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**A novel nonsense mutation in the APTX gene associated with delayed DNA single-strand break removal fails to enhance sensitivity to different genotoxic agents**

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For Peer Review

# A novel nonsense mutation in the *APT*X gene associated with delayed DNA single-strand break removal fails to enhance sensitivity to different genotoxic agents



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Short Title: *APT*X mutation and delayed DNA SSB repair

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**ABSTRACT:** *APT*X is the gene involved in ataxia with oculomotor apraxia type 1 (AOA1), a recessive disorder with early-onset cerebellar ataxia, oculomotor apraxia and peripheral neuropathy. The encoded protein, aprataxin, is a DNA repair protein processing the products of abortive ligations, 5'-adenylated DNA. We describe a novel nonsense mutation in *APT*X, c.892C>T (p.Gln298X), segregating in two AOA1 patients and leading to the loss of aprataxin protein in patient's cells. These cells, while exhibiting reduced catalase activity, are not hypersensitive to toxicity elicited by H<sub>2</sub>O<sub>2</sub> exposure at either physiologic or ice-bath temperature. On the other hand, the rate repair of DNA single-strand-breaks (SSBs) induced in both conditions is always significantly slower in AOA1 cells. By using the alkylating agent methyl methane sulphonate (MMS) we confirmed the association of the *APT*X mutation with a DNA repair defect in the absence of detectable changes in susceptibility to toxicity. These results, while consistent with a role of aprataxin in the repair of SSBs induced by H<sub>2</sub>O<sub>2</sub>, or MMS, demonstrate that other mechanisms may be recruited in AOA1 cells to complete the repair process, although at a slower rate. Lack of hypersensitivity to the oxidant, or MMS, also implies that delayed repair is not *per se* a lethal event.

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**KEY WORDS: *APTX*, AOA1, DNA-SSB Repair, Hydrogen Peroxide, Methyl methane sulphonate**

## INTRODUCTION

Ataxia with oculomotor apraxia type 1 (AOA1) (MIM# 208920) is an autosomal recessive disorder characterized by early-onset cerebellar ataxia, oculomotor apraxia, and peripheral neuropathy often associated with hypoalbuminemia and hyperlipidemia (Le Ber et al., 2003; Tranchant et al., 2003). Additional features, such as cognitive impairment and involuntary movements can also be present overall leading to a wide variability in clinical impact and/or severity of the disease. The disease is due to mutations in the *APTX* gene (MIM# 606350) (Date et al., 2001; Moreira et al., 2001), encoding aprataxin, a 342 aa protein, member of the histidine triad (HIT) superfamily of nucleotide hydrolases and/or transferases. These proteins are involved in the DNA-strand-break-repair machinery (Moreira et al., 2001). Aprataxin can remove adenosine-monophosphate (AMP) from various AMP-linked substrates *in vitro* (Seidle et al., 2005) and also from the 5'-terminus of DNA single- or double-strand breaks (SSBs and DSBs) thus promoting efficient ligation (Ahel et al., 2006; Caldecott, 2008; Daley et al. 2010).

The forkhead-associated (FHA) region at the N-terminus of aprataxin interacts with DNA repair proteins like XRCC1 and XRCC4 among the others (Sano et al., 2004), forming multiprotein complexes of the SSB and DSB-repair machinery respectively (SSBR and DSBR) (Clements et al., 2004; Gueven et al., 2004; Hirano et al., 2007; Caldecott, 2008).

The C-terminal region is a zinc-finger (ZF) domain required for DNA/RNA interaction (Gibson et al., 1988). HIT and ZF domains cooperate to form a high-affinity complex that locks the enzyme onto adenylated DNA lesions (Takahashi et al., 2007; Rass et al., 2008). Most of the mutations identified in AOA1 patients (available at <http://www.hgmd.cf.ac.uk>) fall within the HIT domain or just upstream the C-terminal ZF (see (Caldecott, 2008) for review).

In spite of that, attempts to demonstrate a defect in DNA-SSB repair in AOA1 resulted conflicting for some time. SSBR is known to be impaired in aprataxin knocked-down cells (Hirano et al., 2007) and a defect in cellular SSBR has also been reported in some AOA1 patient's derived cells (Mosesso et al., 2005; Gueven et al., 2007). Aprataxin is indeed recruited at sites of SSBs in an XRCC1-dependent manner and the targeting is mediated by PARP-1, a protein interacting with both XRCC1 and aprataxin and working as key player in SSBs detection (Harris et al., 2009).

Lymphoblast cell lines from an AOA1 patient carrying the T739C mutation were hypersensitive in terms of induction of chromosomal aberrations by CPT (camptothecin), an inhibitor of DNA topoisomerase I causing DNA single-strand breakage (Mosesso et al., 2005). By contrast, no evidence for such a defect was obtained in other studies (Gueven et al., 2004; Reynolds et al., 2009a). However, cells from AOA1 patients with different mutations (689insT/840delT, P206L/P206L, P206L/V263G) were hypersensitive to agents that cause SSBs (methyl methane sulfonate, MMS, and hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>), thereby suggesting a protective role of aprataxin against genotoxic stress (Gueven et al., 2004).

Here we describe a novel aprataxin mutation, p.Gln298X, truncating the protein just upstream the ZF domain and leading to loss of protein expression and activity in the patient's derived lymphoblast cell line (Takahashi et al., 2007; Reynolds et al., 2009b).

These cells display slower rates of resealing of SSBs induced by either H<sub>2</sub>O<sub>2</sub> or MMS. However, no hypersensitivity to toxicity induced by these agents was detected in these cells. Implications of these findings are discussed.

## METHODS

### Clinical Findings

The family pedigree in Figure 1 shows 2 affected siblings: the proband (II-2) and her brother (II-3) both displaying features of AOA1 disease with associated hypoalbuminemia. Detailed clinical description is provided as supplementary material.

