

REVIEW

Mitochondria as a Target of Environmental Toxicants

Joel N. Meyer,^{*1} Maxwell C. K. Leung,^{*} John P. Rooney,^{*} Ataman Sendoel,[†] Michael O. Hengartner,[†] Glen E. Kisby,[‡]
and Amanda S. Bess^{*}

^{*}Nicholas School of the Environment, Duke University, Durham, North Carolina; [†]Institute of Molecular Life Sciences, University of Zurich, Zurich, Switzerland; and [‡]Department of Basic Medical Sciences, Western University of Health Sciences, Lebanon, Oregon

¹To whom correspondence should be addressed at Nicholas School of the Environment, Duke University, Box 90328, A354 LSRC, Research Dr, Durham, NC 27708-0328. Fax: (919) 668-1799. E-mail: joel.meyer@duke.edu.

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Enormous strides have recently been made in our understanding of the biology and pathobiology of mitochondria. Many diseases have been identified as caused by mitochondrial dysfunction, and many pharmaceuticals have been identified as previously unrecognized mitochondrial toxicants. A much smaller but growing literature indicates that mitochondria are also targeted by environmental pollutants. We briefly review the importance of mitochondrial function and maintenance for health based on the genetics of mitochondrial diseases and the toxicities resulting from pharmaceutical exposure. We then discuss how the principles of mitochondrial vulnerability illustrated by those fields might apply to environmental contaminants, with particular attention to factors that may modulate vulnerability including genetic differences, epigenetic interactions, tissue characteristics, and developmental stage. Finally, we review the literature related to environmental mitochondrial toxicants, with a particular focus on those toxicants that target mitochondrial DNA. We conclude that the fields of environmental toxicology and environmental health should focus more strongly on mitochondria.

Key Words: contaminants; mitochondria; mitochondrial DNA; mitochondrial toxicity; mitochondrial disease.

MITOCHONDRIA AND MITOCHONDRIAL DNA

Mitochondria are essential organelles best known for ATP generation and their involvement in apoptosis. They also play critical roles in other key processes including calcium, copper, and iron homeostasis; heme and iron-sulfur cluster assembly; synthesis of pyrimidines and steroids; thermogenesis and fever response; and calcium signaling. The great majority of the ~1000–1500 (Calvo and Mootha, 2010) proteins that carry out these functions are imported from the cytoplasm via mitochondria targeting sequences or other mechanisms (Bolender *et al.*, 2008), and the proteins present vary significantly with tissue

(Johnson *et al.*, 2007). This variability probably reflects extensive variability in function, ranging from energy production in muscle mitochondria to steroid synthesis in adrenal mitochondria (Vafai and Mootha, 2012). A small number (13 in humans) is encoded in the mitochondrial genome, along with the tRNA and rRNAs required for their synthesis. These proteins are components of complexes I, III, IV, and V of the electron transport chain (ETC). Despite their small number, they are essential and are transcribed at high rates: mtRNA represents ~5% of total cellular RNA in many tissues but as high as 30% in heart cells (Mercer *et al.*, 2011).

Mitochondrial morphology also varies with cell type, developmental stage, and environment (Jansen and de Boer, 1998; Rube and van der Bliek, 2004; Vafai and Mootha, 2012) (Fig. 1). Morphology ranges from highly fragmented to highly networked, is regulated via the processes of mitochondrial fusion and fission, and is responsive to a variety of stressors (Jendrach *et al.*, 2008; Twig and Shirihai, 2011). Morphological dynamics are critical for maintenance of mitochondrial function (Green and Van Houten, 2011; Youle and van der Bliek, 2012). The amount of cellular mitochondria and their content are regulated via mitochondrial biogenesis, nuclear signaling-mediated nuclear and mitochondrial transcription, autophagy, and intraorganellar degradation processes (Diaz and Moraes, 2008; Mijaljica *et al.*, 2007; Piantadosi and Suliman, 2012; Scarpulla, 2008). “Mitophagy” can selectively remove damaged mitochondria (Kim and Lemasters, 2011; Kim *et al.*, 2007), and damaged cellular components can be degraded inside the mitochondria or exported to lysosomes and peroxisomes via mitochondria-derived vesicles (Fischer *et al.*, 2012; Nunnari and Suomalainen, 2012). Mitochondria do not merely receive instructions from the nucleus, however, but rather can initiate signaling in a variety of ways. These include “retrograde signaling” (i.e., from the mitochondria to the nucleus; Chae *et al.*, 2013; Liu and Butow,

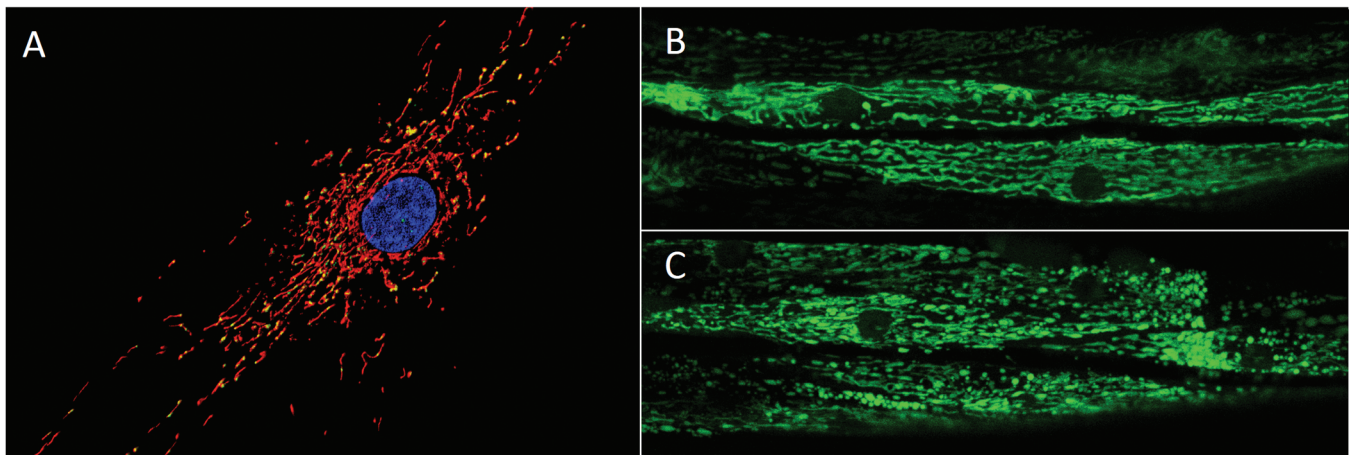


FIG. 1. Mitochondrial morphology. Panel (A) shows the mitochondrial network (MitoTracker Red-stained) containing many mtDNA nucleoids (PicoGreen-stained) surrounding the nucleus (Hoescht-stained) of a human primary fibroblast (photo: Amanda Bess). Panels (B) and (C) show a transgenic strain of *C. elegans* that expresses a mitochondrial matrix-targeted green fluorescent protein in body wall muscle cells. The image in panel (B) is from a control nematode, and the image in panel (C) is from a nematode exposed to 1 μ M carbonyl cyanide *m*-chlorophenyl hydrazone, a potent ionophore and inhibitor of oxidative phosphorylation that leads to mitochondrial fragmentation; the mitochondrial matrix was visualized via expression of green fluorescent protein (photo: John Rooney).

