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SCHOOL OF BIOSCIENCES

Masters by Research

Repair activity of the polymorphic Cys 326 variant of OGG1 under conditions of oxidative stress

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Abstract

Reactive oxygen species (ROS) have damaging effect on cell metabolism and function via various DNA and protein modifications. Cells have evolved many mechanisms to reduce such ROS-induced damage, for instance the base excision repair (BER) pathway. This research emphases the effect of the common Ser326Cys polymorphism in the OGG1 gene, which is an important part of BER to remove mutagenic 8-oxo deoxyguanosine from genomic DNA. Previous studies have highlighted the importance of Ser326Cys polymorphism of OGG1 relating it to many forms of cancer and various degenerative diseases due to variant OGG1 been reported being repair deficient. In the current study, we report the novel application of bimolecular fluorescence complementation (BiFC) to study OGG1 protein complex formation in the native cellular environment and report accumulation of Cys326-OGG1 protein complexes as assessed by confocal microscopy. We observed fluorescence both in and around the nucleus of cells expressing Cys326-OGG1 specifically under conditions of cellular oxidative stress, with increased ROS levels and depletion of GSH following treatment with both buthionine sulfoximine (BSO) and hydrogen peroxide. Furthermore, loss of BiFC fluorescence was seen when cells were treated with thiol reducing agents providing evidence of the role a disulfide bond. In summary, we report for the first time formation of Cys326 OGG1 complexes in the native cellular environment particularly under conditions of oxidative stress. This finding could explain the reduced repair capability of Cys326OGG1 under oxidative stress environment.

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LIST OF ABBREVIATIONS

8-oxo-dG: 7,8-dihydro-8-oxodeoxyguanine

AP-site: apurinic site

ARE: antioxidant response element

BER: base excision repair

BiFC: bimolecular fluorescent complementation

Cys: cysteine

DMEM: Dulbecco's Modified Eagle Medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid

FBS: foetal bovine serum

G: Guanine

GFP: green fluorescent protein

GSH: reduced glutathione

H_2DCF-DA: dichlorodihydrofluoroscein-diacetate

H_2O_2: hydrogen peroxide

hOGG1: human 8-oxoguanine-DNA Glycosylase 1

LB: lutria broth

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromate

NMR: nuclear magnetic resonance

PBS: phosphate buffered saline

ROS: reactive oxygen species

Ser: Serine

YFP: yellow fluorescent protein

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1- INTRODUCTION

1.1 Reactive Oxygen Species

Removal of oxidative damage from genomic DNA is very important to maintain genome stability preventing deleterious mutations, which can lead to various diseases. Because of this various DNA mechanisms have evolved to maintain a state of homeostasis in cells and prevent or eradicate any harmful mutations (Nohmi et al 2005).

It has been estimated that a cell metabolises around 10^{12} oxygen molecules per day and up to 5% of the total molecules metabolised can result in production of Reactive Oxygen Species (ROS)(Nohmi et al 2005). These include hydroxyl radicals (HO), singlet oxygen (O), superoxide (O_2^-) and hydrogen peroxide (H_2O_2). There are numerous potential sources of ROS including endogenous metabolism (electron transport chain) as well as exogenous sources such as ionising radiation and environmental chemicals (Nohmi et al 2005, Murphy 2009, Lyng et al 2001, Harris et al 2011).

Low levels of ROS are needed by the cell to carry out various cell processes for e.g. gene regulation / differentiation, growth pathways (Alder et al 1999). ROS are also involved in redox regulation of many proteins. This can further lead to the activation /deactivation of many phosphates, kinases and transcription factors resulting in altering of protein status and function (Kamata and Hirata 1999). However, excess amounts of ROS have deleterious effects in cells, which

can lead to pre mutagenic lesions, structural and functional alterations by interacting with cellular components (Alder et al 1999).

Cells have evolved various mechanisms to regulate the level of ROS in the intracellular environment. However if the level is disturbed, by excess production of ROS or insufficient removal of ROS, a state of oxidative stress occurs. This can lead to DNA damage, strand breaks, oxidation of DNA bases, nuclear /mitochondria DNA damage or even cell apoptosis (Oka et al 2008). It has been estimated that ROS can cause up-to 10,000-20,000 DNA modifications in each cell per day (Waris and Ahsan 2006, Beckman et al 1997, Ames et al 1993).

Under oxidative stress conditions, ROS is capable of generating various kinds of genetic damages to the DNA (For example single and double DNA strand breaks, oxidation of bases, and formation of abasic site (Demple and Harrison, 1994,Van Gent 2001). This damage if not repaired can lead to mis-sense mutations, cause cell death and is also strongly implicated in the progression of various cancers and other degenerative diseases (e.g. Alzheimer's, heart diseases, diabetes) as well as ageing (Kamata and Hirata 1999, Demple and Harrison 1994, Waris and Ahsan 2006).

1.2 Glutathione –(GSH)

Reduced glutathione (GSH) is a low molecular weight, water-soluble tri-peptide that plays a major role in the protection of cells from various ROS and helps maintain a reduced cellular environment.

GSH detoxifies ROS both in spontaneous and enzyme-catalysed reactions (Townsend et al 2003). For example, GSH detoxifies numerous compounds and peroxides via of glutathione peroxide (GPx) and glutathione-*S*-transferase (GST). It also has an important role in cellular glyoxalase system, reduction of ribonucleotides to deoxyribonucleotides as well as regulation of protein and gene expression via thiol:disulphide exchange reactions (Mullineaur et al 1997).

The tri-peptide exists either as GSSG in its oxidised state or a reduced GSH state. Tight regulation of the ratio of GSH: GSSH is essential to avoid a state of oxidative stress (Araujo et al 2008, Chai et al 1994). GSH regulates its production in a negative feedback manner and any depletion in GSH can lead to increase the rate of GSH synthesis (Meister 1997).Alteration of redox balance via exposure to ROS can also alter the GSH: GSSH ratio, which has been reported to induce gluthathionylation of cysteine by causing oxidation and disulphide exchange reactions at these residues to alter protein conformations. (Lind et al 1998, Klatt et al 1999). Depletion of GSH has been an important tool for studying oxidative cellular stress level conditions. BSO is one such compound which works by inhibition of the rate limiting step in production of GSH and was initially developed for cancer chemotherapy to enhance cytotoxicity and increase cell apoptosis by depleting intracellular GSH levels

and creating cellular oxidative stress conditions (Coleman et al 1998, Green et al 2006, Mitchell et al 1998).

1.3 Oxidative DNA Damage

Oxidative damage has been well documented to be causative for many degenerative disorders as well as been associated with carcinogenesis. ROS induced oxidative stress can damage both nuclear and mitochondria DNA. Oxidative DNA damage if not repaired can be mutagenic and at higher concentrations lead to cell apoptosis (Oka et al 2008). Oxidative DNA damage affects all bases but guanine (G) is particularly susceptibility because of its low redox potential (Brett et al 2000). Oxidation of G has been reported to form at least 2 types of important lesions,2,6 diamino-4-hydroxy-5-formamidopyrimidine (FapyG) , and 7,8-dihydro-8-oxodeoxyguanine (8-oxo-dG)(Yu et al 2008), the repair of latter being main focus of the current research project.

1.4 Base excision repair pathway- (BER)

8-oxo-dG, caused by oxidation of guanine, is a frequently (formed at $1x10^3$ per cell per day) (Fraga C G et al 1990) occurring mutagenic lesion induced by ROS. This pre –mutagenic lesion is found in the genomes of all known life from bacteria's to mammals and is primarily repaired by base excision repair (BER)

(Kunkel T et al 1999, Yu et el 2008, Hills and Evans 2006). BER is highly conserved from bacteria to mammals which highlights it's important for maintaining genomic integrity (Izumi et al2003, Didzaroglu 2005).

BER uses DNA glycosylases, which recognises and cleaves the modified DNA bases leaving an abasic site in DNA. Once an abasic site is generated, this is a substrate for APE1 prior to the activity ofDNA polymeraseβ and XRCC1-DNA ligase IIIα complex to attach and ligate by inserting correct bases (Figure 1)(Nilsen and Krokan, 2001)**.**The human glycosylase enzyme responsible for the removal of 8-oxo-dG is hOGG1 (8-oxo-guanine-DNA glycosylase) (Nohmi et al, 2005).

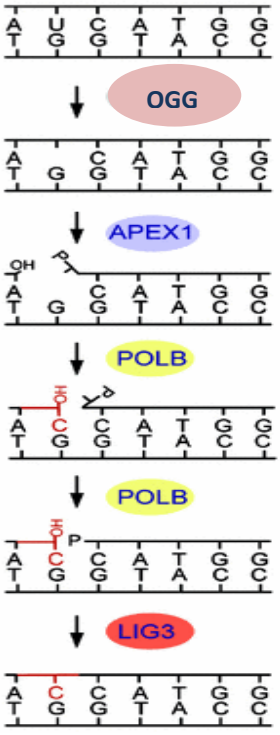


Figure 1. Base excision pathway; 5 step mechanism. Recognition and excision of damaged base. APE1 endonuclease catalyses the incision in the DNA to an AP site. Polβ *acts on an incised AP site created by APE1, inserts correct nucleotideand removes the remaining 5'-deoxyribosephosphate residue. (Robertson et al 2009).*

This pre mutagenic lesion may lead to transversion mutations during DNA replication (figure 2).

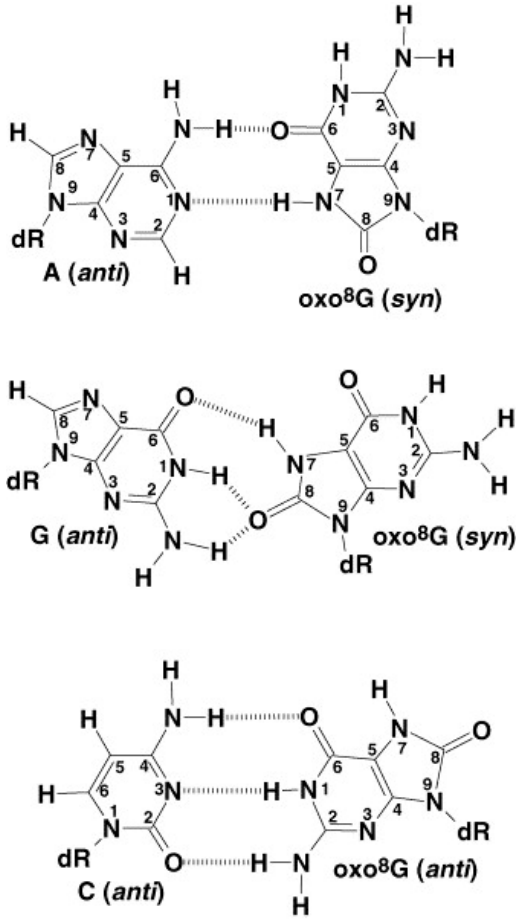


Figure 2. Illustrates the G→T transversion mutations, which occur due to mispairing of 8-oxo-dG. 8-oxo-dG in its anti confirmation base pairs with C, however it can base pair with A during DNA replication while in syn conformation. This further leads to G→T transversions if the change is not repaired. . *Reproduced from Klungland and Bjelland, 2007*

Various studies have observed that G → T transversions are common in tumour related genes such as the ras oncogene, and the p53 tumour suppressor gene although these changes might not be solely due to oxidative DNA damage (Cooke et al 2003, Rodin and Rodin 2000). Previous studies suggest a 10-fold increase of modified nucleotides in smokers with a significant increase in 8-oxo-dG lesions present in their lungs compared to non-smokers (Waris and Ahsan 2005). A significant higher number of 8-oxo-dG has also been linked to progression of various cancer types (Waris and Ahsan 2005).

1.5 8-oxo-guanine –DNA Glycosylase 1

OGG1is a bifunctional DNA glycosylase which possesses both AP lyase and DNA glycosylase activity and repairs DNA by removal of 8-oxo-dG lesions(Fortini et al 2003, Bruner et el 2000). OGG1 possess an α- helix-hairpin helix motif containing a Lys residue (Asp-activated) as an active site which forms a Schiff base and leads to formation of the AP site in DNA (Izumi et al 2003). The human OGG1 gene has been mapped to the 3p26.2 chromosomal region (Fortini et al 20013, Bruner et el 2000) and this region is subject to various monoallelic deletions as well as aberrant OGG1 splicing and these changes are a common feature of many human cancers (Shinmura and Yokota, 2001,Audebert *et al* 2000; Maynard *et al* 2009; Shinmura and Yokota 2001). Knockout study of this mutative OGG1gene in mice and yeast have reported higher level of 8-oxo-dG lesions as well as increase in G→T transversions which demonstrate its potential link in development and progression of cancer. (Klungland et al 1999, Shinmura and Yokota 2001).

The OGG1 gene comprises of 8 exons and is alternatively spliced to result in the formation of two major OGG1 isoforms, α-OGG1 includes exon 1-7 and β-OGG1 (exons 1-6, 8) (Hashiguchi et al 2004, Nishioka K et al 1999). The variants share common N-terminal exons 1-6 (316 amino acids) including a mitochondrial localisation sequences in both isoforms. In contrast,α-OGG1 contains a nuclear localisation sequence in exon 7, which is absent in β-OGG1. Previous studies have suggested that the α-helix in, α-OGG1 is solely responsible for removal of 8-oxo-dG lesions in genomic as well as mitochondrial DNA (Hashiguchi et al 2004). Studies have revealed that targeting OGG1 activity to the mitochondria reveals protection against oxidative stress, which further supports the major role of OGG1 is to reduce the levels of 8-0x0G, therefore minimising the effects of oxidative stress (Dobson et al 2002, Rachek et al 2002).

1.6 OGG1 polymorphism and disease

Although the human OGG1 gene is polymorphic at various sites, the most common polymorphism occurs is a Ser 326 Cys, change in amino acid sequence that is found at varying frequency among different ethnic populations. It arises due to a C→G base substitution mutation at position 1245 of exon 7 which results in altering the codon 326 encoding serine to a cysteine (Kohono et al 1998, Weiss et al 2005, Goode et al 2002). It occurs at a frequency of 0.33-0.45 in Asian population and 0.22-0.27 in Caucasian population, which makes it an

extensively studied polymorphism (Weiss et al 2005, Goode et al 2002, Kohno et al 1998).

The Ser 326 Cys polymorphism has been linked to a repair deficient phenotype (Lee et al 2005). Furthermore, various studies shown that the OGG1 Ser 326Cys polymorphic variant is found at higher frequency in patients with cancers and is a possible risk factor for lung cancer, prostate cancer as well as leukaemia (Li et al 2011, Elahi et al 2002, Hamajima et al 2002, Takezaki et al 2002,Wang et al 2011, Yamane et al 2004).