### Mutation analysis and lymphoblastoid cell lines generation

Blood specimens were obtained from affected and unaffected available members of the family and from control subjects under a protocol of informed consent approved by the ethical committee of E. Medea Institute. Genomic DNA from peripheral blood leukocytes was prepared with the standard high salt extraction method. *APTX* genomic regions are available at the Human Genome Browser Gateway (<http://genome.ucsc.edu/cgi-bin/hgGateway>). We amplified and directly sequenced all 7 exons of the longest transcript using primers designed on the flanking intronic sequences. Sequence and mutation nomenclature is based on RefSeq NM\_175073.1 (considering the A of the ATG as nucleotide 1) and on RefSeq NC\_000009.11 for *APTX* cDNA and genomic sequences respectively, and follows the guidelines of the Human Genome Variation Society (<http://www.hgvs.org/mutnomen/>). Sequences were performed with a Big Dye Terminator Sequencing Kit (version 3.1 Applied Biosystem Foster City, CA, USA), run on an Applied Biosystems ABI 3130 XL Genetic Analyzer (Applied Biosystems). The identified mutation was confirmed on a second PCR performed on a second aliquot of patient DNA. Two hundreds healthy individuals from the Italian population were used as controls. Lymphoblast cells lines from patients and control were established by EBV infection with standard protocol and cultured in RPMI 1640 medium (Invitrogen Life Science, Carlsbad, CA, USA) containing 20% FCS (Hyclone Celbio, Milano, Italy) and supplemented with 2 mM Glutamine and 100U/ml penicillin/streptomycin (Invitrogen).

### Aprataxin protein levels and deadenylation activity in AOA1 lymphoblast cell lines

SDS-PAGE and Western Blot were performed as previously described (Panzeri et al., 2006) with total extracts from two controls (Cr 26 and Cr 48) and EM223/07 lymphoblast cell lines. Aprataxin was revealed with anti-aprataxin Ab (raised against the N-terminal of the protein, residues 1-177) and normalized with anti-actin Ab (both from Abcam, Cambridge, UK). The anti-aprataxin Ab specifically recognizes a single band at 39 kDa as a full-length aprataxin isoform. Deadenylation activity of aprataxin was performed as described (Takahashi et al., 2007; Reynolds et al., 2009b). Details are provided as supplementary material.

### Cytotoxicity assays

The cells ( $3 \times 10^5$ ) were exposed for 15 (4°C) or 30 (37°C) min to  $H_2O_2$  in 2 ml of saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l  $NaHCO_3$  and 0.9 g/l glucose). Treatments with MMS were performed at physiologic temperature in 2 ml of RPMI 1640 culture medium supplemented as detailed above. After treatments, the cells were washed with saline A and re-suspended in pre-warmed complete culture medium, plated into 35 mm tissue dishes and finally incubated at 37°C for up to three days. The number of viable cells was estimated 24, 48 and 72 h after treatments with a hemocytometer, after dilution of the cell suspension (1:1) with 0.4% trypan blue. Under these conditions, cultures were characterised by the presence of viable cells (i.e., excluding trypan blue), cells presenting features of apoptosis (i.e., blebbing, apoptotic bodies) and by the absence of necrotic cells (i.e., taking up trypan blue). In other experiments, however, dead cells were largely detected in the absence of apoptotic cells after a 30 min post-treatment incubation with high concentrations of  $H_2O_2$ .

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##### Measurement of DNA single-strand breakage

The cells ( $3 \times 10^5$ ) were exposed for 15 min ( $4^\circ\text{C}$  or  $37^\circ\text{C}$ ) to increasing concentrations of  $\text{H}_2\text{O}_2$  in 2 ml of saline A. In other experiments, the cells were treated for 1 h ( $37^\circ\text{C}$ ) with increasing concentrations of MMS in 2 ml of RPMI 1640 culture medium supplemented as detailed above. Cells were then analyzed for DNA damage either immediately or after increasing time-intervals of incubation in pre-warmed complete culture medium. SSBs were determined using the alkaline-halo assay developed in our laboratory (Cantoni and Guidarelli, 2008). Details on the processing of fluorescence images and on the calculation of the experimental results are as reported (Cantoni and Guidarelli, 2008). SSBs were quantified by calculating the nuclear spreading factor value, representing the ratio between the area of the halo (obtained by subtracting the area of the nucleus from the total area, nucleus + halo) and that of the nucleus, from 50 to 75 randomly selected cells/experiment/treatment condition. Results are expressed as relative nuclear spreading factor values calculated by subtracting the nuclear spreading factor values of control cells from those of treated cells.

SSBs were also determined using the comet assay, following the procedure detailed by Singh et al. (1988), with minor modifications (Cantoni and Guidarelli, 2008). The extent of DNA strand scission is expressed as tail/nucleus ratio values, calculated according to the method of Müller et al., (1994). Data are typically collected from 50-75 randomly selected cells/experiment/treatment condition.

##### Non-protein thiol assay

Cellular non-protein thiol content was determined as described by Beutler (Beutler, 1984). In brief, cells ( $5 \times 10^6$ ) were washed three times with saline A, centrifuged and the pellet was subsequently re-suspended in 150  $\mu\text{l}$  of a metaphosphoric acid solution [1.67% (v/v) metaphosphoric acid /0.2% EDTA/30% (w/v) NaCl]. After a 5 min incubation in an ice-bath, the cell suspension was centrifuged for 5 min at 10,000 g. The non-protein thiol content was measured spectrophotometrically in the supernatant, at 412 nm, using 5,5'-dithiobis(2-nitrobenzoic acid) ( $\epsilon_{412} = 13600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ).

##### Catalase, glutathione peroxidase and superoxide dismutase activities

Cells were washed twice with saline A, re-suspended in the same medium at a density of  $5 \times 10^6$  cells/ml and finally sonicated three times on ice with a Branson sonifier operating at 20 W for 15 sec. The resulting homogenates were centrifuged for 5 min at  $18,000 \times g$  at  $4^\circ\text{C}$ . Catalase and glutathione peroxidase (Beutler, 1984) and superoxide dismutase (Paoletti et al., 1996) activities were determined spectrophotometrically in the supernatants.

##### Statistical analysis

The results are expressed as means  $\pm$  SD. Statistical differences were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparison or two-way ANOVA followed by Bonferroni's test for multiple comparison. A value of  $p < 0.05$  was considered significant.