2006) to coordinate nuclear transcription with mitochondrial needs via pathways including AMP-activated protein kinase, sirtuins, calcium signaling, peroxisome proliferator-activated receptor γ coactivator 1 α protein, and others (Piantadosi and Suliman, 2012); mitochondrial-nuclear reactive oxygen species (ROS)-mediated signaling (Storz, 2006); cell cycle arrest signaling that may involve proteins with similar functions in the nucleus (Green *et al.*, 2011; Koczor *et al.*, 2009; Kulawiec *et al.*, 2009); and initiation of apoptosis (Tann *et al.*, 2011; Raimundo *et al.*, 2012), as opposed to the better-known amplification of an apoptotic process initiated elsewhere. There is evidence for a “mitocheckpoint” that senses mitochondrial dysfunction and triggers cell cycle arrest (Singh, 2006) although the mechanism for this response is not fully understood.

Mitochondrial DNA (mtDNA) in many animal species is a circular intron-free genome consisting of 14,000–17,000 base pairs (16,569 in humans) although it is much larger and noncircular and contains introns in some species (Anderson *et al.*, 1981; Burger *et al.*, 2003). It is typically maternally inherited and present in multiple copies within each cell; the mtDNAs are grouped in DNA-protein complexes referred to as nucleoids (Bogenhagen, 2012; Kukat *et al.*, 2011) that are anchored to the inner mitochondrial membrane. Most somatic cells in mammals have 10^3 – 10^4 mtDNAs (Shoubridge and Wai, 2007), but this number varies significantly by cell type and developmental stage, as discussed at more length below. mtDNA replication occurs independently of the cell cycle. mtDNA half-life has been reported as 2–4 days (Kai *et al.*, 2006; Lipsky and Pedersen, 1981) but has not been characterized in a wide range of cell types. mtDNA replication and transcription are regulated in part by Tfam (Mitochondrial transcription factor A), a major protein component of the nucleoid (Campbell *et al.*, 2012). From an evolutionary perspective, mtDNA has a high mutation rate compared with nuclear DNA (nDNA). Normally, all of the mtDNAs in all of the cells of an

organism are of the same sequence; the presence of more than one sequence in a cell is referred to as “heteroplasmy.”

Recognition of the key role of mtDNA integrity and mitochondrial function in health has grown greatly in recent years (Chan, 2007; Chan *et al.*, 2006; Christodoulou, 2000; Cohen, 2010; de Moura *et al.*, 2010; DiMauro and Davidzon, 2005; Hirano *et al.*, 2001; Jansen and Burton, 2004; Kwong *et al.*, 2006; Manfredi and Beal, 2000; Nunnari and Suomalainen, 2012; Schapira, 2012; Schmidt, 2010; Shaughnessy *et al.*, 2010; Taylor and Turnbull, 2005; Wallace, 2005), partly due to our increased understanding and awareness of mitochondrial diseases.

MITOCHONDRIAL DISEASES

mtDNA and nDNA Mutations Can Cause Mitochondrial Disease

Leber’s Hereditary Optical Neuropathy and mitochondrial myopathies were in 1988 the first diseases demonstrated to be caused by mtDNA mutations (Holt *et al.*, 1988; Wallace *et al.*, 1988). There are now more than 200, including MELAS (myopathy, encephalopathy, lactic acidosis, stroke-like episodes), MERRF (myoclonus epilepsy with ragged red fibers), and many others (Holt, 2010). Importantly, cellular dysfunction and clinical disease do not occur until a threshold proportion of mtDNAs carrying mutations (heteroplasmy) is reached (DiMauro and Schon, 2003; Rossignol *et al.*, 2003). Together, these diseases affect at least 1/10,000 people clinically (Chinnery *et al.*, 2012), establishing mtDNA mutations as a major cause of disease.

Because most mitochondrial proteins are nuclear encoded, it is not surprising that mutations in many nuclear genes also cause mitochondrial diseases. For example, mutations in DNA polymerase γ , the only mtDNA polymerase and thus

responsible for all mtDNA replication and repair, cause a wide range of mitochondrial diseases including progressive external ophthalmoplegia, Alper's syndrome, and Leigh's syndrome (Copeland, 2012). The disease Friedreich's ataxia is caused by mutations in the gene encoding frataxin, a protein involved in iron-sulfur cluster assembly (Marmolino, 2011).

Although individually rare, diseases caused by mtDNA and nDNA mutations are estimated to collectively have an incidence of ~1/4000 individuals (Chinnery *et al.*, 2004; DiMauro and Davidzon, 2005; Howell *et al.*, 2005; Wallace, 2005). There are currently no pharmaceutical cures for any mitochondrial disease (Nunnari and Suomalainen, 2012).

Mitochondrial Involvement in Common Diseases:

Causation Versus Correlation

The diseases described above are unambiguously caused by mitochondrial dysfunction. There are also many very common diseases for which there is strong correlative evidence, suggesting that mitochondrial dysfunction is at least partially causative (Wallace, 2005). These are frequently diseases of old age or energy homeostasis and include neurodegenerative diseases such as Parkinson's Disease and Alzheimer's Disease (Coskun *et al.*, 2012), many cancers (Brandon *et al.*, 2006; Kulawiec *et al.*, 2010; Penta *et al.*, 2001), diabetes (Rolo and Palmeira, 2006), metabolic syndrome (Mercer *et al.*, 2010), cardiovascular disease (Ballinger, 2005), and others (Schon *et al.*, 2012). Considerable effort is currently devoted to investigate whether these relationships are causal. There is experimental evidence demonstrating that defective mtDNA replication and repair can accelerate organismal aging (Bratic and Larsson, 2013; Trifunovic *et al.*, 2004).

Gene-Environment Interactions

In addition to the genetic diseases described so far, several gene-environment interactions have been identified (Cohen, 2010; Finsterer and Segall, 2010). For example, valproic acid is fatal in the context of some mitochondrial diseases (Krähenbühl *et al.*, 2000; Silva *et al.*, 2008), aminoglycosides (e.g., gentamycin) can cause deafness in the context of specific mtDNA mutations (Guan, 2011), and cigarette smoking and alcohol intake are major risk factors for vision loss in people who are homoplasmic for a mutation causing Leber's hereditary optic neuropathy (Kirkman *et al.*, 2009).