Individuals with a homozygous Cys326-OGG1 genotype have been reported to have a higher risk of lung cancer by up to 2 fold compared to wild type individuals which was further supported by a meta-analysis study in significantly large samples (Kohno et al 2006, Duan et al 2012,Hung et al 2005, Marchand et al 2006, Sugimura et al 1999). A Study conducted to observe effects of smoking in OGG1-Cys326 individuals provided significant results highlighting a 7- fold lower repair defect in OGG1-Cys326 homozygous individuals under oxidative stress conditions. **(**Guixiang Ji et al 2013). Approximate 75% of G→T transversions were found at codon 12 of ras –oncogene in OGG1-/-knockout mice with lung tumour (Xie Y 2004) further supporting a link between OGG1 oxidative stress and carcinogenesis.

1.7 Ser 326 Cys polymorphism and reduced repair

The activity of the Ser 326Cys OGG1-polymorphism has been studied using various biochemical approaches including complementation tests in bacteria, which indicates that CysOGG1 variant has a reduced repair activity (Dherin et al 1999, Hill et al 2006). Functional studies also illustrate that theCys326-OGG1 variant has a 2–3 times lower repair ability compared to wild type OGG1 (Hills and Evans 2006, Zielinska et al 2011). The mechanism of how the polymorphism relates to functional defect of the enzyme has not been fully elucidated; however there is growing evidence of redox regulation on OGG1 activity. The Mechanism basis of repair deficiency and the Cys 326 amino acid position remains unclear due to a lack of crystal structure of the protein beyond the 325th position (Bravard et al 2009).Fusion proteins experiments using western blot have revealed the Cys 326-OGG1 variant produces a slow moving band in gel which indicates that the variant form of the protein may form a dimer but this has yet not been confirmed in live cells (Bravard et al, 2009). Work by Hills and Evans further support Cys326-OGG1 reduced repair ability by demonstrating thatthe purified CysOGG1 variant was found to posses 1.6 lower catalytic efficiency compared to Ser326-OGG1 (Dherin et al 1999, Hills and Evan 2006). Furthermore, 3-4fold lower repair rate and significant loss of fluorescence was seen over time in live cells following targeted DNA damage while using the OGG1-GFP tagged protein to study repair rates of cys326 OGG1 compared to wild type protein (Zielinska et al 2011).

Various hypotheses exist to explain the enzyme's reduced repair capacity. The reduced repair rate of OGG1variant could be a consequence of either loss of

phosphorylation site at serine 326 or incorporation of a new redox sensitive cysteine residuein a highly positive amino acid sequence tendering it liable to oxidation(Bravard et al 2009). Various studies have suggested that repair rate of the OGG1 variant is delayed in cells following Oxidative stress treatment. A study conducted in our lab has provided evidence that OGG1 variant activity was delayed by 12 hours compared to wild OGG1 (Kershaw et al 2012).

1.7.1 Ser326 OGG1 as a potential phosphorylation site:

The cellular function and localisation of OGG1 has been reported to be affected by posttranslational modification of both serine and threonine residues. In particular, the PKC dependant phosphorylation status of serine in OGG1 has been linked to changes in location of variant OGG1 during the cell cycle specifically exclusion from the nucleolus (Dherin 1999, Luna 2005). Furthermore, the exclusion of Cys 326-OGG1from the nucleolus during S phase of cell cycle could be corrected by incorporation of glutamate amino acid at position 326, which, which mimics a phosphoserine (Luna et al 2005). This suggests localisation of OGG1 could be affected by the loss of Serine phosphorylation site in the cysOGG1 variant. The location of OGG1 during DNA repair is very crucial for removing DNA damage, which seems to be dependent on the localisation pattern controlled by phosphorylation of ser326 residue. In variant OGG1, loss of serine could mean loss of phosphorylation site, which could affect the location of the enzyme during cell cycle and be responsible for its reduced repair capacity.

1.7.2 Oxidative modification of incorporated Cys 326 residue

The reduced repair of the polymorphic variant could also be dependent on the oxidative modification of the Cys326 residue. Cys326 lies in a disordered area of the 3D protein structure, which makes it highly susceptible to redox modifications. Furthermore the Cys326 residue is surrounded by positively charged amino acids making the thiol group of the Cysteine more prone to oxidation by formation of a thiolate ion.

In further support of a role of redox regulation, previous studies have shown that Cys326-OGG1 is repair deficient especially under conditions of cellular oxidative stress (Bravard et al 2009). Bravard et al conducted a study, which used reducing agent to treat cells expressing Cys326-OGG1, and demonstrated a significant increase in the repair rate of Cys326-OGG1. In contrast, the wild type enzyme activity was not affected (Bravard et al 2009). This study provides evidence for link between reduced repair capacity and OGG1 oxidative state, where oxidative modification of the redox sensitive Cys326 can lead to formation of disulphide bonds and interfere in enzyme repair capacity.Previous studies have also shown the importance of Cys326 residue for repair activity and its link to possible protein dimerization. Studies in purified OGG1 protein used Cys 326 truncated C- terminally containing residues up to 326 and excluding 326^{th} position indicates that the residue 326 was involved and sufficient for the protein dimerization and possibly responsible for reduced repair ability (Hills and Evans 2006).

1.8 Bimolecular Flourescence Complementation (BiFC)

Bimolecular Fluorescence Complementation (BiFC) is a technique used to visualise protein-protein interaction in cells. It involving transfection of vectors containing the gene of interest fused to two half's of YFP separately into cells, where any interaction between the two proteins would bring the two half's of YFP together and result in refolding of YFP protein and lead to emission of fluorescence which can visualised using confocal microscopy and quantified using flow cytometry analysis. (Figure 3)

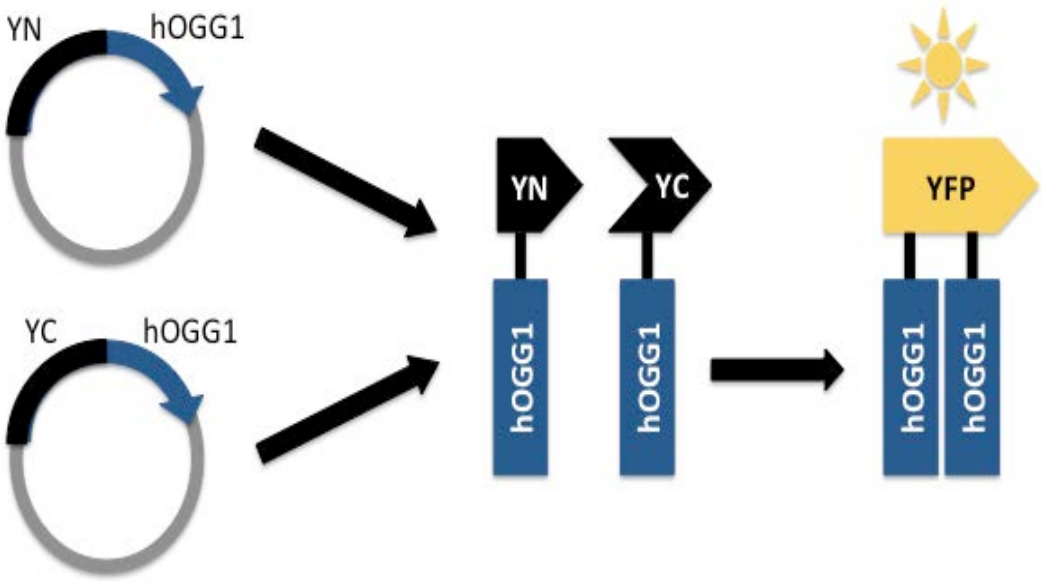


Figure 3. The principle of the BiFC Assay and vector expression used in our study. Cells were transfected with either half of YFP and hOGG1. Interactions between the two hOGG1 would bring two halves of YFP together resulting in YFP fluorescence. (Hu et al, 2005, figure taken from Kaur MP et al 2014)

PROJECT AIMS:

The main aim of the project was to further provide further evidence of OGG1 dimerization in Cys326OGG1 variant under oxidative stress conditions and supporting its involvement in the reduced repair activity compared to wild type Ser 326OGG1. Here we report the novel application of the imaging technique of bimolecular fluorescence complementation (BiFC) to study OGG1 complex formation for the first time in the native cellular environment and show that this only occurs with the Cys326 variant of the protein and is specific to conditions of cellular oxidative stress.

2- MATERIALS AND METHODS

2.1 Cell culture

A Bsb 4a Gelair flow laboratories tissue culture hood was used to maintain sterile conditions for all the cell culture experiments. All the equipment and solutions were sterilised by autoclaving before use. All the solutions were warmed in a 37^0C water bath and cleaned thoroughly with 70% ethanol solution before using them in the sterile hood. A549 lung carcinoma cells (HPA Catalogue number 86012804, ECACCC, HPA) were cultured in Dulbecco's modified Eagle's medium DMEM (Sigma-Aldrich, UK), supplemented with 10%v/v foetal calf serum, 2 mM L- glutamine, penicillin and streptomycin (100µg/ml, PAALaboratories, Ltd.) in T75culture flasks (PAA Laboratories LTd.) at 37^0C in a humidified 5% CO_2incubator (Sanyo CO_2 incubator MCO-17a, Electric Co., Japan). Cells were grown to 80% confluence and passaged for all the experiments using a standard trypsin-EDTA protocol.

2.2 Cell Cryopreservation

Cells grown to confluence were trypsinised and resuspended in 10ml DMEM. Cells were pelleted by centrifugation at 1000xg for 10 minutes (Falcon 6/300, Meadow Rose Scientific). Media was removed and the pellet was resuspended in 1ml of freezing media (FBS containing 10%v/v DMSO). The media was then transferred to cryofreezing vials and stored at -80 ^{0}C for 24 hours before final

long term storage in vapour phase liquid nitrogen -80^{0}C (Scientific ultra low temperature freezer U535-86, New Brunswick).

2.3 Preparation of BiFC vectors

Glycerol stocks of expression vector constructs prepared from Plasmids (pBluescript II KS-) containing either the N- or C – terminus of yellow fluorescent protein (YFP-N and YFP-C) ligated in frame with cDNA fragments of full length Ser 326-OGG1 or Cys326-OGG1 were prepared and characterised previously in our laboratory (NJH personal communication). Vectors containing only YFP fragments were also available and used as a negative control in experiments.

2.4 Plasmid DNA extraction / Preparation

JM 109 super competent *E. coli* cells (1x10^6 CFU/ml, Promega UK) containing the expression vectors were plated onto LB-agar plates (containing100µg/ml ampicillin) and incubated overnight 37 ^{0}C. Well isolated colonies were picked and added to LB broth (15ml, 100µg/ml ampicillin). The culture was then incubated for 24 hour at 37 ^{0}C on a shaking incubator (225rpm). Plasmids were recovered from bacteria using a Qiagen plasmid mini-prep kit according to manufacturer's instructions. The extracted DNA concentration was measured

using Nanodrop and stored at -80 ^{0}C for future experiments. (Scientific ultra-low temperature freezer U535-86, New Brunswick).

2.5 Transfection with OGG1-YFP half vectors

A549 cells were grown to 60% confluence overnight in 6 well plates containing 13mm round glass coverslips (PAA Laboratories ltd). Plasmid DNA extracted from the plasmid mini-prep was used to transfect cells using Turbofect (Thermo-Fisher Scientific Inc.) as per the manufacturer instructions.

Briefly, 4 µg of total plasmid DNA (see table 1) containing either control, Ser326 or Cys326 OGG1 half vectors was used along with 400 µl of serum free media (Sigma-Aldrich, UK) and 6 µl of Turbofect reagent was prepared and incubated for 20 minutes at room temperature and added dropwise to each well. Cells were then incubated for 24 hours prior to analysis.

N-Terminal YFP	C-Terminal YFP
YN (2 µg)	YC (2 µg)
SN (2 µg)	SC (2 µg)
CN(2 µg)	CC (2 µg)

Table 1.Amount of each plasmid type used for transfection. YN and YC contains either N terminal and C terminal of YFP respectively. SN and SC contain either N or C terminal of YFP along with serine326OGG1. CN and CC contain either N or C terminal of YFP along with Cysteine 326 OGG1.

2.6 Transfection of cells with GFP-OGG1

A549 cells were cultured overnight to about 65% confluence on sterile round 13mm glass coverslips (PAA Laboratories Ltd.) Cells were then transfected with (4μg) OGG-GFP expressing constructs using Turbofect (Thermo-Fisher Scientific Inc.) as per the manufactures instructions. After 48 hour cells were fixed with neutral buffered formalin (2ml) and the nuclei counter-stained with Hoechst 33258 (0.8 μg/ml) and analysed by confocal microscopy (Leica TCS SP2 confocal microscope with a oil immersion objective 63x) by exciting fluorophores at 480nm for GFP and 405nm for Hoechst. In addition flow cytometry was used to quantify transfection efficiency. Images analysis of confocal images was carried out using Leicalite software and post processing was done using Adode Photoshop CS5 extended.

2.7 Induction of oxidative stress

Cells transfected for 24h were cultured overnight in 6 well plates prior to treatment with buthionine sulfoximine (BSO). DMEM was removed, and cells were washed with sterile phosphate buffered saline (PBS, 2 ml) (Sigma-Aldrich, UK). Cells were then treated with DMEM containing 0- 1000μM of BSO and incubated for 24 hours before analysis. In some experiments cells were grown to confluence in 6 well plates overnight and treated with (150μM) hydrogen peroxide for 60minutes before analysis.

2.8 MTT assay

To assess cell viability following BSO-induced oxidative stress, mitochondrial activity was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) reduction assay.Cells were grown to confluence in 96 well culture plates (PAA Laboratories Ltd.) and treated with BSO concentrations (0-1000µM). Following treatment cells were washed with 100µl PBS and new media containing MTT (0.5mg/ml) (Sigma-Aldrich, UK) was added and incubated at 37^0C for 2 hours. Next, the media was then removed and DMSO (100 µl) (Sigma-Aldrich, UK) was added to each well and plates were rocked in the dark at room temperature for 30 minutes before analysis using Bio-Tek FL 600 plate reader (Bio-Tek Instruments Inc. USA) to measure absorbance levels at 570nm against DMSO as a blank.

2.9 Quantification of reactive oxygen species

Following treatment with oxidants, 3ml of fresh DMEM along with dichlorodihydrofluorosein-diacetate(H_2DCF-DA) dye (at a final concentration 10 µM) (Sigma, UK) was added per well. . The cells were then incubated at 37^0C for 60 minutes prior to analysis by flow cytometry. Following incubation with H_2 DCF-DA, cells were washed twice with PBS (2ml) and cells were incubated with trypsin solution (1ml) for 15minutes at 37^0C. Next fresh media (1ml) was added to each well and cells were then transferred to an eppendorf

tube and centrifuged at 13000x g for 10 minutes to pellet the cells. Cell pellet was re-suspended in 1 ml PBS and transferred to a flow cytometry tube for analysis.

