

Characterization of OPSIN 3 in Human Skin Cells

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“Mathematic + Science + Heart = Real Human”

-- Oktay Sinanoğlu --

Abstract

Opsins are members of the rhodopsin G-protein coupled receptors (GPCRs) family that mediate phototransduction in both invertebrates and vertebrates. More specifically, humans have nine types of opsins: Blue, green and red opsins (OPN1), OPN2 through 5, peropsin and retinal GPCR (RGR). As critical photoreceptors in several tissues of our own bodies, opsin structures have been extensively characterized, yet functionality of the extra-ocular OPN3 has yet to be elucidated. OPN3 was first discovered in the deep regions of the brain seemingly devoid of light and need for photoreception. In our laboratory, we recently showed that OPN3 is the most highly expressed opsin in human skin. This lends the idea that OPN3 may play a role in dermal light sensing. Furthermore, the presence of other rhodopsin GPCR family members in skin cells raises an intriguing question of whether OPN3 functionally interacts with other skin GPCRs. Here I demonstrated that knocking down OPN3 in human melanocytes leads to an increase in melanin production, intimately linking OPN3 to melanogenic pathways. A key component of melanogenesis is the melanocortin-1 receptor (MC1R), a rhodopsin GPCR family protein. It has been proven numerously that GPCRs, including melanocortin receptors, can form functional heterodimers, which affords the hypothesis that MC1R and OPN3 form a functional heterodimer having a regulatory role in melanin synthesis. To address this hypothesis, I developed several epitope-tagged OPN3 and MC1R constructs and performed co-immunoprecipitation experiments, which showed that MC1R and OPN3 do co-immunoprecipitate in expression systems. Furthermore, these results were confirmed by co-immunoprecipitation of OPN3 and MC1R from human epidermal melanocytes, which endogenously express these proteins. In addition, I demonstrated that expression of OPN3 inhibits the α MSH induced cAMP response of MC1R in a G α i dependent manner. The work presented here identified a novel function of OPN3 in human skin cells, which give much insight into the underrepresented OPN3 and its functional interactions.

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CHAPTER 1. Introduction

Human Skin

Through the evolutionary journey, animal skin developed different types of protective features such as fur, feathers, and scales. However, when it came to development of human skin, nature took a different turn. Unlike their mammalian relatives, humans lost their body hair and were left with bare skin directly exposed to the external environment. This led to the evolution of human skin with unique features; for example, human skin regulates the body temperature under extreme conditions, provides a first line of defense against the invasion of pathogens, and prevents harmful effects of solar radiation [1]. Skin operates this wide spectrum of tasks with the help of its three distinct layers: hypodermis, dermis, and epidermis (Figure 1.1).

The hypodermis is the lower-most layer of the skin that is primarily composed of connective tissue, fat tissue and blood vessels. Fibroblasts, adipocytes and immune cells are the major types of cells found in hypodermis. The dermis is the layer in between the hypodermis and basement membrane, an extracellular matrix structure separates the epidermis and dermis. The dermis is primarily composed of blood vessels, hair follicles and connective tissue. This layer is also rich in structural proteins such as collagen and elastin. Sensory neurons, fibroblasts, and immune cells are major types of cells found in the dermis. The epidermis is the outer-most layer of the skin that is exposed to environmental factors such as pathogens and ultraviolet radiation (UVR), and is composed of immune cells, epidermal melanocytes (HEMs: human epidermal melanocytes) and epidermal keratinocytes (KER). One HEM and approximately 36 neighboring KERs form the epidermal-melanin unit in which the melanocyte produces melanin and transfers it to the keratinocytes (Figure 1.1). Furthermore, the presence of melanocytes in the epidermis is a unique feature of the human skin among higher vertebrates. In other mammals, melanocytes are primarily localized to hair follicles in the dermis, whereas human melanocytes are located primarily in the basal layer of the epidermis.

As the outer protective layer of your body, the epidermis is exposed to a wide spectrum of solar radiation including infrared, visible and UVR that penetrates through the atmosphere. At the surface of the earth, solar radiation is composed of 42% infrared light, 52% visible light (400-750nm) and 6% UVR. Infrared light penetrates the skin more deeply than visible light and UVR. In fair Caucasian skin, infrared light reaches the hypodermis whereas most of the visible light and UVR penetrates the dermis [2]. UVR is composed of 95% long-wavelength UVA (320-400nm) and 5% short-wavelength UVB (280-320 nm). UVA penetrates the skin more deeply than UVB, reaching the top layer of dermis, whereas UVB can only penetrate the epidermis (Figure 1.1). Although the effects of infrared and visible light on skin are not clear, UVR is crucial for skin health; it plays a key role in vitamin D production. On the other hand, though, high doses of UVR can have harmful carcinogenic and photoaging effects [3]. Human skin protects itself against these harmful effects by producing melanin that absorbs excess UVR.

Most of our knowledge about the epidermal melanization process comes from the well-characterized UVB-induced melanin production pathway. UVB causes DNA damage in keratinocytes and triggers a downstream pathway of melanin production in melanocytes. Exposure to UVB leads to highly mutagenic cyclobutane pyrimidine dimers, which cause the upregulation of tumor suppressor gene p53. Transcription of the pro-opiomelanocortin (POMC) gene is stimulated by p53. POMC is cleaved to give rise to alpha melanocyte stimulating hormone (α MSH), which stimulates G α -coupled melanocortin 1 receptor (MC1R) on melanocytes. Activated MC1R stimulates intracellular second messenger cyclic adenosine monophosphate (cAMP) production by activating adenylyl cyclase (AC). Increased cAMP levels activate protein kinase A (PKA), which evokes the cAMP responsive binding element (CREB) phosphorylation. By this way, CREB travels across the nuclear membrane and stimulates transcription of another transcription factor, microphthalmia transcription

factors (MITF). MITF leads to the production of melanogenic enzymes such as tyrosinase and tyrosinase related protein (TYRP1). Melanosomes are the vesicular organelles where the production and storage of melanin take place. Mature and fully melanised melanosomes are transferred to neighboring keratinocytes in the epidermal-melanin unit (Figure 1.2). In keratinocytes, melanin is localized around the nucleus and prevents UVB from reaching DNA and causing DNA damage [4].

While the pigmentation induced by UVB is well characterized, very little is known about other mechanisms used by skin cells to detect and respond to light.

Photoreceptors in Skin

Light perception not only occurs in the eye, but also in non-ocular tissues such as the skin. This ability is important for many organisms across the phylogenetic tree. For example, photoreceptor cells in the skin of pond snails play a role in the withdrawal response in response to light, part of a defense reaction in this animal [5]. In addition, LITE-1, a seven-transmembrane gustatory receptor (GR) homolog found in the skin of the nematode *Caenorhabditis elegans*, mediates avoidance behavior in response to UVR [6-9]. Furthermore, *Drosophila melanogaster* larvae use dermal photosensors to avoid high intensity light. This phototransduction machinery in the fruitfly depends on a seven-transmembrane gustatory receptor called Gr28b [10].

Many higher-order animals, especially aquatic vertebrates, modulate their skin pigmentation in response to light as well. This adaptive behavior provides camouflage and protection from UVR damage. For example, zebrafish larvae display dispersion of melanosomes on the skin surface as a protective response to prevent the harmful DNA-damaging effects of UVR [11, 12]. Similarly, exposure to high-intensity UVR leads to dermal melanization in juvenile hammerhead sharks [13]. In addition, melanopsin (OPN4) mediates the scattering of melanosomes in specialized

dermal pigment cells called melanophores in *Xenopus laevis* [14]. Rhodopsin (OPN2) and neuropsin (OPN5) were found in mouse cultured melanocytes [15].

In addition to that, certain opsins are expressed in mammalian skin cells. Rhodopsin (OPN2) mRNA and protein were found in mouse cultured melanocytes [15]. In addition, neuropsin (OPN5) was also detected in the skin of mouse outer ears [16]. Rhodopsin (OPN2) mRNA and protein were also found in human epidermal melanocytes [17]. Immunostaining human skin samples revealed that long-wavelength cone opsin (OPN1 LW) is expressed in the basal layer of the epidermis, whereas rhodopsin and short-wavelength cone opsin (OPN1 SW) are expressed in the upper layer of the epidermis [18]. Moreover, the photoisomerase RPE65 was also detected in human keratinocytes [19]. Furthermore, our laboratory discovered that HEMs detect UVA via a retinal-dependent and Gq-protein-mediated pathway but the identity of a UVA receptor in HEMs is yet to be found [20]. Our laboratory also found, using high-throughput RNA sequencing (RNA-seq) of HEMs, that these cells express high levels of encephalopsin (OPN3) mRNA [21]. Also, our laboratory showed that expression of OPN1 SW, OPN2, OPN3 and OPN5 mRNA was found in HEMs and KERs by qPCR analysis. Among these opsins, OPN3 showed the highest expression of mRNA [22]. The presence of opsins in human skin cells raises the interesting question of whether opsins are used for light detection in human skin.

The Opsins

Opsins are a member of largest G-protein-coupled receptors (GPCRs) family, the Rhodopsin GPCR Family [23] (Table 1.1). Rhodopsin, the first crystalized GPCR, was first found in rod cells of the bovine retina [24]. The characterization of rhodopsin structure and function revealed that it consists of an apoprotein and a vitamin A-derived chromophore (retinal). In the light, rhodopsin-bound 11-*cis*-retinal absorbs a

photon, which leads to a conformation change from 11-*cis*-retinal to all-*trans*-retinal (Figure 1.3A) and a subsequent conformational change of the rhodopsin apoprotein bound to the retinal. This leads to stimulation of cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE) through the α subunit of the G-protein transducin (Gt). PDE decreases the intracellular cGMP concentration, which leads to closure of cGMP-gated cation (CNG) channels and hyperpolarization of the rod cells. Rhodopsin is a bleaching pigment, which means in the dark this phototransduction process is reversed by reincorporation of a new 11-*cis*-retinal into rhodopsin [25, 26] (Figure 1.3B).

Comparison of the rhodopsin sequence with other opsins revealed highly conserved residues that are structurally and functionally important in defining a protein as an opsin. These highly conserved residues (Figure 1.4A) together with bovine rhodopsin's crystal structure data (Figure 1.4B) [27] revealed that a number of molecular features are needed to fulfill the criteria of a functional opsin. An opsin sequence should possess [27, 28]:

- a lysine residue in the 7th trans-membrane domain (TM7). This lysine is required to bind retinal via a protonated Schiff base linkage. It is necessary for light activation and binding to the retinal chromophore [29]. Bovine rhodopsin possesses a lysine at position 296 (K296).
- a negatively charged residue (counterion) in the 3rd trans-membrane domain (TM3). This residue stabilizes the positively charged and highly unstable Schiff base linkage of lysine and retinal. In bovine rhodopsin, glutamate at position 113 (E113) serves as the counterion.

In addition to that, opsins display the characteristic features of GPCRs. Since these features are not unique to opsins they are not solely used to characterize a protein sequence as an opsin. Such features include [27, 28, 30] (Figure 1.4A):

- serine and threonine residues in the c-terminus. These residues are phosphorylated by protein kinases and they play important roles in the internalization of opsins. Bovine rhodopsin possesses serine residues at position 334, 338, 343 (S334, S338, S343) and threonine residues at position 335, 336, 340, 342 (T335, T336, T340, T342).
- n-linked acetylation site. These residues are important for localization of GPCRs. The n-linked acetylation site of bovine rhodopsin is methionine at position 1 (M1) [30].
- n-linked glycosylation site. As with the n-linked acetylation site, these residues are important for localization of GPCRs. N-linked glycosylation sites of bovine rhodopsin are asparagine at position 2 and 15 (N2, N15) [30].
- palmitoylation site at c-terminus. C-terminal palmitoylation sites of bovine rhodopsin are cysteine 322 and 323 (C322 and 323).
- D/ERY motif in the cytoplasmic surface of 3rd trans-membrane (TM3) and NPxxY motifs in the 7th trans-membrane domain (TM7). These motifs are important for activation of G protein and protein stabilization [31]. The ERY motif of bovine rhodopsin is at 134-136 and the NPxxY motif is at 302-306.

The positions of these molecular features are not necessarily the same in all opsins and such differences often reflect adaptive changes in opsins [32]. For example, changing the location of certain key residues alters the maximal absorption wavelength of the opsins. In bovine rhodopsin, glycine at position 90 and 119 (G90 and G119), serine at position 164 (S164), phenylalanine at position 295 (F265) and alanine at position 295 (A295) are some of these spectral tuning residues [31]. In addition, variations in opsin protein sequences, especially in the intracellular loop areas, are particularly important for opsin-G protein coupling specificities; this is important for opsin functionally since different G protein families lead to different signaling pathways (Table 1.2).

Based on phylogenetic and G protein coupling analyses, the opsin family is divided into eight subfamilies: (1) G α t coupled opsin; (2) Encephalopsin/TMT-opsins; (3) G α s-coupled opsin; (4) G α q-coupled opsins; (5) G α o-coupled opsins; (6) Neuropsins; (7) Peropsins; and (8) Photoisomerases [32-34] (Figure 1.5, Table 1.4).

1. G α t coupled opsin (Vertebrate visual and non-visual opsin subfamily): Cone opsins (OPN1) and rhodopsin (OPN2) are G α t coupled vertebrate visual opsins. OPN1 and OPN2 play a role in photonic vision (daylight vision) and scotopic vision (dim light vision), respectively. Based on their absorption spectra, OPN1 is divided into four subgroups: OPN1 LW (long-wavelength) detects red light (λ max. 557 nm); OPN1 MW (middle-wavelength) detects green light (λ max. 527 nm); OPN1 SW1 (short-wavelength) detects UV/violet light (λ max. 355-445 nm); and OPN1 SW2 detects blue light (λ max. 400-470 nm). Many fish and birds have tetrachromatic color vision, in which all four types of OPN1 are involved. Most mammals have dichromacy (red and blue vision) [35], however, different than other mammals, humans and some closely related primates acquired trichromacy (red, green and blue color vision) with duplication and modification of OPN1 LW to green cone opsin [36, 37]. OPN2 detects green light (type 2 OPN2, λ max. 505 nm). At this point, it is worth mentioning that type 1 OPN2 (microbial rhodopsin) is not classified in this family since it is extinct in vertebrate species. Type 1 OPN2 is a blue-green light (λ max. 480-530 nm)-induced sensor, which functions as an ion channel or pump in unicellular organisms. Human utilization/exploitation of Type 1 OPN2 in expression systems led to the development of the optogenetic field, a biological technique using light to control cell functions.

Pinopsins, parapinopsins, vertebrate ancient opsins (VA-opsins) and parietopsins are the vertebrate non-visual-opsins. Pinopsins are found in lower vertebrates such as birds and lizards [38], but not in mammals [39]. Parapinopsins are found in the pineal and parapineal organ of fish and amphibians [40]. VA-opsins are found in the retina of salmon [41] and teleost fish [42]. Parietopsins are found in the parietal eye, which

contains a complete cornea, lens, and retina and functions as a third eye in non-mammalian vertebrates such as lizards. The function of pinopsin, parapinopsins, vertebrate ancient opsins (VA-opsins) is not well understood, whereas, parietopsins were reported to have green light sensitivity and G α -coupling ability [43].

2. Encephalopsin/TMT-opsin: Encephalopsin (OPN3) and teleost multiple tissue opsin (TMT-opsin) form the encephalopsin/TMT-opsin subfamily, which is closely related to G α t coupled opsin family (Figure 1.5). OPN3 was first found in the mouse brain; therefore, encephalo- (*greek: relating to brain*) prefix was used to name this opsin. Moreover, OPN3 was highly expressed in certain regions of the brain, including the preoptic area, paraventricular nucleus (PVN) of hypothalamus, cerebral cortex, a subset of neurons from the striatum, the ventral horn of spinal cord and the cerebellum. Intriguingly, in the cerebellum, OPN3 is expressed in a radial stripe pattern [44]. in subsequent studies showed that OPN3 is expressed in a wide variety of tissues such as the brain (human, pufferfish, zebrafish), eye (human, pufferfish, zebrafish), skin (human), lung (human), liver (human and pufferfish), heart (zebrafish) and kidney (human and zebrafish) [22, 45-48]. This led to the usage of an alternative name, panopsin, with pan- (*greek: all*) prefix [45]. The first evidence of OPN3 light activity came from light-dependent cellular response measurements from medaka OPN3 expressing Neuro-2A and HEK cells [49]. Following that, the Terakita group showed that mosquito OPN3, a homolog of vertebrate OPN3, forms a functional photopigment with 13-*cis*, 11-*cis* and 9-*cis* retinal and responds to blue-green light (λ max. 490 nm) in a bistable fashion. Bistable pigments intrinsically revert back to their original dark state after light absorption and activation. Mosquito OPN3 also coupled and activated G α i and G α o proteins in a light dependent manner. Intriguingly, a c-terminally truncated version of mosquito OPN3 was used in this study [50, 51]. In addition, the same Terakita group showed that c-terminally truncated zebrafish, pufferfish and chicken OPN3 all absorb blue light (λ max. 465 nm) and suggested

that these opsins have G protein coupling ability [52]. However, the absorption spectra and G protein coupling profile of human OPN3 remained unknown (Table 1.4).

TMT-opsin, a homolog of mammalian OPN3, was found in teleost fish in a wide variety of neuronal and non-neuronal tissues [53]. It was suggested that TMT-opsin plays a role in the entrainment of peripheral circadian rhythm [53] and peripheral light reception in zebrafish [54]. Further studies on pufferfish and medaka TMT-opsins showed that these proteins respond to blue light and couple to Gai and Gao in a light dependent manner [49, 50]. Recently, an OPN3-related gene, which is most similar to TMT-opsins, was found in chicken and this chicken homolog of TMT-opsin showed blue light abortion when expressed in cultured cells [55]. Further studies are required to demystify the absorption spectra and G protein coupling profile of TMT-opsin.

3. Gas-coupled opsin (Cnidops): Box jellyfish cnidop (cnidarian opsins) is the only member of Gas-coupled opsin family [56]. These opsins are found in camera-type lens eyes of box jellyfish species such as *T. cystophora* and *C. rastonii* and activates a Gs-cAMP-dependent signaling cascade in response to green light (λ max. 500 nm) [56, 57]. Phylogenetic studies revealed that Gas-coupled opsin (Cnidops) is near the root of opsin phylogenetic tree and is closer to Gao- and G α q-coupled opsins and photoisomerases rather than Gat-coupled opsins and encephalopsin/TMT-opsin families [58, 59]. Further studies are needed to better understand the function of box jellyfish cnidops.

4. G α q-coupled opsin (G α q-coupled visual pigment and melanopsin): G α q-coupled visual pigment and melanopsin (OPN4) form the G α q-coupled opsin family. G α q-coupled visual pigments are found in the rhabdomeric photoreceptor cells of invertebrates (protostome) [60]. OPN4 and its homologues are expressed in a wide spectrum of species from invertebrates to vertebrates. In mammals, OPN4 is localized in intrinsically photo-sensitive retinal ganglion cells (ipRGCs) and plays a

role in non-image-forming light responses such as regulation of pupil size [61], melatonin production in pineal gland and entrainment of the circadian clock [62]. OPN4 responds to blue light (λ max. 490 nm) in a bistable fashion and this process is independent of rod and cone cell activity [63]. These findings reveal that OPN4 regulates other non-visual light responses of the eye in response to blue light stimulation.

5. Gao-coupled opsin: Gao-coupled opsins were first discovered in the visual cells of scallop [64] and chordate amphioxus [65]. Colocalization of Gao protein with these opsins in scallop visual cells was the first evidence pointing to their G-protein coupling ability. In addition, swapping G-protein coupling areas of Gao-coupled opsin with rhodopsin increased the Go activation in the cells. The absorption spectra of amphioxus Go-coupled opsins revealed a blue light (λ max. 490 nm) absorption profile in HEK cells [65, 66]. Activation of Go-coupled opsins increases cGMP levels in the photoreceptor cells, which open the cGMP-gated potassium channels and hyperpolarize the photoreceptor cells [67].

6. Neuropsin: Neuropsin (OPN5) was first found in the brain, eye, testis and spinal cord of mouse and human [68]. Following studies showed that mouse OPN5, chicken OPN5 and an OPN5 ortholog in ray-finned fish form functional photopigments with 11-*cis*-retinal that, upon UVR (λ max. 380 nm) stimulation, activate the G α i protein signaling pathway [16, 69, 70]. In addition, OPN5 was found in such deep brain areas of quail as the paraventricular organ. Quail OPN5 was reported to be involved in the regulation of seasonal reproduction and response to short-wavelength light (λ max. 420 nm) [71, 72]. Interestingly, chicken and zebrafish OPN5 could function as bistable pigment whereas mouse and human OPN5 could not because of a single amino acid replacement at position 168 [73]. Furthermore, knocking out OPN5 in mice abolished the corneal circadian clock *in vivo* and *ex vivo* [74]. These findings suggest that OPN5 regulates seasonal changes in organisms in response to UVR.

7. Peropsin subfamily: Peropsin is the sole member of the peropsin family. Peropsin was first found in the retinal pigment epithelium cells (RPE) of mammals [75]. Following studies also showed that peropsin homologues are expressed in invertebrates (protostome) like cephalochordate amphioxus [65] and the arthropod spider [76]. Peropsin can bind all-*trans*-retinal and isomerize it to 11-*cis*-retinal. In addition to that, peropsin protein sequence shows NPxxY motifs in the 7th trans-membrane domain, which implies that peropsin might have G protein coupling ability [75, 76].

8. Photoisomerase subfamily: Retinochrome and retinal GPCR (RGR) opsins form the photoisomerase subfamily. RGR was first discovered in mollusks [77] and later discovered to also be in RPE cells of the vertebrate eye [75]. Similar to peropsin, all photoisomerase opsins can bind all-*trans*-retinal and isomerize it to 11-*cis*-retinal [40, 78]. Moreover, enzymatic regeneration of the 11-*cis*-retinal by photoisomerase is critical for function of the neighboring monostable opsins such cone and rod opsins, which relay on the presence of 11-*cis*-retinal [79].

Opsins are not the only rhodopsin GPCR family receptor expressed in skin. MC1R is a member of rhodopsin GPCR family and as mention earlier, it plays crucial role in UVB induced melanization pathway. The presence of other GPCRs in skin cells raises the interesting question whether opsins and other GPCRs affect the activity of each other.

Functional Alteration of GPCRs via Internalization and Dimerization

GPCR function and signal transduction is modulated by the localization of GPCRs to plasma membrane or specific intracellular membrane compartments. Hence, GPCR interacting proteins, signalsomes, play an important role in regulating GPCR activity by fine-tuning receptor trafficking, recycling and availability at the cell surface. GPCR

kinases (GRKs) and arrestins modulate GPCR desensitization, internalization and re-sensitization [80, 81] (Figure 1.6). Among mammalian GRKs (GRK1-7) and arrestins (arrestin1-4), GRK2, GRK3, GRK5, GRK7, β -arrestin1 (arrestin2) and β -arrestin2 (arrestin3) express ubiquitously whereas the rest are only expressed in the eye. The first step of GPCR desensitization is GRK-mediated phosphorylation of the c-terminus of the activated GPCR. This stimulates the recruitment of β -arrestin to the GRK-phosphorylated GPCR and uncoupling of the G protein from the GPCR. β -arrestin facilitates the internalization of GPCRs by promoting the assembly of clathrin-mediated endocytosis. Following that, GPCR phosphatase (GRP) dephosphorylates the internalized GPCR and, at this point, depending on the regulation profile of that particular GPCR, it is either shuttled back to the membrane for another round of activation or to the lysosome for degradation [82]. At this point, Rab GTPases and effector early endosome antigen 1 (EEA1) play a crucial role in the trafficking of GPCRs between early endosomes, recycling endosomes, late endosomes and lysosomes. For example, Rab5 and EEA1 are involved in the formation and fusion of clathrin-coated endosomes in the formation of early endosomes. Rab 4 and Rab 11 mediate the trafficking of recycling endosomes to the plasma membrane. Rab 7 and 9 direct late endosomes towards lysosomes, which express lysosome-associated membrane glycoprotein 1 (LAMP1) [83, 84]. The process described above is not the only regulatory mechanism of GPCRs. There are also arrestin-mediated G protein independent signaling, intracellular signaling and phosphorylation-independent desensitization mechanisms of GPCRs.

GPCR function and signal transduction is also modulated by the interaction of GPCRs with each other [85]. In the GPCR family, there are many examples of heterodimerization-mediated internalization, signaling and ligand binding alterations of GPCRs [86-88] (Table 1.6). For example, melanocortin 3 receptor (MC3R) and ghrelin receptor (GHSR) heterodimerization alters melanocortin and ghrelin induced

intracellular responses in the arcuate nucleus, a brain region which controls energy metabolism. It is important to point out that, as in the case of rhodopsin family members MC3R and GHSR, all known heterodimerization protein pairs belong to the same GPCR family. In addition, it has been shown that homodimerization could partially regulate the spectral tuning of human red cone opsin *in vitro* [89]. Furthermore, opsins were reported to be involved in homodimerization *in vivo* [90]. Further studies are needed to better understand the possible functional role of opsin homodimerization.

Summary

Skin is the largest organ exposed to light. It would be logical that skin then possess an ability to “sense” light. Recent findings from our laboratory indicate that light-sensitive opsins are present in the epidermal layer of human skin. This raises an intriguing question of whether opsins play role in dermal light sensing. Furthermore, human skin cells also express other GPCRs belonging to the rhodopsin GPCR family. This raises another interesting point about possible functional interactions between opsins and other skin GPCRs. In this dissertation, I examined the localization and function of human OPN3 (hOPN3) in human epidermal skin cells. In Chapter 2, we examine the relative mRNA expression levels of opsins in primary human skin cells. This examination revealed high expression of hOPN3 in human skin. In Chapter 3, I determine the localization of heterologously and endogenously expressed hOPN3. In Chapter 4, I examine the function of heterologously and endogenously expressed hOPN3. This study showed that decreasing hOPN3 protein level increases the melanin level in HEMS, and in a heterologous system hOPN3 co-immunoprecipitates with MC1R and inhibits the α MSH induced cAMP response of MC1R in a Gai dependent manner.

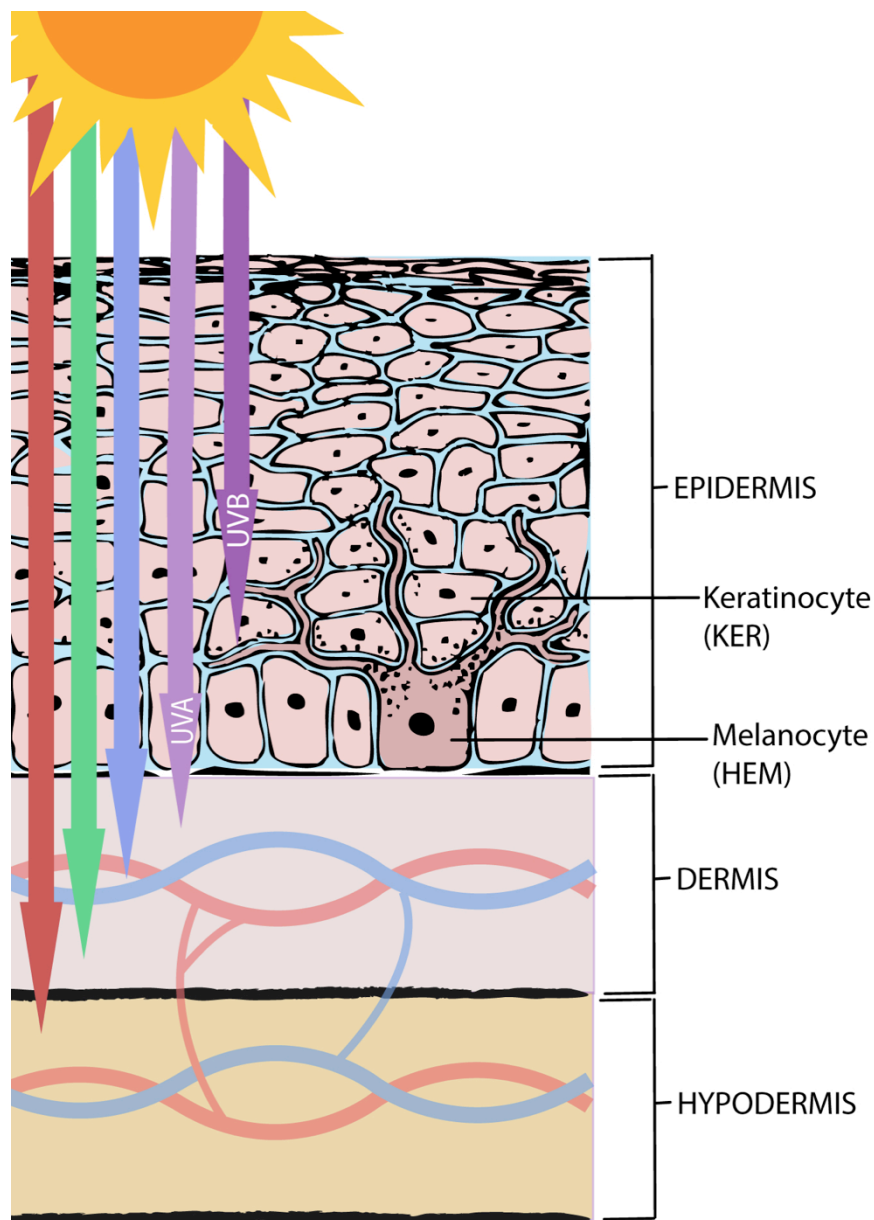


Figure 1.1. Light penetration through human skin and epidermal cells. Human skin is composed of 3 layers: the epidermis (composed of melanocytes and keratinocytes), the dermis, and the hypodermis. Solar light is composed of UVB, UVA, visible and infrared light. Light with a longer wavelength penetrates skin deeper; infrared light penetrates the deepest and UVB penetrates the least. Accordingly, in fair skinned-individuals, UVB and UVA light penetrate the epidermis and dermis, respectively. Visible light penetrates deeper than UVA through the dermis and infrared light reaches the top layer of hypodermis. The blue, green and red arrows represent blue, green and infrared light, respectively.

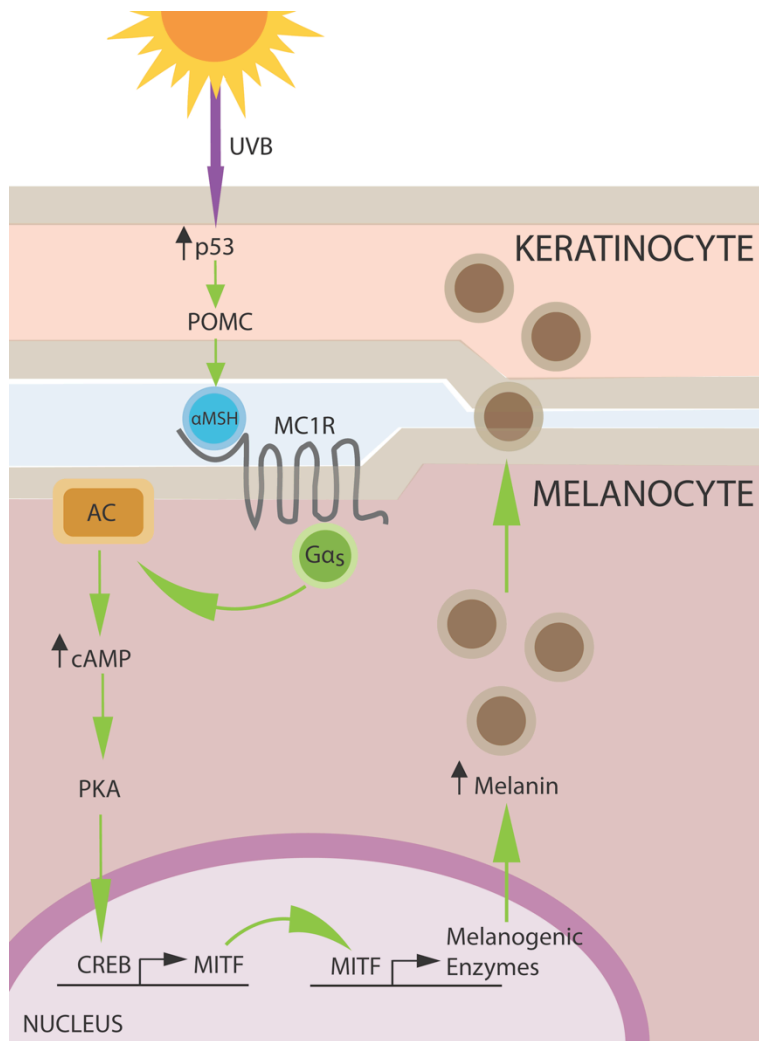


Figure 1.2. The pigmentation mechanism induced by UVB. Human skin protects itself against UVB insults by producing melanin. In keratinocytes, UVB induced upregulation of p53 leads to transcription of the pro-opiomelanocortin (POMC) gene, which is processed to alpha melanocyte stimulating hormone (αMSH). In melanocytes, αMSH activates Gαs coupled melanocortin 1 receptor (MC1R), which increases intracellular second messenger cyclic adenosine monophosphate (cAMP) levels via adenylyl cyclase (AC). cAMP activates protein kinase A (PKA), which phosphorylates cAMP responsive binding element (CREB). CREB stimulates transcription of microphthalmia transcription factor (MITF), a transcription factor of melanogenic enzymes. Melanin is stored in melanosomes and transferred to neighboring keratinocytes to be distributed through the skin.