Lessons from Mitochondrial Diseases

The existence of so many mitochondrial diseases illustrates the critical importance of maintenance of mitochondrial and mtDNA integrity for health. It also raises an important question with implications for environment-mediated mitochondrial toxicity: Why did it take us so long to realize that many diseases are in fact mitochondrial diseases? Part of the answer is surely that these diseases are very complicated. Although they often target tissues that have high energy use or contain non-replicating (and therefore irreplaceable) cells, this is somewhat

oversimplified (Vafai and Mootha, 2012), and there is significant variability in their presentation. Some mtDNA mutations result in single-tissue diseases, others affect a wide spectrum of tissues, and others affect different tissues in different patients, with varying age of onset (Vafai and Mootha, 2012; Nunnari and Suomalainen, 2012). Furthermore, as illustrated by the valproic acid and aminoglycoside examples, the same environmental exposure may be innocuous in some people but deadly in others, depending on genetic background. These complications suggest that identifying the etiology of an environmentally induced mitochondrial dysfunction is likely to be challenging with both laboratory and epidemiological approaches, as borne out by the observation that, currently, the causes of only ~50% of severe cases of mitochondrial dysfunction are identified (Vafai and Mootha, 2012).

DRUG-INDUCED MITOCHONDRIAL AND MTDNA TOXICITY

Drugs Can Cause Many Off-Target Mitochondrial Effects

Recognition that mitochondrial toxicity is a key "off-target" effect of many drugs is also a relatively recent development. Drugs have now been identified that inhibit and uncouple the ETC; inhibit mitochondrial transport pathways, fatty acid oxidation or uptake, the citric acid cycle, mtDNA replication, and mitochondrial protein synthesis (a common mode of toxicity of antibiotics that target bacterial ribosomes, to which mitochondrial ribosomes are similar due to mitochondria's evolutionary origins); generate (or exacerbate generation of) ROS; and more (Begrache *et al.*, 2011; Cohen, 2010; Dykens and Will, 2007; Kovacic *et al.*, 2005; Mehta *et al.*, 2008; Neustadt and Pieczenik, 2008; Pacheu-Grau *et al.*, 2010; Rowe *et al.*, 2001).

One well-studied example is adriamycin (doxorubicin), a chemotherapeutic whose clinical use is limited by the fact that it also causes irreversible and cumulative cardiomyopathy (Wallace, 2007). It appears to act largely by poisoning mitochondria, both via redox cycling to generate ROS and by inhibiting ATP production via uncoupling of oxidative phosphorylation. Although its antineoplastic function is largely a result of DNA intercalation and topoisomerase II inhibition in the nucleus, a substantial amount reaches mitochondria, where it has a high binding affinity for cardiolipin, a lipid found only in the mitochondrial inner membrane (Mustonen and Kinnunen, 1993).

Another example is the nucleoside reverse transcriptase inhibitors (NRTIs) that are used to combat human immunodeficiency virus (HIV) infection. NRTIs act by inhibiting the reverse transcriptase activity required for viral replication. They have been highly successful in treating adults and in preventing transmission of HIV from pregnant mothers to their children, but unfortunately many NRTIs also inhibit the mtDNA polymerase γ . This has resulted mtDNA depletion- and mutation-mediated mitochondrial toxicity, and even death, in patients and in animal models (Benhammou *et al.*, 2007; Blanche *et al.*, 1999; Chan,

2007; Claessens *et al.*, 2003; Divi *et al.*, 2010; Kohler and Lewis, 2007). Similar effects have been observed with nucleoside analogs intended for other viruses as well (McKenzie *et al.*, 1995). Thus, chemicals that damage mtDNA or alter its copy number can have very serious health consequences.

Such toxicities have led to failed clinical trials, “Black Box” warnings and withdrawal of drugs from the market, and have driven the pharmaceutical industry to develop better methods for detection of drug-induced mitochondrial dysfunction (Begriche *et al.*, 2011; Dykens *et al.*, 2007; Marroquin *et al.*, 2007; Nadanaciva *et al.*, 2009; Pereira *et al.*, 2009).

Lessons from Drug-Induced Mitochondrial Toxicity

Mechanisms of drug-induced toxicity offer lessons in terms of how other chemicals might damage mitochondria; these are discussed at more length below. As in the case of mitochondrial diseases, it is instructive to consider why it took us so long to realize that many drugs target mitochondria. One reason is that frequently only a small percentage of people have adverse reactions (Dykens and Will, 2007). For example, only ~1% of

children of HIV-treated mothers showed mitochondrial toxicity (Barret *et al.*, 2003). The reason for this variability is not entirely clear. In some cases, gene-environment interactions are likely the explanation. In others, environment-environment interactions, epigenetic factors (see below), or the stochasticity associated with mtDNA replication and allocation may be involved. In any case, it is sobering from an environmental toxicology perspective that it took years to recognize the mitochondrial toxicity of many drugs because drugs are in general much better studied for toxicity than most environmental contaminants. This suggests that many environmental pollutants may be undiscovered mitochondrial toxicants.

REASONS FOR MITOCHONDRIAL VULNERABILITY, AND FACTORS THAT MAY MODULATE VULNERABILITY

Reasons for mitochondrial vulnerability are presented graphically in Figure 2 and discussed in more detail below.

