Apoptosis-inducing factor (AIF) is a mitochondrial flavoprotein occasionally involved in cell death that primarily regulates mitochondrial energy metabolism under normal cellular conditions. AIF catalyzes the oxidation of NADH in vitro, yet the significance of this redox activity in cells remains unclear. Here, we show that through its enzymatic activity AIF is a critical factor for oxidative stress–induced activation of the mitogen–activated protein kinases JNK1 (c-Jun N-terminal kinase), p38, and ERK (extracellular signal-regulated kinase). AIF–dependent JNK1 signaling culminates in the cadherin switch, and genetic reversal of this switch leads to apoptosis when AIF is suppressed. Notably, this widespread ability of AIF to promote JNK1 signaling can be uncoupled from its more limited role in respiratory chain stabilization. Thus, AIF is a transmitter of extra–mitochondrial signaling cues with important implications for human development and disease.

Mitochondria are key mediators of cell death, survival, and homeostasis. In addition to providing cellular energy via the electron transport chain (ETC) and oxidative phosphorylation (OXPHOS), mitochondria also house pro-apoptotic factors that are released into the cytosol to execute cell death programs following apoptotic stimuli. Mitochondria are significant producers of reactive oxygen species (ROS), which are potentially toxic by-products of cellular respiration that also function as second messengers to control signaling pathways that promote cellular homeostasis. Common to the regulation of these diverse activities is the mitochondrial oxidoreductase known as apoptosis–inducing factor (AIF). This, which, despite the functional implications of its name, is critical to cellular redox balance and metabolic homeostasis. AIF–mediated cell death is essential for limited stimuli and to a narrow range of cell types, and the mechanisms by which AIF mediates caspase–independent cell death have been largely defined. Distinct from its role in cell death, AIF also possesses an intrinsic NADH–oxidase activity that has been linked to the regulation of metabolism and oxidative stress.

The physiological significance of AIF in control of cell homeostasis and survival has been demonstrated in studies of AIF–null mice, targeted AIF deletion experiments, and cases of AIF mutation in humans leading to a spectrum of mitochondrial respiratory chain and redox balance disorders. Mitochondrial malfunction following loss of AIF activity is explained in part by its biochemical role in mediating respiratory chain biogenesis. The ability of AIF to control ETC protein levels is limited to certain cell types and correlates with cellular metabolic preferences, whereas AIF–mediated catalysis balances glycolytic flux with mitochondrial metabolic activity.

A cellular context in which these AIF activities are critical is the environment of tumor cells, which exhibit an elevated dependence upon metabolic and redox balance for growth and survival. Numerous studies have demonstrated AIF overexpression in cancer, suggesting an ability of tumors to exploit AIF activity during pathogenesis. Indeed, we and others have shown that AIF is critical to the growth and/or survival of pancreatic, prostate, and colorectal cancers. The enzymatic activity of AIF is essential for the progression of advanced–stage prostate cancer, but the mechanism of this cancer cell growth support and the molecular players involved remain undefined.

The enzymatic activity of AIF generates superoxide in vitro, yet the significance of AIF–mediated redox control within cells and its relationship to AIF–dependent metabolism is unknown. The observation that AIF promotes ROS formation raises the possibility that this activity functions as a trigger.
for one or more ROS-sensitive signaling pathways that influence tumorigenesis. Concomitantly, AIF is often elevated in cancer (9, 10, 23–29) and may therefore serve as a contributory factor to promote tumorigenic redox signaling. Relative to healthy tissues, cancer cells elevate ROS to levels that benefit tumorigenesis by activating oxidative stress-associated prosurvival and proliferative signaling pathways (31), such as those driven by the activities of the mitogen-activated protein kinase (MAPK) family of proteins (32).

The MAPKs c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) are ROS-responsive signaling molecules whose activation by phosphorylation can control a spectrum of cellular activities ranging from cell death to survival, as well as mediating mitonuclear communication (32–34). Phosphorylation of JNK and p38 is linked to the activities of the AIF-binding proteins X-linked inhibitor of apoptosis (XIAP) and phosphoglycerate mutase 5 (PGAM5), and these physical associations with AIF coordinately regulate redox responses and cell fate decisions (35–40). These findings altogether suggest a possible role for AIF in control of oxidative stress and subsequent downstream MAPK signaling.

Here, we explored the ability of AIF to control JNK-mediated signaling events in a variety of cell types. We show that via an oxidative stress mechanism, AIF signals JNK1 to induce the cadherin switch and promote cell survival. Remarkably, AIF-dependent signal transduction is uncoupled from stabilization of the mitochondrial respiratory chain and cellular metabolism. These findings reveal a novel function for AIF and confirm its role as a pivotal redox signaling molecule.

Results

Enzymatic activity of AIF is required for oxidant-induced MAPK phosphorylation

We have shown previously that AIF promotes the growth and invasiveness of advanced-stage prostate (9) and pancreatic cancer cells (10). A well-characterized system for studying AIF-dependent tumorigenic activity is the setting of PC3 cells, an androgen-independent line of prostate cancer cells commonly used as a model for advanced prostate cancer (41). AIF knockdown substantially reduces the aggressiveness of PC3 cells (9), but the mechanism(s) underlying AIF-dependent growth and survival are presently unclear.

To assess the ability of AIF to regulate signaling pathways associated with these activities, we began by establishing PC3 cells in which AIF expression is stably suppressed using a lentiviral RNAi approach described previously (9, 10). Lentiviruses harboring either control sequences (shLacZ and shGFP) or AIF-targeting sequences (shAIF.1 and shAIF.2) were generated and then used to infect PC3 cells. To reduce the possibility that the results observed were due to off-target effects of a single RNAi construct, we generated two unique AIF knockdown cell lines (shAIF.1 and shAIF.2) through this approach. Following
lentiviral infection, stable suppression of AIF was verified by immunoblot analysis (Fig. 1).