A FACS machine (FACScalibur, BD biosciences USA) was used to measure fluorescence of 10,000cells from each sample using the FITC channel emission wavelength (517-527nm). This was then quantified using unlabelled cells as blank to control for background fluorescence. Weasel software (Walter and Eliza Hall institute of Medical Research Australia) was used to generate histograms of data and mean fluorescence of each population of cells. Data was blank adjusted before statistical analysis.

2.10 Glutathione Assay

2.10.1 Sample preparation

A549 Cells were cultured and grown to confluence in 6 well plates followed by 24-hour BSO treatment or other treatments (DMSO/ DTT/β-ME for other experiments). Following treatment of cells, the media was removed and cells were washed with 1ml PBS and 450µl of Ice cold lysis buffer (0.1 % Triton X-100 in PO_4-EDTA assay buffer) was then added and cells were removed by scraping. The lysate was then transferred to a 1.5ml fresh eppendorf tube. (20µl of the lysate was retained separately for Bradford assay / protein estimation) and 50 µl of protein precipitation buffer (trichloroacetic acid (50 % v/v) in PO_4-EDTA assay buffer**)** was then added to the remaining lysate followed by

centrifugation at13000 x g for 5 minutes. The supernatant was stored at -80 ^{0}C until required for analysis.

2.10.2 Assay procedure

Fluorescence cuvettes (3ml) were used for the all the samples and the GSH standard curve. The GSH standard curve ranged from (0,1,2,4,6,8,10,12,14,16,18,20 µl of 0.1 mg/ml GSH.

1.8 ml of PO_4-EDTA(NaH_2PO_4 (100 mM) + NA_2EDTA (5 mM) adjusted to pH 8.0) was added to all the cuvettes. GSH standard was added to standard curve cuvettes at different volumes to obtain a standard curve. 5 % of (100 µl) TCA was added to all GSH containing cuvettes and 100µl of sample was added to the sample cuvettes followed by addition of O-phthalaldehyde (OPT) (1 mg/ml) in 100% methanol) (100 µl) to all cuvettes. The cuvettes were then covered and incubated at room temperature for 15 minutes before analysis. An excitation wavelength of 350nm and emission wavelength 420nm was used to measure fluorescence of all the samples. Fluorescence values were normalised to protein as estimated by the Bradford assay.

2.11 Fixed cell imaging

Fixed cell imaging was used to visualise YFP- fluorescence in cells treated with BSO using confocal microscope.Cells were grown to 60-70% confluence on sterile 13mm cover slips in 6 well plates and transfected with 4µg DNA for 24 hours followed by BSO (1000µM) treatment for 24 hour (see section 2.5). In

some experiments disulphide reducing agents β-mercaptoethanol (β-ME) (1 mM) or dithiothreitol (DTT) (1 mM) along with DMSO (1*% v/v*) was added for the last hour and in other experiments cells were treated with H_2O_2 (150µl). Following treatment cells were then washed twice in 2ml PBS and fixed with 4% neutral buffered formalin (2ml, pH 7.4) at room temperature for 15 minutes. Next cells were washed twice with PBS (2ml) and then nuclei counterstained using1 ml of PBS containing Hoechst 33258 (0.8 µM, Invitrogen, UK) for 5 minutes. A further 2 washes with PBS (2ml) was performed and cover slips were then carefully removed and mounted cell surface down onto microscope slides using non-fluorescent hydromount vectamount (50 µl, National Diagnostics, UK). Excess mountant was carefully cleaned off slides and the slides were stored at 4^0C in dark for a maximum period of 48 hour prior to analysis. A Leica TCS SP2 confocal microscope was used for image acquisition using an oil immersion objective (63x).Fluorophores were excited using 488nm laser for YFP and 405nm laser for Hoechst. Images were then analysed using Leicalite software and post processing using Adobe Photoshop CS5 extended.

3. RESULTS

3.1 Confirmation of OGG1 location

To confirm nuclear localization of OGG1 protein in this cell line, cells were transfected with GFP-tagged protein and analysed by confocal microscopy and Flow cytometry. OGG-GFP was found to be exclusively nuclear located as confirmed by nuclear counterstained Hoechst dye (Figure 4B). This was consistent with previous studies in MEF cells (Kershaw et al 2012). It was also seen that the nuclear sub-localization of OGG1 was not affected by OGG1 genotype or 24 hour BSO treatment (1000 μM, data not shown). Transfection efficiency was quantified by flow Cytometry (Figure 4A) indicating a transfection efficiency of approximately 50%.

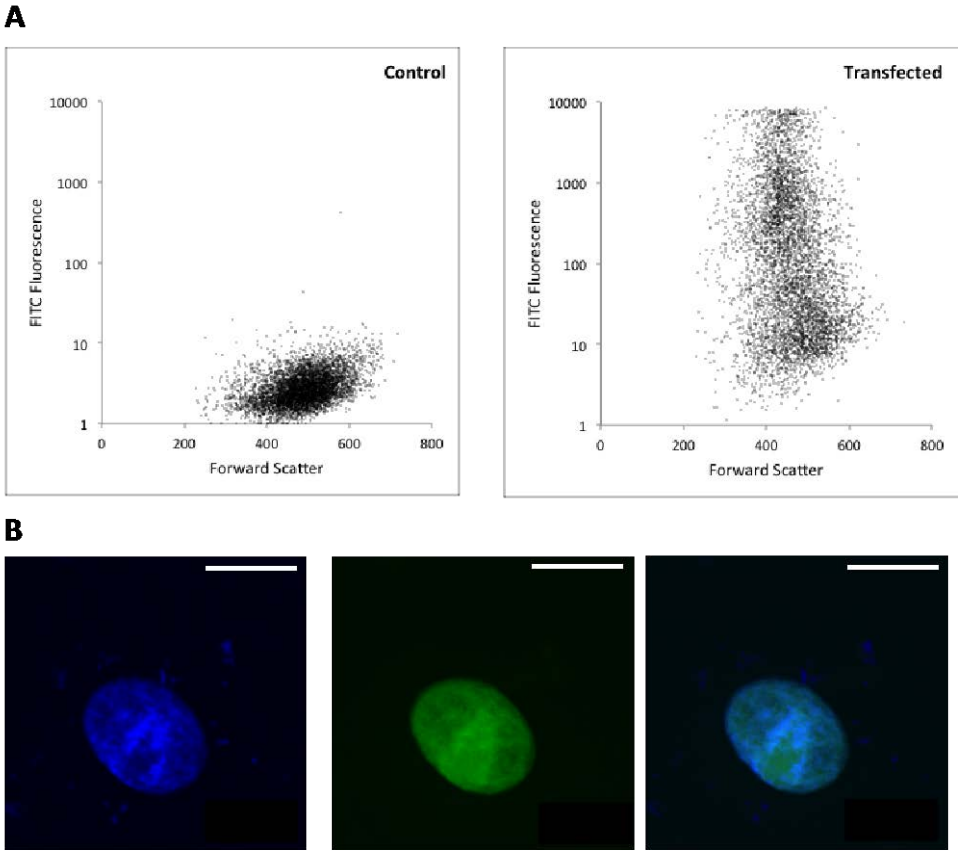


Figure 4: A) Transfection of A549 cells with OGG1-GFP confirmed using flow cytometry analysis and B) Nuclear localization of OGG1 confirmed by confocal microscopic analysis. Scale by bar -10μm. (Figure taken from Kaur et al 2014)

3.2 MTT assay

Different concentration of BSO (50,100,250,500,1000 μM were used to treat cells for 24 hour to identify non-toxic concentration of BSO. MTT is a water soluble dye that crosses cell membraneand is reduced by mitochondrial enzymes producing a purple colour product.

Triplicate measurements of results showed no statistical significant concentration –dependant change in cell viability between BSO treated (0-1000µM) and the control cells as assessed by one way ANOVA (Figure 5).

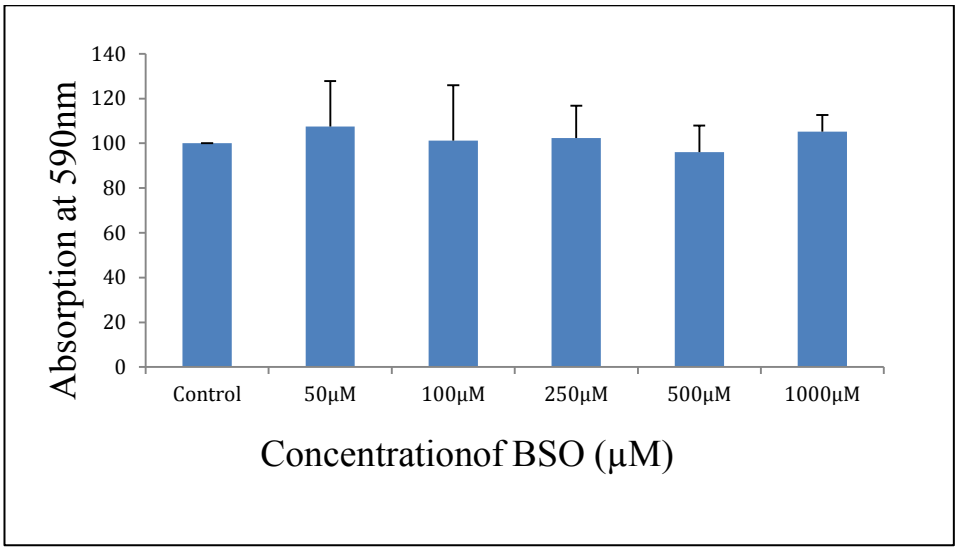


Figure 5: MTT assay performed to analyse cell viability following 24hour BSO treatment at different concentrations (0-1000uM). (±SD, n=3) of all the samples were plotted against untreated control. No significant statistical concentration dependant change was observed as assessed by one –way ANOVA, p-value >0.05.

3.3 Confirmation of oxidative stress

Intracellular ROS levels were measured before and after 24 hour of 1000µM BSO treatment by incubating cells with DCF-DA dye. DCF-DA dye diffuses into cells and is cleaved to produce non florescent H2DCF, which in the presence of ROS is oxidised to form fluorescent DCF. The levels of fluorescent measured by flow cytometry are proportional to the amount of reactive oxygen species oxidising the dye (Wang and Joseph, 1999).

As expected, treatment of A549 cells with BSO (1000 µM) for 24 hours resulted in a statistically significant ($P<0.01$) induction of ROS (Figure 6), which was consistent with previous studies in our laboratory (NJH personal communication).

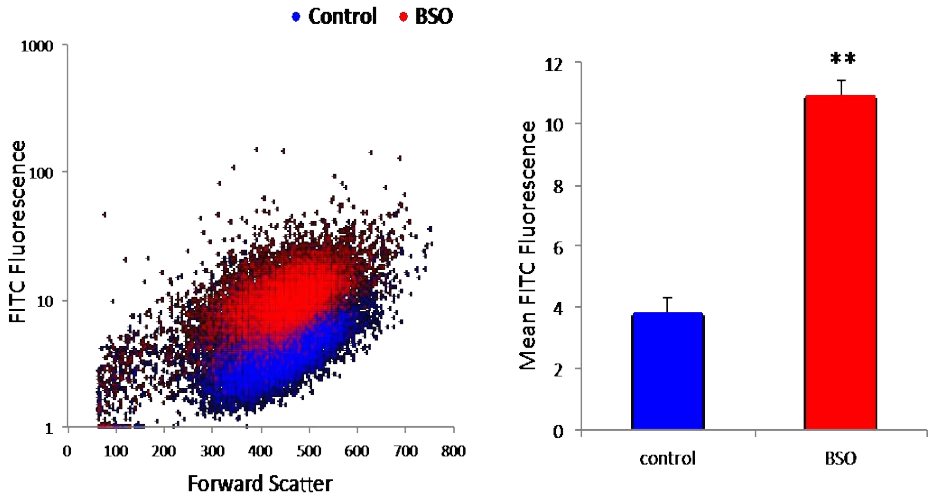


Figure 6: Induction of oxidative stress following treatment of cells with 1000µM BSO. The results represent the mean of four independent experiments (±SD, n=4) carried out in duplicate, significantly different from controls (Student t-test), P < 0.001. (Figure taken from Kaur et al 2014)

3.4 Depletion of glutathione

BSO induces oxidative stress in cells by depleting glutathione levels in cells by inhibiting the rate-limiting step of GSH synthesis. Following 24 hour treatment a concentration dependant reduction in GSH levels was observed (Figure 7)

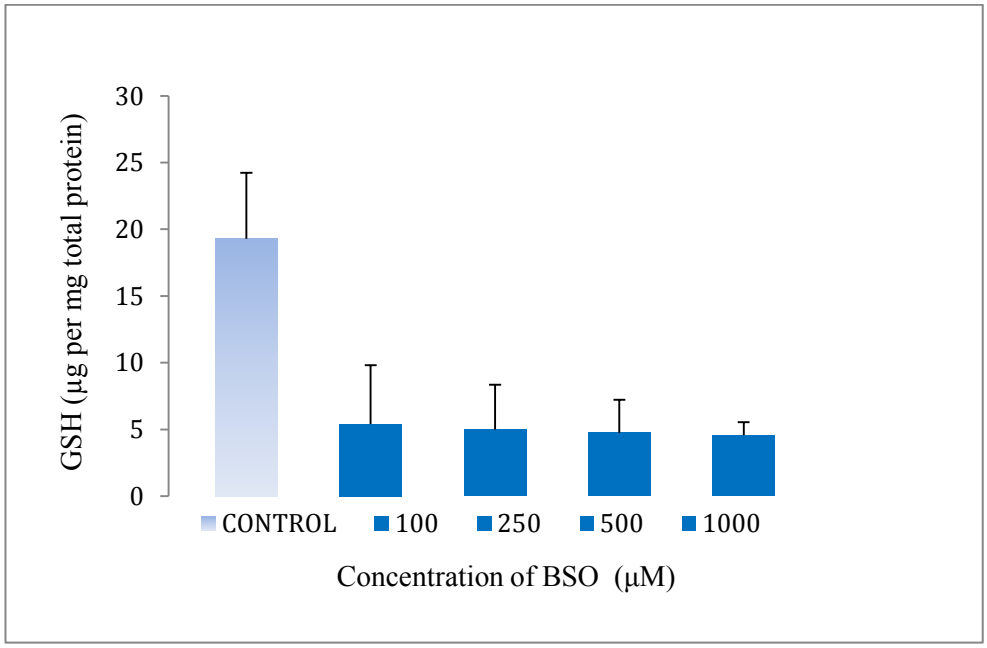


*Figure 7 Cellular levels of GSH in A549 cells after 24 hour treatment of BSO concentrations (100,250,500,1000 µM). ** Significantly different from control (student t-test), p<0.01.*
The results represent the mean of the three experiments carried out in duplicate (n=3)$^{\pm}$SEM.