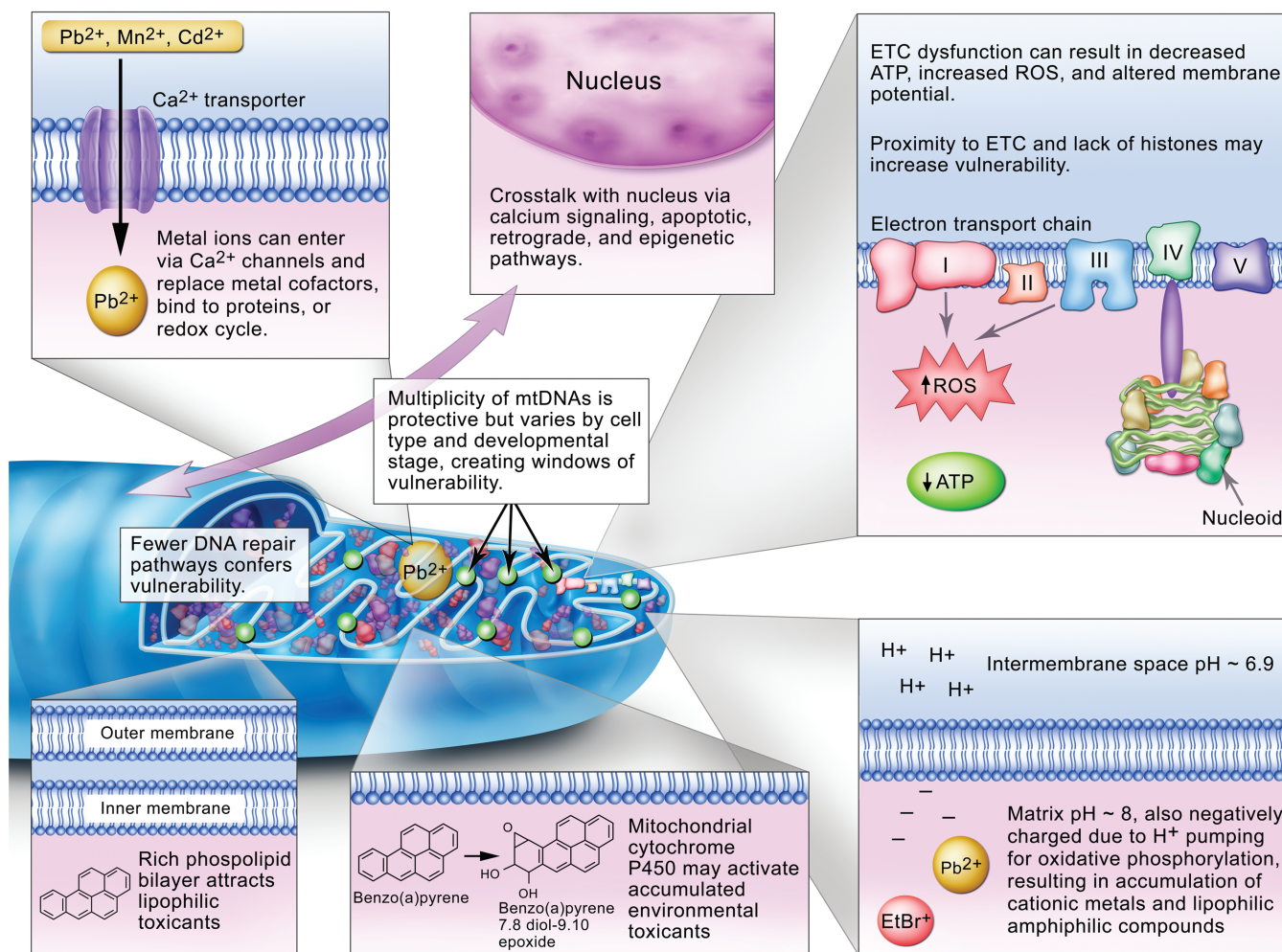


FIG. 2. Factors that affect mitochondrial vulnerability to environmental toxicants, as discussed in the text.

Accumulation of Pollutants

The high lipid content of mitochondrial membranes facilitates accumulation of lipophilic compounds such as polycyclic aromatic hydrocarbons (PAHs) (Backer and Weinstein, 1982) and some alkylating agents (Wunderlich *et al.*, 1972). Cationic metals, such as lead, cadmium, mercury, and manganese, have also been shown to accumulate in mitochondria preferentially (Atchison and Hare, 1994; Bucio *et al.*, 1999; Castellino and Aloj, 1969; Gavin *et al.*, 1992; Sokolova *et al.*, 2005a). These metals may accumulate in mitochondria due to both entry via calcium transporters (i.e., molecular mimicry) and chemical behavior resulting from their interactions with mitochondrial pH and charge. The matrix has a negative charge and slightly alkaline pH (~8 vs. ~6.9 for the intermembrane space) due to the proton pumping associated with oxidative phosphorylation. This also results in accumulation of certain organic chemicals, particularly amphiphilic xenobiotics including well-known mitochondrial toxicants such as ethidium bromide, paraquat, 1-methyl-4-phenylpyridinium (MPP⁺), and others (Cohen, 2010; Mehta *et al.*, 2008). It is also a property that is exploited to target toxic (Modica-Napolitano and Aprille, 2001), diagnostic (Ross *et al.*, 2005), or therapeutic (Murphy, 2008; Murphy and Smith, 2007) chemicals to mitochondria.

Ability to Metabolically Activate Pollutants

Another factor contributing to the mitochondrial susceptibility is the presence of cytochrome P450s in mitochondria, which can activate chemicals that are relatively nonreactive prior to metabolism, such as PAHs and mycotoxins (Dong *et al.*, 2009; Genter *et al.*, 2006; Omura, 2006). In addition, the existence of close contacts between mitochondria and the endoplasmic reticulum (de Brito and Scorrano, 2010) raises the possibility that metabolites generated in the endoplasmic reticulum could reach the mitochondria.

mtDNA Exhibits Unique Vulnerabilities But May Be Buffered by High Copy Number

mtDNA is more vulnerable than nDNA to some environmental genotoxins although the opposite is also true in some cases (see below). A number of explanations for this observation have been presented, including mtDNA's physical location, its reduced protein packaging compared with nDNA, and its reduced repair capacity. These, along with the potential for protection due to high copy number, are described in more detail below.

Physically, mtDNA nucleoids are anchored to the matrix side of the inner membrane, in close proximity to the ETC and many proteins that contain transition metal ions. The ETC normally generates a significant amount of ROS by electron loss; this has been estimated at ~0.1% of O₂ converted to superoxide anion (Beckman and Ames, 1998; Fridovich, 2004) although the amount may decrease with mild uncoupling (Miwa and Brand, 2003). Much of this appears to be produced by Complex I, with release to the matrix, and Complex III, with release to the matrix

and intermembrane space (Brand *et al.*, 2004). Manganese superoxide dismutase (SOD) in the matrix, and copper-zinc SOD in the intermembrane space, convert superoxide anion to the longer lived and uncharged hydrogen peroxide; additional antioxidant enzymes are present and regulated as well (Valle *et al.*, 2005). Mitochondrial H₂O₂ may interact with nearby metals to produce hydroxyl radicals (Giulivi *et al.*, 1995) in close proximity to mtDNA. The many chemicals, disease states, etc., that cause an increase in ROS generation may increase the amount of superoxide produced: This includes redox cycling agents that can intercept electrons such as adriamycin; inhibitors of the ETC such as antimycin A and sodium azide that lead to a super-reduced state of upstream complexes, increasing the tendency to release electrons; dietary and exercise-related ETC dysfunction; hyperoxia and hypoxia-reoxygenation; and others (Bailey *et al.*, 2006; Barrett *et al.*, 2004; Begriche *et al.*, 2006; Davies and Doroshov, 1986; Hasegawa *et al.*, 1990; Indo *et al.*, 2007; Ishiguro *et al.*, 2001; Li *et al.*, 2003a; Powell and Jackson, 2003; Senft *et al.*, 2002a, b; Turrens *et al.*, 1982a, b). Furthermore, the Fenton reaction may be facilitated by redox cycling of transition metal ions that are either chelated (Dean and Nicholson, 1994) or released via ROS-induced degradation of metal-containing proteins (Mladenka *et al.*, 2006).