A

GPCR Families		
Family	Number of Members	Solved Structure
Rhodopsin	701	21
Adhesion	24	0
Frizzled/Taste2	24	1
Glutamate	15	1
Secretin	15	2

B

Rhodopsin GPCR Family			
Group	Receptor Cluster	Number of Members	Example
α -Group	Prostaglandin	15	Prostaglandin E2 receptor
	Amine	40	Dopamine receptor
	Opsin	9	Rhodopsin
	Melatonin	3	Melatonin 1A receptor
	MECA	22	Melanocortin 1 receptor
β -Group		35	Ghrelin receptor
γ -Group	SOG	15	μ -Opioid receptor
	MCH	2	MCH receptor 1
	Chemokine	42	Angiotensin receptor 1
θ -Group	MAS-related	8	MAS1 oncogene receptor
	Glycoprotein	8	Thyrotropin receptor
	Purin	42	fMLP receptor
	Olfactory	460	Olfactory receptor 1 A1

Table 1.1. G-protein coupled receptor (GPCR) families and subgroups of Rhodopsin GPCR family. (A) Human G-protein coupled receptors (GPCR) are classified into five families, which are given with their respective number of members and solved structures. (B) Among the 4 subgroups of the Rhodopsin family, the opsin receptor cluster belongs to the α -group. Prostaglandin and MECA receptor clusters are also classified under this group. MECA stands for Melanocortin, Endothelial differentiation, Cannabinoid and Adenosine binding receptors; SOG for Somatostatin, Opioid and Galanin receptors; MCH for Melanin Concentration Hormone; fMLP for n-formyl-L-Methionyl-L-Leucyl-Phenylalanine. MAS is an oncogene that encodes MAS1 oncogene receptor.

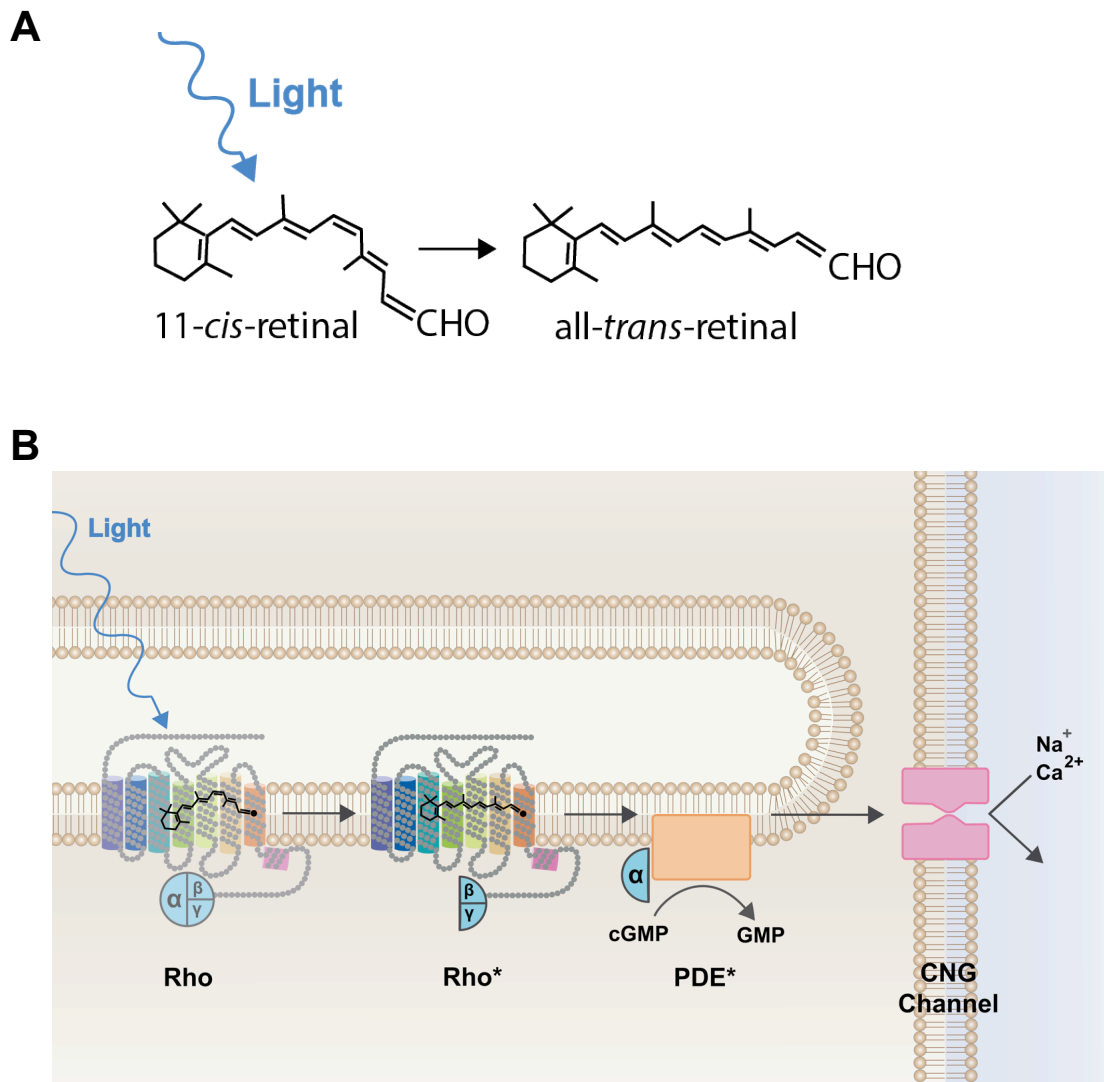
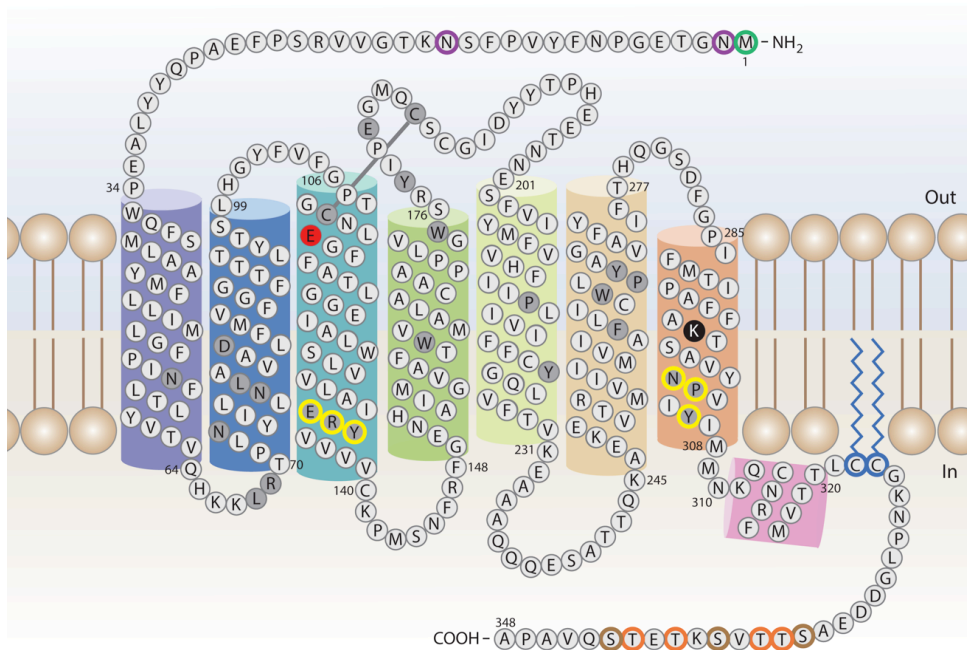


Figure 1.3. Retinal isomerization and phototransduction pathway of bovine rhodopsin. (A) Light stimulated conformational change from 11-*cis*-retinal to all-*trans*-retinal. (B) In rod photoreceptive cells of the retina, light activates G-protein transducin (Gt) coupled bovine rhodopsin through conformational change of 11-*cis*-retinal. The Gt α subunit activates cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE) to decrease intracellular cGMP concentrations. This causes the closure of cGMP-gated cation (CNG) channels and hyperpolarization of rod cells in the retina. Asterisks are used to indicate activated proteins.

A



B

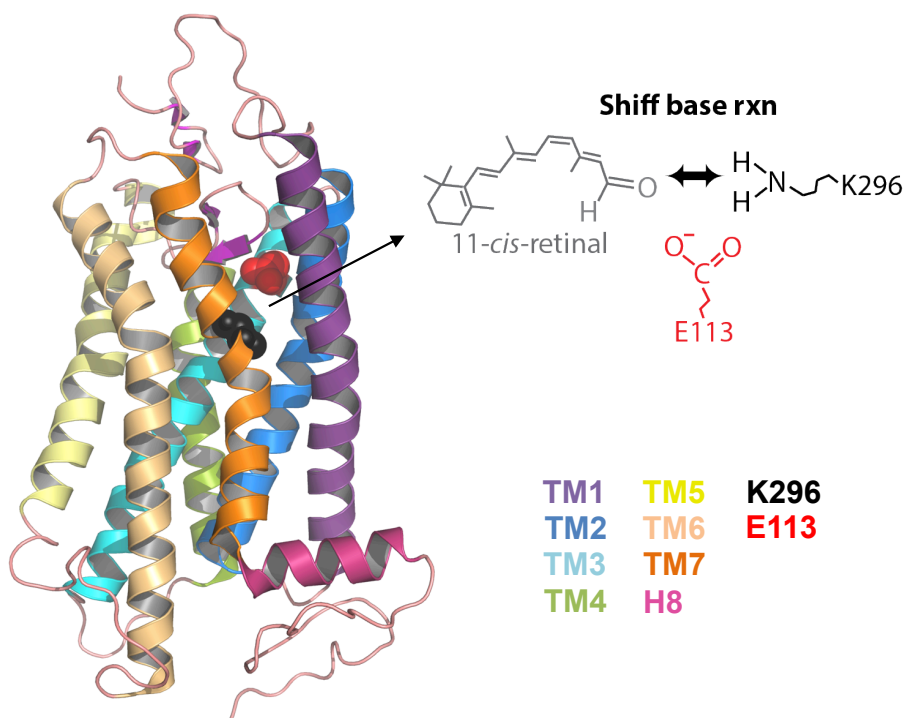


Figure 1.4. Structures of bovine rhodopsin. (A) A two-dimensional model of bovine rhodopsin. Highly conserved residues within the opsin family are shown with a gray infill. Important motifs of bovine rhodopsin are as follows; retinal-binding lysine residue (K296) marked with black infill, the counterion glutamate residue (E113) with red infill, n-terminal acetylation site with green circles, glycosylation sites with purple circles, c-terminal palmitoylation sites with blue circles, c-terminal serine sites with brown circles and c-terminal threonine sites with orange circles. Disulfide bond forming C110 and C187 were connected with a gray line. (B) A three-dimensional model of bovine rhodopsin based on the crystal structure data (Protein DataBank ID: 1U19 [PDB: 1U19]). The Schiff base reaction between 11-*cis*-retinal and K296 is stabilized by E113. TM and H indicates *transmembrane* and *helix* domain.

G Protein Families					
Family	G α_s	G α_i	G $\alpha_q/11$	G $\alpha_{12/13}$	G $\beta\gamma$
Members	G α_s	G $\alpha_{i1/i2/i3}$	G α_q , G α_{11}	G α_{12}	G β
	G α_{sXL}	G α_o , G α_t	G α_{14} , G α_{15}	G α_{13}	G γ
	G α_{olf}	G α_z , G α_{gust}			
Effectors	AC	AC	PLC	Rho GEF	PLC
	$\uparrow$ cAMP	$\downarrow$ cAMP	$\uparrow$ IP $_3$, DAG	Rho	AC
	PKA	PDE	Ca $^{2+}$		Ion Channels
		Ion Channels	PKC		PI3K

Table 1.2. G proteins families and their downstream effectors. Each G protein family plays a different role in the cell. In inactive forms, G proteins are composed of G α , G β and G γ hetero-trimers. When activated by GPCRs, G α detaches from G $\beta\gamma$ complex and transmits the signal to downstream effectors. G α_s and G α_i evoke increases and decreases of cAMP levels, respectively, via adenylate cyclase (AC). cAMP stimulates the activation of protein kinase A (PKA). Furthermore, G α_i can stimulate phosphodiesterase (PDE) activity and subsequently, nucleotide-gated ion channel closure. G α_q activates phospholipase C (PLC), which leads to processing of PIP $_2$ (phosphatidylinositol 4,5-bisphosphate) to IP $_3$ (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). IP $_3$ induces Ca $^{2+}$ release from endoplasmic reticulum (ER). DAG and Ca $^{2+}$ together activate protein kinase C (PKC). G $\alpha_{12/13}$ stimulates GTPase Rho and guanine-nucleotide exchange factors for the GTPase Rho A enzymes. Finally, studies on the G $\beta\gamma$ complex showed that it can modulate activity of PLC, AC, voltage-gated calcium channels, cardiac potassium channels and phosphoinositide 3-kinase (PI3K) [91].



Figure 1.5. Phylogenetic tree of opsins. The opsins are classified into eight subfamilies, which are given on the right hand side of the phylogenetic tree. This image is taken from referred paper [34].

Opsin Subfamilies			
Subfamily	Members	Expression	Absorption (λ)
Gat coupled	OPN1 LW	Vertebrate	Red (~557 nm)
	OPN1 MW	Vertebrate	Green (~527 nm)
	OPN1 SW1	Vertebrate	UV/Violet (~355-445 nm)
	OPN1 SW2	Vertebrate	Blue (~400-470 nm)
	OPN2	Vertebrate	Green (~505 nm)
	Pinopsins	Vertebrate	-
	Parapinopsins	Vertebrate	-
	VA-opsins	Vertebrate	-
	Parietopsins (Gao)	Vertebrate	Green (~522 nm)
Encephalopsin /TMT-opsin	OPN3	Invertebrate & Vertebrate	Blue (~465 nm)
	TMT	Teleost fish	Blue (~460 nm)
Gas-coupled	Box jellyfish cnidops	Box jellyfish	Green (~500 nm)
Gaq-coupled	Gaq visual pigment	Invertebrate	-
	OPN4	Vertebrate & Invertebrate	Blue (~480 nm)
Gao-coupled	Gao visual pigment	Amphioxus & Invertebrate	Blue (~490 nm)
Neuropsin	OPN5	Vertebrate	UV (~380 nm, 420 nm)
Peropsin	Peropsin	Vertebrate & Invertebrate	-
Photoisomerase	Retinochrome	Invertebrate	-
	RGR	Vertebrate	-

Table 1.3. Opsin family members and their expression and absorption wavelength (λ) profile through the animal kingdom. Opsins are classified into eight families, which are given on the left column of the table. Approximate absorption wavelength values given in this table could be variable depending on the specific tissue or specie. OPN stands for opsin; TMT, teleost multiple tissue opsin; LW, long-wavelength; MW, middle-wavelength; SW, short-wavelength.

A

Light Absorption and Expression Profile of Vertebrate OPN3

							
	Human	Mouse	Chicken	Anole	Pufferfish	Zebrafish	Medaka
Stimulus (nm)	-	-	~470	-	~470	465	497
Expression	Brain Eye Skin Kidney Liver	Brain Eye Liver Placenta Testis			Brain Liver Eye	Brain Kidney Heart Eye	

B

Light Absorption, G protein coupling and Expression Profile of Invertebrate OPN3 and TMT-Opsin

	Invertebrate OPN3	TMT-Opsin	
			
	Mosquito	Pufferfish	Medaka
Stimulus (nm)	~500	~460	~450
G protein	Gai/o	Gai/o	Gai/o
Expression		Brain Eye	Brain Eye

Table 1.4. Summary of published results on the light absorption, G protein coupling, and expression of OPN3 and TMT-Opsin. (A) Light absorption and tissue expression of vertebrate OPN3 were studied in human, mouse, chicken, anole (arboreal lizard) and three teleost fish (pufferfish, zebrafish and medaka). G protein coupling of vertebrate OPN3 remains unknown. Recent attempts of light absorption studies on human, mouse and anole OPN3 failed because of low expression of these proteins. (B) Light absorption, G protein coupling and tissue expression of invertebrate OPN3 was studied in only mosquito. Similar studies were conducted on TMT-Opsin by using two teleost fish, pufferfish and medaka.

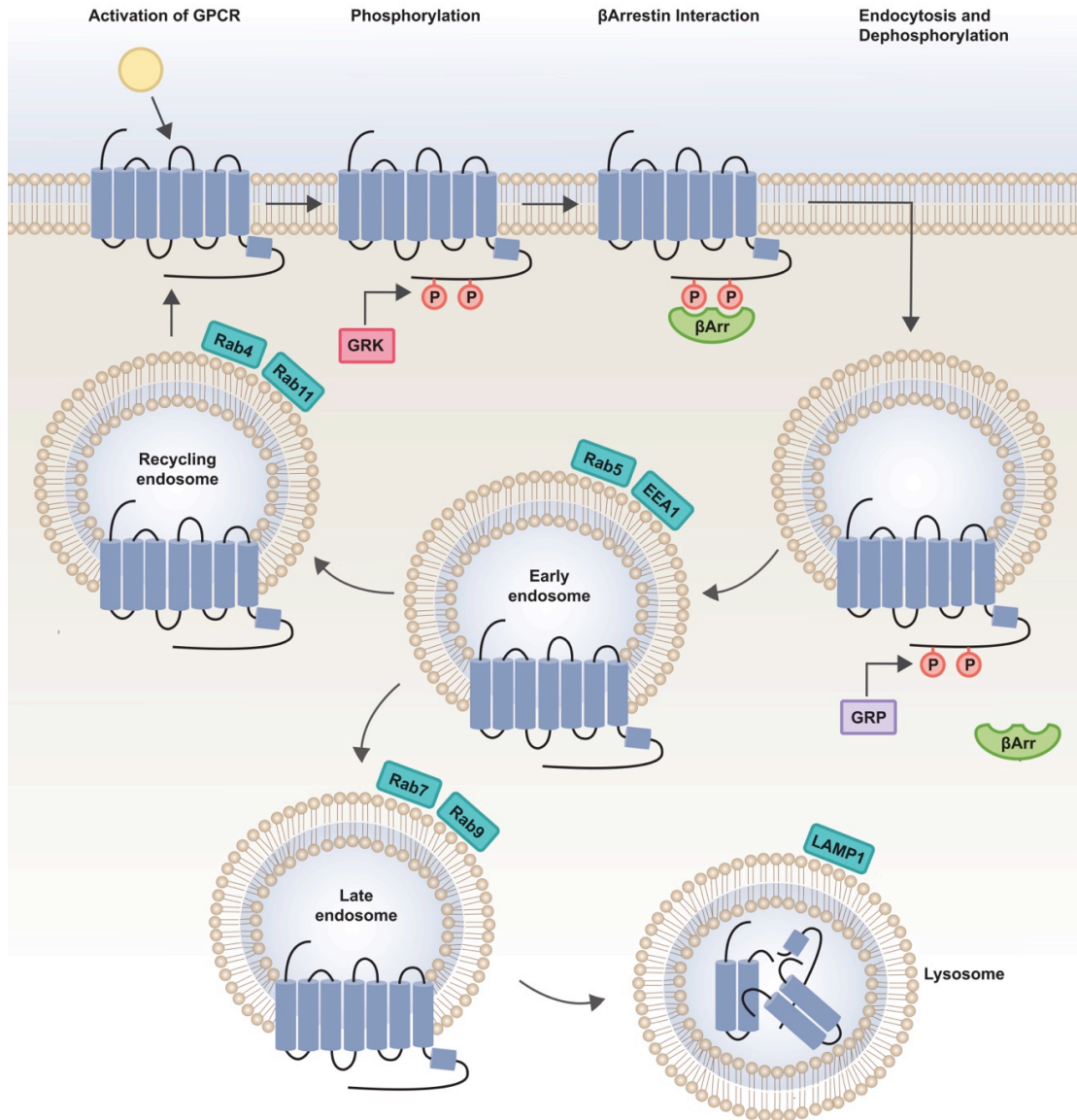


Figure 1.6. Internalization and recycling of G-protein coupled receptors (GPCRs). Activation of a GPCR leads to the c-terminal phosphorylation by GPCR kinase (GRK), which stimulates internalization of the GPCR by binding with β -arrestin (β Arr). GPCR phosphatase (GRP) dephosphorylates the internalized GPCR and it either returns back to membrane through recycling endosomes or is shuttled to the lysosome for degradation through late endosomes. Rab5 and effector early endosome antigen 1 (EEA1) localized at early endosomes, Rab4 and Rab11 at recycling endosomes, Rab7 and Rab9 at late endosomes and lysosome-associated membrane glycoprotein 1 (LAMP-1) at lysosomes [84, 92].

Heterodimer	GPCR Family	Functional Affect	Refs
GABAR _{B1} - GABAR _{B2}	Glutamate	•Transition of localization from ER to plasma membrane	[93-95]
T1R1 - T1R3	Frizzled/Taste2	•Umami recognition	[96-98]
T1R2 - T1R3	Frizzled/Taste2	•Sweet recognition	[96-98]
AT1 – B2	Rhodopsin; γ -Group; chemokine cluster	•Angiotensin II or bradykinin induced IP3 increase	[99, 100]
MOR - DOR	Rhodopsin; γ -Group; SOG cluster	•Met-enkephalin binding affinity reduction •Endomorphin-1 and Leu-enkephalin binding affinity increase •Transition from Gz to Gi coupling	[101-103]
KOR - DOR	Rhodopsin; γ -Group; SOG cluster	•Enhancement of synthetic KOR induced signaling	[104]
OR1 – CB1	Rhodopsin; α -Group; MECA cluster	•CB1 antagonist suppress OR triggered ERK phosphorylation •OR1 antagonist suppress CB1 triggered ERK phosphorylation	[105]
MC3R* – GHSR**	*Rhodopsin; γ -Group; MECA cluster **Rhodopsin; β -Group	•Melanocortin induced cAMP production increase •Ghrelin induced signaling decrease	[106, 107]
D1 - D2	Rhodopsin; γ -Group; amine cluster	•Transition of coupling from Gs to Gq/11	[108, 109]
MT1 - GPR50	Rhodopsin; γ -Group; melatonin cluster	•Melatonin binding decrease •Coupling to Gi, signaling and internalization	[87, 110]
NK1* - MOR**	*Rhodopsin; β -Group **Rhodopsin; γ -Group; SOG cluster	•Internalization profile change	[111]
R1-R4	Rhodopsin; β -Group	•Ca ²⁺ -dependent PKC α and Ca ²⁺ -independent PKC ζ activated ERK phosphorylation increase	[112]
R2 – R4	Rhodopsin; β -Group	•cAMP production decrease via transition from Gs to Gi coupling	[113]
EP1* – β 2AR**	*Rhodopsin; α -Group; prostaglandin cluster **Rhodopsin; α -Group; amine cluster	•EP1 antagonist induced Gs dissociation from bA2R	[114]

Table 1.5. Functional effects of G-protein coupled receptor (GPCR) heterodimerization. The family of the heterodimerizing receptors and the functional effects of this heterodimerization are summarized in this table. GABAR: GABA receptor; T1R: taste receptor; AT1: angiotensin receptor 1; B2: bradykinin receptor 2; MOR: μ -opioid receptor; DOR: δ -opioid receptor; KOR: κ -opioid receptor; OR1: orexin receptor 1; CB1: cannabinoid receptor 1; MC3R: melanocortin receptor 3; GHSR: ghrelin receptor; D1: dopamine receptor 1; MT1: melatonin receptor 1, NK1: tachykinin receptor 1; R1: gonadotropin-releasing hormones receptor 1; EP1: prostaglandin E2 receptor 1; β 2AR: β 2-adrenergic receptor. The table is adapted from referred paper [86].

CHAPTER 2. Opsin Expression in Human Epidermal Skin

Reproduced in part with permission from: Haltaufderhyde, K., Ozdeslik, R.N., Wicks, N.L., Najera N.J. and Oancea, E. (2015) Photochemistry and Photobiology 91: 117–123 [22].

I performed all experiments described in this chapter, with the exception of the following: Figure 2.1, 2.2, 2.5 (Haltaufderhyde, K., Wicks, N.L., Najera N.J.).

Abstract

Human skin is constantly exposed to solar light containing visible and ultraviolet radiation (UVR), a powerful skin carcinogen. UVR elicits cellular responses in epidermal cells via several mechanisms: direct absorption of short-wavelength UVR photons by DNA, oxidative damage caused by long-wavelength UVR, and, as we recently demonstrated, via a retinal-dependent G protein-coupled signaling pathway. Because the human epidermis is exposed to a wide range of light wavelengths, we investigated whether opsins, light-activated receptors that mediate photoreception in the eye, are expressed in epidermal skin to potentially serve as photosensors. Here we show that four opsins—OPN1-SW, OPN2, OPN3 and OPN5—are expressed in the two major human epidermal cell types, melanocytes and keratinocytes, and the mRNA expression profile of these opsins does not change in response to physiological UVR doses. We detected two OPN3 splice variants present in similar amounts in both cell types and three OPN5 splice isoforms, two of which encode truncated proteins. Notably, OPN2 and OPN3 mRNA were significantly more abundant than other opsins and encoded full-length proteins. Our results demonstrate that opsins are expressed in epidermal skin cells and suggest that they might initiate light-induced signaling pathways, possibly contributing to UVR phototransduction.

Results and Discussion

Identification of mRNA corresponding to the retinal-binding site of human opsins in HEMs and KERs

To investigate the ability of human skin to act as a sensory system for ultraviolet and visible radiation, we examined the expression pattern of opsin receptors in human epidermal melanocytes (HEMs) and keratinocytes (KERs) at the mRNA level. We used reverse transcription polymerase chain reaction (RT-PCR) to identify opsin mRNA transcripts expressed in HEMs and KERs, followed by quantitative PCR (qPCR) to determine their relative expression levels.

Functional opsins require a chromophore attached to a lysine residue homologous to K296 in bovine rhodopsin. Hence, we first investigated whether mRNA fragments encompassing the equivalent lysine residue of human OPN1-SW (NM_001708.2), OPN1-M/LW (NM_000513/020061), OPN2 (NM_000539), OPN3 (NM_014322), OPN4 (NM_033282) and OPN5 (NM_181744) are present in HEMs and KERs. Using cDNA obtained through reverse transcription (RT) of mRNA extracted from cultured HEMs or KERs, we PCR-amplified bands of the expected size using primers for OPN1-SW, OPN2, OPN3 and OPN5 from both cell types (Figure 2.1).

Primers specific for OPN1-M/LW did not amplify any transcripts from either HEMs or KERs, but produced a band of the expected size (~300 bp) using cDNA obtained from retinal cells (RET). Primers specific to OPN4 amplified a cDNA fragment of the expected size (~400 bp) from retina (RET), a smaller cDNA fragment from HEMs (~250 bp) and no fragment from KERs (Figure 2.1). Sequencing of the two fragments from RET and HEMs indicated that the amplified retina cDNA corresponded to OPN4, whereas the fragment amplified from HEMs did not. Numerous attempts using different primers and amplification conditions for OPN4 did not yield a fragment corresponding to OPN4 in either HEMs or KERs. On the basis of these results, we

next attempted to amplify full-length cDNAs for OPN1-SW, OPN2, OPN3 and OPN5 from HEMs and KERs.

OPN1-SW (short-wavelength-sensitive cone opsin)

OPN1-SW (short-wavelength-sensitive cone opsin) is one of the three types of cone opsins responsible for normal color vision, having a maximal absorption in the blue-violet range. OPN1-SW cDNA consists of five exons (e1–e5), encoding a 348 amino acid protein (Figure 2.2B). Primers based on the coding sequence of OPN1-SW amplified DNA fragments of the expected size (~1 kb) from both HEM and KER cDNA (Figure 2.2A); these were sequenced and found to correspond to the full-length open reading frame of OPN1-SW.

OPN2 (rhodopsin)

OPN2 (rhodopsin) functions in dim-light photoreception and is highly expressed in retinal rod cells. We have previously shown that OPN2 mRNA and protein are expressed in HEMs [17] and Tsutsumi and colleagues detected rhodopsin immunoreactivity in the upper layer of human epidermis, which consists primarily of keratinocytes [18]. To determine whether full-length OPN2 mRNA is expressed in KERs, we performed PCR using primers based on the coding sequence of OPN2, between its 5' start (ATG) and 3' stop codons, and amplified a transcript of the expected size (1047 bp) from both HEMs and KERs (Figure 2.2C).

The sequenced cDNA fragment corresponded to the five exons of OPN2 (Figure 2.2D) and encodes a full-length protein (red bar). We thus conclude that both HEMs and KERs express full-length OPN2 mRNA. As we previously showed that HEMs

express full-length OPN2 protein [17], we tested if KERs also contain full-length OPN2. Western blot analysis using an anti-OPN2 antibody revealed a protein band of the expected size (~36 kDa) in KERs, similar in size to the band recognized by an anti-HA antibody in HEK293 cells expressing HA-OPN2, and which was absent in untransfected HEKs (Figure 2.2E).

OPN3 (encephalopsin, panopsin)

OPN3 (encephalopsin, panopsin) is a nonvisual opsin of unknown function that is highly expressed in brain, testes and retina [45, 49]. Expression analysis of human OPN3 has shown that it is an ancestral-type opsin, highly conserved across vertebrates [49]. Recently, the pufferfish teleost multiple tissue opsin (TMT opsin) and mosquito OPN3 were shown to have the ability to form a green–light activated photopigment [50]. OPN3 expression or function in skin has not been previously detected.

On the basis of our isolation of an OPN3 fragment containing K299 (equivalent to bovine rhodopsin K296), we sought to amplify larger fragments of OPN3 containing all four coding exons. RT-PCR using a forward primer based on exon 1 and a reverse primer based on exon 4 resulted in two bands from both HEMs and KERs: a longer transcript of ~900 bp and a smaller transcript ≥500 bp (Figure 2.3A). Sequencing identified the longer transcript as a 878 bp sequence encompassing exons 1–4 (Figure 2.3B), and the shorter transcript as a 558 bp sequence containing exons 1, 3 and 4 but lacking exon 2 (320 bp) (Figure 2.3C). This novel splice variant encodes a protein containing a frame-shift that causes truncation after exon 1, and resulting in a 143 amino acid peptide (Figure 2.4) that is presumably nonfunctional.

OPN5 (neuropsin)

OPN5 (neuropsin) is expressed in human brain, testes and retina [68]. Recent studies suggest that OPN5 is expressed in mammalian skin [16, 115], where it functions as a G protein-coupled UVR-sensitive receptor in humans [16] and birds [69, 71]. Together, these findings make OPN5 an attractive candidate for an epidermal UVR-sensitive photoreceptor.

We sought to amplify the full coding sequence of OPN5 (containing seven exons) from HEMs and KERs (Figure 2.5A,B). Repeated attempts to amplify OPN5 with different primer pairs based on exon 1 and exon 7 did not detectably amplify transcripts from either HEM or KER cDNA, but did amplify bands of expected size using brain cDNA (data not shown). Using a forward primer based on the 5' untranslated region (5'-UTR) and a reverse primer based on exon 3, we amplified two transcripts (319 and 408 bp) from both HEMs and KERs (Figure 2.5C). Sequencing identified the shorter of these as the expected 319 bp transcript encompassing exons 1 through 3. The longer transcript corresponded to a sequence containing a novel 89 bp exon between exon 1 and 2 (exon 1a), resulting in a 408 bp splice variant (Figure 2.5D), which truncates in exon 2 due to a frameshift introduced by exon 1a. Interestingly, the relative abundance of the two transcripts varies in HEMs vs KERs (Figure 2.5A), suggesting that this short, and presumably nonfunctional, variant might function to regulate OPN5 expression levels.

Subsequent PCR reactions using primers designed to amplify OPN5 exons 3 through 6 produced a ~750 bp band using KER cDNA (Figure 2.5A, right). Sequencing analysis identified two distinct transcripts: the first corresponded to the expected 612 bp sequence encompassing exons 3–6, whereas the second corresponded to a 681 bp transcript containing a novel 69 bp exon between exon 3 and 4 (exon 3a), resulting in a variant truncated due to a frameshift introduced by exon 3a (Figure

2.5E).

We next assessed the relative expression levels of OPN1-SW, OPN2, OPN3 and OPN5 mRNA in HEMs and KERs using qPCR. We used primer pairs designed to assess total mRNA levels, without discriminating between splice variants. When comparing the levels of OPN1-SW, OPN2, OPN3 and OPN5 to actin levels in HEMs and KERs, we found that OPN2 is expressed at higher levels in HEMs, whereas OPN5 is expressed at higher levels in KERs (Figure 2.6). We measured opsin levels in at least three biological replicates. We observed significant variability (10–100-fold) between HEM and KER lines from different individuals, but did not detect any correlation between the expression levels of different opsins and the pigmentation phenotype of the donor. Surprisingly, we found that OPN3 is expressed in both HEMs and KERs at significantly higher levels than OPN1-SW or OPN5 (Figure 2.6), suggesting that OPN3 might have an important functional role in human skin. We also found that OPN2, which we previously found to be involved in UVR phototransduction in melanocytes [17], is expressed in HEMs at levels comparable to OPN3 and significantly higher than OPN1-SW or OPN5 (Figure 2.6).

