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Genomic instability induced by mutant succinate dehydrogenase subunit D (SDHD) is mediated by $O_2^{\cdot-}$ and H_2O_2

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Abstract

SDHD mutations are associated with human cancers but the mechanisms that may contribute to transformation are unknown. The hypothesis that mutations in SDHD increase levels of superoxide leading to genomic instability was tested using site-directed mutagenesis to generate a truncated SDHD cDNA that was expressed in Chinese hamster fibroblasts. Stable expression of mutant SDHD resulted in 2-fold increases in steady-state levels of superoxide that were accompanied by a significantly increased mutation rate as well as a 70-fold increase in mutation frequency at the *hprt* locus. Over expression of MnSOD or treatment with polyethylene glycol conjugated (PEG)-catalase suppressed mutation frequency in SDHD mutant cells by 50% ($p < 0.05$). Simultaneous treatment with PEG-catalase and PEG-SOD suppressed mutation frequency in SDHD mutant cells by 90% ($p < 0.0005$). Finally, 95% depletion of glutathione using L-buthionine-[S,R]-sulfoximine (BSO) in SDHD mutant cells caused a 4-fold increase in mutation frequency ($p < 0.05$). These results demonstrate that mutations in SDHD cause increased steady-state levels of superoxide which significantly contributed to increases in mutation rates and frequency mediated by superoxide and hydrogen peroxide. These results support the hypothesis that mutations in SDHD may contribute to carcinogenesis by increasing genomic instability mediated by increased steady-state levels of reactive oxygen species.

Keywords

succinate dehydrogenase mutations; mutation frequency; mutation rate; genomic instability; reactive oxygen species

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Introduction

Succinate dehydrogenase (SDH; a.k.a Complex II in mitochondrial electron transport chains) is suggested to act as a tumor suppressor gene [1–3] because several neoplasias, such as paragangliomas and pheochromocytomas, are more prevalent in individuals with germ-line mutations in SDH subunits B, C, and D [1, 4–6]. This is particularly the case with missense and nonsense mutations in SDHD because the majority of human tumors containing SDH mutations involve this subunit [5]. Somatic mutations in SDHB have also been found in papillary thyroid cancers and renal cell carcinomas [7], and a decrease in SDHD expression was found in gastric and colon cancer [8]. Altered expression or mutations in SDH are hypothesized to play a causative role in neoplastic transformation; however, the mechanism is not understood.

Recent studies suggest that mutations in the gene coding for SDHB and SDHC cause Complex II to significantly increase the univalent reduction of O_2 to form superoxide ($O_2^{\bullet-}$) and H_2O_2 [9–13]. Ishii and colleagues showed that a point mutation within the CoEnzyme Q (CoQ) binding site of mev-1, homologous to SDHC, leads to rapid aging and hypersensitivity to oxygen toxicity in *C. elegans* [10]. This same mutation expressed in murine cells was hypothesized to lead to increased $O_2^{\bullet-}$ production and tumorigenesis [11]. In addition, Slane et al. showed that Chinese hamster fibroblasts expressing a truncated SDHC protein exhibited increased steady-state levels of reactive oxygen species (ROS) (i.e., $O_2^{\bullet-}$ and H_2O_2), parameters indicative of oxidative stress, and chromosomal instability [13] that were suppressed by over expression of wild type SDHC. Finally, mutations in SDHB have recently been shown to lead to accelerated aging in *Drosophila* and this is suggested to involve increases in steady-state levels of H_2O_2 [14]. These results allow for the speculation that mutations in SDHB and SDHC subunits may contribute to genomic instability by causing metabolic oxidative stress. However, direct evidence showing that SDHD mutations lead to increases in ROS that are causally involved with the genesis of a mutator phenotype that could contribute to neoplastic transformation is lacking.

