This study reveals a plausible connection between UV radiation, the lipid mediator PAF and chromatin patterns in immune cells of the skin. Although transcriptional regulation of gene expression is commonly associated with histone modifications, the profound effect of PAF on several epigenetic regulators in mast cells is noteworthy. It would be interesting to investigate other genes that may be regulated by PAF and to determine how epigenetic modulation, including histone methylation, is involved. Several other outstanding questions remain as well. They include the signaling route from the G-protein coupled receptor to which PAF binds on mast cells to the induction of histone modifications and binding of transcription factors at the *CXCR4* promoter region. The cell-based assays performed in this study also raise the question how modulation of histone modifying enzymes and epigenetic regulation of *CXCR4* in particular would impact on mast cell migration, UV-induced immunosuppression and skin carcinogenesis *in vivo*. It is now increasingly recognized that environmental stimuli can impact on the epigenome of cells. The demonstration of PAF as a mediator of an environmental factor that induces epigenetic mechanisms of transcriptional regulation could be used as a model for further study. Moreover, the identification of an epigenetic dimension operative in photocarcinogenesis may provide further opportunities for developing strategies to prevent skin cancer.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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Mutational Heterogeneity in Melanoma: An Inconvenient Truth

Gregory A. Chang[1,2] and David Polsky[1,2]

Identification of oncogenic *BRAF* mutations in primary and metastatic melanomas supports a linear model of clonal evolution in cancer. Some mutational studies, however, have failed to identify *BRAF* mutations in metastatic tumors from patients with *BRAF*^{mutant} primary melanomas. Using a combination of methods, Riveiro-Falkenbach *et al.* (2015) assert that technical issues, and not clonal heterogeneity, may explain prior discordant mutational results.

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Clonal evolution of cancers, proposed in 1976 by Nowell (1976), states that malignant tumors are genetically unstable, producing clonal variants with mutations that are either favorable or unfavorable for cell survival. This linear evolution model predicts that metastatic and drug-resistant clones result from an accumulation of several favorable mutations, including the original initiating mutation. With this conceptual framework, a linear model of melanoma tumorigenesis, beginning with the development of a *BRAF*^{mutant} nevus, followed by additional genetic and epigenetic alterations leads to primary melanoma and ultimately to *BRAF*^{mutant} metastatic disease. This model makes sense for *BRAF*^{mutant} melanoma.

Evidence for BRAF mutational heterogeneity in melanoma

Although the findings of many studies are consistent with the model described above, several have failed to identify conservation of *BRAF* mutations in all

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tumor samples analyzed from individual patients (i.e., inter-tumor heterogeneity), results that challenge Nowell's (1976) model (Table 1). For example, Saint-Jean *et al.* (2014) found inter-tumor heterogeneity in 10/74 (14%) patients with primary-metastatic tumor pairs and in 8/43 (19%) patients with more than 2 metastases. Colombino *et al.* (2012) found that of the 20/99 (20%) cases of inter-tumor heterogeneity, 8/20 (40%) had a mutant primary tumor but wild-type metastases, a finding inconsistent with Nowell's model. Similarly, Heinzerling *et al.* (2013) identified 10/53 (19%) patients that had discordant $BRAF^{V600E}$ mutational results when multiple tumors were analyzed using pyrosequencing. Of those 10 patients, 4 exhibited mutant primary tumors with wild-type metastases (Heinzerling *et al.*, 2013). Using a $BRAF^{V600E}$ mutation-specific PCR assay we found that 2/18 (11%) patients had $BRAF^{mutant}$ primary tumors and $BRAF^{wild\text{-}type}$ metastases (Yancovitz *et al.*, 2012). In addition, 5/19 (26%) patients with multiple metastases exhibited inter-tumor heterogeneity for the *BRAF* mutation. We also reported findings of intra-tumor heterogeneity. Using laser microdissection to analyze tumor cells isolated from separate regions of individual primary melanomas, we found that 6/9 (67%) primary melanomas demonstrated substantial variation in the relative abundance of $BRAF^{V600E}$ DNA in different tumor regions. Lin *et al.* (2011) also found evidence of intra-tumor heterogeneity in primary and metastatic tumors using several methods to identify *BRAF* mutations, including analysis of isolated single melanoma cells, mutation-specific PCR assays, or bacterial subcloning and sequencing. The most telling evidence of heterogeneity, however, comes from studies that found both *NRAS* and *BRAF* mutant alleles in multiple tumors from the same patient (prior to the advent of *BRAF* inhibitor therapy) or from the same tumor itself. Colombino *et al.* (2012) identified two patients with an $NRAS^{mutant}$ primary melanoma and a $BRAF^{mutant}$ metastasis. Sensi *et al.* (2006) isolated (via limited dilution cloning) separate $BRAF^{V600E}$ and $NRAS^{Q61R}$ mutant melanoma cells from a short-term culture of an $NRAS^{mutant}$ subcutaneous metastasis providing direct evidence that patient tumors may contain unique clonal subpopulations. Recently, Eriksson *et al.* (2015) identified 4 patients with $BRAF^{V600E}$ primary melanomas and $BRAF^{wild\text{-}type}$ or $BRAF^{non\text{-}V600E}$ mutant metastases. Two of the $BRAF^{wild\text{-}type}$ metastases had *NRAS* mutations.