Because human epidermal skin is physiologically exposed to solar UVR, we tested if UVR stimulation leads to changes in the opsin expression levels in these cells. We exposed melanocytes and keratinocytes to 500 mJ cm² UVR (equivalent to ~10 min of bright solar UVR) to minimize cell death and oxidative damage, and collected RNA at 0, 4, 16 and 24 h after the treatment. The cDNA obtained by RT was used to quantify the levels of each opsin by qPCR. We found that none of the opsins expressed changed their expression levels at any of the time points measured, in neither HEMs nor KERs. In addition, we could not detect OPN4 at later time points after exposure (data not shown), suggesting that its expression is not UVR dependent. These results suggest that opsins are not transcriptionally regulated by UVR exposure, but might contribute to UVR signaling pathways in skin. Alternatively,

their activation mechanism and function in skin could be light-independent.

As the levels of OPN3 mRNA measured in Figure 2.7 consisted of both full-length coding sequence as well as a truncated isoform, we deemed it important to determine their relative expression levels. We found that the ratio of full length to truncated OPN3 mRNA was approximately 1:1 both in HEMs and KERs (Figure 2.6A), suggesting that at least one-half of the OPN3 mRNA detected in these cells (Figure 2.5) encodes the full-length protein (Figure 2.4).

To test whether OPN3 mRNA is translated into a protein of the predicted size in HEMs and KERs, we performed western blot analysis using an anti-OPN3 antibody that recognizes the region between amino acids 181 and 210 of OPN3, present only in the full-length isoform. Anti-OPN3 detected a band of ~45 kDa both in HEMs and KERs, but not in HEK293 cells (Figure 2.7), suggesting that OPN3 protein is expressed in epidermal skin cells.

Intrigued by this finding, we tested by qPCR the levels of a representative opsin, OPN3, in cell lines and primary cells derived from diverse tissues: human lung fibroblasts, primary human neutrophils, buffy coat cells, the leukemia cell line HL60 and human umbilical vein endothelial cells (HUVEC). Compared to HEMs and KERs, OPN3 mRNA was expressed at levels 2- to 40-fold lower in other tested cells (Figure 2.8). Interestingly, western blot with anti-OPN3 antibodies detected a band in HEMs and KERs (as also shown in Figure 2.7B), in fibroblasts, and in buffy coat cells, a weak band in HUVEC and no band in neutrophils or HL60 cells (Figure 2.8). The discrepancy between mRNA and protein expression could be due to specific protein degradation pathways used by different cells. Nevertheless, in addition to expression in cells physiologically exposed to light (HEM and KER), the presence of opsins in primary cells that are not exposed to light raises the question of their activation mechanism and functionality in these cells.

Conclusion

In conclusion, we find that multiple opsin receptors are expressed in human epidermal cells. We demonstrate that OPN1-SW, a cone opsin activated by blue-violet light, is expressed in epidermal skin. In addition, we confirm our previous observation that OPN2 is present in HEMs [17] and show that it is also expressed in KERs. Our findings also agree with previous reports suggesting that OPN5 is expressed in human epidermis [16] and, in addition, identify two novel OPN5 splice variants. The truncated proteins produced by alternative splicing of OPN3 and OPN5 may serve to regulate the expression levels and function of these opsins in skin. We found that OPN3 mRNA and protein is expressed at higher levels than the other opsins in the two major human epidermal cell types and identified a novel splice variant. The expression of opsins in skin suggests that they may function as epidermal photoreceptors, prompting further studies that might uncover their physiological roles. The expression of opsins in other cell types suggests that in the absence of light they could be activated by alternative mechanisms and serve other functions than phototransduction.

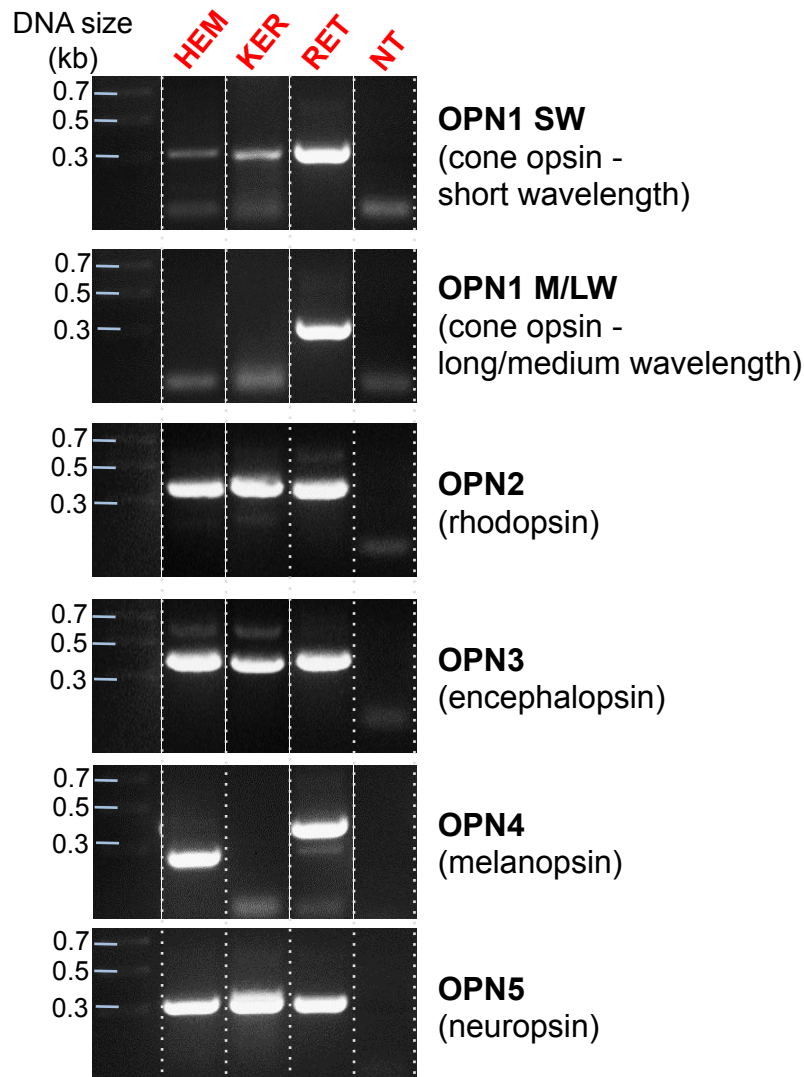
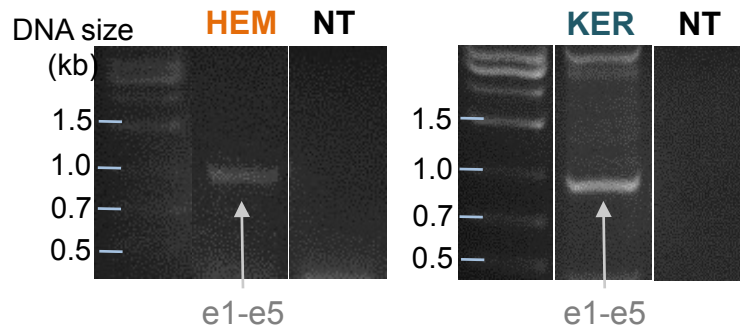


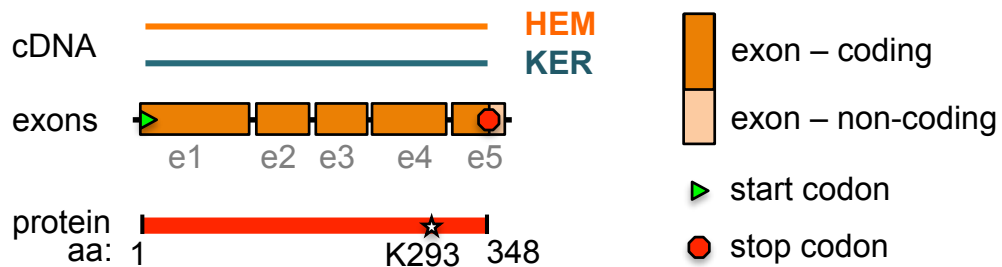
Figure 2.1. Identification of mRNA corresponding to the retinal-binding site of human opsins in HEMs and KERs. PCR screen of HEM, KER, retina (RET) and brain (BR) cDNA with primers encompassing the conserved lysine residue of cone opsin, short wavelength (OPN1-SW), medium and long wavelength (OPN1-M/ LW), rhodopsin (OPN2), encephalopsin (OPN3), melanopsin (OPN4) and neuropsin (OPN5). DNA bands corresponding to OPN1-SW, OPN2, OPN3 and OPN5, but not OPN1-M/LW and OPN4, were amplified from HEMs and KERs. Retina (RET) cDNA was used as a positive control and no amplification occurred in the absence of HEM, KER or retina cDNA (NT, no template). Dotted lines separate the gel lanes continuous and white lines denote different gel lanes joined together.

OPN1 SW

A

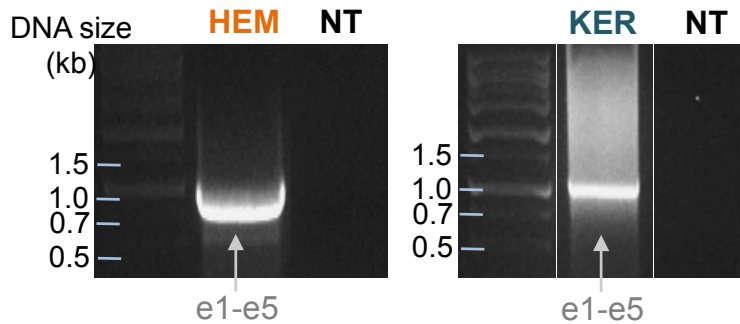


B

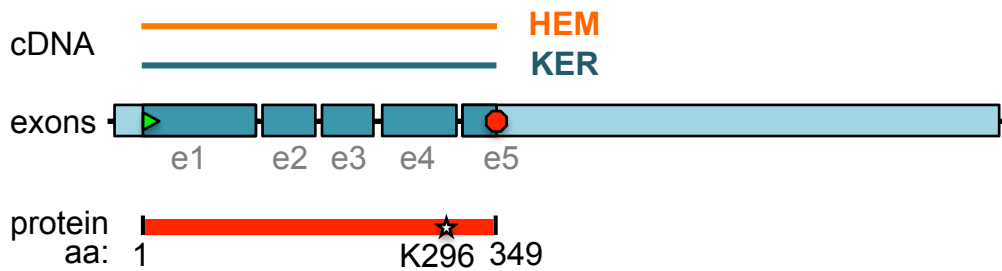


OPN2

C



D



E

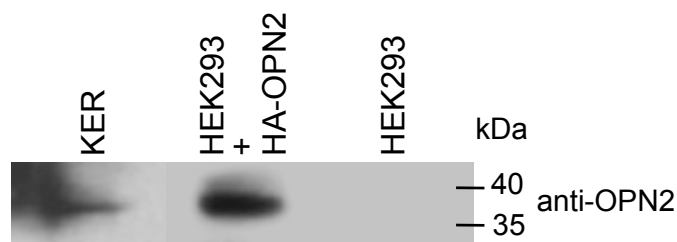
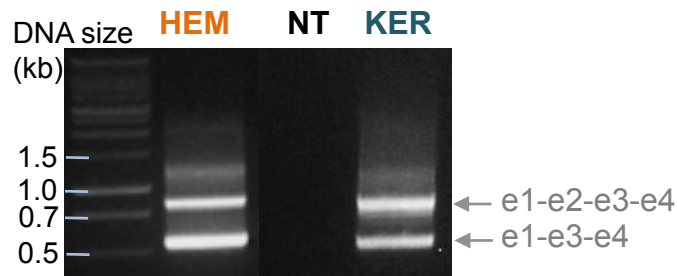


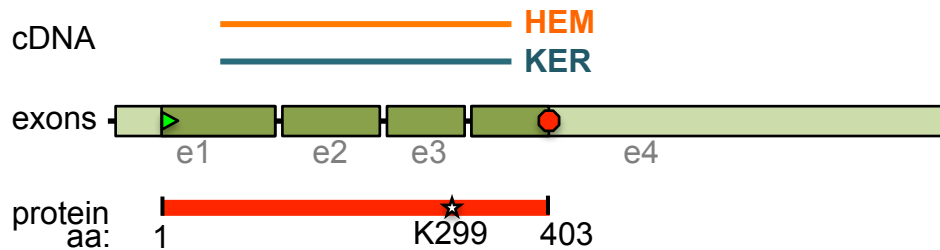
Figure 2.2. Identification of full-length OPN1-SW and OPN2 mRNA in HEMs and KERs. (A) RT-PCR using HEM or KER RNA and primers based on the first and last exon of OPN1-SW amplified a cDNA fragment (e1–e5) corresponding to the full-length OPN1-SW sequence. No amplification occurred in the absence of HEM or KER cDNA (NT, no template). (B) The cDNA amplified from HEM or KER in (A) corresponds to a fragment of the coding sequence of OPN1-SW that encompasses all five exons and results in a 348 amino acids peptide containing the retinal binding residue K293. (C) RT-PCR using HEM or KER RNA and primers based on the first and last exon of OPN2 amplified a cDNA fragment (e1–e5) corresponding to the full-length coding sequence of OPN2. No amplification occurred in the absence of HEM or KER cDNA (NT, no template). (D) The cDNA amplified from HEM or KER in (C) corresponds to the coding sequence of OPN2 that encompasses all five exons and results in a 349 amino acids peptide containing the retinal binding residue K296. (E) Western blot analysis of HEM and HEK293 cell extracts probed with anti-OPN2 antibody shows a ~36 kDa band in HEM and HEK293 cells expressing hemagglutinin (HA)-tagged OPN2, but not in untransfected HEK293 cells. Representative of three independent experiments.

OPN3

A



B



C

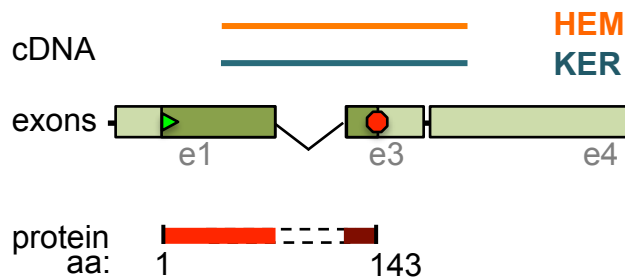


Figure 2.3 Identification of full length and truncated splice variant of OPN3 in HEMs and KERs. (A) RT-PCR using HEM or KER RNA and primers based on exon 1 and exon 4 of OPN3 amplified two bands. (B) The cDNA amplified in the upper band in (A) corresponds to a fragment of the coding sequence of OPN3 that encompasses all four exons and results in a 403 amino acids peptide containing the retinal binding residue K299. (C) The cDNA amplified in the lower band in (A) corresponds to exons 1, 3 and 4 of OPN3, coding for a 143 amino acid peptide that does not contain the K299 residue.



Figure 2.4. Multiple sequence alignment and predicted protein structures of full length and truncated splice variant OPN3 isoforms identified in HEMs and KERs. (A) Multiple sequence alignment was performed by using DNASTAR MegAlign Program. Truncated splice variant shows unique 18 aa sequence (labeled red) after translation of exon1.

OPN5

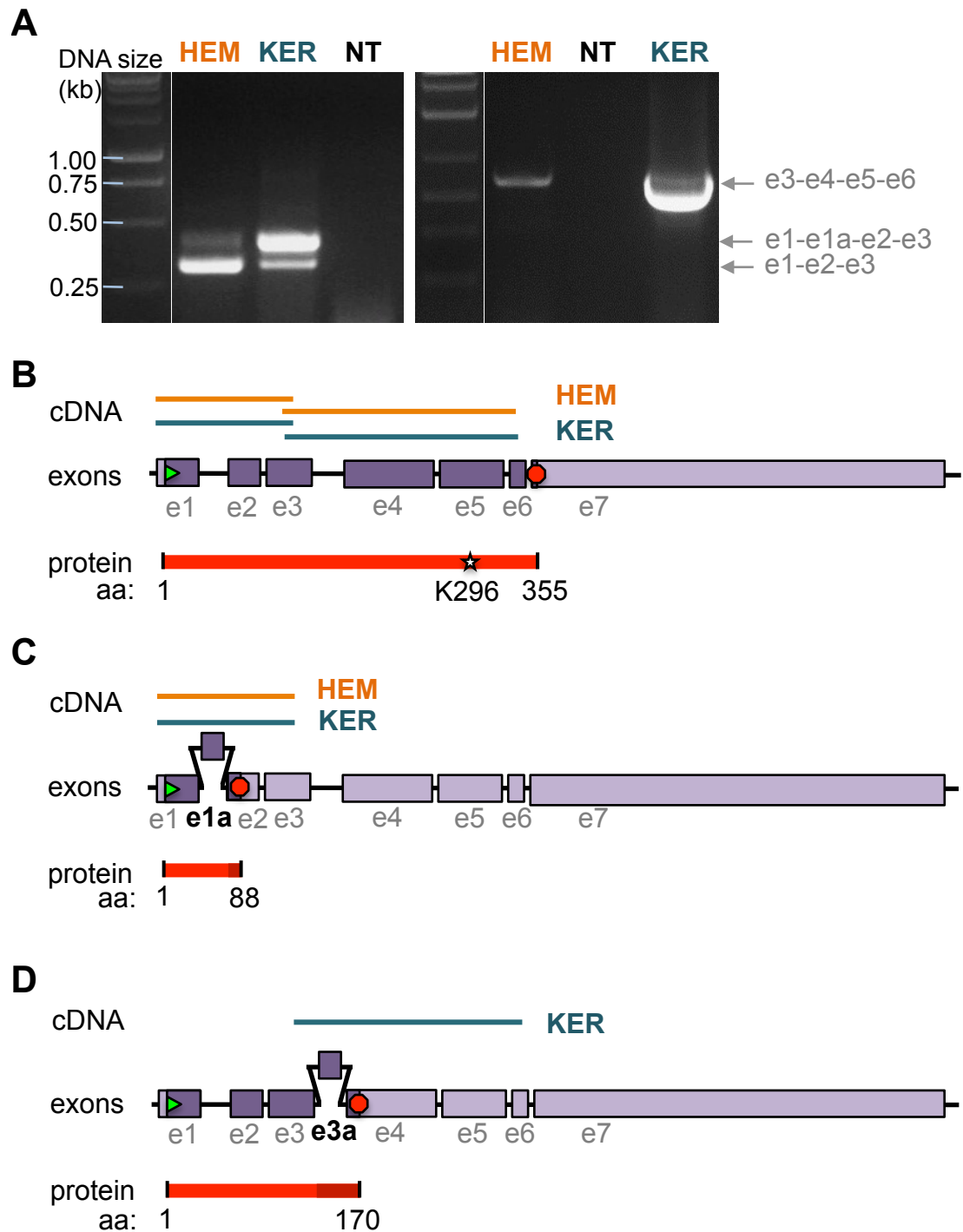


Figure 2.5. Identification of full length and two truncated splice variants of OPN5 in HEMs and KERs. (A) RT-PCR using HEM and KER RNA and primers based on exon 1 and 3 of OPN5 amplified two bands. (B) RT-PCR using HEM or KER RNA and primers based on exon 3 and 6 of OPN5 amplified a band that corresponded to exons 3–6. (C) The lower cDNA bands amplified in (A) and the bands amplified in (B) correspond to exons 1–6 of OPN5, coding for a 355 amino acid peptide containing the retinal-binding site K296. (D) The upper cDNA band amplified in (A) contains an additional 89 bp exon (e3a) between exons 3 and 4.

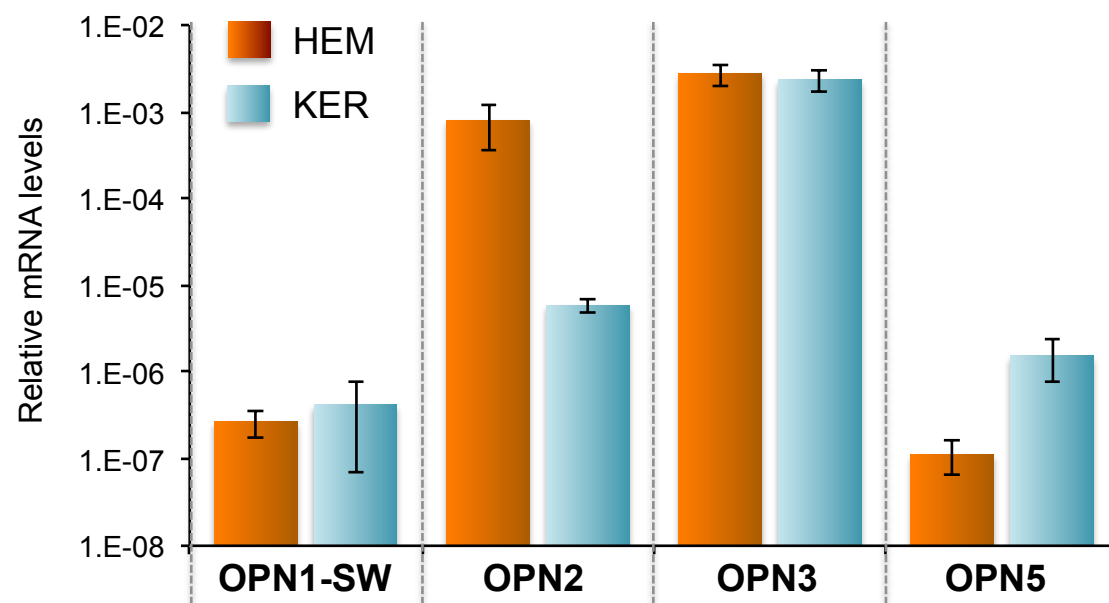


Figure 2.6. Relative expression levels of opsins mRNA in HEMs and KERs. qPCR analysis of the mRNA expression level of OPN1-SW, OPN2, OPN3 and OPN5 in HEM and KER, relative to actin. Bars represent the average of ≥ 4 experiments $\pm$ SEM.

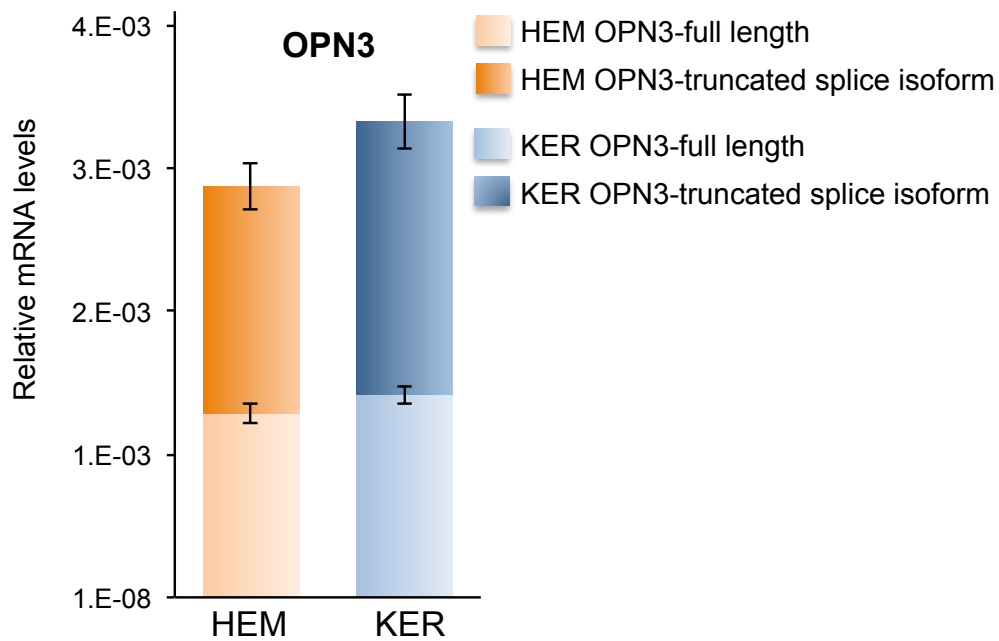
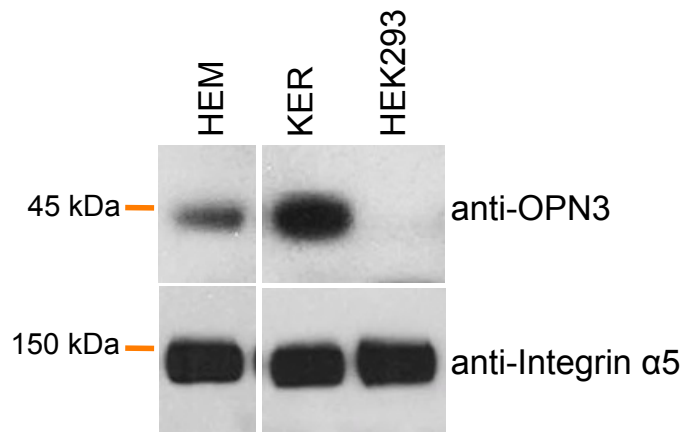
A**B**

Figure 2.7. Relative OPN3 expression in HEMs and KERs. (A) Quantitative PCR analysis of the mRNA expression of full length (light color) and truncated splice variant (dark color) of OPN3 in HEM and KER, using the primers shown in Figure 2.8 and calculated relative to actin levels. Bars represent the average of three experiments $\pm$ SEM. (B) Western Blot analysis of OPN3 protein expression in HEM, KER and HEK293. A 45 kDa band was detected by anti-OPN3 antibodies in HEM and KER, but not in HEK293 cells. Integrin α 5 was used as a positive control.

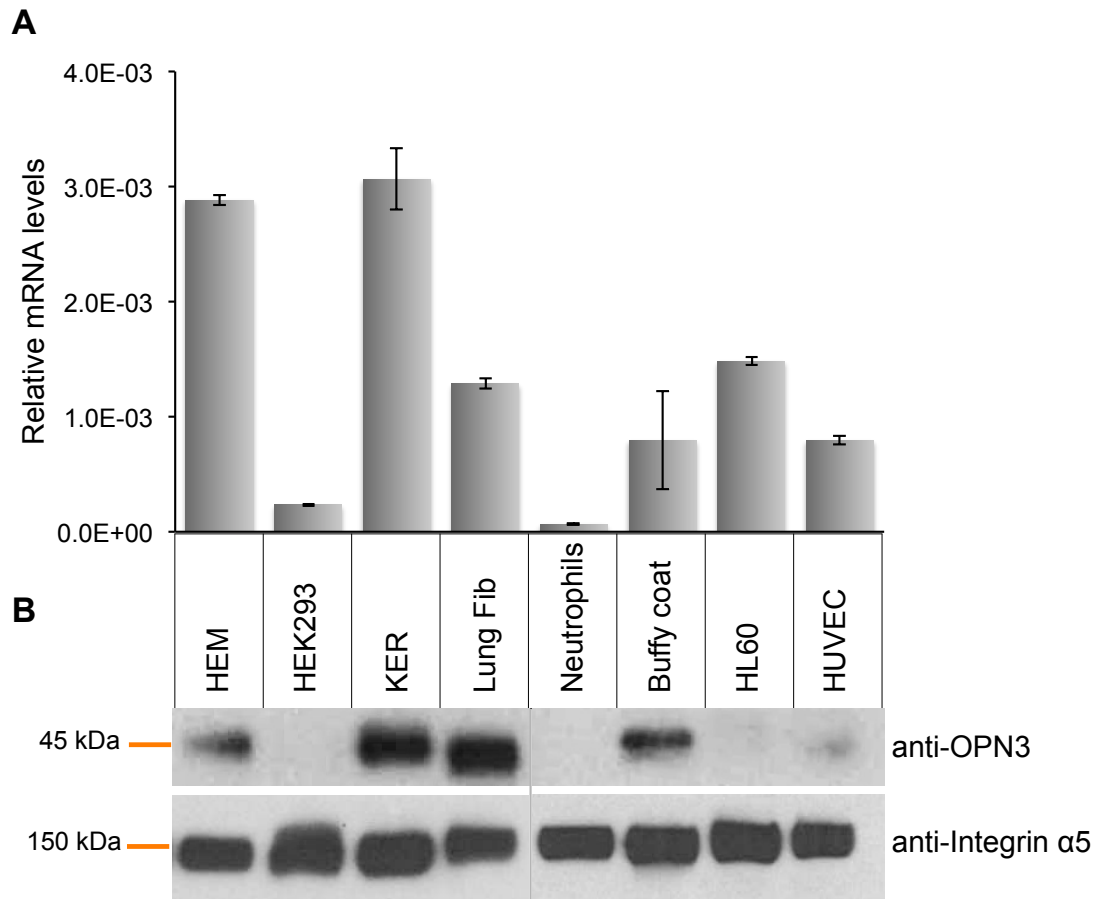


Figure 2.8. qPCR and western blot analysis of OPN3 expression in different human cells. (A) Quantitative PCR analysis of the mRNA expression of OPN3 in HEM, HEK 293, KER, lung fibroblast, neutrophils, buffy coat, HL60 and HUVEC cells using the primers detecting only full-length OPN3 and calculated relative to actin levels. Bars represent the average of three experiments $\pm$ SEM. (B) Western Blot analysis of OPN3 protein expression in different human cells mentioned in (A). A 45 kDa band was detected by anti-OPN3 antibodies in HEM, KER, lung fibroblast, buffy coat, but not in HEK293, neutrophil, HL60 and HUVEC cells. Integrin α 5 was used as a positive control.

CHAPTER 3. Structure and Localization of Human OPN3

Abstract

The localization and function of ocular opsins have been extensively characterized; however, several extra-ocular opsins are waiting to be studied further. In Chapter 2, we demonstrated that OPN3, an extra-ocular opsin, mRNA and protein is expressed at higher levels than the other opsins in human epidermal melanocytes and keratinocytes. To study the function of a protein, first, protein specific antibodies and epitope tagged fusion proteins need to be developed to modify and track the protein easily. However, no studies identifying the working antibodies or epitope tagged constructs of full-length human OPN3 have been published. Here, I characterized the effectiveness of a commercially available human OPN3 antibody and identified the location of an epitope insertion site which does not lead to endoplasmic reticulum retention. I further studied the cellular localization of human OPN3 in HeLa heterologous and HEM endogenous expression systems.

Results and Discussion

Analysis of the human OPN3 sequence

Human OPN3 protein sequence (NM_014322) fulfills the criteria expected of an opsin [44]. Human OPN3 possesses (Figure 3.1A, 3.2A,B):

- a lysine residue at position 299 (K299) in the 7th trans-membrane domain (TM7).
- a negatively charged aspartate residue at position 117 (D117) in the 3rd trans-membrane domain (TM3).

In addition, OPN3 displays other features shared among GPCRs. The human OPN3 sequence includes:

- c-terminal containing 10 serine and 3 threonine residues (S343, S353, S368, S369, S370, S377, S380, S382, S386, S392) and (T365, T376, T389). These residues likely play a role in c-terminal phosphorylation and desensitization of OPN3.
- n-terminal methionine at position 1 (M1). This residue is likely involved in n-linked acetylation of OPN3.
- n-terminal asparagine residues at position 5 (N5). This residue is likely involved in n-linked glycosylation.
- c-terminal cysteine residue at position 325 and 331 (C325 and C331). This residue could be playing role in c-terminal palmitoylation.
- D/ERY motif in the cytoplasmic surface of the 3rd trans-membrane (TM3) and NPxxY motifs in the 7th trans-membrane domain (TM7). The ERY motif of OPN3 is at position 138-140 and an NPxxY motif is at position 305-309.

The novel splice variant of OPN3 mentioned in Chapter 2, encodes a protein containing a frame-shift that causes truncation after exon 1, and results in a 143 amino acid peptide (Figure 2.3, 2.4). Although this protein has 3 transmembrane

regions and a counterion residue at position 117 (D117), it is presumably nonfunctional because of the absence of transmembranes 4-8 and the crucial lysine residue at position 299 (K299) in the 7th trans-membrane domain (TM7) (Figure 3.3A). The hydrophobic alanine (A), isoleucine (I), leucine (L) and valine (V) residues make up the majority of the amino acids in the TM domain accounting for approximately one third (34%) of all residues in α -helical proteins and 28% in β -sheet [116, 117]. The 18 new residues created after frameshift has 1 alanine (A125), 1 isoleucine (I143) and 1 leucine (L127) residue, which make up %16 of the sequence. Although, two-dimensional model, based on the prediction of PredictProtein program, has a complete TM3, this 18-residue sequence does not show any homology with other membrane bound proteins hence it is presumably unstructured as depicted in three-dimensional model prediction by PsiPred program (Figure 3.3B).

Human OPN3 has a unique c-terminal sequence

Comparison of the human OPN3 protein sequence with other light sensitive human opsins revealed that it is highly homologous showing 32-30% amino acid identity and 54-51% amino acid similarity over the full-length of the protein (Figure 3.1B). However, comparison of the human OPN3 protein sequence with that of other opsins and GPCRs also indicates that OPN3 has a unique c-terminal region (Figure 3.1A green outline, 3.2A,B green infill). This region is conserved between OPN3 proteins from different species; however, comparing OPN3 to other membrane-bound proteins outside of the OPN3 subgroup of opsins does not show any homology. The only information we know so far about light absorption and G protein coupling profiles of OPN3 comes from c-terminally truncated OPN3 proteins, which show blue light (λ max. 465 nm) absorption and G α_i coupling [50, 52], but the unique c-terminal sequence of OPN3 could be playing an important role in the function. Hence, studying full-length OPN3 is crucial to fully understand the role of OPN3 in the cells.

