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Dynamic relocalization of hOGG1 during the cell cycle is disrupted in cells harbouring the hOGG1-Cys^{326} polymorphic variant

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ABSTRACT

Numerous lines of evidence support the role of oxidative stress in different types of cancer. A major DNA lesion, 8-oxo-7,8-dihydroguanine (8-oxoG), is formed by reactive oxygen species in the genome under physiological conditions. 8-OxoG is strongly mutagenic, generating G·C→T·A transversions, a frequent somatic mutation in cancers. hOGG1 was cloned as a gene encoding a DNA glycosylase that specifically recognizes and removes 8-oxoG from 8-oxoG:C base pairs and suppresses G·C→T·A transversions. In this study, we investigated the subcellular localization and expression of hOGG1 during the cell cycle. Northern blots showed cell-cycle-dependent mRNA expression of the two major hOGG1 isoforms. By using a cell line constitutively expressing hOGG1 fused to enhanced green fluorescence protein (EGFP), we observed a dynamic relocalization of EGFP-hOGG1 to the nucleoli during the S-phase of the cell cycle, and this localization was shown to be linked to transcription. A C/G change that results in an amino acid substitution from serine to cysteine in codon 326 has been reported as a genetic polymorphism and a risk allele for a variety of cancers. We investigated the cellular localization of the corresponding protein, hOGG1-Cys^{326}, fused to EGFP and observed a dramatic effect on its localization that is explained by a change in the phosphorylation status of hOGG1.

INTRODUCTION

Oxidative damage to DNA is generated by reactive oxygen species that arise both endogenously and from exposure to environmental mutagens or ionizing radiation. Oxidative DNA damage has been implicated in a number of pathophysiological processes, including degenerative diseases, cancer and ageing (1–6). 7,8-Dihydro-8-oxoguanine (8-oxoG) is one of the most important oxidative lesions produced, which has a highly mutagenic effect owing to its bias to mispair with A residues thereby generating G·C→T·A transversion mutations in repair-deficient bacterial and yeast cells (7–10). 8-OxoG is mainly repaired by the base excision repair (BER) mechanism. BER is a multistep process that involves the sequential activity of several proteins, initiated by a DNA glycosylase that recognizes and removes the damaged base (11,12). The human *OGG1* gene encodes a DNA glycosylase/AP-lyase that is responsible for removing 8-oxoG from 8-oxoG:C base pairs in double-stranded DNA. The hOGG1 enzyme has been thoroughly characterized and the crystal structure of the catalytic core of hOGG1 bound to 8-oxoG:C containing DNA as well as the native enzyme have been solved (13–18). The gene is expressed in at least 12 different alternative spliced forms, with products being directed both to the nucleus and to the mitochondria (14,19–23).

High levels of 8-oxoG have been reported in several kinds of human cancer tissues, including lung, renal, breast and colorectal carcinomas, as compared with their non-tumorous counterparts (24–27). Accumulation of up to 7-fold higher levels of 8-oxoG and an increase in spontaneous mutation frequency has been reported for two independently obtained *OGG1*$^{-/-}$ mice (28,29), Furthermore, a predisposition towards the development of lung adenoma/carcinoma has been demonstrated for

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the *OGG1*$^{-/-}$ mice (30). A report on the ability of hOGG1 to suppress G·C→T·A transversions in human cells *in vivo* strengthens the idea that hOGG1 is a critical factor in preventing mutations in human cells (31). Therefore, defects in hOGG1 that affect its repair capability might have an involvement in carcinogenesis by enhancing the mutation rates of key genes, such as oncogenes or tumour suppressor genes.

Among several *HOGG1* alleles described, a C/G polymorphism at position 1245 in exon 7 of the *HOGG1* gene results in the substitution of a serine for a cysteine in codon 326. Among several studies carried out to determine whether hOGG1 is a risk factor for several cancer types (32–45), few have indicated that the hOGG1-Cys^{326} might be a risk allele for a variety of cancers (38–40,43–45). Ambiguous results have been obtained on the effect of such an amino acid change on the DNA repair activity of the resulting mutant hOGG1-Cys^{326} protein compared with the wild-type hOGG1 protein. On the basis of an *Escherichia coli* complementation assay, one study showed that the hOGG1 protein encoded by the wild-type allele exhibited substantially higher DNA repair activity than the hOGG1-Cys^{326} variant (20), while a second study showed that both forms of the enzyme played an equal role (33). Furthermore, no differences in catalytic activities were observed in two independent studies (33,46).

Although the biochemical properties of hOGG1 are well defined, the biological role of *OGG1* in mammalian cells and its possible involvement in carcinogenesis is still poorly understood. In order to elucidate the cellular role of hOGG1 we have investigated both the expression of hOGG1 and its cellular distribution. Using enhanced green fluorescent protein (EGFP) as a reporter gene we show that hOGG1 is localized in the nucleoli during the S-phase of the cell cycle. Furthermore, the nucleolar localization of EGFP-hOGG1 is linked to transcription. We also investigated the localization of the polymorphic form of hOGG1 harbouring the serine to cysteine mutation at codon 326 and show that there is a dramatic change on the subcellular localization of the mutant protein as it is transported to the nucleus but is excluded from the nucleoli. We have previously shown that hOGG1 is associated with the soluble chromatin and the nuclear matrix during interphase and with condensed chromatin during mitosis (47). These associations are also disrupted in the mutant protein. Interestingly, this serine residue lies within a predicted site of potential phosphorylation, close to the nuclear localization signal. Finally, by mimicking a hOGG1 protein phosphorylated at Ser-326 we demonstrate that the regulation of the nucleolar localization, soluble chromatin, nuclear matrix and condensed chromatin association of hOGG1 are mediated through the phosphorylation of Ser-326.

MATERIALS AND METHODS

Synchronization of HaCat cells, mRNA isolation and northern blot hybridization were carried out as described previously (48).

Site-directed mutagenesis of hOGG1

Full-length hOGG1 cDNA cloned into pEGFP-N1 vector (pHOGH1-EGFP-12) as described previously (14), represents the wild-type hOGG1 construct, referred to as pEGFP-hOGG1. The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce the Ser^{326}Cys (pEGFP-hOGG1-Cys^{326}), the Ser^{326}Ala (pEGFP-hOGG1-Ala^{326}) and the Ser^{326}Glu (pEGFP-hOGG1-Glu^{326}) mutations with pHOGH1-EGFP-12 construct as the parental vector according to the manufacturer's protocol. The cloned fragments were sequenced and the desired mutations were thus verified. The pEYFP-hOGG1 plasmid was constructed by ligating the hOGG1 cDNA fragment from pEGFP-hOGG1 into the pEYFP-N1 vector. The pECFP-PCNA and the HcRed-PCNA are described in (49) and (50), respectively.

