Received: 29 January 2021 | Revised: 25 March 2021 | Accepted: 13 April 2021

DOI: 10.1111/pcmr.12980

REVIEW

WILEY

The α-melanocyte-stimulating hormone/melanocortin-1 receptor interaction: A driver of pleiotropic effects beyond pigmentation

Cecilia Herraiz[1] | Idoya Martínez-Vicente[1] | Vittoria Maresca[2]

[1]Department of Biochemistry, Molecular Biology and Immunology, School of Medicine, University of Murcia and Instituto Murciano de Investigación Biosanitaria (IMIB), Murcia, Spain

[2]Laboratory of Cutaneous Physiopathology, San Gallicano Dermatological Institute IRCCS, Rome, Italy

Correspondence
Cecilia Herraiz, Department of Biochemistry, Molecular Biology and Immunology, School of Medicine, University of Murcia and Instituto Murciano de Investigación Biosanitaria (IMIB), 30120 Murcia, Spain.
Email: ceciliahs@um.es

Vittoria Maresca, Laboratory of Cutaneous Physiopathology, San Gallicano Dermatological Institute IRCCS, Via Elio Chianesi 53, 00144 Rome, Italy.
Email: vittoria.maresca@ifo.gov.it

Abstract

Melanocortin-1 Receptor (MC1R), when stimulated by alpha-melanocyte-stimulating hormone (α-MSH), is a driver of eumelanogenesis. Brown/black eumelanin is an effective filter against ultraviolet radiation (UVR) and is a scavenger of free radicals. Several polymorphic variants of MC1R are frequent in red-head people. These polymorphisms reduce the ability of MC1R to promote eumelanogenesis after its activation and spontaneous pheomelanogenesis take place. Since pheomelanin can act as an endogenous photosensitizer, people carrying MC1R polymorphisms are more susceptible to skin cancer. Here, we summarize current knowledge on the biology of MC1R beyond its ability to drive eumelanogenesis. We analyze its capacity to cope with oxidative insult and consequent DNA damage. We describe its ability to transduce through different pathways. We start from the canonical pathway, the cAMP/protein kinase A (PKA) pathway mainly involved in promoting eumelanogenesis, and protection from oxidative damage, and we then move on to describe more recent knowledge concerning ERK pathways, phosphoinositide 3-kinase (PI3K) pathway/AKT, and α-MSH/Peroxisome proliferators activated receptor-γ (PPAR-γ) connection. We describe MC1R polymorphic variants associated with melanoma risk which represent an open window of clinical relevance.

KEYWORDS

MC1R, melanocytes, melanogenesis, melanoma cells, αMSH

1 | INTRODUCTION

1.1 | MC1R: more than just the main regulator of melanogenic enzymes

Skin pigmentation depends on the proliferation and differentiation of the pigment-producing cells, melanocytes (Bennett & Lamoreux, 2003), as well as on epigenetic control mediated by other adjacent cell types, especially dermal fibroblasts and epidermal keratinocytes (Kunisada et al., 2000). One of the main signaling pathways regulating melanin production involves the melanocortin peptide α-MSH. Melanocortins form a family of neuroendocrine peptides originally characterized as regulators of cutaneous pigmentation and cortisol production. This family includes MSH peptides (α-MSH, β-MSH, and γ-MSH) and adrenocorticotropic hormone (ACTH). α-MSH derives from proteolytic processing of proopiomelanocortin (POMC) (Lonati et al., 2020). α-MSH is involved in the regulation of melanogenesis and pigmentation by binding to the Melanocortin-1 Receptor (MC1R). ACTH is also an agonist for human MC1R, in addition to α-MSH (Abdel-Malek et al., 1995). In response to UVR, p53

is accumulated in epidermal keratinocytes and induces the expression of *POMC* and, subsequently, increases the levels of α-MSH and ACTH, which regulate the function of human melanocytes (Abdel-Malek et al., 2008).

MC1R is a G protein-coupled receptor (GPCR) expressed in melanocytes and melanoma cells (MCs) (García-Borrón et al., 2014; Herraiz et al., 2017). Low levels of expression have been detected in other cell types, such as epidermal keratinocytes, fibroblasts (Roberts et al., 2006), and cells of the immune system (Böhm et al., 2006), although these cells do not seem to express a functional MC1R. Activated MC1R plays a key role as a driver of melanogenesis. Some MC1R variant alleles are associated with red hair color and fair skin, known as the RHC (Red Hair Color) phenotype, as well as increased skin cancer risk (see below). After α-MSH/MC1R interaction, the $G\alpha_s$ subunit dissociates from MC1R and stimulates adenylyl cyclase, which generates cyclic adenosine 3′,5′-monophosphate (cAMP). cAMP activates protein kinase A (PKA) which phosphorylates the cAMP Response Element Binding Protein (CREB). CREB binds to gene promoters containing the CRE (cAMP-responsive element) sequence. CREB activates "Microphthalmia-associated Transcription Factor" (MITF), which upregulates expression of the melanogenic enzymes Tyrosinase (TYR), Tyrosinase-related protein-1 (TRP1), and Tyrosinase-related protein-2 (TRP2), also known as dopachrome tautomerase (DCT) (Busca & Ballotti, 2000; D'Orazio and Fisher, 2011; Wolf Horrell et al., 2016). This pathway is the driver of eumelanogenesis.

Brown/black eumelanin is an effective filter against UVR. It can also act as a scavenger of free radicals, whereas red pheomelanin can act as an endogenous photosensitizer. Both melanin types are generated by tyrosinase-catalyzed oxidation of tyrosine and, until the formation of dopaquinone, they share the same biosynthetic pathway. In eumelanogenesis, a series of reactions catalyzed by enzymes (TYR, TRP1, TRP2) lead to the formation of brown/black melanin. Instead, in pheomelanogenesis, dopaquinone immediately reacts with sulfhydryl groups to produce cysteinyl-DOPA and then, quinone, which is further converted into benzothiazine and benzothiazol. These products polymerize to generate red pigment (d'Ischia et al., 2015; Ito & Wakamatsu, 2003). Thus, melanins synthesized inside the melanosomes must be then conveyed toward the ends of the dendrites in order to be transferred to the surrounding keratinocytes. MITF is involved in promoting the transfer of melanosomes to the cell periphery (Adelmann et al., 2020; Hume et al., 2007; Passeron et al., 2004).