To determine whether AIF plays a role in regulating intracellular signal transduction, we then evaluated the activation status of MAPK signaling molecules following AIF ablation. MAPKs are activated by phosphorylation, allowing activity levels to be assessed by immunoblot. When AIF expression was suppressed, phosphorylation levels of three MAPKs (JNK, p38, and ERK), as well as the JNK target c-Jun, were markedly reduced under basal conditions (Fig. 1, A and B). When upstream factors (MLK1 and MKK4/SEK1) of the MAP2K and MAP3K families were assessed, neither protein levels nor phosphorylation was altered. We additionally evaluated protein levels of the endogenous MAPK inhibitor-dual specificity phosphatase 4 (DUSP4). DUSP4 dephosphorylates JNK, p38, and ERK to suppress their activities, but it is inactivated and degraded under conditions of oxidative stress (42). Notably, DUSP4 protein levels increased 2-fold following AIF ablation (Fig. 1, A and B). This observation may be an indicator of decreased oxidative stress levels and a possible explanation for MAPK impairment when AIF expression is suppressed.

We further explored the AIF-dependent phosphorylation capacity of MAPKs by overexpressing apoptosis signal-regulating kinase 1 (ASK1), a redox-sensitive MAP2K that phosphorylates both JNK and p38 under conditions of oxidative stress (43) but that is activated in a ROS-independent manner when overexpressed (40). HA-tagged ASK1 was transfected into PC3-derived cells followed by immunoblot for HA to verify overexpression (Fig. 1C). When ASK1 was overexpressed, phosphorylation of JNK and p38 increased both in the presence and absence of AIF (Fig. 1C). This strongly suggests that although AIF ablation impairs MAPK activation, the intrinsic activity of neither JNK nor p38 is inherently compromised.

Based upon its ROS-generating enzymatic activity (30) and having observed that AIF influences multiple levels of MAPK signaling pathways, we then questioned whether AIF influences signal transduction under conditions of redox stimulation, concentrating our efforts on the JNK pathway. We therefore assessed the effects of AIF ablation on oxidative stress-induced phosphorylation of JNK in a broad panel of cell types derived from both normal and cancerous tissues: PC3 (prostate cancer); HPAC (pancreatic cancer); MRC-5 (normal lung); HCT 116 (colorectal cancer); and MIA PaCa-2 (pancreatic cancer). Following acute, nonlethal redox stimulation with increasing concentrations of tert-butylhydroquinone (tBHQ), we assessed levels of phosphorylated JNK. Although the exact patterns of JNK phosphorylation with respect to oxidative stress sensitivity varied among cell lines, AIF was required for this oxidant-induced phosphorylation in all cells tested (Fig. 2).

AIF-dependent changes in JNK phosphorylation were observed under basal conditions, under oxidative stress conditions, or both. Cell lines exhibited variable JNK sensitivity to...
tBHQ, with some cells readily phosphorylating JNK at low doses and others requiring substantially higher tBHQ concentrations, possibly reflecting differences in endogenous activators (MAP2Ks and MAP3Ks) and/or inhibitors (MAPK phosphatases). Moreover, detection levels of basal phospho-JNK differed among cell types. In PC3 and HCT 116 cells, modest basal phospho-JNK was observed and was further increased with treatment; when AIF was suppressed, cells were unable to phosphorylate JNK following tBHQ stimulation. HPAC cells exhibited low to no basal JNK phosphorylation but underwent extensive phosphorylation following stimulation in an AIF-dependent manner. Conversely, in MIA PaCa-2 and MRC-5 cells, JNK was relatively insensitive to the treatment conditions used yet showed AIF-dependent changes at the highest levels of tBHQ tested. However, despite cell type-specific differences in basal phospho-JNK levels and pre-existing differences in sensitivity to tBHQ, AIF was critical for all cell types to phosphorylate JNK. This suggests the ability of AIF to regulate JNK phosphorylation is widespread among cell types.

To determine whether JNK phosphorylation depends on the NADH-oxidase activity of AIF, we next generated AIF restoration cell lines stably expressing either WT AIF (AIFWT) or a catalytically inactive AIF mutant (AIFTVA) containing the substitutions T263A and V300A. As shown in Fig. 3A, restoration of AIF-deficient PC3, HPAC, and RWPE-1 cells with AIFWT was enriched from PC3-derived cells treated with 0 or 2 mM tBHQ by immunoprecipitation (IP) using an isoform-nonspecific P-JNK antibody, and precipitated material was analyzed by immunoblotting for JNK1 and JNK2. Phospho-JNK was quantified by normalizing precipitated material to JNK1 or JNK2 and β-actin of input samples. Data are presented as average ± S.D.* p < 0.05.

Figure 3. Enzymatic activity of AIF promotes oxidant-induced phosphorylation of JNK1. A, AIF-deficient (shAIF.1) PC3, HPAC, and RWPE-1 cells restored with either empty vector, AIFWT, or AIFTVA were treated with tBHQ as follows: 0 or 2 mM (PC3), 0 or 0.25 mM (HPAC), or 0.5 mM (RWPE-1); treatment was followed by immunoblotting for phosphorylated JNK. Replicate immunoblots were quantified and normalized to total JNK and β-actin. B, total phosphorylated JNK was enriched from PC3-derived cells treated with 0 or 2 mM tBHQ by immunoprecipitation (IP) using an isoform-nonspecific P-JNK antibody, and precipitated material was analyzed by immunoblotting for JNK1 and JNK2. Phospho-JNK was quantified by normalizing precipitated material to JNK1 or JNK2 and β-actin of input samples. Data are presented as average ± S.D.* p < 0.05.
(54 kDa) and short (46 kDa) molecular mass variants for all three JNK species. JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is expressed primarily in the brain and to a lesser extent in the heart and testis (44). To determine which JNK isoform responds to AIF activity, we used an isoform-nonspecific antibody to precipitate total phosphorylated JNK from tBHQ-treated cells followed by isoform-specific immunoblotting for JNK1 and JNK2. Only JNK1 was phosphorylated in response to acute oxidant stimulation, suggesting JNK1 is the target of tBHQ. Additionally, this phosphorylation event was AIF-dependent (Fig. 3B), demonstrating that AIF is required for the activity of JNK1 but not JNK2.