> Mutational heterogeneity, a feature of tumor evolution models, challenges current treatment strategies for melanoma.

These reports demonstrating inter- and intra-tumor mutational heterogeneity can be accommodated in Nowell's tumor evolution paradigm when one considers the modifications described in 1982 by Fidler and Hart (1982). They noted that primary tumors are heterogeneous, polyclonal neoplasms comprised of tumor cell subpopulations with varying biological characteristics, including metastatic potentials. As such, more than one subclone may have the ability to form metastases, which could explain the findings described above, assuming that *BRAF* mutations are not required for metastatic spread of melanoma. Further support for the polyclonality of human tumors is evidenced by the different organotropisms of metastatic disease, a prominent feature of melanoma and other malignancies (Figure 1). Currently, this polyclonal model of cancer best explains discordant inter- and intra-tumor mutational results and the organotropic characteristics of melanoma metastases.

Challenging the concept of mutational heterogeneity

In the current issue, this concept is challenged by Riveiro-Falkenbach *et al.* (2015). They use immunohistochemistry (IHC) to compare expression of mutant $BRAF^{V600E}$ to DNA-based $BRAF^{V600}$ mutation detection in paired primary and metastatic melanomas from 140 patients. The Ventana VE1 anti-$BRAF^{V600E}$ antibody (Ventana Medical Systems, Tucson, AZ) was used to conduct the IHC analysis. Two DNA-based methods were used: the cobas 4800 BRAFV600 Mutation Test, and Sanger sequencing.

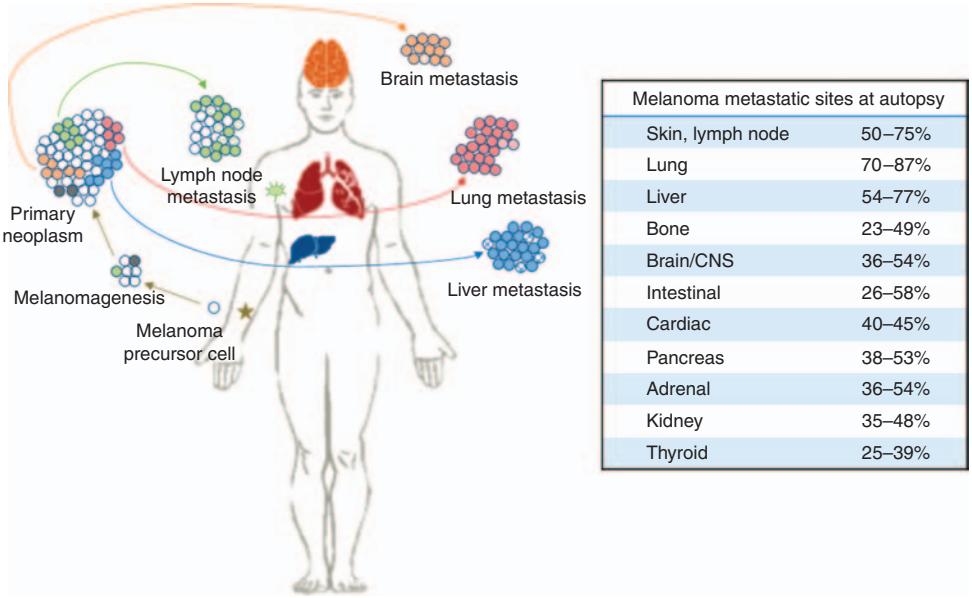
The cobas 4800 BRAFV600 Mutation Test is a Taqman-based real-time PCR mutation assay used to detect V600 mutations in formalin-fixed, paraffin-embedded human melanoma tissue. The test mainly detects *V600E* but is known to cross-react with *V600K/D*. This FDA-approved test is part of the standard of care for melanoma patients with advanced disease to determine eligibility for *BRAF* inhibitor therapy: patients with the mutation are eligible for treatment, whereas patients lacking the mutation are not. The manufacturer's package insert states that the cobas 4800 has an over 95% positive detection rate in pre-clinical and clinical investigations. Additional validation studies demonstrate that the cobas 4800 test is able to detect more *V600E* mutations than Sanger sequencing, and that Sanger has more false-positive results than cobas when using an additional detection method to confirm results (Halait *et al.*, 2012). Importantly, adequate tumor content is an essential requirement for any DNA-based mutational assay (Sensi *et al.*, 2006). In tissue samples with $<10\%$ tumor content (i.e., $>90\%$ normal tissue), the chances of finding a mutation are diminished and the cobas test often fails to attain consistent results (Halait *et al.*, 2012). In addition, there always exists the possibility of sampling error if cut sections are not representative of a genetically polyclonal tumor.

