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AIF suppresses chemical stress-induced apoptosis and maintains the transformed state of tumor cells

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Apoptosis-inducing factor (AIF) exhibits reactive oxygen species (ROS)-generating NADH oxidase activity of unknown significance, which is dispensable for apoptosis. We knocked out the *aif* gene in two human colon carcinoma cell lines that displayed lower mitochondrial complex I oxidoreductase activity and produced less ROS, but showed increased sensitivity to peroxide- or drug-induced apoptosis. AIF knockout cells failed to form tumors in athymic mice or grow in soft agar. Only AIF with intact NADH oxidase activity restored complex I activity and anchorage-independent growth of *aif* knockout cells, and induced *aif*-transfected mouse NIH3T3 cells to form foci. AIF knockdown in different carcinoma cell types resulted in lower superoxide levels, enhanced apoptosis sensitivity and loss of tumorigenicity. Antioxidants sensitized AIF-expressing cells to apoptosis, but had no effect on tumorigenicity. In summary, AIF-mediated resistance to chemical stress involves ROS and probably also mitochondrial complex I. AIF maintains the transformed state of colon cancer cells through its NADH oxidase activity, by mechanisms that involve complex I function. On both counts, AIF represents a novel type of cancer drug target.

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Introduction

A single X-linked mammalian gene *aif* encodes a 57-kDa mitochondrial flavoenzyme, apoptosis-inducing factor, homologues of which are present in human, mouse, *Caenorhabditis elegans* and probably yeast (Susin *et al*, 1999; Joza *et al*, 2001; Lipton and Bossy-Wetzel, 2002; Wissing *et al*, 2004) apoptosis-inducing factor (AIF) is released from mitochondria to the cytoplasm of many mammalian cells induced to undergo apoptosis, where it combines with cyclophilin A to form an active DNase, translocates to the nucleus and contributes to nuclear DNA fragmentation and chromatinolysis (Loeffler *et al*, 2001; Cande *et al*, 2004a). This death pathway is conserved in *C. elegans*, where the AIF homologue WAH-1 functionally associates with the mitochondrial DNA endonuclease CPS-6/Endo G. Inhibition of WAH-1 delayed the normal progression of apoptosis during development (Wang *et al*, 2002). Knockout of the *aif* gene in mouse embryonic stem (ES) cells is lethal, being associated with a defect in sculpting of the early embryo and reduced susceptibility of the cells to serum withdrawal (Joza *et al*, 2001). Thus, AIF/WAH-1 appears to play crucial roles in programmed cell death during early animal development.

The crystal structures of mouse and human AIF revealed two important regions: a domain required for DNA fragmentation (a putative DNA-binding site) and a domain with homology to a bacterial NADH-dependent ferredoxin reductase that is structurally similar to the eukaryotic glutathione reductase family of enzymes (Mate *et al*, 2002; Ye *et al*, 2002). In functional *in vitro* assays, AIF acts as an NADH oxidase, accepting electrons from NADH, and transferring them to molecular oxygen to form the superoxide (O_2^-) free radical, which subsequently undergoes dismutation to H_2O_2 (Miramar *et al*, 2001). The NADH oxidase and DNA fragmenting activities of AIF are completely independent, suggesting that the NADH oxidase activity of AIF is not required for apoptosis (Ye *et al*, 2002).

The first evidence that AIF has a protective function in some cell types came from studies on the Harlequin mutant mouse, which has an 80% reduction in AIF, but appears normal except for premature neurodegeneration and increased peroxide sensitivity in specific subsets of neurons in adult animals (Klein *et al*, 2002). The precise mechanism of action of AIF in free radical scavenging and counteracting oxidative stress in this mouse model remains to be established. More recently, it has been shown that a redox-active domain of AIF and reduced glutathione are required for the inhibition of cytoplasmic stress granule formation under conditions of chemical stress, suggesting that AIF is involved in an adaptive response (Cande *et al*, 2004b). Interestingly, AIF deficiency compromises oxidative phosphorylation (OXPHOS) by inhibiting respiratory chain complex I *in vitro* and *in vivo*, revealing a 'life' function for AIF (Vahsen *et al*, 2004). However, the mechanistic connections between the redox activity of AIF, OXPHOS and cell survival remain unclear.

Here, we investigate the role of the NADH oxidase activity of AIF by knocking out the gene in different human colon cancer cell lines, and analyzing the phenotype of AIF knockout cells re-expressing either AIF or mutant AIF deficient in NADH oxidase activity.

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Results

Generation of AIF-deficient carcinoma cell lines

We generated viable AIF-null human colon carcinoma cell lines by modifying the single X-linked locus of the human *aif* gene using bipartite promoter-less targeting vectors (Jallepalli *et al*, 2001) (Figure 1A). The human colon cancer cell lines HCT116 and DLD-1 are near diploid with a single X chromosome and have been used extensively for gene targeting (Sedivy *et al*, 1999). $aif^{-/y}$ knockout clones were identified by PCR analysis of genomic DNA using two unique sets of primers. One primer set detected only the correct integration of the targeting vector reporter gene (hygromycin, *hyg*) together with the deletion of the first exon, while the other set detected only the original configuration of the human *aif* gene (Figure 1B and C). Lack of *aif* expression in two independent clones derived from both cell lines was further confirmed by RT–PCR of the exon 1–exon 2 boundaries of *aif* mRNA (Figure 1D). Western blot analysis demonstrated AIF protein in the isogenic control cell lines and the complete absence of AIF in all knockout cell lines (Figure 1E).

To extend our studies to other tumor types, we generated stable AIF knockdown cell lines via siRNA in colon cancer cell line SW480, breast cancer line MCF-7 and lung cancer cell line A549. Two independent siRNA knockdown clones for each cell line showed 50–80% reduction in AIF protein (Supplementary Figure S1A).

Decreased superoxide (O_2^-) and reactive oxygen species in AIF-deficient tumor cell lines

AIF has been reported to exhibit *in vitro* NADH oxidase activity that generates O_2^- and subsequently H_2O_2 by dismutation of O_2^- (Miramar *et al*, 2001), and therefore the lack of AIF might be expected to lower reactive oxygen species (ROS) levels. On the other hand, AIF has been reported to behave as