## RESULTS

##### Genetic analysis and 5'-AMP SSB repair in mutant lymphoblastoid cell line

The proband (EM223/07) of the AOA1 family was screened for mutations in the *APTX* gene and a novel homozygous nonsense mutation (c.892C>T) in exon 7 was identified leading to a premature protein interruption, p.Gln298X (Fig. 1A). The mutation was also found in the affected brother, while the two healthy brothers (II-1, II-4) were heterozygous carriers. The mutation was not found in 200 controls. The parents were not available for analysis. The mutation is predicted to cause complete loss of the aprataxin zinc-finger domain, important for

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3 DNA/RNA interaction (Fig. 1C). Aprataxin protein levels were analyzed by Western Blot in controls and  
4 EM223/07 lymphoblast cell lines with an anti-aprataxin Ab raised against the N-terminal of the protein (Fig. 1B).  
5 While control cells (Cr 26 and Cr 48) expressed the 39 kDa full-length aprataxin isoform, EM223/07 cells failed to  
6 express the 34 kDa truncated p.Gln298X protein. This suggests that the p.Gln298X mutation results in loss of  
7 aprataxin protein. We also checked the presence of any residual aprataxin deadenylation activity in EM223/07  
8 lymphoblastoid cell lines, incubating increased concentration of Cr 48 and EM223/07 cell extracts with a duplex  
9 substrate carrying a 5'-AMP end SSB for 1 hour. While the control (Cr 48) efficiently repairs the SSBs, EM223/07  
10 extract does not show any residual activity (Fig. 1S).  
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### 13 14 Lymphoblast cell lines sensitivity to hydrogen peroxide and methyl methane sulphonate

15  
16 In order to investigate the impact of the p.Gln298X mutation on SSB repair and on toxicity induced by H<sub>2</sub>O<sub>2</sub>,  
17 preliminary studies were performed to determine possible confounding factors, which may affect the above  
18 responses independently of aprataxin expression. Changes in the redox status of the cells, regardless of their  
19 dependence on the mutation, may indeed provide misleading results.

20 Cells from the AOA1 patient EM223/07 were therefore analysed for their glutathione peroxidase and  
21 superoxide dismutase activities, as well as for their non-protein-thiol pool, obtaining results comparable with those  
22 derived from a normal age-matched control (Cr 48) (Table 1S). Further analyses, however, provided evidence for a  
23 significantly reduced activity of catalase, the major H<sub>2</sub>O<sub>2</sub>-detoxifying enzyme.

24 In order to assess whether loss of aprataxin delays SSB repair, we employed a strategy minimising the  
25 contribution of catalase activity in the accumulation of the initial damage induced by H<sub>2</sub>O<sub>2</sub>. For this purpose, cells  
26 were treated for 15 min with increasing concentrations of H<sub>2</sub>O<sub>2</sub> in an ice bath, i.e., at a temperature remarkably  
27 slowing down all enzymatic activities. Under these conditions (Fig. 2A), the dose-response curves relating the  
28 degree of DNA strand scission to the concentration of H<sub>2</sub>O<sub>2</sub> were virtually superimposable in normal and  
29 aprataxin-deficient cells. This notion was established using two separate techniques for the assessment of DNA  
30 damage, the alkaline-halo and comet assays, based on different principles but nevertheless characterised by  
31 identical sensitivities (Cantoni and Guidarelli 2008). Both techniques involve the use of an alkaline pH, converting  
32 alkali labile sites in frank DNA SSBs. EM223/07 and Cr 48 cells were next treated on ice with 50 μM H<sub>2</sub>O<sub>2</sub> and  
33 subsequently incubated at 37°C to investigate the rate of SSB resealing. Importantly, data obtained in these  
34 experiments (Fig. 2B), represent the observed net frequency, i.e., the result of a dynamic balance between the  
35 breaks introduced by repair endonucleases in response to various types of DNA damage and the sealing of all  
36 types of breaks by polymerisation and ligation. In both cells types, a time-dependent increase in the rate of SSB  
37 removal was consistently observed, thereby suggesting that the expected exponential kinetics are masked by an  
38 early formation of endonuclease-dependent DNA SSBs restricted to the first 15-30 minutes of incubation at 37°C.  
39 The rate of SSBR was however significantly slower in EM223/07 cells (t<sub>1/2</sub> = 25.8 min) as compared to Cr 48  
40 cells (t<sub>1/2</sub> = 17 min). On the other hand, SSBs were nevertheless completely resealed in both cell types.  
41 Importantly, the results presented in Fig. 2B were obtained using the alkaline-halo as well as the comet assays,  
42 with identical outcomes.  
43

44 There was no evidence of toxicity in either of the cell types under investigation, as assessed by visual inspection  
45 as well as by release of lactate dehydrogenase or trypan blue uptake (data not shown). We also assessed the  
46 proliferation rate of these cells over three days of growth after H<sub>2</sub>O<sub>2</sub> treatment on ice: the kinetics were  
47 superimposable for both cell types (Fig. 2C and D), regardless of the treatment received (vehicle vs H<sub>2</sub>O<sub>2</sub>). Hence,  
48 the observed delay in SSB repair does not appear to be dependent on non-specific toxic effects of the oxidant and  
49 is most likely attributable to lack of aprataxin expression.

50 We next addressed the question of whether the repair defect was associated with delayed hypersensitivity to the  
51 toxic effects of H<sub>2</sub>O<sub>2</sub>. For this purpose, the cells were treated with concentrations of the oxidant higher than those  
52 employed in DNA repair studies. The proliferation rates of normal or AOA1 cells were however similarly affected  
53 by H<sub>2</sub>O<sub>2</sub> (Fig. 2C and D) so that identical toxicity curves were obtained for the two cell types (Fig. 2E). It is  
54 important to note that these experiments measure the number of viable cells, which decreases after exposure to  
55 H<sub>2</sub>O<sub>2</sub> as a result of both growth inhibition and cell killing, taking place by apoptosis, as determined by visual  
56 inspection (cell blebbing and chromatin condensation, not shown) and activation of caspases (not shown).  
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3 It should be emphasised that these results were obtained in cells exposed to H<sub>2</sub>O<sub>2</sub> at ice bath temperature and  
4 should therefore reflect the exclusive action of chemical/free-radical processes. However, the biological effects of  
5 H<sub>2</sub>O<sub>2</sub> at 4°C or 37°C may not be necessarily mediated by the same mechanisms. At 37°C, biochemical/enzymatic  
6 processes (which are not operative at 4°C) may become important for H<sub>2</sub>O<sub>2</sub> metabolism and the mechanisms of  
7 toxicity may be altered.

8 We therefore decided to perform the experiments at 37°C; consistently with the observed reduced catalase  
9 activity (Table 1S), we obtained results indicating that AOA1 cells are hypersensitive to H<sub>2</sub>O<sub>2</sub>-induced DNA strand  
10 scission induced at physiologic temperature (Fig. 2F). Note that treatments were for 15 min and that, under these  
11 conditions, remarkably lower concentrations of the oxidant were necessary to produce SSBs levels comparable to  
12 those obtained at ice-bath temperature, as assessed with both the alkaline-halo and comet assays. The results  
13 illustrated in Fig. 2G indicate that equivalent levels of SSBs induced by H<sub>2</sub>O<sub>2</sub> exposure at 37°C in control (10 μM)  
14 and AOA1 (5 μM) cells are repaired with identical kinetics, superimposable on those observed in cells oxidatively-  
15 injured at ice-bath temperature.