3.5 Assessment of BiFC by flow cytometry following BSO treatment

Weasel software was used to analyse the results from the BiFC flow Cytometry experiments. The cell population was plotted in a dot plot and gated to calculate average fluorescence (Figure 8A) and the histogram of fluorescence (Figure 8B) was used to calculate the mean fluorescence of each cell population.

A) B)

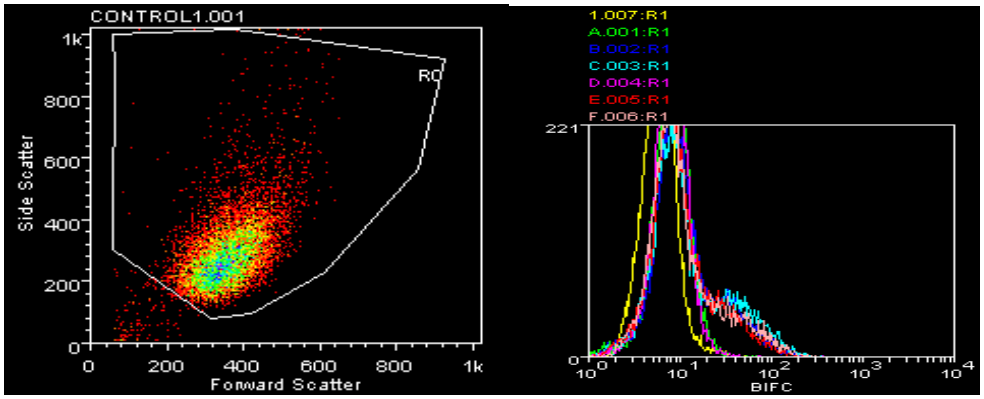


Figure 8A) Represents and an example of dot plot for cell population for the control YC/YN vector .The gate RO was used to separate viable cells from debris which was then used to calculate mean fluorescence of cells. B) Example of a histogram overlay showing fluorescence profile of cells in the presence and absence of BSO treatments.

The mean fluorescence values were blanked for background fluorescence using un-transfected control cells. Average fluorescence values (3 repeat duplicate experiments) of the samples were then plotted (Figure 9). Although BiFC fluorescence was detectable, no statistically significant difference between control and the OGG1-expressing YFP vectors was observed. Furthermore, there was no statistically significant difference between control and BSO treated cells.

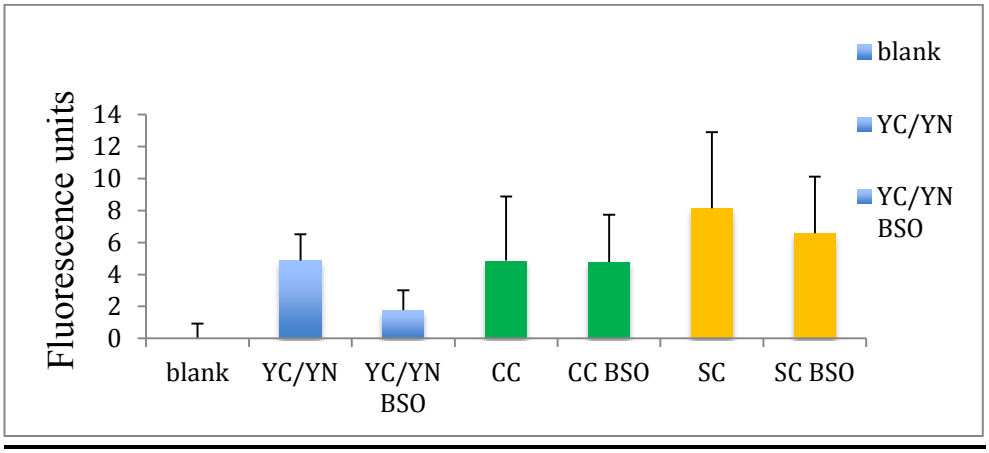


Figure 9: Results represent average BiFC fluorescence (±SD, n=3) of YC/YN; Cys326OGG1; ser326OGG1 untreated and BSO treated samples blanked against unlabelled cells. No statistically significant change was observed between the untreated and BSO treated vectors as assessed by student t-test, P>0.05.

3.6 Detection of BiFC by confocal microscopy

To study protein interactions in cells, we used BiFC a powerful technique to visualise direct protein interactions and find possible evidence of dimer between OGG1.

Cells were transfected with either Cys326OGG1 or Ser326OGG1 half YFP vectors. Cells transfected with only YFP half vectors were used as control to check for any background fluorescence. Cells were treated for 24 hours with BSO to induce oxidative stress and deplete GSH levels. Fluorescence results for possible protein interactions were assessed using confocal microscopy.

Little or no BiFC fluorescence was seen in Ser326OGG1 or YFP transfected cells before or after the 24 hour BSO treatment. In contrast, in cells transfected with Cys326OGG1 illustrate no evidence of BiFC before treatment. However there was clear BiFC fluorescence in Cys326-OGG1 transfected cells after 24 hour treatment with BSO.Hoechst 33258 was used for counterstaining the

nucleus and indicated the BiFC fluorescence appeared as discrete foci mainly located in the nucleus (Figure10). There was evidence of fluorescence in peri-nuclear regions. Figure 11A show higher magnification of BiFC fluorescence in peri-nuclear region. Figure11B illustrates a Z –scan of Bifc fluorescence, which overlaps with the nuclear stained Hoechst 33258 in the same plane and hence confirms its location in the nucleus. Furthermore, No BiFC was seen in co-transfection experiment with cells with YFP-N-Cys326-hOGG1 and YFP-C-Ser326-hOGG1 or YFP-N-Ser326-hOGG1 and YFP-C-Cys326-hOGG1 constructs (Data not shown), which further supports that the BiFC is specific to Cys326-OGG1 expressing cells.

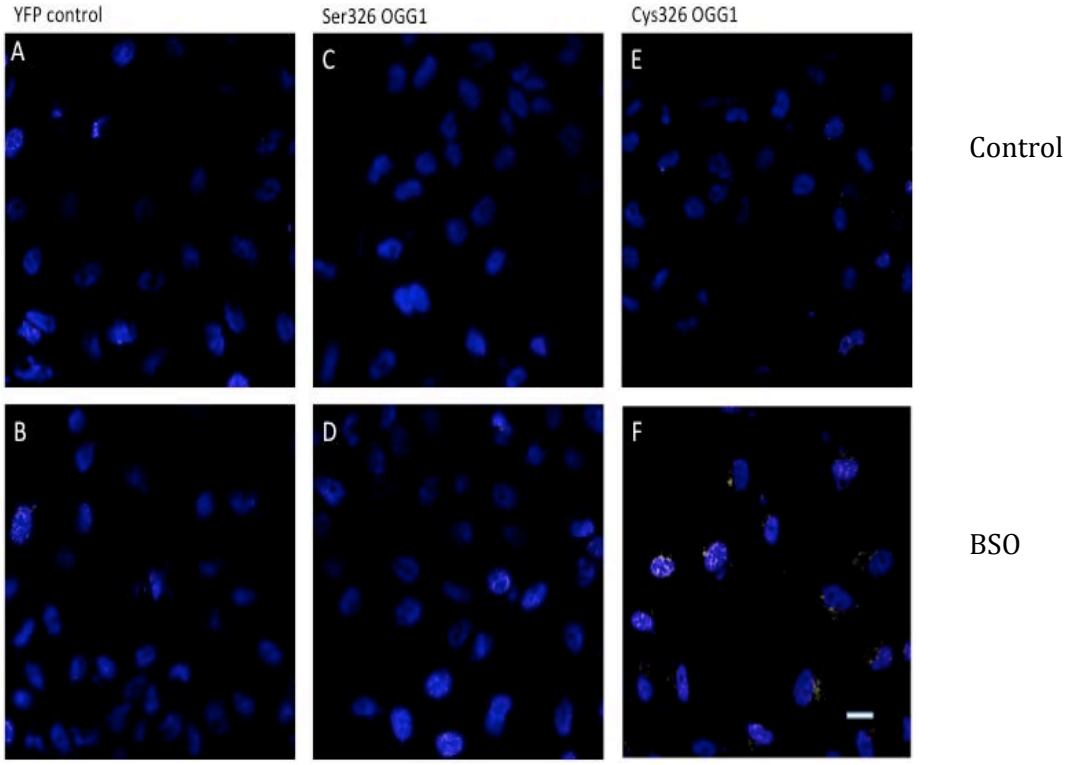
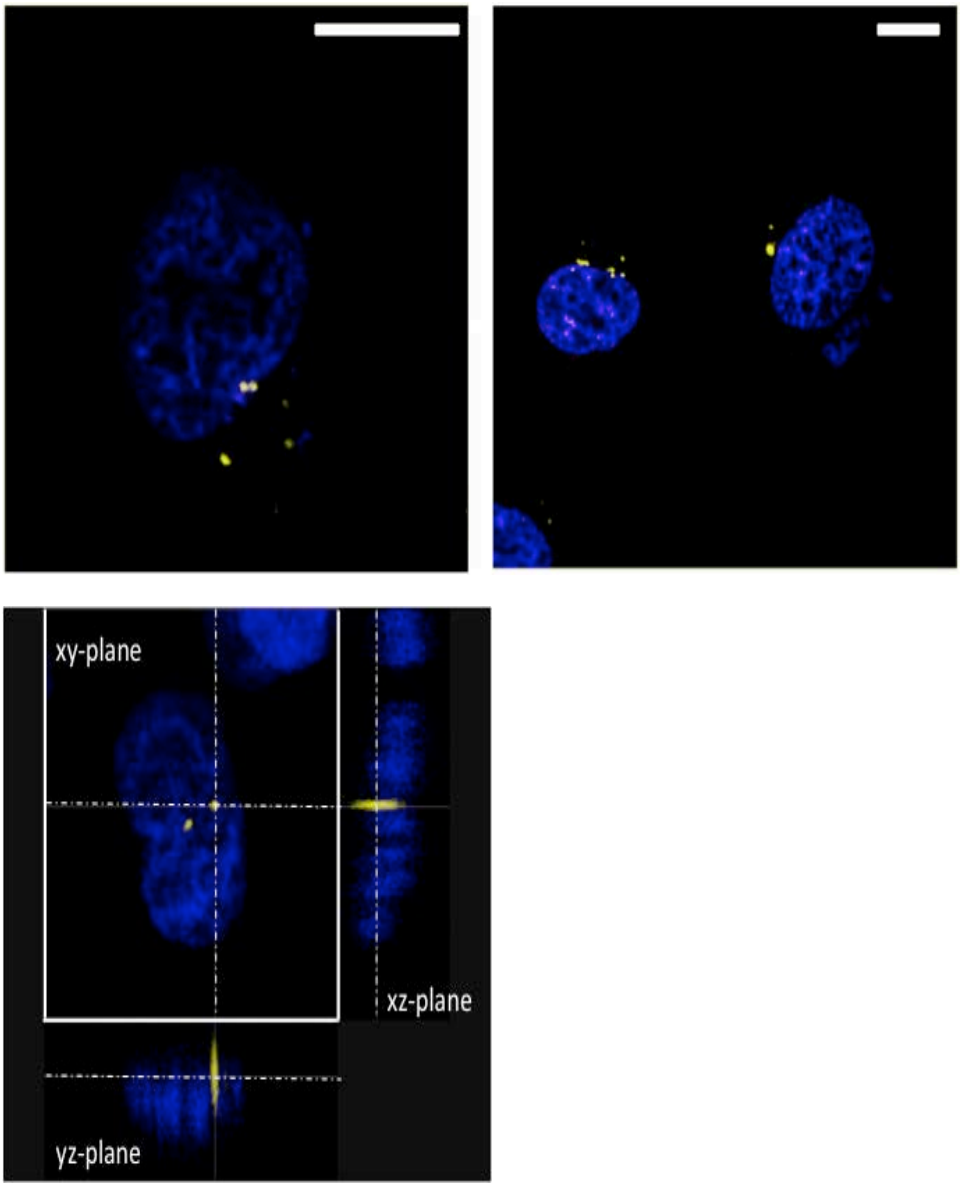


Figure 10. No or little BiFC fluorescence was seen in cells transfected with only YFP A), B) or Ser326-OGG1 C), D) before or after 24 hour BSO treatment. Cells with Cys 326-OGG1 show no

fluorescence in untreated cells E). However clear foci of discrete fluorescence was seen in cells transfected with Cys326-OGG, F) after 24 hour BSO treatment. Nuclear DNA is counterstained with Hoechst 33258 and scale bar is 1010µm. in all images. (Figure taken from, Kaur et al 2014).

Cys326 OGG1 cells treated with BSO and plotted as an average number of BiFC fluorescence foci per cell i.e. 10.5

A)



B)

Figure 11: A) Figure represent BiFC fluorescence images of cells transfected with Cys326 N1/C1following 24 hour BSO treatment (1000 µM) B) Illustrates three-dimensional

distribution of BiFC fluorescence in nucleus of cells transfected with Cys326-OGG1 following 24hour BSO treatment. In All figures, nuclear DNA is counterstained with Hoechst 33258. (Figuretaken from, Kaur et al 2014).Scale bar is 10 µm

3.7 BiFC following treatment of cells with hydrogen peroxide

In contrast to BSO, hydrogen peroxide directly induces the production of ROS in cells without the need for depletion of GSH. After treatment of cells for 60minute hydrogen peroxide (100µM) induced oxidative stress was confirmed by oxidation of DCF-DA as assessed by flow cytometry (Figure 12A). Despite induction of ROS, there was no change in levels of reduced GSH in cells (Figure 12B).

