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Human Mut T homolog 1 (MTH1)

A roadblock for the tumor-suppressive effects of oncogenic RAS-induced ROS

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Oncogenic RAS-induced reactive oxygen species (ROS) trigger barriers to cell transformation and cancer progression through tumor-suppressive responses such as cellular senescence or cell death. We have recently shown that oncogenic RAS-induced DNA damage and attendant premature senescence can be prevented by overexpressing human MutT Homolog 1 (MTH1), the major mammalian detoxifier of the oxidized DNA precursor, 8-oxo-dGTP. Paradoxically, RAS-induced ROS are also able to participate in tumor progression via transformative processes such as mitogenic signaling, the epithelial-mesenchymal transition (EMT), anoikis inhibition and PI3K/Akt-mediated survival signaling. Here we provide a preliminary insight into the influence of MTH1 levels on the EMT phenotype and Akt activation in RAS-transformed HMLE breast epithelial cells. Within this context, we will discuss the implications of MTH1 upregulation in oncogenic RAS-sustaining cells as a beneficial adaptive change that inhibits ROS-mediated cell senescence and participates in the maintenance of ROS-associated tumor-promoting mechanisms. Accordingly, targeting MTH1 in RAS-transformed tumor cells will not only induce proliferative defects but also potentially enhance therapeutic cytotoxicity by shifting cellular response away from pro-survival mechanisms.

Keywords: reactive oxygen species (ROS), MTH1, 8-oxoguanine (8-oxoG), DNA damage, proliferation, E-cadherin, epithelial-mesenchymal transition (EMT), oncogene-induced senescence (OIS), Akt activation

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The RAS oncogene, found in approximately 25% of all cancers, confers multiple tumor-promoting characteristics including unrestrained proliferation, survival signaling, resistance to anchorage loss-dependent cell death (anoikis), increased migration and invasiveness, and angiogenesis.[1,2] Several of these features are known to be mediated by reactive oxygen species (ROS). In particular, hyperactivated RAS signaling elevates cellular ROS levels,[3] through Rac-GTP signaling-mediated NADPH oxidase (Nox) activity as well as by inducing mitochondrial dysfunction.[4,5] Oncogenic RAS-induced mitogenic signaling is inhibited by the antioxidant, N-acetylcysteine,[3] and its ability to confer tumorigenicity, anoikis resistance and angiogeniccapability is functionally dependent on NADPH oxidase 1 (Nox1)-generated superoxide radicals.[6,7] The PI3K/Akt pathway, a downstream RAS effector of survival signaling,[8] is stimulated by ROS through oxidative inactivation of Akt-inhibitory phosphatases such as PTEN.[9] Oncogenic RAS also induces the cell invasion-promoting epithelial-mesenchymal transition (EMT) through RacGTPase activity.[10] Rac1 activates Nox1-dependent ROS generation[11] which has been reported to enhance production of matrix metalloprotease-9 (MMP-9),[12] an effector of EMT-induced invasion and migration. Thus, although ROS generation by oncogenic RAS causes oxidative DNA damage[4,13] resulting in cell senescence[13-15] or cell death,[16,17] it is also essential to its transformative and tumor-promoting functions.

Hence, RAS-transformed cells must deal with the damaging effects of ROS without eliminating ROS production entirely. One way for RAS-transformed cells to accomplish this outcome is to compensate for elevated RAS oncoprotein

signaling by increasing expression of redox-protective proteins. Such an adaptation would serve to uncouple the tumor-promoting effects of ROS from their tumor-suppressive consequences. A proteomics analysis study indicates that proteins involved in cellular redox balance are among the relatively small number significantly upregulated upon RAS-mediated transformation.[18] Furthermore, disabling the glutathione system has been found to selectively induce ROS-mediated death in RAS-transformed ovarian cells,[19] providing concrete evidence that redox-regulatory proteins play a functionally protective role in these cells. Even more strikingly, it has been demonstrated that enhancing expression of glutathione S-transferase (GST) in RAS-transformed murine cells abrogates the pro-apoptotic p38alpha-mediated response to RAS-induced ROS without affecting the pro-survival/pro-proliferation PI3K/Akt pathway.[20] More recently, oncogenic KRAS was reported to increase expression of Nrf2, a critical redox-regulatory transcription factor that controls antioxidant response element (ARE)-mediated gene expression, with Nrf2 ablation reducing oncogenic KRAS-mediated proliferation and tumorigenesis in vivo.[21] Thus, in effect, the redox-protective proteins discussed above comprise a "non-oncogene addiction" in RAS-transformed cells, in that their inhibition could significantly sensitize such cells to tumor-suppressive responses despite their not having a direct role in cell transformation. As such, identifying additional members in this class of proteins is likely to lead to clinically valuable therapeutic targets and/or prognostic markers for activated RAS-sustaining cancers.