In the current study site directed mutagenesis was utilized to generate a cDNA coding for a truncated form of SDHD, the mutant cDNA was over expressed in fibroblasts, and the effect this had on $O_2^{\bullet-}$ production as well as mutation frequencies was determined. Expression of the mutant SDHD cDNA was found to cause a 2-fold increase in steady-state levels of $O_2^{\bullet-}$ and significant increases in mutation frequency and mutation rate. Over expression of manganese superoxide dismutase (MnSOD) and pretreatment with PEG-catalase and PEGSOD was utilized to demonstrate the relative contribution of $O_2^{\bullet-}$ and H_2O_2 to this phenotype. Selenium supplementation and glutathione (GSH) depletion were also utilized to assess the contribution of GSH metabolism to the increased mutation frequency observed in SDHD mutant cells. The results provide strong evidence supporting the hypothesis that genomic instability demonstrated by mammalian cells expressing an SDHD mutation was caused by increased steady-state levels of $O_2^{\bullet-}$ and H_2O_2 . These results also provide proof-of-principle evidence favoring a potential mechanism by which SDHD mutations may contribute to neoplastic transformation *via* induction of a mutator phenotype.

Experimental Procedures

Cells and culture conditions

Immortalized Chinese hamster lung fibroblasts (B1 cells, a gift from Dr. Immo Scheffler University of California San Diego, [15]) were cultured as previously described [13]. B1 cells were assayed between passages 40 to 70.

Site-directed mutagenesis of hSDHD

A premature stop codon was created at the 66th codon in human hSDHD using site-directed mutagenesis. The hSDHD cDNA was inserted into a pcDNA 3.1 plasmid (Invitrogen, Eugene, Oregon) and a point mutation was induced using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, California) using the forward primer (5'-TGCATCTCTCCACTGAACTAGCG AGAGGGTTG-3') and reverse primer (5'-CAACCCTCTCGCTAGTTCAGTGGAGAGATGCA-3'). The vectors were cloned into competent *E. coli* and isolated using the Spin Miniprep kit (QIAGEN, Valencia, California). The mutation was verified by sequencing at the University of Iowa's DNA Core.

Mutated hSDHD and a vector control were stably transfected into B1 cells according to the Superfect Transfection Reagent protocol (QIAGEN). The stably transfected cells were selected in 800 µ/ml of G418 (GIBCO). Colonies of stably transfected B1 clones were isolated using the paper disk method [16].

Expression levels of W66X hSDHD mutant

To verify the expression of the W66X hSDHD gene in the B1 clones, RNA was isolated from the cells with QIAGEN's RNAeasy Isolation kit (QIAGEN), using the RNase-free DNase (QIAGEN). The RNA was quantified on a biophotometer (Eppendorf, Westbury, NY) and 1 µg of RNA was reverse transcribed into random cDNAs using the AdvantageTM RT-for-PCR kit (Clontech, Palo Alto, CA). A real time PCR single nucleotide polymorphism (SNP) assay was run to determine the expression levels of W66X hSDHD. As an endogenous control, β-actin expression was determined using a single-plex amplification reaction using a SYBR green master mix (Applied Biosystems, Foster City, CA).

Protein expression levels of W66X hSDHD mutant

To verify protein expression, samples were enriched for mitochondria using a method adapted from previous reports [17, 18]. Briefly, dry cell pellets were homogenized using a Dounce homogenizer in 0.25 M Sucrose, 5 mM HEPES, 0.1 mM EDTA pH 7.25 and centrifuged at 1000 × g for 10 min. Supernatant from this sample was then centrifuged at 10,000 × g for 10 min. Crude membrane fractions (CMFs) were then obtained from the mitochondrially enriched samples by two repeated freeze thaw cycles followed by low intensity sonication and 10,000 × g centrifugation. The supernatant (matrix) portion of this sample was also kept for study. Mutant protein expression was then verified via Western Blot analysis using Bio-Rad reagents and equipment (Hercules, CA) unless otherwise specified. 20 µg of each sample was boiled for 60 minutes in the presence of Laemlli buffer with 20 mM dithiothreitol (DTT) and then run on a 4–20% gradient Ready Gel. Blotting was done using a polyclonal, full length recognizing SDHD antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and an amino terminus recognizing voltage dependent anion channel (VDAC) antibody (Cell Signaling, Danvers, MA).

Steady-state O₂^{•-} levels

Dihydroethidium (DHE) (Invitrogen) oxidation was used to measure intracellular O₂^{•-} levels as previously described [13]. To determine if changes in DHE fluorescence were due to O₂^{•-}, cells were incubated with 100 U/ml PEG-SOD (Sigma, St. Louis, Missouri) for 2 h prior to labeling with DHE. The DHE fluorescence with and without PEG-SOD was measured and compared (see Supplemental Figure 1). The SOD inhibitable mean fluorescent intensity (MFI) of DHE was calculated by subtracting the MFI in the presence of PEG-SOD from the MFI without PEG-SOD.