mtDNA lacks histones, which has frequently been cited as a potential reason for increased vulnerability. However, recent work (Bogenhagen, 2012) has shown that in fact a large number of proteins associate with mtDNA, some of which are explicitly protective (Valle *et al.*, 2005), so mtDNA vulnerability to reactive chemicals due to "nakedness" may be less than previously speculated. Recent work has shown that nucleoid structure is not static but can also be remodeled in response to exposure to DNA intercalators including ethidium bromide and doxorubicin (Ashley and Poulton, 2009a, b) and oxidative stress (Rebelo *et al.*, 2009). Rebelo *et al.* (2009) presented evidence suggesting that different areas of the mitochondrial genome were differentially packaged, so different regions may be more or less "naked." The nucleoid remodeling observed by Ashley and Poulton (2009a) was protective against mtDNA depletion that resulted from intercalator exposure. Furthermore, the remodeling was p53 mediated, increasing the evidence for a role of p53 in mitochondrial homeostasis (Szczepanek *et al.*, 2012). mtDNA occurs in a surprising array of structures in cells from a wide range of tissues and species (Kolesar *et al.*, 2012). This may reflect to some extent significant variation in the packaging function of proteins such as Tfam (Campbell *et al.*, 2012) and the transcriptional status of the individual genomes (Kolesar *et al.*, 2012). Finally, Chen *et al.* (2005) showed that aconitase is a nucleoid component that assists in mtDNA maintenance and protection against ethidium bromide exposure, raising the possibility that nucleoid repackaging may respond to bioenergetics and redox perturbations via aconitase mobilization (Shadel, 2005). Thus, mtDNAs likely have differential susceptibility to damage, both in a cell type-specific fashion and even within single cells.

A very large difference between nDNA and mtDNA is the relative lack of repair pathways present in mitochondria, at least in humans (there is species variability). Although our understanding of mtDNA repair pathways is evolving and includes more proteins and pathways than previously recognized (Croteau *et al.*, 2012; Kamenisch *et al.*, 2010; Liu and Demple, 2010; Scheibye-Knudsen *et al.*, 2012; Sykora *et al.*, 2011; Valentin-Vega *et al.*, 2012), many types of damage are either irreparable or only very slowly repaired. Base excision repair is present in mitochondria, but nucleotide excision repair (NER) is not, and recombinational and mismatch repairs are either absent or quite limited (Druzhyňa *et al.*, 2008; Gredilla *et al.*, 2010; Kazak *et al.*, 2012; Kraysberg *et al.*, 2004). Furthermore, mtDNA is a major target for oxidative stress (Van Houten *et al.*, 2006), despite the relatively robust capacity for repair of oxidative damage. The sole replicative mtDNA polymerase, DNA polymerase γ , appears to have very little translesion synthesis capacity (Graziewicz *et al.*, 2004, 2007; Kasiviswanathan *et al.*, 2012), so that this damage tolerance mechanism is also lacking or very limited. The absence of NER is of particular relevance from an environmental toxicology perspective because it means that many important environmental genotoxicants including some PAHs, mycotoxins, and ultraviolet light will cause damage that is irreparable in mtDNA. Furthermore, because a subset of DNA damage caused by ROS is repaired by NER (Brooks, 2008; Cline *et al.*, 2010), any of the many factors that increase ROS in mitochondria (see above) could also create some highly persistent damage.

What might the effects of persistent mtDNA damage be? In addition to inhibiting replication, there is strong evidence that such damage inhibits RNA transcription (Cline, 2012) and mitochondrial function (Bess *et al.*, 2012; Brar *et al.*, 2012; Furda *et al.*, 2012b; Leung *et al.*, 2013; Rachek *et al.*, 2009). Although most of the measures of mitochondrial function so far have focused on energetics (ATP levels, oxygen consumption, etc.), it may be useful to consider other endpoints. For example, might mitochondrial dysfunction in the context of steroid-producing cells result in endocrine disruption? mtDNA damage likely contributes to generation of mtDNA mutations although this process is currently poorly understood (Copeland, 2012; Krishnan *et al.*, 2008; Kulawiec *et al.*, 2010; Loeb *et al.*, 2005). Improved sequencing methods support the relatively high (compared with the rest of the mtDNA) mutability of the D loop region (Schmitt *et al.*, 2012) and will improve our understanding of both mutagenesis and normal occurrence of heteroplasmy, which is now detectable at much lower levels than previous methods permitted. The “vicious cycle” hypothesis suggests that mitochondrial damage can lead to greater mitochondrial dysfunction, in turn leading to greater release of ROS and damage, etc. (Yakes and Van Houten, 1997).

On the other hand, the mitochondrial genome is present in multiple copies, potentially conferring buffering via redundancy. As described earlier, mitochondrial dysfunction resulting from mtDNA mutations is not observed until a certain threshold of

heteroplasmy is reached. This likely results from the combination of multiple copies and the fact that mitochondrial fusion permits swapping of contents, sometimes referred to as complementation or functional buffering. Presumably, such buffering would also occur for mtDNA damage. We recently found that similar levels of mtDNA damage led to much greater inhibition of mitochondrial function in live *Caenorhabditis elegans* than cells in culture (Bess *et al.*, 2012, 2013b; Leung *et al.*, 2013). Although there are multiple possible explanations for this observation, it could result from the much higher mtDNA copy number in human cells in culture compared with *C. elegans*. The high copy number also means that irreparable—or even repairable—DNA damage can be handled by degrading damaged mtDNAs by one of a number of possible mechanisms including degradation of the genomes themselves or of entire mitochondria along with mtDNA (i.e., autophagy or mitophagy) (Bess *et al.*, 2012, 2013a, b; Rouzier *et al.*, 2012; Shokolenko *et al.*, 2009). mtDNAs that have been removed can then be replaced by mtDNA replication. The high copy number and potential functional buffering also suggests that mtDNA damage may be an excellent early biomarker of mitochondrial damage: High levels of mtDNA damage will occur and persist and therefore be detectable, yet will precede late-stage collapse of mitochondrial function associated with clinical detection.

Further complicating the picture is evidence that inhibition of mitochondrial function may, at intermediate levels and under some circumstances, have at least some beneficial effects. For example, mild inhibition of mitochondrial function extends lifespan and increases resistance to a variety of stressors in *C. elegans* (Dillin *et al.*, 2002; Rea *et al.*, 2007) and mice (Liu *et al.*, 2005). In many cases, this effect is only observed if the inhibition occurs during development and not if it occurs later in life. However, it is important to note that greater inhibition leads to pathological effects, and in general, developmental exposures that alter programming of mitochondrial function later in life are not always be beneficial (Knudsen and Green, 2004).