16 These findings therefore indicate that loss of aprataxin expression delays the rate of SSB repair, regardless of  
17 whether the initial damage was induced at 37° or 4°C. As previously noted for experiments involving treatments at  
18 4°C, there were no signs of toxicity under the conditions of exposure at 37°C (data not shown), thereby  
19 emphasizing the specificity of the repair defect, most likely dependent on the lack of aprataxin expression. A  
20 significant toxicity, also characterised by biochemical and morphological features of apoptosis (not shown), was  
21 however observed by doubling the exposure time to 30 min and post-incubating the cells in fresh culture medium  
22 for up to 72 h (Fig. 2H and I). The resulting toxicity curves were identical for control and AOA1 cells (Fig. 2L), in  
23 contrast to AOA2 EM518 cells, remarkably more sensitive to H<sub>2</sub>O<sub>2</sub> (Airoldi et al., 2010).

24 Taken together, these results lead to the conclusion that, despite their hypersensitivity to DNA strand scission  
25 (probably due to reduced catalase activity) and slow rate of SSB removal (likely due to lack of aprataxin  
26 expression), EM223/07 cells are not hypersensitive to toxicity elicited by H<sub>2</sub>O<sub>2</sub> exposure at physiologic  
27 temperature. This notion was also established by studies in which cells were incubated for 30 min (37°C) with high  
28 concentrations of H<sub>2</sub>O<sub>2</sub> and subsequently analysed for viability with the trypan blue assay after a 30 min  
29 incubation in fresh culture medium (Fig. 2S). By contrast, the AOA2-EM518 cell line was hypersensitive also  
30 under these conditions. It is interesting to note that the results illustrated in Fig. 2S provide an estimate of the  
31 number of dead cells, trypan blue positive, and are therefore indicative of necrosis, as expected, since toxicity  
32 arises very rapidly in response to high concentrations of the oxidant. There was no evidence of apoptosis in any of  
33 the cell types employed.

34 These results confirm and extend the above findings, indicating the existence of a dichotomy between the repair  
35 defect and sensitivity to toxicity, by showing identical outcomes even under conditions in which massive SSB  
36 induction is associated with a necrotic mode of death.

37 The final set of experiments involved the use of another SSB inducer, the alkylating agent MMS. The results  
38 obtained are in line with those previously described in cells treated with H<sub>2</sub>O<sub>2</sub> at 4°C. Control and AOA1 cells  
39 were indeed equally sensitive to DNA strand scission (Fig. 3A) and toxicity (Fig. 3C-E) induced by increasing  
40 concentrations of MMS but nevertheless displayed remarkably different rates of SSB rejoining (Fig. 3B). As  
41 previously noted for H<sub>2</sub>O<sub>2</sub>, also MMS-induced DNA cleavage was repaired more slowly in EM223/07 cells than in  
42 Cr 48 cells.

43 Collectively, our results indicate that aprataxin-deficient cells display reduced rates of repair of MMS- or H<sub>2</sub>O<sub>2</sub>-  
44 induced SSBs. These data demonstrate also that this event is not associated with hypersensitivity to toxicity.

## 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

A large variety of proteins is involved in the repair of DNA SSBs, the commonest lesions arising in cells from  
different sources (Caldecott, 2008). Most of these lesions are repaired by a rapid-global-SSB-repair process,  
SSBR. Additional pathways exist all considered sub-pathways of SSBR, with an extensive overlap between the  
enzymes used (Caldecott, 2008). Two of the proteins involved in the end-processing step are APTX and TDP1  
(Caldecott, 2008), involved in neurodegenerative disease, AOA1 (Date et al., 2001; Moreira et al., 2001), and  
SCAN1 (Takashima et al., 2002), respectively. As previously shown for TDP1 (Takashima et al., 2002), recent  
studies indicate that aprataxin catalyzes the hydrolytic release of DNA end-blocking lesions with APTX being

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3 specific for end-blocking 5'-adenylate and TDP1 for 3'-tyrosyl termini (El-Khamisy et al., 2005; Rass et al.,  
4 2007a; Rass et al., 2007b; Rass et al., 2008). A clear defect in cellular SSB repair, however, has only been reported  
5 for mutations in *TDP1*, as the attempts to demonstrate a similar defect in AOA1 failed to give consistent results.  
6 While aprataxin knock down appears to delay resealing of SSBs accumulated under conditions of GSH depletion  
7 (Hirano et al., 2007), other studies failed to detect slower repair kinetics of lesions caused by H<sub>2</sub>O<sub>2</sub> in AOA1 cells  
8 carrying different *APTX* mutations (Gueven et al., 2004) or in *APTX* knock out chicken DT40 cells, *APTX* -/  
9 mouse fibroblasts, AOA1 human fibroblasts and AOA1 lymphoblast cell lines (Reynolds et al., 2009a). It was thus  
10 hypothesized that lack of consistent defects in SSB repair in aprataxin-defective cells, may reflect either the low  
11 abundance of 5'-AMP breaks or the availability of other factors for repair of these types of lesions (El-Khamisy et  
12 al., 2009). More recently evidence for both direct and indirect modulating functions for aprataxin on base excision  
13 repair were provided (Harris et al., 2009).

14  
15 To gather more information on the repair of SSBs induced by different agents in aprataxin-deficient cells, we  
16 employed an AOA1 patient's derived lymphoblast cell line carrying a novel nonsense mutation in aprataxin,  
17 leading to loss of aprataxin expression and activity. These cells exhibited a delayed rate of SSB rejoining (Figs 2B,  
18 2G and 3B). In order to appreciate the significance of our findings, it is important to note that the above phenotype  
19 was not associated with poor viability, as experiments comparing DNA repair in normal and AOA1 cells involved  
20 the use of exposure paradigms devoid of intrinsic toxicity. In addition, the genotoxic agents employed, H<sub>2</sub>O<sub>2</sub> and  
21 MMS, are two well-characterised DNA-damaging agents widely used as reference compounds in DNA repair  
22 studies. Conditions were also used producing identical levels of initial damage (Fig. 2A and 3A), falling within a  
23 range in which two different techniques accurately measure the extent of DNA strand scission. As a final note,  
24 exposure to H<sub>2</sub>O<sub>2</sub> was performed at ice-bath temperature, a condition allowing the accumulation of identical levels  
25 of DNA lesions in response to the same concentration of the oxidant in the two cell types (Fig. 2A), despite the  
26 reduced catalase activity expressed by AOA1 cells. This technical aspect is critical to validate the outcome of  
27 results which are potentially affected by a variety of mutation-unrelated factors.