**AIF promotes the cadherin switch**

We next sought to determine downstream effects of AIF-mediated JNK signaling. As a central signaling hub at the convergence of cellular programs regulating death, survival, proliferation, and differentiation (32), JNK contributes to a variety of tumorigenic processes when phosphorylated. An important cellular activity influenced by JNK (as well as p38 and ERK) in cancer is the epithelial-to-mesenchymal transition (EMT) (45), a large-scale series of gene expression changes that increase the metastatic proclivity of cells. A key event of EMT is the cadherin switch, whereby cells reduce expression of the tumor suppressor E-cadherin and elevate N-cadherin to invade distal tissues (46). To determine whether AIF and JNK are involved with control of EMT, we assessed expression levels of EMT-associated genes in AIF-deficient PC3 cells via RT-PCR. AIF ablation did not globally alter EMT transcripts, but we observed a striking elevation in E-cadherin mRNA (Fig. 4A) that was confirmed at the protein level by immunoblot in PC3 and MRC-5 cells (Fig. 4, B and C). To further determine the role of AIF in the cadherin switch, we probed lysates for N-cadherin and observed decreased levels following AIF ablation in both cell types (Fig. 4, B and C). The effects of AIF upon the cadherin switch were also examined in LNCaP and MIA PaCa-2 cells (Fig. 4, B and C). As a cell line derived from a less aggressive (androgen-sensitive) prostate cancer (47), LNCaP cells do not exhibit a cadherin switch, expressing high levels of E-cadherin and low N-cadherin. Because pre-existing cadherin levels in LNCaP cells are similar to those associated with AIF ablation in PC3 and MRC-5 cells, cadherin levels expectedly did not change with AIF knockdown in LNCaP cells (Fig. 4, B and C). This is consistent with our previous observations that LNCaP cells are metabolically insensitive to the loss of AIF (9). In contrast, MIA PaCa-2 cells displayed a cadherin profile similar to PC3 and MRC-5 cells but that was unaffected by suppression of AIF (Fig. 3, B and C). We previously demonstrated that MIA PaCa-2 cells do not rely on either their mitochondria or AIF to survive and grow (10); thus, it was not surprising that cadherin levels were unaffected by AIF ablation in this cell type.

**AIF ablation and JNK suppression converge to similar molecular phenotypes**

To determine whether JNK is responsible for the cadherin switch in PC3 cells, we suppressed JNK activity by two independent approaches: pharmacological inhibition or genetic knockdown. For chemical inhibition experiments, AIF-proficient and -deficient PC3 cells were treated with the pan-JNK inhibitor SP600125 (48). AIF-deficient cells exhibit subtle differences in morphology when compared with controls that may represent a more epithelial phenotype (9). Notably, exposure to SP600125 induced morphological changes in AIF-proficient cells, including increased size and length, converging on the appearance of PC3–shAIF cells (Fig. 5A). JNK inhibition by SP600125 was confirmed by immunoblot for phospho-c-Jun (Fig. 5B). c-Jun phosphorylation in AIF-deficient cells was expectedly reduced prior to treatment and further impaired by SP600125 to a greater extent than in AIF-proficient cells; this corresponds to our observations that AIF is an upstream effector of JNK (Figs. 1 and 2). Furthermore, and similar to the biochemical effects of AIF ablation, SP600125 increased E-cadherin and decreased N-cadherin protein to levels comparable with those associated with AIF deficiency (Fig. 5, B and C), demonstrating their regulation both by AIF and JNK.

To further assess the involvement of JNK in the cadherin switch and to define isoform specificity, we next generated PC3 cells stably deficient in JNK1 or JNK2 by lentivirus-mediated shRNA induction (Fig. 5D). In agreement with the effects of SP600125, we observed changes in E-cadherin and N-cadherin following JNK knockout that were isoform-specific. Knockdown of JNK2 decreased N-cadherin expression, whereas the loss of JNK1 phenocopied the previously observed effects of AIF ablation, triggering both an elevation in E-cadherin and a reduction in N-cadherin levels (Fig. 5, D and E). Taken together, these data suggest that AIF functions upstream of JNK, and through JNK activity the AIF can control the expression of E-cadherin and N-cadherin.

**AIF-mediated signal transduction triggers the cadherin switch via JNK1**

Collectively, these data show that AIF enzymatic activity induces JNK1 signaling and that AIF and JNK1 share E-cadherin as a target. The ability of AIF to promote both JNK1 activation and the cadherin switch raised the possibility that if JNK1 functions as a conduit for AIF-mediated signaling, then restoration of JNK activity in AIF-deficient cells would reverse E-cadherin to control levels. To restore JNK activity in the absence of AIF, we employed constitutively active forms of JNK (CA-JNK) fused with the JNK activator MKK7 and tagged with a FLAG epitope that have been characterized previously (49). Plasmids encoding CA-JNK1 or CA-JNK2 were transfected into PC3–shLacZ and PC3–shAIF cells followed by immunoblot for FLAG and phospho-c-Jun (Fig. 5F), demonstrating both expression and activity of each CA-JNK protein. When CA-JNK1 was introduced into PC3–shAIF cells, E-cadherin reversed to near-control (shLacZ) levels (Fig. 5, F and G), confirming that AIF regulates the cadherin switch by activating JNK1.