Multiple antagonists of MC1R are known. They include agouti-signaling protein (ASIP) and human β-defensin 3 (HBD3). ASIP acts as suppressor of melanogenesis and is considered an inverse agonist of MC1R (Walker & Gunn, 2010). HBD3 prevents the binding of both α-MSH and ASIP to MC1R (Nix et al., 2013) hindering both the increase in cAMP and the activation of TYR in melanocytes (Swope et al., 2012).

More recent insights demonstrated that MC1R signaling is able to activate both ERK1/2 and phosphoinositide 3-kinase (PI3K)/AKT pathways (Cao et al., 2013; Castejón-Griñán et al., 2018; García-Borrón et al., 2014; Herraiz et al., 2017). A connection among α-MSH and Peroxisome proliferator-activated receptor-γ (PPAR-γ) has been highlighted (Maresca et al., 2013) involving phospholipase C (PLC)-dependent calcium fluxes (Maresca et al., 2013; Motiani et al., 2018).

MC1R expression is upregulated by paracrine factors basic fibroblast growth factor, endothelin-1, α-MSH, and ACTH synthesized by epidermal keratinocytes, whose synthesis is increased upon UVR (Scott et al., 2002).

Moreover, MC1R signaling is regulated by several intracellular molecules that interact physically with the receptor. These include cytosolic beta-arrestins (ARRB) responsible for MC1R desensitization and internalization (Abrisqueta et al., 2013), the negative regulator of the AKT pathway phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Cao et al., 2013) and the RING Finger domain-containing E3 ubiquitin ligase Mahogunin Ring Finger-1 (MGRN1) (Perez-Oliva et al., 2009), which inhibits signaling from MC1R to cAMP, most likely due to a physical interaction of MGRN1 and MC1R, competitive with respect to the Gs protein. Information on MC1R available in the literature is helping to change the view on its functionality. MC1R is not only the principal regulator of melanogenesis, but it also performs different functions, thus contributing to cell well-being.

1.2 | The MC1R receptor: molecular characterization

MC1R gene is located on chromosome 16q24.3 and has 4 exons, giving rise to several intra- and intergenic splice variants. Two forms of intergenic splicing yielding MC1R-TUBB3 chimerae and at least two forms of alternative splicing of the *MC1R* gene have been described (Dalziel et al., 2011; Herraiz et al., 2015; Martínez-Vicente et al., 2020; Tan et al., 1999). In all cases, the proteins encoded by the non-canonical mRNAs preserve the general architecture of GPCRs and differ from canonical MC1R by the presence of a longer cytosolic extension. Since the regions of the MC1R molecule involved in ligand binding and functional coupling to G proteins are present in all these splicing products, their functional properties have been analyzed to determine the effect of the additional C-terminal extension in MC1R splice variants compared with canonical MC1R (Herraiz et al., 2015; Martínez-Vicente et al., 2020). All the non-canonical proteins have been found to display reduced signaling to the cAMP pathway, although most of them are still able to activate the ERKs. Canonical MC1R belongs to the class A subfamily of GPCRs and is preferentially expressed in the surface of melanocytes and MCs. MC1R is a seven-transmembrane-helix protein with an extracellular N terminus, 7 transmembrane (TM) regions, three extracellular loops, three intracellular loops, and an intracellular C terminus. The N-terminal functions as a non-cleavable signal anchor directing traffic of the protein to the endoplasmic reticulum (ER) membrane (Wallin and Heijne, 1995). This region contains two N-linked glycosylation sites, ^{15}NSTP^{18} and ^{29}NQTG^{32}, occupied

by structurally and functionally different glycans (Herraiz, Journe, et al., 2011; Herraiz, Sánchez-Laorden, et al., 2011). MC1R glycosylation regulates the availability of receptor molecules at the plasma membrane, improving forward trafficking and decreasing internalization. There is a conserved cysteine residue located at the first TM domain (Cys35) which is essential for receptor function (Frändberg et al., 2001; Sánchez-Laorden, Sánchez-Mas, Martínez-Alonso, et al., 2006; Sánchez-Laorden, Sánchez-Mas, Turpín, et al., 2006; Zanna et al., 2008). The C-terminal extension of MC1R is formed by 19 amino acids, 6 of which are conserved in all MCRs. Deletion of the last five amino acids of canonical MC1R resulted in intracellular retention and decreased cell surface expression (Sánchez Más et al., 2005). A recent report showed that MC1R was palmitoylated at residue Cys315, increasing signaling downstream of MC1R (Chen et al., 2017). This palmitoylation was promoted upon UVR and impaired in a significant number of natural hypomorphic variants (Chen et al., 2017). This region also contains key residues for the retrograde route in receptor intracellular trafficking, Thr308 and Ser316, whose phosphorylation by GPCR kinases regulates receptor desensitization and internalization (Sánchez-Laorden et al., 2007).

2 | CAMP-MEDIATED EFFECTS BEYOND PIGMENTATION

2.1 | MC1R: a cornerstone against oxidative stress in melanocytes and other cell types

Ultraviolet radiation, melanogenesis, and pheomelanin are important sources of reactive oxygen species (ROS). Among ROS, hydrogen peroxide (H_2O_2) reaches all cellular compartments (Calabrese et al., 2019) and reacts with transition metal ions such as Fe^{2+} and Cu^{2+} giving rise to extremely reactive hydroxyl radical (Arosio & Levi, 2010).

Catalase is the main enzyme responsible for H_2O_2 neutralization (Gebica, 2020). In normal human melanocytes (NHMs), levels and activity of catalase are proportional to melanin content and expression of TYR (Maresca et al., 2008). After UVR irradiation, NHMs stimulate the generation of H_2O_2, which correlated with a decrease in catalase activity (Song et al., 2009). Pretreatment with α-MSH protected melanocytes from oxidative DNA damage, reducing H_2O_2 production (Haycock et al., 2000; Kadekaro et al., 2005, 2010), decreasing the generation of 7,8-dihydro-8-oxyguanine (8-oxodG), and increasing protein levels of catalase and ferritin (Song et al., 2009). Maresca et al. (2010) showed that α-MSH-mediated MC1R stimulation induces both the activity and overexpression of catalase. α-MSH-dependent catalase induction was downstream of cAMP/PKA pathway, but independent of melanogenic process. Catalase induction was neither MITF dependent or due to a transcriptional regulation and was, in turn, dependent on a post-transcriptional regulation, involving mRNA 5′ Cap ribose reversible methylation, which is known to increase protein translation efficiency (Schmidt et al., 2002). Furthermore, Maresca et al. (2010), demonstrated that, in response to α-MSH, catalase was also conveyed to melanosomes. Therefore, the melanosome was protective because it carried both melanin and catalase (Figure 1).