We recently found that overexpressing the human 8-oxo-dGTPase MutT Homolog1 (MTH1) in normal human skin fibroblasts suppresses total cellular 8-oxoguanine (8-oxoG) levels, the DNA damage response (DDR), and cell senescence induced by oncogenic RAS expression, without affecting oncogenic RAS-induced ROS levels.[13] Our study provides the first indication that detoxification of oxidative damage to DNA precursors is an important downstream mediator of oncogenic ROS-induced cellular responses. Examination of ONCOMINE tumor data sets indicates a definite correlation between high MTH1 levels and the presence of oncogenic RAS mutations in human cancers, specifically pancreatic and lung cancers.[22,23] In accordance with this fact, we found that oncogenic RAS overexpression itself upregulated MTH1 levels in both normal and tumorigenic cells.[13] Furthermore, MTH1 suppression in isogenic pairs of breast epithelial cells lines (MCF7/MCF7-RAS and HMLE/HMLE-RAS) selectively led to elevated total cellular 8-oxoguanine (8-oxoG) and reduced proliferation in the RAS-transformed counterparts.[13]

The MTH1 gene promoter contains multiple consensus sequences corresponding to the Ets family of transcription factors,[24] known to regulate gene expression in response to RAS signaling.[25] The MTH1 promoter also contains binding sites for NFkappaB and AP-1, both of which regulate gene transcription in response to oxidative stress.[24] In addition, AP-1 and Ets sequences form composite RAS-responsive elements (RRE) that can amplify RAS signaling-mediated transcriptional events.[25] Collectively, these observations are consistent with MTH1 being transcriptionally upregulated by oncogenic RAS signaling and/or RAS activation-induced oxidative stress. Therefore, MTH1 falls into the putative class of proteins that protect RAS-transformed cells from ROS-induced tumor suppressor effects. However, due to its defined and well-characterized role in preventing incorporation of oxidized precursors into the genomic DNA,[24] MTH1 has an advantage over redox-regulatory thiol proteins as a therapeutic target, because the latter often exhibit pleiotropic or dose-dependent effects on cellular functions. Furthermore, as normal untransformed cells do not suffer the chronically high levels of oxidative stress and DNA damage that afflict oncogenically transformed tumor cells,[13,26] they are unlikely to exhibit the same degree of reliance on MTH1 function for proliferation and survival. Accordingly therapeutic inhibitors of MTH1 are predicted to show high selectivity for tumor cells and to possess fewer undesirable off-target effects.

Oxidative DNA damage can trigger either cell senescence or cell death, depending on the degree of damage and on which downstream damage response pathways are activated.[27] Therefore, understanding the genetic or epigenetic context under which the different antitumor responses are activated is critical for effectively utilizing MTH1 as a potential therapeutic target. Whereas MTH1 loss led to elevated senescence markers in the p53-competent MCF7-Ras cells, an unexpected finding from our study was the antiproliferative effect of MTH1 suppression on HMLE-RAS cells,[13] which no longer have active senescence induction pathways due to the presence of SV40 Large T Antigen. In these cells, we found that MTH1 suppression reduced cell numbers via a G_1/S arrest but did not appear to induce cell death. This result indicates that, despite its role in modulating the DNA damage response (DDR) and p53-based cell senescence,[28] MTH1 loss can exert tumor-suppressive effects even in RAS-transformed cells that have lost p53 function. This class of tumor cells is often intractable to chemotherapeutic regimens and therefore in dire need of novel druggable targets.

