Targeting the Mitochondria Activates Two Independent Cell Death Pathways in Ovarian Cancer Stem Cells

Ayesha B. Alvero, Michele K. Montagna, Jennie C. Holmberg, Vinicius Craveiro, David Brown, and Gil Mor

Abstract
Cancer stem cells are responsible for tumor initiation and chemoresistance. In ovarian cancer, the CD44+/MyD88+ ovarian cancer stem cells are also able to repair the tumor and serve as tumor vascular progenitors. Targeting these cells is therefore necessary to improve treatment outcome and patient survival. The previous demonstration that the ovarian cancer stem cells are resistant to apoptotic cell death induced by conventional chemotherapy agents suggests that other forms of targeted therapy should be explored. We show in this study that targeting mitochondrial bioenergetics is a potent stimulus to induce caspase-independent cell death in a panel of ovarian cancer stem cells. Treatment of these cells with the novel isoflavone derivative, NV-128, significantly depressed mitochondrial function exhibited by decrease in ATP, Cox-I, and Cox-IV levels, and by increase in mitochondrial superoxide and hydrogen peroxide. This promotes a state of cellular starvation that activates two independent pathways: (i) AMPK pathway leading to mTOR inhibition; and (ii) mitochondrial MAP/ERK kinase/extracellular signal-regulated kinase pathway leading to loss of mitochondrial membrane potential. The demonstration that a compound can specifically target the mitochondria to induce cell death in this otherwise chemoresistant cell population opens a new venue for treating ovarian cancer patients.

Introduction
Epithelial ovarian cancer (EOC) is the most lethal of the gynecologic malignancies and accounts for about 15,000 deaths every year (1). A major obstacle in the successful treatment of ovarian cancer is the development of chemoresistance. Chemoresistant cancer cells typically have a high threshold for activation of the caspase-dependent apoptotic cascade, mostly brought about by overexpression of antiapoptotic genes (2, 3). Several approaches have been used to overcome resistance to apoptosis. These include the use of small molecules directed against antiapoptotic proteins such as X-linked inhibitor of apoptosis protein or the use of chemosensitizers such as phenoxodiol (4–7). Pretreatment of chemoresistant EOC cells with phenoxodiol lowers the Glc₅₀ for carboplatin, paclitaxel, topotecan, and gemcitabine and allows these agents to activate caspases and induce cell death (6, 8). Although the use of chemosensitizers was found to be successful in vitro and in mouse in vivo studies, it did not prove to be effective in clinical trials. Following an initial response, tumors recurred and became unresponsive to the addition of chemosensitizers (9). This suggests that certain cancer cell populations possess a mechanism of chemoresistance, which is not associated with the inhibition of apoptosis. Therefore, in these patients, there is a need for other forms of targeted therapy.

Cancer stem cells are defined as cells that possess the capacity to self-renew and generate the heterogeneous lineage of cancer cells that comprise the whole tumor (10, 11). Current evidence suggests that cancer stem cells are the putative mediators of chemoresistance and tumor progression (10). It is thought that they are able to survive conventional chemotherapeutic treatments, which usually target fast dividing cells, and give rise to recurrent tumors that are even more chemoresistant and more aggressive.

In ovarian cancer, the CD44+/MyD88+ cells possess attributes described for cancer stem cells. These cells have tumor-initiating properties, express stem cell markers, and are extremely chemoresistant (12). Addition of phenoxodiol does not alter their resistance to carboplatin nor paclitaxel. On the other hand, CD44-/MyD88- EOC cells can not form tumors in mice in limiting dilution assays, do not express stem cell markers, and can respond to carboplatin and paclitaxel when pretreated with phenoxodiol. In addition, the capacity to serve as tumor vascular progenitors and the capability of tissue repair is limited to the CD44+/MyD88+ ovarian cancer stem cells.
stem cells (13). Taken together, these data suggest that targeting the ovarian cancer stem cells is necessary to improve treatment outcome and patient survival. The demonstration that ovarian cancer stem cells do not respond to sensitizers of the apoptotic pathway suggest that other forms of cell death should be explored.