Measurements of genomic instability

To test for point mutations and deletions in nuclear DNA at the hypoxanthine-guanine phosphoribosyl transferase (hprt) locus, cells were grown in 40 μ M 6-thioguanine (6-TG; Sigma) and seeded for clonogenic survival as previously described [19]. Mutation frequency was determined by the following expression: # colonies/ #cells plated in 6-TG $\div$ # colonies/ # cells plated in the absence of 6-TG.

To determine mutation rate, cells were selected in 1x HAT (hypoxanthine; aminopterin; thymidine) media (Cellgro, Manassas, VA) for three weeks to eradicate any cells that harbored preexisting mutations in hprt. HAT selected cells were then passed out of selection media and plated for clonogenic survival in complete DMEM media containing 40 μ M 6-TG at 0, 3, 6, and 9 d after selection as described by Glaab and Tindall [20]. Mutation frequency as a function of time following HAT selection was plotted and the slope of the linear regression through the data points was used to calculate mutation rate.

To determine if ROS played a role in the mutation frequency, cells were pretreated with either 100 MOI of adenovirus containing no target gene (AdEmpty) or MnSOD (AdMnSOD) (Viraquest; North Liberty, IA, U.S.A.), 18 μ M PEG (Sigma), 100 U/ml PEG-Cat (Sigma), 100 U/ml PEG-SOD, 50 U/ml PEG-Cat and 50 U/ml SOD, 250 μ M L-buthionine-[S,R]-sulfoximine (BSO) (Sigma), or 30 nM selenium (Se) (Sigma) for 72 h and assayed for 6-TG resistance as described above. Duplicated treatment dishes were collected to analyze for MnSOD, catalase, CuZnSOD, and glutathione peroxidase (GPx) activity and intracellular glutathione levels as described below.

Antioxidant Analysis

SOD activity was measured in cell homogenates using the activity assay developed by Spitz and Oberley [21]. MnSOD activity was measured using 5 mM sodium cyanide to inhibit CuZnSOD (Fisher Scientific, Fair Lawn, New Jersey). Catalase activity was determined in cell homogenates 24 h after PEG-catalase treatment by measuring the disappearance of H₂O₂ at 240 nm in a spectrophotometric assay described by Beers and Sizer, and analyzed according to Aebi [22, 23]. Total GSH levels were determined in a recycling assay that spectrophotometrically measures the reduction of dTNB (Sigma) to TNB in the presence of glutathione reductase (GR) (Sigma) as described by Griffith and Anderson [24, 25]. Glutathione peroxidase 1 (GPx 1) was measured as described previously by monitoring NADPH oxidation in the presence of reduced GSH (Sigma), GR, and H₂O₂ [26]. Biochemical data was normalized to protein content as described previously [21, 27].

Statistical analyses

One-way analysis of variance was utilized for experiments with three or more groups and significance between individual groups was determined using Tukey's HSD post-hoc analysis ($p < 0.05$). T-tests were used to determine significance in experiments with only 2 experimental groups ($p < 0.05$). All statistical analyses were accomplished using SPSS 10.0 software.

Results

Expression of mutant hSDHD

Site-directed mutagenesis was used to create a nonsense, point mutation that resulted in tryptophan 66 being mutated to a premature stop codon (W66X) in human SDHD (hSDHD) truncating 94 amino acids from the C terminus (Fig 1A and B). This mutation would be expected to disrupt the CoQ binding site of the SDH complex potentially resulting in increased one electron reductions of O₂ to form O₂^{•-} (Fig 1C and D). The mutant hSDHD

plasmid was sequence verified to have a nonsense mutation in the 66th codon that changes a tryptophan to a stop codon (TGG→TGA) (Figure 1E). Clonally isolated vector control cell lines (V3 and V4) as well as a mutant hSDHD clone (D4) were analyzed for mRNA expression of the mutant gene using real-time PCR (Fig 1F). The vector controls did not express W66X SDHD, while the D4 clone clearly demonstrated SDHD mutant gene expression (Fig 1F).