In investigating the basis of the observed proliferative defect in MTH1-suppressed HMLE-RAS cells, we noticed an increased number of epithelial islets in the shMTH1-transduced cells (~20–30% by visual examination of the confluent bulk population) relative to the shGFP-transduced control cells (**Fig. 1A**). These epithelial islets are similar in appearance to the parental HMLE cells (**Fig. 1A**) whereas the HMLE-RAS shGFP cells clearly exhibit the predominantly mesenchymal phenotype conferred by oncogenic RAS expression[29,30] (**Fig. 1A**). As E-cadherin downregulation is a critical effector of the EMT phenotype in HMLE-RAS cells,[29] we analyzed total E-cadherin protein levels and found that the total levels are 1.49 (±0.11)-fold higher in shMTH1-suppressed HMLE-RAS cells relative to the shGFP HMLE-RAS cells (normalized to the loading control actin; **Fig. 1B**). These findings suggest that MTH1 suppression partially inhibits the EMT phenotype, putatively by preferentially reducing proliferation of the mesenchymal subpopulation[30] that has

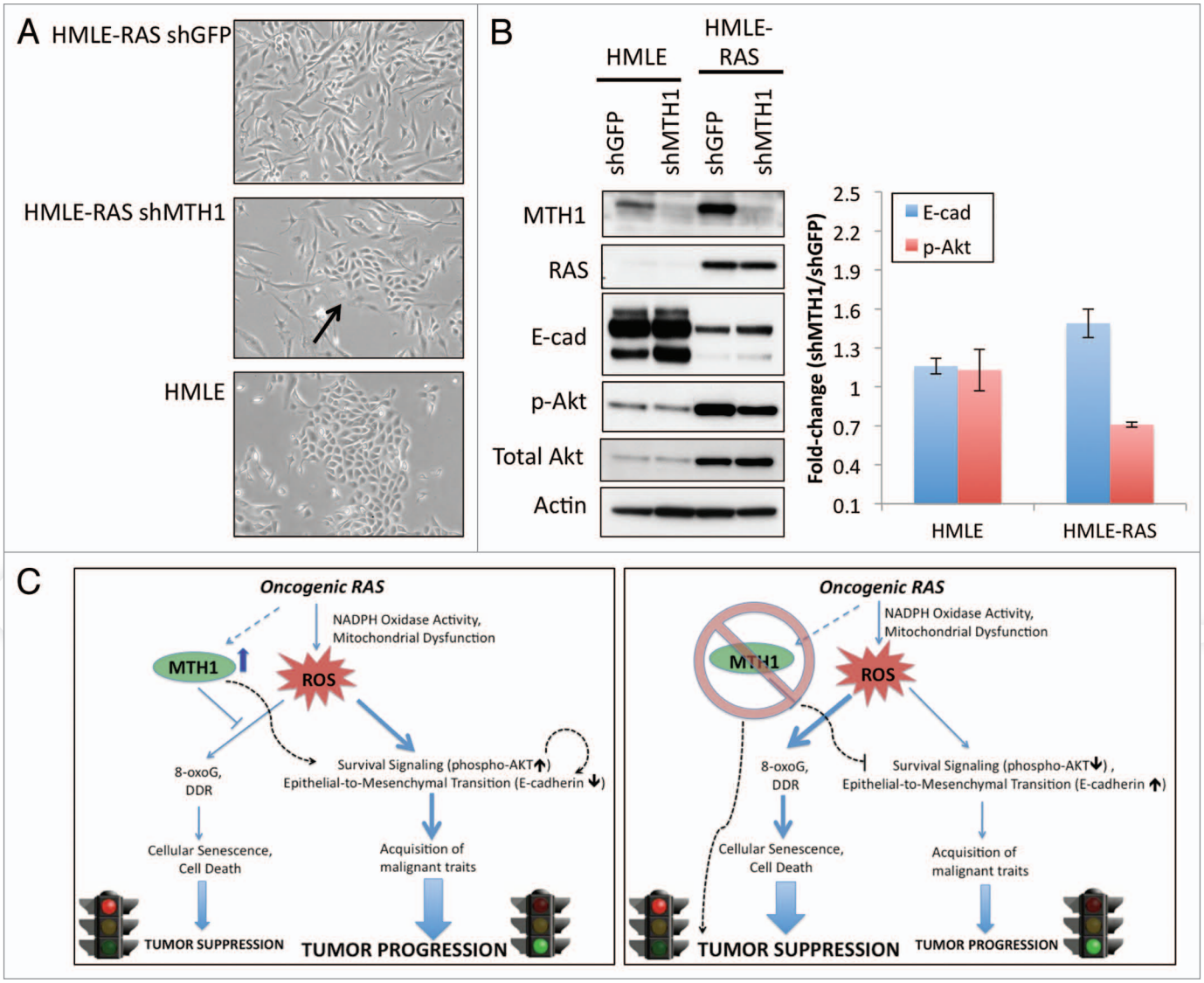

Figure 1. MTH1 suppression reduces oncogenic RAS-induced EMT and survival signaling. (A) Representative images of HMLE-RAS tumorigenic breast epithelial cells and the parental HMLE cells. Cells cultured as previously described in reference 13. The arrow indicates epithelial morphology in shMTH1-transduced cells. Note that the HMLE-RAS shGFP cells are largely mesenchymal and the HMLE cells are epithelial in appearance. In comparison, the shMTH1 have a mixed morphology comprising both mesenchymal scattered cells and epithelial islets. (B) Western blotting indicates that E-cadherin is upregulated and phospho-Akt/total-Akt ratio is downregulated by MTH1 suppression in RAS-transformed HMLE cells. Approximately 35 μg of protein lysates from the indicated HMLE and HMLE-RAS samples were run on a 4–12% Bis-Tris gradient gel (Nupage, Invitrogen). The resulting immunoblot was probed with the indicated antibodies at the following concentrations: MTH1, RAS and actin (as described previously in ref. 13), E-cadherin (1:4,000, BD Transduction Laboratories), p-Akt and total-Akt (1:2,000, Cell Signaling). Western blot bands were quantified using ImageJ 1.42q software (National Institutes of Health) for densitometry, and the areas of all bands were normalized to the actin signal. All three bands on the immunoblot detected by the E-cadherin antibody are specific to E-cadherin, as confirmed by shRNA knockdown.