Recently we reported that the phenyl-substituted isoflavone compound, NV-128, can induce caspase-independent cell death in all EOC cells tested (14). NV-128-induced cell death was promoted by two apparently independent events—mitochondrial depolarization and mTOR inhibition. The mitochondrial effect was characterized by loss of mitochondrial membrane potential (MMP) as early as 1 hour posttreatment. Loss of MMP however, was not associated with caspase activation but instead resulted in nuclear translocation of endonuclease G leading to caspase-independent DNA fragmentation (14). NV-128 also inhibited both complexes of mTOR protein. Cells treated with NV-128 had significantly lower levels phosphorylated-p70 ribosomal S6 kinase (pS6K; target of mTOR complex 1), and phosphorylated-AktSer473 (pAkt; target of mTOR complex2). As a result of mTOR inhibition, NV-128 treated cells acquired autophagic vacuoles and showed increase levels of the autophagic marker, LC3-II (14). Thus, the combined loss of MMP and inhibition of the mTOR pathway potently lead to caspase-independent cell death.

The objectives of this study are two-fold: first is to determine the efficacy of NV-128 in the ovarian cancer stem cells, and the second is to elucidate a link between the observed mitochondrial changes and the inhibition of the mTOR pathway. We show that by targeting mitochondrial energetic function, NV-128 is able to create a state of cellular starvation and activate two independent, noncanonical pathways to induce death in the ovarian cancer stem cells. NV-128 is able to activate the 5′-AMP kinase (AMPK)-mTOR pathway, and the extracellular signal-regulated kinase (ERK)-Bax pathway to potently induce death in the apoptosis-resistant ovarian cancer stem cells.

Materials and Methods

Cell lines, culture conditions, and reagents

CD44+/MyD88+ ovarian cancer stem cells were isolated from either tumor tissue or ascites obtained from patients diagnosed with stage III/IV serous ovarian carcinoma as previously described (12, 15, 16). Sample collection was carried out with patient consent and approved by the Human Investigations Committee of Yale University School of Medicine. Purity of the cultures were tested before each experiment by measuring the levels of CD44 and MyD88 by flow cytometry. The epithelial nature of the isolated cells was determined by staining for Ck18. Ovarian cancer stem cells were propagated as previously described (12, 13). The EOC cell lines A2780 and CP70 were obtained from Dr. T. C. Hamilton in 2002 and propagated as previously described (15, 16). The epithelial nature of the cell lines was determined by immunostaining with Ck7 and authenticated on a regular basis (at least every two months) based on their differential response to carboplatin.

Cell viability assay and determination of cell morphology

Cell viability was determined as previously reported (16). Briefly, cells (5 × 10⁵) were plated in triplicate wells in a 100 μl volume per well of a 96-well microtiter plate (BD Biosciences/Pharmingen). The cells were grown to 70% confluence and then incubated in reduced serum phenol red-depleted Opti-MEM medium (Invitrogen-GIBCO) for 4 hours before treatment. NV-128 (Novogen, Ltd.) and rapamycin (Sigma Aldrich) were added to the medium from 10 mg/ml and 10 mmol/L stock, respectively, to give the final concentrations described in the results section. Following treatment for 24 hours, cell viability was evaluated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corporation) according to the manufacturer’s instructions. Optical densities of the samples were measured at 490 nm using an automatic microplate reader (Model 550, Bio-Rad). The values from the treated cells were compared with the values generated from the untreated control and reported as percent viability. Each experiment was done in triplicate. For studies with inhibitors, U0126 (Sigma Aldrich) and MnTBAP (Alexis Biochemicals) were added 1 hour before NV-128 treatment. Cellular morphology was assessed using Incucyte (Essen Instruments).

Analysis of mitochondrial function

Levels of ADP and ATP were measured using ApoSensor ADP/ATP ratio assay kit (Biovision Inc.). Accumulation of mitochondrial superoxide and cellular hydrogen peroxide were measured using MitoSOX Red and CM-H2DCFDA, respectively (Invitrogen). Mitochondrial membrane potential was determined using Mitocapture apoptosis detection kit based on JC1 dye staining (Biovision Inc.) and Mitotracker Red CMXRos (Molecular Probes). Mitochondrial mass was quantified using Mitotracker Green FM (Invitrogen). Flow cytometry data were acquired using BD FACSCalibur and analyzed using CellQuest (BD Biosciences).