A. B.

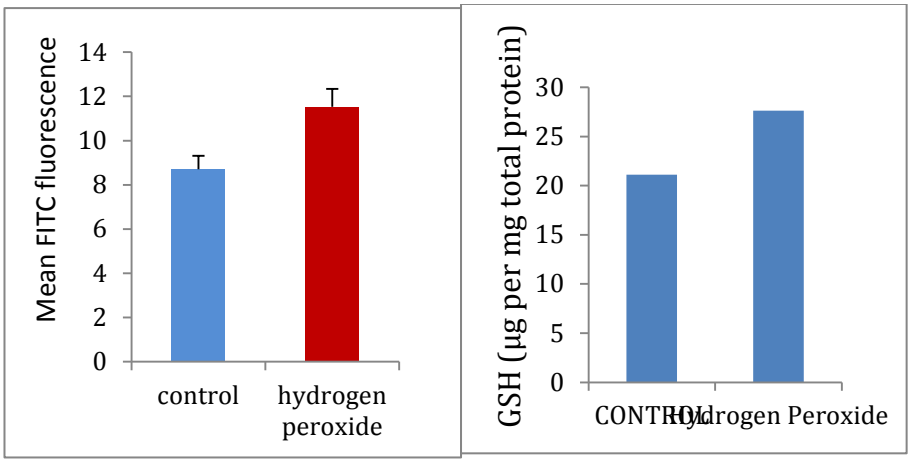
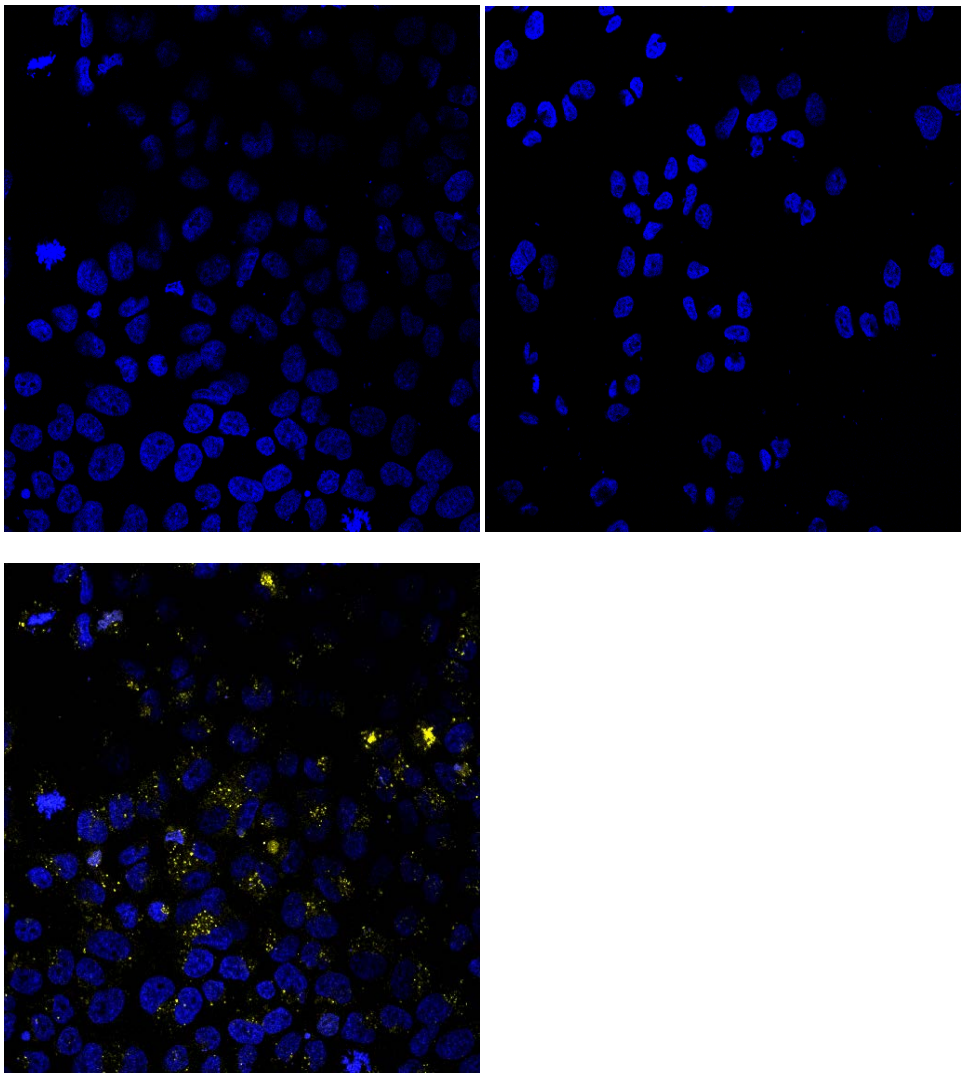


Figure 12A) Results represent average ROS levels in untreated and hydrogen peroxide 150µM treated A549 cells (1 hour treatment) (±SD, n=3) show a statistically significant increase in ROS levels after treatment as investigated by students t-test, p <0.05). B) Result represent average of duplicate GSH depletion experiments between control and hydrogen peroxide 150µM treated cells. Statistically no difference between the GSH was seen as assessed by student t-test, p>0.01.

3.8 Treatment with H_2O_2 results in BiFC fluorescence

Cells transfected and treated with hydrogen peroxide for 1 hour support the results seen using BSO to induce oxidative stress. Discrete foci of BiFC fluorescence were specific to Cys326OGG1 BSO treated cells (Figure 13). This supports our previous results using BSO and suggests that BiFC fluorescence was not dependant on the type of oxidant use to treat the cells.

Control Ser326-OGG1



Cys 326-OGG1

Figure 13.BiFC fluorescence was only observed in cells transfected with Cys326- OGG1. Control and Ser326- OGG1 show no evidence of fluorescence after 1 hour hydrogen peroxide treatment (150μM), nuclear DNA is counterstained with Hoechst 33258.

Cys326-OGG1 cells treated with hydrogen peroxide plotted as an average number of BiFC fluorescence foci per cell i.e. 85.0

3.9 Treatment of cells with anti-oxidants

3.9.1 Cellular ROS Levels following treatment with β-me and DTT and DMSO.

Cells treated with 1000μM of BSO treatment for 24 hour were subjected to treatment with 1mM DTT or β-me or DMSO for the final 4 hours of treatment and cellular ROS levels were analysed by flow cytometry (Figure 14).

A statistically significant increase in levels of ROS was found between cells treated with BSO and untreated cells compared to control. However, surprisingly no statistically significant difference between ROS levels was seen in cells treated with BSO and BSO and anti-oxidants (Figure14).

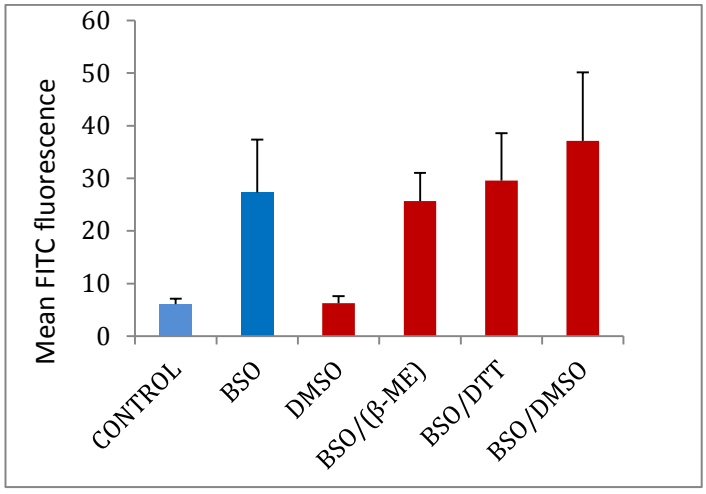


Figure 14: Results show average ROS levels from the triplicate replicate experiments (±SD, n=3) In cells treated with DTT and β-ME for the last 4 hour of the 24hour BSO Treatment were

plotted against untreated and the cells treated with 24 hour BSO treatment and addition of DMSO for last hour of treatment in BSO/DMSO treatment. Significant increase in ROS levels was found between the untreated vs. BSO treated cells and BSO treated vs. BSO+DMSO treated cells, while no significant difference of ROS levels was found between cells treated with BSO and (BSO+ β-mercaptoethanol, BSO=DTT) as assessed by students T-test, $p < 0.05$, $p > 0.05$ respectively).

3.9.2 Cellular Glutathione Levels in cells treated with Reducing Agents

GSH levels in cells treated with reducing agents and anti-oxidants were also measured to investigate any changes in intracellular levels of reduced glutathione. Glutathione levels in cells treated withDMSO and BSO/DMSO together and reducing agents DTT and β-ME were measured and plotted against untreated cells and cells treated with 24hour BSO treatment (Figure15). Following treatment with eitherβ-ME or DTT there was a small but statistically significant increase in the levels of GSH compared to cells treated with BSO alone (Figure15).

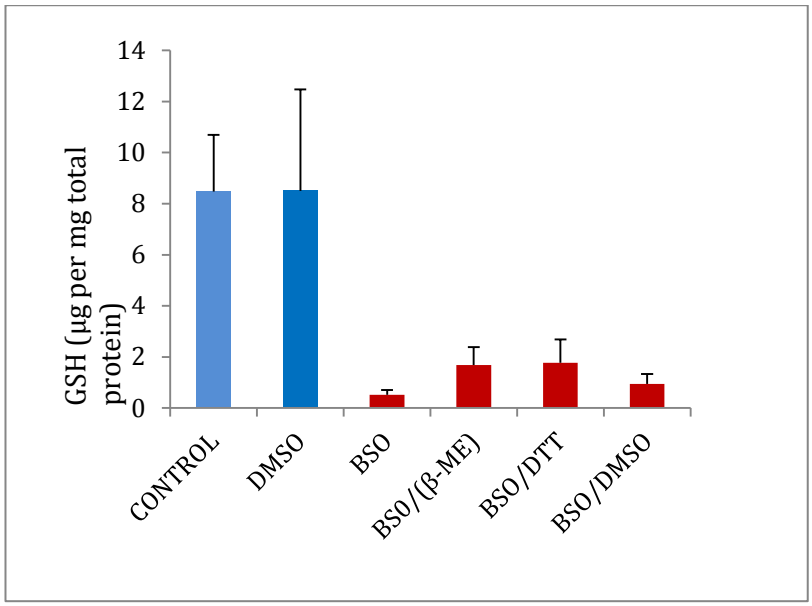


Figure 15: Results show average GSH levels from the replicate experiments(±SD, n=3). GSH level in cells treated with DTT and β-ME for the last 4 hour of the 24hour BSO Treatment was plotted against untreated and the cells treated with 24 hour BSO treatment and addition of DMSO for last hour of treatment in BSO/DMSO treatment. Significant depletion of GSH levels was found between the untreated vs. BSO treated cells, while no significant difference of GSH Level was found between cells treated with BSO and (BSO+ β-mercaptoethanol, BSO+DTT) as investigated by students T-test, $p < 0.05$, $p > 0.05$ respectively).

3.9.3 Reducing Agents inhibit BiFC florescence in OGG1-Cys36 cells.

Antioxidants inhibit BiFC fluorescence

The disulphide reducing agents β -ME and DTT were added for last 4 hour of oxidative stress treatment in cells expressing Cys326-OGG1 to further investigate the basis of OGG1 BiFC fluorescence. As expected, BSO-induced BiFC fluorescence in Cys326-OGG1 cells but this was significantly reduced number of BiFC foci per cell after treatment with reducing agents (Figure 16A and Figure 16B). This further supports our hypothesis of the formation of a disulphide bond been responsible for OGG1 complex formation in cells. Cells treated with DMSO (a ROS scavenger) also show significant loss of fluorescence, which indicates importance of ROS levels in cells for generation of BiFC fluorescence (Figure 16).

A

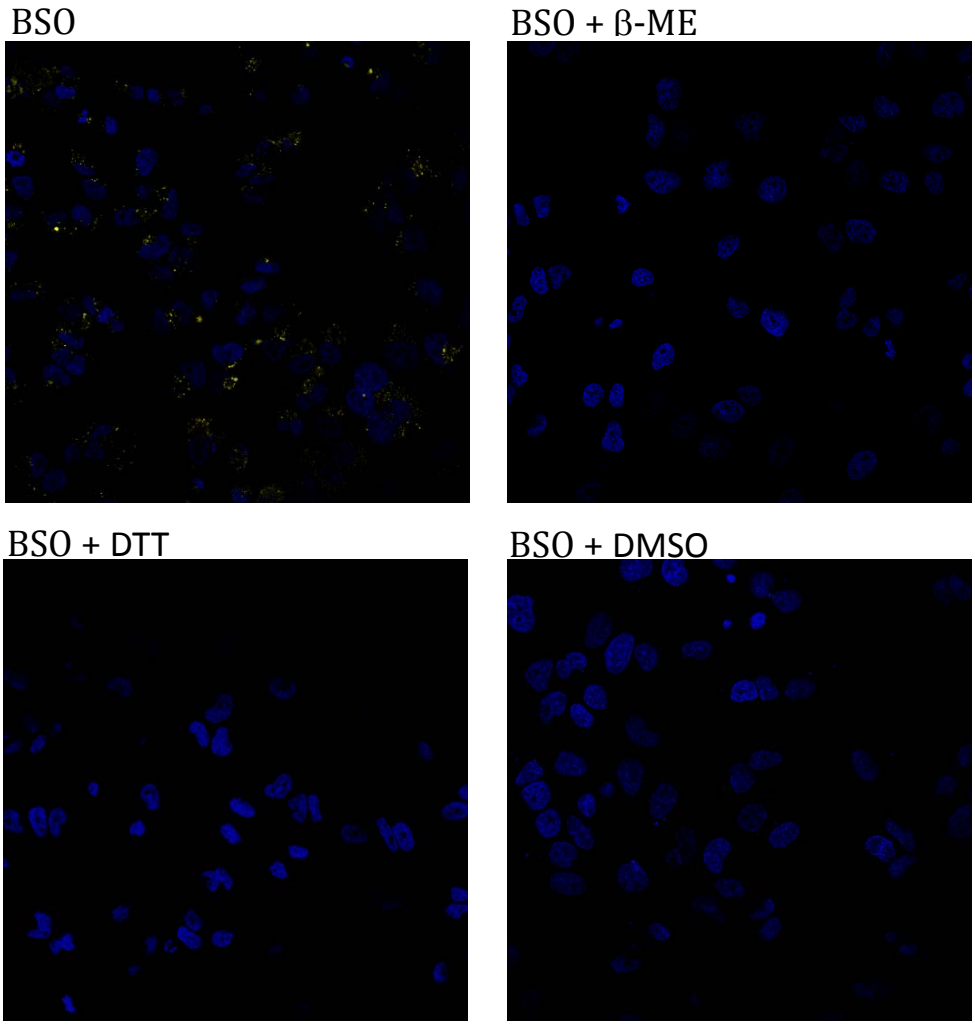


Figure 16: A) Confocal microscopy images of Cys326 OGG1 transfected cells treated with BSO for 24 hour followed by co-incubation with thiol reducing agents (1mM, DTT or β-ME) or anti oxidant DMSO (1%v/v) for final four hours of treatment. Loss of BiFC fluorescence was observed with all the above listed treatment.