**Failure to induce E-cadherin causes AIF-deficient cells to undergo apoptosis**

We have shown previously that AIF ablation severely impairs tumorigenesis (9, 10). Seeking to link these results to our current data defining an AIF-dependent signaling pathway, we employed the use of a Matrigel-based assay as an *in vitro*....
JNK redox signaling requires AIF
approximation of molecular responses that may occur in vivo. We began by growing PC3–shLacZ and PC3–shAIF cells in Matrigel™ followed by extraction from substrate after 3 days. Similar to our data acquired from cells grown under nutrient-rich conditions in vitro, phosphorylation of JNK was impaired in AIF-deficient cells grown in Matrigel™ (Fig. 6, A and B). This suggests that AIF-mediated JNK phosphorylation occurs under physical and chemical environments encountered by tumor cells in vivo.

Because we observed that AIF ablation leads to a loss of JNK1-mediated control of E-cadherin, we then explored the effects of reverting E-cadherin to control levels in AIF-deficient cells. Lenti-viruses harboring either control scramble shRNA or shRNA against E-cadherin (50) were generated and then used to infect PC3–shLacZ and PC3–shAIF cells. Stable knockdown of E-cadherin in AIF-deficient PC3 cells (PC3–shAIF/shE-cadherin) increased levels of N-cadherin (Fig. 6, C and D), suggesting N-cadherin levels can be altered by E-cadherin (50) in an AIF-dependent manner.

To determine whether E-cadherin is involved with these changes, we next subjected PC3–shLacZ/scramble, PC3–shAIF/scramble, and PC3–shAIF/shE-cadherin cells to Matrigel™.
Cells were seeded at three different densities (low, medium, and high) and then incubated at 37 °C for 7 days (Fig. 6E). In agreement with our previous observations (9), AIF-proficient cells (PC3–shLacZ/scramble) demonstrated population increases ranging from 4- to 8-fold, indicating an ability to survive growth stress conditions. As expected, PC3–shAIF/scramble cells showed no increase in total viable numbers, with seed populations remaining essentially unchanged over the duration of the experiment. Notably, this AIF-deficient phenotype was further exacerbated in PC3–shAIF/shE-cadherin cells, which exhibited net reductions of 25–75% compared with controls (Fig. 6E).

During this incubation, significant fractions of PC3–shAIF/scramble cells condensed and detached from the substrate (hallmarks of cell death), and the severity of this phenotype increased for PC3–shAIF/shE-cadherin cells, which exhibited net reductions of 25–75% compared with controls (Fig. 6E). Our data demonstrate that AIF-mediated signaling promotes a JNK1-dependent cadherin switch. In addition to this activity, AIF is essential for mitochondrial homeostasis under healthy cellular conditions (12, 13, 20). AIF controls both protein levels within the mitochondrial complex I and metabolic flux, yet the role of AIF-mediated redox control in these meta-
bolic activities remains unclear. Moreover, it is presently unclear whether the ability of AIF to influence mitochondrial metabolism (through complex I protein expression) and redox/ROS signaling are related properties, or whether these are separate features of AIF activity that govern distinct cellular responses. To begin addressing this question, we evaluated AIF-mediated control of complex I and mitochondrial activity (potential sources of intracellular ROS) in a broad panel of human cell lines. Suppression of AIF protein by lentivirus-mediated RNAi was verified by immunoblot (Fig. 7A), and following ablation of AIF we observed cell line–specific losses of complex I subunits NDUFA9 and NDUFB8 (Fig. 7A). Previously, we have tested the effect of AIF ablation on respiratory chain protein levels (9, 10) in some cell types employed here (PC3, DU145, LNCaP, PANC-1, BxPC-3, HPAC, HPAF-II, and MIA PaCa-2), which are consistent with our current data. Following AIF ablation, 7 of the 13 cell lines (MRC-5, PC3, DU145, PANC-1, BxPC-3, HPAC, and PL45) exhibited reductions in levels of complex I subunits NDUFA9 and NDUFB8 to variable extents, whereas in HeLa cells a reduction of only NDUFB8, but not NDUFA9, was observed. In the remaining five cell lines (RWPE-1, HCT 116, LNCaP, HPAF-II, and MIA PaCa-2), levels of neither NDUFA9 nor NDUFB8 were affected following suppression of AIF (Fig. 7A), indicating
AIF is not universally required to maintain expression of respiratory chain proteins. Because some cell types exhibited complex I deficiency following AIF ablation, we evaluated the mitochondrial status in our panel of cell lines to determine whether mitochondrial function and/or biogenesis (possible triggers for oxidative stress signaling) was impaired. Citrate synthase, a mitochondrial matrix enzyme and marker for mitochondrial abundance, was unchanged when AIF was suppressed (Fig. 7A). Furthermore, assessment of mitochondrial status by staining with tetramethylrhodamine ester (TMRM, an indicator of mitochondrial ΔΨm) and MitoTracker^TM^ Red (an indicator of mitochondrial abundance) suggests that AIF ablation does not substantially dissipate the mitochondrial electrochemical gradient nor decrease mitochondrial abundance in any cell types tested (Table 1). It is therefore likely that although AIF controls complex I in some cell types, AIF is not critical for mitochondrial fitness. Strikingly, all cell types examined required AIF for JNK signaling (Fig. 2) regardless of whether AIF deficiency led to changes in complex I (Fig. 7A).