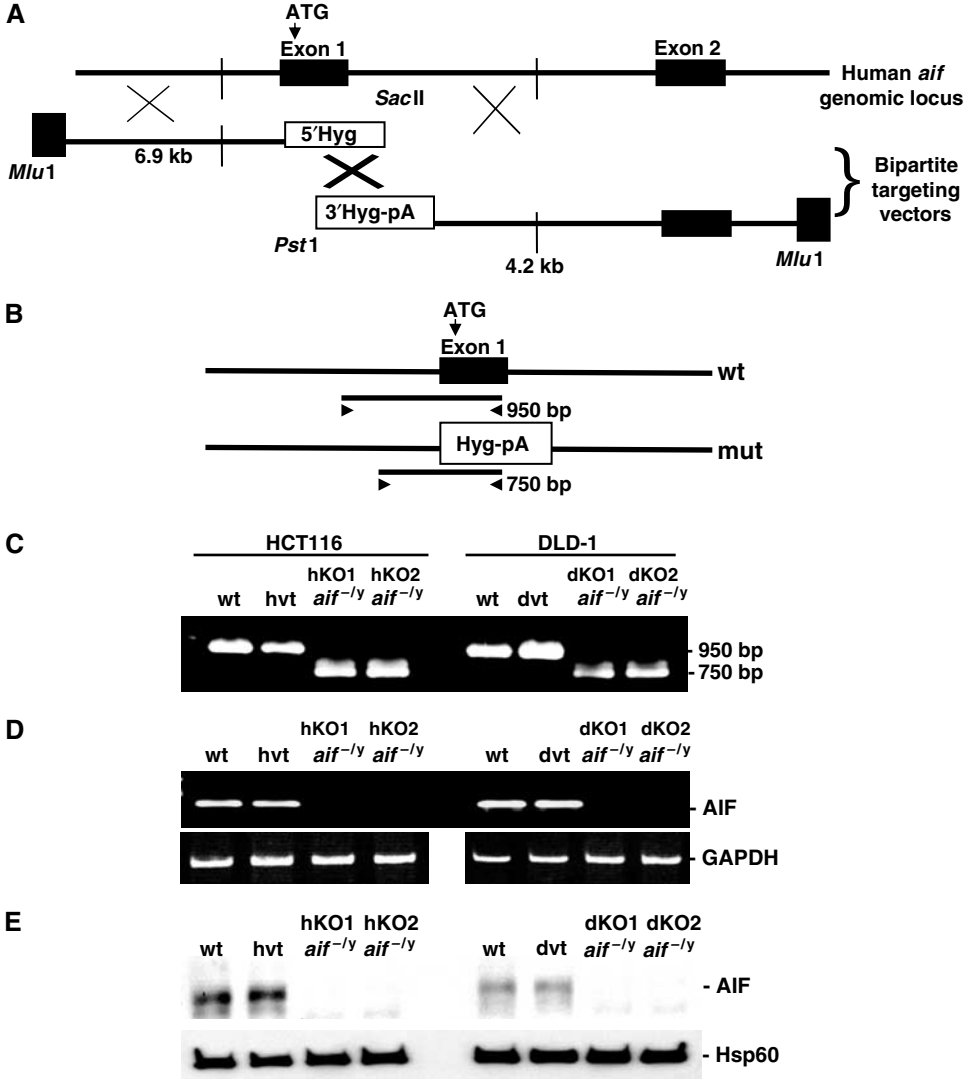

Figure 1 Generation of AIF knockout cell lines by homologous recombination. (**A**) Schematic representation of bipartite promoter-less targeting vector. Hyg, hygromycin; p-A, poly(A). (**B**) Two different primer pairs for genomic DNA PCR assay for correct gene targeting. (**C**) Genomic DNA from HCT116 and DLD-1 cells was analyzed by PCR using the primers described in panel B. As a control, the wild-type locus of AIF was amplified together with the diagnostic PCR for integration of the promoter-less Hyg-pA cassette. hvt and dvt refer to vector controls for HCT116 and DLD-1 cells, respectively. hKO and dKO refer to HCT116 and DLD-1 knockout cells, respectively. (**D**) *aif* mRNA-specific RT–PCR analysis of exon 1–exon 2 boundary confirming lack of *aif* expression in HCT116 and DLD-1 clones. (**E**) Western blot analysis with human AIF antibodies to confirm the absence of AIF in knockout cells. Hsp60, a mitochondrion-specific protein, was used as a loading control.

an antioxidant protein that suppresses oxidative stress in neurons (Klein *et al*, 2002). AIF is required for the proper assembly or function of mitochondrial complex I (Vahsen *et al*, 2004), and its loss might affect ROS levels as a result of altered leakage of O_2^- from a defective complex I. To explore these possibilities in carcinoma cells, we first assessed intracellular O_2^- rigorously by employing three different methods to overcome limitations associated with measuring the short-lived O_2^- radical with one method only. Using assays for aconitase (which is inhibited by O_2^-), independent clones of both AIF knockout cell lines exhibited higher aconitase activity than the parental and vector control cell lines, reflecting 20–40% less intracellular O_2^- (Figure 2A). Similar results were obtained when O_2^- production was assessed with lucigenin (Figure 2B) and NBT reduction (data not shown). Similar decreases in intracellular O_2^- were also observed in the three different AIF knockdown cell types (Supplementary Figure S1B). Consistent with the O_2^- data, we demonstrated corresponding decreases in ROS in both AIF knockout colon cancer cell lines (Figure 2C) and in AIF knockdown colon and breast tumor cell lines (Supplementary Figure S1C). Thus in human carcinoma cells, AIF contributes significantly to

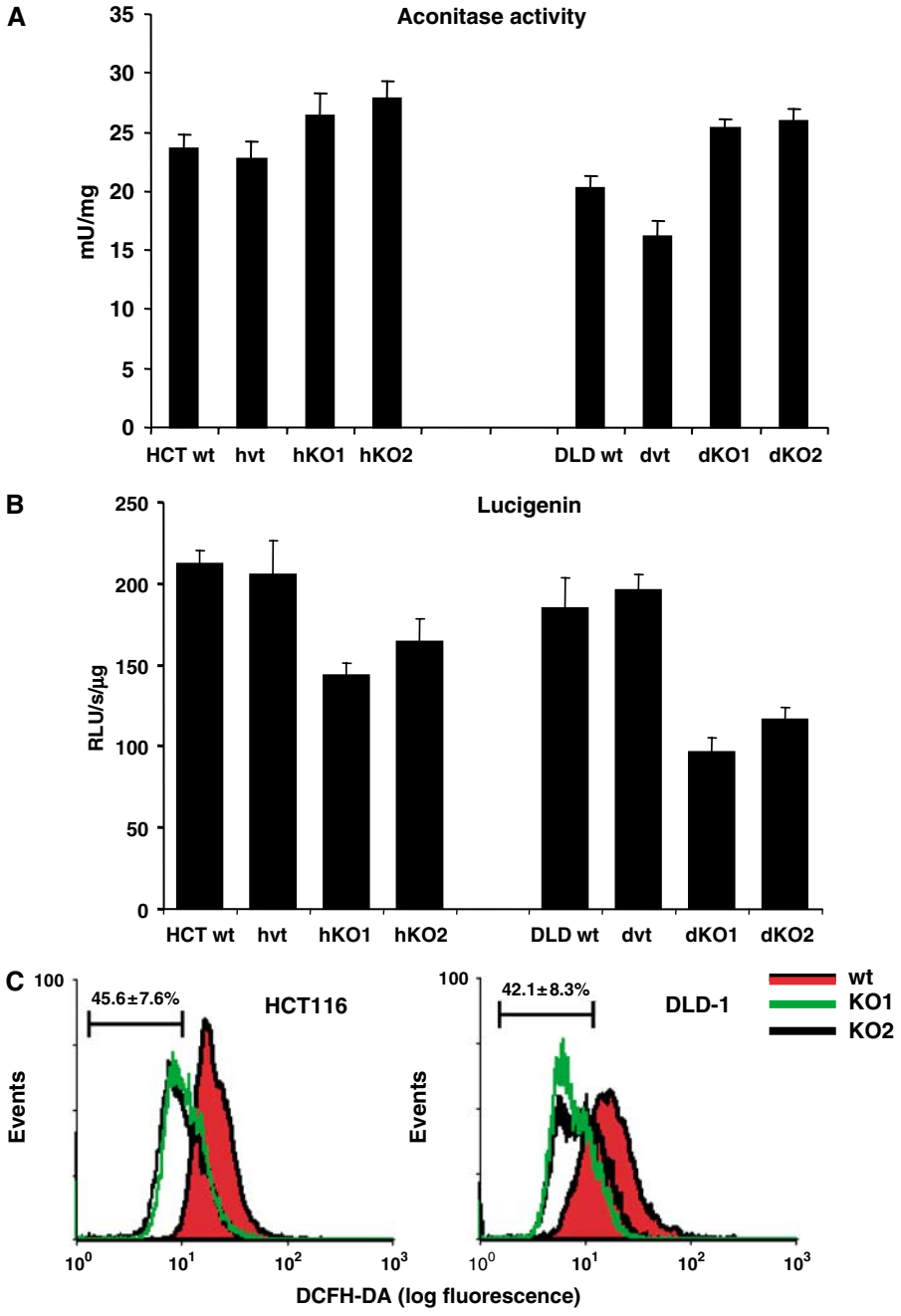

Figure 2 Reduction in O_2^- and ROS in AIF knockout cell lines. Relative O_2^- levels were measured using aconitase (**A**) and lucigenin (**B**) assays in AIF knockout (hKO, dKO) clones derived from HCT116 and DLD-1 cell lines, and are means ± s.d. of three independent experiments (note that increased aconitase activity but decreased lucigenin activity both reflect reduction in O_2^-). (**C**) Flow cytometry analysis of relative intracellular ROS levels detected by measuring oxidized dichlorodihydrofluorescein diacetate (H_2DCFH-DA) in two independent knockout clones compared to the wild-type HCT116 and DLD-1 cells. Values represent % of AIF knockout cells having decreased ROS compared to wild-type cells. Graphs are representative of three independent measurements.

intracellular O_2^-, either directly through its NADH oxidase activity or indirectly through leakage of O_2^- from AIF-mediated mitochondrial complex I function (or both). Either way, the generated O_2^- would undergo dismutation to H_2O_2 in most cell lines, accounting for the elevated ROS.