Furthermore, we describe an unusual mechanism of antioxidant protection, involving the eumelanin intermediate 5,6-dihydro xyindole-2-carboxylic acid (DHICA). DHICA can contribute to skin defense mechanisms not only as a source of melanin pigments in melanocytes, but also as a diffusible bioactive messenger, acting on the neighborhood keratinocytes (Figure 1). Treatment of primary cultures of human keratinocytes with DHICA induced cell differentiation, preserved the overall cell survival, and counteracted the peroxidation of cell membrane lipids. Finally, cell death after UVA irradiation was significantly reduced in the presence of DHICA. Moreover, DHICA was able to enhance both the activity and protein expression of antioxidant enzymes catalase and superoxide dismutase (Kovacs et al., 2012).

α-MSH upregulates the expression of Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), as well as Nrf-dependent gene expression, such as heme oxygenase-1, γ-glutamylcysteine-synthetase, and glutathione-S-transferase Pi in keratinocytes and melanocytes, blocking the inhibitory effect of UVB radiation (Kokot et al., 2009). α-MSH also acts as an inhibitor of TNF-α-stimulated NF-κB transcription factor, responsible for expression of several inflammatory and immune system genes, in MCs and melanocytes (Haycock et al., 1999), in human glioma (Ichiyama et al., 1999), and in cells of the immune system (Manna & Aggarwal, 1998).

A375 MCs harboring a natural hypomorphic variant of MC1R stimulated with the synthetic analog of α-MSH, NDP-MSH, showed increased *SOD1* expression (Castejón-Griñán et al., 2018). In human MCs (hMCs) expressing wild-type (WT) MC1R, stimulation with NDP-MSH caused a time-dependent increase in *catalase* (*CAT*), *a* faster stimulation of *superoxide dismutase* (*SOD1*), and a weaker increase in *glutathione peroxidase* (*GPx1*) expression compared with unstimulated hMCs, which were impaired by adenylyl cyclase inhibition with DDA, indicating the involvement of the cAMP pathway (Castejón-Griñán et al., 2018). Improved antioxidant defenses in WT MC1R MCs contribute to the protective effect of MC1R activation. In WT MC1R-expressing keratinocytes HaCaT cells, ROS production induced by a NOXA1 mechanism upon UVA irradiation is almost completely abolished after α-MSH treatment by a PKA-dependent mechanism involving phosphorylation of NOXA1 (Henri et al., 2012).

All these data contribute to define MC1R as a cornerstone of antioxidant protection, not only for melanocytes but also for the surrounding keratinocytes.

2.2 | Preservation of DNA integrity: Induction of DNA repair pathways downstream of MC1R

Upon UVR exposure, several events occur within cells: (a) the induction of DNA photoproducts, mainly cyclobutane pyrimidine dimers (CPDs), the most frequent UVR-induced lesions in cellular DNA (Kielbassa et al., 1997), and pyrimidine (6-4) pyrimidone

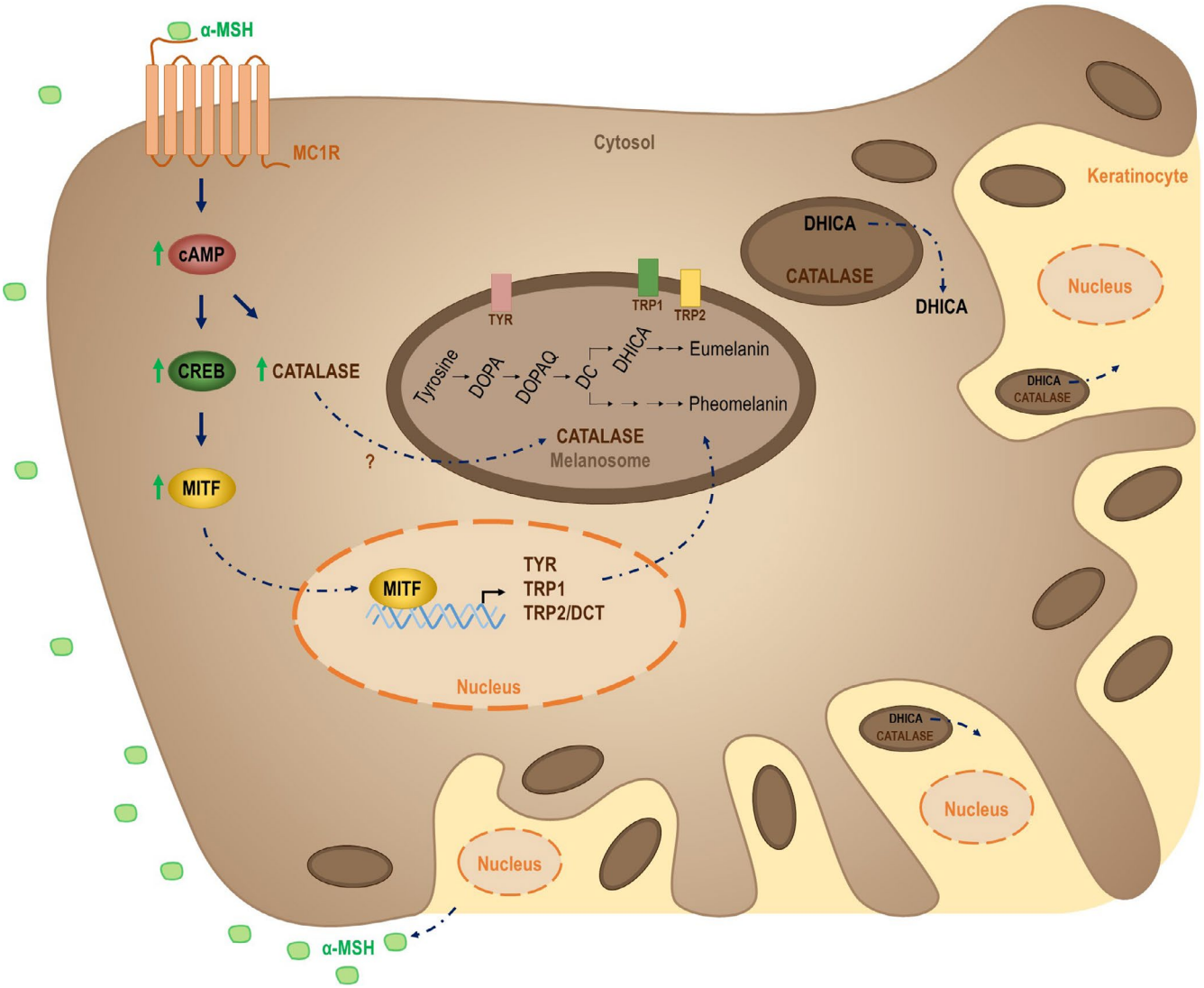

FIGURE 1 Activated MC1R is a cornerstone of antioxidant protection, not only for melanocytes but also for surrounding keratinocytes. In response to α-MSH, catalase is induced and it is also conveyed to melanosomes. Therefore, the melanosome is protective because it carries both melanin and catalase. Furthermore, DHICA contributes to skin defenses, not only as an intermediate of eumelanin synthesis, but also as an antioxidant. The antioxidant function of DHICA is exerted both inside and outside the melanosomes, thanks to its capacity to diffuse across cell membranes

