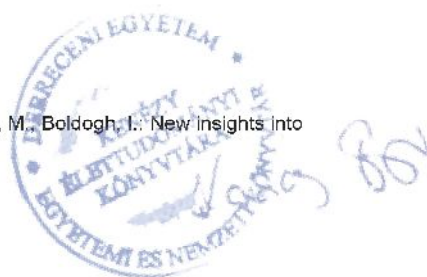
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4. **Hajas, G.**, Zsiros, E., László, T., Hajdu, P., Somodi, S., Réthi, B., Gogolák, P., Ludányi, K., Panyi, G., Rajnavölgyi, É.: New phenotypic, functional and electrophysiological characteristics of KG-1 cells.
Immunol. Lett. 92 (1-2), 97-106, 2004.
DOI: <http://dx.doi.org/10.1016/j.imlet.2003.11.021>
IF:2.136

List of other publications

5. Sárga, L., Hart, N., Koch, L.G., Britton, S.L., **Hajas, G.**, Boldogh, I., Ba, X., Radák, Z.: Aerobic endurance capacity affects spatial memory and SIRT1 is a potent modulator of 8-oxoguanine repair.
Neuroscience. 252, 326-336, 2013.
DOI: <http://dx.doi.org/10.1016/j.neuroscience.2013.08.020>
IF:3.327
6. **Hajas, G.**, Bácsi, A., Aguilera-Aguirre, L., Germán, P., Radák, Z., Sur, S., Hazra, T.K., Boldogh, I.: Biochemical identification of a hydroperoxide derivative of the free 8-oxo-7,8-dihydroguanine base.
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7. Szaniszló, P., Germán, P., **Hajas, G.**, Saenz, D.N., Woodberry, M.W., Kruzel, M.L., Boldogh, I.: Effects of Colostrinin on gene expression-transcriptomal network analysis.
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9. Márkász, L., Hajas, G., Kiss, A., Lontay, B., Rajnavölgyi, É., Erdődi, F., Oláh, É.: Granulocyte colony stimulating factor increases drug resistance of leukaemic blast cells to daunorubicin. *Pathol. Oncol. Res.* 14, 285-292, 2008.

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10. Gogolák, P., Réthi, B., Hajas, G., Rajnavölgyi, É.: Targeting dendritic cells for priming cellular immune responses.

J. Mol. Recognit. 16 (5), 299-317, 2003.

DOI: <http://dx.doi.org/10.1002/jmr.650>

IF:2.013

Total IF of journals (all publications): 31,656

Total IF of journals (publications related to the dissertation): 15,859

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

24 March, 2015



8. Keywords

8-Oxoguanine, 8-Oxoguanine DNA glycosylase-1 (Ogg1), Base excision repair (BER), Cell signaling, NADPH oxidase 4, Oxidative stress, Rac1 GTPase, Ras GTPase.

Tárgyszavak

8-Oxoguanin, 8-Oxoguanin DNS glikoziláz-1 (OGG1), Bázis-kivágó javítás (BER), Jelátvitel, NADPH oxidáz 4, Oxidatív stressz, Rac1 GTPáz, Ras GTPáz.

9. Acknowledgement

Although the cover page of this thesis shows only one author, several individuals have contributed to its content. I am thankful all those people for their help and support.

I would like to thank my advisor Dr Attila Bácsi for all his help with our research papers, for shaping this dissertation into its final form and aiding the administrative part of my PhD.

I would like to express my special appreciation and thanks to Professor Dr. István Boldogh, my supervisor at UTMB who supported my work in the United States. His wide theoretical and practical knowledge and visionary thinking were crucial to my projects. He taught me that science requires undivided focus to get through obstacles and challenges. As a good mentor he shared not only his scientific views but also his reach life experience.

I would like to thank all my colleagues and collaborators I worked with during my 6 years at UTMB, for their assistance and contribution to my research.

I would also like to thank Professor Dr. Éva Rajnavölgyi for her support during my PhD student time at the Department of Immunology in Debrecen.

I would also like to thank all of my friends who motivated me on my journey and reminded me of my values.

I would like to say thank you to my parents and my son who support and believe in me no matter where I am in my life.