AIF-deficient cell lines show increased sensitivity to DNA-damaging agents and oxidative stress

Surprisingly, all AIF knockout clones derived from HCT116 and DLD-1 showed a three- to five-fold increased sensitivity to the DNA-damaging agents campthothecin and etoposide, as well as the oxidative stress agents H_2O_2 and *t*-butylhydroperoxide, as compared to the AIF-expressing cell lines (Figure 3A and B). Likewise, *aif* siRNA knockdown colon, lung and breast carcinoma cell lines were markedly more sensitive to etoposide and *t*-butylhydroperoxide than the parental cell lines (Supplementary Figure S2A and B). Evaluation of apoptosis using caspase-3 assays and annexin-V or morphological staining using Sytox-Hoechst dyes confirmed the greatly increased sensitization of both AIF knockout colon

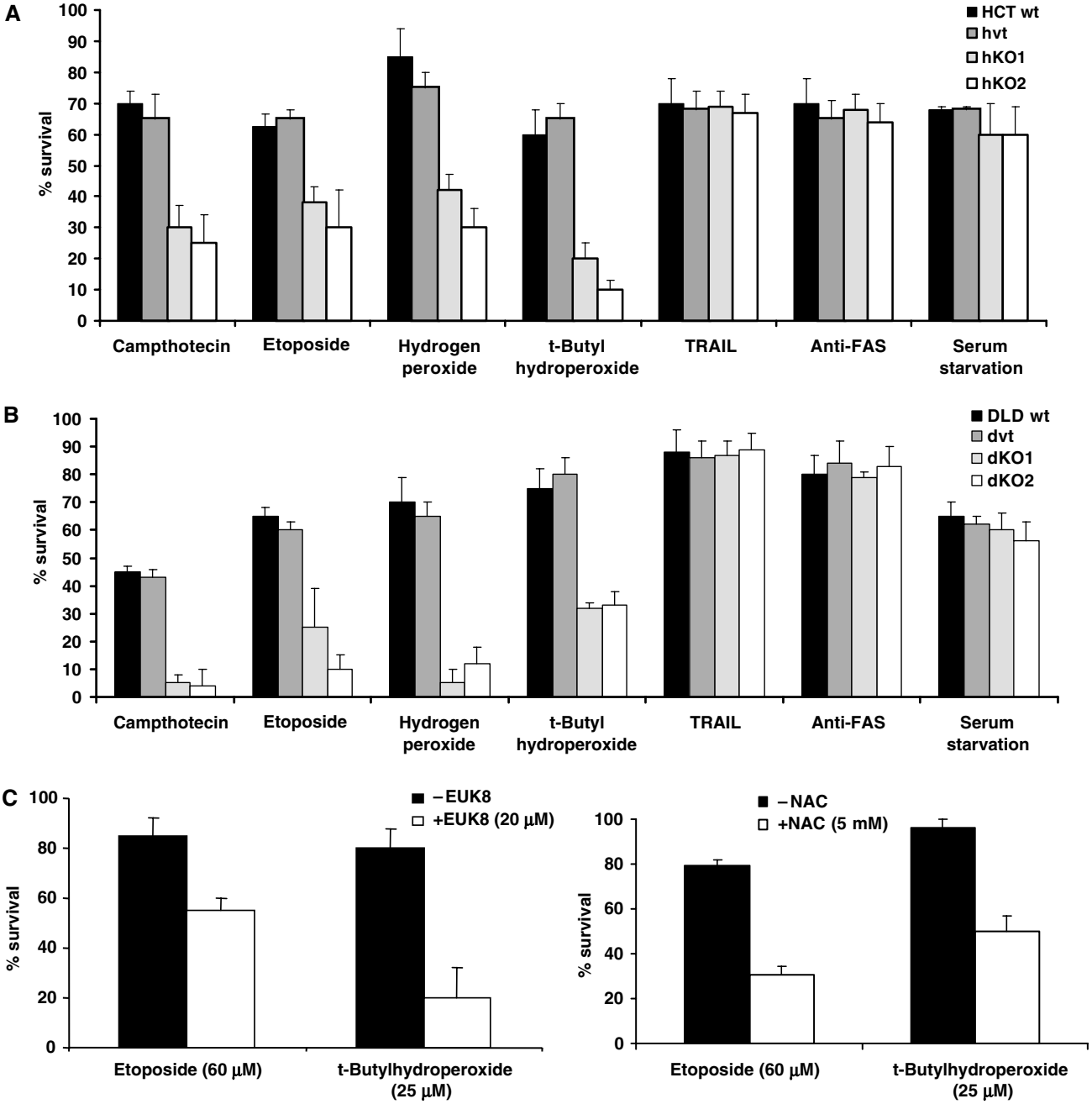

Figure 3 Increased sensitivity of AIF knockout cell lines to cancer drugs and oxidative stress. (**A**) Cytotoxicity assays for HCT116 wild-type and vector control (hvt) cells, and counterpart AIF knockout cell lines (hKO1, hKO2) after exposure to 60 μM campthothecin, 60 μM etoposide, 500 μM hydrogen peroxide, 25 μM *t*-butylhydroperoxide, 25 ng/ml TRAIL, 0.5 μg/ml anti-FAS or 4 days serum starvation. (**B**) Cytotoxicity assays for DLD-1 wild-type and vector control (dvt) cells, and counterpart AIF knockout clones (dKO1, dKO2) after exposure to 5 μM campthothecin, 2.5 μM etoposide, 250 μM hydrogen peroxide, 12.5 μM *t*-butylhydroperoxide, 25 ng/ml TRAIL, 0.5 μg/ml anti-FAS or 4 days serum starvation. (**C**) Cytotoxicity assays for HCT116 cells pre-exposed to EUK-8 (20 μM) and NAC (5 mM) overnight prior to treatment with 60 μM etoposide or 25 μM *t*-butylhydroperoxide. In panels A–C, surviving cells were detected by incubation with MTS/PES (Promega Cell Titer Proliferation Assay) and expressed as a % of untreated cells and are means ± s.d. of three independent experiments.

carcinoma cell lines to drug and oxidative stress-induced apoptosis (Figure 4A–C). In contrast, the knockout cells showed no alterations in cell death sensitivity with TRAIL, anti-Fas or serum starvation (Figure 3A and B), or with staurosporine or actinomycin D (data not shown).

To further investigate the link between decreased O_2^-/ROS and increased sensitivity to DNA-damaging agents and oxidative stress, we exposed parental HCT116 cells to the general antioxidant *N*-acetylcysteine (NAC) and the MnSOD mimetic (O_2^- scavenger) EUK-8, which decreased intracellular ROS by 23% (±5%) and O_2^- by 30% (±6%), respectively. This is comparable to the reduction in O_2^-/ROS observed in AIF-deficient cells (Figure 2A–C). Exposure of HCT116 cells pretreated with NAC or EUK-8 to etoposide or *t*-butylhydroperoxide resulted in increases in sensitivity in the 1.5- to 3-fold range (Figure 3C), which reiterates the effects of curbing AIF expression, and establishes a closer link between a pro-oxidant state and resistance to apoptosis in these tumor cells.

Dramatic loss of tumorigenicity of AIF-deficient carcinoma cell lines

To assess tumorigenicity, we grew the AIF knockout cells in anchorage-independent conditions using the standard soft agar assay. Remarkably, the four independent AIF knockout clones derived from HCT116 and DLD-1 cells showed a substantial 5- to 20-fold decrease in colony growth in soft agar compared to the AIF-expressing cells (Figure 5A and B). Comparable large decreases in soft agar colony formation were observed with AIF siRNA knockdown clones from the colon, lung and breast cancer cell lines (Supplementary Figure S2C). However, the antioxidant NAC and the O_2^- scavenger EUK-8 both failed to curb growth of HCT116 or DLD-1 cells in soft agar at concentrations that cause a 30% or greater reduction in ROS (data not shown). Subsequently, we injected the HCT116 and DLD-1 wild-type and AIF knockout cells subcutaneously into nude mice to determine the *in vivo* tumorigenicity of the AIF knockout cell lines. The two independent clones from both knockout cell lines exhibited dramatic reduction or absence of tumor growth *in vivo* compared to the wild-type and vector control cell lines, even when the AIF-expressing and knockout cells were injected on opposite flanks of the same animal (Figure 5C). Measurements of tumor weight and volume showed an overall 6- to 10-fold decrease in tumor sizes for the AIF knockout cells (Figure 5D). These data demonstrate a dramatic loss of tumorigenicity of AIF-deficient carcinoma cells that does not appear to depend on an overall reduction in cellular ROS.

Deficiency in mitochondrial complex I in AIF knockout cells

As previously reported for ES and HeLa cells (Vahsen *et al*, 2004), the absence of AIF in HCT116 and DLD-1 cells resulted in partial reduction (HCT116) or loss (DLD-1) of several complex I proteins (Figure 6A). In agreement and as measured by three independent assays, mitochondrial complex I activity (an indirect indicator of AIF function) was 40–60% lower in AIF knockout HCT116 and DLD-1 cells (Figure 6B and Supplementary Figure S3). In contrast, citrate synthase activity, which assesses mitochondrial redox status independently of OXPHOS, was unchanged in independent clones representing both AIF knockout cell lines (Figure 6C). These results indicate serious defects in complex I in AIF-null colon cancer cell lines.