We then questioned whether the AIF-dependent signaling factors (e.g. ROS) regulating JNK are directly produced by AIF or an indirect effect of AIF-mediated control of mitochondrial complex I activity. If re-establishment of mitochondrial respiration in AIF-deficient cells restores the signaling effects observed above, then this would suggest that AIF drives redox signaling through complex I. Therefore, we sought to restore functionality of ETC/OXPHOS while maintaining knockdown of AIF. To accomplish this, we expressed an epitope-tagged form of NDI1 in AIF-proficient and -deficient cells. NDI1 is a single-subunit NADH:ubiquinone oxidoreductase expressed by yeast and known to enhance and/or restore mitochondrial respiratory activity in human cells with complex I defects. NDI1 can therefore be used to determine whether an AIF-deficient phenotype is due to the specific loss of AIF or the more general loss of complex I function (51–53). Control vectors and NDI1 plasmids were transfected into PC3-derived cells and then assessed for NDI1 expression. Immunoblot of mitochondrial extracts for the FLAG epitope showed that NDI1 protein is expressed in transfected PC3-derived cells (Fig. 7B). A critical metabolic effect of AIF ablation is an increase in glucose consumption that supports energy production required for cell survival (9, 10). When NDI1 was transfected into AIF-deficient PC3 cells, glucose consumption decreased significantly (Fig. 7B) and indicates an improvement in mitochondrial metabolic activity resulting from the introduction of a functional NDI1 protein.

Despite restoration of the ETC with NDI1, oxidant-induced JNK phosphorylation remained unaffected by respiratory enhancement (Fig. 7, C and D), and changes in the cadherin switch were not altered when AIF-deficient cells were transfected with NDI1 (Fig. 7, E and F). Therefore, in addition to the finding that JNK requires AIF enzymatic activity across cell types regardless of AIF-dependent complex I status, these data demonstrate that AIF redox signaling is uncoupled from its roles in control of complex I and cellular metabolism.

**Discussion**

Collectively, our data provide a mechanism for AIF-mediated promotion of tumor growth and survival (9–11), suggesting that through its catalytic function AIF primes MAPK activity by moderating cell redox state. It is likely that the biological consequences of AIF-induced signaling are diverse and dependent upon intracellular contexts (9, 10). For example, although AIF-dependent JNK activity is widespread, it is unrelated to the cadherin switch in some cell types (LNCaP and MIA PaCa-2). Oncogene status, cellular metabolic activity, and levels of oxidative stress proteins (e.g. superoxide dismutase) and MAPK regulators are potential factors in the variability of AIF-mediated redox signaling.

The NADH-oxidase activity of AIF has been studied in vitro (30), but the biochemical consequences of AIF-mediated redox control within cells and the contribution of AIF to cell signaling have remained largely unclear. A number of studies have indicated that AIF is up-regulated in tumors, albeit typically not more than 2-fold (9, 10, 23–29). The ability of AIF to regulate cellular redox signaling provides further insight into overexpression levels, which may explain why AIF elevation in cancer rarely exceeds 2-fold compared with normal cells (9, 10). Rather than AIF nuclear death activity, it may instead be AIF promotion of stress signaling, uncoupled from the “classic” death mechanism, which must be held in check by tumors.

Importantly, AIF-dependent control of cellular metabolism (unique to specific cell types) is dissociable from its role in facilitating a pro-tumor cellular oxidant state. A mechanistic justification for variable AIF-mediated complex I control among cell types remains elusive but may be explained by different levels of CHCHD4 (21), AIF isoforms (8), or other yet to be identified factors. Regardless of whether cells exhibited respiratory chain deficiency following AIF ablation, JNK phosphorylation was impaired in all cell types tested. Although restoration of mitochondrial respiratory activity reversed the AIF-deficient metabolic phenotype, AIF-dependent signaling effects were unaffected. Together these observations suggest that in some cells AIF regulates respiratory chain protein levels, but in all cells tested the enzymatic activity of AIF leads to the activa-

**Table 1**

| Cell Type | ΔΨm (%) | p-value | Change in abundance (%) | p-value |
|-----------|---------|---------|-------------------------|---------|
| HeLa      | 20.9    | 0.020   | 9.7                     | 0.587   |
| HPAC      | 16.3    | 0.007   | 34.2                    | 0.002   |
| MRC-5     | 15.5    | 0.007   | 19.6                    | 0.026   |
| DU145     | 13.9    | 0.064   | 23.4                    | 0.147   |
| MIA PaCa-2| 13.7    | 0.128   | −13.8                   | 0.212   |
| HCT 116   | 12.6    | 0.079   | 21.7                    | 0.315   |
| PL45      | 8.5     | 0.149   | 30.2                    | 0.120   |
| BxPC-3    | 8.5     | 0.001   | 26.2                    | 0.004   |
| Panc-1    | 5.6     | 0.178   | −12.7                   | 0.007   |
| LNCaP     | 4.7     | 0.553   | −13.2                   | 0.376   |
| PC3       | −2.0    | 0.595   | 2.6                     | 0.272   |
| RWPE-1    | −7.7    | 0.040   | −2.6                    | 0.168   |
| HPAF-II   | −10.5   | 0.389   | −0.2                    | 0.987   |
| STS       | −59.3   | 0.000   | −30.1                   | 0.045   |
tion of redox-sensitive signaling molecules that include the MAPK family of proteins. Therefore, AIF is crucial to the regulation of not only metabolism but also oxidative stress, and AIF-dependent redox activity is independent of respiratory chain status. This explains how AIF can influence signaling events outside the mitochondria to alter nuclear gene expression.

The reversal of the cadherin switch following AIF ablation in some cells raises the possibility that AIF may influence metastasis (9) by triggering E-cadherin suppression and N-cadherin elevation. Experiments presented here focus primarily on the cadherin switch in the context of altered AIF/JNK activity, but all MAPKs (JNK, p38, and ERK) have been implicated in the cadherin switch and metastatic progression (45); whether and how AIF can regulate cadherin levels through p38 and ERK represent significant future questions. Interestingly, we observed that although AIF promotes the cadherin switch via JNK1 activation, reversing this switch in AIF-deficient cells is lethal under Matrigel™ growth conditions. This suggests that induction of the tumor suppressor E-cadherin following AIF ablation is a potential survival adaptation in the absence of AIF-mediated mitochondrial ATP production (9). This might occur to slow cell growth to rates that can match metabolic availability of macromolecules and cell fuel sources (54), altogether demonstrating the roles of AIF, JNK, and E-cadherin as double-edged swords in cancer (55–57). Cancer cells may use AIF to activate pro-tumor signaling pathways (such as JNK1-mediated suppression of E-cadherin), but their activation in the absence of AIF can in some situations be lethal, possibly due to altered AIF-dependent metabolism (9, 10, 20).