28  
29 The results obtained lead to the univocal conclusion that the mutation associated with loss of aprataxin  
30 expression delays resealing of SSBs induced by H<sub>2</sub>O<sub>2</sub> or MMS. The same conclusion is reached from studies in  
31 which cells were treated with H<sub>2</sub>O<sub>2</sub> at physiologic temperature. Under these conditions, EM223/07 cells were  
32 hypersensitive to SSB induction but nevertheless the repair kinetics were identical in AOA1 cells and in the  
33 normal counterpart (Fig. 2F and G). While the different sensitivity to the initial damage is most likely dependent  
34 on reduced catalase activity expressed by AOA1 cells, it is also important to observe that the repair kinetics of  
35 SSBs induced by H<sub>2</sub>O<sub>2</sub> at 4° (Fig. 2B) or 37°C (Fig. 2G), in either of the two cell types, are also identical. This is  
36 an unexpected finding, as we previously detected remarkably slower repair kinetics for the DNA lesions induced  
37 by the oxidant at 37°C vs 4°C in Chinese Hamster Ovary cells (Cantoni et al., 1986).

38  
39 A second important point emerging from our studies is that SSBs, although with slower rates, were nevertheless  
40 completely resealed in AOA1 cells, thereby implying that aprataxin is dispensable for SSB resealing. (Figs. 2B,  
41 2G and 3B). A recent study indeed provided results consistent with the notion that long-patch repair compensates  
42 for defective short-patch repair in AOA1 cells (Reynolds et al., 2009a).

43  
44 The third relevant point we can make from our experiments is that delayed removal of SSBs is not associated  
45 with hypersensitivity to toxicity. Using intrinsically toxic concentrations of H<sub>2</sub>O<sub>2</sub> at 4°C (up to 10-fold greater than  
46 those employed in DNA repair studies), we indeed obtained identical toxicity curves in normal and AOA1 cells  
47 (Fig. 2E). The same outcome was provided by studies using significantly lower H<sub>2</sub>O<sub>2</sub> concentrations (Fig. 2L),  
48 however given to the cells at physiological temperature, permissive for both signalling events associated with  
49 toxicity and for enzymatic H<sub>2</sub>O<sub>2</sub> metabolism (i.e., detoxification). These experiments measured the number of  
50 viable cells after increasing time intervals, a parameter potentially affected by growth inhibition and by cell death.  
51 H<sub>2</sub>O<sub>2</sub> induced apoptosis in both EM223/07 and Cr 48 cells (not shown) thereby confirming the notion that  
52 functional aprataxin is dispensable for survival under these conditions. Importantly, the same assay was  
53 successfully employed to demonstrate that AOA2 cells are hypersensitive to H<sub>2</sub>O<sub>2</sub> (Fig. 2L and Airoidi et al.,  
54 2010). As a final note, identical results were also obtained with the alkylating agent MMS (Fig. 3A).

55  
56 Our findings therefore provide unambiguous evidence for lack of hypersensitivity of AOA1 cells to apoptosis  
57 induced by H<sub>2</sub>O<sub>2</sub>. Additional studies provided results in keeping with the notion that the same is true for necrosis.  
58 We indeed found that high concentrations of H<sub>2</sub>O<sub>2</sub> rapidly promote an identical accumulation of trypan blue  
59 positive EM223/07 and Cr 48 cells, whereas hypersensitivity was once again detected in AOA2 cells (Fig. 2S).

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Lack of hypersensitivity is therefore further established and even appears somewhat surprising, as catalase activity in AOA1 cells is about 25% lower than that detected in the normal counterpart. Future studies should investigate whether reduced catalase activity is a general feature of the AOA1 phenotype; previous studies providing evidence for hypersensitivity to H<sub>2</sub>O<sub>2</sub> may have employed AOA1 cells with remarkably low catalase activity. The notion that AOA1 lymphoblast cells display reduced rates of SSB removal, but are nevertheless as sensitive as normal cells to H<sub>2</sub>O<sub>2</sub> or MMS may be explained by the lack of contribution of SSBs in the molecular events mediating toxicity. Indeed SSBs are normally associated with poor toxicity in non-neuronal cells, in particular those caused by H<sub>2</sub>O<sub>2</sub>, continuously produced in oxygenated tissues. In this perspective, it should be reminded that the AOA1 neurological phenotype is not associated with gross damage in non-neuronal tissues. However, inefficient repair of SSBs continuously produced by reactive oxygen species promotes the accumulation of these lesions in non-replicating post-mitotic neurons, thereby interfering with transcription (Wilson and Mattson, 2007) and causing neurodegeneration (Caldecott, 2003).

In conclusion, although the exact contribution of delayed repair of SSBs to the AOA1 pathology is still to be demonstrated, we herein provide experimental evidence that aprataxin is required for the normal repair rate of SSBs induced by H<sub>2</sub>O<sub>2</sub> or MMS.

**Acknowledgements**

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## Figure legends

## Figure 1. Pedigree of the AOA1 family and the APTX mutation

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20 (A) Diamond symbols are for anonymity reasons. Stars indicate subjects whose DNA is available.  
21 Electropherograms of the mutant and control sequence are shown on the right, with a box indicating the mutant  
22 nucleotide. (B) Aprataxin protein levels in EM223/07 lymphoblastoid cells. Total extracts prepared from  
23 EM223/07 and two control (Cr 26 and Cr 48) lymphoblastoid cell lines were run on a 15% SDS-polyacrilamide  
24 gel. Endogenous senataxin was revealed with anti aprataxin Ab, raised against the N-terminal of the protein (amino  
25 acids 1-177), and normalized with anti actin Ab. While control cells expressed aprataxin at the expected 39 kDa  
26 size, no signal is observed in EM223/07 at the size of 34 kDa corresponding to the truncated p.Gln298X protein.  
27 The results shown are representative of three reproducible experiments. (C) A schematic representation of  
28 aprataxin protein domains is shown below with the novel mutation identified (p.Q298X) falling upstream the zinc  
29 finger domain. (FHA: forkhead-associated, NLS: nuclear localization signal, HIT: histidine triad, ZF: zinc-finger  
30 domain, XRCC-1: X-ray cross-complementing protein 1; XRCC-4: X-ray cross-complementing protein 4;  
31 SSBR/DSBR: single/double strand break repair).