Cell culture, DNA transfection and selection of stable cell lines

HeLa S3 cells were cultivated in DMEM with 4.5 g/l glucose, 10% FCS, 0.3 mg/ml glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a 5% CO_2 air atmosphere. Transfections of HeLa S3 cells were performed using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals) according to the manufacturer's recommendations. To generate stable cell lines, 8×10^5 HeLa S3 cells were plated in 100 mm culture dishes. After overnight incubation, cells were transfected with pEGFP-hOGG1, pEGFP-hOGG1-Cys^{326}, pEGFP-hOGG1-Glu^{326} and pEGFP-N1. After 2 days, each plate was subcultured into six 100 mm culture dishes. Cells were grown overnight and 800 μg/ml geneticin was added. Single green fluorescent clones were isolated after 20 days.

Nuclear protein extracts, western blot and assay for faPy DNA glycosylase activity

Nuclear extracts from HeLa-EGFP, HeLa-EGFP-hOGG1, HeLa-EGFP-hOGG1-Cys^{326} and pEGFP-hOGG1-Glu^{326} were prepared as described previously (28). For western blot analysis, 20 μg nuclear protein extracts were separated by SDS–PAGE and transferred onto a nitrocellulose membrane. To detect EGFP and EGFP-hOGG1 (and the mutants) the membrane was probed with a polyclonal anti-GFP antibody (ab290-50; Abcam), and to detect EGFP-hOGG1 (and the mutants) a monoclonal anti-human OGG1 antibody (clone 7E2; IBL) was used. FaPy DNA glycosylase assay using increasing amounts of nuclear extracts was carried out as described previously (48).

Cell-cycle synchronization, flow cytometry and immunofluorescence

HeLa-EGFP-hOGG1 and HeLa-EGFP were synchronized at the G_1/S boundary by two cycles of thymidine blockage and at the G_2/M boundary by nocodazole treatment. Briefly, exponentially growing cells were blocked for 16 h in 2 mM thymidine containing DMEM, released for 9 h in thymidine-free DMEM and blocked again with 2 mM thymidine containing DMEM for another 16 h. Synchronization at G_2/M was achieved by culturing the cells with nocodazole (40 ng/ml) for 16 h. After the double-thymidine block and the removal of nocodazole, the cells were allowed to progress under normal conditions and harvested at different time points after release. The cell-cycle phase distribution of a cell population was determined by measuring cell DNA contents by flow cytometry as follows: cells (1×10^6) were trypsinized, washed and fixed in 75% ice-cold ethanol and stored at 4°C. Immediately before flow cytometric analysis, the samples were

treated with RNase A for 30 min at room temperature and then stained with propidium iodide (final concentration 50 μg/ml) for 30 min at 37°C. The samples were analysed by using a BD LSR flow cytometer (Becton Dickinson, NJ). For immunofluorescence, cells were grown on glass coverslips and fixed with 4% paraformaldehyde for 20 min at room temperature at different time points after release. Cellular DNA was stained with 0.25 μg/ml 4′,6′-diamino-2-phenylindole (DAPI) for 10 min at room temperature. Coverslips were mounted with fluorescent mounting medium (DAKO) and visualized using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss, Jena, Germany).

Immunostaining and confocal analysis

DNA labelling and staining with 5-bromo-2′-deoxyuridine (BrdU labelling and detection kit I; Boehringer Mannheim, Germany) was performed according to the manufacturer's instructions. The nucleoli were detected with an anti-nucleolin antibody (clone 3G4B2; Upstate biotechnology, Lake Placid, NY) on cells fixed in 2% formalin for 10 min followed by permeabilization with ice-cold methanol for 30 min. The secondary antibody used for both BrdU and nucleoli staining was a rhodamine (tetra-methyl)-conjugated goat anti-mouse antibody (T-2762) from Molecular Probe (Eugene, OR). The cells were examined in a Zeiss LSM 510 laser scanning microscope equipped with a Plan-Apochromate 63×/1.4 oil immersion objective. We used the 488 nm laser line for excitation of EGFP (detected at 505 nm < λ_{EGFP} < 530 nm) and the 543 nm laser line for rhodamine (tetra-methyl) (detected at $\lambda_{Rhodamine}$ > 560 nm). ECFP fusion protein was excited with a 458 nm laser line (detected at 480 nm < λ_{ECFP} < 520 nm), EYFP fusion protein was excited with a 514 nm laser line (λ_{EGFP} > 560 nm) and HcRed fusion protein was excited with a 543 nm laser line and detected at >585 or 650 nm. The images were from 1 μm thick slices of the cells. The images were exported into Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Actinomycin D treatment

For actinomycin D treatment, HeLa-EGFP-hOGG1 cells were grown on chamber slides and synchronized at the G_1/S boundary by two cycles of thymidine blockage. Cells were allowed to enter S-phase and actinomycin D was added 1 h after block release and incubated for 4 and 5 h, respectively. Cells were then washed twice with phosphate-buffered saline (PBS), fixed with 96% ethanol (anti-nucleolin) for 10 min at room temperature or 4% PFA (anti-fibrillarin and anti-RPA135) for 20 min and permeabilized with 0.5% Triton X-100 for 5 min at 4°C and washed again with PBS. Cells were air-dried before incubation with primary and secondary antibodies diluted in PBS with 1% BSA. As primary antibody monoclonal anti-nucleolin (clone 3G4B2; Upstate Biotechnology), RPA40 (N-20) (sc-17700; Santa Cruz), RPA135 (N-17) (sc-17913; Santa Cruz) and anti-fibrillarin (AFB01, cytoskeleton) were used and incubated overnight at room temperature. Incubation with secondary antibody (Alexa 594, 590/617; Molecular Probes) was carried out for 30 min at room temperature. DNA was stained with DAPI for 10 min at room temperature. Slides were mounted using the DAKO fluorescence mounting medium and examined using a Zeiss Axioplan 2 fluorescence microscope. Images were obtained using CoolSNAP (Photometrics) and then processed for publication using Jasc Pain Shop Pro (Jasc software).

Nuclear matrix preparation

Nuclear matrix proteins were fractionated from the indicated cells as described previously (47). For western blot analyses, equal cell equivalents from each fraction were subjected to 8% SDS–PAGE and probed with appropriate antibodies: rabbit polyclonal anti-GFP antibody (ab 290-50; Abcam), goat polyclonal anti-lamin A/C antibody (N-18; Santa Cruz), goat polyclonal anti-histone H1 antibody (C-17; Santa Cruz), monoclonal anti-human OGG1 antibody (clone 7E2; IBL) and rabbit polyclonal anti-phosphoserine antibody (poly-Z-PS1; Zymed Laboratories).