Protein preparation and subcellular fractionation

Protein was extracted and measured as previously described (16). For separation of the cytoplasmic and mitochondrial fractions, cell pellets were processed using the ApoAlertTM Cell Fractionation Kit (BD Biosciences) according to the manufacturer’s instructions.

SDS-PAGE and Western blots

SDS-PAGE and Western blots were carried out as previously described (16). Antibodies used were: mouse antiphospho-ERK1/2 T204 (Santa Cruz Biototechnology, Inc.), rabbit anti-ERK1/2 (Millipore), rabbit antiphospho-Akt Ser473 (Cell Signaling Technology), rabbit anti-Akt...
(Cell Signaling Technology), rabbit antiphospho-p70 S6 kinase Thr412/Thr389 (Millipore), rabbit antiphospho-AMPKα1/2 T174/T172 (Cell Signaling), rabbit anti-Cox-IV (Cell Signaling Technology), mouse anti-Cox-I (MitoSciences Inc.), mouse anti-Cox-III (MitoSciences Inc.), mouse anti-Bax (BD Biosciences), and rabbit anti-actin (Sigma Aldrich).

**Statistical analysis**

Data are expressed as mean ± standard error. Statistical significance (P < 0.05) was determined using either two tailed unpaired t-tests or Mann–Whitney U test for nonparametric data. Unless stated otherwise, all experiments were carried out in triplicate and repeated at least three times.

**Results**

**NV-128 induces degradation of Cox-I and Cox-IV, loss of ATP, and upregulation of mitochondrial superoxide**

We previously showed the cytotoxic activity of NV-128 on EOC cells and showed that its effect was partly associated with inhibition of the mTOR pathway (14). We then determined if the cytotoxic effect of NV-128 is maintained in the ovarian cancer stem cells. Figure 1A shows that NV-128 is able to significantly reduce the viability of all ovarian cancer stem cells tested. Moreover, NV-128 had a very potent cytotoxic effect such that the addition of carboplatin or paclitaxel did not have any additive effect (Supplementary Fig. S1).

Interestingly, all EOC cells tested are resistant to rapamycin (a specific mTOR complex 1 inhibitor; Fig. 1A). The observation that NV-128 can induce cell death in rapamycin resistant cells and the previous demonstration that it can inhibit both mTOR 1 and 2 complexes (14) suggest that NV-128 does not act like a rapalogue.

In addition to mTOR inhibition, another event observed during NV-128-induced cell death is the loss of mitochondrial membrane potential (14). It is thus possible that this effect on the mitochondria, coupled with mTOR inhibition, act in concert to induce death in the rapamycin-resistant ovarian cancer stem cells. To test this hypothesis, we first carried out further characterization of NV-128’s effect on mitochondrial function. Evaluation of the components of the electron transport chain such as Cox-I, Cox-III,
and Cox-IV showed a significant and time-dependent decrease in the levels of Cox-I and Cox-IV, but not Cox-III, after NV-128 treatment (Fig. 1B). To further show the specific effect on the members of the electron transport chain, we stained NV-128-treated ovarian cancer stem cells with MitoTracker Red and MitoTracker Green. MitoTracker Red quantifies polarized mitochondria whereas MitoTracker green staining is equivalent to mitochondrial mass. Flow cytometry results showed that NV-128-induced loss of MMP was not associated with loss of mitochondrial mass (Fig. 1C), suggesting that the decrease in the electron transport chain complexes is not due to mitophagy.

Due to the presence of Cox-I and -IV degradation, we next determined the impact of NV-128 on energy production by measuring ADP and ATP levels. Figure 1D shows that ovarian cancer stem cells treated with NV-128 had significantly lower levels of ATP and accumulated ADP after 2 and 4 hours of treatment. Because an accompanying event in the uncoupling of oxidative phosphorylation is the production of reactive oxygen species (ROS), we next measured the levels of mitochondrial superoxide and cellular hydrogen peroxide. Flow cytometry results using the MitoSox and CM-H2CFDA showed a significant increase in the fluorescence intensity of both dyes in NV-128-treated cells (Fig. 1E). Thus, NV-128 is able to significantly inhibit mitochondrial ROS production (Fig. 2B), and more importantly, MnTBAP pretreatment abrogated NV-128-induced phosphorylation of mitochondrial ERK 1/2 (Fig. 2C). This suggests that NV-128-induced ROS is the cause of mitochondrial ERK activation. Our next objective was to determine the role of NV-128-induced mitochondrial ERK activation on the observed effect on mTOR and MMP.