Steady-state $O_2^{\bullet-}$ levels in cell lines expressing the W66X SDHD protein

Western blotting of crude mitochondrial membrane fractions (Fig. 2A; CMF) and crude mitochondrial soluble fractions (Fig. 2A; Solu), for immuno-reactive SDHD protein revealed a ~7 kD band (the approximate molecular weight of the truncated protein) present in CMF isolated from the D4 cell line expressing the mutant mRNA (Fig. 1F) but not in the V4 vector control that did not express the mutant mRNA (Fig. 1F). It also appeared that there was much more wild type protein being expressed relative to mutant protein (Fig. 2A). Equal loading of mitochondrial membrane protein content was verified via immuno-blotting for VDAC (Fig. 2A). The previously described DCIP method [18] of measuring mitochondrial membrane bound SDH activity was used to verify that SDH activity was similar in the cell lines (Figure 2B) which was not surprising given that equal amounts of wild type protein were being expressed in the V4 and D4 clones. Oxidation of DHE to its fluorescent product was measured to estimate steady-state level of $O_2^{\bullet-}$. Figure 2C shows, that the D4 clone expressing the mutant had a 2.3-fold increase in DHE oxidation as compared to the vector controls and wild-type ($p < 0.0005$). There was no significant difference in DHE oxidation between the vector controls (V3 and V4) and the wild-type B1 cells (Fig 2C). To determine if the oxidation of the DHE was specifically due to $O_2^{\bullet-}$, cells were incubated with PEG-SOD prior to labeling. This analysis confirmed a 2.3-fold increase in SOD inhibitable DHE oxidation (Fig 2D) ($p < 0.01$), clearly showing that the cell line expressing the W66X SDHD mutation demonstrated increased steady-state levels of intracellular $O_2^{\bullet-}$.

Genetic instability and mutator phenotype induced by the expression of mutant SDHD

The mutation frequency in nuclear DNA was assayed by clonogenic survival in 6-TG which detects mutations at the hprt locus. D4 cells expressing the SDHD mutation demonstrated a 70-fold increase in survival in 6-TG as compared to vector controls, indicating a 70-fold increase in mutation frequency in these cell populations ($p < 0.0005$ vs. controls) (Fig 3A). To determine if the mutation frequency in the D4 cells was increased; B1, V3, V4, and D4 clones were selected in HAT media to eradicate any cells in the population harboring hprt mutations. Cells were then plated for clonogenic survival in 6-TG at various times out of HAT selection. Mutation frequency was calculated as a function of time following removal from HAT selection (Fig. 3B). There was a linear increase in the mutation frequency in D4 cells up to nine days out of HAT selection with a slope of 119×10^{-8} mutations/day, which represents the mutation rate in populations of cells expressing the SDHD mutation (Fig 3B, Table 1). In contrast to D4, B1 (8×10^{-8} mutations/day), V3 (16×10^{-8} mutations/day), and V4 (4×10^{-8} mutations/day) cells had significantly lower mutation rates. (Fig 3B, Table 1). These results show that hprt mutations developed in the D4 cell populations as the result of a dynamic process that occurred at a significantly higher rate than in the parental or vector controls (Table 1) ($p < 0.0005$).

To determine if the increased mutation frequency in D4 cells was causally related to the increase in steady-state levels of ROS, cells were pretreated with adenovirus MnSOD, PEG-catalase, PEG-SOD, or PEG-catalase + PEG-SOD in combination, 3 d prior to analysis with 6-TG. AdMnSOD increased MnSOD activity 8-fold and decreased the mutation frequency of D4 cells by 50%, compared to D4 AdEmpty ($p < 0.05$) (Fig 4A). PEG-catalase treatment

increased catalase activity 4.5-fold and decreased the mutation frequency of D4 cells by 50% compared to PEG alone control. ($p < 0.005$) (Fig 4B). PEG-SOD caused a slight decrease in the mutation frequency of D4 cells that did not reach statistical significance; however, simultaneous treatment with PEG-catalase and PEG-SOD significantly decreased mutation frequency to a greater extent (91%) than either pegylated-enzyme alone ($p < 0.0005$) (Fig 4B). PEG alone treatment had no effect on mutation frequency, and treatment with either AdMnSOD, or PEG-catalase had no significant effect on the mutation frequency of vector control cells. These data show that the increase in *hprt* mutations seen in the D4 cells is at least in part caused by increases in ROS in cells expressing the SDHD mutation.