Figure 2. Formation/repair of SSBs, and toxicity, induced by H<sub>2</sub>O<sub>2</sub> exposure at ice-bath or physiologic temperature.

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36 Cells were exposed for 15 min to increasing concentrations of H<sub>2</sub>O<sub>2</sub> at 4°C (A) or 37°C (F) and immediately  
37 analysed for DNA strand scission with either the alkaline halo (circles) or the comet (squares) assays, as detailed in  
38 the Methods section. (B) Cells were pre-exposed (15 min) to 50 µM H<sub>2</sub>O<sub>2</sub> (4°C), and analysed with the alkaline  
39 halo (circles) or the comet (squares) assays either immediately or after increasing time intervals of post-incubation  
40 in complete culture medium at 37°C. (G) The cells were pre-exposed (15 min) to 10 (Cr 48) or 5 µM (EM223/07)  
41 H<sub>2</sub>O<sub>2</sub> at 37°C and analyzed for SSB repair with the alkaline halo or the comet assays as in B. Results represent the  
42 means ± SD calculated from three to five separate experiments. \*P < 0.01; \*\*P < 0.001 compared to Cr 48 cells  
43 (two-way ANOVA followed by Bonferroni's test).

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45 (C, D) Counts of Cr 48 and EM223/07 cells exposed to increasing concentrations of H<sub>2</sub>O<sub>2</sub> for either 15 min at  
46 4°C (C-E) or for 30 min at 37°C (H-L) and post-incubated for increasing time intervals in fresh culture medium.  
47 (E, L) The results obtained at the 72 h time point are normalized vs the respective untreated control. The results  
48 obtained with EM518 cells, derived from an AOA2 patient (Airoldi et al., 2010), are also shown to provide a  
49 positive control. Results represent the means ± SD calculated from three to five separate experiments. \*\*P < 0.001  
50 compared to Cr 48 cells (two-way ANOVA followed by Bonferroni's test).

## Figure 3. Formation/repair of SSBs, and toxicity, induced by MMS exposure at physiologic temperature.

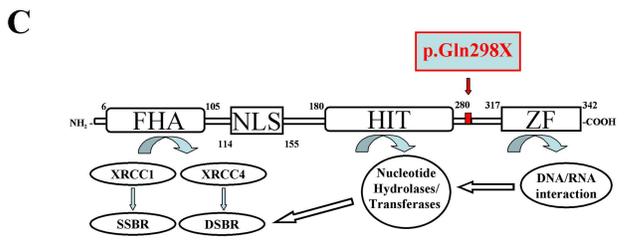
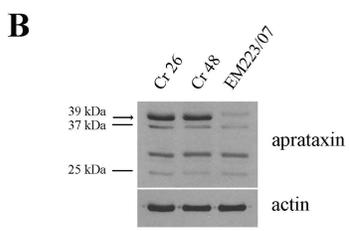
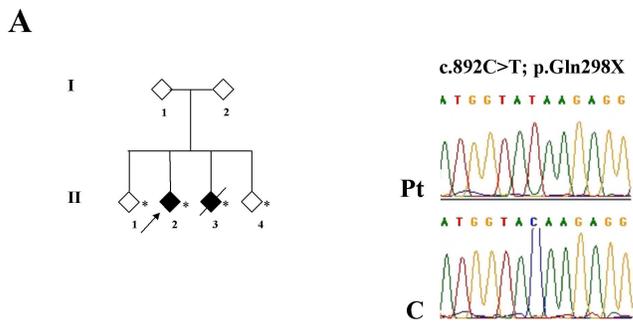
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53 (A) Cells were exposed for 1h to increasing concentrations of MMS at 37°C and immediately analysed for  
54 DNA strand scission with either the alkaline halo (circles) or the comet (squares) assays, as detailed in the  
55 Methods section. (B) Cells were pre-exposed (1h) to 50 µg/ml MMS and analysed with the alkaline halo (circles)