**NV-128-induced mTOR inhibition is independent of the MEK/ERK pathway**

Thus, we used the specific MEK1/2 inhibitor, U0126, which is known to inhibit phosphorylation of ERK.

**Previous studies have shown that upregulation of ROS can induce activation of the mitogen-activated protein/ERK kinase/ERK (MEK/ERK) pathway (17). Evaluation of the phosphorylation status of ERK 1/2 in ovarian cancer stem cells treated with NV-128 showed a significant increase in the levels of phosphorylated-ERK1/2 (pERK) in the mitochondria (Fig. 2A). This was however, not associated with an increase in total mitochondrial ERK 1/2 (tERK) suggesting that NV-128 does not induce translocation of pERK into the mitochondria, but instead induces phosphorylation of resident mitochondrial ERK 1/2. Indeed, baseline mitochondrial ERK is mostly in the nonphosphorylated state and only becomes phosphorylated after treatment with NV-128 (Fig. 2A).

To determine if the increase in pERK is a direct consequence ROS production, ovarian cancer stem cells were pretreated with the cell permeable superoxide dismutase mimetic, MnTBAP, 1 hour before NV-128 treatment. MnTBAP is able to inhibit NV-128-induced mitochondrial ROS production (Fig. 2B), and more importantly, MnTBAP pretreatment abrogated NV-128-induced phosphorylation of mitochondrial ERK 1/2 (Fig. 2C). This suggests that NV-128-induced ROS is the cause of mitochondrial ERK activation. Our next objective was to determine the role of NV-128-induced mitochondrial ERK activation on the observed effect on mTOR and MMP.
Pretreatment of ovarian cancer stem cells for 1 hour with U0126 was able to abolish NV-128-induced upregulation of mitochondrial pERK (Fig. 3A). However, U0126 was not able to prevent NV-128-induced mTOR inhibition. Levels of pS6K and pAkt remained low in NV-128 treated cells even in the presence of U0126 (Fig. 3A). This suggests that NV128-induced mTOR inhibition is independent of MEK/ERK activation.

Interestingly, analysis of cellular morphology showed that inhibition of the MEK/ERK pathway is able to delay NV128-induced cell death (Fig. 3B). This suggests that although NV-128-induced mTOR inhibition is independent of the MEK/ERK pathway, the activation of ERK still has a significant contribution to NV128-induced cell death.

Because another event observed in NV-128 treated cells is the loss of MMP, we next determined the association between MEK/ERK activation and loss of MMP.

**NV128-induced extracellular signal-regulated kinase activation controls mitochondrial membrane potential through Bax**

To determine the association between ERK activation and loss of MMP, ovarian cancer stem cells were pretreated with U0126 before NV-128 treatment. Flow cytometry analysis using the JC1 dye showed that inhibition of ERK with U0126 is able to reverse the effect of NV-128 on MMP. As shown in Fig. 4A, pretreatment with U0126, significantly reduced the percentage of cells that lost MMP (59% vs. 23% for NV-128 alone and NV128 with U0126, respectively). These results suggest that NV-128-induced loss of MMP is dependent on the activation of the MEK/ERK pathway.

MMP is controlled in part by the Bcl2 family of proteins (18). To determine if NV-128-induced activation of the MEK/ERK pathway affects the status of Bcl2 family members, we analyzed mitochondrial fractions of cells treated with NV-128 in the presence or absence of U0126. Our results showed that NV-128 was able to upregulate the proapoptotic Bcl2 family member, Bax (Fig. 4B). More importantly, pretreatment of ovarian cancer stem cells with U0126 prevented NV-128-induced upregulation of mitochondrial Bax. Thus, ERK activation after NV-128 treatment contributes to cellular loss of MMP through Bax.