To determine if hydroperoxide metabolism by the glutathione/glutathione peroxidase system played a significant role in the accumulation of *hprt* mutations, cells were pretreated with BSO, a relatively specific inhibitor of GSH synthesis, to deplete GSH [28, 29]. Treatment with BSO decreased intracellular total GSH levels by 95% and significantly increased the mutation frequency of D4 cells 4-fold ($p < 0.05$), but did not affect mutation frequency in V4 controls (Fig 4C). As a further test of the role of the glutathione/glutathione peroxidase system in the formation of *hprt* mutations, cells were grown in the presence and absence of 30 nM Se which is required for the active site of glutathione peroxidase (GPx) and was found to increase GPx activity 2 to 3-fold. Se-supplementation resulted in a 60% decrease in mutation frequency in the D4 cells ($p < 0.0005$) but did not affect mutation frequency in the V4 cells (Fig 4D). Overall, the results in Figure 4 clearly demonstrate that manipulations of ROS scavengers can significantly alter the mutation frequency of cells harboring the SDHD mutation and strongly support the hypothesis that the mutator phenotype seen in these cells is caused by increased steady-state levels of $O_2^{\bullet-}$ and H_2O_2 .

Discussion

Mutations in genes coding for mitochondrial electron transport chain (ETC) proteins are hypothesized to lead to dysfunctional mitochondrial metabolism resulting in increased steady-state levels of $O_2^{\bullet-}$ and H_2O_2 , that contribute to genetic instability and carcinogenesis [30]. Mutations in SDHD are some of the most prevalent germline mutations in genes coding for ETC proteins that are associated with human tumor formation [5], but the mechanism(s) by which these mutations might contribute to tumorigenesis are not clearly defined. In the current study a premature stop codon resulting in a truncation mutation, expected to disrupt the CoQ binding site, was introduced into the human gene coding for SDHD. When this mutant SDHD cDNA was stably expressed in hamster fibroblasts, increased steady-state levels of $O_2^{\bullet-}$ and a mutator phenotype characterized by increased mutation frequency and increased mutation rate was induced despite the continued presence of the wild type protein. Furthermore, antioxidant enzymes specific for scavenging $O_2^{\bullet-}$ and H_2O_2 (SOD and catalase, respectively) were able to significantly suppress the increased mutation frequency in the cells expressing the SDHD mutation and both enzymes together were more effective than either enzyme alone. In addition, increasing non-specific hydroperoxide metabolizing enzyme activity (i.e., GPx) via Se supplementation also suppressed mutation frequency and depleting GSH (an essential cofactor for GPx activity) dramatically increased mutation frequency in the SDHD mutants. These results unambiguously show that mutations in the gene coding for SDHD in mammalian cells are capable of resulting in increased steady-state levels of $O_2^{\bullet-}$ which causes a persistent heritable mutator phenotype that is mediated by both $O_2^{\bullet-}$ and H_2O_2 .

The most logical explanation for the fact that PEG-SOD and PEG-catalase together were more effective than either enzyme alone in SDHD mutants involves the reaction of $O_2^{\bullet-}$ and H_2O_2 commonly known as the Haber-Weiss reaction [31, 32]. In this scenario, excess $O_2^{\bullet-}$ produced by the mutant SDHD containing ETC Complex II, could reduce redox active metal

ions [i.e., Fe(III) or $\text{Cu(II)} + \text{O}_2^{\bullet-} \rightarrow \text{Fe(II)}$ or $\text{Cu(I)} + \text{O}_2$] which could then readily react with H_2O_2 (also formed by the dismutation of $\text{O}_2^{\bullet-}$) to produce hydroxyl radicals ($\bullet\text{OH}$) capable of causing DNA damage [31, 33]. In addition $\text{O}_2^{\bullet-}$ is not only capable of reducing metal ions; it has also been shown to result in the mobilization of Fe from metal storage proteins such as ferritin and Fe-S complexes, leading to an increase in labile iron that is capable of diffusing to critical cellular targets such as DNA and catalyzing damaging reactions [31, 34]. Therefore, increasing SOD activity in the SDHD mutants would be expected to remove $\text{O}_2^{\bullet-}$ inhibiting the reduction and release of metal ions while increasing catalase activity would be expected to remove H_2O_2 . The combination of SOD and catalase would be expected to act in at least an additive fashion by inhibiting the Haber-Weiss reaction.