The known roles of AIF in cell death and survival exemplify the duality of mitochondria in life/death decisions; nuclear translocation of AIF is occasionally observed under conditions of cell death (3), whereas under nonstress conditions AIF is essential for mitochondrial energy production through regulation of the respiratory chain and glucose metabolism (9, 10, 20).

The contribution of AIF enzymatic activity to cellular homeostasis has remained largely elusive, and the ability of mitochondria-localized AIF to control intracellular redox signaling has long been speculated (11); yet to date, no studies have definitively shown the existence of such pathways. Although actively respiring mitochondria are an important ROS source for pro-oxidant signaling, our experiments suggest that AIF itself regulates signaling through its catalytic activity in addition to regulating respiratory chain biogenesis, and these functions are independent of each other. Experiments described here show that AIF is required for cell signaling, and it is conceptually satisfying that this activity controls signaling pathways well-known for both pro- and anti-apoptotic downstream effects.

Experiments presented here underscore the role of AIF in mediating the balance of cell death and survival and its dysregulation in cancer. We and others have demonstrated that in addition to its nuclear activity in cell death, AIF controls mitochondrial energy production by regulating respiratory chain protein levels, whereas its NADH-oxidase activity functions as a redox conduit that senses and responds to the metabolic and oxidative environment. The unification of metabolism, oxidative stress, MAPK signaling, and the cadherin switch by AIF activity raises numerous possibilities that include roles in normal physiology, development, redox balance, and disease. Altogether, we demonstrate a cellular role for AIF-mediated catalysis and the existence of AIF-dependent redox pathways that can be uncoupled from ETC control, implicating AIF as a fundamental control point for cellular oxidant signaling.

**Experimental procedures**

**Materials**

MEM, DMEM, RPMI 1640, DMEM/F-12, Glutamax, horse serum, insulin, transferrin, epidermal growth factor, trypsin, 4–12% bis-tris polyacrylamide gels, nitrocellulose membranes, fetal bovine serum (FBS), phosphate-buffered saline (PBS), and Pierce ECL 2 Western blotting substrate were from Thermofisher Scientific; IgG-Sepharose was from Amersham Biosciences; Matrigel™ was from BD Biosciences; Matrigel recovery solution was from Corning; protease inhibitor tablets were from Roche Applied Science; bVAD-fmk was from Enzyme Systems Products; QuantiChrom™ glucose assay kit was from BioAssay Systems; all other materials were from Sigma.

**Plasmids**

Lentiviral plasmids FG12–shLacZ–GFP, FG12–shGFP–GFP, FG12–shAIF.1–GFP, FG12–shLacZ–puro, and FG12–shAIF.1-puro were described previously (9, 10, 58, 59). FG12–shAIF.2–GFP, a second lentiviral plasmid for RNAi suppression of AIF that contains the targeting sequence CTTGG–TCCAGCGATGCCC, was generated as described (9). To generate RNAi-resistant AIF restoration pSL4-hygro plasmids, AIF cDNA was subcloned from pEBB–siMut–AIFTVA and pEBB–siMut–AIFVTA (9) into pSL4-hygro (60). Lentiviral packaging plasmids pHCMV-G, pRRE, and pRSV-rev are as described (61). Additional constructs were kind gifts obtained as follows: pcDNA3–ASK1–HA (62) from Dr. Jonathan D. Ashwell; pcDNA3–FLAG–MKK7–JNK1α1 and pcDNA3–FLAG–MKK7β2–JNK2α2 (49) from Dr. Roger Davis (Addgene plasmids 19726 and 19727); and pMXs–NDI1 (63) from Dr. David Sabatini (Addgene plasmid 72876). To generate pEBB–NDI1–FLAG, NDI1 cDNA was amplified by PCR from pMXs–NDI1 using primers containing NotI and KpnI restriction sites (sequences available upon request) and then inserted into the pEBB–FLAG backbone using standard cloning techniques. Lentiviral plKO plasmids were obtained as follows: pSPAX2 and pMD2.G-vsv-G from Dr. Didier Trono (Addgene plasmids 12260 and 12259); pLKO.1–puro-scramble (64) from Dr. David Sabatini (Addgene plasmid 1864); pLKO.1–puro–shE-cadherin (50) from Dr. Robert Weinberg (Addgene plasmid 18801); and pLKO.1–puro–shJNK1 (DTRCN000010580) and pLKO.1–puro–shJNK2 (TRCN000000945) were from Dharmacon. All shRNA sequences have been rigorously assessed for off-target effects and used as described previously (9, 10, 50, 58, 65–69).

**Antibodies**

Antibodies were obtained as follows: anti-AIF (Santa Cruz Biotechnology, sc-13116); anti-NDUFA9 (Invitrogen, 459100); anti-NDUF8 (Invitrogen, 459210); anti-citrate synthase (Cell Signaling, 14309); anti-β-actin (Sigma, A5316); and anti-MLK1
**JNK redox signaling requires AIF**

(With the text from the image)