**NV-128-induced loss of ATP leads to mTOR inhibition**

Our data so far showed that NV-128-induced ROS production activates the MEK/ERK/Bax axis leading to loss of MMP. This was however, not associated with NV-128-induced mTOR inhibition. As there is significant loss of ATP in NV-128-treated ovarian cancer stem cells, our next objective was to determine the association between ATP loss and mTOR. We hypothesized that loss of ATP may create a state of cellular starvation that can potentially inhibit mTOR. To test this hypothesis we compensated for reduced ATP levels in NV-128-treated cells by adding 20% FBS. Figure 5A shows that NV-128-induced ATP loss was partially inhibited

![Figure 3. NV-128-induced inhibition of mTOR is independent of the MEK/ERK pathway. A, ovarian cancer stem cells were pretreated with U0126 (10 μmol/L) for 1 hour before NV-128 treatment and the effect on ERK and mTOR activity determined using Western blotting analysis. C, control. B, effect on cell morphology analyzed using a real-time video imaging system. Results shown are for ovarian cancer stem cell 1. Similar results were observed with other cells tested. n = 3.](image-url)
upon the addition of FBS. More importantly, abrogation of ATP loss is able to rescue the mTOR pathway. Treatment of ovarian cancer stem cells with NV-128 in the presence of FBS was able to prevent NV-128-induced decrease in pS6k (Fig. 5B). This suggests that inhibition of ATP production by NV-128 triggers mTOR inhibition. The addition of FBS also abrogated NV-128-induced increase in pERK. This further shows that the mitochondrial effects are upstream of ERK activation.

**NV-128 activates AMPKα1**

AMPKα1 is an energy sensor, which is responsive to levels of cellular AMP (19) and is therefore activated when ATP production is low. Previous studies have linked ATP loss and mTOR inhibition through AMPKα1 (20). AMPKα1 can inhibit mTOR by phosphorylating TSC2 resulting in the activation of the TSC1/TSC2 complex and subsequent inhibition of mTOR (21). Therefore we hypothesized that inhibition of ATP production by NV-128 may activate AMPKα1. Indeed, analysis of phosphorylation status of AMPKα1 showed that NV-128 is able to activate the AMPK pathway in a time dependent manner (Fig. 5C). Interestingly, activation of AMPKα1 is not abrogated with the ROS scavenger, MnTBAP (Fig. 2C).

**Discussion**

We show in this study that by disrupting oxidative phosphorylation, NV-128 can induce cell death in the ovarian cancer stem cells through two independent non-canonical pathways. NV-128-induced degradation of Cox-I and IV can lead to accumulation of ROS and decrease ATP production. Elevated ROS activates the MEK/ERK/Bax axis leading to loss of MMP, and the resulting decrease in ATP levels creates a low energy...
DNA fragmentation (Fig. 6; ref. 14). Induce autophagic cell death and caspase-independent correlates with the previous observation that NV-128 can ing events lead to ovarian cancer stem cell death and ovarian cancer stem cells probably share this characteristic.

Therefore it is not surprising that elevated mTOR activity is a characteristic of many human cancers, rendering mTOR active even in conditions of growth factor withdrawal (25, 26). As aberrant mTOR activation is a common molecular event in cancer, there is much interest in developing and testing mTOR inhibitors as antitumor agents. To date, there are completed and ongoing clinical trials testing the efficacy of rapamycin or its analogs using mTOR inhibitors and upon direct inhibition of mTOR, alternative pathways are activated (28, 29). Therefore, it is necessary to elucidate alternative approaches that can inhibit all the signals originated and related to the mTOR pathway. In this study, we show that by inducing energy stress, through the inhibition of mitochondrial energy production, we were able to trigger an inhibitory cascade, mediated by AMPKα1, which can potently suppress mTOR activity.

Under conditions of energy depletion, the highly conserved energy sensing protein kinase AMPK (5' AMP-activated protein kinase) is activated and phosphorylates TSC2, which by still unclear mechanisms, enhances the ability of the TSC1-TSC2 complex to turn off the mTOR pathway (21). In this study, we show that the NV-128-induced decrease in ATP is able to create a depleted energy status, which activates the energy sensing AMPK leading to inhibition of mTOR. This inhibitory effect is sufficiently potent to induce autophagic cell death in the ovarian cancer stem cells.