The potential mechanisms by which mutations in SDHD (or other ETC proteins) could result in increased one electron reductions of O_2 to form $\text{O}_2^{\bullet-}$ are a subject of intense interest in cancer biology and aging. Recent studies with SDHC and SDHD missense mutations in the CoQ binding site have shown that the yeast expressing the mutant proteins become sensitive to cell killing mediated by hyperoxia and respiratory directed substrates such as galactose as well as showing evidence of increased $\text{O}_2^{\bullet-}$ production [12]. Consistent with these findings in yeast, our previous work in mammalian cells with SDHC truncation mutants and the current work with SDHD truncation mutants (both thought to disrupt the CoQ binding site) have led us to speculate that mutations in ETC proteins may cause an increase in the probability of one electron reduction of O_2 to form $\text{O}_2^{\bullet-}$. In cases where SDHD or SDHC mutations result in the decreased or increased expression of specific ETC protein subunits, increased $\text{O}_2^{\bullet-}$ could be the result of stoichiometric mismatches between complexes resulting in increased residence time of electrons at sites that are normally accessible to O_2 . In cases where truncation mutations or missense mutations that disrupt the tightly coupled flow of electrons from one subunit to another (as in the CoQ binding site) are expressed, increases in the accessibility of electrons to O_2 would be expected to lead to excess $\text{O}_2^{\bullet-}$ production. In either case the rate at which one electron reductions of O_2 occur would be expected to increase. In the current work we speculate that by truncating the SDHD protein at codon 66 (Fig 1C and D), only one and a half of the three transmembrane domains will be translated and inserted into the inner membrane of the mitochondria. We speculate that this truncated SDHD subunit could either cause the destabilization of Complex II such that it isn't anchored properly in the membrane coupling Complex II to CoQ or open up the CoQ binding site thereby increasing the accessibility of O_2 to undergo one electron reductions. These changes in Complex II structure would be anticipated to make the Fe-S clusters in SDHB or the heme binding site at the interface of SDHC and SDHD more accessible to O_2 increasing the probability of $\text{O}_2^{\bullet-}$ formation.

Several studies have associated mutations in SDH with oxidative stress, cancer, and aging [10, 11, 13, 14]. To our knowledge, the current study is the first to show that increased steady-state levels of $\text{O}_2^{\bullet-}$ and reactive oxygen species (ROS) derived from $\text{O}_2^{\bullet-}$ (i.e., H_2O_2 and/or organic hydroperoxides) in cells expressing SDHD mutations are directly responsible for causing a mutator phenotype in mammalian cells. This provides a potential mechanism for how mutations in SDHD may contribute to neoplastic transformation by leading to ROS-mediated cellular damage and genomic instability [35–38]. Consistent with the mutator phenotype hypothesis [39] and the fact that in general tumor cells have much higher mutation frequencies than their normal cell counterparts [40], the propagation of persistent genomic instability via mutations in genes coding for ETC proteins may be a common feature leading to genomic instability and carcinogenesis. Also this paradigm potentially supports a general mechanism by which mutations in genes involved with oxidative metabolic processes may contribute to the well known mechanistic link between neoplastic transformation and aging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Construction and expression of the mutant SDHD cDNA by site directed mutagenesis. Site-directed mutagenesis was used to create a nonsense, point mutation that resulted in a tryptophan mutated to a premature stop codon (W66X, tryptophan → stop at the 66th codon, truncating 94 amino acids from the C terminus) in human SDHD. A. SDHD contains 4 exons and the third exon was chosen as the site for a premature stop codon. B. Amino acid sequence of wild-type and W66X hSDHD protein. Amino acids at the CoQ binding site are in bold italics. Heme b binding site (H65) is underlined. C. Schematic of wild type SDHC and SDHD proteins in the inner mitochondrial membrane. Amino acids binding sites for heme b and CoQ are shown in SDH-D. D. Schematic of wild type SDHC and mutant SDHD proteins in the inner mitochondrial membrane. The mutation disrupts the CoQ binding site. E. The mutant hSDHD plasmid was sequence verified to have a nonsense mutation in the 66th codon that changes a tryptophan to a stop codon (TGG→TGA). Chromatogram of the wild-type SDHD sequence with the 66th codon being tryptophan (TGG) and the mutated SDHD sequence with a stop site at codon 66 (TGA). F. Real-time PCR verification of W66X expression in D4 cells. Vector controls (V3 and V4) do not express W66X hSDHD.