The monoclonal $BRAF^{V600E}$ mutation-specific antibody, named VE1, binds to an 11-amino-acid sequence in the $BRAF^{V600E}$ mutant protein between amino acids 596–606 (Capper *et al.*, 2011). Studies investigating VE1 have demonstrated the high sensitivity (80–97%) and specificity (98–100%) of the antibody to distinguish the V600E mutant protein from V600K/R/Q (Capper *et al.*, 2011; Pearlstein *et al.*, 2014). One of the values of IHC analysis is that normal tissue contamination, which can limit the sensitivity of DNA-based assays, is not a major concern. Quantifying the homogeneity

Table 1. Selected publications describing tumor heterogeneity

Authors	Saint-Jean *et al.* (2014)	Colombino *et al.* (2012)	Heinzerling *et al.* (2013)	Yancovitz *et al.* (2012)	Busam *et al.* (2013)	Riveiro-Falkenbach *et al.* (2015)
Disease type	Melanoma	Melanoma	Melanoma	Melanoma	Melanoma	Melanoma
Samples analyzed	• 278 patients • 367 tumors	• 99 patients • 291 tumors (102 primaries paired with 165 metastases; 24 unpaired brain metastases)	• 187 patients • 300 tumors	• 73 patients • 112 tumors	• 44 metastases (22 w/o BRAFV600E, 22 w/o BRAFV600E) • 20 primaries	• 140 patients • 311 tumors
Analysis tools	• Sanger sequencing • Cobas • Therascreen BRAF RGQ assay	• Sanger sequencing	• Pyrosequencing • IHC	• Sanger sequencing • MS-PCR • Laser microdissection • SNaPshot	• IHC • Sequenom mass spectrometry	• IHC • Cobas • Sanger sequencing
Paired	74 patients with multiple tumor samples	53 patients with matched primary-single metastasis 46 patients with primary-multiple metastases	53 patients with multiple tumor samples	18 patients with matched primary-metastases 19 patients with multiple metastases	1 patient with primary-metastatic tumor pair	110 patients with primary+1 metastasis; 29 patients with primary+2 metastases; 1 patient with primary+3 metastases
Inter- and intra-tumor heterogeneity observed	Inter-tumor heterogeneity found in 10/74 patients, including 8/43 patients with $\geqslant 2$ metastases	Inter-tumor heterogeneity detected in 20 cases 2 cases carrying different mutations in paired samples (NRAS and BRAF) All other exhibiting mutant-wild-type mismatches	Inter-tumor heterogeneity found in 10/53 patients who had multiple metastases Intra-tumor heterogeneity shown using IHC analysis	Inter-tumor heterogeneity detected in 2/18 V600E primary-metastatic pairs and 5/19 V600E metastases-only pairs Intra-tumor heterogeneity found in 6/9 tumors using laser microdissection	Intra-tumor heterogeneity of BRAF expression in 6/22 V600E metastases and 2/7 V600E primaries Inter-tumor heterogeneity not identified; only one patient with a paired sample analyzed	Inter-tumor heterogeneity not detected Intra-tumor heterogeneity not detected
Key findings with respect to heterogeneity	Analysis of additional samples leads to greater probability of discordance between tumors	NRAS mutant primaries from 2 patients gave rise to BRAF mutant metastases providing significant evidence of inter-tumor heterogeneity	Inter- and intra-tumor heterogeneity with respect to BRAF mutational status and expression	Relative differences in abundance of BRAF mutant DNA in distinct microdissected regions of the same tumor sample	High sensitivity and specificity of VE1 suggests that focal staining of tumors may be evidence of heterogeneity	IHC analysis shows that inter- and intra-tumor heterogeneity not present and that IHC analysis is more sensitive than DNA-based techniques