RESULTS

Cell-cycle-dependent expression of *HOGG1* gene

The cell-cycle-dependent expression of *HOGG1* was followed by northern blot hybridization of poly(A)$^+$ RNA isolated from synchronized HaCat cells. Progression throughout the cell cycle was monitored by [^{3}H]thymidine incorporation, which started to increase 12 h after serum addition and reached a peak at 25 h (Figure 1A). Northern blots revealed two mRNA bands of ~2.0 and 2.4 kb, representing the transcripts of the

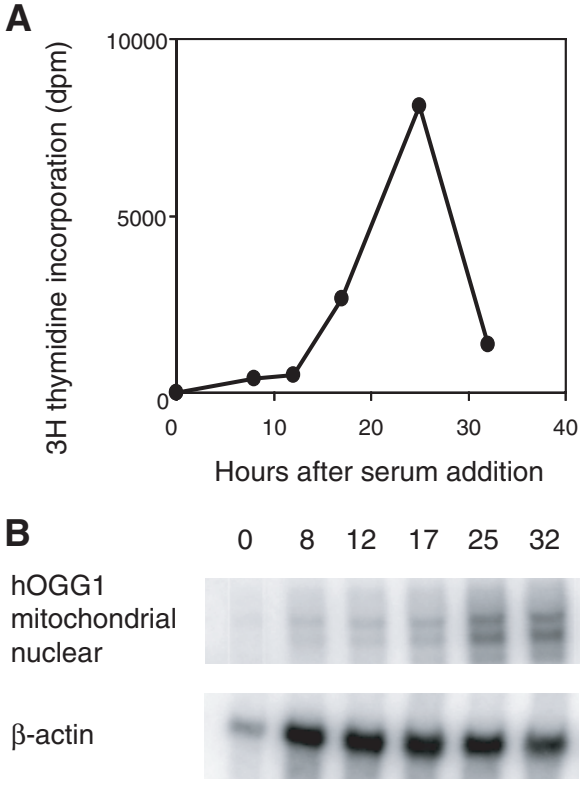

Figure 1. Cell-cycle-dependent expression of hOGG1 mRNA in synchronized HaCat cells. DNA synthesis and mRNA levels were measured at 0, 8, 12, 17, 25 and 32 h after serum addition to serum-starved HaCat cells. (**A**) Incorporation of ^{3}H-labelled thymidine during 25 min incubation. (**B**) Northern blot analysis of hOGG1 and β-actin expression as indicated.

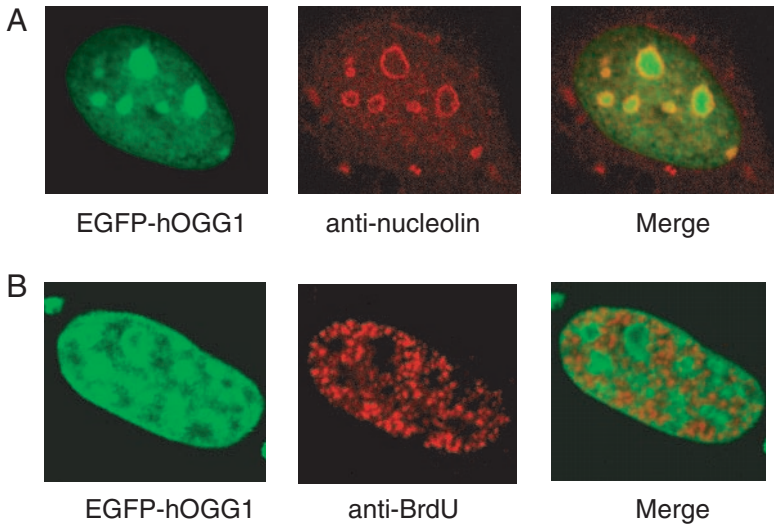

Figure 2. Confocal laser scanning microscopy of EGFP-hOGG1. HeLa cells were transiently transfected with pEGFP-hOGG1 and fixed 24 h after transfection. (**A**) Nucleoli were detected by staining with anti-nucleolin. Merged images show the localization of EGFP-hOGG1 in the nucleoli. (**B**) Replication foci were visualized by pulsing the cells with BrdU. Merged images show that EGFP-hOGG1 is localized in the nucleoli during S-phase.

nuclear and mitochondrial isoforms, respectively (Figure 1B). Expression of both isoforms increased during S-phase and into G_2.

hOGG1 is sorted to the nucleoli during S-phase

Studies of transiently transfected HeLa cells with the reporter protein EGFP fused to the C-terminal of hOGG1 have previously shown that the enzyme is transported to the nucleus (14). These studies revealed a heterogeneous population of cells displaying either homogeneous or reminiscent nucleolar localization of hOGG1. To confirm the nucleoli localization of EGFP-hOGG1, cells were fixed and nucleoli were visualized by confocal microscopy using antibodies against nucleolin. When the signals from EGFP-hOGG1 and anti-nucleolin were merged, EGFP-hOGG1 was found to be concentrated predominantly within the nucleoli as shown in Figure 2A. Furthermore, we established stable HeLa cell lines carrying either EGFP alone or EGFP-hOGG1 fusion proteins to further study the nuclear sublocalization of hOGG1 during the cell cycle. Overexpression of EGFP and EGFP-hOGG1 in the stable cell lines was examined in nuclear extracts by western blot using anti-OGG1 and anti-GFP specific antibodies. EGFP (30 kDa), EGFP-hOGG1, EGFP-hOGG1-Cys^{326} and EGFP-hOGG1-Glu^{326} (68 kDa) were recognized by the anti-GFP specific antibody (Figure 3A) while only EGFP-hOGG1, EGFP-hOGG1-Cys^{326} and EGFP-hOGG1-Glu^{326} (68 kDa) were recognized by the anti-OGG1 specific antibody (Figure 3B). Nuclear extracts from HeLa-EGFP and HeLa-EGFP-hOGG1 were tested for faPy DNA glycosylase activity to demonstrate the functionality of the fusion protein product. Results showed that hOGG1 fused to the EGFP was catalytically active (Figure 3C).