Epitope insertion site affects the localization of OPN3 in HeLa cells

First, I sought to determine the steady-state localization of full-length human OPN3 in HeLa cells. Localization of human OPN3 constructs with epitope tags inserted at either the n-terminal end, c-terminal end or in-frame within the intracellular cytosolic loop 1 (see Figure 5.1) were examined in HeLa cells after transient transfection. GPCRs, most commonly found on the cell membrane, must first attain their native conformation in order to exit from the endoplasmic reticulum (ER) where they are assembled and begin their translocation to the membrane [118, 119]. To determine if the OPN3 constructs properly folded, I tested the colocalization of tagged OPN3 protein with labeling for an ER marker, Calnexin. The ratio of overlap between OPN3 and Calnexin was quantified using the equation, $\text{Percent Overlap} = \text{Overlap Area} / \text{Calnexin Area}$. Two proteins were considered completely colocalized if this ratio was higher than 50% and not colocalized if this ratio was lower than 10% [120].

N-terminal green fluorescent protein (GFP) and hemagglutinin (HA) tagged full-length OPN3 constructs (OPN3-nGFP and OPN3-nHA, respectively) showed $59.7 \pm 13.9\%$ and $28.1 \pm 2.29\%$ overlap, respectively, with labeling for Calnexin (Figure 3.4A, a-f). N-terminal GFP tagged OPN3 splice variant (OPN3SV-nGFP) showed $32.5 \pm 7.2\%$ overlap with Calnexin labeling (Figure 3.4A, g-i). OPN3-nGFP showed complete ER localization, whereas OPN3-nHA and OPN3SV-nGFP overlapped partially with labeling for Calnexin. This could be because of possible blockage of n-linked acetylation and n-linked glycosylation sites, which plays an important role in GPCR trafficking.

Next, intracellular loop 1 was chosen as a tagging area because intracellular loop 2 and 3 are known to have a critical role in G protein activation. Intracellular loop 1 (iL1) mCherry (MCh) tagged full-length OPN3 (OPN3-iL1MCh) and iL1 GFP tagged OPN3 splice variant (OPN3SV-iL1GFP) showed $32.5 \pm 9.2\%$ and $30.9 \pm 9.1\%$ overlap,

respectively with labeling for Calnexin (Figure 3.4A, j-o). OPN3-iL1MCh and OPN3SV-iL1GFP overlapped partially with Calnexin. ER retention of OPN3-iL1MCh and OPN3SV-iL1GFP might be because of bulky GFP and MCh groups, which might lead to incomplete folding or misfolding of the OPN3 proteins. In addition to that, a single leucine residue within intracellular loop 1 was reported as a crucial component for endoplasmic reticulum export of GPCRs. This leucine residue is also conserved in OPN3 at position 72 (L72) and insertion of GFP and MCh, in between 69-70 amino acids, might affect the function of this leucine residue.

C-terminal yellow fluorescent protein (YFP) and FLAG tagged full-length OPN3 (OPN3-cYFP and OPN3-cFLAG) showed $3.6 \pm 2.4\%$ and $3.5 \pm 2.6\%$ overlap, respectively with Calnexin (Figure 3.4A, p-u). OPN3-cYFP and OPN3-cFLAG did not show ER localization. Since c-terminal tagged OPN3 constructs were the only constructs that were able to exit the endoplasmic reticulum (ER) as expected from a properly folded GPCR, I used these constructs to further investigate the localization of OPN3.

OPN3 localize to plasma membrane and recycling endosomes in HeLa cells

Next, I sought to determine where exactly c-terminal tagged full-length human OPN3 localizes in HeLa cells. I tested the colocalization of c-terminal YFP tagged full-length OPN3 (OPN3-cYFP) with early endosome, late endosome, recycling endosome, lysosome and plasma membrane markers (Percent Overlap = $\text{Overlap Area} / \text{OPN3-cYFP Area}$). First, I examined colocalization of OPN3-cYFP with an early endosome marker, early endosome antigen 1 (EEA1) (Figure 3.5A, a-c). OPN3-cYFP showed $2.5 \pm 1.7\%$ overlap with n-terminal red fluorescent protein (RFP) tagged EEA1 (EEA1-nRFP). This result indicates that OPN3-cYFP does not localize with early endosomes. Next, I examined colocalization of OPN3-cYFP with late endosome markers, Rab7

and Rab9 (Figure 3.5A, d-i). OPN3-cYFP showed $1.4 \pm 1.3\%$ and $3.3 \pm 0.7\%$ overlap with n-terminal RFP tagged Rab7 (Rab7-nRFP) and n-terminal *Discosoma sp.* red fluorescent protein (DsRed) tagged Rab9 (Rab9-nDsRed), respectively. This result indicates that OPN3-cYFP does not localize to late endosomes. Following that, I examined colocalization of OPN3-cYFP with a recycling endosome marker, Rab11 (Figure 3.5A, j-l). OPN3-cYFP showed $28.1 \pm 9.2\%$ overlap with n-terminal DsRed tagged Rab11 (Rab11-nDsRed). This result shows that OPN3-cYFP partially localizes to recycling endosomes. In addition, I examined colocalization of OPN3-cYFP with a lysosome marker, lysosome-associated membrane glycoprotein 1 (LAMP1) (Figure 3.5A, m-o). OPN3-cYFP showed $3.9 \pm 1.0\%$ overlap with labeling for LAMP1. This result indicates OPN3-cYFP does not localize with lysosomes. Generally, melanosomal proteins localize to late endosomes or lysosomes when expressed in expression systems [121-123]. Since OPN3-cYFP localized neither at late endosomes nor lysosomes, it does not follow melanosomal protein localization pattern.

Lastly, I examined colocalization of OPN3-cYFP with a plasma membrane marker, MEM (Figure 3.5A, p-r). MEM is a 20 amino acid-long peptide originating from the n-terminus of GAP-43, also known as Neuromodulin. Notably, MEM contains cysteine residues at position 3 and 4 (C3 and C4). Posttranslational palmitoylation of these cysteine residues targets MEM-bound proteins to cellular membranes, especially the plasma membrane [124]. OPN3-cYFP showed $54.1 \pm 2.4\%$ overlap with c-terminal DsRed tagged MEM (MEM_cDsRed). Based on this result, OPN3-cYFP showed complete plasma membrane localization.

Additionally, I used förster resonance energy transfer (FRET) to investigate potential recruitment of β -arrestin1 or β -arrestin2 to OPN3. Human β -arrestin1 and β -arrestin2 are ubiquitously expressed members of the arrestin protein family; arrestins are well-known modulators of GPCR desensitization and internalization. Two arrestin

constructs were made to this end, both c-terminal CFP tagged: β -arrestin1-cCFP and β -arrestin2-cCFP. However, β -arrestin1-cCFP and β -arrestin2-cCFP were localized in the endoplasmic reticulum (Data not shown). Although there are examples of c-terminal FLAG and GFP tagged arrestins [125-128] there is a chance that c-terminal CFP might lead to misfolding of arrestin. Bioluminescence resonance energy transfer (BRET) between c-terminal *Renilla* luciferase tagged GPCRs and n-terminal Venus (a derivative of GFP) tagged β -arrestin2 was used to test the recruitment of arrestin to activated muscarinic acetylcholine receptor 2 (M2), dopamine receptor 1 (D1), dopamine receptor 2 (D2), and β 2-adrenergic receptor (β 2AR) [129]. A similar approach could be taken to investigate role of arrestin in the internalization of OPN3.

The effect of helix 8 and the c-terminus on localization

I also tested the effect of helix 8 and the unique c-terminus on the localization of OPN3 using colocalization of truncated constructs. I measured colocalization of helix 8 and c-terminus truncated, c-terminal GFP tagged OPN3 (OPN3TM1to7-cGFP) with full-length OPN3 (Percent Overlap = $\text{Overlap Area} / \text{OPN3TM1to7-cGFP Area}$). OPN3TM1to7-cGFP showed $53.6 \pm 5.7\%$ overlap with c-terminal MCh tagged full-length human OPN3 (OPN3-cMCh) (Figure 3.6A, a-c). This data shows that OPN3TM1to7-cGFP completely overlaps with OPN3-cMCh. In addition, c-terminus truncated c-terminal 1D4 tagged OPN3 (OPN3TM1to7-c1D4), which is similar to c-terminally truncated OPN3 proteins showing blue light (λ max. 465 nm) absorption and Gai coupling [50, 52], showed the same results as OPN3TM1to7-cGFP (Data not shown). Furthermore, OPN3TM1to7-cGFP showed $50.4 \pm 8.3\%$ overlap with a c-terminal MCh tagged OPN3 construct containing only helix 8 and the unique c-terminus (OPN3H8&cterm-cMCh) (Figure 3.6A, d-f). Based on the results, OPN3TM1to7-cGFP also completely colocalizes with OPN3H8&cterm-cMCh. Lastly, a c-terminal MCh tagged construct of the OPN3 c-terminus alone (OPN3cterm-cMCh) showed cytosolic localization without a nuclear exclusion (Figure 3.6A, g-i). I

therefore interpret these results to suggest that the cellular localization of OPN3 does not require the presence of helix 8 and the unique c-terminus. Also, helix 8 and the c-terminus are sufficient for intracellular vesicular localization, which relies on the presence of helix 8 since the c-terminal only construct spread through the cytosol without vesicular localization. The c-terminal cysteine residues (C325 and C331) could play a role in c-terminal palmitoylation but in this case presumably it was not enough to localize to the plasma membrane.

OPN3 localizes to intracellular vesicles in human epidermal melanocytes

I next examined the steady-state localization of endogenous OPN3 in human epidermal melanocyte. First, I investigated the specificity of a commercial OPN3 antibody (Santa Cruz Biotechnologies, H-76), which is raised against the last 10 amino acids of human OPN3. OPN3 labeling showed complete colocalization with OPN3-cFLAG and OPN3-cYFP; $53.2 \pm 12.2\%$ and $51.4 \pm 10.8\%$ overlap, respectively (Figure 3.7A,B). Western blot analysis of the OPN3 antibody in HeLa cells transiently transfected with empty vector (Mock, as negative control), OPN3-cHA, OPN3-nHA, and c-terminal truncated, n-terminal HA tagged OPN3 (OPN3 Δ C-nHA) shows specific detection of OPN3 by the OPN3 antibody (Figure 3.7C). Anti HA and OPN3 staining of OPN3-cHA and OPN3-nHA showed the same size bands matching the expected 45 kDa size of OPN3. As expected, HA staining detected OPN3 Δ C-nHA around 40 kDa, which is missing the 5 kDa c-terminus of OPN3. Since the OPN3 antibody binds to the c-terminal end of OPN3, OPN3 staining did not detect OPN3 Δ C-nHA.

Next, I sought to determine where exactly endogenous OPN3 localizes in HEMs. I tested the colocalization of OPN3 with lysosome and melanosome markers (Percent Overlap = Overlap Area/ α -OPN3 Area). First, I examined the colocalization of OPN3 with a lysosome marker, LAMP1 (Figure 3.8A, a-c). OPN3 showed $2.3 \pm 2.0\%$ overlap

with LAMP1. As in OPN3-cYFP transiently transfected HeLa cells (Figure 3.5A, m-o), OPN3 did not show lysosomal localization in HEMs. Next I examined colocalization of OPN3 with melanosomal marker, tyrosinase related protein (TYRP1) and melanosomal dark spots seen in brightfield (BF) images (Figure 3.5A, d-f and g-i). OPN3 showed $7.6 \pm 5.3\%$ and $6.2 \pm 4.2\%$ overlap labeling for TYRP1 and melanosomal dark spots, respectively. Hence, OPN3 does not show melanosomal localization in HEMs. Further studies are required to identify the exact identity of the intracellular organelle where OPN3 localizes to in HEMs.

OPN3 mRNA is highly expressed in human and mouse tissues not accessible to environmental light

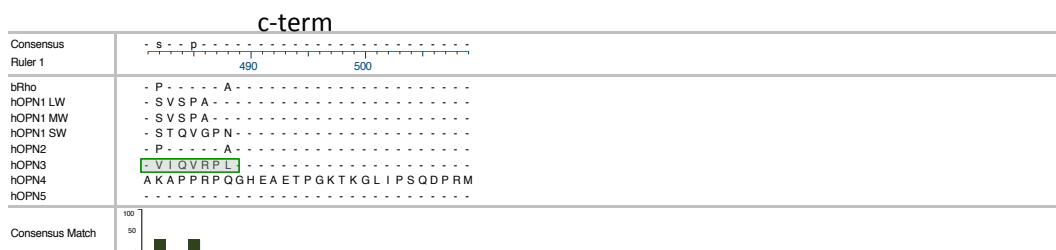
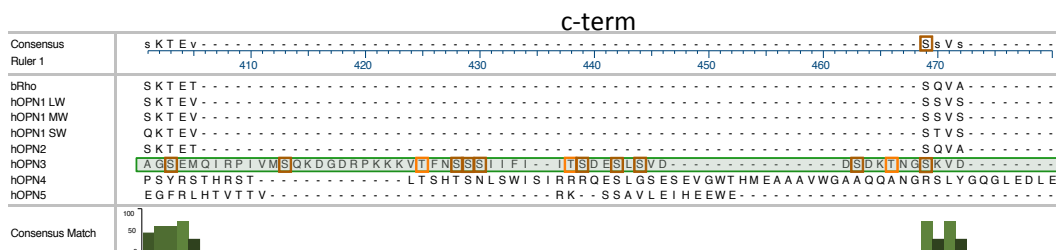
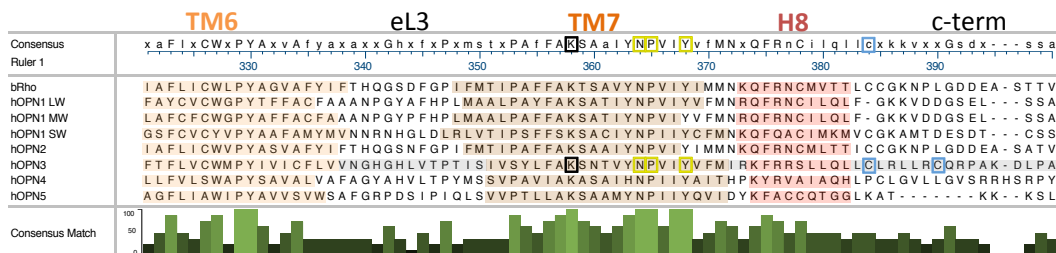
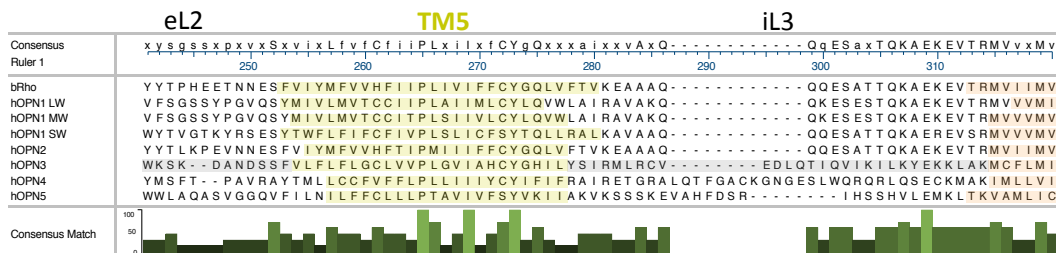
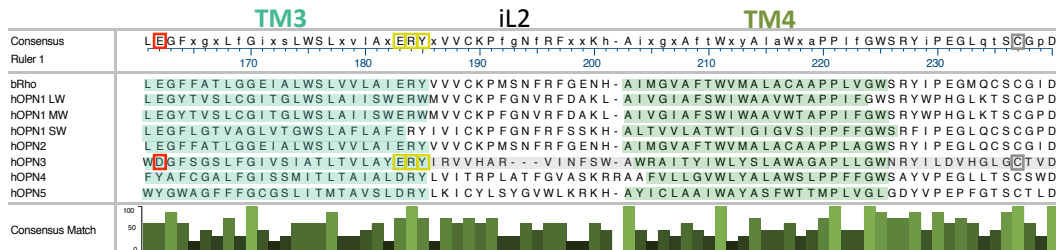
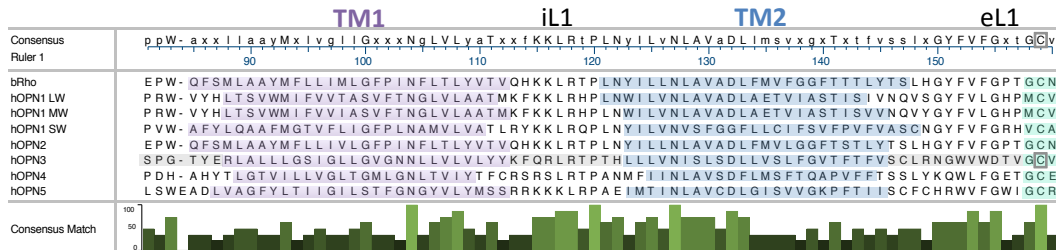
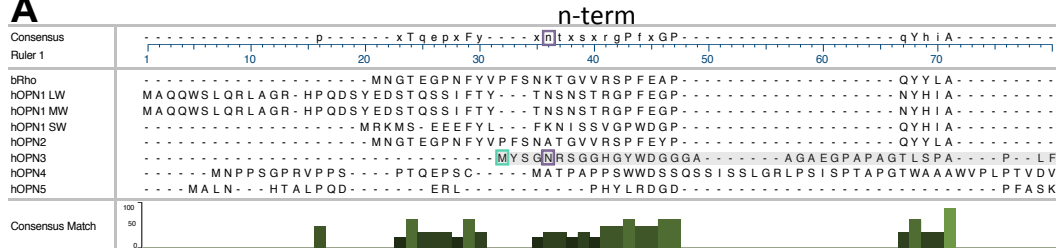
Among all the tissues screened in a tissue array, the highest human OPN3 mRNA expression was found in the placenta. In sequence; rectum, spleen, lung, lymph node and thymus also exhibited high OPN3 mRNA expression (Figure 3.9A). The highest mouse OPN3 mRNA expression was found in ovary, followed by testis and adipose tissue (Figure 3.9B). As member of the light-receptive opsin family, it is surprising that in both of the tissue arrays OPN3 expression is highest in areas of the body not readily accessible to environmental light.

Conclusion

In conclusion, I found that human OPN3 has a unique c-terminal region, which does not show homology with other membrane bound proteins but is conserved between OPN3 sequences from different species. In addition, the epitope insertion site in OPN3 construct design effects cellular localization of OPN3 in a way that n-terminal and intracellular tags lead to ER retention whereas c-terminal tagged OPN3 is targeted to the cell surface and recycling endosome. Furthermore, absence of helix 8 and the c-terminus of OPN3 did not affect the localization and the presence of only

OPN3 helix 8 and c-terminus was enough for intracellular vesicular localization similar to full-length OPN3. In human epidermal melanocytes, endogenous OPN3 was found in intracellular vesicles, the identity of which warrants further study. Lastly, OPN3 mRNA is highly expressed in areas of the body not thought to be readily accessible to environmental light in both human and mouse. These results suggest that OPN3 might possess alternative activation mechanisms quite different than light activation.

A



B

Level of amino acid identity and homolog to human OPN3 (%)						
	hOPN1 LW	hOPN1 MW	hOPN1 SW	hOPN2	hOPN4	hOPN5
Identity	32	31	30	32	31	30
Homology	53	52	54	54	53	51

Figure 3.1. Multiple sequence alignment of human OPN3 with bovine rhodopsin and other light sensitive human opsins. (A) Human and bovine opsin protein sequences were aligned using CLUSTALW. Bovine rhodopsin (bRho, NP_001014890.1), human opsin1 long-wavelength (hOPN1LW, NM_020061), human opsin1 medium-wavelength (hOPN1MW, NM_000513), human opsin1 short-wavelength (hOPN1SW, NM_001708.2), human opsin2 (hOPN2, NM_000539), human opsin3 (hOPN3, NM_014322), human opsin4 (hOPN4, NM_033282), human opsin5 (hOPN5, NM_181744). Transmembrane regions of opsins were predicted by PredictProtein program [130]. The unique c-terminal sequence was labeled with a green outline. Predicted motifs of hOPN3 are as follows: Transmembrane domain (TM) 1 to 7 and helix (H) 8, purple to red infill; retinal-binding lysine residue (K299), black outline; the counterion glutamate residue (D117), red outline; n-terminal acetylation site, green outline; glycosylation sites, purple outline; c-terminal palmitoylation sites, blue outline; c-terminal serine sites, brown outline; c-terminal threonine sites, orange outline, D/ERY and NPxxY motifs, yellow outline. iL stands for *intracellular* domain; eL, *extracellular* domain; n-term, n-terminal region; c-term, c-terminal region. (B) Human OPN3 protein sequence was compared via NCBI's Protein BLAST [131] to hOPN1LW, hOPN1MW, hOPN1SW, hOPN2, hOPN4, hOPN5.

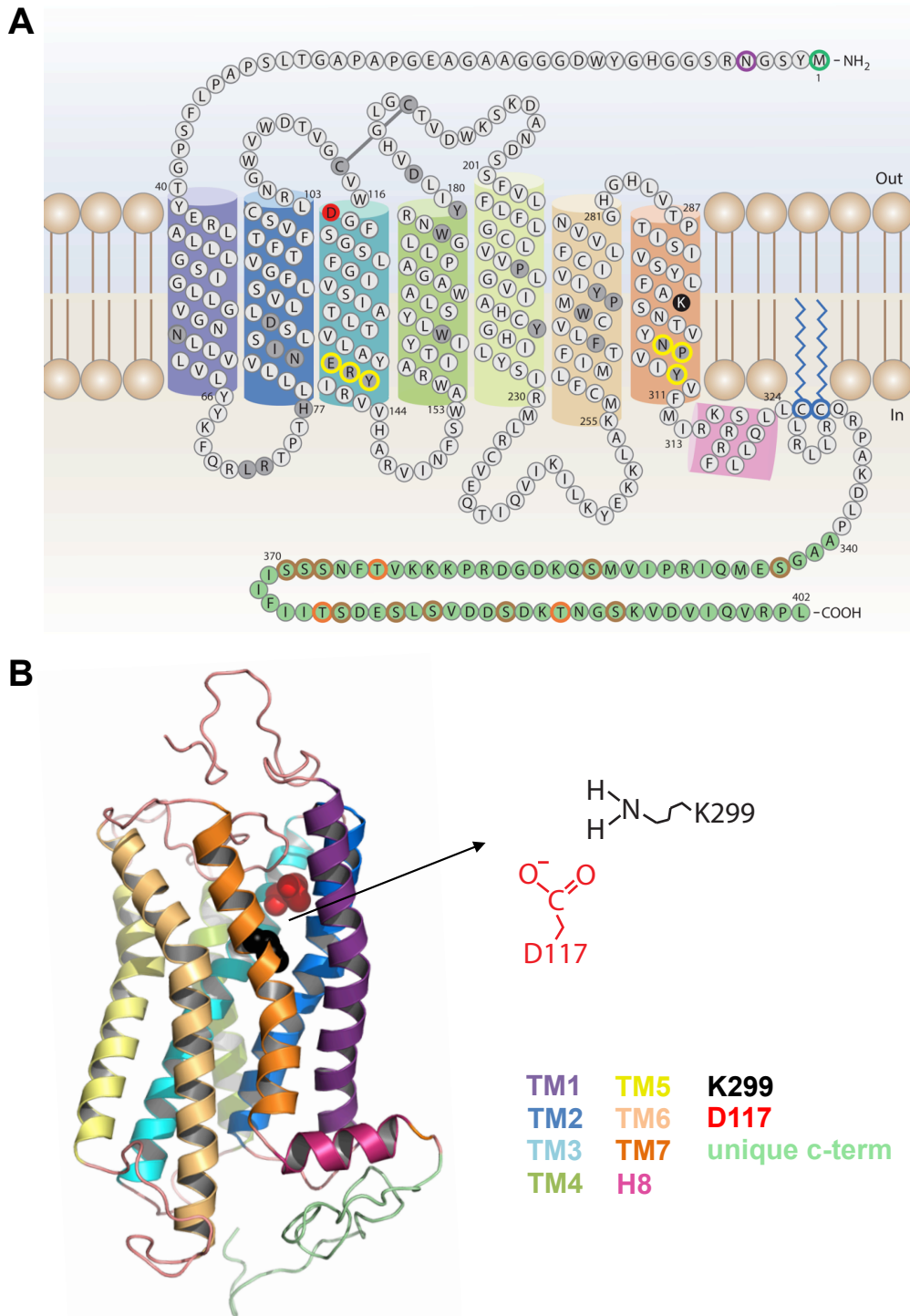


Figure 3.2. Predicted structure of human OPN3. (A) A two-dimensional model of human OPN3 based on the prediction of PredictProtein program [130]. Highly conserved residues of opsin family are shown with a gray infill. The unique c-terminal sequence was labeled with a green infill. Predicted motifs of hOPN3 are as following; transmembrane domain (TM) 1 to 7 and helix (H) 8, purple to red cylinder; retinal-binding lysine residue (K299), black infill; the counterion glutamate residue (D117), red infill; n-terminal acetylation site, green outline; glycosylation sites, purple outline; c-terminal palmitoylation sites, blue outline; c-terminal serine sites, brown outline; c-terminal threonine sites, orange outline, D/ERY and NPxxY motifs, yellow outline. Disulfide bond forming C114 and C188 were connected with a gray line. (B) A three-dimensional model of OPN3 based on the prediction of PsiPred program [132].

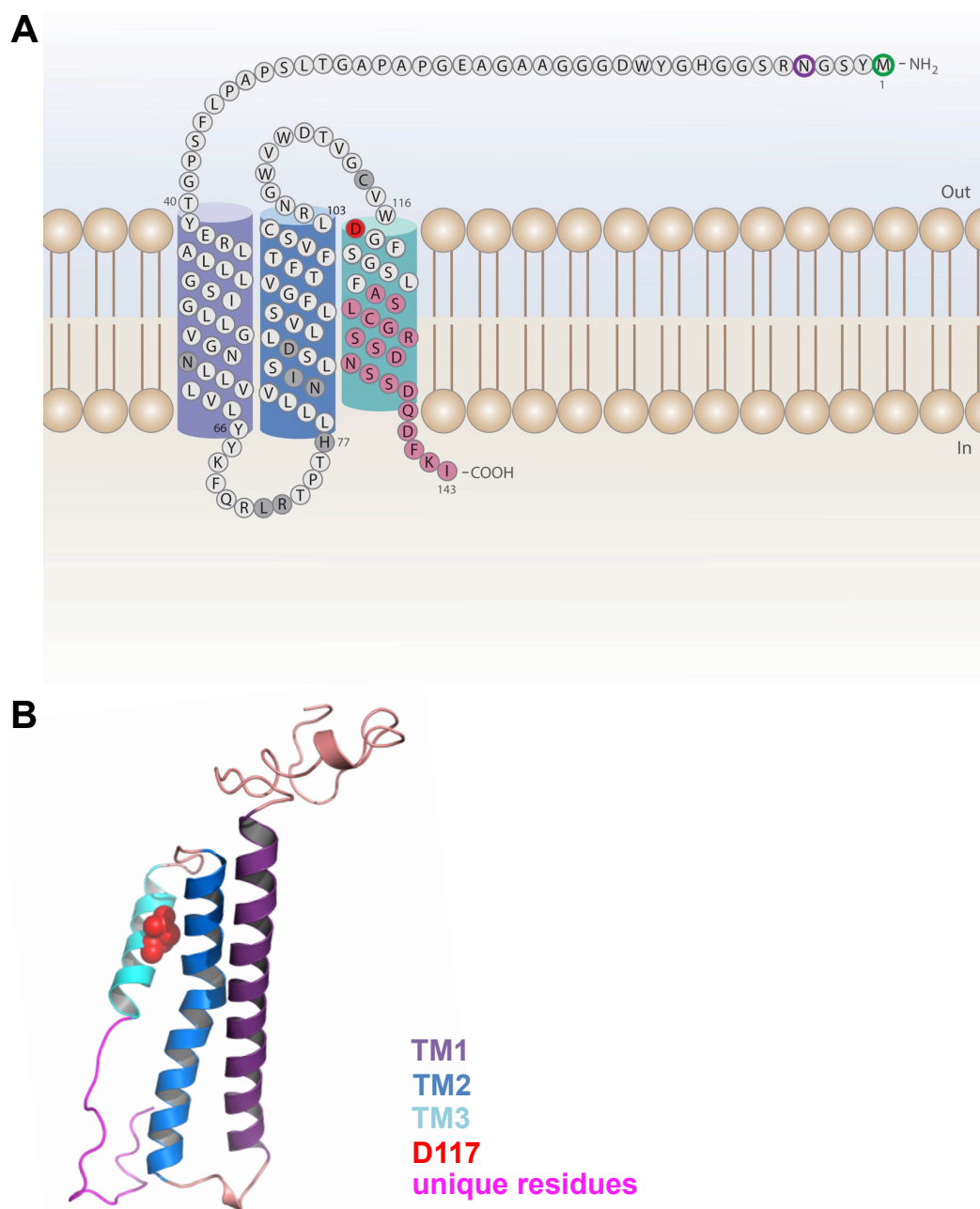
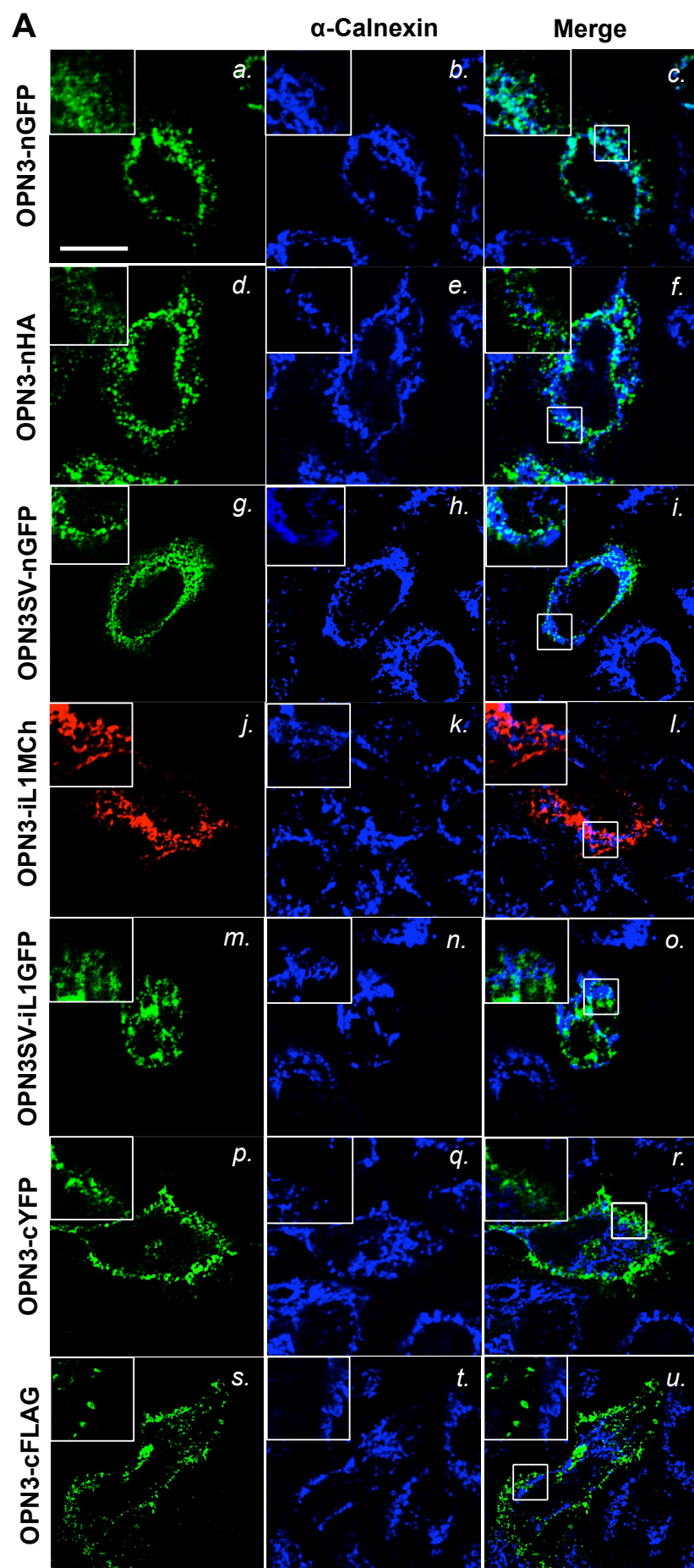


Figure 3.3. Predicted structure of human OPN3 splice variant. (A) A two-dimensional model of the novel human OPN3 splice variant based on the prediction of PredictProtein program [130]. Highly conserved residues present in the opsin family are shown with a gray infill. Unique residues created with excision of exon2 were labeled with a magenta infill. The predicted motifs of the human OPN3 splice variant are as follows; the counterion glutamate residue (D117) marked with red infill, n-terminal acetylation site with green circles, glycosylation sites with purple circles, (B) A three-dimensional model of the human OPN3 splice variant based on the prediction of PsiPred program [132].