Cell Type Modulates Sensitivity

Mitochondria vary dramatically from tissue to tissue (Calvo and Mootha, 2010; Vafai and Mootha, 2012), so it is not surprising that evidences from mitochondrial disease and mitochondrial drug toxicity indicate that different cell types are differentially susceptible to mitochondrial toxicants. There are many factors that may sensitize specific cell types to mitochondrial toxicity. For example, high reliance on mitochondrial function may mean that dysfunction more easily causes cell death (e.g., as often seen in mitochondrial diseases that target high energy-use cells) because the increased function may result in higher ROS production in some circumstances. An interesting example here is the aforementioned case of adriamycin, which causes principally mitochondrial toxicity in heart, where much electron flow occurs in mitochondria, and much more endoplasmic reticulum damage in liver, where electron flow is much higher in the ER (Berthiaume and Wallace, 2007; Brown and Borutaite, 2012). Certain cell types (e.g., some neurons) also have low antioxidant

defenses and/or high inherent oxidative stress (Crouch *et al.*, 2007). Proliferating cells, both cancerous and noncancerous, generate a great deal of ATP via aerobic glycolysis rather than oxidative phosphorylation, apparently in order to preferentially utilize carbon for biosynthesis required for cell division (Vander Heiden *et al.*, 2009). Any functional deficits associated with mitochondrial dysfunction in such cells may result more from loss of biosynthetic than energy-generating capacity. Finally, there is evidence that mtDNA repair capacity varies with cell type, with brain tissue in particular showing low repair (Karahalil *et al.*, 2002; Szczesny *et al.*, 2010). Nonetheless, it is clear that the full spectrum of factors that result in cell-type sensitivity is not well understood, and short-term effects may differ from long-term effects, in part due to the occurrence of secondary responses (Vafai and Mootha, 2012).

Developmental Stage Modulates Sensitivity

Some developmental stages are likely more sensitive to mitochondrial toxicants due to age-dependent differences in mitochondrial content, mitochondrial metabolism, and mitochondrial defense/repair mechanisms.

Mitochondrial count/volume and mtDNA copy number vary dramatically with age in humans and other species that have been examined. Although most cells thus far examined have 10^3 – 10^4 copies of mtDNA, somatic and primordial germ cells in the early embryo may contain as few as 10s or 100s of copies, constituting a “bottleneck” in mtDNA copy number and/or transferral (Carling *et al.*, 2011; Jansen, 2000; Jansen and de Boer, 1998; Pikó and Taylor, 1987; Shoubridge and Wai, 2007). Lower copy number is likely to reduce the protective effect of redundant copies described above. However, the bottleneck may also offer an opportunity for removal of severely damaged mitochondria or aberrant mtDNAs (Fan *et al.*, 2008; Stewart *et al.*, 2008), although it is not entirely clear when this process of purifying selection acts or how it may vary by mutation type (Freyer *et al.*, 2012), in part because the mitochondrial genome is not believed to be very active early in development when the bottleneck occurs. Furthermore, although newly fertilized eggs have a very high mtDNA copy number ($\sim 10^5$ (Shoubridge and Wai, 2007); but highly variable (May-Panloup *et al.*, 2007)), the evidence suggests that in many species those mtDNAs are simply allocated into daughter cells during early development, without additional replication, resulting in much lower copy number after initial rounds of cell division (Artuso *et al.*, 2012; Bratic *et al.*, 2009; Chase and Dawid, 1972; Leung *et al.*, 2013; Rubenstein *et al.*, 1977; Tsang and Lemire, 2002; Wai *et al.*, 2008). Because mtDNA allocation into daughter cells at mitosis (“mitotic segregation”) is apparently random, and the bottleneck phenomenon results in low copy number at least in some cell types (most have not been examined), it is possible for some cells to end up with a high percentage of damaged or mutant mtDNAs after multiple cycles of cell division. This high level of heteroplasmy or damaged mtDNAs would then be inherited by all of the cells derived from the progenitor cell. As previously

pointed out in other contexts (Krishnan *et al.*, 2008), mitochondrial alterations such as mutations in cell types that do undergo a bottleneck would not manifest until the second (in the case of somatic cells) or even third (in the case of primordial germ cells) generation, relative to an exposed mother. The effects might be somatic alterations or fertility difficulties in the children of the exposed mother or mitochondrial defects in the grandchildren. There is also a lower copy number in old age (Hebert *et al.*, 2010; Meyer *et al.*, 2007), suggesting vulnerability in aged individuals.

Age-related changes in mitochondrial metabolism are also well documented. Early life stages often display more anaerobic metabolism and spherical morphologies, and older individuals typically show signs of mitochondrial dysfunction although these effects are frequently tissue specific (Braeckman *et al.*, 2002; Brand *et al.*, 2004; Brys *et al.*, 2007, 2010; Dillin *et al.*, 2002; Hebert *et al.*, 2010; Jansen and de Boer, 1998; Knudsen and Green, 2004; Meyer *et al.*, 2007; Tsang and Lemire, 2003; Tsang *et al.*, 2001; Yasuda *et al.*, 2006). How reduced aerobic mitochondrial function will influence the effect of mitochondrial toxicants is not certain. On the one hand, reduced dependence on oxidative phosphorylation can result in less sensitivity to mitochondrial toxicity, as observed in at least some cells in culture (Marroquin *et al.*, 2007). On the other hand, reduced capacity to generate energy might inhibit repair processes and stress responses. Furthermore, the age dependence of nonenergy-related mitochondrial functions (calcium homeostasis, heme and iron-sulfur cluster assembly, synthesis of pyrimidines and steroids, etc.) is less well understood. Finally, there is evidence for an age-related decrease in base excision repair capacity in mtDNA (Anson *et al.*, 2006; Druzhyzna *et al.*, 2008; Gredilla *et al.*, 2010; Maynard *et al.*, 2009; Szczesny and Mitra, 2005; Szczesny *et al.*, 2003).

We speculate that these early-life vulnerabilities make mitochondria important candidates for playing a role in diseases and pathologies resulting from early environmental exposures, an important area of current environmental health research (Kai *et al.*, 2006; Knudsen and Green, 2004; Landrigan *et al.*, 2005). Similarly, late-life vulnerabilities suggest that environmental toxicants may play an important role in the mitochondrial dysfunction commonly observed in aging and diseases of aging (Ames *et al.*, 1995; Chomyn and Attardi, 2003; Fridovich, 2004; Melov *et al.*, 1995; Nicholls, 2002).

Conclusion: Vulnerability and Robustness

Many of the physical, chemical, and biological characteristics of mitochondria make them vulnerable to toxicant exposure (Fig. 2). At the same time, mitochondria are generally robust (Vafai and Mootha, 2012) and are protected in a number of ways, including great redundancy of content, ability to replace defective components, and complementation. However, genetic deficiencies in the mitochondrial processes responsible for these defenses may sensitize some people to exposures (Meyer and Bess, 2012).