Cells were synchronized by nocodazole, which causes the cells to arrest at the G_2/M boundary, or by the double-thymidine block, which arrest the cells at G_1/S boundary. Cell samples were taken at different time points after release and cell-cycle progression was followed by flow cytometry. Samples were fixed and examined under a fluorescence

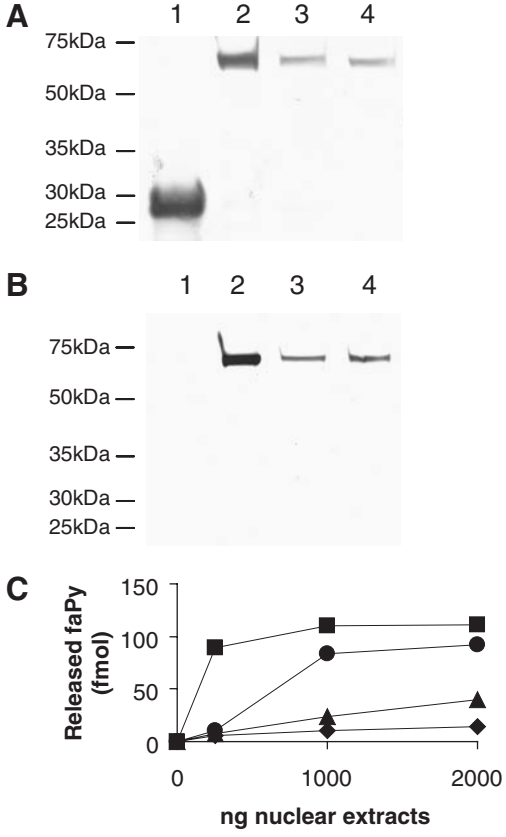

Figure 3. Expression of EGFP, EGFP-hOGG1, EGFP-hOGG1-Cys^{326} and EGFP-hOGG1-Glu^{326}. An aliquot of 20 µg nuclear extracts from HeLa-EGFP (lane 1), HeLa-EGFP-hOGG1 (lane 2), HeLa-EGFP-hOGG1-Cys^{326} (lane 3) and HeLa-EGFP-hOGG1-Glu^{326} (lane 4) cell lines were examined by western blot analysis using (**A**) anti-GFP antibody and (**B**) anti-OGG1 antibody. Anti-GFP detected EGFP (30 kDa), EGFP-hOGG1 and all mutant proteins (68 kDa). Anti-OGG1 detected EGFP-hOGG1 and the mutant proteins (68 kDa). (**C**) Formamidopyrimidine excision by increasing amounts of nuclear extracts from HeLa-EGFP (closed diamonds), HeLa-EGFP-hOGG1 (closed squares), HeLa-EGFP-hOGG1-Cys^{326} (closed triangles) and HeLa-EGFP-hOGG1-Glu^{326} (closed circles).

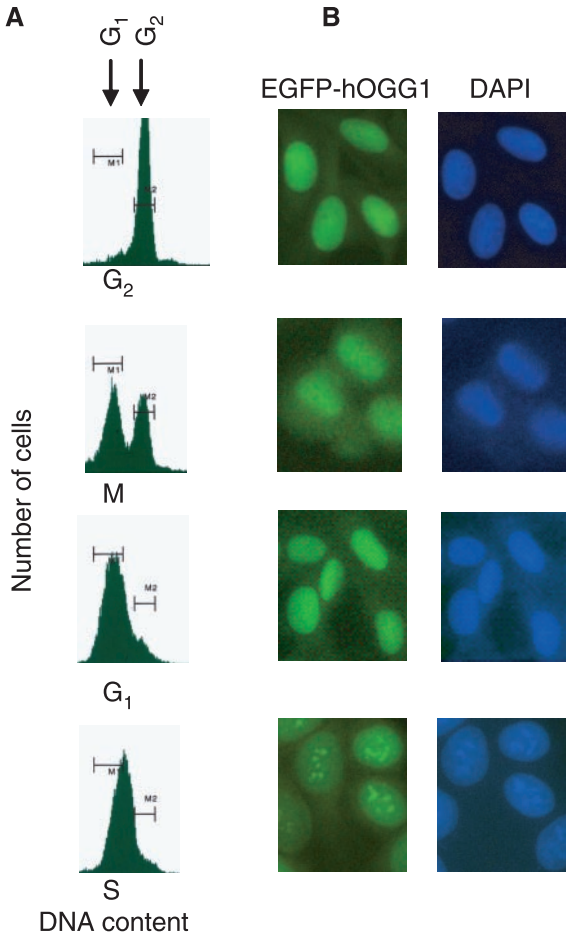

Figure 4. Dynamic subcellular relocalization of EGFP-hOGG1 during the cell cycle in HeLa-EGFP-hOGG1 cells. HeLa-EGFP-hOGG1 parallel cell cultures were synchronized by either nocodazole or double-thymidine block treatment. Samples were harvested immediately after the removal of nocodazole (most of the cells are in G_2 phase), 2 h after nocodazole removal (cells are in M-phase), at the time of double-thymidine block release (cells are in G_1 phase) and 4 h after double-thymidine block release (cells are in S-phase). (**A**) Determination of cell cycle phases by flow cytometry. (**B**) Subcellular distribution of EGFP-hOGG1 analysed using an Axioplan 2 fluorescence microscope (Carl Zeiss). DNA was labelled with DAPI staining.

microscope. Immediately after the removal of nocodazole most of the cells are in the G_2 phase. At this time, EGFP-hOGG1 is mainly distributed evenly throughout the nucleus (Figure 4). Within the next 2 h, cells enter the M-phase, the nuclear envelope disassembles and EGFP-hOGG1 associates with chromatin (Figure 4). At 2–8 h after nocodazole removal (data not shown), and at the time of double-thymidine block release, the cells are in G_1, and EGFP-hOGG1 is mainly homogeneously distributed to the nucleus (Figure 4). However, as the cells progress through the S-phase, 4 h after double-thymidine block release, in most of the cells, EGFP-hOGG1 accumulates within the nucleoli (Figure 4). In addition, to confirm the localization of EGFP-hOGG1 to the nucleoli during S-phase, we visualized sites of replication using anti-BrdU antibodies. As shown in Figure 2B, EGFP-hOGG1 is associated with the nucleoli during S-phase. Control cells carrying only EGFP stained green both in the cytoplasm and in the nucleus throughout the cell cycle (data not shown).

The localization of EGFP-hOGG1 to the nucleoli is linked to transcription

Actinomycin D specifically inhibits RNA polymerase I at low concentrations (51). EGFP-hOGG1 cells were synchronized at the G_1/S boundary, released and allowed to enter S-phase before being treated with 0.04 μg/ml actinomycin D for 4 h. In addition to nucleolin, we examined the distribution of fibrillarin and RNA polymerase I (RPA135) relative to EGFP-hOGG1 in cells with or without actinomycin D. The staining of nucleolin, fibrillarin and RPA135 overlapped with the green fluorescence signal of EGFP-hOGG1 in S-phase cells (Figure 5A–C, G–I and M–O). The staining of RNA polymerase I and fibrillarin revealed dot-like structures in the nucleoli (Figure 5H and N) that represent the fibrillar components of the nucleolus: the fibrillar centre (FC) together with the surrounding dense fibrillar component (DFC). The FC together with the surrounding DFC has been considered as a functional unit for rRNA synthesis and processing (52,53). Treatment with actinomycin D leads to the formation of crescent-shaped structures (or caps) in nucleoli, as there is a relocalization of nucleolar proteins whereby the fibrillar components are segregated from the granular components (54–56). After actinomycin D treatment, both RPA135 and fibrillarin were redistributed as crescent-shaped structures in the segregated nucleoli, as was EGFP-hOGG1 (Figure 5J–L and P–R) while most nucleolin and some EGFP-hOGG1 were dispersed into the nucleoplasm (Figure 5D–F).