[29] Data shown is representative of three separate data sets. Quantitation of fold-changes in protein expression from shMTH1 cells relative to shGFP cells are shown to the right. The corresponding fold-change in oncogenic RAS protein levels in shMTH1 vs. shGFP HMLE-RAS cells is 1.12 ± 0.1. (C) Schematics depicting the known (solid lines) and putative (dashed lines) roles for MTH1 in modulating the tumor-promoting vs. the tumor-suppressive effects of oncogenic RAS-induced ROS.

been reported to exist in the HMLE-RAS culture.

Previous research has found that E-cadherin downregulation is necessary for EMT-associated anoikis resistance and increased metastatic capability in HMLE-RAS cells.[29] While MTH1 suppression in HMLE-RAS cells does not, in of itself, induce cell death,[13] we wanted to determine if it adversely impacted maintenance of other survival mechanisms besides the EMT. We found that Akt activation (measured as the fraction of phospho-Akt/total Akt), a key survival mechanism in cancer cells, is also reduced following MTH1 suppression (0.71 ± 0.02-fold-change in shMTH1 HMLE-RAS vs. shGFP HMLE-RAS cells; **Fig. 1B**). The differences in E-cadherin and p-Akt protein levels between the shMTH1 HMLE and shGFP HMLE cell were minor

(**Fig. 1B**). Therefore, in addition to inhibiting cell proliferation, MTH1 loss appears to negatively affect survival and pro-malignancy pathways in HMLE-RAS cells. Because Akt signaling is also linked to RAS-mediated cell cycle progression,[31] the observed decrease in phospho-Akt levels may be a contributing factor to the shMTH1-induced G_1/S arrest in these cells. However enhanced Akt signaling has been reported to mediate both oncogene-induced senescence and cell death via elevation of ROS levels.[32] Therefore, alternatively, the reduced phospho-Akt levels observed upon MTH1 suppression could occur as the result of some feedback mechanism responding to shMTH1-induced elevation of oxidative DNA damage in cell subpopulations with a high degree of Akt signaling.