MITOCHONDRIAL SUSCEPTIBILITY TO ENVIRONMENTAL TOXICANTS

The pathologies associated with mitochondrial disease and drug-induced toxicity highlight the importance of mitochondrial function for health. The demonstrated mitochondrial effects of many drugs along with the theoretical considerations described above lead to the concern that mitochondria may be an important target for many environmental toxicants, presumably via many or all of the same mechanisms described above for drugs. The possibility that environmental contaminants may target mitochondria has been noted previously (Shaughnessy *et al.*, 2010). Nonetheless, which contaminants are important is far from clear (Schmidt, 2010). We review some of the literature on pollutants that target mitochondria, focusing in particular on mtDNA as a target. We also discuss promising research approaches and propose important directions for future research.

Environmental Mitochondrial Toxicants

Although environmental contaminants have in general been less well studied than drugs from the mitochondrial toxicity perspective, there are nonetheless some well-understood examples. Paraquat, for example, appears to act largely by redox cycling in a fashion analogous to adriamycin (Martinez and Greenamyre, 2012). Rotenone is a Complex I inhibitor that results in increased ROS production, mtDNA damage, and apoptosis (Ishiguro *et al.*, 2001; Li *et al.*, 2003b; Shokolenko *et al.*, 2009). Carbon monoxide and cyanide act as Complex IV inhibitors (Ninomiya-Tsuji, 2008). An example of an environment-environment interaction is provided by the unfortunate co-occurrence of folate deficiency and exposure to either cyanide or methanol, which appears to have resulted in mitochondrial toxicity-mediated optic neuropathy in ~50,000 people (Sadun, 1998). There are also many other contaminants that are less well characterized as mitochondrial toxicants but are reported to cause at least some of their effects via mitochondrial toxicity. These include particulate matter (Janssen *et al.*, 2012; Hou *et al.*, 2010; Xia *et al.*, 2004), lipopolysaccharide (Suliman *et al.*, 2003), cadmium and copper (Garceau *et al.*, 2010; Sokolova *et al.*, 2005a, b), PAH quinones (Babu *et al.*, 2005), dioxin (Biswas *et al.*, 2008; Shertzer *et al.*, 2006), acrolein (Jia *et al.*, 2007), acrylamide (Lee *et al.*, 2012), perfluorinated compounds (Starkov and Wallace, 2002; Walters *et al.*, 2009), usnic acid (Guo *et al.*, 2008; Joseph *et al.*, 2009), methoxychlor (Gupta *et al.*, 2006), 3-nitropropionate (Sabri, 1998), cigarette smoke (Ballinger *et al.*, 1996; Cakir *et al.*, 2007; Westbrook *et al.*, 2010), manganese and lead (Bowman *et al.*, 2011; Sabri, 1998; Zheng *et al.*, 1998), arsenic (Dopp *et al.*, 2008; Echaniz-Laguna *et al.*, 2012; Naranmandura *et al.*, 2011), mercury (Belyaeva *et al.*, 2012; Farina *et al.*, 2012; O'Hara *et al.*, 2002), and pentachlorophenol (Valmas *et al.*, 2008). It is important to note that many if not all of the exposures are likely to affect targets other than mitochondria, and in some cases, mitochondrial effects may be secondary to effects elsewhere.

However, although many of these chemicals cause effects other than mitochondrial toxicity, the importance of the mitochondrial effects is in some cases supported by the fact that there is overlap in the pathologies observed after exposures and mitochondrial diseases. For example, the optic neuropathy described by Sadun (1998) was very similar to Leber's Hereditary Optic Neuropathy, and rotenone, paraquat, and manganese are all associated with Parkinson's Disease or related symptoms (Cannon and Greenamyre, 2011; Tanner *et al.*, 2011).

Environmental Mitochondrial Genotoxicants

Supplementary table 1 lists studies that have directly compared nDNA with mtDNA damage after chemical exposure. A few studies are included that examined only mtDNA damage or deletions. Studies that have examined chemical effects on mtDNA copy number only, while important, were too numerous to tabulate. Although a full review of the observed effects is beyond the scope of this review, it is important to note that chemical exposures have resulted in both decreased and increased mtDNA copy number, and this may depend not just on the chemical but also on the dose and time point assessed. For example, oxidative stress appears to be able to stimulate mitochondrial biogenesis (including mtDNA replication) at low doses but depletion at higher doses. This may be a function of competing effects of ROS, resulting in both removal of damaged genomes (Shokolenko *et al.*, 2009) and induction of biogenesis (Suliman *et al.*, 2005). In addition, short-term depletion can result in compensatory responses (Suliman *et al.*, 2003; Leung *et al.*, 2013) over the longer term.

Some genotoxicants cause more damage in mtDNA (e.g., PAHs and oxidative stress), whereas others (e.g., methylmethanesulfonate) cause more damage in nDNA (Supplementary table 1). In some cases (e.g., cisplatin, aflatoxin B₁), both have been reported (Supplementary table 1). For some toxicants, the degree of differential vulnerability has been quite variable in different reports. In a dramatic example, PAHs and aflatoxin B₁ have been reported to cause more mtDNA than nDNA damage, but the ratio has been as low as less than 2:1 and as high as 500:1 (Supplementary table 1). Similarly, mtDNA:nDNA damage ratios after cisplatin exposure have varied from much less than 1 to several hundred. The reasons for these large differences are unclear but may reflect different experimental designs, methods for measuring DNA damage, or cell type differences such as nucleoid packaging (Campbell *et al.*, 2012). Of note, the number of genotoxicants that have been tested for relative mitochondrial versus nuclear genotoxicity is relatively limited.

The differential mtDNA versus nDNA susceptibilities likely reflect a combination of the different factors reviewed earlier: toxicokinetics (i.e., where the contaminant and/or its metabolites compartmentalize within the cell), differential packaging of the two genomes, lack of repair of some types of damage in mtDNA, etc.

Approaches for Investigating mtDNA Damage

As can be seen from the examples above, it can be challenging to determine that a specific chemical causes toxicity only via mitochondrial effects. Most chemicals, even those that clearly directly cause mitochondrial toxicity, will also cause some other effects. In some cases, mitochondrial toxicity may be a secondary effect. How then can we isolate the effects of mitochondrial or mtDNA toxicity?