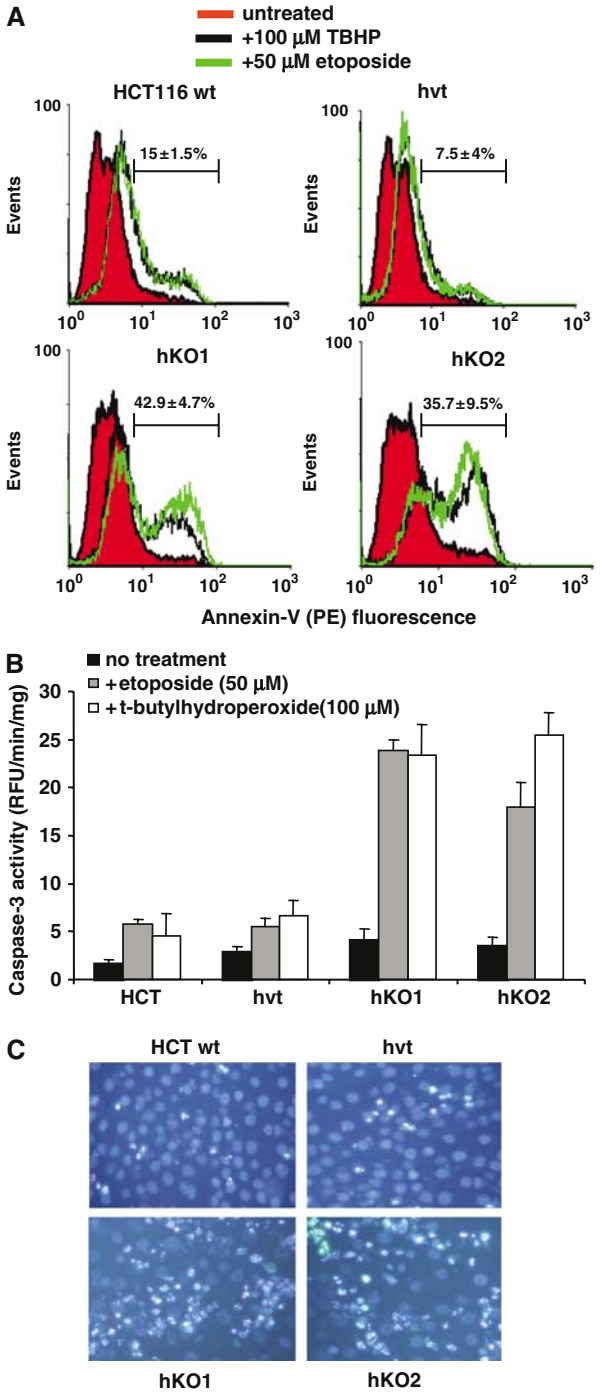

Figure 4 Increased sensitivity of *aif*$^{-/y}$ HCT116 cells to apoptosis. (**A**) Flow cytometry analysis of HCT116 wild-type cells, vector control (hvt) cells and two AIF knockout clones stained with Annexin-V (PE) after 16 h exposure to 100 μM *t*-butylhydroperoxide or 50 μM etoposide. (**B**) Caspase-3 activity in HCT116 and two counterpart AIF knockout clones after 16 h exposure to 100 μM *t*-butylhydroperoxide or 50 μM etoposide. (**C**) Fluorescence microscopy of HCT116 cells and two counterpart AIF knockout clones stained with Sytox/Hoechst dye after exposure to 50 μM etoposide for 48 h.

NADH oxidase activity of AIF is required to restore tumorigenicity and complex I activity in AIF knockout cells

Exhaustive attempts to generate stable knockout cell lines re-expressing AIF were unsuccessful. We attributed this to the

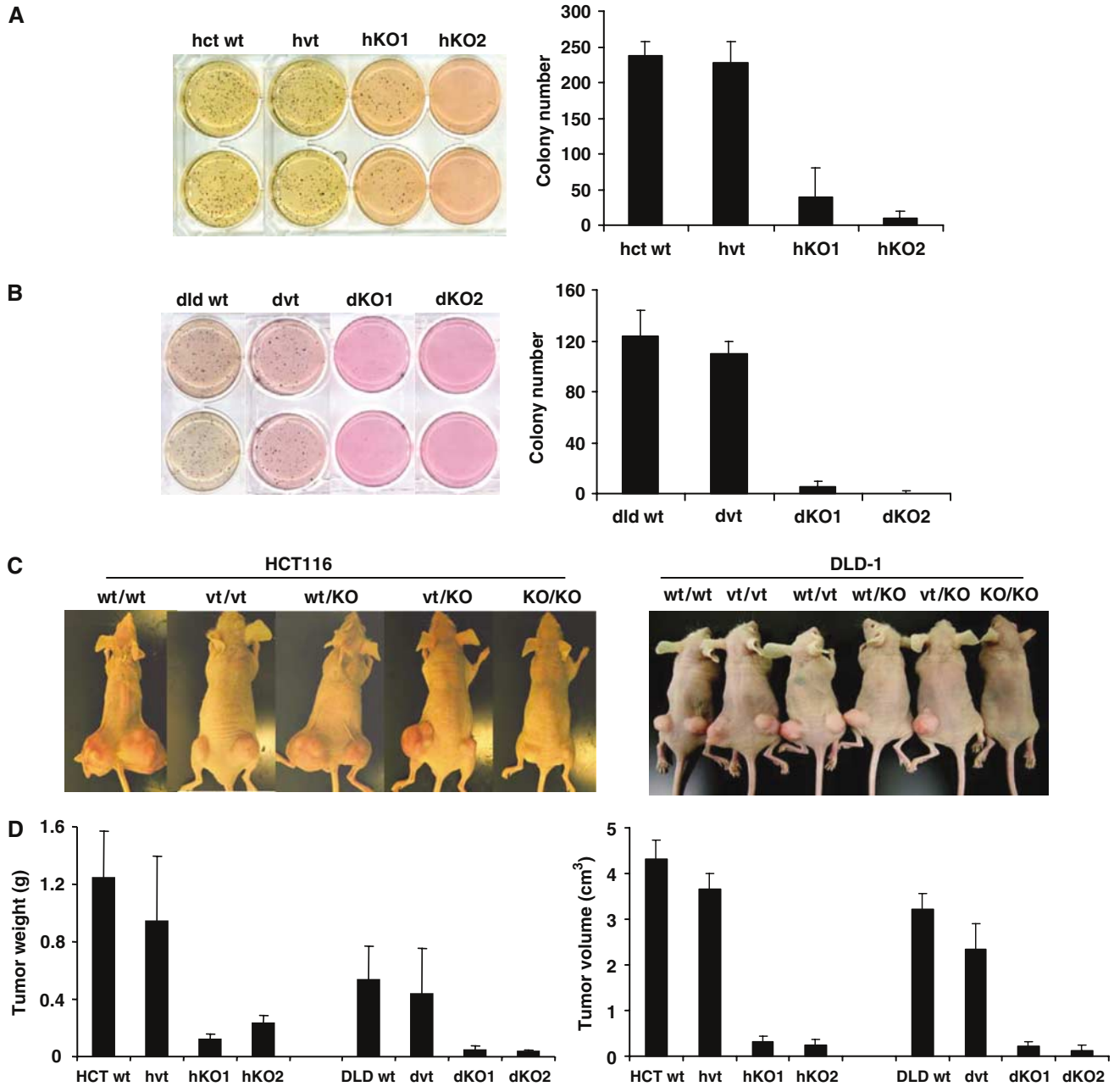

Figure 5 Dramatic loss of *in vitro* anchorage-independent growth and *in vivo* tumorigenicity of *aif*$^{-/y}$ colon cancer cells. (**A, B**) Representative soft agar assays in duplicate (14 days incubation) for HCT116 cells (A, left panels) and DLD-1 cells (B, left panels) and their respective counterpart AIF knockout (KO) cells. Histograms (A and B, right panels) show numbers of colonies formed and are means ± s.d. of three independent experiments. (**C**) HCT116 and DLD-1 cells and their counterpart AIF knockout (KO) cells were injected as xenografts in nude mice, which were killed after 3 weeks (HCT116) and 5 weeks (DLD-1). (**D**) Histograms show data points representing average tumor weights (left panel) and volumes (right panel) from 15 mice (except dvt = 10 mice) ± s.d.

pro-apoptotic property of AIF, since we found that overexpressed AIF was not localized only to mitochondria, but distributed overwhelmingly in the cytoplasm, where it can readily translocate to the nucleus and initiate DNA degradation (data not shown). A similar phenomenon was reported in HeLa cells expressing exogenous AIF in which mitochondrial AIF leaked spontaneously into the cytoplasm on long-term culture (Loeffler *et al*, 2001). To circumvent the apoptotic function of AIF and test the requirement for its NADH oxidase activity in tumorigenicity, we stably expressed two different classes of mutant AIF-GFP molecules in the AIF knockout cell line hKO1 derived from HCT116. One class had mutations in one or other of the (DNA-binding) domains known to be required for AIF-mediated apoptosis (mDNA1 and mDNA2; Figure 7A). The other class denoted 'double mutants' had the DNA-binding mutations as well as mutations in two amino acids implicated in NADH binding (mDNA1 plus NADH mut; Figure 7A), which compromised the NADH oxidase function of AIF (Supplementary Figure S4) (Mate *et al*, 2002).