Abbreviation: IHC, immunohistochemistry.
Several studies in melanoma have demonstrated intra- and inter-tumor heterogeneity using a wide range of techniques including cobas and IHC. The table below shows how the study by Riveiro-Falkenbach *et al.* (2015) compares with others.



Melanoma metastatic sites at autopsy	
Skin, lymph node	50–75%
Lung	70–87%
Liver	54–77%
Bone	23–49%
Brain/CNS	36–54%
Intestinal	26–58%
Cardiac	40–45%
Pancreas	38–53%
Adrenal	36–54%
Kidney	35–48%
Thyroid	25–39%

Figure 1. Model of primary intra-tumor heterogeneity giving rise to organotropic metastases characterized by intra- and inter-tumor heterogeneity. Fidler and Hart (1982) note that genetic instability, as discussed by Nowell (1976), provides a mechanism for tumors to evolve in response to selective pressures. They suggest this evolution leads to biological diversity rather than a reduction in the number of tumor subclones. Wan *et al.* (2013) review recent studies that suggest that subclones within the primary tumor may possess intrinsic genotypic and phenotypic differences in their abilities to spread to different metastatic sites. The illustration above depicts how the theories of Nowell (1976), Fidler and Hart (1982), and Wan *et al.* (2013) work together to model the path to polyclonality and organ tropisms via genetic instability. Metastatic melanoma data from Hwu *et al.* (2003).

of staining, however, can be highly subjective. There may be difficulties in identifying rare events in tissue sections (e.g., stained or unstained single cells), or distinguishing true staining from artifacts such as the presence of melanophages or necrotic tissue.

Overall, Riveiro-Falkenbach *et al.* (2015) found that 52% of tumors had $BRAF^{V600}$ mutations according to the cobas test. When analyzed for inter-tumor heterogeneity of *BRAF* mutations they found that 117/140 (83.6%) primary-metastatic pairs were concordant for the presence or absence of the *BRAF* mutation. They used IHC on a subset of the concordant pairs ($n = 61$) and confirmed that those pairs were 100% concordant using VE1. When using IHC to test 23 cobas-discordant primary-metastatic tumor pairs, they found no evidence of discordance. They suggested that IHC is more sensitive than DNA-based mutation detection methods. There were several cases of positive VE1 staining in samples with $<10\%$ tumor content and Sanger sequencing and cobas testing yielded wild-type results. In addition, the high specificity of the VE1 antibody, evidenced by the lack of staining in all tumor samples harboring a V600K mutation, mediates concerns of possible false-positive results. Ultimately,Riveiro-Falkenbach *et al.* (2015) conclude that the cobas assay is less sensitive and accurate in calling mutant positive samples than either Sanger sequencing or IHC, and that the existence of tumor heterogeneity in published studies may be owing to technical issues rather than actual tumor heterogeneity.