photoproducts (6-4PPs) (Douki, 2013) and (b) the generation of ROS that result in DNA damage, such as 8-oxodG and DNA strand breaks, as well as in lipid and protein peroxidation. In human melanocytes, exposure to UVR causes chemical excitation of fragments of the pigment melanin, resulting in the formation of additional CPDs (Premi et al., 2015). α-MSH has been shown to protect against the UVR-induced oxidative stress by several pigment-independent mechanisms, including induction of antioxidant defenses (see above) and activation of DNA repair pathways (Figure 2). UVR-induced DNA damage in the form of CPDs and 6-4PPs is mainly repaired through the Nucleotide Excision Repair (NER) system, which recognizes large distorsions in the helical structure of DNA and involves over two dozen different protein factors (Povey et al., 2007). Irradiation with solar UVR induces DNA damage in keratinocytes and increases α-MSH synthesis. The two DNA repair proteins, DDB1 and PCNA, were shown to be reduced by UVR and increased by α-MSH, partially reversing the inhibitory effects of UVR (Kadekaro et al., 2010). Microarray data also suggested that α-MSH altered gene expression and antagonized the effects of UVR on many genes, such as those regulating oxidative stress and DNA repair (Kadekaro et al., 2010). Regulation of XPC and DDB2 protein expression levels have been reported on WT MC1R melanocytes grown in cocultures with keratinocytes after treatment with NDP-MSH and UVR exposure (Wong et al., 2012). Abdel-Malek's group also showed that treatment of human melanocytes with α-MSH increased the levels of XPC, induced phosphorylation of ataxia telangiectasia and Rad3-related kinase (ATR) and ataxia telangiectasia mutated (ATM), and their respective substrates checkpoint kinases 1 and 2, and increased phosphorylated H2AX