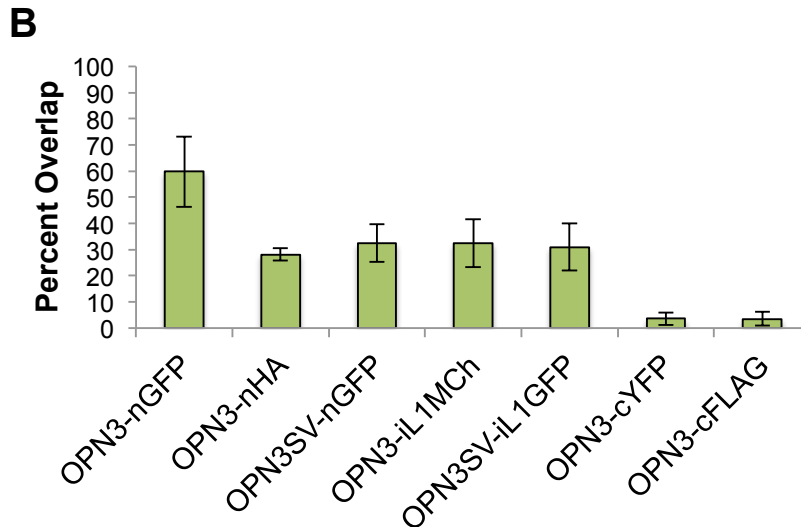
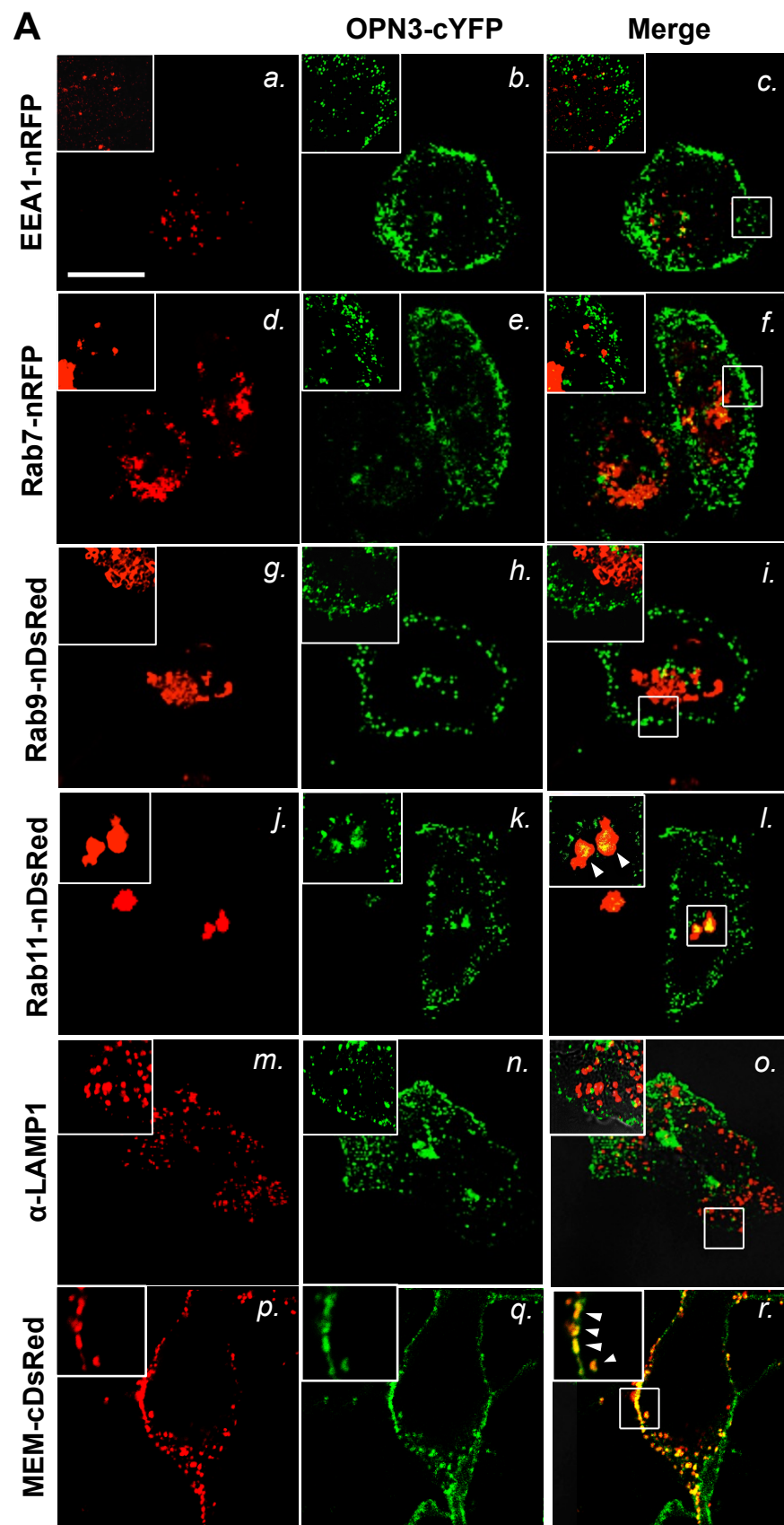


Figure 3.4. Localization of n-terminal, intracellular loop1, and c-terminal tagged human OPN3 in HeLa. (A) HeLa cells expressing epitope tagged human OPN3 protein, were labeled with Alexa647 fluorochrome-conjugated Calnexin, an endoplasmic reticulum (ER) marker. OPN3-nHA and OPN3-cFLAG were labeled with primary and fluorochrome-conjugated secondary antibody. Confocal images represent day 1 post transfection of OPN3 expression of HeLa cells kept in the dark. Bar, 10 μ m. (B) Calculation of colocalization was conducted in ImageJ (Percent Overlap = Overlap Area/ α -Calnexin Area). Results showed that OPN3-nGFP (a-c) protein extensively colocalizes with Calnexin, whereas very little overlap was observed for c terminal tagged OPN3 proteins (p-u). Other n-terminal tagged and intracellular loop 1 tagged OPN3 proteins (d-o) showed approximately %30 overlap with Calnexin. Bars represent the average of 30 images taken from three independent experiments. Tagged OPN3 proteins are named as the following: OPN3-nGFP, n terminal GFP tagged full-length OPN3; OPN3-nHA, n terminal HA tagged full-length OPN3; OPN3SV-nGFP, n terminal GFP tagged splice variant OPN3; OPN3-iL1MCh, full-length OPN3 tagged with MCh at intracellular loop 1; OPN3SV-iL1GFP, splice variant OPN3 tagged with GFP at intracellular loop 1; OPN3-cYFP, c terminal YFP tagged full-length OPN3, OPN3-cFLAG, c terminal FLAG tagged full-length OPN3 (See Fig. 5.1).



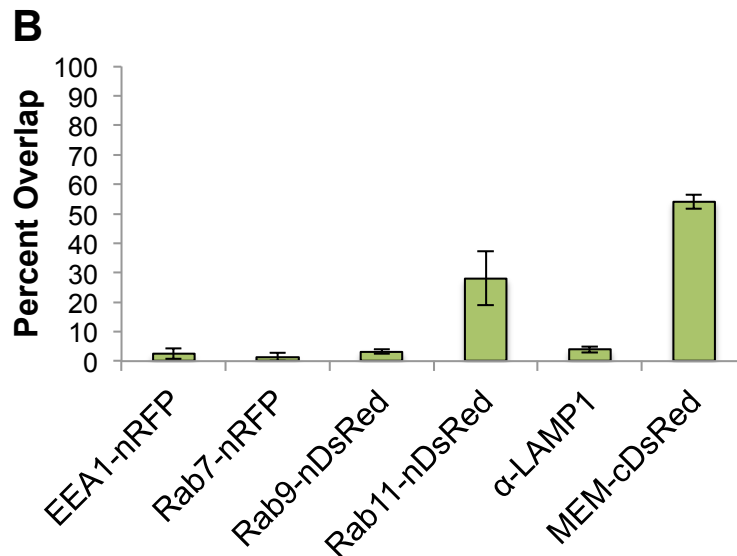


Figure 3.5. Localization of c-terminal YFP tagged human OPN3 in HeLa. (A) Epitope tagged an early endosomes marker, early endosome antigen 1 (EEA1); late endosomes marker, Rab7; late endosomes marker, Rab9; recycling endosomes marker, Rab11; a plasma membrane marker, MEM and OPN3 protein were expressed in HeLa cells. Lysosome-associated membrane glycoprotein 1 (LAMP1) was labeled with primary and fluorochrome-conjugated secondary antibody. Confocal images represent day 1 post transfection of OPN3 expression of HeLa cells kept in the dark. Bar, 10 μ m. (B) Calculation of colocalization was conducted in ImageJ (Percent Overlap = Overlap Area/ OPN3-cYFP Area). Results showed that OPN3-cYFP extensively colocalize with MEM-cDsRed, whereas very little overlap was observed for EEA1-nRFP, Rab7-nRFP, Rab9-nDsRed and LAMP1 proteins and Rab11-nDsRed showed partial overlap with c-terminal YFP tagged OPN3 protein. Bars represent the average of 30 images taken from three independent experiments. Tagged proteins named as following: OPN3-cYFP, c terminal YFP tagged OPN3; n terminal RFP tagged EEA1, EEA1-nRFP; n terminal RFP tagged Rab7, Rab7-nRFP; n terminal DsRed tagged Rab9, Rab9-nDsRed; n terminal DsRed tagged Rab11, Rab11-nDsRed; c terminal DsRed tagged MEM, MEM-cDsRed (See Figure 5.1).

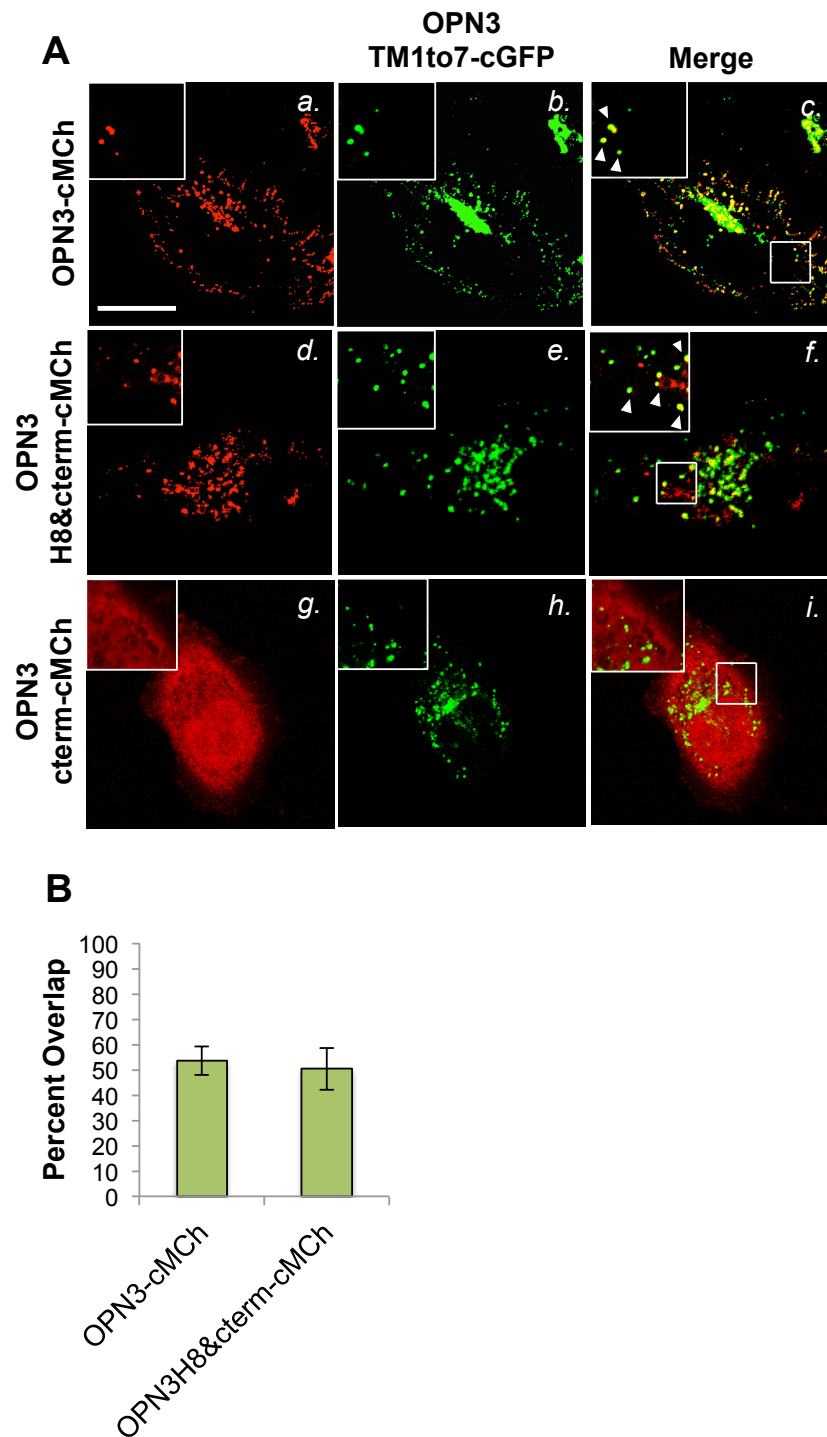


Figure 3.6. Role of helix 8 and c terminus of human OPN3 in localization. (A) Epitope tagged full-length, transmembrane 1-7, helix 8-c terminus and c terminus of OPN3 protein were expressed in HeLa cells. Confocal images represent day 1 post transfection of OPN3 expression of HeLa cells kept in the dark. Bar, 10 μ m. (B) Calculation of colocalization were conducted in ImageJ (Percent Overlap = Overlap Area/ OPN3TM1to7-cGFP Area). Bars represent the average of 20 images taken from three independent experiments. Tagged proteins named as following: OPN3-cMCh, c terminal mCherry tagged OPN3; OPN3TM1to7-cGFP, c terminal GFP tagged transmembrane 1 to 7 of OPN3; OPN3H8&cterm-cMCh, c terminal mCherry tagged helix 8 and c terminus of OPN3; OPN3cterm-cMCh, c terminus of OPN3 (See Figure 5.1).

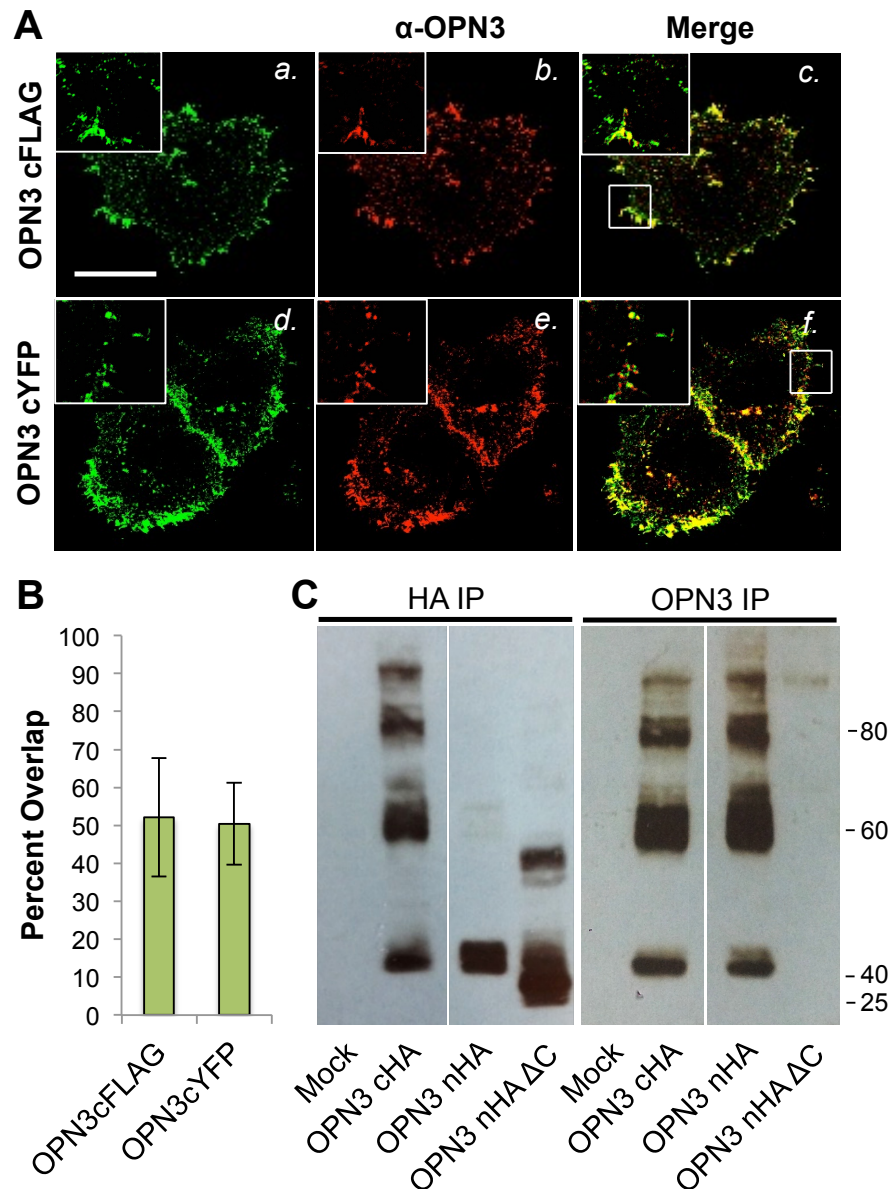


Figure 3.7. Immunohistochemical and western blot identification of human OPN3 by anti-OPN3 antibody. (A) HeLa cells expressing OPN3-cFLAG OPN3-cYFP were labeled with anti-OPN3 primary antibody and fluorochrome-conjugated secondary antibody. Confocal images represent day 1 post transfection of OPN3 expression of HeLa cells kept in the dark. Bar, 10 μ m. (B) Calculation of colocalization was conducted in ImageJ (Percent Overlap = Overlap Area/ α -OPN3 Area). Results showed that c terminal tagged OPN3 proteins extensively colocalize with α -OPN3. Bars represent the average of 30 images taken from three independent experiments. (C) Western blot analysis of hemagglutinin (HA) and OPN3 antibody staining on AD293 cells transfected with empty vector, c-terminal HA tagged OPN3, n-terminal HA tagged OPN3 and n-terminal HA tagged c-terminal truncated OPN3 constructs. Results are representative of four independent experiments. Tagged OPN3 proteins named as following: OPN3-cFLAG, c terminal FLAG tagged OPN3; OPN3-cYFP, c terminal YFP tagged OPN3; OPN3-cHA, c terminal HA tagged OPN3; OPN3-nHA, n terminal HA tagged OPN3; OPN3 Δ C-nHA, n-terminal HA tagged c-terminal truncated OPN3 construct (See Figure 5.1).

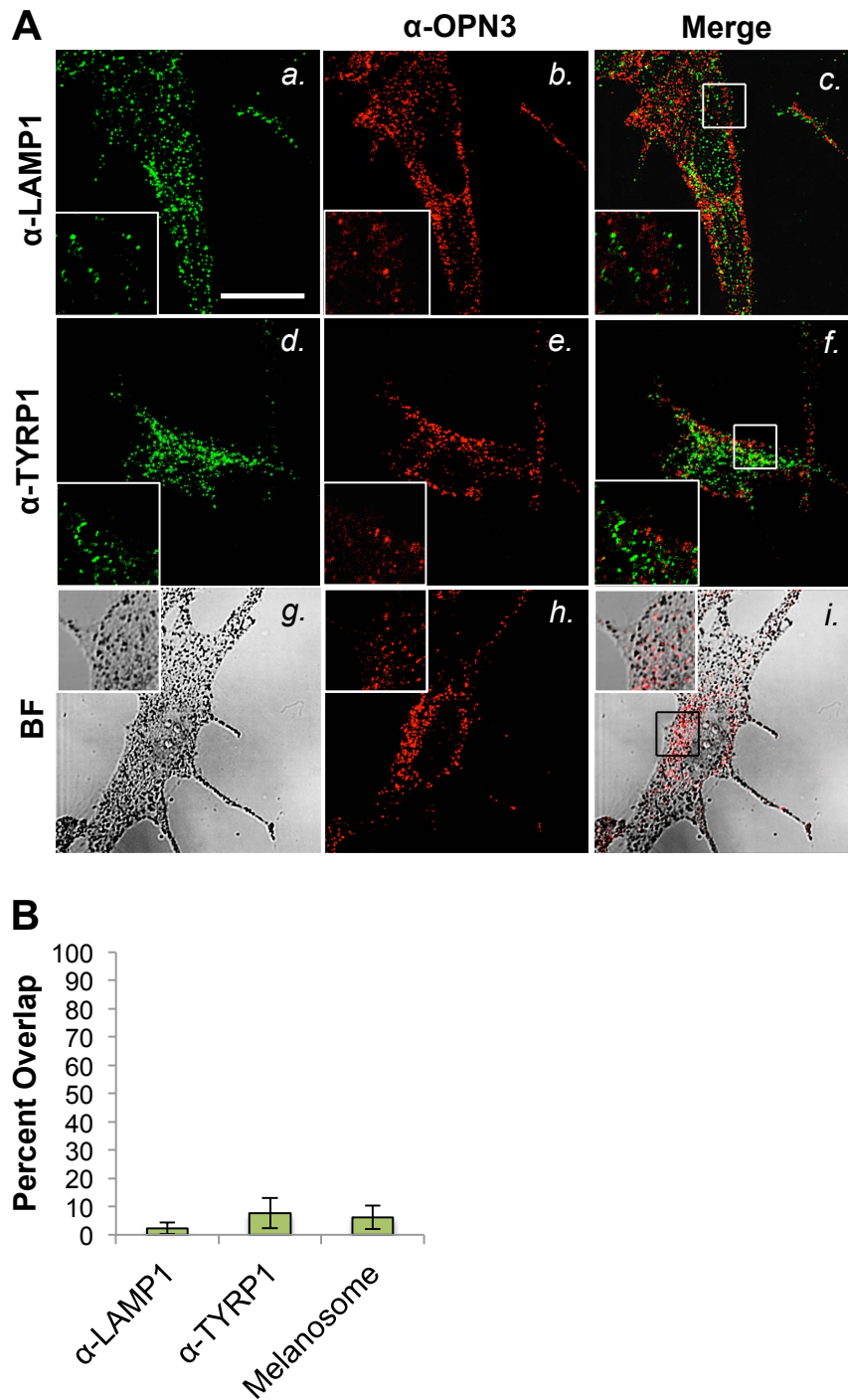


Figure 3.8. Localization of OPN3 in human epidermal melanocytes (HEMs). (A) HEMs were labeled with anti-OPN3 and anti-LAMP1 or anti-TYRP1 primary antibody and fluorochrome-conjugated secondary antibody. LAMP1 is a lysosomal marker and TYRP1 is a melanosomal marker. Melanosomes of HEMs could also be seen in brightfield (BF) images. Confocal images represent OPN3 expression of HEMs kept in the dark. Bar, 10 μ m. (B) Calculation of colocalization was conducted in ImageJ (Percent Overlap = Overlap Area/ α -OPN3 Area). Results showed that endogenous OPN3 protein does not colocalize with LAMP1, TYRP1 and melanosomal dark spots. Bars represent the average of 20 images taken from three independent experiments.

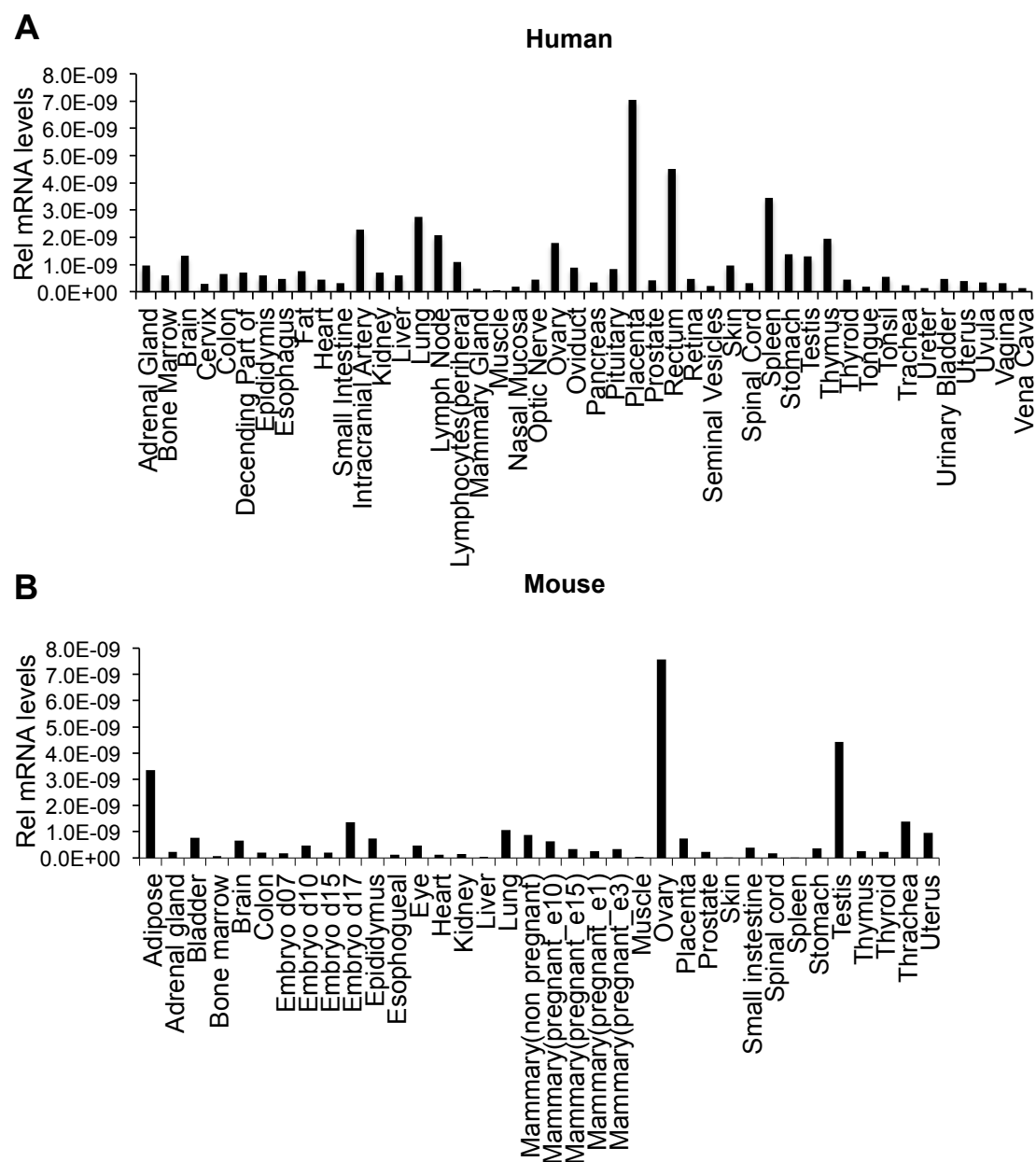


Figure 3.9. Relative mRNA expression of OPN3 in mouse and human tissues. (A) Quantitative PCR analysis of the mRNA expression of full length OPN3 in human tissues, (B) Quantitative PCR analysis of the mRNA expression of full length OPN3 in mouse tissues, using primers detecting only full-length OPN3 and calculated relative to actin levels. Bars represent the average of three experiments.

CHAPTER 4. Functionality of Human OPN3

Abstract

The localization and function of ocular opsins are characterized extensively; however, several extra-ocular opsins lack data supporting their physiological function. In Chapter 2, we demonstrated that OPN3 is expressed at higher levels than other opsins in human epidermal melanocytes and keratinocytes. In Chapter 3, I characterized the cellular localization of human OPN3 in HeLa and HEM cells acting as heterologous and endogenous expression systems, respectively. Using these developed tools, in this chapter I examine the function of human OPN3 in human skin cells. I found that knock down of OPN3 leads to melanin level increase in HEMs. Furthermore, OPN3 and MC1R co-localize and co-immunoprecipitate in HeLa and HEMs. Lastly, overexpression of OPN3 in MNT-1 human melanoma cells inhibits the α MSH response of endogenously expressed MC1R in a Gai dependent manner. These results suggest that OPN3 may function to inhibit the MSH-induced cAMP response of MC1R in a Gai dependent manner in order to limit melanin production in human skin. In addition to that, OPN3 might play role in the internalization and desensitization of MC1R, as well as, inhibition of Gas and MC1R interaction through its uniquely long c terminus.

Results and Discussion

Comparison of human OPN3 with OPN3 sequences from different animals

OPN3 has been studied in a variety of animals including human, mouse, chicken, anole, pufferfish, zebrafish, medaka and mosquito. Comparison of OPN3 protein sequences show that the unique c terminal sequence is highly conserved among vertebrates (Figure 4.1A). Following this, as the only invertebrate in the list, mosquito OPN3 sequence shows slight changes in the size and amino acid composition of its c terminus. Multiple sequence alignment of human OPN3 with blue light absorbing OPN3 sequences (chicken, pufferfish and zebrafish OPN3) showed 81%, 76% and 76% sequence homology, and 70%, 61% and 60% sequence identity, respectively (Figure 4.1B). Although high homology and identity values suggest that human OPN3 might have a blue light absorption ability, it is a known fact that changing the location of certain key residues alters the maximal absorption wavelength of the opsins, hence, it is very hard to guess the absorption spectra of human OPN3 based on sequence alignments. Spectroscopic experiments will be needed to understand the exact spectral profile of human OPN3.

Knock down of OPN3 increases melanin level in human epidermal melanocytes

To test the effect of OPN3 reduction in human epidermal melanocytes (HEMs), I transduced HEMs with lentiviral control miRNA and OPN3-targeted two miRNA (miRNA 1 and 2) constructs. miRNA 1 and miRNA 2 target a 20 bp sequence in exon 2 and exon 4 of OPN3 sequence. Transduced cells were detected by co-cistronic expression of mCherry (MCh) reporter in these constructs. After blasticidin antibiotic selection (10 days) and amplification of cells (20 days), qPCR, western blot, melanin analysis, and immunofluorescent imaging were performed on control, miRNA 1 and miRNA 2 transduced HEMs.

Compared to control miRNA-treated cells, OPN3-targeted miRNA 1 and miRNA 2 decreased mRNA expression by 74% and 76% (Figure 4.2A) and protein expression by 61% and 63%, respectively (Figure 4.2C and D). The cells with reduced OPN3 expression had more melanin (appeared darker) than control cells (Figure 4.2B). Using a well-established melanin assay, I found that miRNA 1 or 2 treated cells showed 3.6 or 2.8 times more melanin, respectively, when compared to control miRNA-treated cells (Figure 4.2E). Immunofluorescent imaging of control (Fig. 4.2F, a-d), miRNA 1 (Figure 4.2F, e-h) and miRNA 2 (Figure 4.2F, i-l) treated cells also showed that OPN3-targeted miRNA cells appeared darker than control cells. Quantification of melanosome level, which was normalized against Calnexin level (Percent = Melanosome/Calnexin Area), showed that miRNA 1 or 2 treated cells has 3.5 or 3.1 times more melanin, respectively, when compared to control miRNA-treated cells (Figure 4.2E). In addition to that, quantification of OPN3 labeling level (Percent = α -OPN3/Calnexin Area), showed that miRNA 1 or 2 treated cells had 88% and 86% less OPN3 labeling, respectively, when compared to control miRNA-treated cells (Figure 4.2H). I interpret these results to suggest that the decrease in OPN3 level leads to melanin increase in HEMs.

OPN3 and MC1R colocalize in HeLa and human epidermal melanocytes

I investigated the steady-state localization of full-length human OPN3 and MC1R in HeLa cells. OPN3 with YFP epitope tag inserted at the c-terminal end (OPN3-cYFP) and MC1R with HA epitope tag inserted at n-terminal end (MC1R-nHA) were detected in HeLa after transient transfection. I tested the colocalization of tagged OPN3 protein with MC1R by using the Percent Overlap = Overlap Area/MC1R-nHA Area ratio. Two proteins were considered completely colocalized if this ratio was higher than 50% and not colocalized if this ratio was lower than 10% [120]. OPN3-

cYFP and MC1R-nHA showed $50.0 \pm 10.1\%$ (Figure 4.3A, a-c and Figure 4.3B); hence, OPN3-cYFP and MC1R-nHA overlapped completely. Intriguingly, colocalization was seen mostly in intracellular vesicles.

Next, I used prostaglandin E2 receptor (EP2), a G α s coupled member of rhodopsin GPCR family like MC1R, to test the specificity of OPN3 and MC1R colocalization. OPN3-cYFP and EP2 with a HA epitope tag inserted at the n-terminal end (EP2-nHA) (see Figure 5.1) were detected in HeLa after transient transfection. OPN3-cYFP and EP2-nHA showed $6.7 \pm 1.3\%$ (Figure 4.3A, d-f and Figure 4.3C); hence, OPN3-cYFP and EP2-nHA did not show colocalization. I therefore interpret these results to suggest that the OPN3 specifically colocalizes with MC1R in intracellular vesicles.