Independently isolated clones of all these various mutants gave similar levels of mitochondrial AIF-GFP expression in

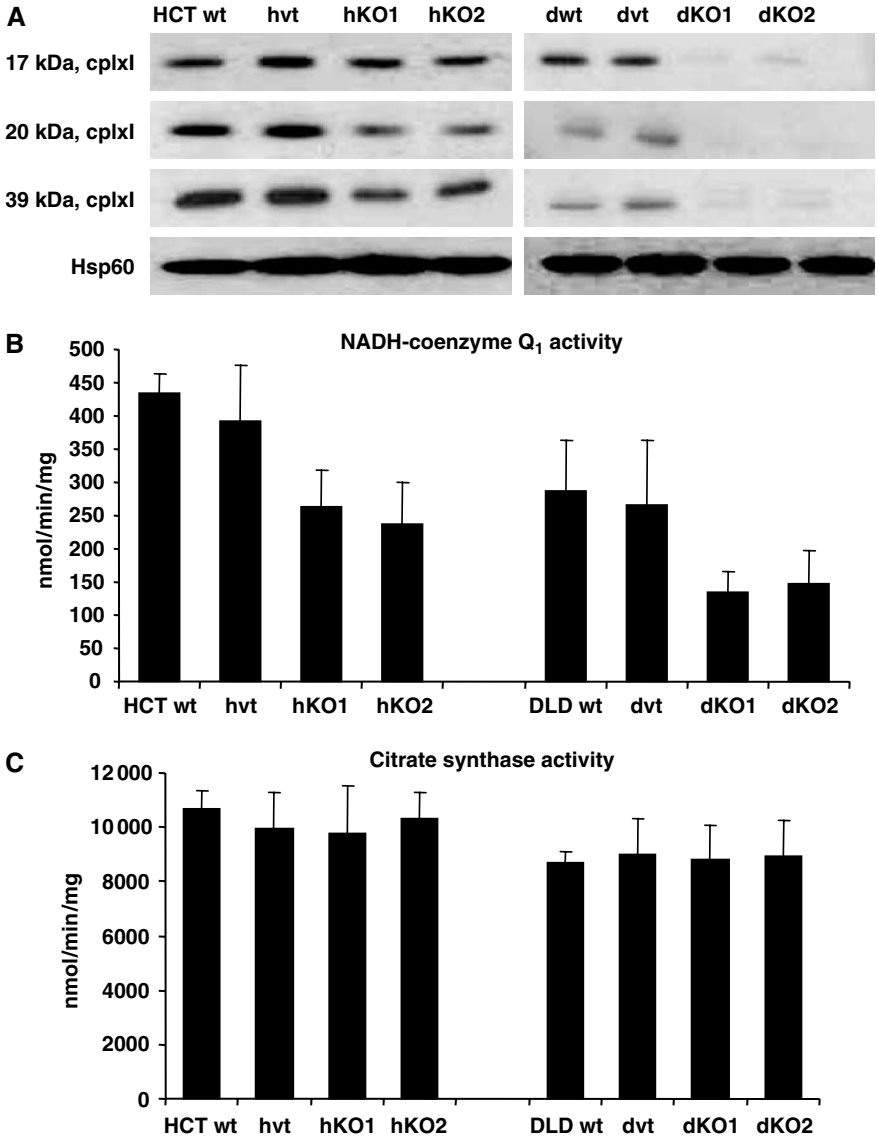

Figure 6 Mitochondrial complex I is compromised in HCT116 and DLD-1 knockout (KO) cells. (**A**) Representative protein analysis showing decrease in yields of three mitochondrial complex I subunits, which were performed using 5 μg of mitochondrial proteins. The loading control is mitochondrial Hsp60. (**B**) Decrease in NADH-coenzyme Q_1 oxidoreductase activity in AIF knockout cell (KO) cells. Graph shows means ± s.d. of three independent experiments. (**C**) Citrate synthase activity in isolated mitochondria from AIF KO cells represented as means ± s.d. of three independent experiments.

aif$^{-/y}$ HCT116 cells (Figure 7B). The nonapoptotic DNA-binding mutants ('DNA muts') were able to restore anchorage-independent growth in soft agar comparable to parental HCT116 cells, which is in sharp contrast to the GFP control or the double mutants ('NADH muts') harboring amino-acid substitutions in the NADH-binding domain as well as in the DNA-binding domain (Figure 7C). Mitochondrial complex I proteins as well as complex I activity (measured by two methods) were re-established in *aif*$^{-/y}$ HCT116 cells stably transfected with the DNA-binding mutants, but not with the double mutants deficient in NADH binding or with GFP alone (Figure 7D and E, and Supplementary Figure S5A). Similar to the independent clones, recovery of colony formation in soft agar was observed with pools of *aif*$^{-/y}$ HCT116 cells stably re-expressing DNA-binding mutants, but not the double mutants defective in NADH binding (Supplementary Figure S5B and C). These data altogether show that NADH oxidase activity of AIF is necessary for its tumorigenic properties and important for mitochondrial complex I activity in HCT116 cells.

NADH oxidase activity of AIF is required for foci formation in NIH3T3 cells

To further investigate whether AIF has transforming properties, we overexpressed the various AIF-GFP mutants in the premalignant mouse cell line NIH3T3, and selected stable cell lines that synthesized similar levels of AIF-GFP protein (Figure 8A). NIH3T3 cells transfected with the non-apoptotic DNA-binding mutants mDNA1 and mDNA2 formed obvious foci, which were 5- to 10-fold higher in number compared with cells transfected with either GFP alone or the double mutants with compromised NADH oxidase activity (Figure 8B and C). NIH3T3 cells transfected with parental AIF coupled to GFP did not form significantly more foci than GFP-expressing cells with background levels of foci