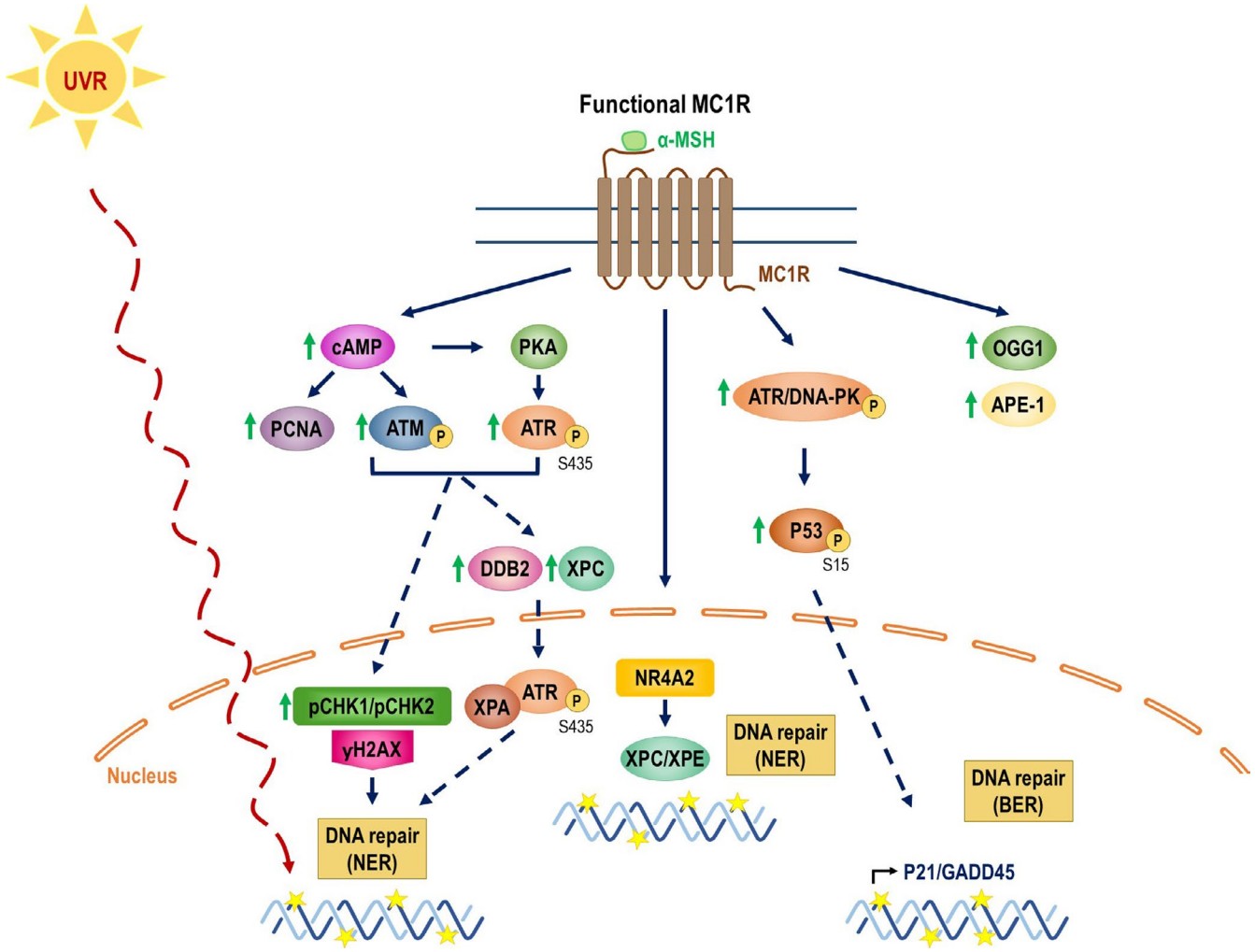

FIGURE 2 In melanocytic cells, α-MSH/MC1R signaling promotes DNA integrity by pigment-independent mechanisms upon UVR exposure. In human melanocytes, MC1R activation by α-MSH triggers cAMP second messenger cascade that mediates the increase in protein levels of PCNA and the phosphorylation of ATM and ATR. ATM and ATR lead to the activation of CHK1/2 and promote the formation of γH2AX, leading to clearance of photoproducts. In addition, ATM and ATR mediate the increase in DDB2 and XPC, and PKA-dependent ATR phosphorylation at Ser435 recruits XPA to sites of the nuclear photodamage, promoting repair photolesions. Moreover, induction of NR4A2 downstream of MC1R activated by α-MSH leads to translocation of this nuclear receptor to sites of DNA damage and colocalization with DNA repair factors XPC and XPE at the sites of the lesion. α-MSH enhances the expression of BER enzymes OGG1 and APE-1, and the phosphorylation of upstream activators of p53, ATR, and DNA-PK. Activated p53 translocates to the nucleus to induce the expression of p21 and GADD45, contributing to the repair of oxidative DNA damage

(γH2AX) formation, leading to repair of DNA photoproducts (Swope et al., 2014). MC1R allelic variants hypomorphic for the cAMP pathway were not able to activate this DNA damage response (Swope et al., 2014). One year later, another research group demonstrated that cAMP-dependent PKA activation induced by α-MSH/MC1R led to ATR phosphorylation at Ser435, which actively recruited XPA to sites of nuclear UVR photodamage, promoting clearance of UVR-induced photolesions and reducing mutagenesis (Jarrett et al., 2014, 2015; Swope et al., 2020). Moreover, MC1R activation by α-MSH was shown to result in a rapid and transient induction of all three family members of the nuclear receptor subfamily 4 (NR4A), which was impaired in melanocytes that were homozygous for MC1R RHC variant alleles (Smith et al., 2008). Importantly, induction of NR4A2 downstream of MC1R led to translocation of this nuclear receptor to sites of DNA damage in a p38-dependent manner and colocalization with DNA repair factors XPC and XPE at the sites of the lesion (Jagirdar et al., 2013).

In contrast with large helix-distorting DNA lesions, oxidative DNA damage is mainly repaired through the Base Excision Repair (BER) system. In normal melanocytes, incubation with α-MSH prior to UVR exposure increased levels of phosphorylated p53 on Ser15, leading to stabilization and activation of p53, a major sensor of DNA damage (Kadekaro et al., 2012). Upon α-MSH stimulation, p53 translocated to the nucleus to induce the expression of p53 targets p21 and GADD45 (Kadekaro et al., 2012). Moreover, α-MSH enhanced the phosphorylation of upstream activators of p53, ATR, and DNA-PK, and the expression of BER enzymes OGG1 and APE-1.