In addition, I tested the endogenous localization of full-length OPN3 and MC1R in HEMs. Immunostaining for OPN3 with the antibody described in Figure 3.7 (α -OPN3) and labeling for MC1R with the antibody used in literature [133-135] (N-19, Santa Cruz Biotechnology) (α -MC1R) were detected in HEMs. I tested the colocalization of labeled OPN3 with MC1R by using Percent Overlap = Overlap Area/ α -MC1R Area ratio. α -OPN3 and α -MC1R staining showed $46.6 \pm 14.1\%$ (Figure 4.3A, g-i and Figure 4.3C). α -OPN3 and α -MC1R did not show complete colocalization since the overlap ratio was lower than 50%; however, results suggest that α -OPN3 and α -MC1R labeling showed high level of overlap in intracellular vesicles.

Furthermore, I used Förster resonance energy transfer (FRET) to investigate potential heterodimerization of OPN3 with MC1R. For thorough and critical evaluation of heterodimers, three criteria are proposed as a set of standards [88]. One of these criteria is “Colocalization and Interaction of Proteins” which mandates the usage of colocalization studies, proximity-based techniques such as FRET, and co-immunoprecipitation assays in expression systems and native cells to address this

criterion. I designed c-terminal CFP tagged MC1R (MC1R-cCFP) to cotransfect with OPN3-cYFP; however, MC1R-cCFP was localized in the endoplasmic reticulum (Data not shown). ER retention of MC1R-cCFP might be because of bulky CFP groups, which might lead to incomplete folding or misfolding of the MC1R proteins. As an alternative approach to FRET, proximity ligation assay (PLA) has been used to investigate the heterodimerization of two proteins. PLA is a chromogenic in situ protein-protein interaction assay, which uses antibodies to detect target protein rather than tagging with epitopes. For example, the HER2 and HER3 heterodimerization [136] and vascular endothelial growth factor (VEGF) ligand-induced VEGFR2 and VEGFR3 heterodimerization [137] were proven by using this technique. A similar approach could be taken to investigate the possible heterodimerization of MC1R and OPN3.

OPN3 and MC1R co-immunoprecipitate in HeLa and human epidermal melanocytes

I sought to investigate the co-immunoprecipitation of full-length human OPN3 and MC1R in HeLa cells. After transient transfection with c-terminal FLAG tagged OPN3 (OPN3-cFLAG) and n-terminal HA tagged MC1R (MC1R-nHA), HeLa cell lysate was treated with agarose conjugated anti-FLAG or anti-HA primary antibodies and immunoprecipitated samples were analyzed with western blotting using anti-HA staining. As expected, HA tagged MC1R (app. 30 kDa) in cell lysate was detected by anti-HA staining. Similarly, in anti-HA antibody immunoprecipitated (HA IP) samples, HA tagged MC1R was detected by anti-HA staining. In the experimental condition, anti-FLAG antibody immunoprecipitation (FLAG IP) followed by anti-HA staining, OPN3 and MC1R proteins pulled-down together, which could be seen in OPN3-cFLAG + MC1R-nHA lane (Figure 4.4A).

Next, I investigated the co-immunoprecipitation of endogenous OPN3 and MC1R in HEM cells. HEM cell lysate was treated with agarose conjugated anti-OPN3 antibody. Immunoprecipitated samples were analyzed with western blotting using anti-MC1R staining (Figure 4.4A). Specificity of the anti-MC1R antibody was tested by comparing the HA staining and MC1R staining of MC1R-nHA expressing HeLa cell lysate. Bands of the same size, app. 30 kDa corresponding to the molecular weight of MC1R, were detected from both stainings, which suggests that the MC1R antibody specifically detects MC1R protein. In HEM cell lysate, in the anti-OPN3 antibody immunoprecipitated (HEM OPN3 IP) sample, MC1R was detected by anti-MC1R staining (Figure 4.4B). The co-immunoprecipitation of OPN3 and MC1R suggests that these receptors may form a heterodimer, higher order heterooligomeric complex or a scaffold-mediated protein complex in heterologous or endogenous expression system.

I also investigated the co-immunoprecipitation of OPN3-cFLAG with n-terminal HA tagged MC2R (MC2R-nHA), MC3R (MC3R-nHA), MC4R (MC4R-nHA) and MC5R (MC5R-nHA) in HeLa cells. As in the MC1R co-immunoprecipitation case, HeLa cell lysate was treated with agarose conjugated anti-FLAG or anti-HA primary antibodies and immunoprecipitated samples were analyzed with western blotting using anti-HA staining. In cell lysate samples, HA tagged MC2R (app. 39 kDa), MC3R (app. 36 kDa), MC4R (app. 40 kDa) and MC5R (app. 40 kDa) were detected by anti-HA staining. In the experimental condition, which consisted of anti-FLAG antibody immunoprecipitation (FLAG IP) followed by anti-HA staining, OPN3 pulled down with MC2R, MC3R and MC4R proteins, which could be seen in OPN3-cFLAG + MC2R-nHA, +MC3R-nHA and + MC4R-nHA lanes, respectively (Figure 4.5A,B). These results show that OPN3 immunoprecipitates with MC2R, MC3R and MC4R protein. Lower protein expression of MC5R could be the reason of the failure of immunoprecipitation with OPN3.

Lastly, I tested the importance of helix 8 and the c-terminus of OPN3 in co-immunoprecipitation of OPN3 and MC1R. After HeLa transient transfection with either MC1R-nHA and helix 8 and c-terminus truncated, c-terminal GFP tagged OPN3 (OPN3TM1to7-cGFP) or MC1R-nHA and only helix 8 and the unique c-terminus, c-terminal mCherry tagged OPN3 (OPN3H8&cterm-cMCh), cell lysate was treated with agarose conjugated anti-GFP or anti-MCherry antibody. Results showed that OPN3TM1to7-cGFP co-immunoprecipitates with MC1R, whereas OPNH8&cterm-cMCh does not (Figure 4.6). I therefore interpret these results to suggest that the interaction between MC1R and OPN3 does not require the presence of helix 8 and c terminus.

OPN3 decrease the cAMP response to α MSH in a Gai-dependent manner

I explored whether over-expression of OPN3 modulates MC1R response to α MSH, a MC1R agonist. For this purpose, I could not use HEM primary cells, which are highly difficult to transfect by using conventional transfection methods. Instead, MNT-1 cells, a highly pigmented human melanoma cell line, were used because of their ease of transfection and endogenous MC1R and prostaglandin expression. As expected, OPN3-cMCh localized on the plasma membrane and intracellular vesicles in MNT-1 cells (Figure 4.7A). After transient transfection with cAMP indicator Epac-H187 and c-terminal mCherry tagged OPN3 (OPN3-cMCh) or only mCherry tagged (cMCh) constructs, MNT-1 cells were treated with α MSH and imaged with a fluorescent microscope (Forskolin + IBMX used to normalize cAMP response). OPN3-cMCh expression decreased the α MSH induced cAMP response in MNT-1 cells (Figure 4.7B), more specifically OPN3-cMCh reduced α MSH induced cAMP response by 76% (Figure 4.7C). To test the specificity of OPN3-cMCh mediated reduction of α MSH induced cAMP response, I used prostaglandin, an agonist of Gas coupled

prostaglandin receptor. Both OPN3-cMCh and cMCh gave the same approximate level of cAMP response, which suggests that OPN3 does not affect the functionality of prostaglandin (Figure 4.7D,E). Lastly, I examined the affect of pertussis toxin (PTX, a Gai inhibitor) treatment on OPN3-cMCh mediated reduction of α MSH induced cAMP response. Interestingly, OPN3-cMCh did not affect the α MSH induced cAMP response of PTX treated MNT-1 cells (Figure 4.7F,G). This finding strongly suggests that OPN3 might inhibit the melanogenic pathway of MC1R via Gai activation (Figure 4.10). If this is true, the increase in melanin content in miRNA knock down HEMs could be due to decreased inhibition of cAMP production by OPN3.

Characterization of K299G and W268F mutants of OPN3

First, I sought to determine the effect of retinal binding sites in the localization of full-length human OPN3 in HeLa cells. For this purpose, I designed lysine 299 to glycine (K299G) and tryptophan 268 to phenylalanine (W268F) mutants of OPN3. Mutation of rhodopsin K296 residue (human OPN3 homolog is K299) had been used extensively to abolish its light sensitivity [138, 139]. Based on this, K299 of OPN3 might play a role as retinal binding residue in OPN3. Presumably from the rhodopsin studies, my K299G mutation of OPN3 abolishes the light sensitivity of OPN3, if OPN3 is in fact light-sensitive. However, further studies demonstrated that mutations of K299 leads to constitutive activity of the rhodopsin pigment [140]. For this reason, alternative mutations sites have been proposed, which would not affect the functionality of the opsin beside its light sensitivity. For example, mutation studies of rhodopsin showed that W265F, Y, A alter the binding dynamic of retinal in a way that it does not reach completion. Results suggested that theses mutations reduce the affinity of 11-*cis*-retinal for the altered binding pockets in the opsins [141, 142]. This highly conserved W265 group of rhodopsin is found at position 268 (W268) in OPN3

protein sequence and it is positioned in close proximity of the hypothetical retinal binding site of OPN3 (Figure 4.8A).

Localization of human OPN3 K299G and W268F constructs with c terminal FLAG tags (OPN3K299G-cFLAG and OPN3W268F-cFLAG, respectively) were examined in HeLa cells after transient transfection. I tested the colocalization of OPN3K299G-cFLAG and OPN3W268F-cFLAG, with c terminal mCherry tagged full-length OPN3 (OPN3-cMCh). The ratio of overlap between mutants and full-length protein was quantified using the equation, Percent Overlap = Overlap Area/ OPN3-cMCh. Two proteins were considered completely colocalized if this ratio was higher than 50% [120]. OPN3K299G-cFLAG and OPN3W268F-cFLAG showed $57.6 \pm 5.9\%$ and $60.1 \pm 9.3\%$ overlap, respectively, with OPN3-cMCh (Figure 3.4B,C); hence OPN3K299G-cFLAG and OPN3W268F-cFLAG showed complete overlap with OPN3-cMCh. In addition, OPN3K299G-cFLAG and OPN3W268F-cFLAG showed co-immunoprecipitation with MC1R like the full-length OPN3 protein. At this point, alteration of hypothetical retinal binding site of OPN3 has not been detected as a modulator of OPN3 functionality. Furthermore, Ca^{2+} response was examined in HEMs in response to UVR, green and blue light. But, there was no difference between control, and OPN3 miRNA 1 and 2 (Figure 4.9A,B). Furthermore, cAMP imaging of OPN3 overexpressing MNT-1 cells in response to light has not demonstrated a light dependent response of human OPN3 (Data not shown). These findings and the high expression level of OPN3 in internal organs (Figure 3.9) suggest that human OPN3 might have a light independent function in the cells.

Predicted OPN3 and MC1R interaction models

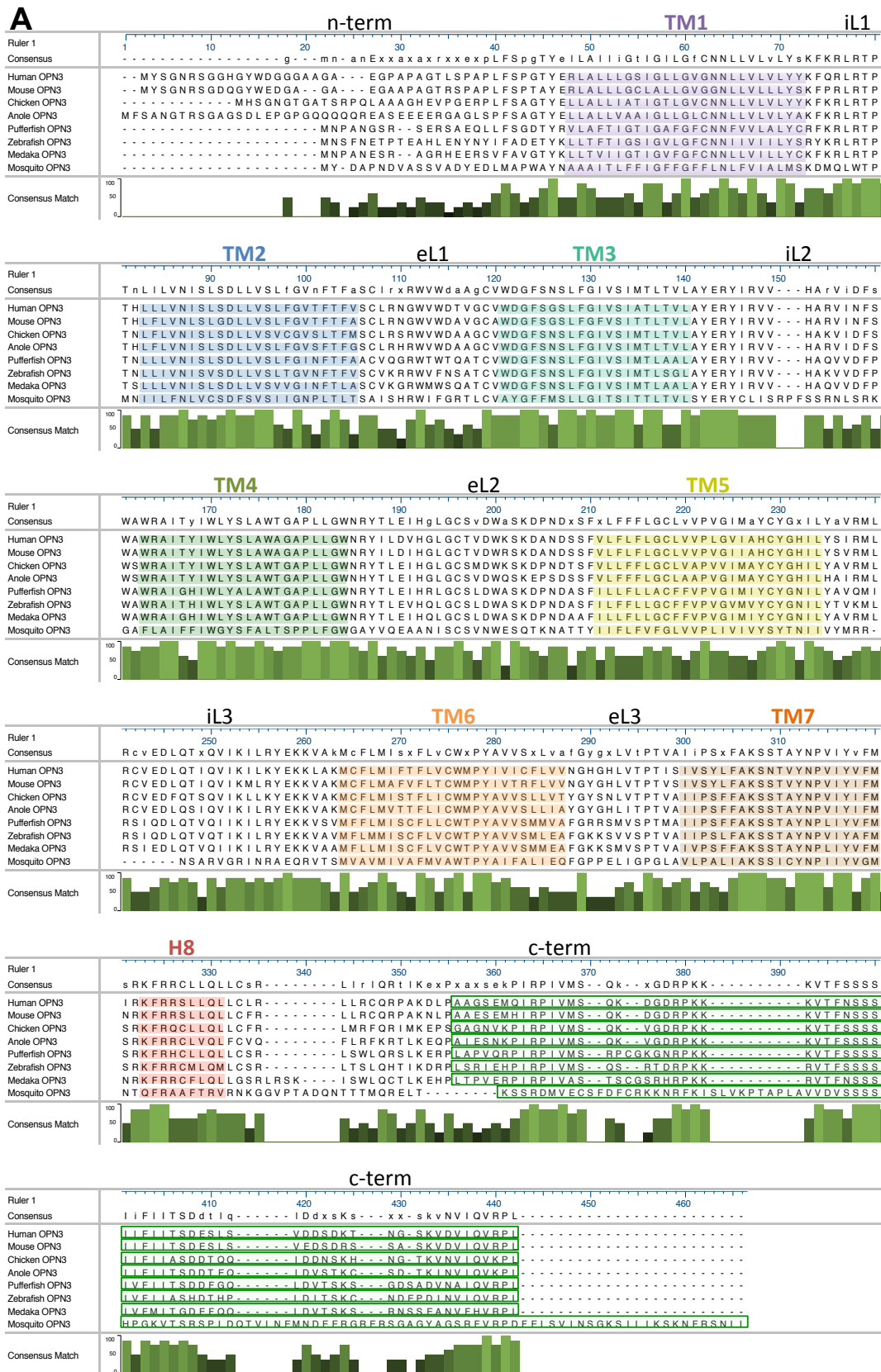
Based on the finding of this chapter, I hypothesized three models for OPN3 and MC1R interaction. First, OPN3 might affect the function of MC1R at the G protein

level. In the case of OPN3 mediated G α i activity, adenylyl cyclase (AC) activity is inhibited in a way that MC1R mediated G α s activity cannot increase intracellular cAMP level as effectively (Figure 4.10A). Second, interaction of OPN3 might alter the function of MC1R by enhancing its internalization. The long c terminal sequence of OPN3 has 13 possible phosphorylation residues. In the case of direct protein-protein interaction, arrestin mediated internalization of OPN3 might also lead to removal of MC1R from the cell surface. Given the fact that the main agonist of MC1R, α MSH, cannot cross the plasma membrane, this kind of internalization would decrease the efficiency of MC1R function (Figure 4.10B). Lastly, OPN3 might physically inhibit the interaction of G α s and MC1R through its long and unique c terminus (Figure 4.10C). These last two models are compatible with co-immunoprecipitation findings whereas the first model explains the G α i dependent decrease of cAMP response of MC1R cAMP response in presence of OPN3.

Conclusion

In conclusion, I found that the unique c terminal region is conserved between OPN3 sequences from different species and that human OPN3 sequence show high homology with previously studied chicken, zebrafish and pufferfish OPN3, which showed blue light absorption ability. In addition, knock down of OPN3 leads to melanin level increase in HEMs. Furthermore, OPN3 and MC1R co-localize and co-immunoprecipitates in HeLa and HEMs. I therefore interpret these findings to suggest that these receptors may form a heterodimer, a higher order heterooligomeric complex or a scaffold-mediated protein complex. Lastly, overexpression of OPN3 in MNT-1 cells inhibits the α MSH response of endogenously expressed MC1R. Furthermore, this inhibition of α MSH response is alleviated with the treatment of PTX. These results suggest that OPN3 might inhibit α MSH induced cAMP response of

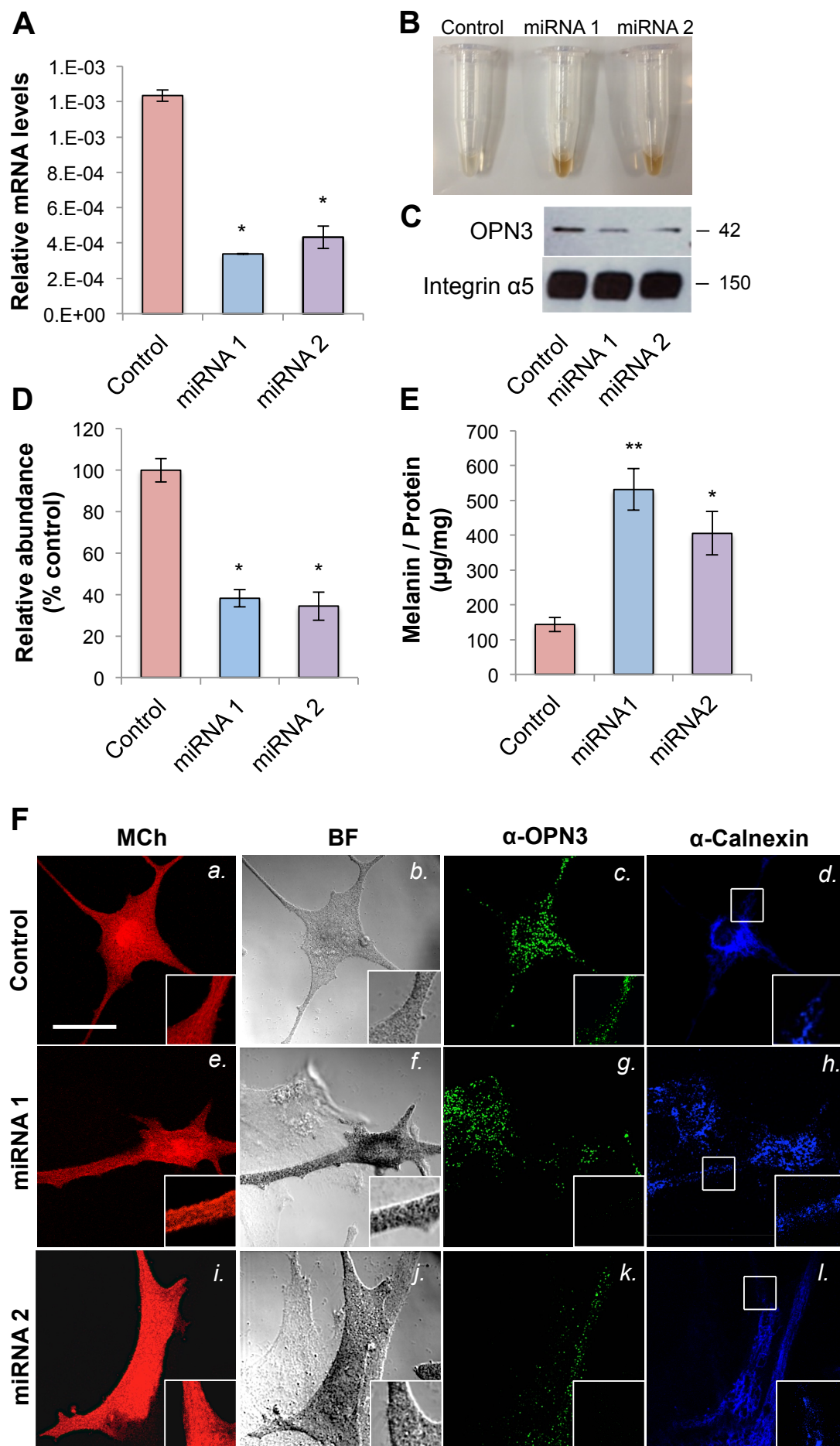
MC1R in a G α i dependent manner. In addition to that, OPN3 might play role in the internalization and desensitization of MC1R, as well as inhibition of G α s and MC1R interaction through its long c terminus. Further studies will clarify the true nature of the MC1R and OPN3 interaction.



B

Level of amino acid identity and homolog to human OPN3 (%)							
	Mouse	Chicken	Anole	Pufferfish	Zebrafish	Medaka	Mosquito
Identity	86	70	72	61	60	59	36
Homology	91	81	84	76	76	73	55

Figure 4.1. Multiple sequence alignment of human OPN3 with OPN3 sequences from different animals. (A) Human and other animal OPN3 protein sequences were aligned using CLUSTALW. Human OPN3 (NM_014322), mouse OPN3 (NP_034228.1), chicken OPN3 (XP_426139.2), anole OPN3 (XP_008122094.1), pufferfish OPN3 (XP_003963715.1), zebrafish OPN3 (NP_001104634.1), medaka OPN3 (NP_001292332.1) and mosquito OPN3 (BAN05625.1). Transmembrane regions of opsins were predicted by PredictProtein program [130]. The unique c-terminal sequence was labeled with a green outline. Predicted transmembrane domain (TM) 1 to 7 and helix (H) 8 of the OPN3s are labeled with purple to red infill. iL stands for *intracellular* domain; eL, *extracellular* domain; n-term, n-terminal region; c-term, c-terminal region. (B) Human OPN3 protein sequence was compared via NCBI's Protein BLAST [131] to mouse OPN3, chicken OPN3, anole OPN3, pufferfish OPN3, zebrafish OPN3, medaka OPN3, mosquito OPN3.



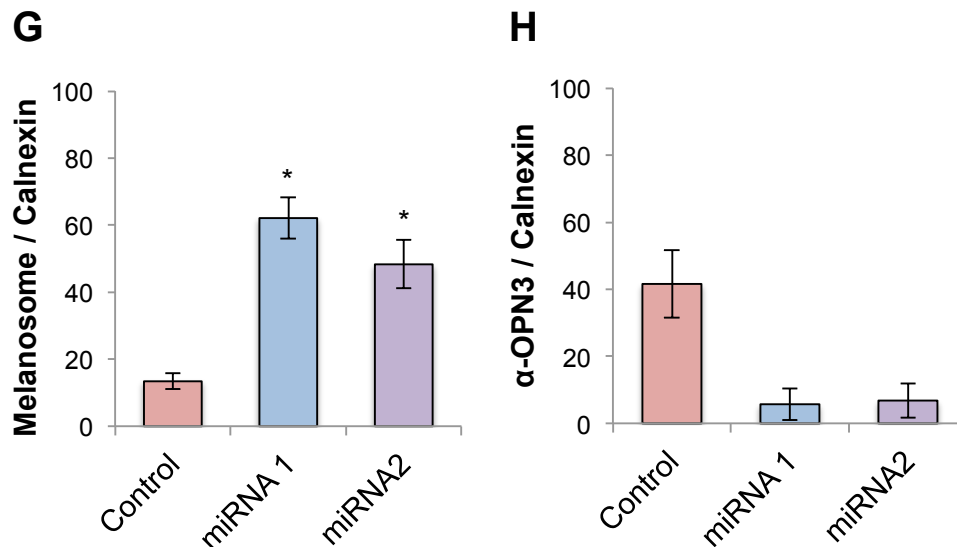


Figure 4.2. Knock down of OPN3 leads to melanin level increase in HEMs. (A) Quantitative PCR analysis of the mRNA expression of OPN3 control and OPN3-targeting miRNAs (miRNA 1 and miRNA 2) transduced HEMs, using primers detecting only full-length OPN3 and calculated relative to actin levels. miRNA 1 and miRNA 2 show significantly lower level of OPN3 mRNA compared to control. Bars represent the average of three experiments. (B) The appearance of the cell pellet after centrifugation. The cells with reduced OPN3 expression had more melanin (appeared darker) than control cells. (C) Western blot analysis of OPN3 antibody staining on control, OPN3 miRNA 1 and 2 transduced HEMs. Results are representative of two independent experiments. Integrin $\alpha 5$ was used as a positive control. (D) Quantification of western blot analysis show that miRNA 1 and miRNA 2 transduced HEMs show significantly lower level of OPN3 protein compared to control cells. Bars represent the average of three independent experiments, calculated relative to Integrin $\alpha 5$ levels. (E) Melanin analysis of control, miRNA 1 and 2 transduced HEMs revealed that miRNA 1 and 2 cells contain significantly more melanin compared to control cells. For normalization, melanin amount was divided by total protein level. Bars represent three independent experiments. (F) miRNA 1 and 2 transduced HEMs were labeled with anti-OPN3 and anti-Calnexin primary antibody and fluorochrome-conjugated secondary antibody. Transduced cell were detected by co-cistronic expression of mCherry (MCh). Dark spots in the brightfield images (BF) present the melanosomes. Confocal images represent day >20day post transduction of miRNA constructs. Bar, 10 μ m. (G) Calculation of relative melanosome levels was conducted in ImageJ (Percent Overlap = Melanosome/Calnexin Area). Results showed that miRNA 1 and miRNA 2 transduced HEMs has significantly more melanosome than control cells. Bars represent the average of 15 images taken from three independent experiments. (H) Calculation of relative melanosome levels was conducted in ImageJ (Percent = α -OPN3/Calnexin Area). Results showed that miRNA 1 and miRNA 2 transduced HEMs has less OPN3 labeling than control cells. Bars represent the average of 15 images taken from three independent experiments. **P < 0.01 and *P < 0.05.

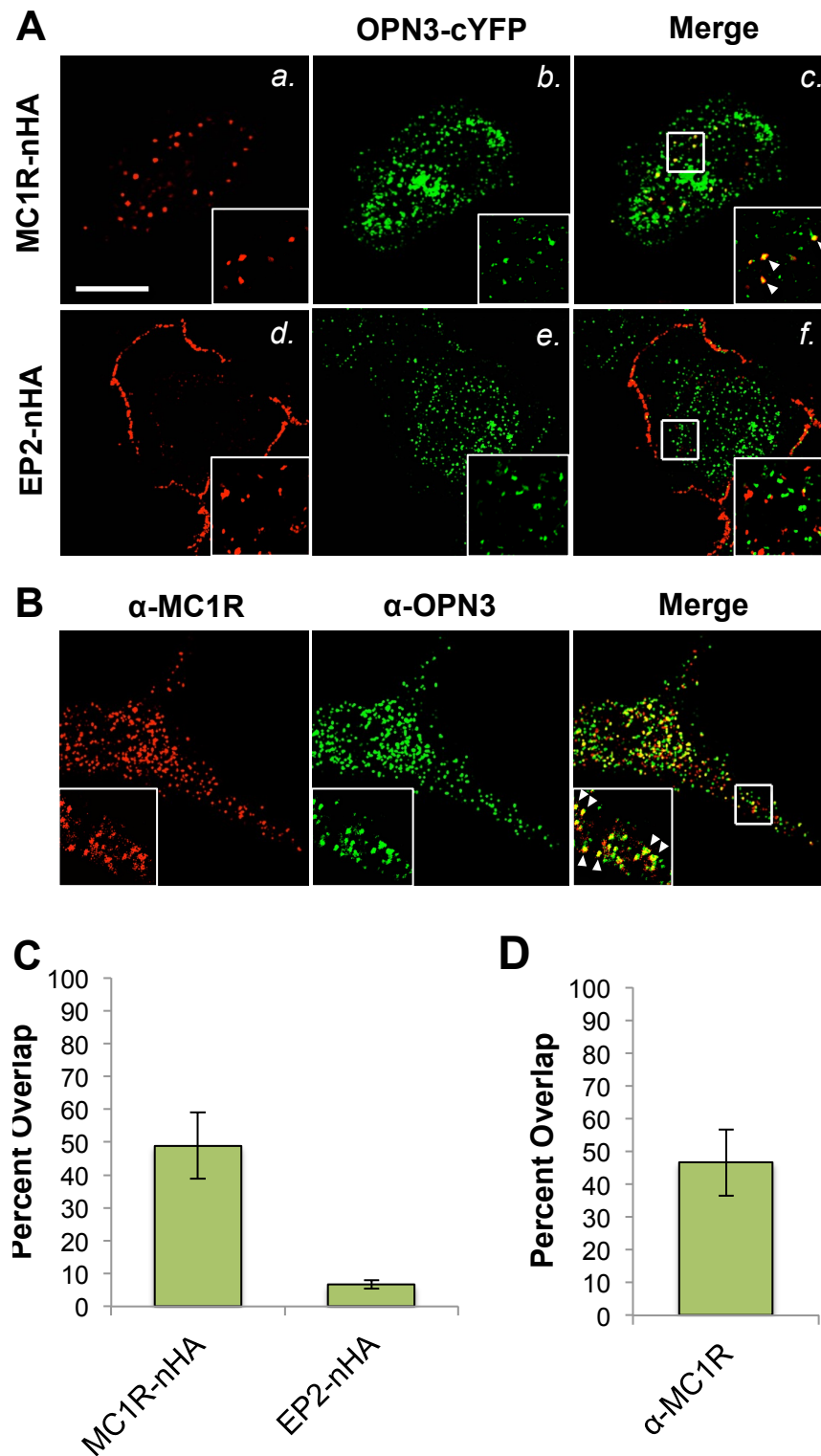


Figure 4.3. Localization of OPN3 with MC1R in HeLa and Human Epidermal Melanocytes. (A) Epitope tagged human OPN3, MC1R and prostaglandin E2 (EP2) protein, were expressed in HeLa cells. MC1R-nHA and EP2-nHA were labeled with anti-HA primary and fluorochrome-conjugated secondary antibody. Confocal images represent day 1 post transfection of OPN3 expression of HeLa cells kept in the dark. Bar, 10 μ m. (B) HEM cells, which endogenously express OPN3 and MC1R, were labeled with anti-OPN3 and anti-MC1R primary and fluorochrome-conjugated secondary antibody. (C) Calculation of colocalization was conducted in ImageJ (Percent Overlap = Overlap Area/ OPN3-nYFP Area). Results showed that MC1R-

nHA (a-c) protein extensively colocalizes with OPN3-nYFP, whereas no overlap was observed for EP2-nHA (d-f). Bars represent the average of 20 images taken from three independent experiments. (D) Calculation of colocalization was conducted in ImageJ (Percent Overlap = $\text{Overlap Area} / \alpha\text{-OPN3 Area}$). Results showed that α -MC1R labeling extensively colocalizes with α -OPN3. Bars represent the average of 20 images taken from three independent experiments. Tagged proteins are named as the following: OPN3-cYFP, c terminal YFP tagged full-length OPN3; MC1R-nHA, n terminal HA tagged full-length MC1R and EP2-nHA, n terminal HA tagged full-length EP2.

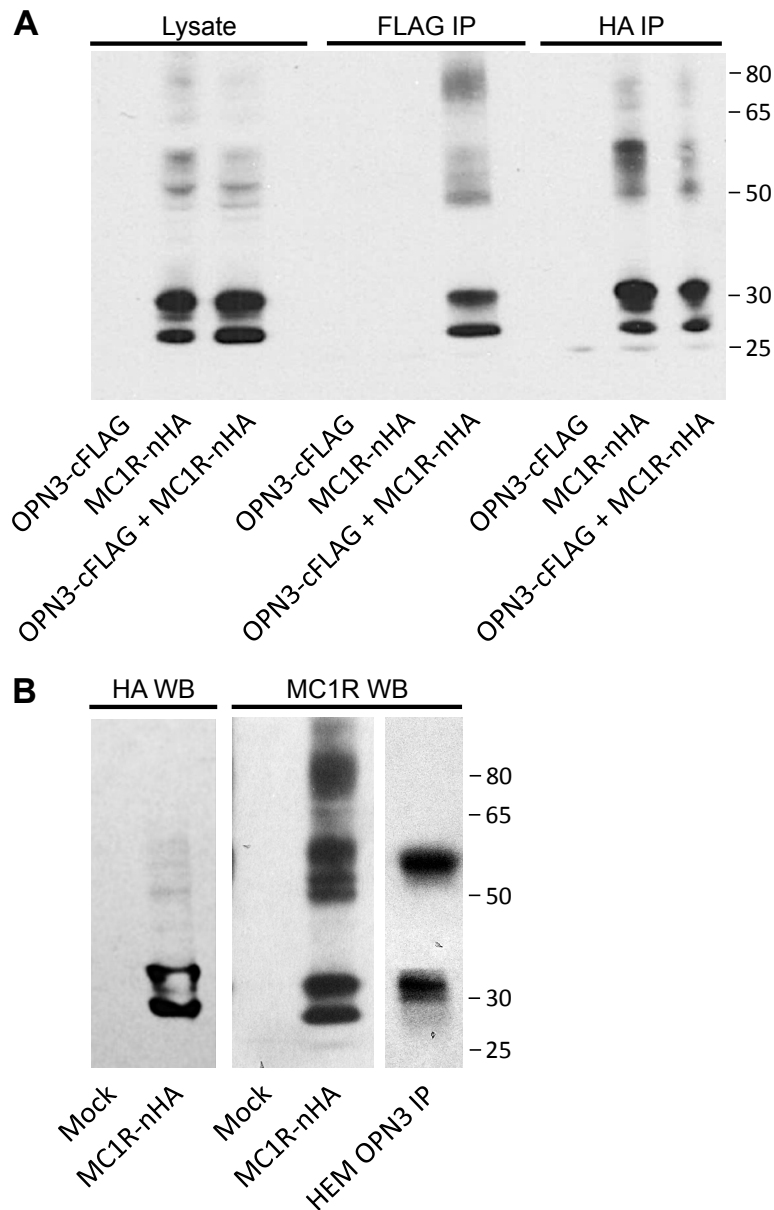


Figure 4.4. Co-immunoprecipitation of OPN3 with MC1R in HeLa and Human Epidermal Melanocytes. (A) Epitope tagged human OPN3 and MC1R or OPN3+MC1R expressing HeLa cell lysates were used for co-immunoprecipitation by anti-FLAG (FLAG IP) and anti-HA antibody (HA IP), followed by HA staining. In the experimental condition, FLAG IP OPN3-cFLAG + MC1R-nHA lane, OPN3 and MC1R protein pulled-down together, which suggest that OPN3 and MC1R heterodimerize, higher order heterooligomeric complex or a scaffold-mediated protein complex. Western blot results represent day 2 post transfection of OPN3 and MC1R expression of HeLa. (B) Epitope tagged human MC1R expressing HeLa cell lysates were used for testing the specificity of anti-MC1R antibody by comparing the HA staining (HA WB) and MC1R staining (MC1R WB) of MC1R-nHA expression HeLa cell lysate. Both staining gave same size bands, app. 30 kDa, which suggests that MC1R antibody specifically, detected MC1R protein. HEM OPN3 IP, co-immunoprecipitation by anti-OPN3 (OPN3 IP) followed by MC1R staining, showed that endogenously expressed OPN3 could pull down MC1R in HEMs. Tagged proteins are named as the following: OPN3-cFLAG, c terminal FLAG tagged full-length OPN3; MC1R-nHA and n terminal HA tagged full-length MC1R.