Although the VE1 antibody has proven to be a highly sensitive and V600E-specific reagent in several studies (Capper *et al.*, 2011; Pearlstein *et al.*, 2014) including Riveiro-Falkenbach *et al.* (2015), their conclusions regarding the existence (or lack thereof) of inter-tumor heterogeneity needs to be interpreted cautiously. Close inspection of their results reveals that among the 23 patients with cobas-discordant mutational results, 5 patients had V600K mutant tumors. These cases should not be included among the discordant cases as the cobas assay is not primarily designed to detect this mutation. Besides, these cases were concordant using Sanger sequencing. In addition, 1 patient had two primary melanomas, of which the thicker one was not tested via cobas, and it was determined to be mutationally concordant with its paired metastasis using Sanger sequencing. Finally, there were 2 cases where the cobas test was repeated and subsequently yielded results concordant with other samples from that patient. Removing these 8 cases from the analysis leaves 15 cobas-discordant cases. Fortunately, Riveiro-Falkenbach *et al.* (2015) included Sanger sequencing as a second DNA-based mutation detection method. When analyzing the tumor sets for inter-tumor heterogeneity using Sanger sequencing, only 3/15 (20%) cases had discordant mutational results. In all three of the discordant cases, the discordant, wild-type samples contained $\leqslant 15\%$ tumor content prior to macrodissection. The authors note that all samples were macrodissected prior to DNA extraction; however, enriching samples with very low tumor content using macrodissection can be technically challenging as

this is performed on unstained sections. It may still result in low (or no) tumor content in the dissected sample if not carefully performed.

If we consider VE1 IHC to be the most sensitive V600E mutation detection method employed in this study, then their results suggest that Sanger sequencing is more sensitive than the FDA-approved cobas assay. This finding contradicts previous reports that demonstrated that the cobas assay is more sensitive than Sanger sequencing (Halait *et al.*, 2012). This raises concerns about their cobas assay results. If we consider their Sanger sequencing results to be the more accurate DNA-based mutation method in this study, we find a lack of inter-tumor heterogeneity among this sample set. The authors, therefore, cannot conclude that prior reports of inter-tumor heterogeneity—of which several were based on Sanger sequencing or other methodologies using tumor rich samples—are solely due to artifact.

With respect to intra-tumor heterogeneity, Riveiro-Falkenbach *et al.* (2015) reported homogenous staining of all 137 positively stained tumor samples, and they assert that their results provide evidence that intra-tumoral heterogeneity does not exist. They suggest that artifacts and necrotic tissue may have a role in the heterogeneous results reported by others. These results, in fact, contradict the findings of other groups including Busam *et al.* (2013), Heinzerling *et al.* (2013) and Eriksson *et al.* (2015) who demonstrated heterogeneous staining using the same VE1 antibody. Busam *et al.* (2013) found that 2/10 (20%) superficial spreading melanomas exhibited focal staining of a subpopulation within the tumor suggestive of intra-tumoral heterogeneity. In addition, they found that 6/22 (27%) $BRAF^{mutant}$ metastases had both $BRAF^{V600E}$-immunopositive and -immunonegative populations within the tumor (Busam *et al.*, 2013). Heinzerling *et al.* (2013) found strong VE1 staining in one part of a mutation-positive lymph node whereas adjacent tumor cells did not react with the antibody. Eriksson *et al.* (2015) described heterogeneous staining within 10/200 (5%) tumors, most of which were primary melanomas. Clearly, the conclusion that all heterogeneity is due to artifact or technical issues must be explored in future studies.

Altogether, the findings by Riveiro-Falkenbach *et al.* (2015) support the potential use of the VE1 antibody as a triage tool to identify the V600E mutation. IHC negative results, however, still require DNA testing to rule out *V600K* and other rare *V600* mutations, as patients with those less common *BRAF* mutations may still benefit from *BRAF* inhibitor therapy (Klein *et al.*, 2013).

The inconvenient truth of tumor heterogeneity in melanoma

Ultimately, heterogeneity with respect to *BRAF* mutations is part of a larger story when considering polyclonal variations in gene expression, or analysis of additional mutations to identify tumor subclones. Several studies in breast, prostate, pancreatic, and renal cancers have contributed to the growing body of evidence for tumor evolution leading to biological diversity and heterogeneity. Recent studies in melanoma using next-generation sequencing methods have made similar findings. Ding *et al.* (2014) identified tumor subclones in 11/15 melanomas, as well as evolutionary mutation relationships between four anatomically separate metastases in two different patients. They also showed that mixed treatment responses were associated with different subclonal populations possessing (or not) a *BRAF* resistance gene mutation. Other studies analyzing tumors removed from patients who progressed on *BRAF* inhibitor therapy have also revealed mutational tumor heterogeneity. Van Allen *et al.* (2014) found multiple resistance gene alterations in a single metastatic tumor providing evidence of continued divergent evolution that supports Fidler and Hart's (1982) polyclonal model. Shi *et al.* (2014) analyzed 100 metastatic tumors from 44 patients and found that 9/44 (20%) patients had $\geqslant 2$ resistance mechanisms in their tumors, and 13/16 (81%) patients with multiple tumors had multiple mechanisms of resistance.