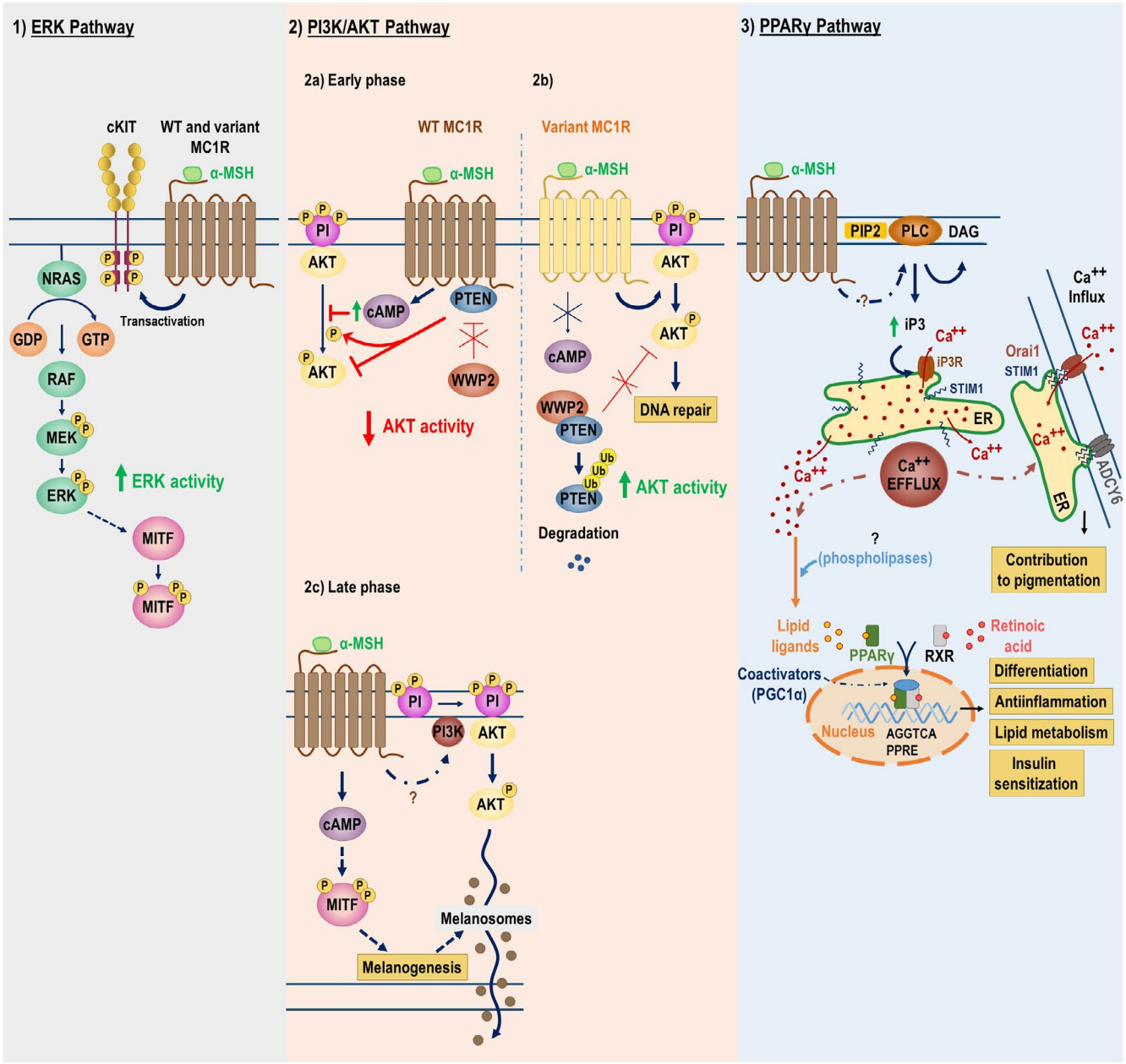

FIGURE 3 Activated MC1R is able to transduce through different pathways. (1) ERK pathway. α-MSH binding to WT and most natural allelic variants of MC1R triggers ERK phosphorylation and activation through a mechanism involving transactivation of the RTK c-KIT, independently of cAMP. (2a) Early phase PI3K/AKT pathway. In MC1R-WT melanocytic cells, stimulation with α-MSH leads to an increase in cAMP levels and inhibition of AKT phosphorylation. Upon UVR, WT MC1R interacts with PTEN, protecting PTEN from WWP2-mediated degradation and contributing to AKT inhibition. In melanocytic cells carrying variant-MC1R hypomorphic for cAMP, non-canonical signaling from MC1R leads to AKT phosphorylation and activation of DNA repair. After exposure to UVR, interaction between PTEN and variant MC1R is impaired, PTEN is degraded by WWP2 and this contributes to increased AKT activity. (2b) Late phase PI3K/AKT pathway. After stimulation with α-MSH for prolonged times, increased pAKT was observed. In this late phase, PI3K contributes to the transport of melanin to the extracellular environment. (3) PPAR-γ Pathway. α-MSH induces the release of Ca^{2+} from ER by a PLC-dependent mechanism. ER Ca^{2+} efflux is connected, in turn, with the translocation of PPARγ into the nucleus, where it promotes the transcription of gene clusters. The involvement of PPAR-γ and PGC-1α, in response to α-MSH exposure, underlies the role of α-MSH in promoting energy metabolism and cellular well-being even through these mediators. Moreover, the ER Ca^{2+} efflux mediates the recruitment of STIM1 protein at the ER-PM junction. At this junction, STIM1 interacts with ADCY6, activating it and contributing to pigmentation. Furthermore, at the ER-PM junction, STIM1 activates Orai-1 mediated Ca^{2+} influx, in order to reestablish ER calcium

Pharmacological inhibition of p53 or siRNA-mediated p53 abolishment impaired the protective effects of α-MSH and increased oxidative stress (Kadekaro et al., 2012).

Overall, these studies demonstrate that stimulation of MC1R after UVR leads to overexpression and activation of multiple repair proteins that ultimately contribute to the protection of DNA integrity.

3 | ADDITIONAL PATHWAYS AND THEIR EFFECTS

3.1 | ERK pathway

ERK cascade is a highly regulated mitogen-activated protein kinase (MAPK) pathway responsible for the regulation of basic cellular processes, including cell proliferation, differentiation, and melanogenesis (Katz et al., 2007). The small GTPase RAS and the protein kinases RAF, MEK, and ERK form the ERK pathway. Activating mutations in N-RAS and B-RAF are very frequent in melanoma (~25% for N-RAS and ~60% for B-RAF), causing cell cycle and proliferation dysregulation (Colombino et al., 2012; Dhomen and Marais, 2009; Hodis et al., 2012, Krauthammer et al., 2012,). The ERK pathway is sequentially activated by multiple stimuli such as growth factors, cytokines, viruses, GPCR ligands, and oncogenes, resulting ultimately in ERK1/2 phosphorylation. Once activated, ERK1/2 can phosphorylate cytoplasmic and cytoskeletal proteins, and translocate to the nucleus to regulate different transcription factors, such as c-FOS, c-JUN, ELK-1, c-MYC, and ATF-2 (Murphy & Blenis, 2006), and mediate cell growth, migration, and differentiation. Importantly, ERK1/2 can phosphorylate MITF (Hemesath et al., 1998), decreasing its protein levels and resulting in a negative regulation of melanogenic enzymes and inhibition of melanogenesis (Xu et al., 2000). In human melanocytic cells, non-canonical signaling from MC1R to the ERKs upon α-MSH binding is cAMP-independent and occurs through transactivation of c-KIT (Figure 3, panel 1), a receptor tyrosine kinase (RTK) crucial for melanogenesis, proliferation, migration, and survival of the pigment-producing cells (Herraiz et al., 2009; Herraiz, Journe, et al., 2011; Herraiz, Sánchez-Laorden, et al., 2011). Importantly, this was the first report of activation of c-KIT by signaling from a GPCR. Moreover, most of the frequent allelic variants with strongly impaired functional coupling to the cAMP canonical pathway are still able to activate the non-canonical ERK pathway (Herraiz et al., 2012), consistent with a cAMP-independent mechanism for ERK activation in human melanocytic cells.

In summary, functional coupling of the human MC1R to the ERK pathway is differentially regulated from the cAMP pathway and involves transactivation of the RTK c-KIT and many natural MC1R variants hypomorphic for the cAMP pathway efficiently activate the ERK signaling cascade.

3.2 | PI3K/AKT pathway

PI3Ks form a kinase family that phosphorylate inositol phospholipids. Canonical signaling is initiated by activation of growth factor receptor protein tyrosine kinases, resulting in autophosphorylation on tyrosine residues. Activated PI3K phosphorylates the phosphatidylinositol-4,5-bisphosphate (PIP_2) leading to the production of the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP_3). PIP_3 recruits a subset of signaling proteins, including AKT/Protein kinase B (PKB), regulating cellular processes such as cell survival and cell cycle progression (Vanhaesebroeck et al., 2016). PTEN functions in the cell as a phosphatidylinositol-3-phosphatase, whose primary role is the conversion of PIP_3 to PIP_2, thus acting as an antagonist of the PI3K/AKT pathway (Li et al., 1997).

The role of activated MC1R in influencing PI3K/PTEN signaling was investigated. Upon UVB exposure, WT MC1R interacted with PTEN, preventing its WWP2-mediated proteolytic degradation and resulting in AKT inactivation. On the contrary, RHC MC1R allelic variants showed an impaired ability to interact with PTEN, thus increasing AKT signaling and predisposing melanocytes to melanomagenesis (Cao et al., 2013) (Figure 3, panels 2a-b). In retinal pigment epithelium (RPE) cells expressing functional MC1R, α-MSH activated AKT/mammalian target of rapamycin (mTOR) and ERK1/2 signaling, protecting RPE cells from H_2O_2-induced apoptosis and reducing the risk of developing age-related macular degeneration (Cheng et al., 2014).