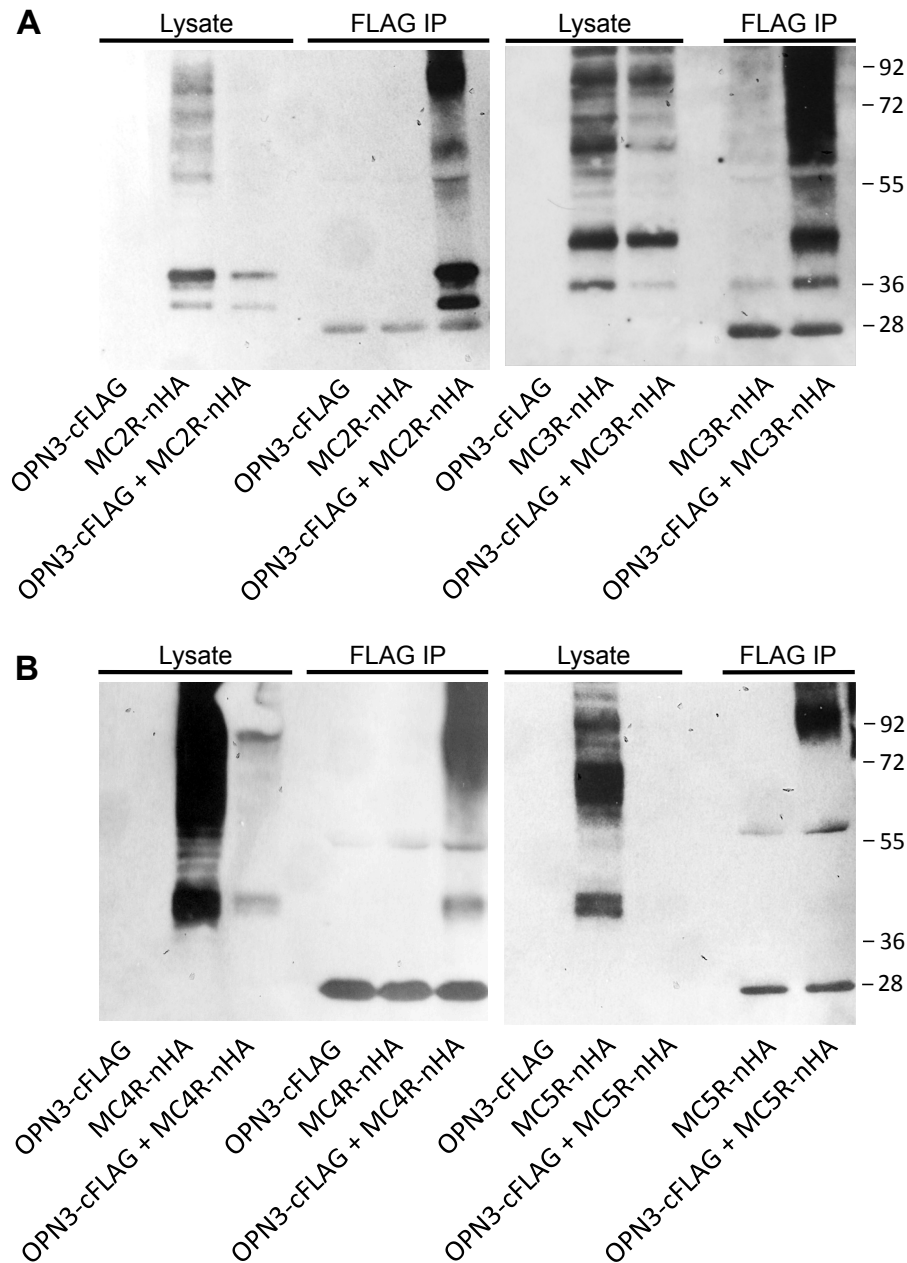


Figure 4.5. Co-immunoprecipitation of OPN3 with MC2R, MC3R, MC4R and MC5R in HeLa. Epitope tagged human OPN3, melanocortin receptor (MCXR) and OPN3+MCXR expressing HeLa cell lysates were used for co-immunoprecipitation by anti-FLAG (FLAG IP), followed by HA staining. Western blot results represent day 2 post transfection of OPN3 and MCXR expression of HeLa. In the experimental condition, FLAG IP OPN3-cFLAG + MCXR-nHA lane, (A) OPN3 and MC2R and MC3R protein pulled-down together, which suggest that OPN3 and MC2R or MC3R heterodimerize, higher order heterooligomeric complex or a scaffold-mediated protein complex. (B) OPN3 and MC4R protein pulled-down together, which suggest that OPN3 and MC4R heterodimerize, higher order heterooligomeric complex or a scaffold-mediated protein complex. MC5R did not show co-immunoprecipitation. Tagged proteins are named as the following: OPN3-cFLAG, c terminal FLAG tagged full-length OPN3; MC2R-nHA, n terminal HA tagged full-length MC2R; MC3R-nHA, n terminal HA tagged full-length MC3R; MC4R-nHA, n terminal HA tagged full-length MC4R; MC5R-nHA, n terminal HA tagged full-length MC5R.

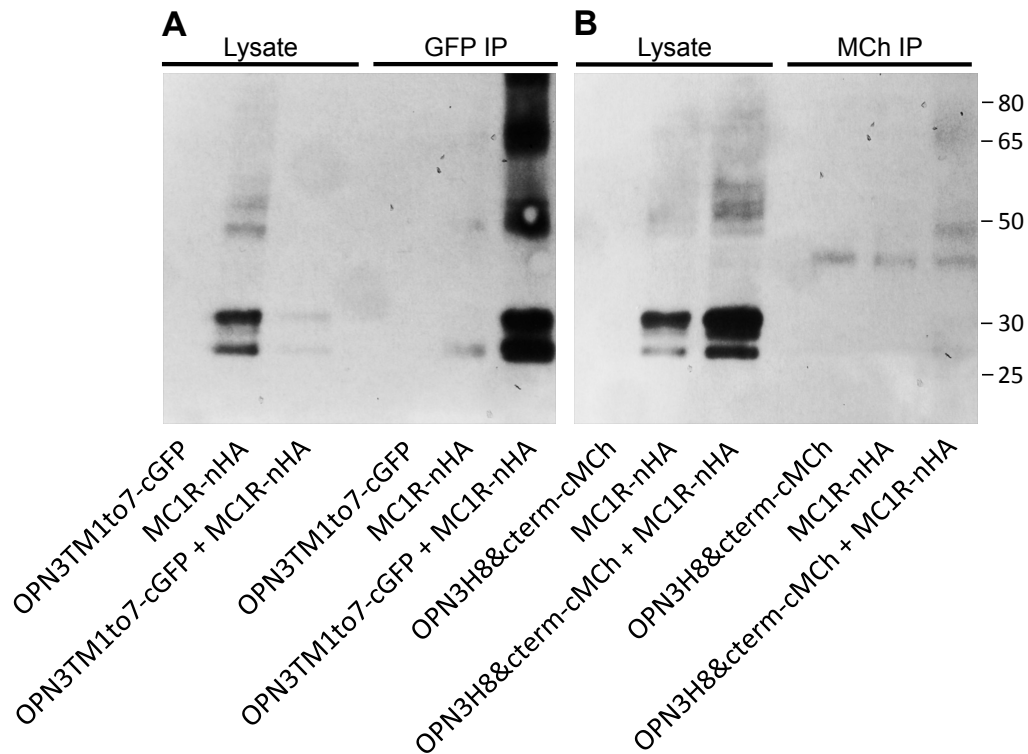


Figure 4.6. Co-immunoprecipitation of helix 8 and c terminus of human OPN3 with MC1R in HeLa. (A) MC1R and OPN3TM1to7-cGFP expressing HeLa cells lysates were used for co-immunoprecipitation by anti-GFP (GFP IP), followed by HA staining. Western blot results represent day 2 post transfection. In the experimental condition, GFP IP OPN3TM1to7-cGFP + MC1R-nHA lane, OPN3TM1to7-cGFP and MC1R protein pulled-down together, which suggest that OPN3TM1to7-cGFP and MC1R heterodimerize, higher order heterooligomeric complex or a scaffold-mediated protein complex. (B) MC1R and OPN3H8&cterm-cMCh expressing HeLa cell lysates were used for co-immunoprecipitation by anti-mCherry (MCh IP), followed by HA staining. Western blot results represent day 2 post transfection. In the experimental condition, MCh IP OPN3H8&cterm-cMCh + MC1R-nHA lane, OPN3H8&cterm-cMCh and MC1R protein did not pulled-down together, which suggest that OPN3H8&cterm-cMCh and MC1R do not interact. Tagged proteins named as following: OPN3TM1to7-cGFP, c terminal GFP tagged transmembrane 1 to 7 of OPN3; OPN3H8&cterm-cMCh, c terminal mCherry tagged helix 8 and c terminus of OPN3 (See Figure 5.1); MC1R-nHA, n terminal HA tagged full-length MC1R.

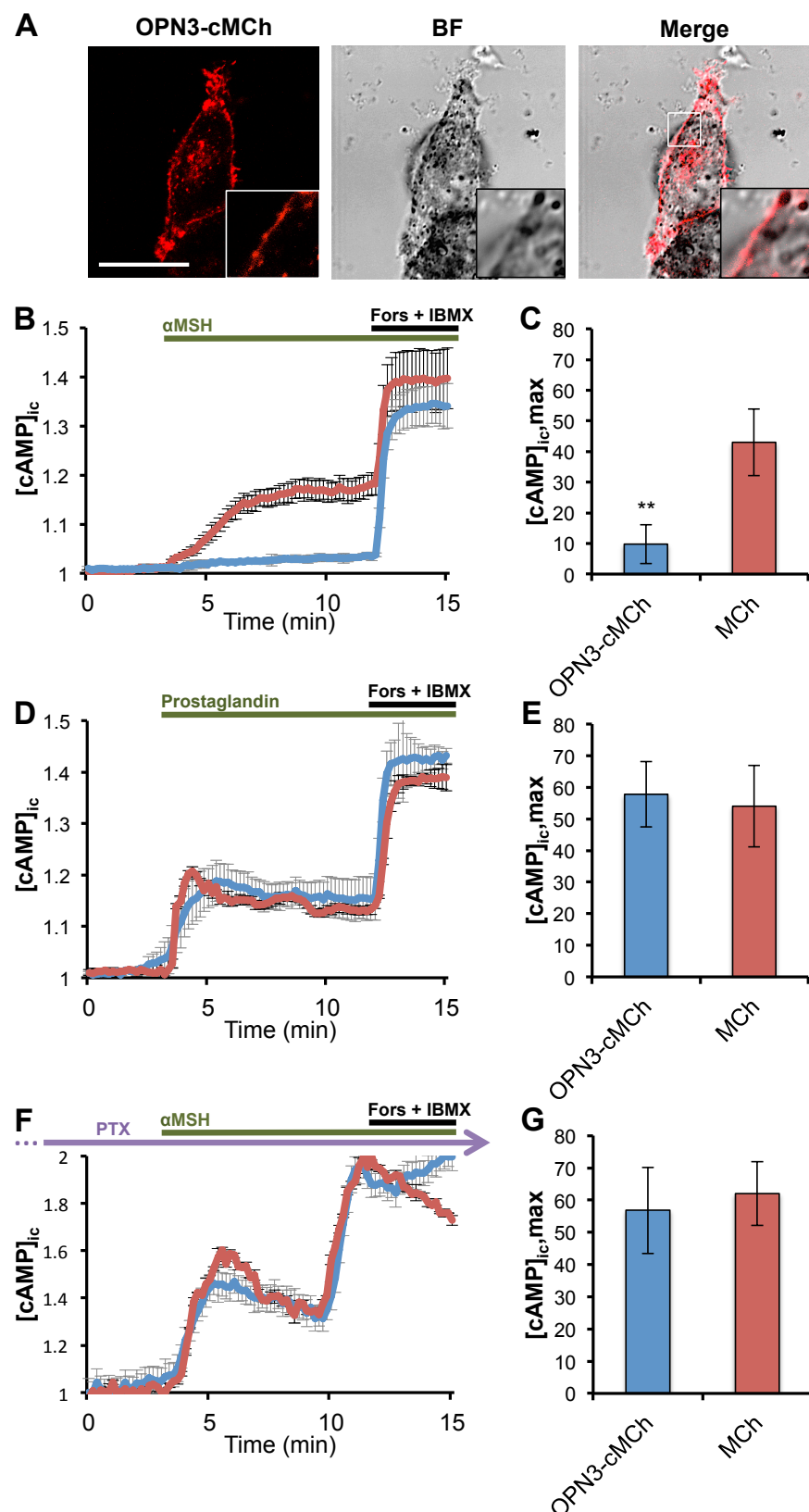


Figure 4.7. OPN3 decreases the cAMP response to α MSH in MNT-1 in Gai dependent manner. (A) MNT-1 cells expressing OPN3-cMCh were imaged with confocal microscope 2 days post transfection. OPN3-cMCh showed plasma membrane and intracellular vesicle localization. Bar, 10 μ m. (B) cAMP imaging of OPN3-cMCh and only MCh expressing MNT-1 cells stimulated with MSH (agonist of MC1R, a G α s coupled GPCR). OPN3-cMCh significantly decrease the α MSH

induced cAMP (intracellular cAMP response, $[cAMP]_{ic}$) response of MC1R (C) Average amplitude of cAMP responses to α MSH stimulus compared to Forskolin + IBMX response (Percent = α MSH induced cAMP response/ Forskolin + IBMX induced cAMP response*100) (intracellular maximum cAMP response, $[cAMP]_{ic,max}$). Bars represent the average of 3 experiments $\pm$ SEM. (D) cAMP imaging of OPN3-cMCh and only MCh expressing MNT-1 cells stimulated with prostaglandin (agonist of prostaglandin receptor, a G α s coupled GPCR). OPN3-cMCh did not affect the prostaglandin induced cAMP response of prostaglandin receptor (E) Average amplitude of cAMP responses to prostaglandin stimulus compared to Forskolin + IBMX response (Percent = prostaglandin induced cAMP response/ Forskolin + IBMX induced cAMP response*100). Bars represent the average of 3 experiments $\pm$ SEM. (F) cAMP imaging of OPN3-cMCh and only MCh expressing MNT-1 cells were treated with PTX (G α i inhibitor) and then stimulated with α MSH. OPN3-cMCh significantly did not affect the α MSH induced cAMP response of PTX treated MNT-1 cells. This finding suggest G α i play role in OPN3 mediated inhibition of MC1R response demonstrated in B. (G) Average amplitude of cAMP responses to α MSH stimulus compared to Forskolin + IBMX response (Percent = α MSH induced cAMP response/ Forskolin + IBMX induced cAMP response*100). Bars represent the average of 3 experiments $\pm$ SEM. **P < 0.01.

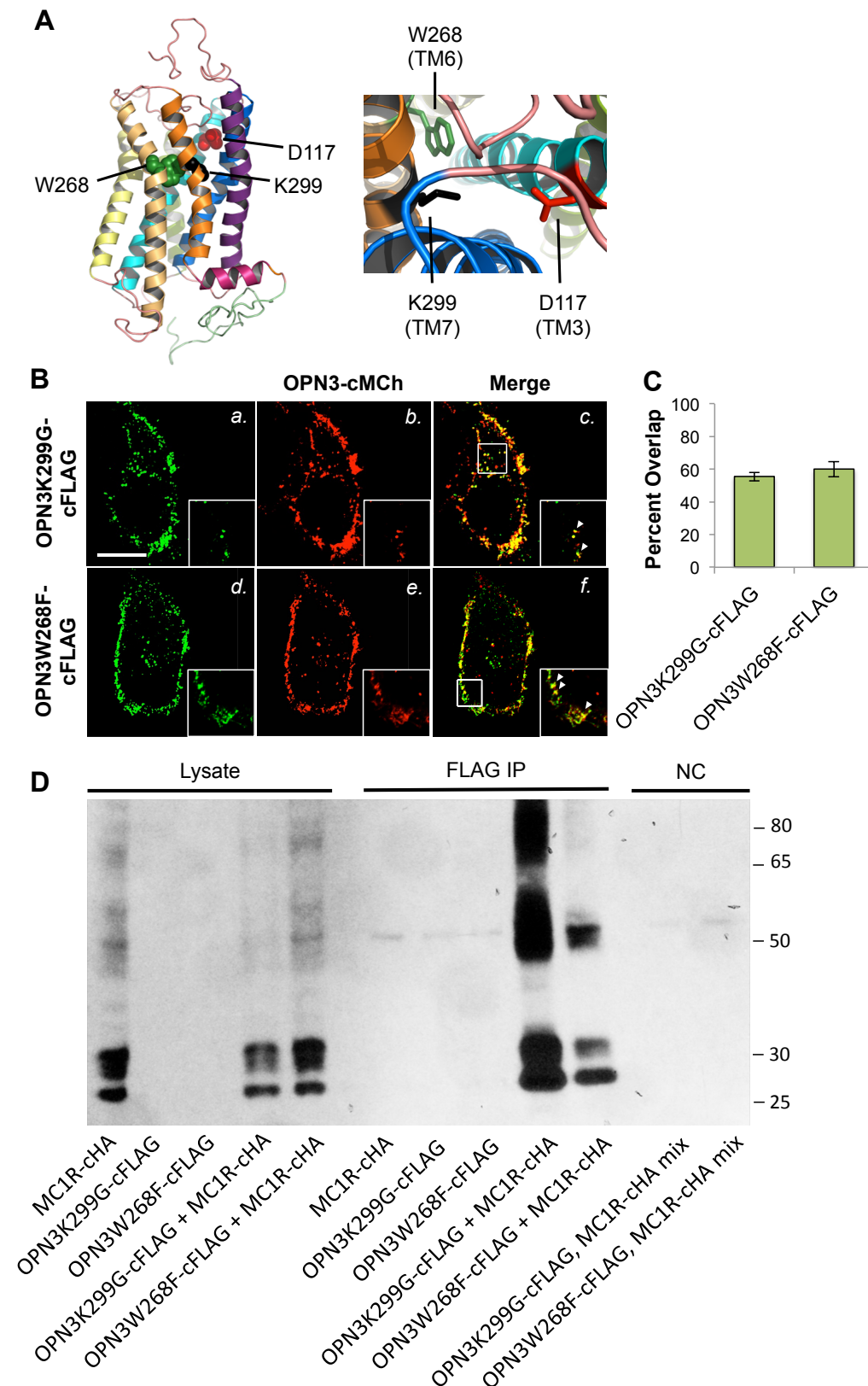


Figure 4.8. Localization and MC1R interaction of OPN3 K299G and W268F. (A) Localization of K299 and W268 residues are shown in the predicted structure of OPN3. W268 residue is in close proximity of hypothetical retinal binding site of human OPN3. (B) HeLa cells expressing OPN3-cMCh, OPN3K299G-cFLAG and OPN3W268F-cFLAG were imaged with confocal microscope. Images represent day 1 post transfection of HeLa cells kept in the dark. Bar, 10 μ m. (C) Calculation of

colocalization was conducted in ImageJ ($\text{Percent Overlap} = \text{Overlap Area} / \text{OPN3-cMCh Area}$). Results showed that OPN3K299G-cFLAG and OPN3W268F-cFLAG proteins extensively colocalize with OPN3-cMCh. Bars represent the average of 20 images taken from three independent experiments. (D) MC1R-nHA and OPN3K299G-cFLAG or OPN3W268F-cFLAG expressing HeLa cell lysates were used for co-immunoprecipitation by anti-FLAG (FLAG IP), followed by HA staining. Results shows that K299G and W268F mutation do not affect the interaction of OPN3 with MC1R. Western blot results represent day 2 post transfection. Tagged proteins are named as the following: OPN3K299G-cFLAG, c terminal FLAG tagged full-lenght OPN3 with mutation of lysine (K) 299 to glycine (G); OPN3W268F-cFLAG, c terminal FLAG tagged full-lenght OPN3 with mutation of tryptophan (W) 268 to phenylalanine (F) (See Figure 5.1); MC1R-nHA and n terminal HA tagged full-lenght MC1R.

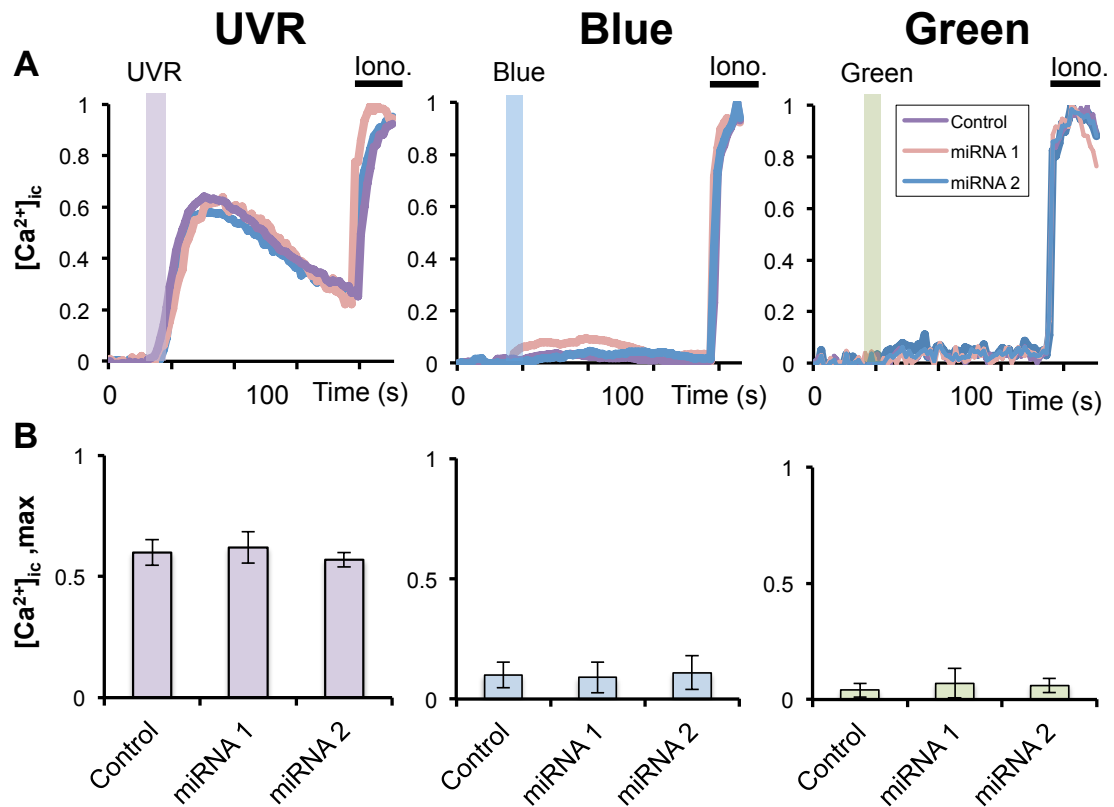


Figure 4.9. Ca^{2+} response of control and OPN3-targeted miRNA transduced HEMs in response to UVR, blue and green light. (A) Ca^{2+} imaging of control, miRNA 1 or miRNA 2 transduced HEMs stimulated with 200 mJ/cm² ultraviolet radiation (UVR, max. λ 360 nm), blue (max. λ 450 nm) or green light (max. λ 550 nm). lonomyacin (lono) responses were used to normalize Ca^{2+} responses. (B) Average amplitude of HEM Ca^{2+} responses to UVR, blue and green light exposure. Results suggest that decrease of OPN3 level does not affect the Ca^{2+} response to UVR in HEMs. Also, there is no Ca^{2+} response to blue or green light in presence or absence of OPN3. Bars represent the average of 5 experiments $\pm$ SEM.

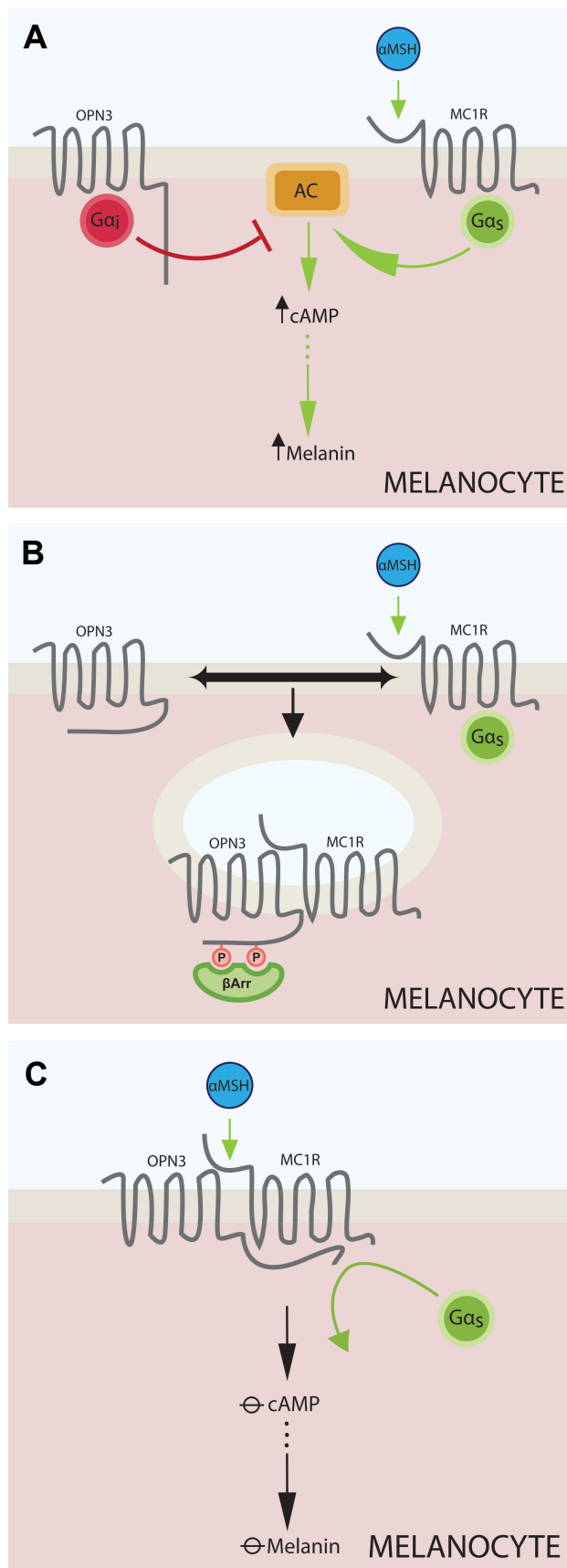


Figure 4.10. Three hypothetical models of OPN3 and MC1R interaction.

CHAPTER 5. Experimental Methods

Cell culture, Transfection and Transduction

Cell culture reagents were purchased from ThermoFisher Scientific, unless stated otherwise. HeLa and HEK 293 cells were maintained under standard conditions in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and streptomycin. MNT-I cells were maintained under standard conditions in DMEM supplemented with 18% FBS, 10% AIM-V®, 100 units/ml penicillin and streptomycin. Primary human epidermal melanocytes (HEM) obtained from neonatal foreskin (Cascade Biologics) were maintained under standard conditions in Medium 254 supplemented with 1% human melanocyte growth supplement (HMGS-2, Cascade Biologics), 100 units/ml penicillin and streptomycin. HEM cells and propagated for a limited number of cell divisions (≤ 20). Primary human keratinocytes (KER; Lifeline Cell Technology) were cultured in keratinocyte media with keratinocyte growth factors (Lifeline Cell Technology) and propagated in an undifferentiated state for a limited number of cell divisions (usually ≤ 10). Human lung fibroblasts CCD-19Lu (ATCC) cells were cultured in EMEM/FBS. Neutrophils and buffy coat cells extracted from blood were kindly provided by Dr. Reichner's laboratory (Brown University) and used without further culturing. Human Umbilical Vein Endothelial Cells (HUVEC) were cultured in EGM-2 (Lonza) and HL60 cells in IMDM with 20% FBS.

For cellular localization experiments, HeLa cells were transiently transfected with 1 μ g of DNA in 35 mm cell culture plate (Corning Incorporated) using Lipofectamine 2000. For cAMP imaging experiments, MNT-I cells were transiently transfected with OPN3 c-mCherry or only mCherry constructs using U-019 Nucleofector® Program with Nucleofector™ Kits for Human Melanocytes (Lonza).

For microRNA (miRNA) experiments, HEM cells were transduced with either targeted (miRNA1 and miRNA2) or scrambled miRNAs (Invitrogen) using BLOCK-IT™

Lentiviral RNAi expression system (Invitrogen) according to manufacturers' instructions. miRNA 1 target exon 2 (CTCCTGGGATGGAACAGGTAC) and miRNA 2 target exon 3 (CCTGGTCTGTTGGATGCCTTA) of human OPN3. I changed the GFP marker of purchased targeted or scrambled miRNA constructs to mCherry. The transduction rates were app. 60% with both OPN3 miRNA and control miRNA as detected by co-expression of mCherry. Three days after transduction, cells were treated with Blasticidin for antibiotic selection. Cells were analyzed in melanin assay experiments 14 days after transduction.

DNA Constructs

cDNAs encoding full length human OPN3 were generated by RT-PCR from RNAs extracted from HEM cells. Expression vectors for the various constructs containing OPN3, MC1R and Arrestin cDNA sequences depicted in Figure 5.1 were assembled as follows; gene-specific and amplification site-specific oligonucleotide primers were designed for PCR amplification of different OPN3 regions. Full length OPN3 cDNA was used as a template for PCR reactions. PCR products were digested with restriction enzymes, gel purified (Qiagen) and ligated into expression vectors. Mutations were introduced by site-directed mutagenesis using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene). Full list of DNA constructs with their respective amplification primers and restriction sites was shown at Table 6.1. The human n3xHA-MC1R and n3xHA-EP2 expression vectors were purchased from cDNA Resource Center. All designed DNA constructs were checked by sequencing and tested in at least three different series of transfection experiments. Image analysis was carried out using Zeiss Zen software and Image J software.

RNA extraction, Reverse Transcription, PCR

HEM and KER cDNA was obtained by reverse transcription (RT) using SuperScript III kit (ThermoFisher Scientific) with total RNA extracted from cultured cells using the RNeasy Plus kit (Qiagen).

For PCR amplification of full-length OPN3 transcript, following protocol was used: 95°C for 45 s, 56°C for 45 s and 72°C for 1.5 min (32 cycles). Among other polymerase options, GoTaq® DNA polymerase (Promega) was found much efficient when it comes to cloning OPN3, which has a n-terminal G/C rich area. PCR products were sequenced directly, or cloned into a plasmid vector (TOPO-TA, ThermoFisher Scientific) before sequencing. For experiments described in Chapter 2; mostly touchdown PCR protocol was used with the primers shown at Table 5.2 [22].

Quantitative PCR

Quantitative PCR (qPCR) was performed with the primers shown at Table 5.3. Template dose–response curve was used to measure the primer efficiency. Only primers with slopes within 10% of the theoretical slope were used for amplification. Reactions were prepared according to manufacturer's instructions using Power SYBR Green (Applied Biosystems) and cycled on a 7500 Real-Time PCR System (Applied Biosystems). Actin was used as an internal control and all reactions were run in triplicate. mRNA levels were determined by calculating $2^{-\Delta Ct}$ values for each opsin. In chapter 3, I investigated high OPN3 expressing tissues using a commercially available human tissue qPCR array (Origene).