With advanced methodologies in molecular pathology, the polyclonal nature of malignant neoplasms including melanoma is becoming more apparent. Although this phenomenon may challenge our current ability to cure patients with melanoma, additional investigations into the mechanisms driving clonal diversity may lead to greater understanding of the molecular pathogenesis of various melanoma subtypes and the development of more effective therapies.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Beyond Red Hair and Sunburns: Uncovering the Molecular Mechanisms of MC1R Signaling and Repair of UV-Induced DNA Damage

Pamela B. Cassidy[1], Zalfa A. Abdel-Malek[2] and Sancy A. Leachman[1]

Scientists at the University of Kentucky are unraveling the details of DNA-damage repair in the melanocyte, with an eye towards finding druggable targets for melanoma prevention. Jarret *et al.*, (2015, this issue) report in this issue three new assays that can yield mechanistic information about nucleotide excision repair (NER) stimulated by cAMP-dependent signaling downstream of the melanocortin-1 receptor (MC1R).

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Molecularly-targeted prevention

Protecting the lives of individuals and increasing the productivity of society by stopping disease before it causes harm is of the greatest aspirations of medicine; and as the molecular etiologies of cancer are discovered, there will be opportunities to apply targeted molecular strategies to its prevention, much as they have been applied to therapeutics. Consider the development of targeted therapy for melanoma using BRAF inhibitors (Puzanov and Flaherty, 2010). This accomplishment resulted directly from the elucidation of the molecular mechanism of melanocyte transformation (V600E mutation of BRAF) and subsequent identification of a drug that could specifically antagonize the mutated protein. To develop targeted prevention agents, it will be necessary to elucidate targetable molecular pathways that predispose a cell to transformation and then to identify drugs that can successfully and safely target these pathways. Melanoma is a good candidate disease for the development of targeted prevention agents because several melanoma predisposition genes have been identified. One of the most common and well characterized of these is *MC1R* (Abdel-Malek *et al.*, 2014), the target of investigation in an article from this issue by Jarrett *et al.*, (2015).

MC1R and DNA-damage repair

Epidemiological studies have found a strong correlation between the carriage of loss-of-function mutations in *MC1R* (which encodes a 7-pass transmembrane G-protein-coupled receptor) and both the red-hair phenotype and melanoma risk (Pasquali *et al.*, 2015). In cell culture, treatment of melanocytes that express the wild-type MC1R (Figure 1), with the agonist alpha–melanocyte-stimulating hormone (α-MSH), elicits a variety of responses, including synthesis of eumelanin, reduction of UV-induced oxidative stress, stimulation of adenylyl cyclase and cAMP-dependent signaling, and enhancement of DNA repair via base-excision repair and NER mechanisms (Abdel-Malek *et al.*, 2014). NER repairs UV-photoproducts such as cyclobutane pyrimidine dimers and 6′-4′-pyrimdine-pyrimidone photoproducts. If not repaired properly, cyclobutane pyrimidine dimer formation can result in C>T transitions, which are a signature mutation in melanomas (discussed in (Jarrett *et al.*, 2014)). Thus, impaired NER in the skin cells of individuals with loss-of-function *MC1R* mutations likely contributes to their vulnerability to melanoma, and it is therefore a rational target for prevention. Until recently, the mechanistic details (i.e., demonstrable, quantifiable and targetable molecular events) linking MC1R activation to NER have been hazy. Then, it was reported last year that pre-treatment of melanocytes with α-MSH augmented their DNA-damage response by increasing phosphorylation of DNA-damage sensing proteins ataxia telangiectasia and Rad3-related protein (ATR, at serine 428) and ataxia telangiectasia mutated (at serine 1981) and enhancing formation of phosphorylated γH2AX at nuclear sites of DNA repair (Swope *et al.*, 2014). And now, in

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