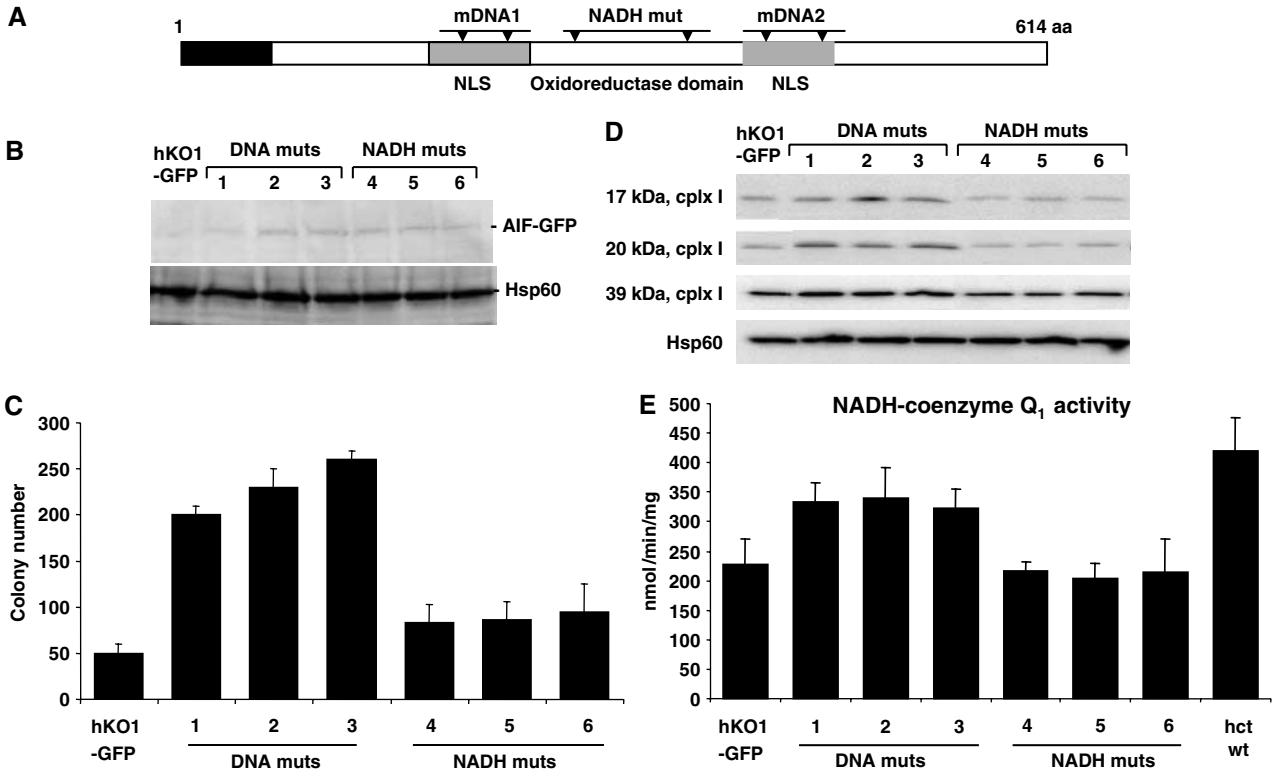

Figure 7 NADH oxidase activity of AIF mutants expressed in *aif*$^{-/y}$ HCT116 (hKO1) cells is required for restoration of anchorage-independent growth and mitochondrial complex I activity. (**A**) Locations of AIF DNA-binding and NADH oxidase mutations. Non-apoptotic DNA-binding mutants mDNA1 and mDNA2 and NADH oxidase-deficient mutant (NADH mut) all have two amino-acid substitutions (arrowed). (**B**) Western blot analysis of mitochondrial AIF-GFP expression levels in *aif*$^{-/y}$ HCT116 (hKO1) cells detected using anti-GFP antibody. DNA muts are three independent clones containing AIF mDNA1 and mDNA2 mutations, NADH muts are three independent clones transfected with *aif* harboring NADH-binding and mDNA1 DNA-binding mutations. hKO1-GFP represents *aif*$^{-/y}$ HCT116 (hKO1) cells transfected with EGFP vector. (**C**) Colony formation in soft agar assays for hKO1 cells transfected with AIF-GFP mutants as above. Histogram shows numbers of colonies formed and are means$\pm$s.d. of three independent experiments. (**D**) Increased expression of mitochondrial complex I proteins in hKO1 cells transfected with DNA-binding mutants but not NADH-binding mutants. (**E**) NADH-coenzyme Q_1 activity in parental HCT116 cells and in hKO1 cells transfected with AIF-GFP mutants or GFP alone. Histogram shows enzyme activities normalized against amounts of total proteins and are means$\pm$s.d. of three independent experiments.

(Figure 8C). Fluorescence microscopy demonstrated 100% colocalization of AIF-GFP mutants (but not GFP alone) with the foci (Figure 8D). These experiments (and those involving reintroduction of *aif* into AIF knockout HCT116 cells; Figure 7) suggest that AIF has latent transforming properties revealed either by artificial mutagenesis or inherent suppression of its pro-apoptotic function in tumor cells. These latent transforming properties are a reflection of the involvement of AIF in the maintenance of tumorigenicity of established cancer cells. Moreover, the NADH oxidase activity of AIF is required for foci formation in NIH3T3 cells.

Discussion

AIF contributes to pro-oxidant state of carcinoma cells, and NADH oxidase activity of AIF is important for mitochondrial complex I activity

Dying neurons of the AIF-deficient Harlequin mutant mouse exhibited increased oxidative stress, and it was proposed AIF acts as a free radical scavenger (Klein *et al*, 2002). No differences in oxidative damage or ROS levels were found in wild-type and *aif*$^{-/y}$ mouse ES cells, and it was concluded that AIF does not behave as an antioxidant protein in these cells (Vahsen *et al*, 2004). In contrast, we showed that knockout or knockdown of the *aif* gene in tumor cell lines of various tissue origins generally resulted in a marked reduction in both O_2^- and ROS levels. AIF contributes to the activity of mitochondrial complex I and consequently to energy production via OXPHOS (Vahsen *et al*, 2004). In agreement, we found that complex I activity was much reduced in AIF knockout cells, but was restored with stable transfection of *aif* cDNA encoding mutant AIF lacking pro-apoptotic function. Complex I activity was not, however, restored with NADH-binding mutants of AIF, indicating that the NADH oxidase activity of AIF is important for complex I function. Thus, AIF normally contributes a significant amount of O_2^- in various carcinoma cell types, which could be derived directly from the NADH oxidase activity of AIF (Miramar *et al*, 2001) or indirectly as a result of AIF-dependent complex I activity causing leakage of O_2^- from the respiratory chain (or both) (Vahsen *et al*, 2004).

AIF suppresses apoptosis of carcinoma cell lines—role of a pro-oxidant state and complex I

We showed that AIF-deficient cell lines (representing colon, breast and lung tumors) were all sensitized to apoptosis induced by peroxides and some cancer drugs. We found no evidence for an essential cell death promoting function for

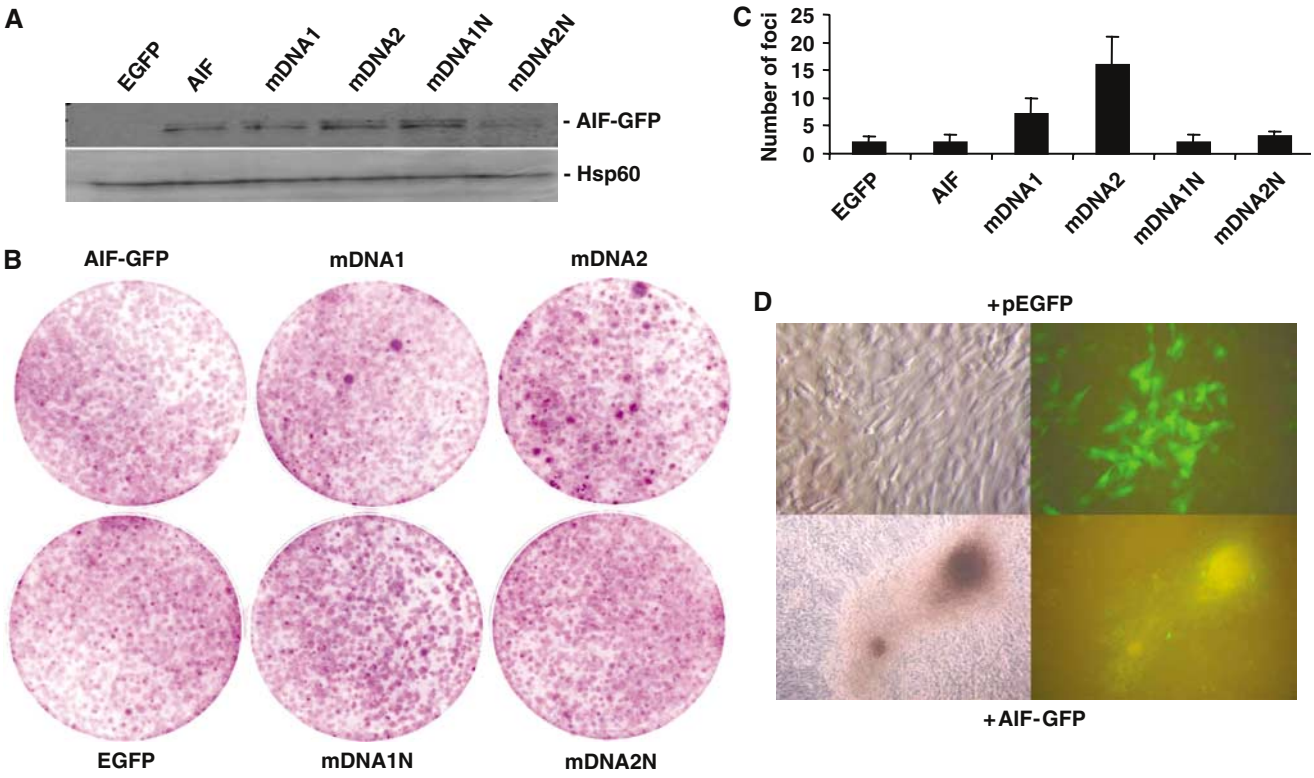

Figure 8 Stable transformation of NIH3T3 cells by AIF-GFP DNA-binding mutants with NADH oxidase activity. (**A**) Expression of AIF-GFP mutants stably transformed in NIH3T3 cells detected by Western blot using anti-GFP antibody. mDNA1 and mDNA2, non-apoptotic mutants; mDNA1N and mDNA2N, nonapoptotic plus NADH oxidase mutants. (**B**) Representative plates showing foci formation in NIH3T3 cells stably transfected with AIF-GFP mutants. (**C**) Histogram shows numbers of foci formed in panel B. Values are means $\pm$ s.d. of three independent experiments. (**D**) Phase-contrast and fluorescence microscopy of GFP and AIF-GFP mutants showing colocalization of AIF-GFP expression and foci only in AIF-GFP-transfected NIH3T3 cells.