EGFP-hOGG1-Cys^{326} is excluded from the nucleolus during S-phase

EGFP fused to the C-terminal of hOGG1-Cys^{326} was transiently transfected into HeLa cells to compare its cellular localization with that of EGFP-hOGG1. As shown in Figure 6, the Ser→Cys^{326} polymorphism has a dramatic effect on the nuclear localization of the protein. EGFP-hOGG1-Cys^{326} is excluded from the nucleoli as seen when cells were fixed and nucleoli were visualized with anti-nucleolin antibody. A new mutant, EGFP-hOGG1-Ala^{326}, was produced to address the question of whether the absence of the serine residue or the presence of a cysteine residue was responsible for the dramatic difference in the localization of the polymorphic allele. Transient transfection of HeLa cells with EGFP-hOGG1-Ala^{326} showed the distribution to be identical to that of EGFP-hOGG1-Cys^{326} indicating that the presence of the serine residue is important for the correct localization of hOGG1 to the nucleoli (data not shown).

The associations with interphasic chromatin and nuclear matrix, and with mitotic condensed chromosomes are disrupted in EGFP-hOGG1-Cys^{326}

To determine the distribution of EGFP-hOGG1-Cys^{326} during mitosis, we created stable cell lines overexpressing EGFP-hOGG1-Cys^{326}. Nuclear extracts were tested for faPy DNA glycosylase activity and results showed the fused enzyme to be active (Figure 3C). EGFP-hOGG1-Cys^{326} was excluded from condensed chromosomes during mitosis (Figure 7A, example of cells in pro-metaphase, metaphase and telophase, respectively) as evidenced by counterstain with DAPI, in contrast to EGFP-hOGG1, which has been previously

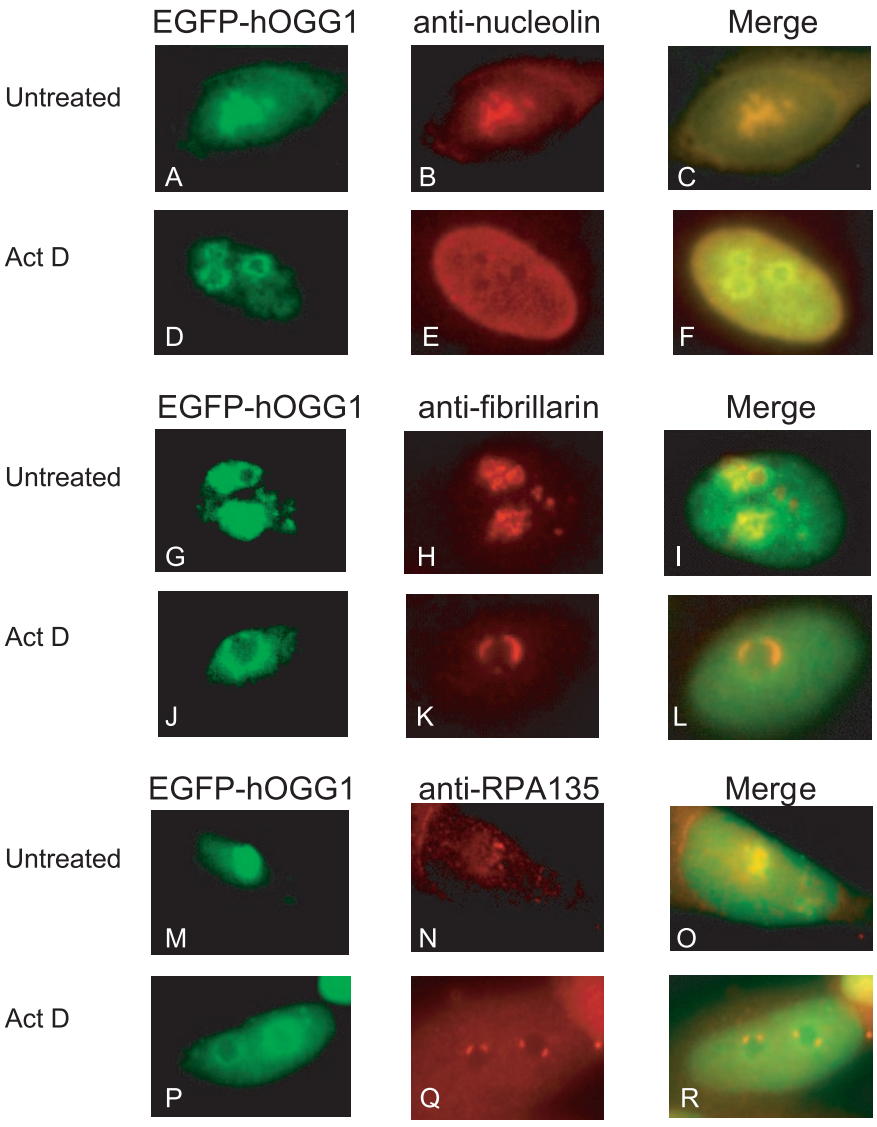

Figure 5. Effect of transcription inhibition by actinomycin D on the cellular distribution of EGFP-hOGG1. HeLa-EGFP-hOGG1 cells were grown on coverslips, synchronized by double-thymidine block, allowed to enter the S-phase and treated with 0.04 μg/ml actinomycin D before being fixed and subjected to immunofluorescence staining with the indicated antibodies. The cells were analysed using an Axioplan 2 fluorescence microscope (Carl Zeiss).