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3 or the comet (squares) assays either immediately or after increasing time intervals of incubation in complete  
4 culture medium at 37°C. Results represent the means ± SD calculated from three to five separate experiments. \*P  
5 < 0.01; \*\*P < 0.001 compared to Cr 48 cells (two-way ANOVA followed by Bonferroni's test).  
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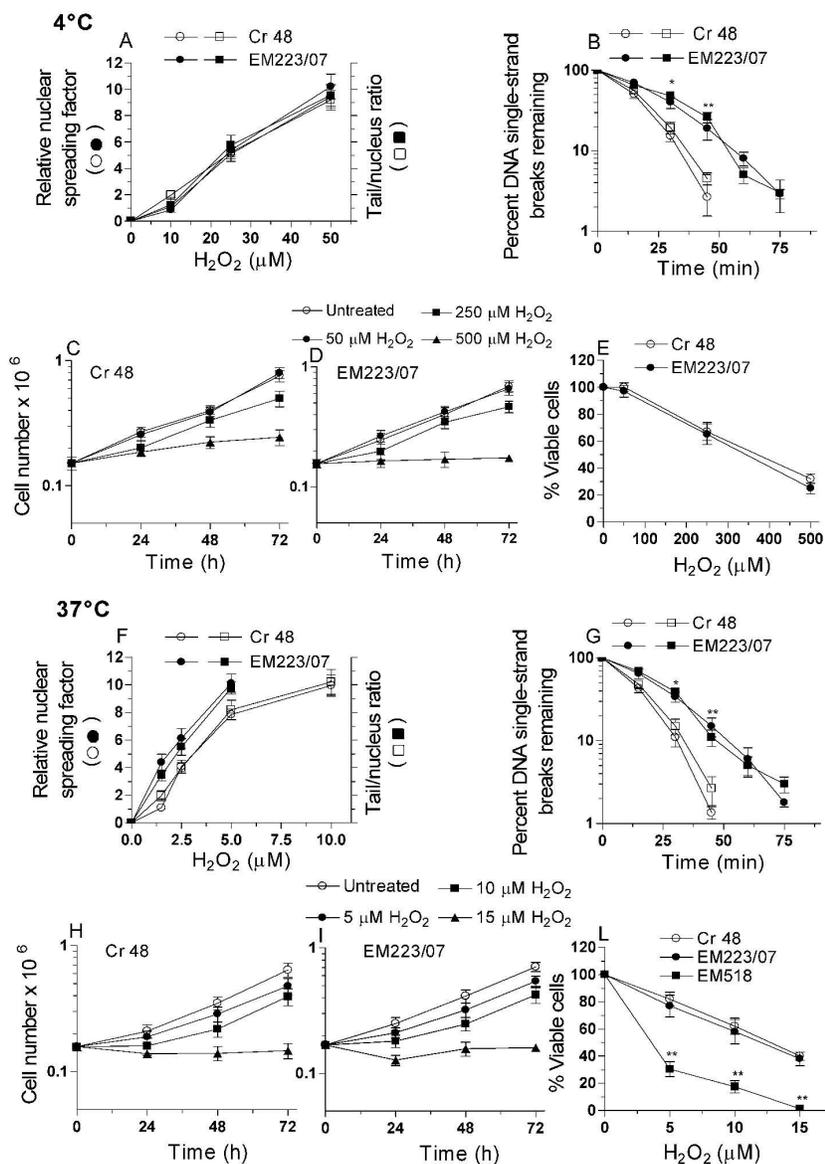
7 (C, D) Counts of Cr 48 and EM223/07 cells exposed for 1h to increasing concentrations of MMS and post-  
8 incubated for increasing time intervals in fresh culture medium. (E) The results obtained at the 72 h time point are  
9 normalized vs the respective untreated control. Results represent the means ± SD calculated from three to five  
10 separate experiments.  
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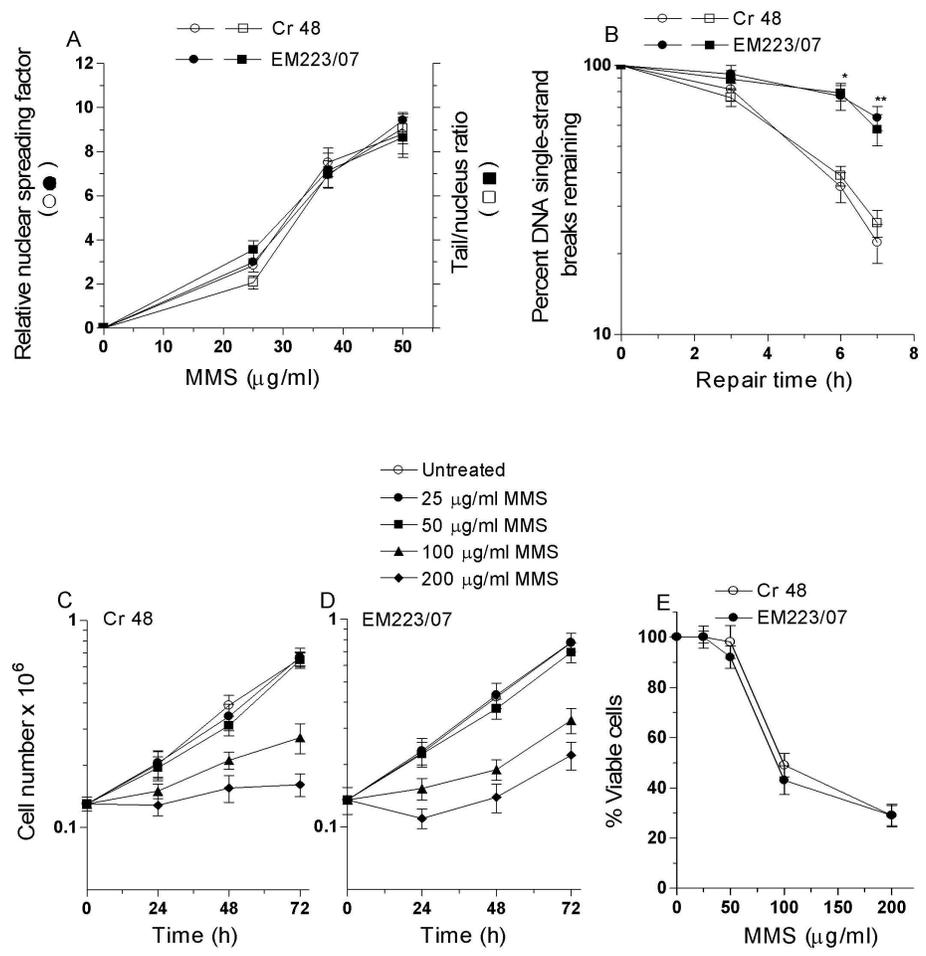


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## Supplementary material

### Clinical description of the AOA1 family showed in Fig 1.

The family pedigree in figure 1 shows 2 affected siblings: the proband (II-2) and her brother (II-3). The proband is a 53 years old female born to non-consanguineous parents, at term following an uncomplicated pregnancy. From the age of 6 she presented slurred speech, frequent falls, progressively more severe walking difficulties, leading in 2 years to the need of assistance in walking and to complete loss of ambulation by the age 18.

Neurological examination at the age of 14 showed ataxic gait possible only with bilateral help, gross dysmetria of voluntary movements, diffuse distal leg and hand hypotrophy, absence of deep tendon reflexes (DTR), pes cavus, with intact strength and sensation. There was marked dysarthria and difficulties in visual pursuit with ocular esotropia. Cognition was intact. The disease progressed slowly and at the last examination (at 52 years of age) the patient presented flaccid tetraplegia, severe hypotrophy, hypotonia and areflexia. The difficulties in visual pursuit and the dysarthria were unchanged.

Blood exams were remarkable for hypoalbuminemia (1.6 g/l, n.v. 3.4-4.8), hypercholesterolemia (344mg/dL, n.v. <200), high  $\alpha$ 1-fetoprotein (12.1ng/ml, n.v. <10)

Neurophysiology showed severe axonal neuropathy more severe at the lower limbs. Needle EMG was compatible with severe chronic denervation. Acoustic and visual evoked potential were within the normal range. MRI imaging showed moderate to severe cerebellar atrophy.