AIF in carcinoma cells; on the contrary, AIF appears to suppress cell death in the carcinoma cell types we tested. Although much evidence exists for an evolutionarily conserved pro-cell death function for AIF in development (Joza *et al*, 2001; Wang *et al*, 2002), and for a proapoptotic signaling role for AIF in nuclear chromatinolysis (Cande *et al*, 2004a), it is likely that in some scenarios, the survival function of AIF is dominant over its death-inducing activity. Consistent with these conclusions and our results, only certain neurons in the AIF-deficient Harlequin mouse were more prone to H_2O_2-induced cell death (Klein *et al*, 2002). Thus, the precise involvement of AIF in regulating the life–death balance may be rather specific for the cell type and death inducer.

How might a reduction in intracellular ROS (especially O_2^-) be reconciled with the increased susceptibility of AIF-deficient tumor cells to stress-induced apoptosis that we demonstrated? Physiological levels of some ROS, particularly O_2^- and H_2O_2, are variously required for cell adhesion, immune functions and certain growth factor-dependent survival pathways (Droge, 2002; Reth, 2002; Finkel, 2003). Many tumor cells exist in a pro-oxidant state that is not necessarily detrimental and does not equate with lethal oxidative stress (Cerutti, 1985; Szatrowski and Nathan, 1991; Oberley, 2002; Go *et al*, 2004). Thus, AIF-negative transformed cells may be more vulnerable to stress if, for example, lower O_2^- (and consequently lower H_2O_2) lessens survival signaling mediated by Ras, NF-KB or AP-1 (Irani *et al*, 1997; Gupta *et al*, 1999; Finkel, 2003). It is known that modest reductions in ROS (O_2^- in particular) can promote apoptosis of diverse tumor cell types (Clement and Stamenkovic, 1996; Lin *et al*, 1999; Pervaiz *et al*, 1999; Vaquero *et al*, 2004). NAD(P)H oxidases are usually the source of protective ROS in these reports, which is in line with our findings (Pervaiz *et al*, 2001; Clement *et al*, 2003; Vaquero *et al*, 2004). In our study, the antioxidant NAC (which reduced ROS) and a SOD mimetic (which reduced O_2^-) both strongly enhanced drug- or peroxide-induced apoptosis of AIF-expressing cells.

Deficiency in mitochondrial respiratory chain complex I (confirmed in the present study) and consequent dependence on the less efficient anaerobic ATP generation (Vahsen *et al*, 2004) provide another not necessarily mutually exclusive explanation for the raised sensitivity of AIF-deficient carcinoma cells to chemical stress-mediated cell death. OXPHOS deficiencies in animals can lead to cell death, particularly in energy demanding cells (Klein *et al*, 2002; Atorino *et al*, 2003; Vahsen *et al*, 2004). Therefore, tumor cells, which are heavily reliant on mitochondrial ATP production, may be more vulnerable to drug and oxidative stress when AIF is absent. Moreover, cells lacking complex I are less able to effect mitochondrial detoxification of H_2O_2 (Atorino *et al*, 2003; Zoccarato *et al*, 2004), which likely contributes to the increased peroxide sensitivity of AIF-deficient cells in our study.

In sum, our findings suggest that AIF helps to counteract chemical stress by mechanisms dependent on a pro-oxidant state and probably also a properly functioning mitochondrial complex I.

AIF maintains the transformed state via its NADH oxidase activity—role of complex I

We found that AIF knockout clones from two distinct colon cancer cell lines, differing in p53 and oncogenic H-ras status, displayed very similar properties characteristic of the loss or reversal of the transformed state. These are lack of anchorage-independent growth in soft agar and extremely poor development of tumors in athymic nude mice. The failure of anchorage-independent growth of AIF-deficient cells was confirmed by siRNA knockdown in SW480 colon carcinoma cells and representative lung and breast carcinoma cells. Anchorage-independent growth was only restored in AIF knockout HCT116 cells by AIF mutants with intact NADH-binding domains. Thus, AIF maintains the tumorigenicity of various carcinoma cell types regardless of p53 function or oncogenic H-ras, and the NADH oxidase activity of AIF is essential for maintaining the transformed state of HCT116 colon carcinoma cells.

NIH3T3 cells transformed with the non-apoptotic AIF mutant formed foci in culture, whereas NIH3T3 cells transformed with AIF harboring deleterious mutations in both the pro-apoptotic and NADH-binding domains did not, which provides additional evidence that the NADH oxidase activity of AIF functions in cancer. Overall, the ability of transfected DNA-binding mutants of AIF (but not unmodified AIF) to stimulate foci formation in nonmalignant NIH3T3 cells and generate soft agar colonies in AIF knockout HCT116 cells indicates a latent transforming effect of AIF that is a reflection of its important role in maintaining the transformed state.

How does the NADH oxidase activity of AIF contribute to the transformed phenotype? There is ample evidence that a pro-oxidant state is associated with the development and maintenance of the transformed phenotype of many tumors, but the source of ROS is unknown (Cerutti, 1985; Szatrowski and Nathan, 1991; Dreher and Junod, 1996; Irani *et al*, 1997; Ha *et al*, 2000; Oberley, 2002). One possible source is the superoxide-generating Nox1 NAD(P)H oxidase, a Ras-inducible gene that is functionally required for oncogenic Ras-mediated transformation of fibroblasts and maintenance of their malignant phenotype (Suh *et al*, 1999; Lambeth, 2004; Mitsushita *et al*, 2004). Dismutation of O_2^- to H_2O_2 was proposed to mediate the increased cell growth and transformation caused by *nox1* overexpression (Arnold *et al*, 2001).

In our study, although reduction in ROS correlated with loss of tumorigenicity, both O_2^- and ROS scavengers failed to block tumorigenicity at concentrations that significantly reduced cellular ROS, which argues against a prominent role for ROS in maintaining the transformed state of colon cancer cells. Alternatively, complex I function might be linked to tumorigenicity, which is supported by our data showing that the NADH oxidase activity of AIF is required both for full complex I activity and for the transformed state. Interestingly, complex I has been linked to carcinogenesis, since the classical specific complex I inhibitor, rotenone, is known to potently block the formation of spontaneous or chemically induced carcinomas of the tongue, liver and colon in rodents (Cunningham *et al*, 1995; Yoshitani *et al*, 2001; Tanaka *et al*, 2002, and references therein).

Together, our data and these considerations suggest that AIF NADH oxidase-dependent complex I function may be important for tumorigenicity in carcinoma cells rather than ROS *per se*.

Inhibitors of AIF as potential cancer drugs?

Targeting mitochondrial AIF in cancer is attractive. First, the enhanced rates of glucose uptake and glycolysis of transformed cells enable small molecule inhibitors to accumulate at higher concentrations in the mitochondria of cancer cells (Don and Hogg, 2004). Second, blocking the NADH oxidase activity of AIF should leave its extramitochondrial pro-apoptotic function intact (Ye *et al*, 2002). Third, whereas in our study an incomplete reduction in AIF expression inhibited anchorage-independent growth, an 80% reduction in AIF in the Harlequin mouse had no adverse effects, except in a subset of neurons and in the retina in the adult animal (Klein *et al*, 2002). Therefore, specific inhibitors of the NADH oxidase function of mitochondrial AIF that do not cross the blood–brain barrier should be considered as a novel approach to the therapy of colorectal cancers and other carcinomas. We speculate that inhibitors of the NADH oxidase activity of AIF would be differentially toxic to tumor cells and both sensitize colon cancer cells to cancer drugs and block or reverse their tumorigenicity regardless of their p53 or H-ras status.

Materials and methods

Cell culture and materials

HCT116 and DLD-1 colon carcinoma cells were maintained in McCoy's 5A Medium (Sigma-Aldrich), SW480 colon carcinoma cells and NIH3T3 cells in DMEM (Sigma-Aldrich) and A549 colon carcinoma cells and MCF7 breast carcinoma cells in RPMI medium. All media were supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT) and penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma-Aldrich).