A study carried out on NHMs expressing a WT MC1R demonstrated that when PI3K pathway is inhibited, α-MSH reduces its capacity to protect DNA integrity and becomes ineffective in protecting against apoptosis (Kadekaro et al., 2005). Within hMCs, NDP-MSH stimulation of RHC MC1R allelic variants activated DNA repair pathways through a mechanism mediated by AKT activation and independently of cAMP (Castejón-Griñán et al., 2018) (Figure 3, panel 2b). Several lines of evidence supported this conclusion: (a) NDP-MSH stimulation of MC1R variants activated clearance of ROS-generated strand breaks and 8-oxodG (marker of oxidative DNA damage) in an AKT-dependent manner; (b) NDP-MSH failed to promote cAMP signaling in variant MC1R as demonstrated by lack of stimulation of cAMP levels or MITF gene expression; (c) AKT-activating phosphorylation was increased upon NDP-MSH treatment of hMCs carrying RHC MC1R variants and blocking AKT signaling with LY294002 (a PI3K inhibitor) and MK-2206 (an AKT inhibitor) abolished variant MC1R-dependent activation of DNA repair. On the other hand, NDP-MSH treatment of WT MC1R-activated DNA repair and antioxidant defenses in a cAMP-dependent fashion and decreased AKT phosphorylation (Figure 3, panel 2a).

Working on murine melanoma cell line B16-F10, Mosca et al. (2021), investigated the α-MSH-dependent modulation of pAKT/AKT (as a key element of PI3K pathway) over time (starting from minutes of stimulation until 48 hr). In agreement with previous studies (Khaled et al., 2003; Castejón-Griñán et al., 2018), the authors observed an initial phase in which AKT was not phosphorylated in response to the hormone. Moreover, analysis at later timepoints (6–24 hr) showed that AKT was phosphorylated. This effect resulted mainly evident after 24 hr. The authors confirmed the same effect also on NHMs. On B16-F10, they also explored the functional influence of α-MSH-mediated PI3K/AKT pathway on melanogenesis. As expected, all parameters of melanogenic activity (MITF, TYR expression, and activity, intracellular melanin amount) induced by α-MSH were more increased by combined treatment with α-MSH plus LY294002 (as an inhibitor of PI3K pathway). In the B16-F10 cell lines, as well as in NHMs and in ex vivo skin biopsies explants, treatment

with α-MSH plus LY294002 caused an evident phenomenon of melanin retention, underlining the crucial role of α-MSH-dependent PI3K pathway in mediating the release of pigment outside the cell (Figure 3, panel 2c).

In summary, even if the mechanisms that modulate PI3K pathway after α-MSH stimulation are only partially known, the available literature data show that this pathway downstream of α-MSH/MC1R, plays an important role in maintaining redox equilibrium, in protecting against DNA damage and apoptosis. Moreover, α-MSH-dependent PI3K pathway seems to exert a crucial role in favoring the release of melanin outside melanocytes.

3.3 | α-MSH-PPAR-γ connection

The functions promoted by α-MSH overlap with those promoted by inducers of PPAR-γ (e.g., promotion of pigmentation and/or antioxidant and DNA repair systems) (Grabacka et al., 2008; Lee et al., 2007; Okuno et al., 2010; Polvani et al., 2012). PPAR-γ belongs to the Peroxisome proliferator-activated receptors (PPARs) family, which includes three isoforms: PPAR-α, PPAR-β/δ, and PPAR-γ. PPARs act as nuclear receptors and are activated by lipids released from cell membranes in response to physiological or pathological stimuli (Varga et al., 2011; Wagner & Wagner, 2020). After activation, PPARs translocate into the nucleus, form heterodimers with retinoic X receptors, and promote transcription of downstream target genes involved in lipid metabolism, adipogenesis, maintenance of metabolic homeostasis, and inflammation and inducing also anticancer effects in a variety of human tumors (Fanale et al., 2017; Varga et al., 2011). A connection between α-MSH and PPAR-γ has been identified both in MCs and NHMs. Both the α-MSH-dependent translocation of PPAR-γ to the nucleus, and its activity as a transcription factor, were not reproduced by the cAMP inducer Forskolin and they rather relied on a Ca^{2+}/PLC-dependent mechanism (Maresca et al., 2013) (Figure 3, panel 3).

In agreement with this study, Motiani et al. (2018) demonstrated that α-MSH induces a release of Ca^{2+} from ER. Moreover, they showed that this release is associated with the recruitment of STIM1 protein at the ER–plasma membrane (PM) junction. At this junction, STIM1, an ER Ca^{2+} sensor, interacts with Adenylyl cyclase 6 (ADCY6), activating it and contributing to promote pigmentation. Moreover, at the same junction, STIM1 activates Orai-1 mediated Ca^{2+} influx, in order to reestablish ER calcium (Figure 3, panel 3). This influx seems to be crucial in regulating melanocytes hyper-proliferation. Since MC1R is a GPCR that couples to Gs, the authors speculated that PLC activation is most likely achieved through cAMP-dependent activation of PKA and/or through exchange protein directly activated by cAMP (EPAC). Literature data showed that both PKA and EPAC can induce IP_3 generation, by activating PLCβ (Luo et al., 1999) and PLCε (Schmidt et al., 2001), respectively.

When activated, both GPCRs and PPAR-γ can influence mitogenic signals (Bar-Shavit et al., 2016; Law et al., 2016; van Jaarsveld et al., 2016). However, the literature regarding potential roles of MC1R in their type of regulation is still scarce. Melanocyte differentiation in the skin, with the induction of TYR and melanosome maturation, did not preclude their mitotic division (Jimbow et al., 1975). In vitro studies carried out on NHMs confirmed these early data and now is unequivocal that the cAMP pathway, activated by α-MSH, stimulates melanocyte proliferation (Abdel-Malek et al., 1995; Im et al., 1998; Novosadova et al., 2016). Sporadic and very old studies show that transformed cells respond to α-MSH by proliferating or down-regulating proliferation, according to the degree of pigmentation and the progression of the pathology (Halaban and Lerner, 1997; Pawelek, 1979). Flori, Mastrofrancesco, et al. (2017) and Flori, Rosati, et al. (2017) explored the link between α-MSH stimulation and proliferative behavior in B16-F10 and human MCs, which expressed a wild-type MC1R. α-MSH promoted decreased proliferation in these melanoma cell lines, and this effect was mainly due to a PLC-dependent mechanism, employing PPAR-γ as an effector element.