Figure 2.

Expression of W66X SDHD protein does not diminish SDH activity however steady-state levels of $O_2^{\bullet-}$ are increased in cells expressing the SDHD mutation. A. Western blot for SDHD using a polyclonal antibody that recognizes both the wild type SDHD protein in V4 and D4 crude mitochondrial membrane fractions (CMFs) as well as the ~ 7 kD mutant protein in only the D4 CMFs. Antibodies to VDAC were used as a mitochondrial membrane protein loading control. B. Isolated mitochondria were used to measure membrane bound complex II activity by the DCIP method as previously described [17, 18]. Error bars represent ± 1 SD from 3 separate experiments. The differences were not statistically significant ($p > 0.05$), indicating complex II activity was intact in the D4 cells. C. Background fluorescence in DMSO controls was subtracted from fluorescence of DHE labeled cells in the experimental groups. Data points represent the mean of each clone normalized to the vector control ± 1 SEM, $\dagger P < 0.0005$ as compared to V4. D. DHE oxidation measured as in panel A with and without 100 U/ml PEG-SOD. Superoxide specific DHE oxidation (MFI) was calculated by subtracting the MFI with PEG-SOD from the MFI without PEG-SOD. The data prior to doing this subtraction to calculate the superoxide specific MFI is shown in supplemental Figure 1. Data represent the mean of each clone normalized to vector control ± 1 SEM, $* P < 0.01$ as compared to vector control.

Figure 3.

Cells expressing the SDHD mutation have increased mutation frequency and mutation rate as determined by 6-thioguanine (6-TG) resistance. Clones were grown in 40 μ M 6-TG for 14 d and assayed for clonogenic survival. A. 6-TG resistance before selection in HAT media. Data represent the mean mutation frequency for each clonal population $\pm$ 1 SEM, $\dagger$ $P < 0.0005$ as compared to V4. B. Mutation frequency determined as a function of time after selection for 3 weeks in HAT media. Cells were grown in HAT free media for 0 to 9 d before being analyzed for clonogenic survival in 6-TG. Data points represent the mean mutation frequency $\pm$ 1 SEM at each time point.

Figure 4.

Increasing antioxidant enzyme activity decreased the mutation frequency and depletion of glutathione increased the mutation frequency in cells expressing the SDHD mutation. Antioxidants were added 3 d prior to treatment with 6-TG. A. Treatment of V4 and D4 cells with 100 MOI AdMnSOD was compared to cells treated with 100 MOI of empty adenovirus (AdEmpty). MnSOD activities of infected cells was reported as U/mg protein. Data represents the mean for each treatment normalized to its appropriate control $\pm$ 1 SEM, ND = non-detectable, * $P < 0.05$ as compared to AdEmpty. B. Treatment of V4 and D4 cells with 100 U/ml PEG-catalase, 100 U/ml PEG-SOD, or 50 U/ml PEG-Cat and 50 U/ml PEG-SOD was compared to cells treated with 18 μ M PEG. Catalase activities were reported as mk units/mg protein. Total SOD activities were reported as U/mg protein. Data represents the mean for each treatment normalized to its appropriate control $\pm$ 1 SEM, ND = non-detectable, † $P < 0.005$, ‡ $P < 0.0005$ as compared to PEG alone, * $P < 0.05$ as compared to PEG-Cat. C. Treatment of V4 and D4 cells with 250 μ M BSO was compared to untreated control. Total cellular glutathione levels were reported as nmol/mg protein. Data represents the mean for each treatment normalized to its appropriate control $\pm$ 1 SEM, * $P < 0.05$ as compared to no BSO control. D. Treatment of V4 and D4 cells with 30 nM selenium was compared to untreated control. Selenium dependent glutathione peroxidase activities were reported as mU/mg protein. Data represents the mean for each treatment normalized to its appropriate control $\pm$ 1 SEM, ‡ $P < 0.0005$ as compared to no selenium control.

Table 1

Mutation rates in cell populations following HAT selection

Clone	Mutation Frequency/day [*]
B1	7.9×10^{-8}
V3	7.9×10^{-8}
V4	4.0×10^{-8}
D4	$119.0 \times 10^{-8}^{\dagger}$

^{*} Mutation rate (mutation frequency/day after HAT selection was calculated from the slope of the linear regression in Figure 3B

[†] $p < 0.05$, as compared to vector control