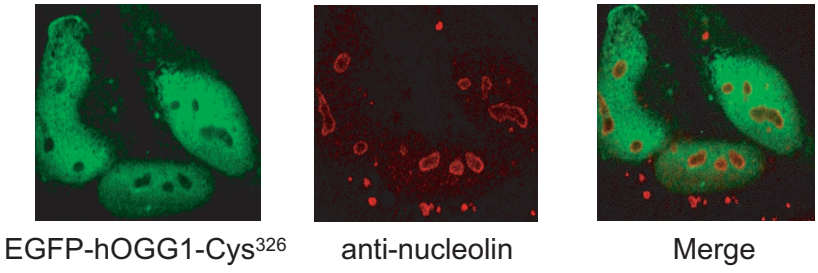

Figure 6. Confocal laser scanning microscopy of EGFP-hOGG1-Cys^{326}. HeLa cells were transiently transfected with pEGFP-hOGG1-Cys^{326} and fixed 24 h after transfection. Nucleoli were detected by staining with anti-nucleolin. Merged images showed that the EGFP-hOGG1-Cys^{326} is excluded from the nucleoli.

shown to co-localize with condensed chromatin in mitotic cells (47). As EGFP-hOGG1 has been shown to be associated with the soluble chromatin and the nuclear matrix during interphase (47), we wanted to investigate whether these associations were maintained for the EGFP-hOGG1-Cys^{326} mutant protein. Accordingly, asynchronously dividing HeLa-EGFP-hOGG1-Cys^{326} cells were sequentially extracted to obtain cytoplasm, soluble chromatin and nuclear matrix.

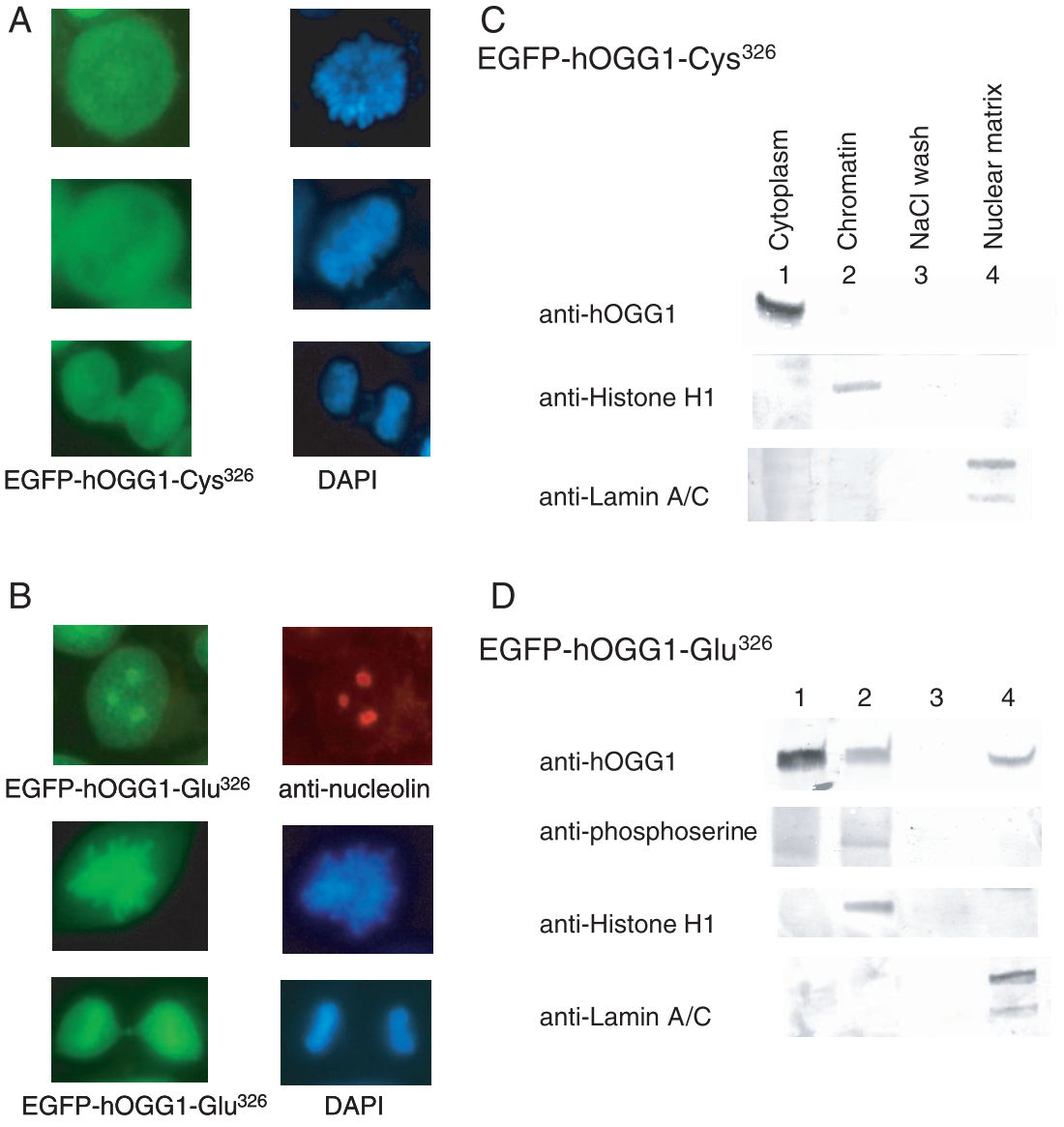

Figure 7. EGFP-hOGG1-Cys^{326} is excluded from mitotic chromosomes and its association with chromatin and nuclear matrix is disrupted, while EGFP-hOGG1-Glu^{326} is associated with the nucleoli, with the soluble chromatin fraction and the nuclear matrix. (**A**) HeLa-EGFP-hOGG1-Cys^{326} cells were grown on coverslips fixed and analysed using an Axioplan 2 fluorescence microscope (Carl Zeiss). EGFP-hOGG1-Cys^{326} was excluded from mitotic chromosomes. DAPI stain for DNA showed the condensed chromosomes in the mitotic cells. (**B**) HeLa-EGFP-hOGG1-Glu^{326} cells were grown on coverslips fixed and analysed using an Axioplan 2 fluorescence microscope (Carl Zeiss). Nucleoli were detected by staining with anti-nucleolin. DAPI stain for DNA showed the condensed chromosomes in the mitotic cells. EGFP-hOGG1-Glu^{326} is associated with the nucleoli, with the soluble chromatin and the nuclear matrix. (**C**) HeLa-EGFP-hOGG1-Cys^{326} and (**D**) HeLa-EGFP-hOGG1-Glu^{326} cells were sequentially extracted to obtain a cytoplasmic fraction, a chromatin fraction, a high salt wash fraction and the nuclear matrix. Proteins from equal cell equivalent from each fraction were analysed by western blotting with the indicated antibodies.

The protein extracts were analysed by using western blotting. The fractionation procedure was controlled by immunoblotting with antibodies directed against lamin A/C, a nuclear matrix-associated protein, and histone H1, a chromatin-associated protein (Figure 7C, lanes 2 and 4). Immunoblotting with anti-hOGG1 antibody revealed EGFP-hOGG1-Cys^{326} to be present only in the cytoplasmic fraction (Figure 7C, lane 1). This probably reflects protein leakage from the nucleus during the cell lysis procedure. However, no EGFP-hOGG1-Cys^{326} could be detected in the soluble chromatin or in the nuclear matrix fractions (Figure 7C, lanes 2 and 4).