B)

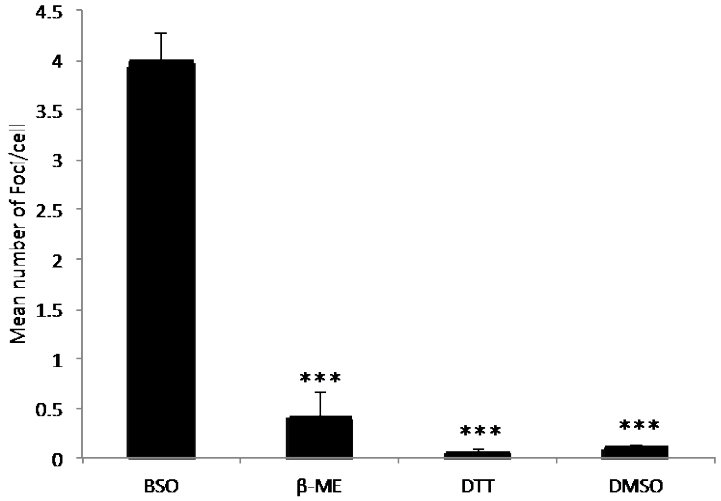


*Figure16B) Represents the bar graph of three different views of field and plotted as an average number of BiFC fluorescence foci per cell i.e. 57.3 ± 13.33. *** Which was statistically significantly different from BSO treated cells alone(±SD, n=3) (P < 0.001, t-test) (Figure taken from, Kaur et al 2014).*

4- DISCUSSION

In a previous study in our lab we have used an oligonucleotide molecular beacon, to visualise the location of OGG1 repair activity in peri-nuclear regions as well as the nucleus (Mirbahai et al 2010). Regulation of nuclear localization of OGG1 has been previously linked to phosphorylation of Ser326, with the Cys326 variant of OGG1 been excluded from the nucleolus during S-phase of the cell cycle (Luna et al 2005, Dantzer et al 2002, Campalans et al 2007 have shown that following UVA irradiation, OGG1 is relocated from the nuclear matrix to form complexes that co-localise in "nuclear speckles" and it has been suggested that they represent foci of OGG1 and other BER components at sites of active repair of oxidative DNA damage. Although similar to the fluorescence observed in the current study, localisation of OGG1 to nuclear speckles described by Campalans et al 2007 were observed in cells expressing wild type protein OGG1-GFP protein. In contrast, BiFC fluorescence was observed in the current study in cells expressing Cys326-OGG1 YFP vectors and only under conditions of oxidative stress.

BSO was used in this study to induce oxidative stress by depleting glutathione levels and increasing ROS levels in cells. The concentration of BSO used in this study (1000μM), has been shown to deplete glutathione and this leads to an increase ROS levels in cells compared to untreated cells. Flow cytometry analysis showed no statistically significant difference in BiFC fluorescence

between the control and BSO treated cells expressing YFP variants of OGG1. Although it was apparent that the levels of BiFC fluorescence observed in OGG transfected cells was substantially higher than in cells transfected with the control vector. This suggested that there was a specific interaction between OGG1 in cells and that dimer formation may well be important in regulating function and activity. One possible confounding factor that might explain the apparent lack of specificity in the flow cytometry data is difference in transfection efficiencies between the experiments. To try and control for this, cells were transfected together in a 60mm dish and later split into different well plates for treatment. The BiFC results for experiments controlling transfection efficiency were similar to experiments where cells were transfected in different wells (results not shown) suggesting that variation in transfection efficiency did not explain the failure to see treatment related effects. Furthermore, it was confirmed by the MTTassay that there was no cytotoxicity following treatment with1000μm BSO. A longer term aim could be to generate stable cell lines which will mitigate the possibility of the data being confounded by variations in transfection efficiencies. Furthermore, in contrast to confocal microscopy, flow cytometry measures the bulk fluorescence of a population of cells and if only a small proportion of the cells respond and exhibit BiFC fluorescence it may not be sensitive enough to detect the small change in fluorescence after treatment.

In contrast to flow cytometry, BiFC fluorescence as assessed by confocal microscopy in fixed cells showed that cells transfected with control YFP vectors or wild type OGG1-Ser 326 YFP vectors exhibited very little fluorescence either

before or after 24 hour BSO treatment. However, we report BiFC fluorescence specifically in cells expressing Cys326 OGG1-YFP only under conditions of oxidative stress. These results supported previous preliminary results seen before in our lab (NJH personal communication) and support the hypothesis of possible protein interactions between Cys326 OGG1 under oxidative stress conditions.

Interestingly, we observed BiFC fluorescence in the form of discreet foci within the nucleus as well as cytoplasmic suggesting that the BiFC fluorescence observed represents the accumulation of Cys326-OGG1 complexes at sites of active DNA repair but further work is required to confirm this. Regardless of the exact nature of Cys326-OGG1 at sites of nuclear BiFC fluorescence, in regions of active repair it seems likely that the concentration of OGG1 protein would be relatively high facilitating the formation of BiFC fluorescent complexes. Alternatively as discussed below, BiFC fluorescence may represent sites where oxidatively damaged Cys326-OGG1 has accumulated in the cell.

BiFC fluorescence foci were also seen outside of the nucleus in cells. It could suggest impaired nuclear localisation of the Cys326-OGG1 variant. Nevertheless, various possible explanations could explain this observation. Firstly, the BiFC fluorescence foci could be at site of protein synthesis in cells where redox sensitive Cys326 OGG1 complexes could have formed. On the other hand it could represent sites where inactive or mis-folded Cys326OGG1 could have been assigned for degradation. Previous studies support the second hypothesis suggesting possible evidence of base excision repair proteins being targeted for proteasomal degradation by ubiquitin ligases activity in cells.

(Parsons et al 2008, 2009, Meisenberg et al 2012) which could be a possible pathway to regulate OGG1 protein.

One possible explanation for the reduced repair activity of OGG1 under oxidative stress conditions could be a accumulation of degraded damaged protein and previous results in our lab have demonstrated reduced activity of OGG1 and protein expression levels using pro-oxidant treatment (sodium dichromate)(Hodges et al 2002).Reduced repair of damage by Cys326-OGG1 under conditions of oxidative stress could be associated with redox modification of the Cys326. Studies by Hill and Evans showed that purified Cys326OGG1 exists mainly as a homo-dimer, which is dependent on Cys326 for its formation.It has also been previously observed that Cys326-OGG1 possess a 2-4 times reduced DNA repair rate compared to wild type protein (Bravard et al 2009,2010, Hill et al 2006, Zielinska et al 2011,Dherin et al 1999) and this is consistent with the possible formation of dimer between Cys326 which could reduce OGG1 catalytic activity by up-to 2 fold due to reduced access to substrate.A recent mass spectrometry study proposes the possible formationof a redox sensitive disulfide bond between Cys28 and Cys326 under conditions of oxidative stress. It suggests altered protein folding under oxidative stress conditions which allows disulfide bond formation between Cys28 and Cys326 amino acids which is likely not possible in its native folded stage due to large bond distance between the residues. (Simonelli et al 2013).

It is possible that under conditions of oxidative stress that a inter-molecular Cys28-Cys326 disulfide bond could also form which would explain the

occurrence of discreet foci of fluorescence under oxidative stress conditions and these foci would signify accumulation of mis-folded protein which could account for OGG1 Cys 326 variant reduced repair capacity particularly under oxidative stress conditions (Lee et al 2005). Another possibility could be formation of an inter-molecular disulfide bond between two Cys326 resides and further work is required to distinguish these two possibilities.

BiFC fluorescence was seen only in cells expressing Cys326-OGG1 specifically under the conditions of oxidative stress following GSH depletion, which rely on the Cys326- OGG1 redox dependant complex formation. Various studies suggest that disulfide bond formation is dependent on the type of oxidant used to induce cellular oxidative stress (Cumming et al 2004). Therefore, the effects of another proxidant (hydrogen peroxide, a direct acting oxidant) on OGG1BiFC was also assessed.Hydrogen peroxide was shown to increase ROS levels but had no effect on GSH levels in cells. Interestingly cells treated with hydrogen peroxide show increased BiFC fluorescence which suggests that under these conditions at least, the BiFC fluorescence observed was more likely to be as a result of increase in ROS levels and direct modification of OGG1 protein rather than an indirect result of GSH depletion and subsequent protein thiol oxidation.To further investigate the mechanism of BSO-induced fluorescence observed in cells, DMSO, a ROS scavenger was added for the final four hours of incubation in cells.Little or no BiFC fluorescence as assessed by confocal microscopy was observed after the treatment of cells with BSO and DMSO. This suggests that ROS alone may be enough for the production of BiFC fluorescence

/ disulfide bond formation in OGG1 Cys326. DTT and β-ME are both strong thiol reducing agents, capable of reducing protein disulfide bonds in cells. To investigate whether the BiFC fluorescence observed in OGG1 Cys326 expressing cells was the result of disulfide bond formation cells were treated with either DTT or β-ME after 24-hour treatment of BSO to induce cellular stress and dimer formations.The results show that cells treated with both reducing agents, showed little or no BiFC fluorescence. This supports the hypothesis, for formation of a redox sensitive disulfide bond involving the OGG1 Cys326 variant under oxidative stress conditions and is in agreement with the previous studies of Hill and Evans in purified OGG1 protein (Hill and Evan 2006).

In summary, our findings demonstrate the accumulation of Cys326-OGG1 protein complexes in a redox dependant manner and provide evidence that this is dependent on ROS-induced modification of the Cys326 residue.Importantly this is first time that evidence for OGG1 complex formation has been observed in intact cells. Our study also provides a novel example of applying BiFC to study a component of base excision repair pathway.

Future work

This study provides evidence for the first time of the formation of cys326 OGG1 dimers in the nuclei of cells specifically under conditions of oxidative stress. Interesting we also report the existence if foci of fluorescence in cytoplasmicregions of the cell as well. It would be interesting to visualise OGG1 dimers in live cells by BiFC as this could be used to test whether formation is reversible and the kinetic relationship between nuclear and cytoplasmic foci of fluorescence in real time. In addition, mass spectrometry analysis could be undertaken to characterise the nature of the OGG1 complexes observed and investigate whether they are the result of either Cys326-Cys326 or Cys28-Cys326 disulfide bond formation. It would also be interesting to further investigate the nature of the cytoplasmic YFP-foci in the Cys 326-OGG1 variant. Specifically whether these represent impaired protein folding of OGG1 and targeting for proteosomal degradation for example by ubiquitinylation.

A more detailed understanding of regulation of OGG1 repair activity in cells by oxidative stress may have important implications in both understanding an individual susceptibility to disease such as cancer as well as potentially improving existing treatments. For example, a better understanding of regulation of DNA repair activity in cancer cells may allow therapeutic opportunities to improve the effectiveness of both radio- and chemotherapy.

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Appendix 1

List of Publications

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Cellular accumulation of Cys326-OGG1 protein complexes under conditions of oxidative stress

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ABSTRACT

The common Ser326Cys polymorphism in the base excision repair protein 8-oxoguanine glycosylase 1 is associated with a reduced capacity to repair oxidative DNA damage particularly under conditions of intracellular oxidative stress and there is evidence that Cys326-OGG1 homozygous individuals have increased susceptibility to specific cancer types. Indirect biochemical studies have shown that reduced repair capacity is related to OGG1 redox modification and also possibly OGG1 dimer formation. In the current study we have used bimolecular fluorescence complementation to study for the first time a component of the base excision repair pathway and applied it to visualise accumulation of Cys326-OGG1 protein complexes in the native cellular environment. Fluorescence was observed both within and around the cell nucleus, was shown to be specific to cells expressing Cys326-OGG1 and only occurred in cells under conditions of cellular oxidative stress following depletion of intracellular glutathione levels by treatment with buthionine sulphoximine. Furthermore, OGG1 complex formation was inhibited by incubation of cells with the thiol reducing agents β-mercaptoethanol and dithiothreitol and the antioxidant dimethylsulfoxide indicating a causative role for oxidative stress in the formation of OGG1 cellular complexes.

In conclusion, this study has provided for the first time evidence of redox sensitive Cys326-OGG1 protein accumulation in cells under conditions of intracellular oxidative stress that may be related to the previously reported reduced repair capacity of Cys326-OGG1 specifically under conditions of oxidative stress.

1. Introduction

Reactive oxygen species (ROS) are ubiquitous in the intracellular environment and are generated by numerous endogenous processes including, for example the mitochondrial electron transport chain, reviewed by Murphy [1]. In addition, exposure to both physical (e.g. ionising radiation) [2] and chemical agents (e.g. toxic metals) [3] can further increase ROS formation inside cells. DNA is susceptible to a range of oxidative modifications including oxidation of guanine to 7,8-dihydro-8-guanine (8-oxo G), which, if not repaired has the capacity to mis-pair during DNA replication resulting in the formation of GC to TA transversion mutations [4,5] and oxidative modifications to DNA are considered to contribute to the aetiology of a range of human pathological conditions including cancer [6–8]. The major pathway for the repair of 8-oxo G in genomic DNA is base excision repair (BER), which is initiated by the DNA glycosylase 8-oxoguanine glycosylase 1 (OGG1). The human OGG1 (*hOGG1*) gene is located on chromosome 3p26.2 [9], a region subject to deletion and loss of heterozygosity in human cancers. Furthermore, OGG1 knockout mice have been shown to accumulate elevated levels of 8-oxo G as they age and are more susceptible to a range of chemical stressors [10–14]. hOGG1 is polymorphic in the human population with a relatively common single nucleotide polymorphism – allele frequency of 0.22–0.27 in Caucasian populations – occurring at position 1245 in exon 7 resulting in a serine to cysteine amino acid substitution at position 326 (Ser326Cys) [15–17]. There is also evidence that post-translational modification to hOGG1 including: nitrosylation [18], phosphorylation [19–22], acetylation [23], ubiquitination [24] and redox modifications [25,26] modulate both the cellular localisation of hOGG1 and its catalytic activity and mechanistic studies focused on the Ser326Cys polymorphism indicate that the Cys326 variant is repair deficient, that this deficiency is enhanced under conditions of cellular oxidative stress [27] and that reduced repair activity may be related to oxidative modification of the Cys326 amino acid which resides in a positively charged sequence of amino acids (ADLRQ[ser326cys]RHAQ) rendering the thiol group labile to oxidation [28]. As part of these data, work by Hill and Evans [29] has

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demonstrated that purified Cys326 hOGG1 exists predominantly as a homo-dimer dependent on the Cys326 amino acid. Despite this, there is little if any direct evidence of OGG1 complex formation in cells and specifically how this is related to OGG1-genotype and to the cellular redox environment. Here we report the novel application of the imaging technique bimolecular fluorescence complementation (BiFC) to study OGG1 complex formation for the first time in the native cellular environment and show that this only occurs with the Cys326 variant of the protein and is specific to conditions of cellular oxidative stress.