It is not clear from our present results whether the shMTH1-induced changes in E-cadherin and phospho-Akt occur independently of each other or whether one is upstream of the other. PI3K/Akt signaling has been implicated in EMT induction;[33] its activation represses E-cadherin transcription in epithelial cells and modulates subcellular localization of the residual protein.[34] Akt inhibition in oral squamous carcinoma cells has been shown to revert the EMT phenotype and its attendant invasive traits and restore E-cadherin expression.[35] However, a recent study reported that E-cadherin inhibits ovarian cancer cell growth through PTEN expression-mediated repression of Akt signaling,[36] putting E-cadherin upstream of Akt signaling. Thus, the relative contributions of E-cadherin and Akt to shMTH1-mediated HMLE-RAS proliferative defect and EMT inhibition are likely to be complex.

The molecular mechanism(s) by which MTH1 suppression is able to partially inhibit the EMT phenotype also remain to be determined. The simplest explanation would be that MTH1 suppression selects for a subset of cells with low oncogenic RAS levels and concomitantly reduced cytoskeletal remodeling. This does not appear to be the case in the bulk population, as both the shGFP and shMTH1 HMLE-RAS cells exhibit equivalent RAS oncoprotein expression (the fold-change in shMTH1 vs. shGFP HMLE-RAS cells is 1.12 ± 0.1; representative data shown in **Fig. 1B**). However, in the absence of segregating the mesenchymal from the epithelial populations and comparing RAS and MTH1 levels within each subset, this finding does not preclude the possibility that there may be variability in RAS expression depending on MTH1 expression at the individual cell level.

Furthermore, as ROS production is downstream of oncogenic RAS signaling, a related possibility that we have not yet explicitly tested involves inhomogeneity in cellular ROS levels through variable Nox1 activity and/or antioxidant levels within the HMLE-RAS culture. A subpopulation with unusually high oxidant levels may be able to more effectively provoke Akt activation[9] or be associated with enhanced levels of Akt signaling.[32] Such a subpopulation is equally likely to induce the EMT via Rac1/Nox1 signaling[10] or suppress E-cadherin levels via hypermethylation.[37] We postulate that this subpopulation would have greater need for the redox-protective function of MTH1 and thus be most prone to shMTH1-induced proliferative defects. Such a scenario could potentially explain why MTH1 suppression retards but does not fully inhibit cell proliferation in HMLE-RAS cells,[13] and why it enriches for cells with an epithelial phenotype and lower levels of Akt activation (**Fig. 1A and B**).

A second possibility arises from the reported effect of the EMT phenotype on enriching tumor-initiating or cancer "stem" cells in breast epithelial cells.[30] Resistance to DNA damaging agents via enhanced DNA repair mechanisms appears to be one of the hallmarks of such tumor-initiating cells.[38,39] Thus, it is tempting to speculate that the tumor-initiating subpopulation of HMLE-RAS cells, enriched by the oncogenic RAS-induced EMT, preferentially upregulates MTH1 expression to minimize oxidative DNA damage produced by oncogenic RAS-induced ROS. MTH1 suppression would then be predicted to selectively abolish or reduce this mesenchymal progenitor-like subpopulation,[30] leading to an increased number of epithelial-appearing differentiated cells (**Fig. 1A**). Loss of such progenitor-like cells and concomitant decreased transit amplification in the HMLE-RAS culture could also potentially explain why we observed reduced cell proliferation upon MTH1 suppression even in the absence of functional senescence pathways.[13] Comparing the extent of non-EMT breast cancer stem cell markers, such as the fraction of high CD44/low CD24 subpopulation,[40] in control vs. MTH1-suppressed HMLE-RAS cells would shed further light on this possibility.