The EGFP-hOGG1-Glu^{326} mutant displays a similar cellular distribution to the wild-type protein

Since the Ser-326 is a potential site of phosphorylation as predicted by the NetPhos 2.0 Protein Phosphorylation Prediction Server (57), we wanted to examine whether the aberrant localization of EGFP-hOGG1-Cys^{326} could be due to the phosphorylation status of the serine. As glutamate can mimic phosphoserine (58,59), we constructed a pEGFP-hOGG1-Glu^{326} mutant. A HeLa cell line stably expressing EGFP-hOGG1-Glu^{326} was generated and the subcellular localization was examined throughout the cell cycle (Figure 3). Nuclear extracts from these cells were tested for faPy DNA

glycosylase and results showed that the fused protein was active (Figure 3C). As described for the wild-type protein, EGFP-hOGG1-Glu^{326} was transported to nucleoli during S-phase (Figure 7B). Furthermore, during mitosis, from pro-metaphase to telophase, the EGFP-hOGG1-Glu^{326} signal was co-localized with the condensed chromosomes (Figure 7B shows examples of cells in pro-metaphase and in telophase). Finally, we wanted to examine whether EGFP-hOGG1-Glu^{326} would also be associated with the soluble chromatin and the nuclear matrix. By sequentially extracting the cells to obtain cytoplasm, soluble chromatin and the nuclear matrix, we were able to show that EGFP-hOGG1-Glu^{326} was associated with both the soluble chromatin and the nuclear matrix fractions (Figure 7D, lanes 2 and 4). The fractionation procedure was controlled as described previously (Figure 7D). Interestingly, the anti-phosphoserine recognized a phosphorylated form of the enzyme in both EGFP-hOGG1 and EGFP-hOGG1-Glu^{326} chromatin fractions.

EGFP/EYFP-hOGG1, EGFP-hOGG1-Cys^{326} and EGFP-hOGG1-Glu^{326} localization during S-phase in live cells

One of the advantages of using fluorescent fusion proteins to examine the subcellular localization of proteins is that live cells can be viewed and followed with time-lapse imaging. We investigated the fate of EYFP-hOGG1 during S-phase in living cells using PCNA as a marker for replication foci as it has been shown that fluorescent-labelled-PCNA forms distinct foci in S-phase cells that co-localize completely with endogenous PCNA and bromo-deoxyuridine incorporation (50,60). We transiently co-transfected HeLa S3 cells with EYFP-hOGG1 and ECFP-PCNA and followed the cells for 20 h. Throughout S-phase, EGFP-hOGG1 is localized around the nucleoli as shown in Figure 8A, which shows a cell during S-phase. To compare the localization of EGFP-hOGG1, EGFP-hOGG1-Cys^{326} and EGFP-hOGG1-Glu^{326} in living cells during S-phase, stable cell lines were transiently transfected with HcRed-PCNA. As shown in Figure 8B, EGFP-hOGG1-Cys^{326} is not associated with the nucleoli while the localization of EGFP-hOGG1-Glu^{326} looks similar to the wild-type protein.

DISCUSSION

An increasing number of DNA repair proteins have been shown to have cell-cycle-dependent expression, including UNG2, RAD51, RAD52, APE, hNTH1, ANPG and hMYH (48,61–65). Northern blot hybridization of synchronized

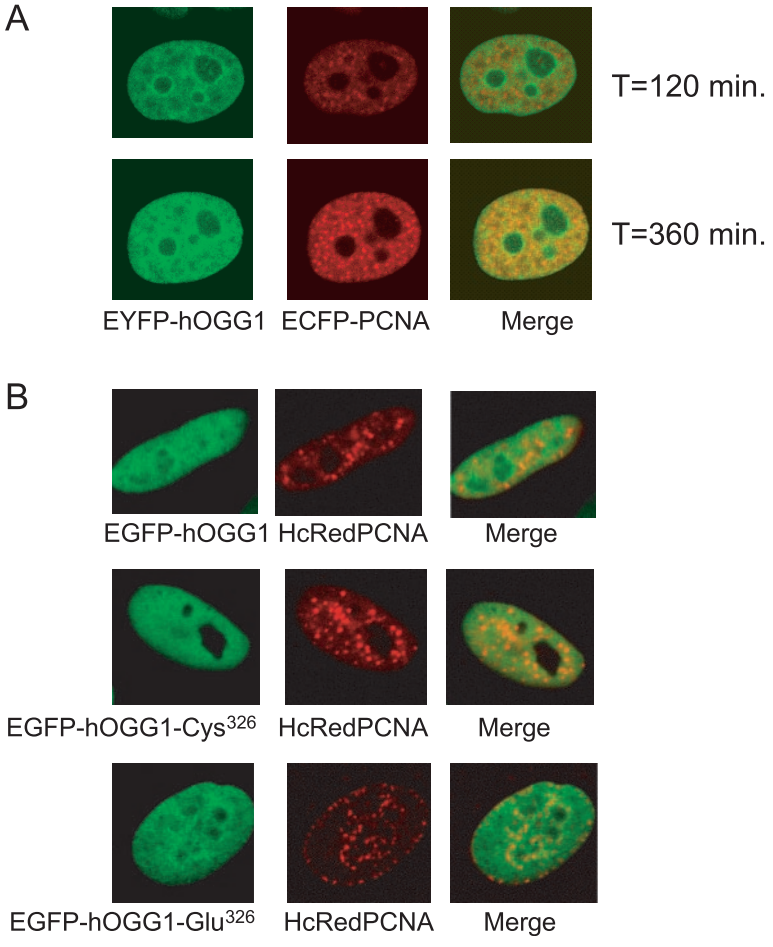

Figure 8. HeLa cells were transiently transfected with pEYFP-hOGG1 and pECFP-PCNA to visualize replication foci, and individual cells were examined by time-lapse fluorescence microscopy. (**A**) A representative cell shown early in S-phase ($T = 120$ min) and 4 h later ($T = 360$ min). (**B**) HeLa-EGFP-hOGG1, HeLa-EGFP-hOGG1-Cys^{326} and HeLa-EGFP-hOGG1-Glu^{326} cells were transiently transfected with HcRed-PCNA to visualize replication foci.