2. Materials and methods

2.1. Preparation of BiFC vectors

Plasmids (pBluescript II KS-) containing either the N- or C-terminus of yellow fluorescent protein (YFP-N and YFP-C) were a generous gift from Dr. Saverio Brogna (University of Birmingham, B15 2TT). Full length Ser326- and Cys326-hOGG1 cDNA was amplified from *hOGG1* containing pcDNA3© plasmids [30] by PCR (Forward primer: 5′-GAGAGGATCCATGCCTGCCCGCGCGCTTCTG-3′ Reverse primer: 5′-GGCAGGATCCTTACTAGCCTTCCGGCCCTTTG-3′). Following overnight digestion with *BamHI* hOGG1 cDNA fragments were ligated into the pBluescript II vector in frame with YFP-N or YFP-C. Next hOGG1-YFP cDNA constructs were sub-cloned into the pcDNA3.1/Hygro© (+) mammalian expression vector (Invitrogen, USA) to generate: YFP-N-Ser326-hOGG1, YFP-C-Ser326-hOGG1, YFP-N-Cys326-hOGG1 and YFP-C-Cys326-hOGG1 constructs. Vectors containing only the YFP fragments were prepared in the same manner. All vectors were confirmed by sequencing and plasmids were propagated using standard protocols in super competent JM109 *Escherichia coli* (Promega, UK) with antibiotic selection (ampicillin 100 μg/ml). Plasmids were recovered from bacteria using a Qiagen plasmid mini-prep kit according to manufacturer's instructions.

2.2. Cell culture

A549 lung carcinoma cells (HPA catalogue number 86012804) were cultured at 37 °C in T_{75} flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v foetal calf serum, glutamine (2 mM), penicillin (100 μg/ml) and streptomycin (100 μg/ml). Cells were grown to approximately 80% confluence and passaged using trypsin–EDTA either into new T_{75} flasks or well culture plates for experimentation as appropriate.

2.3. Assessment of oxidative stress and GSH levels

Cells were cultured in 6-well plates to confluence before treatment with buthionine sulphoximine (BSO, 1000 μM) for 24 h. Following treatment dichlorodihydrofluoroscein-diacetate (H_2DCF-DA, final concentration 10 μM) was added and cells incubated at 37 °C in the dark for 60 min. Cells were washed with PBS (2 × 2 ml), trypsin (1 ml) added and cells incubated for 10 min at 37 °C. Next PBS (1 ml) was added and cells transferred to centrifuge tube (15 ml). Cells were pelleted by centrifugation at 1500*g* (10 min), the supernatant removed and the cell pellet re-suspended in PBS (2 ml) and transferred to flow cytometry tubes for flow cytometry analysis (FACScalibur, BD Biosciences, USA). For each treatment the FITC fluorescence (517–527 nm) of

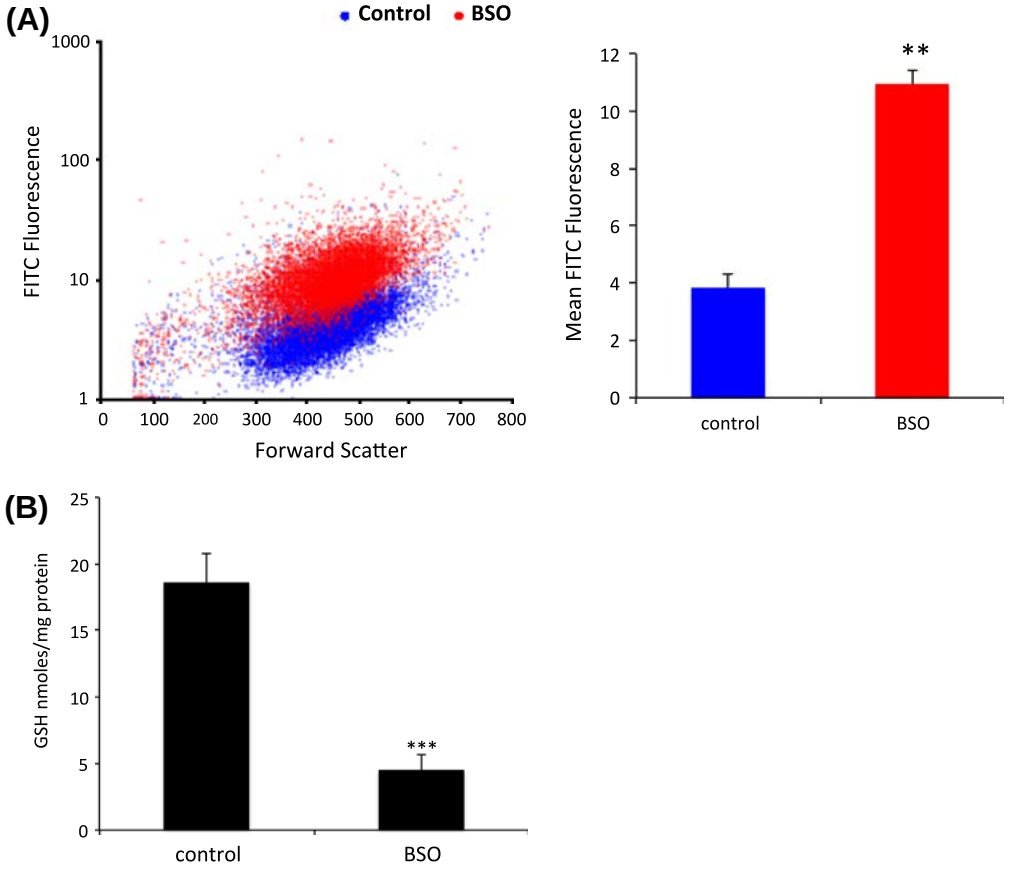

Fig. 1. (A) Induction of oxidative stress and (B) depletion of reduced glutathione (GSH) in A549 cells following treatment with BSO (1000 μM, 24 h). The results represent the mean of four independent experiments (±SD, $n = 4$) carried out in duplicate, *** and ** significantly different from controls (Student *t*-test), $P < 0.001$ and 0,01 respectively.

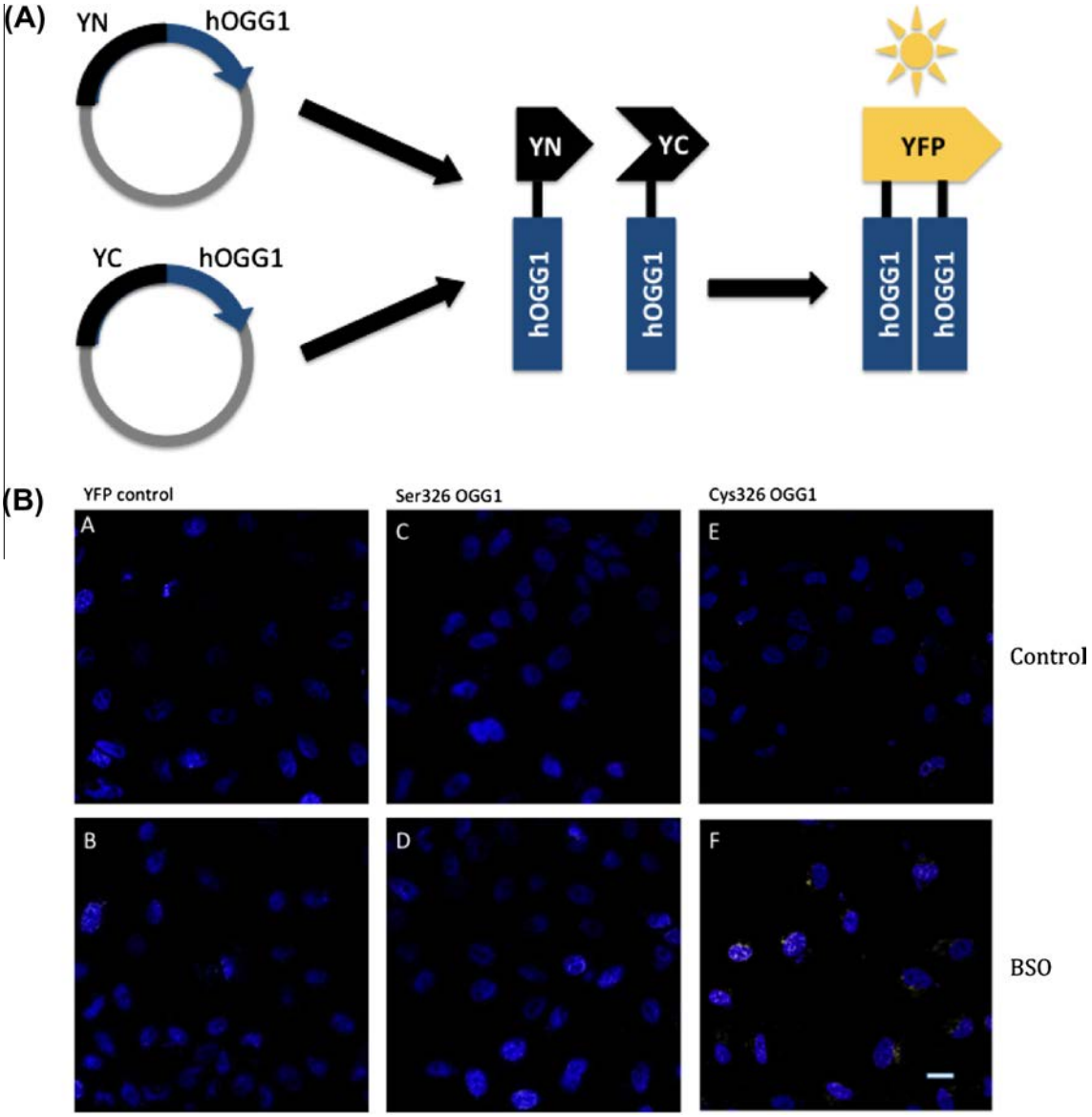

Fig. 2. (A) Principle of the BiFC assay: Cultured A549 cells were co-transfected with YC- and YN-YFP hOGG1 constructs. In the event of hOGG1 dimer formation the YC and YN fragments of YFP come into close physical proximity resulting in YFP fluorescence, which can be detected by confocal microscopy. (B) BiFC fluorescence is only observed in cells transfected with Cys326 N/C and only under conditions of oxidative cellular oxidative stress. (A) and (B) control vectors, (C) and (D) Ser326 N and C, (D) and (E) Cys326 N and C. (A), (C) and (E) untreated, (B), (D) and (E) treated with BSO (1000 μM, 24 h). Scale bar is 10 μm. In all images, nuclear DNA is counterstained with Hoechst 33258.

10,000 cells were quantified with unlabelled cells used as a blank to control for background fluorescence. Weasel software (Walter and Eliza Hall Institute of Medical Research, Australia) was used for the analysis of results and to calculate the mean fluorescence of each population of cells. Total reduced GSH was measured in cells using the method of Hissin and Hilf [31], as described previously [32].

2.4. Transfection of cells with GFP-OGG1

A549 cells were cultured on sterile round 13 mm glass coverslips (PAA Laboratories Ltd.) until approximately 65% confluent before being transfected with OGG-GFP constructs [33] using Turbofect (Thermo-Fisher Scientific Inc.) according to the manufactures instructions. 48 h later cells were fixed and analysed by confocal microscopy and flow cytometry to confirm location of OGG1-GFP and transfection efficiency respectively.

2.5. Detection of BiFC fluorescence

Cells were cultured on sterile round 13 mm glass coverslips as above. At approximately 65% confluence cells were transfected with pairs of BiFC constructs (4 μg each) described above using Turbofect according to the manufacturers instructions (Thermo-Fisher Scientific Inc.), 24 h after transfection cells were treated with BSO (1000 μM, 24 h). In some experiments the disulphide reducing agents β-mercaptoethanol (1 mM) or dithiothreitol (1 mM) were added for the final four hours of incubation. In other experiments cells were co-incubated with BSO (1000 μM) and the antioxidant DMSO (1% v/v). Following treatment the media was removed, cells washed with PBS (2 ml) and fixed at room temperature (15 min) with neutral buffered formalin (10%, pH 7.4, 2 ml). Following further washing with PBS (2 × 1 ml) cells were counterstaining with 1 ml of PBS containing Hoechst 33258(0.6 μM, Invitrogen, UK) for 5 min, washed again with PBS (2 × 1 ml) before been

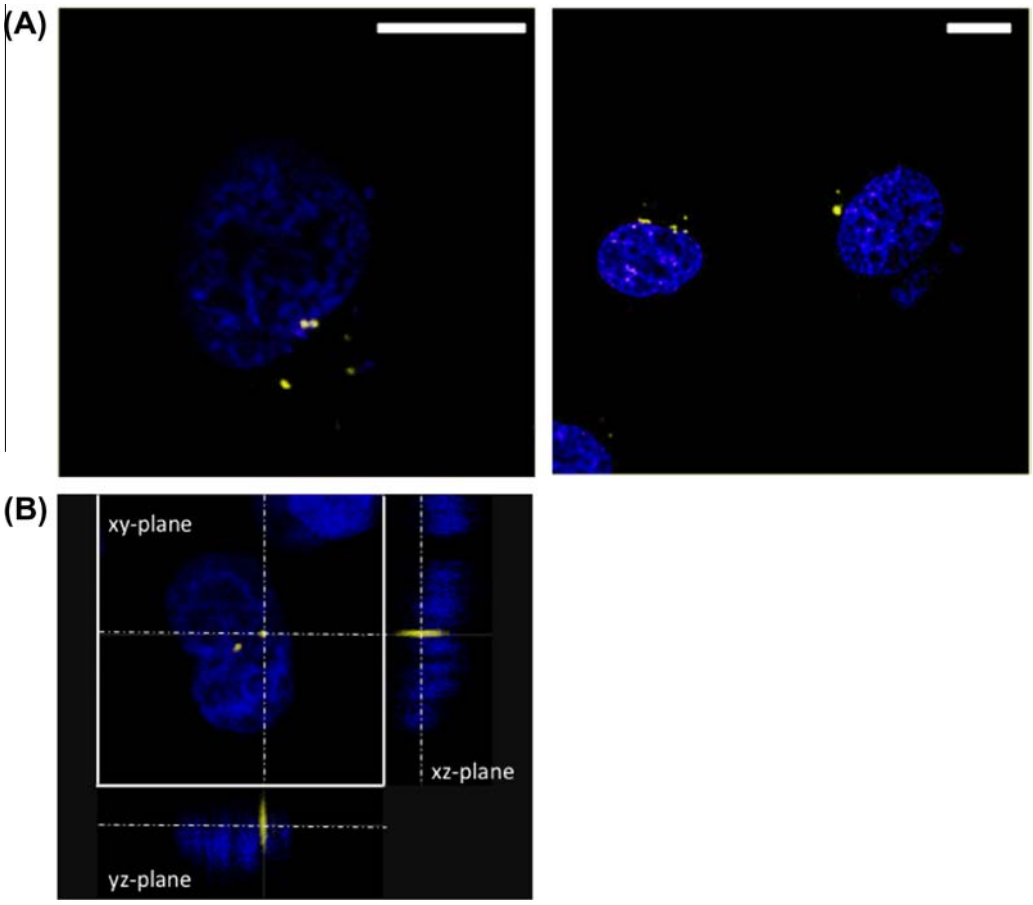

Fig. 3. (A) Higher magnification representative images of BiFC fluorescence in Cys326 N/C transfected cells following treatment with BSO (1000 μM, 24 h). Scale bar is 10 μm, nuclear DNA is counterstained with Hoechst 33258. (B) Three-dimensional distribution of yellow BiFC fluorescence in the nucleus of a cell transfected with Cys326-OGG1 vectors following treatment with BSO (1000 μM, 24 h). Nuclei are counterstained with Hoechst 33258.

mounted onto a glass microscope slide with Hydromount Vecta Mountant (approximately 50 μl, National Diagnostics, UK). Excess mountant was carefully removed by dabbing with a paper towel and slides were stored at 4 °C in the dark for a maximum period of 48 h prior to analysis by confocal microscopy. Image acquisition was performed using a Leica TCS SP2 confocal microscope (Leica Microsystems) using an oil immersion objective (63x). Fluorophores were excited using a 488 nm laser for YFP and 405 nm laser for Hoechst 33258. Images were analysed using Leicalite software and post-processing was carried out using Adobe Photoshop CS5 extended.