II-3. The clinical history of the second born of this family was quite similar to the elderly sister, presenting his first motor and coordination problems at school age, and progressing to flaccid tetraplegia by the age of 25. When first examined at age 46 he was tetraplegic, with severe hypotrophy and hypotonia, absent DTRs. Cognition and sensation were preserved. His oropharyngeal and laryngeal function was severely impaired, resulting in dysarthria, dysphonia and dysphagia causing recurrent respiratory infections. Ocular movements were mildly impaired, with slow saccades and minimal nystagmus. MRI imaging at age 36 showed moderate cerebellar hypotrophy. Blood tests showed hypoalbuminemia (2.4 g/l, n.v. 3.4-4.8) and elevated  $\alpha$ 1-fetoprotein (15.5, n.v. <10). The patient died for respiratory infection at age 49.

## Supplementary Methods

### DNA deadenylation reaction

For analysis of mutant aprataxin deadenylation activity (removal of an adenylated residues at 5'-end of a single strand break, SSB) we used a 5'-AMP and 3'-FAM labeled 23-mer oligonucleotide (AMP-23-FAM; 5'-App-TCTAGCACTTGAGGCTATCCATG-FAM-3'), a 3'-hydroxyl 21-mer oligonucleotide (21-OH; 5'-CTACGTCAGATCTGCGGATGT-3') and a complementary 45-mer oligo (45-mer; 5'-CATGGATAGCCTCAAGTGCTAGAGACATCCGCAGATCTGACGTAG-3') (Eurogentec, Seraing, Belgium) as described (Takahashi et al., 2007, Reynolds et al., 2009b). Oligonucleotides were annealed to produce a duplex substrate with an adenylated single strand break and a 1-bp gap with a 3'-hydroxyl terminus (OH).

Cell extracts were prepared from Cr 48 and EM223/07 lymphoblast cell lines. Cells lysis was performed in 20 mM Tris-HCl pH 7.5, 10 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail (Sigma-Aldrich, San Louis, MO, USA), by incubation on ice for 10 minutes and then clarified by centrifugation. For DNA adenylate hydrolysis assay 0,1  $\mu$ g, 1  $\mu$ g or 5  $\mu$ g of lymphoblast cell extracts were incubated with 100 nM of annealed oligonucleotides for 1 h at 30°C in 25 mM Hepes pH 8.0, 130 mM KCl, 1 mM DTT, 10 mM MgCl<sub>2</sub>, 100  $\mu$ M dNTPs and 1 mM ATP. Reactions were stopped by addition of an equal volume of formamide gel loading buffer and incubation at 95°C for 5 min. Products were fractionated on a 15% denaturing polyacrylamide/urea gel and images were acquired with a GelDoc-It-UVP (EuroClone, Milano, Italy) using an UV transilluminator and an emission filter (513-557 nm).

**Table 1S** Non-protein thiol content and catalase, glutathione peroxidase or superoxide dismutase activities in Cr 48 and EM 223/07 cells

	<b>Cr48</b>	<b>EM223/07</b>
<b>Non-protein thiols</b> (nmol/mg protein)	21.9 $\pm$ 2.2	19.8 $\pm$ 1.0
<b>Catalase</b> (U/mg protein)	4.08 $\pm$ 0.2	3.07 $\pm$ 0.4*
<b>Glutathione peroxidase</b> (mU/mg protein)	41.8 $\pm$ 3.9	43.2 $\pm$ 4.7
<b>Superoxide dismutase</b> (U/mg protein)	12.2 $\pm$ 1.3	12.5 $\pm$ 1.4

Results are means  $\pm$  S.E.M. from five separate experiments. \* P < 0.01 as compared to Cr 48 cells (one-way ANOVA followed by Dunnett's test).

### Legends to supplementary figures

**Figure 1S. 5'-AMP single strand break (SSB) repair in control and AOA1 lymphoblastoid cell lines.** (Top) Cartoon of the duplex substrate used in the experiment with a 5'-AMP terminus, a 1-bp gap and a 3'-hydroxyl terminus. (Bottom) 0,1  $\mu$ g, 1  $\mu$ g or 5  $\mu$ g of Cr 48 or EM223/07 lymphoblast cell extracts were incubated with a FAM labeled 5'-AMP SSB substrate for 1h at 30°C. Reaction products were fractionated on a 15% denaturing polyacrilamide/urea gel and image was acquired with a GelDoc-It-UVP. The AMP-23-FAM oligonucleotide was fractionated in parallel as size marker (Mk). The position of the repaired 45-FAM oligonucleotide is indicated. The position 23-FAM indicates both AMP-23-FAM and 23-FAM generated from AMP adenylation in Cr 48 extracts, as shown in the lower part of the gel as separated bands with a longer gel run.

### Figure 2S. Toxicity induced by high concentrations of H<sub>2</sub>O<sub>2</sub> in Cr 48, AOA1-EM223/07 and AOA2-EM518 cells.

Cells were exposed to increasing concentration of H<sub>2</sub>O<sub>2</sub> for 30 min in saline A at 37°C and post-incubated for 30 min in fresh culture medium. Toxicity was then assessed by using the trypan blue exclusion assay. Results represent the means  $\pm$  SD calculated from three to five separate experiments. \*P < 0.001 as compared to Cr 48 cells (one-way ANOVA followed by Dunnett's test).

Figure 1S

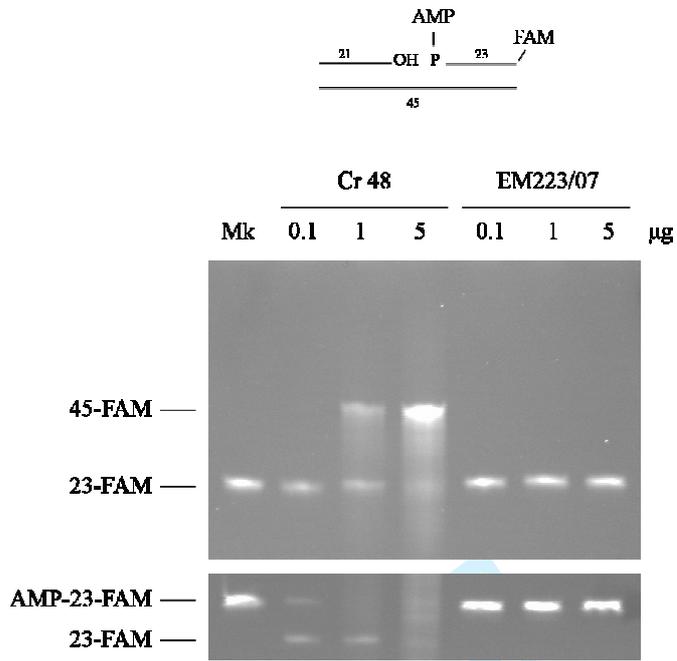


Figure 2S