A third possibility entails that the elevated cellular 8-oxoG levels and/or DNA damage stemming from MTH1 suppression in HMLE-Ras cells[13] reduces the EMT phenotype. While this issue has not been comprehensively addressed in RAS-transformed cells, at least one study indicates that the MMP 3-induced EMT in mouse mammary epithelial cells is mediated by ROS and associated with significantly increased cellular 8-oxoguanine levels.[41] This finding suggests that elevated 8-oxoguanine levels per se need not inhibit the EMT phenotype. Similarly, existing research on the effect of genomic instability and DNA damage signaling proteins on the EMT indicates that these potentiate acquisition of mesenchymal traits rather than inhibit them.[41,42] Thus, it seems unlikely that oxidative DNA damage produced by MTH1 suppression is directly affecting the EMT phenotype. It is also possible that shMTH1-induced 8-oxoGTP participates in and interferes with small GTPase signaling. Functional inhibition by exogenously added 8-oxoGTP on Rac1 activation has been previously reported in reference 43; however, this result has not yet been verified under cell-physiologic concentrations of 8-oxoGTP and needs to be further validated.

The potential relevance of these various mechanisms, the implications of shMTH1-induced E-cadherin upregulation on anoikis and motility, and the existence of similar interplay between MTH1 levels, Akt signaling and the EMT in other RAS-transformed epithelial tumor cells are being further investigated in our laboratory (Giribaldi M and Rai P, manuscript in preparation). Nevertheless, regardless of the specific underlying mechanisms, the preliminary results presented here potentially point to an emerging novel role for MTH1 in maintenance of

the malignant EMT phenotype, in addition to its inhibition of oncogene-induced senescence (**Fig. 1C**).

Additionally, should further investigations bear out our preliminary findings regarding shMTH1-induced mitigation of pro-survival mechanisms, auxiliary chemotherapeutic-induced stresses that induce oxidant damage or selectively targets cells that have undergone an EMT,[44] could potentially synergize with MTH1 loss to trigger pervasive cell death. This is an important consideration because the tumor-suppressive efficacy of antiproliferative measures such as senescence is likely to be optimal in fast-growing tumors, and in the short-term. In the longer term or in slow-growing cancers, there is a strong likelihood that subpopulations able to evade this proliferative barrier through selection or mutation will emerge, resulting in a more malignant and treatment-resistant tumor. Indeed, evidence for this phenomenon has been observed in mouse models of oncogene-induced senescence, and in human tumors whereby more advanced tumor grades exhibit progressively fewer senescence markers when compared with pre-neoplastic or early neoplastic tissue (reviewed in ref. 45). Accordingly, improved tumor clearance is likely to result from combinatorial treatments with a secondary stressor that is able to induce cell death while tumor proliferation is held in check via a growth arresting mechanism. Thus, with its putative maintenance of EMT-associated survival factors (**Fig. 1**) and its inhibition of oxidative DNA damage-induced antiproliferative effects,[13,28] MTH1 represents a promising drug development target for oncogenic RAS-sustaining tumors.

Previous studies have established that complex relationships exist between intracellular ROS levels and survival signaling pathways in cancer.[46] However, the challenge of identifying clearly defined roles for oxidants in these processes is often confounded by the many pleiotropic effects that can be ascribed to ROS, for instance, oxidant signaling or oxidative post-translational modifications. The putative involvement of MTH1 indicates that detoxification of ROS-mediated damage to DNA and its precursors is a specific contributor to the regulation of EMT and Akt signaling. Our published[13] and preliminary results herein suggest that other oxidative DNA damage repair proteins may selectively affect the proliferation and survival of RAS-transformed cells. A promising candidate in this regard is the apurinic/apyrimidinic endonuclease, APE1/Ref 1. APE1 participates in oxidative DNA damage repair via the base excision repair (BER) pathway, and also functions as a transcriptional coactivator to modulate cellular redox states and gene expression.[47] Furthermore, it has been reported to increase H-RAS expression and potentiate H-RAS-mediated PI3K/Akt signaling.[48] Additionally, other nucleotide-sanitizing enzymes such as NUDT16, a deoxyinosinediphosphastase whose deficiency also engenders DNA single strand breaks and proliferative arrest[49] may also potently influence ROS-mediated cell fates in RAS-transformed cells. Thus, further exploring the effect of DNA oxidation-responsive enzymes on oncogenic RAS-induced cellular outcomes has the potential to open new and informative avenues of research.

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