3. Results

3.1. Confirmation of oxidative stress

As expected and consistent with previous studies, treatment of A549 cells with BSO (1000 μM) for 24 h resulted in a statistically significant ($P < 0.01$) induction of reactive oxygen species (Fig. 1A) as assessed by the oxidation of DCF as well as depletion of glutathione (Fig. 1B) in the absence of cytotoxicity as assessed by the MTT assay (data not shown). To confirm nuclear localisation of OGG protein in this cell line, cells were transfected with GFP-tagged proteins and imaged by confocal microscopy. OGG-GFP was found to be nuclear located (Fig. S1) and consistent with previous studies in MEF cells [34], the nuclear sub-localisation of OGG1 was not influenced by OGG1 genotype and was not affected by treatment with BSO (1000 μM) for 24 h.

3.2. Oxidative stress induces BiFC fluorescence only in cells expressing Cys326-OGG1

BiFC is a powerful technique for the visualisation of direct protein–protein interactions (Fig. 2A). To investigate possible OGG1 protein complex formation, A459 cells were transfected with either Ser326-OGG1 half YFP vectors, Cys326 half YFP vectors or YFP only half vectors as a control prior to treatment with 1000 μM BSO (24 h) to induce oxidative stress and deplete intracellular GSH before BiFC fluorescence was assessed by confocal microscopy. Little to no BiFC fluorescence was observed in YFP control or Ser326-OGG1 transfected cells either before or after treatment with BSO (1000 μM, Fig. 2B panels A, B and C, D). In contrast, although there was no evidence of BiFC fluorescence in cells transfected with Cys326-OGG1 in the absence of treatment with BSO (Fig. 2B panel E) there was clear BiFC fluorescence following treatment with BSO (1000 μM, 24 h, Fig. 2B panel F). BiFC fluorescence appeared as discrete foci that were mainly located to the nucleus as mapped by counterstaining with the nuclear stain Hoescht 33258. YFP fluorescence was also apparent in peri-nuclear regions; this is shown at higher magnification in Fig. 3A. A similar pattern of fluorescence was observed when cells were treated with the pro-oxidant hydrogen peroxide (data not shown). The presence of BiFC fluorescence inside of the nucleus was confirmed by a z-scan that clearly shows yellow BiFC fluorescence staining in the same plane as the Hoechst 33258 counterstained nucleus (Fig. 3B). Co-transfection experiments with YFP-N-Cys326-hOGG1 and YFP-C-Ser326-hOGG1 or YFP-N-Ser326-hOGG1 and YFP-C-Cys326-hOGG1 constructs did

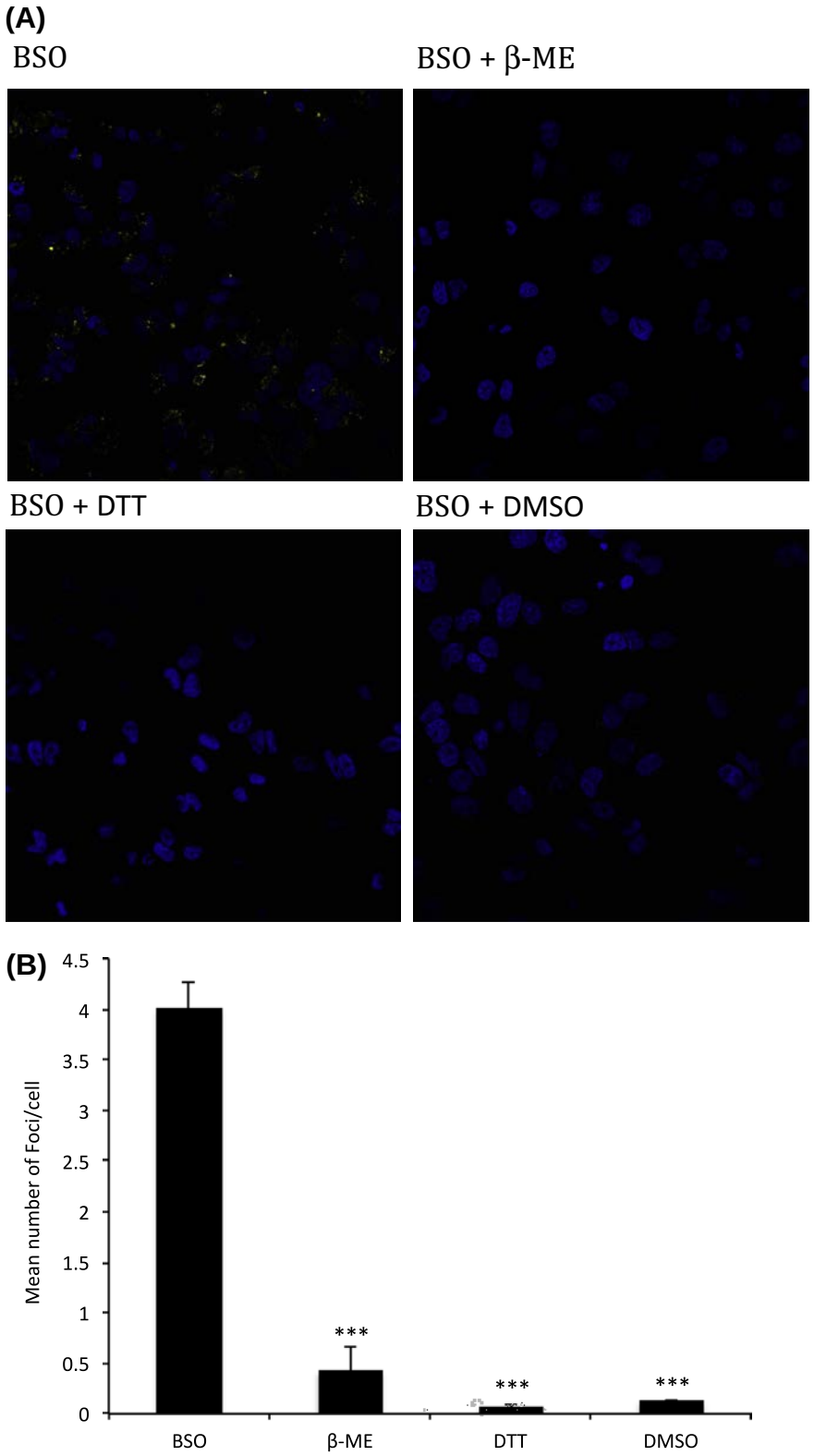

Fig. 4. (A) Inhibition of BiFC fluorescence in Cys326 OGG1 transfected cells treated with BSO (1000 μM), 24 h after co-incubation with the antioxidant DMSO (1% v/v) or addition of the disulphide reducing agents β-mercaptoethanol (1 mM) of dithiothreitol (1 mM) for the final four hours of incubation. (B) The bar graph represents the average number of BiFC fluorescent foci per cell ± SD for three independent fields of view; the average number of cells per field was 57.3 ± 13.33. *** Significantly different from BSO treated cells alone ($P < 0.001$, *t*-test).

not result in any BiFC fluorescence (data not shown) suggesting that BiFC fluorescence was entirely dependent on Cys326-OGG1 protein and did not involve an interaction between Ser326-OGG1 and Cys326-OGG1.

3.3. Antioxidants inhibit BiFC fluorescence

To further investigate the role of disulphide bond formation in the observed BiFC fluorescence in Cys326-OGG1 expressing cells following oxidative stress cells, the disulphide reducing agents β-mercaptoethanol and dithiothreitol were added for the final four hours of culture. Under these experimental conditions the number of BSO-induced fluorescence foci per cell was significantly reduced (Fig. 4A and B), strongly supporting our hypothesis that OGG1 complex formation within cells is dependent on the formation of a disulphide bond. Furthermore, co-incubation of cells with BSO and the antioxidant DMSO for 24 h also inhibited BiFC fluorescence suggesting that ROS generation is also important in the generation of BiFC fluorescence (Fig. 4A and B).

4. Discussion

Regulation of nuclear localisation of OGG1 has been putatively linked to phosphorylation of Ser326, with the Cys326 variant of OGG1 apparently excluded from the nucleolus during S-phase of the cell cycle [19,21]. Campalans et al. [35] have shown that following UVA irradiation, OGG1 is relocated from the nuclear matrix to form complexes that co-localise in "nuclear speckles" and it has been suggested that they represent foci of OGG1 and other BER components at sites of active repair of oxidative DNA damage. Although similar to the fluorescence observed in the current study, localisation of OGG1 to nuclear speckles described by Campalans et al. [35] were observed in cells expressing wild type OGG1-GFP protein. In contrast, we report BiFC fluorescence specifically in cells expressing Cys326 OGG1-YFP only under conditions of oxidative stress. Interestingly, we observed BiFC fluorescence in the form of discreet foci within the nucleus suggesting that the BiFC fluorescence observed represents the accumulation of Cys326-OGG1 complexes at sites of active DNA repair but further work is required to confirm this. Regardless of the exact nature of Cys326-OGG1 at sites of nuclear BiFC fluorescence, in regions of active repair it seems likely that the concentration of OGG1 protein would be relatively high facilitating the formation of BiFC fluorescent complexes of OGG1. Alternatively as discussed below, BiFC fluorescence may represent sites where oxidatively damaged Cys326-OGG1 has accumulated in the cell.

Interestingly, BiFC fluorescent foci were also present outside of the nucleus of cells. There are several possible explanations that could account for this observation – it may represent redox sensitive Cys326-OGG1 complex formation at sites of protein synthesis in the cell. Alternatively it could represent accumulation of misfolded or inactive Cys326-OGG1 protein that has been targeted for degradation. In support of this second hypothesis there is emerging evidence that base excision repair proteins are regulated and targeted for proteasome-mediated degradation by the activity of ubiquitin ligases [36–38] and it is possible that similar pathways may act to regulate OGG1 protein. Increased turnover and degradation of damaged Cys326-OGG1 protein would account for the reduced repair activity of this form of OGG1 observed under conditions of oxidative stress and we have observed previously that treatment of cells with the pro-oxidant sodium dichromate results in reduced levels of OGG1 protein expression and activity [39].

At the molecular level there is emerging evidence that reduced activity of Cys326-OGG1 under oxidative conditions may be related to redox modification of the Cys326 amino acid. Hill and Evans [29] have shown that purified Cys326-OGG1 exists predominantly as a homo-dimer whose formation is dependent on the short c-terminal loop containing the Cys326 residue. Studies in cellular systems indicate that DNA repair rates of Cys326-OGG1 are reduced 2–4-fold compared to wild type protein [25,29,33,40,41] and the existence of a Cys326-OGG1 dimer might be expected to reduce catalytic activity by approximately 2-fold because only one protein of each dimer would have access to the substrate at a time. A recent study [42] has utilised mass spectrometry to identify the formation of redox sensitive disulphide bond formation in Cys326-OGG1 involving Cys28 and Cys326 amino acids but conclude that this is only possible under conditions where folding of the protein is altered because the bond distance between Cys326 and Cys28 residues is too large to form a intra-molecular disulphide bridge when OGG1 is in its native folded state. It is possible that under conditions of oxidative stress Cys326-OGG1 becomes at least partially unfolded and an intra-molecular Cys28-Ser326 disulphide bond is formed. This would explain the occurrence of discreet foci of fluorescence, which may represent accumulation of mis-folded Cys326-OGG1 under conditions of oxidative stress and would also explain the reduced repair capacity of Cys326-OGG1 specifically under conditions of oxidative stress observed previously [27]. It is also possible that an inter-molecular disulphide bridge between two Cys326 residues may be formed in cellular environments and that the BiFC fluorescence observed represents Cys326-dependent OGG1 homo-dimer formation. Consistent with a role for redox dependent Cys326-OGG1 complex formation, BiFC fluorescence was specifically observed in cells expressing Cys326-OGG1 and only under conditions of oxidative stress and GSH depletion. The observation that incubating cells with disulphide reducing agents inhibits BSO-induced BiFC fluorescence also strongly supports the hypothesis that Cys326-OGG1 complex formation in cells is dependent on the formation of a redox sensitive disulphide bond possibly between two Cys326 amino acids and is in agreement with the previous studies of Hill and Evans in purified OGG1 protein [29]. Furthermore, the observation that BSO-induced BiFC was also inhibited by co-incubation with the antioxidant DMSO appears to confirm that BSO-mediated generation of ROS is also important for Cys326-OGG1 BiFC fluorescence. In conclusion, this study has demonstrated for the first time the application of BiFC to study a component of the BER pathway and provided further insight into the molecular mechanisms of the reduced repair capacity Cys326-OGG1. The novel finding of redox dependent Cys326-OGG1 complex accumulation and possible dimer formation within the native cellular environment provides further support to an increasing body of data implicating redox modification and disulphide dimer formation in the mechanism of the reduced repair capacity of Cys326-OGG1 observed.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.044.

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