Cell culture

Cells were cultured in an atmosphere of 95% air and 5% CO₂ at 37 °C. All media formulations were supplemented with 2 mM GlutaMAX, except for K-SFM as described below. HEK293T, MRC-5, PANC-1, PL45, and HeLa cells were cultured in DMEM supplemented with 10% FBS; PC3, DU145, LNCaP, BxPC-3, and HCT 116 cells in RPMI 1640 medium were supplemented with 10% FBS; HPAF-II cells in MEM were supplemented with 10% FBS; MIA PaCa-2 cells in DMEM were supplemented with 10% FBS and 2.5% horse serum; and HPAC cells in a 1:1 mixture of DMEM and Ham's F-12 medium were supplemented with 5% FBS, 1% FBS, 2 μg/ml insulin, 1 μg/ml transferrin, 40 ng/ml hydrocortisone, and 10 ng/ml EGF. RWPE-1 cells were cultured in K-SFM (ThermoFisher Scientific, 17005042) supplemented with 0.05 mg/ml bovine pituitary extract and 5 mg/ml transferase inhibitor mixture tablet per 10 ml. HEK293T cells were cultured in K-SFM (ThermoFisher Scientific, 17005042) supplemented with 0.05 mg/ml bovine pituitary extract and 5 mg/ml transferase inhibitor mixture tablet per 10 ml. HEK293T cells were cultured in 1 ml DMEM supplemented with 10% FBS; PC3, DU145, LNCaP, BxPC-3, and HCT 116 cells in RPMI 1640 medium were supplemented with 10% FBS; HPAF-II cells in MEM were supplemented with 10% FBS; MIA PaCa-2 cells in DMEM were supplemented with 10% FBS and 2.5% horse serum; and HPAC cells in a 1:1 mixture of DMEM and Ham's F-12 medium were supplemented with 5% FBS, 2 μg/ml insulin, 1 μg/ml transferrin, 40 ng/ml hydrocortisone, and 10 ng/ml EGF. RWPE-1 cells were cultured in K-SFM (ThermoFisher Scientific, 17005042) supplemented with 0.05 mg/ml bovine pituitary extract and 5 mg/ml transferase inhibitor mixture tablet per 10 ml. HEK293T cells were cultured in K-SFM (ThermoFisher Scientific, 17005042) supplemented with 0.05 mg/ml bovine pituitary extract and 5 mg/ml transferase inhibitor mixture tablet per 10 ml.

Lentiviral production and stable infection of cell lines

To establish cell lines stably suppressing or overexpressing AIF, lentiviral particles were produced by transfecting equal amounts of pHCMV-G, pRRE, pRSV-rev, and FG12- or pSL4-derived plasmids into HEK293T cells using the calcium phosphate method (70). Following incubation at 37 °C for 48 h, supernatants were collected, filtered using 0.45-μm pore size Millex HV PVDF filter units (Millipore), and concentrated by centrifugation at 20,000 × g. Viral pellets were then resuspended in PBS at 4 °C overnight. Resuspended virus was added to cells in the presence of 4 μg/ml Polybrene for 4 h at 37 °C in an environment of 93% air and 7% CO₂. Cell lines used for stable RNAi targeting of AIF were infected with either control lentivirus or lentivirus harboring an AIF target sequence as follows: shLacZ-puro or shAIF.1-puro (PC3, HPAC, MRC-5, RWPE-1, HeLa, PANc-1, BxPC-3, PL45, and HPAF-II) followed by selection with 1 μg/ml puromycin, shLacZ-GFP, or shAIF.1-GFP (DU145, LNCaP, HCT 116, and MIA PaCa-2); shLacZ-GFP or shAIF.2-GFP (PC3 and HPAC); and shGFPP-GFP or shAIF.2-GFP (MRC-5). To establish “restoration” cell lines (PC3, HPAC, and RWPE-1) expressing either WT AIF or a catalytically impaired mutant, cells were infected with empty vector, AIFWT, or AIFTVA and then selected with 500 μg/ml hygromycin B.

To establish “restoration” cell lines (PC3, HPAC, and RWPE-1) expressing either WT AIF or a catalytically impaired mutant, cells were infected with empty vector, AIFWT, or AIFTVA and then selected with 500 μg/ml hygromycin B.

For establishment of cell lines stably suppressing JNK1, JNK2, or E-cadherin, pLKO.1-based lentiviruses were generated by transfecting HEK293T cells with pMD.2, pSpAX2, and pLKO-shRNA using the calcium phosphate method. Viral supernatant was collected at 48 and 72 h post-transfection and then filtered using 0.45-μm pore size Millex HV PVDF filter units. Target cells were then incubated with viral supernatant and 8 μg/ml Polybrene for 24 h. Stably infected cells were selected using 1 μg/ml puromycin.

**Cell lysis, fractionation, immunoprecipitation, and immunoblot analysis**

Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) lysis buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 1 mM PMSF, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 protease inhibitor mixture tablet per 10 ml) or Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol). Mitochondrial extracts were prepared as described (71). Briefly, cells were resuspended in buffer A (250 mM sucrose, 20 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 1 protease inhibitor mixture tablet per 10 ml) and equilibrated on ice for 20 min, followed by homogenization with a Dounce tissue grinder (Kimble™ Kontes™). Samples were then centrifuged at 400 × g for 10 min at 4 °C. Supernatants were then collected and further centrifuged at 10,000 × g for 10 min at 4 °C. Precipitated material (constituting the mitochondrial fraction) was then washed in buffer A and resuspended in Nonidet P-40 lysis buffer (1% Nonidet P-40, 10% glycerol, 25 mM HEPES, pH 7.9, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 protease inhibitor tablet per 10 ml).

For immunoprecipitation experiments, RIPA cell lysates were normalized for protein content and then incubated with the indicated antibodies for 2 h at 4 °C. Protein G-coupled agarose beads were then added and incubated for 1 h. Agarose beads were then recovered by centrifugation and washed in RIPA buffer. Precipitated protein was eluted by adding lithium dodecyl sulfate sample buffer followed by heating samples at 95 °C for 5 min. Samples were then analyzed by immunoblot as described below.

For immunoblot experiments, protein samples were separated by SDS-PAGE using 4–12% gradient SDS-polyacrylamide gels followed by electrotransfer to nitrocellulose membranes. Membranes were blocked with 5% milk or BSA in TBS containing 0.02 to 0.2% Tween 20 and then incubated with the indicated primary antibodies. Membranes were then washed three times and incubated with HRP-conjugated anti-mouse or anti-rabbit followed by visualization using enhanced chemiluminescence.
Replicate immunoblots were quantified at multiple exposures for linearity using myImage Analysis™ software (ThermoFisher Scientific). Band intensities for phosphorylated proteins were divided by intensities for the corresponding total proteins; all protein values and phospho-protein/total protein ratios were then normalized to β-actin values. To determine fold changes among lanes, all ratios were then normalized to control lanes.