Inactivation of the human aif gene by homologous recombination

Gene targeting in diploid male cell lines HCT116 and DLD-1 via homologous recombination has been described previously (Sedivy *et al*, 1999). We utilized this strategy to modify the single X-linked locus of the human *aif* gene by adapting the design for promoter-trap vectors (Sedivy and Dutriaux, 1999) in construction of the targeting vector. Briefly, PCR amplifications of a 0.6 kb region upstream of exon 1 and 5.2 kb region downstream of exon 1 from the human *aif* locus of HCT116 genomic DNA were cloned into the pGEM-T vector (Promega, Madison, WI). Exon 1 was replaced by the *hyg* gene (with a polyadenylation signal) exactly at the start codon of the human *aif* gene (Figure 1). To further reduce background selection of hygromycin-resistant clones due to nonhomologous recombination, the bipartite method was utilized (Jallepalli *et al*, 2001). The conventional promoter-trap vector described above was digested with *Mlu*1, *Sac*II and *Pst*1 (New England Biolabs, Beverly, MA) to generate vectors with overlapping regions of the truncated *hyg* gene. The vectors were gel-purified and transfected at 1 μg/10^6 HCT116 or DLD-1 cells via electroporation using the Bio-Rad Gene Pulser system (250 V, 960 μF). Transfected cells were plated at 10^3 cells/well in 96-well plates and selected in McCoy's 5A medium (Sigma-Aldrich) and 0.3 mg/ml hygromycin (Clontech) for 2–3 weeks. The 96-well plates were screened for individual clones, which were isolated and analyzed by PCR analysis of genomic DNA using the following primers: PgAFA: TTG GGT GGG AAA GAG CCG GCA ACT GGT AAA AG; Hyg-Rev: AGA TTC TTC GCC CTC CGA GAG CTG CAT CAG GT. Candidate $aif^{-/y}$ clones were confirmed by additional PCR analysis for the loss of the 950 bp wild-type junctions with the following primers: primer A: GAG TCT GCG TAA TGT GCG TGT GAA GAG AGA CTG G; primer B: TTG CCT GGA ATG GGT CAG TCA CCT GGG AG. To further validate gene targeting of the human *aif* gene, we analyzed clones using RT–PCR by preparing oligo-dT primed cDNA from clones and analyzed with the following mRNA specific PCR primers for AIF and GAPDH: AFRTA: CGG TCG CCG AAA TGT TCC GGT GTG GAG; AFRTB: ACG CGG CCT TTT TCT GTT TCT GTT CTG G; GPDA: CCA ATA TGA TTC CAC CCA TGG C; GPDB: TTC TCC ATG GTG GTG AAG AC. Clones

negative for *aif* mRNA were analyzed for protein expression using Western blotting.

Generation and transfection of AIF mutants

The pEGFP-C1 vector (Clontech) alone and vector containing the human *aif* coding region in-frame with *gfp* cDNA were routinely transfected into 2×10^6 cells using Fugene 6 (Roche) transfection reagent. Stable expressing cells were selected using 1 mg/ml G418 (geneticin; Invitrogen). Mutagenesis of *aif* was performed using the transformer site-directed mutagenesis kit (Clontech). Mutations introduced were as follows: mDNA1: K255A, R265; mDNA2: K510A, K518A; mDNA1N: K255A, R265A, T263A, V300A; mDNA2N: K510A, K518A, T263A, V300A (Mate *et al*, 2002; Ye *et al*, 2002).

Western blot

Western blotting was performed with 10 μg protein as described (Towbin *et al*, 1979) using anti-AIF polyclonal antibody (N-1, 1:2000) (QED Biosciences, San Diego, CA) or anti-GFP polyclonal antibody (Clontech) and horseradish peroxidase-conjugated goat anti-rabbit IgG (New England Biolabs) as secondary probe. The transfer conditions were 10 mM $NaHCO_3$, 3 mM $NaCO_3$ and 20% methanol pH 9.9 for AIF, and 192 mM glycine, 20% methanol and 25 mM Tris pH 8.3 for complex I proteins and GFP. All blots were washed with phosphate-buffered saline and 0.5% Tween 20 (Sigma-Aldrich) and developed using enhanced chemiluminescence (Amersham Biosciences).

Aconitase activity and lucigenin assays for O_2^-

O_2^- measurement using the aconitase assay was as described (Gardner *et al*, 1995). A total of 10^6 cells were lysed using M-PER (Pierce, Rockford, IL) solution and protein concentration measured using Bio-Rad protein assay solution. A 30 μg portion of proteins was incubated with 50 mM Tris–HCl pH 7.4, 0.6 mM $MnCl_2$, 20 mM $NADP^+$, 250 mM sodium citrate pH 7.0 and 1 U isocitrate dehydrogenase (Sigma-Aldrich) in a 200 μl reaction volume. Aconitase activity was quantified by measuring the rate of NADPH formation during citrate isomerization to isocitrate at 340 nm using a TECAN microplate reader (Maennedorf, Switzerland) and expressed as mU/mg protein, where mU is nmol/min computed from the NADPH molar extinction coefficient of 6.22 $mM^{-1}\,cm^{-1}$. Lucigenin-based chemiluminescence measurement of intracellular O_2^- has been described (Clement and Stamenkovic, 1996). A total of 2×10^6 cells were lysed in 400 μl ATP-releasing solution (Sigma-Aldrich) and pelleted at 12 000 *g*. Together with the supernatant, 100 μl of 850 μM lucigenin (Sigma-Aldrich) was immediately added and chemiluminescence was measured for 20 s using a Turner TD-20e Luminometer. Data are expressed as relative light units/s and normalized against total protein content.

Flow cytometric analysis of intracellular ROS

Intracellular ROS levels were determined by staining with H_2DCFH-DA (Molecular Probes, Oregon), which is oxidized to dichlorofluorescein (DCF) by ROS. Cells were exposed to 5 μM H_2DCFH-DA at room temperature for 1 h, analyzed by flow cytometry using a Becton Dickinson FACScan machine and analyzed using the CellQuest program.

Cytotoxicity and apoptosis assays

A total of 5×10^3 cells were plated in 96-well plates and exposed to different concentrations of drugs: etoposide, campthothecin, staurosporine, doxorubicin and actinomycin D (Sigma-Aldrich); oxidative stress agents *t*-butylhydroperoxide and hydrogen peroxide (Sigma-Aldrich); and apoptosis inducers TRAIL (Biomol, Philadelphia) and anti-FAS (Roche). Cells were incubated for up to 4 days at 37°C in 5% CO_2. Survival was measured by processing cells with 20 μl of CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS); Promega, Madison, WI) for 2 h and measurement of color reaction absorbance at 485 nm using a TECAN microplate reader, which was expressed as a fraction of untreated cells. Apoptosis was measured using Annexin-V staining, Sytox-Hoechst stain and caspase-3 activity assay as previously described (Hentze *et al*, 2003; Li *et al*, 2004). For Annexin-V staining, 5 μl of Annexin-V (PE) (Pharmingen) was incubated with 10^5 pretreated cells for 1 h and analyzed by flow cytometry using a Becton Dickinson FACScan machine and using the CellQuest program.

Measurement of mitochondrial complex I and citrate synthase activities

The NADH-coenzyme Q_1 oxidoreductase activity of complex I was measured using mitochondria isolated using the Mitochondrial Isolation Kit (Pierce, Rockland, IL). Assays were performed as described (Estornell *et al*, 1993). Mitochondria were freeze-thawed three times and incubated immediately with 50 mM Tris–HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, 2 mM KCN, 150 μM NADH and 100 μM coenzyme Q_1. All reagents were obtained from Sigma-Aldrich. A time course for NADH oxidation was measured at 340 nM using a TECAN microplate reader at 37°C and calculated using an extinction coefficient of 5.5 $mM^{-1}\,cm^{-1}$. Citrate synthase activity was measured using the Citrate Synthase Assay Kit (Sigma-Aldrich) and performed according to the manufacturer's instructions. Protein concentration measurements were performed using the Bio-Rad Protein Assay Solution.

Colony formation in soft agar

Cells were trypsinized and resuspended in McCoy's 5A medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.3% noble agar (Difco). A total of 10^3 cells/well were plated in six-well plates containing solidified medium with 1% noble agar. Plates were incubated at 37°C in 5% CO_2 and fed with fresh media every 5 days. After 14 days, cells were incubated with 1 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) for 1 h.

Tumorigenesis in athymic mice

Cells (5×10^6) were subcutaneously injected into the flanks of 4- to 5-week-old athymic nude mice (Animal Resource Centre, Australia) and incubated for 3 weeks. Tumors were excised, weighed and dimensions were measured using calipers. Tumor volume (cm^3) was determined using the standard formula $a^2 \times b/2$, where a is the width and b is the length of the horizontal tumor perimeter. A total of 10–15 athymic nude mice were used for each cell line in two experiments, and the data were represented by the mean values. All animal experiments were performed strictly in accordance with Institute of Molecular and Cell Biology and A*Star regulations.

Foci formation

To demonstrate the transforming properties of human AIF, NIH3T3 cells were transfected with cDNA encoding AIF-GFP or various mutants, and the cells were sorted as described above. Cells were continuously cultured for 3–6 weeks and monitored for foci formation. Plates were analyzed after 6 weeks of incubation by staining with crystal violet (0.3% crystal violet, 30% methanol), and only dense colonies more than 2 mm in diameter were scored as foci.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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