HaCat cells showed a cell-cycle-dependent mRNA expression of hOGG1 with increased transcription of the major nuclear and mitochondrial isoforms during S/G_2 (Figure 1). Among the predicted DNA-binding sites for transcription factors in the *HOGG1* promoter are two consensus sequences that potentially bind NF-Y (position 1884–1897, accession no. AJ131341) and E2F (position 1862–1869, accession no. AJ131341). Both NF-Y and E2F have been shown to be crucial regulators of the cell-cycle-dependent expression of several genes such as cdc2, CDC25, cyclin E, cyclin A, cyclin B, topoisomerase IIα and A-myb transcription factor (66–70). Moreover, although the murine and human OGG1 cDNAs are well conserved (85% over the ORF), there is little homology between the promoter regions of the genes (46% over 2 kb upstreams of the translation start). However, the mouse *OGG1* gene also contains putative binding sites for NF-Y (position 2028–2040) and E2F (positions 967–974 and 231–240). In contrast, a recent study in which the promoter of *HOGG1* was fused to the firefly luciferase gene carried out in HeLa cells showed that the expression of the reporter gene was not modulated during the cell cycle (71). As these experiments have been carried out in a different cell line, the participation of tissue-specific transcription factors in the transcriptional cell-cycle-dependent regulation of *HOGG1* cannot be excluded.

We and others have tried to examine the intracellular distribution of endogenous hOGG1 in different cell lines. However, endogenous hOGG1 is barely detected using the antibodies that are presently available [(23) and L. Luna, M. Bjørås, V. Rolseth and E. Seeberg, unpublished data]. We, therefore, used the EGFP tag to study the subcellular localization of hOGG1. Using this approach we showed that EGFP-hOGG1 was relocalized during the cell cycle and it was associated with the nucleoli during S-phase and with condensed chromosomes during mitosis. The method by which cells were fixed (formalin, PFA or ethanol) had an effect on the localization of EGFP-HOGG1 within the nucleoli as can be seen when comparing Figures 2 and 5–8. In live cells, EGFP-hOGG1 appeared to be concentrated around the nucleoli while in fixed cells it appeared to have 'leaked' into the nucleolus. This has also been observed in other proteins like the high-mobility group box proteins 1 and 2 (HMGB1 and HMGB2) (72).

Some insights into the biological role of hOGG1 might be gained by examining putative role(s) of hOGG1 in the nucleolus. Ribosomal DNA is relatively GC-rich and it has been shown to be very sensitive to ionizing radiation (73). However, only a few studies have been carried out to investigate both the extent and the repair of DNA damage of rDNA in mammals. The severe hereditary progeroid disorder Cockayne syndrome is a consequence of a defective transcription-coupled repair (TCR) pathway. Some key players in TCR, such as the Cockayne syndrome A (CSA) and B (CSB) proteins have been identified (74). Recently, Egly and co-workers (75) showed that CSB is found in the nucleolus within a complex that includes RNA pol I, TFIIH and XPG. At the same time, Bohr and co-workers (76) reported a functional cross-talk between CSB and hOGG1. As neither a functional nor a physical direct interaction was detected between these two proteins, the authors hypothesize a protein complex containing among others CSB and hOGG1. Likewise, the protein coded for the *XRCC1* gene participates in DNA single-strand breaks and in BER by its interaction with several of the enzymes involved in these processes. XRCC1 has been shown to be localized to the nucleolus and to physically interact with hOGG1 (77–80). Thus, we can picture a situation where multi-protein complexes composed of various combinations of polypeptides coexist in the nucleoli. The composition of these complexes depends on transient protein–protein interactions that most probably vary during cell-cycle progression and/or under specific conditions such as post-translational modifications. The actinomycin D inhibition results that show relocalization of EGFP-hOGG1 together with fibrillarin and polI to the caps in the segregated nucleoli strengthens the idea of a multimodular complex. This brings us to the question: is hOGG1 part of a complex of proteins that have a role in maintaining the integrity of rDNA, or are nucleoli simply a storage place for these proteins. Thus, the nucleolus might be a sequestering compartment of hOGG1 to avoid the accumulation of GC→TA transversions owing to the removal of 8-oxoG from 8-oxoG:A. Post-replicative repair of DNA mispairs is critical for effective maintenance of the genome. Thus, compartmentalization of hOGG1 to the nucleoli during S-phase might increase the repair efficiency of A:8-oxoG mispairs that arise during DNA replication, as dCMP or dAMP can be selectively incorporated opposite 8-oxoG (7). Accordingly, it has been shown that hMYH, an adenine-specific glycosylase responsible for removal of the mispaired adenine, localizes to the nucleus and during S-phase associates specifically with the replication foci for immediate post-replicative repair of adenine mispaired with 8-oxoG (64).

Nuclear localization signals (NLSs) are short stretches of amino acids that mediate the transport of nuclear proteins into the nucleus. NLSs are classified into three categories; the best studied are mono- and bi-partite motifs (81). Several sequences in a different number of proteins have been defined as nucleolus localization signals (NoLSs). In contrast to NLSs, there are no simple NoLSs and it has been proposed that localization of proteins to the nucleolus may occur through a variety of mechanisms (82). The nuclear localization of hOGG1 has shown to be driven by an NLS situated in the C-terminal segment of the protein (21). The serine to cysteine 326 mutation did not affect the nuclear localization of the protein; however, the association with the nuclear matrix and the chromatin, the nucleoli localization during S-phase and co-localization to the condensed chromosomes during mitosis were altered. As there appears to be no NoLS consensus similar to a mitochondrial or peroxisomal localization sequences, a scenario has emerged in which a nucleolar protein might have an interaction that causes it to be localized to the nucleolus. Thus, we suggest that the Ser-326 has to be phosphorylated to interact with a protein during S-phase that will translocate EGFP-hOGG1 to the nucleolus and to the condensed chromosomes during mitosis. Supporting this idea is the restoration of wild-type localization of the EGFP-hOGG1-Glu^{326} mutant protein that mimicked a phosphorylated serine. A link between aberrant protein localization and cancer has been established by several studies that include BRCA1, p53, APC and RCAS1 (83–87). A recent report has shown that the hOGG1-Cys^{326} protein has a lower ability to suppress mutations than the hOGG1-Ser^{326} protein in human cells *in vivo* (88). We suggest that the difference in the mutation suppressive ability between the two polymorphic

hOGG1 proteins is due to the subnuclear localization brought about by a phosphorylation of the Ser-326.

Finally, we had identified a phosphorylated form of EGFP-hOGG1 in the chromatin fraction by western blotting using a phosphoserine-specific antibody (47). Interestingly, this form was still present in the chromatin fraction isolated from HeLa-EGFP-hOGG1-Glu^{326} but it was clear that this antibody did not recognize the mimicked phosphorylated Ser-326. We, therefore, suggest that an additional phosphorylation on a different serine is responsible for the association of the protein with the chromatin fraction. Further studies are needed to elucidate the complete phosphorylation status of hOGG1 during the cell cycle.

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