**Transfections**

Plasmids were transfected into cells using Lipofectamine™ 2000 as described by the manufacturer. Cells were then harvested 48–72 h following transfection and either lysed as described below or quantified by Coulter™ counting.

**Drug treatments**

Cells were subjected to chemical treatments as follows: tBHQ at 0–2 mM for 1 h, SP600125 at 0–40 μM for 72 h, stauroporine at 1 μM for 16 h, and bVD-fmk at 50 μM at time of seeding and then every 48 h for 7 days. Following treatment, cells were harvested and assessed in assays as described below.

**Quantitative RT-PCR**

Gene expression analysis was carried out as described (72) using the relative standard curve method for quantification. Briefly, real time PCR was carried out on the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Total RNA was isolated from the indicated cell lines using TRIzol reagent according to the manufacturer’s instructions. 30 μg of RNA isolated from each sample was treated with 30 units of DNase I (Promega) for 30 min at 37 °C. After DNase I digestion, RNA was purified using an RNeasy mini purification kit (Qiagen) following the manufacturer’s protocol. An oligo(dT) primer was used in cDNA synthesis. 200 ng of RNA was reverse-transcribed in a total volume of 50 μl using the TaqMan reverse transcription reagents kit (Applied Biosystems). 200 ng of RNA was treated with 30 units of DNase I (Promega) for 30 min at 37 °C. After DNase I digestion, RNA was purified using an RNeasy mini purification kit (Qiagen) following the manufacturer’s protocol. An oligo(dT) primer was used in cDNA synthesis. 200 ng of RNA was reverse-transcribed in a total volume of 50 μl using the TaqMan reverse transcription reagents kit (Applied Biosystems). To make a standard curve, serial dilutions of RNA from one sample (160, 40, 10, and 2.5 ng/μl final concentrations) were added to the RT reaction. Aliquots (3.5 μl) of cDNA were added to a 31.5-μl reaction mixture containing 17.5 μl of 2× SYBR®Green PCR Master Mix (Applied Biosystems) and 200–400-nm primers; quadruplicate samples were prepared for each RNA source. Absence of DNA contamination was verified by performing amplification from cDNA without reverse transcriptase. The primers for PCR were designed with IRT PrimerQuest software (Integrated DNA Technologies, Inc.) and are as follows: GAPDH forward, 5′-CCCCACTCTCCACCTTGAC-3′, and reverse, 5′-TGTGGCTGTAGCCTAACCCT-3′; β-actin forward, 5′-GCGGGAATCTGTCGTTACAT-3′; and reverse, 5′-GATGGAGTTGAAGGTAGTTTCGTG-3′; AIF forward, 5′-AATCGGAAGGCAGTGGTTAGTATG-3′; and reverse, 5′-CAAGGGGTTTTCCGCTGC-3′; Zeb1 forward, 5′-TGC- ACTGAGTGTTGAAAGCA-3′, and reverse, 5′-TGGGTGATGGCAAGAGACTG-3′; N-cadherin forward, 5′-GAAAGCCCATATCCACGCAACC-3′; and reverse, 5′-CTCTGCTCACCACTACA-3′; TwistI forward, 5′-GGAGTCCGCGTCTTGACGACCTTG-3′, and reverse, 5′-TCTGGAGGACCTGTTAGAGG-3′; Met forward, 5′-GGTCAATTCCGAGAATCTC-3′; and reverse, 5′-CAAGGGGTTTTCCGCTGC-3′; Zeb2 forward, 5′-CAAGGGGTTTTCCGCTGC-3′; and reverse, 5′-GGTGGGAATATTCCGTTACCT-3′; E-cadherin forward, 5′-ACGCATGATCCGACATACA-3′, and reverse, 5′-CGTGGACGATGATCCGACATACA-3′. Primers to either β-actin or GAPDH were used to determine normalization factors between AIF-proficient (shLacZ) and AIF-deficient (shAIF) cell lines, and variation in β-actin/GAPDH levels between cell lines was less than 10% in all cases.

**Phase contrast microscopy**

Images were captured by phase contrast microscopy using the ×10 objective of a Nikon TS100F microscope equipped with a Nikon DS-Fi1 digital camera detection system and NIS Elements 4.0 software.

**Matrigel™ experiments**

Equal volumes of cold Matrigel™ were added to each well in 24-well plates and then allowed to solidify at 37 °C for 1 h. Cells were added to Matrigel™ layers at equal densities and exposed to substrate conditions for 3–7 days in the absence or presence of bVD-fmk. Detached cells were collected and quantified by Coulter™ counting; attached cells were extracted from substrate using Matrigel recovery solution (Corning) and either lysed as described above or quantified by Coulter™ counting. Percent death was defined as the number of detached cells divided by total cells.

**Measurements of mitochondrial ΔΨm and abundance**

Staining for mitochondrial membrane potential was carried out as described previously (73) by resuspending harvested cells in PBS containing 200 nM TMRM followed by flow cytometry. Staining for mitochondrial abundance was carried out by incubating cells with 100 nM MitoTracker™ Green for 37 °C for 20 min. Cells were then harvested and resuspended in PBS, followed by assessment of stain intensity using an Accuri C6 flow cytometer.

**Glucose consumption measurements**

Following transfections, fresh media were replenished, and cells were grown at 37 °C for 72 h. Media glucose levels were then assessed using the QuantiChrom™ glucose assay kit (BioAssay Systems). Cells in each sample were quantified by Coulter™ counting and used to determine glucose consumption levels per cell.

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