Several approaches have been used. One is to remove mtDNA from the picture altogether via the use of rho zero (mtDNA-depleted) cells (Brar *et al.*, 2012; Davermann *et al.*, 2002; Huang *et al.*, 2012). This approach is clean but has the disadvantage of creating cells that are highly abnormal. A second approach is to create mtDNA damage by targeting a restriction endonuclease to the mitochondria, so that it will only cut mtDNA. This is also a clean approach, with the disadvantage that it only permits the study of doublestrand breaks, rather than any of the types of DNA damage that are more commonly caused by toxicant exposure. A third approach is genetic; for example, Tann *et al.* (2011) recently used siRNA to specifically deplete the mtDNA repair enzyme ExoG, Green *et al.* (2011) studied the effects of p53 deficiency on cellular response to rotenone, and Simsek *et al.* (2011) compared the rescue capacity of nuclear- versus mitochondrial-targeted DNA Ligase III in DNA Ligase III-deficient mice. Several groups have demonstrated mtDNA-mediated effects by targeting DNA repair enzymes to mitochondria, improving DNA repair, and reducing or rescuing the toxic effects observed (Cai *et al.*, 2005; Koczor *et al.*, 2009; Racheck *et al.*, 2009; Shokolenko *et al.*, 2010). The use of a suite of toxicants that all impact a similar mitochondrial target (e.g., ETC complex I) has also been employed (Kulawiec *et al.*, 2009). Finally, it is possible to use a genotoxicant that causes much more damage to mtDNA than nDNA (Anson *et al.*, 1998, 2006; Herraiz *et al.*, 2003; Racheck *et al.*, 2009; van der Eb *et al.*, 2003) or that is repaired in nDNA but not in mtDNA (Bess *et al.*, 2012; Leung *et al.*, 2013). These approaches are more environmentally realistic but less clean because it is never possible to entirely avoid some damage to macromolecules other than mtDNA.

A methodological prerequisite for such studies is an unbiased method for comparing DNA damage in the two genomes. One approach is to measure specific adducts after extracting and isolating two genomes. However, this may lead to artifacts (Anson *et al.*, 2000). An alternative approach is to use a sequence-based approach that permits analysis of damage in the two genomes without differential extraction. The two best described methods are the Southern blot (Anson *et al.*, 2006) and QPCR (Furda *et al.*, 2012a; Hunter *et al.*, 2010; Meyer, 2010) assays.

Another critical methodological consideration, especially for *in vitro* (cell culture) work, is ensuring that the cells are actually using their mitochondria. Marroquin *et al.* (2007) showed that cells grown on typical high glucose media, which derive nearly all of their ATP from anaerobic metabolism, show much less susceptibility to a variety of mitochondrial toxicants than do the same cells grown on galactose, which forces them to use

oxidative phosphorylation. This also means that the typical concerns about using cancer cells are especially pertinent because many cancer cells are not only grown on glucose but exhibit very abnormal mitochondrial metabolism in any case (Nakajima and Van Houten, 2012).

Future Directions

There are important areas of basic mitochondrial biology that are growing rapidly and represent important opportunities for interdisciplinary research for environmental health scientists. For example, the growing understanding of nuclear-mitochondrial signaling and coordination described earlier has important implications for mitochondrial responses to stressors. Three additional emerging research areas are addressed briefly below.

Our understanding of repair and removal of mtDNA damage has increased greatly. Nonetheless, much remains to be learned about repair pathways *per se* (Gredilla *et al.*, 2010; Liu and Demple, 2010), how mtDNA damage leads to mtDNA mutations (Copeland, 2012; Krishnan *et al.*, 2008; Loeb *et al.*, 2005), and the role of mitochondrial dynamics in mtDNA maintenance. Recent results demonstrate that in addition to functioning in removing mtDNA damage (Bess *et al.*, 2012, 2013a, b; Rouzier *et al.*, 2012) and mtDNA mutations (Fan *et al.*, 2008; Stewart *et al.*, 2008), mitochondrial dynamics processes may be critical even for normal function of DNA repair pathways that are present in the mitochondria (Rouzier *et al.*, 2012) and for maintenance of nDNA integrity (Qian *et al.*, 2012). As discussed above, a key future area of research will be to investigate how these functions vary by cell type and developmental stage.

Second, there is growing understanding of the importance of epigenetic-mitochondrial interactions (Chinnery *et al.*, 2012; Wallace and Fan, 2010). Most directly, there is now evidence for methylation and hydroxymethylation at some CpGs in mtDNA, probably carried out via a mitochondrial-targeted form of DNA methyltransferase 1 (DNMT1), where it may serve as both a *de novo* and maintenance methyltransferase (Shock *et al.*, 2011). Furthermore, the levels of mitochondrial DNMT1 were regulated by stress-responsive transcription factors involved in mitochondrial biogenesis (Shock *et al.*, 2011), and one study reports that exposure to metal-rich particulate matter can alter mtDNA methylation (Byun *et al.*, in press). It will be important to clarify both how common pollutant-mediated alterations in mtDNA methylation are and what the functional consequences of mtDNA methylation are. More indirect evidence for a mitochondria-epigenetics relationship was provided by Carugno *et al.* (2012), who found in an epidemiological study that benzene exposure resulted in increased mtDNA copy number, and that this correlated with altered methylation patterns in four nuclear loci (*L1NE-1*, *Alu*, *p15*, and *MAGEA1*). There are also examples of nuclear-encoded mitochondrial proteins whose epigenetic regulation affects mitochondrial homeostasis and mitochondrial changes affecting nDNA methylation.

For example, *in vitro* methylation of the Tfam promoter site led to reduced transcription of mtDNA (Choi *et al.*, 2004); Tfam function is absolutely required for mtDNA maintenance and mitochondrial function, as shown using various mouse Tfam knockout strains (Dogan and Trifunovic, 2011). Methylation of the mtDNA polymerase γ regulates mtDNA copy number (Kelly *et al.*, 2012). Loss of mtDNA altered nDNA methylation at multiple specific loci in cancer cell lines (Smiraglia *et al.*, 2008; Xie *et al.*, 2007). The mechanisms for mitochondrial-nuclear influences are unclear but may include retrograde signaling (Bungard *et al.*, 2010), oxidative stress-induced alterations in DNA methylation (Chia *et al.*, 2011), and alterations in mitochondrial function that reduce availability of mitochondria-produced molecules required and potentially rate limiting for epigenetic modification (e.g., methyl groups for DNA methylation or cofactors such as flavin adenine dinucleotide, NAD⁺, acetyl-CoA, and α -ketoglutarate required for histone modification; Cyr and Domann, 2011; Donohue and Bultman, 2012; Minocherhomji *et al.*, 2012).

Finally, in addition to laboratory work, we hope that epidemiologists will consider mitochondrial endpoints. Although there are diseases where we know mitochondrial dysfunction is causal, more research is needed for many more where there is only correlative evidence or where such association may be theoretical only. Epidemiological work may be particularly difficult in the context of mitochondrial dysfunction for a variety of reasons, including (1) the unusual patterns of heritability, (2) heteroplasmy and the requirement that a threshold be reached prior to clinical detection of effects, (3) the potential for latency of effects, especially when a damaging event occurs early in development, and (4) potential epigenetic influences.

In conclusion, we argue that mitochondria are likely an underappreciated target of environmental contaminants and hope that future work from the environmental toxicology and environmental health communities will focus more on mitochondria.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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