Another characteristic of cancer cells is their dependence on the glycolytic pathway to fulfill their energy needs (30). This observation leads to the hypothesis that cancer cells have inefficient or suboptimal oxidative phosphorylation capacity or that their antioxidant system is suppressed (31, 32). Both were later shown true with the demonstration that under basal conditions, several human carcinoma cell lines produce high levels of hydrogen peroxide (33). In addition, it has been shown that tumor cells have lower levels of catalase and dismutase activity to counteract ROS levels (34, 35). Taken together, this suggests that the highly energy-dependent cancer cells are under persistent oxidative stress. Interestingly, the elevated level of ROS in cancer cells is insufficient to induce cell death suggesting that cancer cells are able to withstand oxidative stress. Instead, elevated ROS have been shown to be favorable for tumor growth. Persistently high ROS levels in cancer cells can contribute to oxidative DNA damage and the induction of prosurvival transcription factors such as NFκB, the activation of oncopogens, and suppression of tumor suppressor genes (36, 37).

In hematopoietic cancer stem cells, it was previously reported that low basal ROS is a requirement for maintaining self-renewal potential (38). Minute changes in ROS levels induce either senescence or apoptosis in these cells. The redox state in ovarian cancer stem cells remains to be elucidated. However, the demonstration that a compound like NV-128, which can disrupt oxidative phosphorylation and increase mitochondrial ROS, can induce cell death in ovarian cancer stem cells suggests that the ovarian cancer stem cells probably share this characteristic.

Given the highly oxidative status of cancer cells compared with normal cells, one can argue that cancer cells are primed for cell death induced by agents that can target the electron transport chain. Minute perturbation in oxidative phosphorylation can possibly induce cell death in the primed cancer cells without harming the surrounding normal stroma. Indeed, we show in this study that NV-128, by disrupting the electron transport chain can cause increase in mitochondrial ROS leading to the activation of mitochondrial ERK.

ERK1/2 are conserved serine/threonine protein kinases, which are classically known as mediators of proliferation and transformation (47). As a response to cell stress, such as oxidative stress, AMPK is often activated (48). Such activation of AMPK leads to the phosphorylation of ERK1/2, which in turn promotes cell survival (49). However, recent studies have shown that AMPK activation reduces ROS production, which in turn decreases the oxidative stress, leading to the cell death. This suggests that AMPK activation in combination with oxidative stress can induce cell death in cancer cells.
growth and survival (39). Ligation of receptor tyrosine kinases with growth factors results in the activation of MEK/ERK pathway and nuclear translocation of ERK1/2, where it activates multiple transcription factors leading to cell proliferation, differentiation, and survival (40). On the other hand, although not as well characterized as nuclear ERK, mitochondrial ERK has been described to be involved in the regulation of mitochondrial function, as well as cell death decisions. Activation of ERK by the redox cycling dopamine analog, 6-hydroxydopamine (6-OHDA) results in mitochondrial localization of pERK, mitophagy, and neuronal cell death (17). Moreover, activation of ERK in response to cisplatin is required for cisplatin-induced apoptosis in renal cells (41). In this study, we show that in ovarian cancer stem cells treated with NV-128, the ROS-dependent activation of ERK is able to disrupt mitochondrial function through Bax-mediated loss of MMP. The ERK-related mitochondrial changes observed however, did not involve mitophagy as seen in the neuronal model.

In summary, we show that disruption of mitochondrial function by NV-128, activates two independent cell death pathways in the ovarian cancer stem cells. Our previous data, using an ovarian cancer xenograft model, showed that NV-128 is able to inhibit tumor growth without inducing toxicity in mice (14). This suggests that a sufficient therapeutic window exist that will allow NV-128 to be safely administered to patients. Therefore, the studies described here provide sufficient proof of concept study to initiate clinical trial in ovarian cancer patients.

Disclosure of Potential Conflicts of Interest

David Brown is an employee of Marshall Edwards Inc.

Acknowledgments

The authors would like to thank Dr. Gerald S. Shadel for assistance with the analysis of mitochondrial function.

Grant Support

This study was supported by the NIH grants RO1CA118678 and RO1CA127913, and The Janet Burros Memorial Foundation and the Discovery to Cure Research Program (G. Mor).

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Received January 17, 2011; revised May 31, 2011; accepted June 7, 2011; published OnlineFirst June 15, 2011.

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Molecular Cancer Therapeutics

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doi:10.1158/1535-7163.MCT-11-0023

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