PPAR-γ activity is regulated by co-activators and co-repressors. The PPAR-γ coactivator-1α (PGC-1α) belongs to a small family of transcriptional co-activators which interact with other transcription factors and nuclear receptors and are involved in the regulation of mitochondrial biogenesis, liver, and brown adipose tissue metabolism and detoxification by ROS (Shoag et al., 2013; Villena, 2015). Both α-MSH and cAMP were shown to increase strongly PGC-1α expression, both in MCs and NHMs. PGC-1α and also the related PGC-1β-stimulated MITF and TYR expression, as well as melanin production (Shoag et al., 2013). These studies highlight an important link between pigmentation and metabolism.

Overall, the knowledge regarding the involvement of phospholipases and lipid mediators in MC1R transduction is limited and sporadic. However, the involvement of PPAR-γ and PGC-1α, in response to α-MSH exposure, underlies the role of α-MSH in promoting energy metabolism and cellular well-being even through these mediators.

3.4 | Clinical relevance perspectives of studying MC1R polymorphic variants associated with melanoma risk

Due to the high prevalence of alleles encoding for partial loss-of-function variants, MC1R is an important gene predisposing to cutaneous melanoma (García-Borrón et al., 2014; Tagliabue et al., 2015). Human *MC1R* is highly polymorphic (García-Borrón et al., 2014; Herraiz et al., 2017), with more than 300 variants. Thus, it is considered the major genetic determinant of cutaneous phototype and the best established susceptibility gene for melanoma. Valverde et al. (1995) first reported the association between *MC1R* polymorphisms and the pale skin, red hair, freckles, and inability to tan phenotype, the RHC phenotype. RHC MC1R alleles have been classified according to their penetrance as high (R) or low (r) variants. MC1R "R" variants include D84E, R142H, R151C, R160W, and D294H. People with these MC1R allelic variants are

at highest risk of developing melanoma and non-melanoma skin cancers (Kennedy et al., 2001; Palmer et al., 2000; Sánchez Más et al., 2002; Sturm et al., 2003; Valverde et al., 1995;). The allelic variants V60L, V92M, and R163Q showed a weaker association with the RHC phenotype and are designated as "r" alleles. Interestingly, R variants R142H, R151C, R160W, and D294H, along with r allele V60L, are present in around 30% of individuals of northern European descent and, overall, they account for more than 60% of all individuals with red hair (Healy et al., 2001). MC1R coupling to the ERK and cAMP pathways is differentially regulated by MC1R genotype (Herraiz et al., 2012). Both R and r MC1R variants show decreased or undetectable functional coupling to cAMP pathway in response to MC1R agonists in heterologous systems, hMCs or NHMs of defined genotype (García-Borrón et al., 2014; Newton et al., 2007). The main cause of functional impairment for the major RHC alleles R151C, R160W, I155T, and D84E may be a decreased cell surface expression (Beaumont et al., 2007; Herraiz et al., 2012; Pasquali et al., 2015), due to deficient anterograde trafficking or increased desensitization and internalization (Beaumont et al., 2005; Sánchez-Laorden et al., 2009; Sánchez-Laorden, Sánchez-Mas, Martínez-Alonso, et al., 2006; Sánchez-Laorden, Sánchez-Mas, Turpín, et al., 2006). On the contrary, certain alleles show decreased functional coupling in spite of a normal plasma membrane density. On the other hand, most RHC alleles are able to positively couple to ERK1/2 in response to their agonists in NHMs or hMCs, as well as when transfected in heterologous systems (Herraiz et al., 2009). Thus, most RHC variants should be considered as imbalanced signaling forms rather than as loss-of-function mutants. Genome-wide association studies and meta-analyses have widely demonstrated the association of RHC variants with increased risk of melanoma (Amos et al., 2011; Chatzinasiou et al., 2011; Pasquali et al., 2015; Raimondi et al., 2008; Williams et al., 2011) and non-melanoma skin cancers (Bastiaens et al., 2001; Dwyer et al., 2004; Han et al., 2006; Kennedy et al., 2001; Pasquali et al., 2015). This association can be only partially explained by the protective effect of pigmentation. Increased TYR activity in response to WT MC1R activation by α-MSH stimulates the synthesis of black/brown eumelanin pigments, whereas minimal receptor activity, as in RHC allelic variants, produces red/yellow pheomelanins. Pheomelanin has weak shielding capacity against UVR relative to eumelanin and has been shown to amplify UVA-induced ROS. Thus, an increased ratio of photoprotective eumelanins to pro-oxidant pheomelanins provides an effective shield against mutagenic UVR (Maresca et al., 2015). Moreover, considering the link existing between α-MSH and PPAR-γ (see above), it has been shown that specific PPAR-γ modulators provide photoprotective effect in keratinocytes harboring *MC1R*-inactivating mutations (Flori, Mastrofrancesco, et al., 2017; Flori, Rosati, et al., 2017). However, genetic epidemiological studies showed that a significant association of MC1R variants and melanoma persists after stratification for pigmentation, and carrying MC1R allelic variants also increases melanoma risk in dark-skinned population of European origin, thus pointing to pigment-independent actions of MC1R (Bastiaens et al., 2001; Chatzinasiou et al., 2011; Gerstenblith et al., 2007; Kennedy et al., 2001; Landi et al., 2005; Palmer et al., 2000; Pasquali et al., 2015; Raimondi et al., 2008; Stratigos et al., 2006; Sturm, 2002; Tagliabue et al., 2015), such as induction of antioxidant defenses and DNA repair mechanisms, discussed above.

In conclusion, MC1R allelic variants are imbalanced signaling forms with frequent impaired cAMP production, resulting in an increased ratio of pheomelanin/eumelanin content, and efficient signaling through the ERKs pathway. The increase in pheomelanin may partially account for the association with increased melanoma risk (Mitra et al., 2012). The inability to activate the cAMP pathway also impairs downstream signaling to cAMP-mediated DNA repair events induced by UVR. However, stimulation of variant MC1R after oxidative stress induces a pigment-independent pathway that contributes to DNA repair by a cAMP-independent and AKT-dependent mechanism. Further analysis would be important to elucidate the precise nature of this DNA repair pathway.

ORCID

Vittoria Maresca https://orcid.org/0000-0001-9239-6978

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How to cite this article: Herraiz C, Martínez-Vicente I, Maresca V. The α-melanocyte-stimulating hormone/melanocortin-1 receptor interaction: A driver of pleiotropic effects beyond pigmentation. *Pigment Cell Melanoma Res*. 2021;00:1–14. https://doi.org/10.1111/pcmr.12980