To quantify the mRNA level of full-length and truncated isoforms of OPN3, primers based on exon 2 and 3 to detect full-length OPN3 exclusively, and primers based on exons 3 and 4 to detect both the full-length and truncated isoforms were designed.

We calculated the expression level of the truncated isoform by subtracting the expression of the full-length measured with the first primer pair from the expression of both isoforms measured with the second primer pair. Localization of these qPCR primers on OPN3 DNA sequence was shown at Figure 5.2.

Western Blot

Western Blot reagents were purchased from ThermoFisher Scientific, unless stated otherwise. Cells were rinsed briefly in cold PBS before the addition of 500 μ l lysis buffer [300mM NaCl, 50mM Tris-HCl (pH 7.4), 1% Triton X100, and protease inhibitor mixture (Roche)]. Cells were scraped from plates and homogenized using a 22G syringe. Lysates were rotated end-over-end for 1 h at 4°C then centrifuged at 14,000 rpm for 30 min at 4°C to remove cell debris.

Total protein content was determined using the Pierce BCA Protein Assay Kit. Equal amounts of protein were loaded onto each lane, separated by electrophoresis on NuPAGE Bis-Tris gels (Life Technologies), and then transferred to PVDF membranes (Roche Applied Science). Membranes were blocked at room temperature for 1 h and incubated overnight at 4°C with primary antibody followed by 1 h at RT with HRP-conjugated secondary antibody. The full list of western blot antibodies with their respective dilution ratios was shown at Table 5.4. Immunoreactive protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate and visualized with CL-XPosure Film. For experiments described in Chapter 2; different than the protocol described above, cells were homogenized in ice-cold buffer containing: 300mM sucrose, 35mM Tris (pH 7.4), 10mM EDTA, 10mM EGTA [22].

Immunoprecipitation Assay

Cells were rinsed briefly in cold PBS before the addition of 500 μ l lysis buffer [300mM NaCl, 50mM Tris-HCl (pH 7.4), 1% Triton X100, and protease inhibitor mixture (Roche)]. Cells were scraped from plates and homogenized using a 22G syringe. Lysates were rotated end-over-end for 1 h at 4°C then centrifuged at 14,000 rpm for 30 min at 4°C to remove cell debris. 15 μ L of 50% (wt/vol) protein A/G- or protein A-agarose beads (Santa Cruz Biotechnology) were added to 400 μ L of supernatant and rotated for 30 min to preclear the samples. Samples were centrifuged at 14,000 rpm for 5 min, and the agarose pellet was discarded. Samples were split into two 200 μ L aliquots and mixed with either 25 μ L of pre-conjugated HA-protein A agarose beads (Santa Cruz Biotechnology) or 25 μ L of pre-conjugated FLAG-protein A agarose beads and rotated overnight at 4°C. The immunoprecipitates were collected by centrifugation at 7,000 rpm for 5 s, washed three times with wash buffer [300mM NaCl, 50mM Tris-HCl (pH 7.4), and 0.1% Triton X100], and solubilized in a mixture of 10 μ L elution buffer [100mM Tris-HCl, 1% SDS, 10mM DTT] and 5 μ L SDS-PAGE sample buffer (ThermoFisher Scientific). After this point, the western blot protocol is followed.

Immunocytochemistry

Cells were seeded on coverslips and maintained as described above. The cells were fixed by incubation with 4% paraformaldehyde at room temperature for 10 min. After blocking with blocking solution [0.1% BSA, 0.2 % saponin, 0.02% sodium azide in PBS] for 1h, cells were incubated overnight with the primary antibody. For visualization, secondary fluorescent antibodies (Life Technologies) were used. The full list of immunocytochemistry antibodies with their respective dilution ratios was

shown in Table 5.5. Imaging was performed using a LSM510 Meta confocal microscope (Zeiss) with a 63X objective.

Quantification of colocalization was carried out using ImageJ software, as previously described [143]. Fluorescent images were converted to binary images for each channel by using Split Channel function. Using Subtract Background function eliminated the structures smaller than 5 pixels. The overlap area of binary images was quantified by using Image Calculator function at multiply setting. Threshold of each channel was set at 50 by default. Analyze Particle function was used to calculate the area of and the overlap of images and fluorescently tagged or stained OPN3 protein. The ratio of overlap was quantified using Percent Overlap = Overlap Area/OPN3 Area. Two proteins are considered colocalized if this ratio was higher than 50% [120].

Ca²⁺ imaging

Fluo-4 AM (Life Technologies), a cell-permeant fluorimetric Ca⁺⁺ indicator, was used for Ca⁺⁺ imaging. Fluo-4 shows excitation at $\lambda_{\text{max}} = 494\text{nm}$, emission at $\lambda_{\text{max}} = 516\text{nm}$ and has an equilibrium dissociation constant (K_d) around 345nM.

The day before the experiment, cells were plated on 12mm coverslips (Fisher Scientific). After this point every step of the protocol was performed under a dim red light. In experiments requiring retinal treatment, cells were incubated for 20 min in 12 μM all-*trans*-retinal (Sigma-Aldrich). Coverslips were then incubated in Ringers' solution [150 mM NaCl, 5mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 10mM HEPES, 10mM D-glucose] and 2 μM Fluo-4 and 250 μM sulfinpyrazone (uridine 5'-diphosphoglucuronosyltransferase inhibitor, Sigma-Aldrich) for 20 min. Sulfinpyrazone was used to prevent the loss of Fluo-4 from cells by inhibiting multidrug resistance-associated protein activity. Time-lapse fluorescence images were acquired every 2

sec using a GFP light cube on an inverted microscope (Olympus IX71) controlled with MetaMorph software (Molecular Devices). After acquiring 25 baseline images (50 s), 200 mJ/cm² UVR (λ_{max} 360 nm), blue (λ_{max} 460 nm) or green (λ_{max} 560 nm) light was applied. After acquiring 75 images (150 s), 25 μ M ionomycin (Sigma-Aldrich) was added to elicit maximal Fluo-4 fluorescence, to be used for normalization. Normalized fluorescence intensities were quantified using $F_{\text{norm}}(t) = (F_{\text{cell}}(t) - F_{\text{min}})/(F_{\text{iono}} - F_{\text{min}})$ formula [F_{cell} is the fluorescence of an intracellular region of interest, F_{iono} is the maximal fluorescence with ionomycin, and F_{min} is the baseline fluorescence before stimulation]. Light-induced changes in fluorescence intensity were quantified using MetaMorph (Molecular Devices), MATLAB (MathWorks), and Excel software (Microsoft).

UVR was applied by using a 400 nm short pass and 280 nm long pass filters (Newport) attached to a 200 W Hg-Xe arc lamp. For blue and green light, 460 and 560 nm LED light sources were attached to an LED current controller (Prizmatix). For all experiments, 20mW/cm² light power was applied on cells for 10 seconds, which gives 200 mJ/cm² energy ($E = P \times t$, energy (E), power (P), time (t)). An optic power meter (Newport) was used to measure light power at the imaging surface.

All-*trans*-retinal was prediluted in 100% ethanol (ThermoFisher Scientific) under dim red light and stored in amber glass at -20°C. Fluo-4, sulfinpyrazone and ionomycin were prediluted in DMSO (Sigma-Aldrich). For each tested condition and standard, three replicate experiments were performed and results were averaged. Light scattering of Ringers' solution was negligible.

cAMP imaging

Epac-H187, a FRET-based cAMP indicator transgenic protein, was used for cAMP imaging. Epac-H187 respond to cAMP based on conformational change of Epac

which separates n-terminal Turquoise (ex. 430 nm; em. 470nm) and c-terminal Venus (ex. 470 nm; em. 530nm) fluorophore groups; hence, decreased FRET reflect the increased cAMP level (Figure 5.3).

The 2 days before the experiment, MNT-1 cells were transfected with Epac-H187 construct [144, 145] and plated on 12mm coverslips (Fisher Scientific). As explained in Chapter 4, instead of hard-to-transfect HEM primary cells, MNT-1 cells were used for cAMP imaging experiments. MNT-1 cells can be transfected easily and they endogenously express MC1R and prostaglandin receptor, which respond to α MSH and prostaglandin stimulation, respectively.

The day before the experiment, cell media was changed to OPTI-MEM (ThermoFisher Scientific) to increase the MC1R level on the cell membrane by starving the cells. In the case of PTX treatment, cells were incubated in 200ng/ml PTX for 4 hours before the experiment. After this point every step of the protocol was performed under a dim red light. Coverslips were transferred to an imaging chamber, and covered with 1ml Ringers' solution [150 mM NaCl, 5mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 10mM HEPES, 10mM D-glucose]. Time-lapse fluorescence images were acquired every 10 sec using CFP and FRET light cubes on a inverted microscope (Olympus IX71) controlled with MetaMorph software (Molecular Devices). Cells were exposed to an excitation wavelength at 430 nm and emission of CFP and YFP was detected simultaneously through 470 $\pm$ 20 and 530 $\pm$ 25 band-pass filters of the CFP and FRET light cube. After acquiring 18 baseline images (3 min), 1 μ M NDP- α MSH (Sigma-Aldrich) or 5 μ M prostaglandin (Sigma-Aldrich) was applied. After acquiring 54 images (9 min), 25 μ M forskolin (Sigma-Aldrich) and 100 μ M IBMX (3-isobutyl-1-methylxanthine, Sigma-Aldrich) were added to elicit maximal cAMP fluorescence, to be used for normalization. Fluorescence intensities were quantified using $F = f_{\text{CFP}}/f_{\text{YFP}}$ ratio [f_{CFP} is the emission intensity of CFP, f_{YFP} is the emission intensity of YFP]. Normalized fluorescence intensities were quantified using $F_{\text{norm}}(t) = (F_{\text{cell}}(t) -$

$F_{\min})/(F_{f+i} - F_{\min})$ formula [F_{cell} is the fluorescence of an intracellular region of interest, F_{f+i} is the maximal fluorescence with forskolin and IBMX, and $F_{\min}$ is the baseline fluorescence before stimulation]. Light-induced changes in fluorescence intensity were quantified using MetaMorph (Molecular Devices), and Excel software (Microsoft).

NDP- α MSH, prostaglandin, forskolin and IBMX were prediluted in DMSO (Sigma-Aldrich). After addition of chemicals, the final DMSO concentration in the imaging chamber did not exceed 1‰ (v/v), which was well tolerated by cells. For each tested condition and standard, three replicate experiments were performed and results were averaged.

Melanin Assay

Cells were harvested by scraping and proteins were prepared by standard methods [146]. Lysates were centrifuged to separate melanin from solubilized protein. Melanin pellets were solubilized in boiling 1 M NaOH for 10 min. Spectrophotometric analysis of melanin content was performed at 405 nm absorbance. To calculate melanin/protein ration, protein content was measured using PierceTM BCA protein assay kit (ThermoFisher Scientific).

Statistical Analysis

All data are given as means $\pm$ SEM. Statistical differences among the experimental groups were analyzed by two-sided Student t test when comparing two experimental groups. Significance was defined as **P < 0.01 and *P < 0.05.

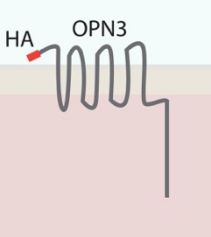
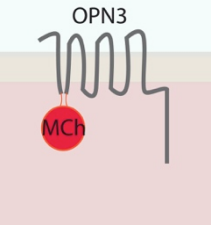
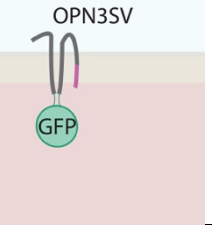
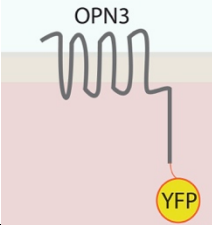
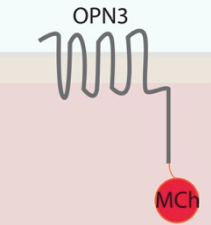
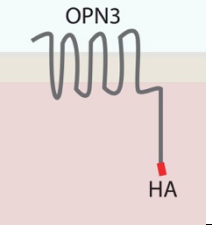
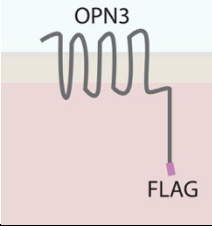
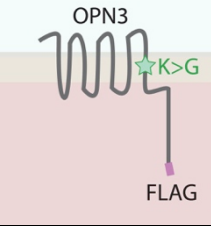
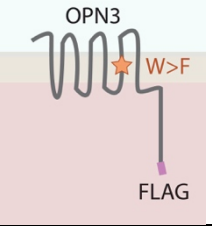
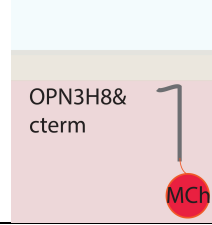
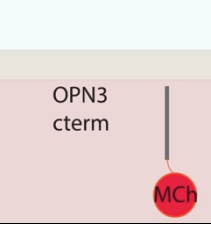
N-terminus & Intracellular loop1 tagged	OPN3-nGFP 	OPN3-nHA 	OPN3SV-nGFP 
	OPN3ΔC-nHA 	OPN3-iL1MCh 	OPN3SV-iL1GFP 
C-terminus tagged	OPN3-cYFP 	OPN3-cMCh 	OPN3-cHA 
	OPN3-cFLAG 	OPN3K299G-cFLAG 	OPN3W268F-cFLAG 
	OPN3TM1to7-cGFP 	OPN3TM1to7-c1D4 	
	OPN3H8&cterm-cMCh 	OPN3cterm-cMCh 	

Figure 5.1. List of OPN3 constructs. List of OPN3 constructs with their respective tagging epitope. GFP and MCh were shown in green and red circle, respectively. HA, FLAG and 1D4 epitope sequence are shown in red, purple and blue line, in sequence. SV, splice variant; ΔC, c terminal truncated; iL, *intracellular* domain; TM, *transmembrane* domain; H, helix; c-term, c-terminal region.

Name	Primer (5'-3')	Restriction site	Plasmid
OPN3-nGFP	F: CATCATGAATTCATGTACTCGGGG AACCGCAGCG R: CATCATCTCGAGCTACAAAGGAC GAACTTGGATTACATCAACTTTG	EcoRI - XhoI	pcDNA4 TO N-GFP
OPN3-nHA	F: CATCATGAATTCGGATGTACTCGG GGAACCGCAGCG R: CATCATCTCGAGCTACAAAGGAC GAACTTGGATTACATCAACTTTG	EcoRI - XhoI	pcDNA4 TO N-HA
OPN3SV-nGFP	F: CATCATGAATTCATGTACTCGGGG AACCGCAGCG R: ATGATGCTCGAGTCATATTTTAAA ATCTTGATCACTTGAATTGTCTGA	EcoRI - XhoI	pcDNA4 TO N-GFP
OPN3-cYFP	F: CATCATGGATCCATGTACTCGGG GAACCGCAGCG R: ATGATGCTCGAGCAAAGACGAAC TTGGATTACATCAACTTTG	BamHI - XhoI	pcDNA3.1 C-YFP
OPN3-cMCh	F: CATCATAAGCTTATGTACTCGGGG AACCGCAGC R: ATGATGCTCGAGCAAAGACGAAC TTGGATTACATCAACTTTG	HindIII - XhoI	pcDNA4 TO C-MCh
OPN3-cHA	F: CATCATGAATTCATGTACTCGGGG AACCGCAGCG R: ATGATGCTCGAGCAAAGACGAAC TTGGATTACATCAACTTTG	EcoRI - XhoI	pcDNA4 TO C-HA
OPN3-cFLAG	F: CATCATAAGCTTATGTACTCGGGG AACCGCAGC R: ATGATGCTCGAGCAAAGACGAAC TTGGATTACATCAACTTTG	HindIII - XhoI	pcDNA3.1 C-FLAG
OPN3K299 G-cFLAG	F: CTATTGTTTCGTACCTCTTTGCTG GATCGAACACTGTATACAATC R: GATTGTATACAGTGTTTCGATCCAG CAAAGAGGTACGAAACAATAG		pcDNA3.1 C-FLAG
OPN3W268 F-cFLAG	F: GATATTACCTTCCTGGTCTGTTT CATGCCTTATATCGTGATCTGC R: GCAGATCACGATATAAGGCATGAA ACAGACCAGGAAGGTGAATATC		pcDNA3.1 C-FLAG
OPN3ΔC- nHA	F: CATCATGAATTCATGTACTCGGGG AACCGCAGCG R: CATCATCTCGAGCTGCATTTCACT TCCAGCTGCTGGTAG	EcoRI - XhoI	pcDNA TO C-GFP
OPN3TM1to 7-cGFP	F: CATCATGAATTCATGTACTCGGGG AACCGCAGCG R: CATCATCTCGAGTCTGATCATGAA	EcoRI - XhoI	pcDNA4 TO C-GFP

	GACATAAATCACTGGATTGTAT		
OPN3TM1to 7-c1D4	F: CATCATGAATTCATGTACTCGGGG AACCGCAGCG R: ATGATGCTCGAGTTAGGCTGGAG CCACCTGGCTGGTCTCTCTGATCAT GAAGACATAAATCACTGGATTGTA	EcoRI - XhoI	pcDNA4 TO
OPN3H8&ct erm-cMCh	F: CATCATGATATCAGAAAGTTTCGA AGATCCCTTTTGCAGC R: ATGATGCTCGAGCAAAGACGAAC TTGGATTACATCAACTTTG	EcoRV - XhoI	pcDNA3.1 C-MCh
OPN3cterm- cMCh	F: CATCATGATATCCTGTGCCTCCGA CTGCTGAGGTG R: ATGATGCTCGAGCAAAGACGAAC TTGGATTACATCAACTTTG	EcoRV - XhoI	pcDNA3.1 C-MCh

F, forward primer; R, reverse primer

Table 5.1. List of primers used for making OPN3 constructs. List of OPN3 constructs with their respective amplification primers and restriction sites. Restriction sites and 1D4 epitope sequence are shown in red and blue, respectively. Mutation sites of site mutagenesis primers are labeled with gray.

Gene	Primer (5'-3')	exon span
OPN1-SW	F: GTGGGGCCGTGGGATGGGCCTCAGTACCAC R: CCTCAGTTGGGGCCAACTTGGGTAGACGAGACAG	e1 - e5
OPN1-SW	F: GGARGTGASSCGCATGGTGGTKGTGATG R: CCTCAGTTGGGGCCAACTTGGGTAGACGAGACAG	e4 - e5
OPN1-MW/LW	F: GGARGTGASSCGCATGGTGGTKGTGATG R: TCATGCAGGCGATACCGAGGACACAGATGAGAC	e4 - e5
OPN2	F: ATGAATGGCACAGAAGGCCCTAACTTCTACGTGCC R: TTAGGCCGGGGCCACCTGGCTCGTC	e1 - e5
OPN3	F: CGGCAACAACCTGCTGGTGCTCGTCCTCTAC R: CCTGTCCCCATCTTTCTGTGACATCACAATGG	e1 - e4
OPN3	F: GGTCATGGTCACCTGGTCACTCCAACAATATC R: CCTGTCCCCATCTT TCTGTGACATCACAATGG	e3 - e4
OPN5	F: TTCAGATCCCTCGTGGTTCGAGAACAGA R: GCAAGCCGTTCAACCATCATCTCTTGCTTTTGTAC	e1 - e3
OPN5	F: GGTGCCAACCCTACTTGCAAAATCTGCAGC R: GCAGAAGACTTCCTGACTGTGGTTACGGTGTGC	e5 - e6

F, forward primer; R, reverse primer

Table 5.2. List of PCR primers. List of primer pairs used for PCR with gene target, primer sequence and amplified sequence's exon span information.

Gene	Primer (5'-3')	exon span
OPN1-SW	F: TGTGCCTCTCTCCCTCATCT R: GGCACGTAGCAGACACAGAA	e3 - e4
OPN2	F: GAGTCAGCCACCACACAGAA R: CATGAAGATGGGACCGAAGTTGGAG F: CGGAGGTCAACAACGAGTCT R: TCTCTGCCTTCTGTGTGGTG	e4 e3 - e4
OPN3	F: CAATCCAGTGATTTATGTCTTCATGATCAGAAAG R: GCATTTCACTTCCAGCTGCTGGTAGGT F: CATTGCTATGGCCATATTCTATATTCCATTCTG R: ATCATTA AAAAGCACATTTTGGCCAGTTTC	e3 - e4 e2 - e3
OPN5	F: CTAGACGAAAGAAGAAGCTGAGACC R: GCGGTGACAAAAGCAAGAGA	e2 - e3

F, forward primer; R, reverse primer

Table 5.3. List of qPCR primers. List of primer pairs used for qPCR with gene target, primer sequence and amplified sequence's exon span information.

Product Name	Dilution	Source	Company
α -OPN3 (H-76)	1:2000	Rabbit polyclonal	Santa Cruz Biotechnology
α -OPN3 (ab75285)	1:500	Rabbit polyclonal	Abcam
α -MC1R (N-19)	1:2000	Goat polyclonal	Santa Cruz Biotechnology
α -Rhodopsin (Clone 1D4)	1:500	Mouse monoclonal	Chemicon
α -HA (Clone 3F10)	1:1000	Rat monoclonal	Roche
α -GFP (ab6556)	1:2000	Rabbit polyclonal	Abcam
α -FLAG (Clone M2)	1:1000	Mouse monoclonal	Sigma Aldrich
α -FLAG – HRP (Clone M2)	1:1000	Mouse monoclonal	Sigma Aldrich
α -Integrin α 5 (H-104)	1:500	Rabbit polyclonal	Santa Cruz Biotechnology
α -Rabbit – HRP (65-6120)	1:10000	Goat polyclonal	ThermoFisher Scientific
α -Mouse – HRP (62-6520)	1:10000	Goat polyclonal	ThermoFisher Scientific
α -Rat – HRP (62-9520)	1:2000	Goat polyclonal	ThermoFisher Scientific
α -Goat – HRP (sc-2020)	1:5000	Donkey polyclonal	Santa Cruz Biotechnology

Table 5.4. List of western blot antibodies. List of western blot antibodies with their respective dilution ratios.

Product Name	Dilution	Source	Company
α -OPN3 (H-76)	1:400	Rabbit polyclonal	Santa Cruz Biotechnology
α -MC1R (N-19)	1:400	Goat polyclonal	Santa Cruz Biotechnology
α -EP2 (E-13)	1:100	Goat polyclonal	Santa Cruz Biotechnology
α -Calnexin Alex647 (AF18)	1:200	Mouse monoclonal	Abcam
α -TYRP1 [Ta99] (sig38150-10)	1:50	Mouse monoclonal	Covance
α -LAMP1 (H4A3-s)	1:10	Mouse monoclonal	Hybridoma Bank
α -HA (Clone 3F10)	1:100	Rat monoclonal	Roche
α -FLAG (Clone M2)	1:1000	Mouse monoclonal	Sigma Aldrich
α -Rabbit Alex564	1:2000	Goat polyclonal	ThermoFisher Scientific
α -Rabbit Alex488	1:2000	Mouse polyclonal	ThermoFisher Scientific
α -Mouse Alex488	1:2000	Goat polyclonal	ThermoFisher Scientific
α -Mouse Alex564	1:2000	Goat polyclonal	ThermoFisher Scientific
α -Rat Alex564	1:2000	Goat polyclonal	ThermoFisher Scientific
α -Goat Alex564	1:2000	Donkey polyclonal	ThermoFisher Scientific

Table 5.5. List of immunocytochemistry antibodies. List of immunocytochemistry antibodies with their respective dilution ratios.

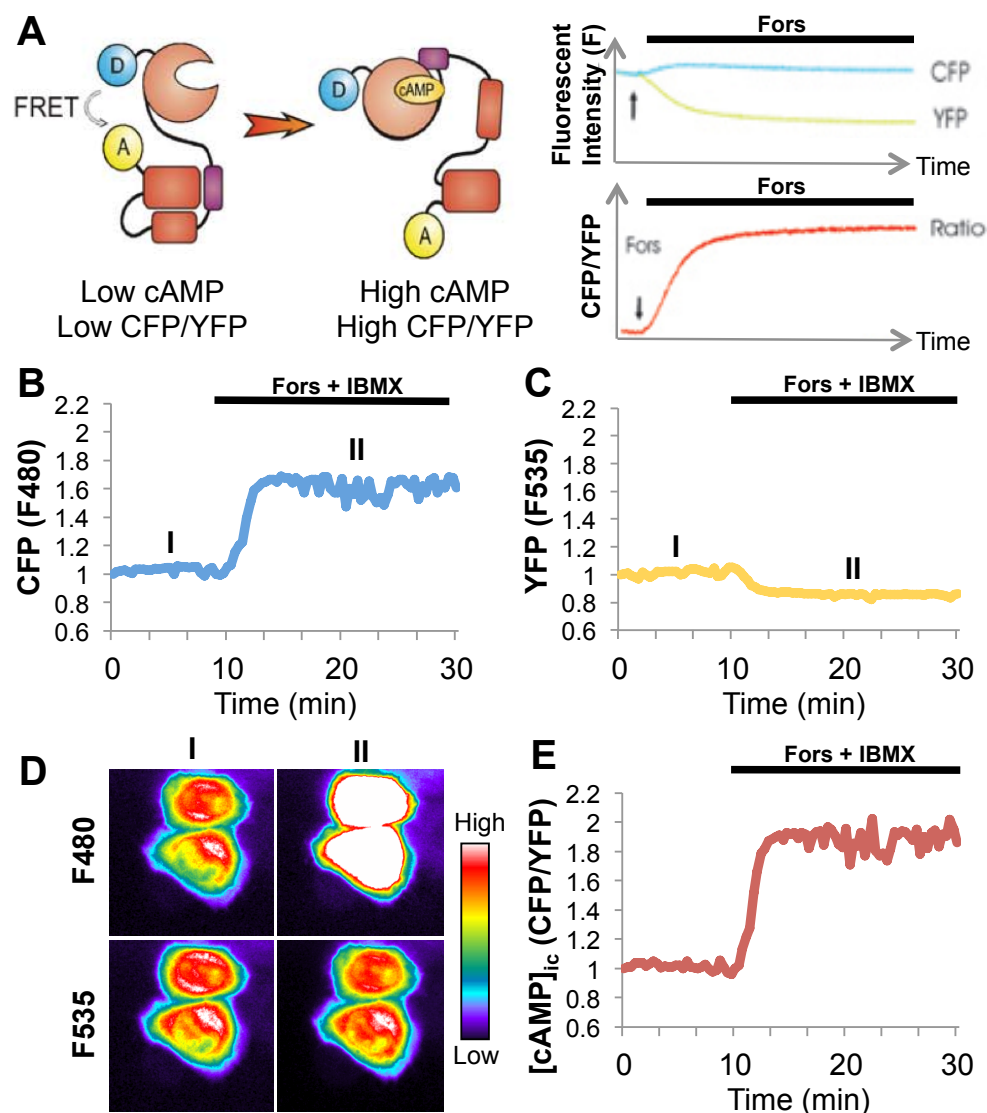


Figure 5.3. cAMP response of Epac-H187 expressing HeLa cells. (A) Conformational change model of Epac following binding of cAMP. FRET between the donor fluorophore, CFP, and acceptor fluorophore, YFP, tags allows detection of the conformational change of Epac. Greater levels of cAMP give higher levels of CFP over YFP emission (CFP/YFP). D, donor fluorophore; A, acceptor fluorophore; Fors, Forskolin. This image is modified from referred paper [144]. (B) CFP fluorescence (excitation at 430 nm; fluorescence detection at 480 nm (F480) through 470±20 band-pass filter) of Epac-H187 expressing HeLa cells stimulated with Forskolin + IBMX. Increase of intracellular cAMP with Forskolin + IBMX leads to increase in CFP emission. (C) YFP fluorescence (excitation at 430 nm; fluorescence detection at 535 nm (F535) through 530±25 band-pass filter) of Epac-H187 expressing HeLa cells stimulated with Forskolin + IBMX. Increase of intracellular cAMP with Forskolin + IBMX leads to decrease in YFP emission. (D) Pseudo-color images from baseline (I) and after Forskolin + IBMX (II) of B and C graph. (E) Relative intracellular cAMP level of cell was calculated based on CFP/YFP (F480/F535) ratio. Increase of intracellular cAMP with Forskolin + IBMX leads to increase in CFP/YFP ratio.

CHAPTER 6. Discussion and Future Direction

Summary

In this dissertation project, I investigated a novel human opsin, OPN3, with respect to its cellular localization and function in human skin cells. First, we investigated the expression of opsins in epidermal skin cells and found OPN1-SW, OPN2, OPN3 and OPN5 mRNA expression in human epidermal melanocytes (HEM) and keratinocytes. Furthermore, these results revealed that OPN3 is highly expressed compared to other human opsins [22]. Following that, I examined the localization of OPN3 in heterologously and endogenously OPN3 expressing HeLa and HEM cells, respectively. In addition, I studied the function of OPN3 in heterologously and endogenously expressing cells. Findings of this study revealed that a decrease in OPN3 level leads to an increased melanin level in human epidermal melanocytes. Furthermore, OPN3 co-immunoprecipitates with MC1R in heterologously and endogenously expressing cells and lastly, OPN3 modulates the α MSH induced cAMP response of MC1R and this functional interaction is blocked by inhibition of Gai activation. I therefore interpret these results to propose that OPN3 alters MC1R activity at the G protein level.

In this section, I explore how my dissertation findings contribute to the human opsin field, more specifically to our understanding of human OPN3's role in skin cells. In the first part of this, I interpret the results of Chapter 2, 3 and 4 with respect to findings from the literature. Then, I finish the section by exploring the possible trajectories for future research projects, which would enhance our understanding of human OPN3 function and its physiological affects in cells.

Expression and Function of OPN3 in Human Skin Cells

Opsin Expression in Human Epidermal Skin

- Presence of light sensitive opsins in skin raises the interesting question about a retinal source for these opsins in the case of light dependent activation. All-*trans*-retinol also known as vitamin A, is a reduced version of all-*trans*-retinal and it can be carried in the blood serum with the help of adaptor proteins such as serum retinol-binding protein (RBP) [147, 148]. Retinol dehydrogenase (RDH) is an enzyme that converts retinol to retinal and it is expressed in many tissues such as retina, liver and skin [149, 150]. Hence, blood serum retinol could function as a possible retinal source for skin. At this point, it is worth mentioning that fetal bovine serum (FBS), which is added as a supplement into cell culture media, has substantial amount of retinol, which could act as a source of retinal for cultured cells. Furthermore, all-*trans*-retinal and 13-*cis*-retinal are two stable isomers in thermodynamic equilibrium [151, 152]; thus, 13-*cis*-retinal can also be found in tissues that have access to all-*trans*-retinal. Since, the Terakita group found that mosquito OPN3, a homolog of vertebrate OPN3, forms a functional photopigment with 13-*cis*-, 11-*cis* and 9-*cis* retinal, the presence of 13-*cis*-retinal in the OPN3 expressing human skin cells would suggest that human OPN3 might function as a 13-*cis*-retinal bound photopigment in the skin. In addition, the expression of photoisomerase RPE65 [19], which converts all-*trans*-retinal to 11-*cis*-retinal, in keratinocytes suggests that 11-*cis*-retinal could be supplied to OPN3 expressing melanocytes by neighboring keratinocytes.
- Terakita groups also showed that OPN3 responds to blue-green light (λ max. 490 nm) in a bistable fashion. This raises an interesting question of whether OPN3 expressed in human epidermal melanocyte is exposed to blue light. As described in Figure 1.1 blue light penetrate the epidermis and reaches upper layer of dermis; hence, epidermal melanocytes are exposed to blue light coming from sun,

which would suggest that OPN3 could function as a blue light responsive photopigment.

- The novel splice variant of OPN3 (OPN3SV) mentioned in Chapter 2, has three transmembrane regions and a counterion residue at position 117 (D117), it is presumably nonfunctional because of the absence of transmembranes 4-7 and the crucial lysine residue at position 299 (K299) in the 7th trans-membrane domain (TM7) (Figure 3.3A). However, OPN3SV has an intact transmembrane 1 and the Yau group showed that rhodopsin dimerization relies on homotypic transmembrane 1 (TM1) and helix 8 (H8) [90]. I therefore interpret these results to suggest that OPN3SV might act as a dominant negative peptide role in cells. It might interact with other GPCRs and prevent their interaction with full-length OPN3.

Structure and Localization of Human OPN3

- Intracellular localization of OPN3 in HeLa and HEMs (Figure 3.7, 3.8) raises an intriguing question of whether OPN3 could function internally. In direct exposure, light easily can pass through the cells hence unlike ligand activated GPCRs opsins do not have to be on the plasma membrane to interact with their stimulus. For example, rhodopsins are localized in intracellular sacs of rod cell and they effectively function as GPCR [153]. Furthermore, recent findings demonstrate that internalized GPCRs can continue stimulating or inhibiting cAMP production in a sustained manner. According to these findings, internally functional GPCRs internalize with their cognate G protein subunit and adenylyl cyclase. Once internalized, the GPCRs could produce cellular responses different than the ones elicited at the plasma membrane [154]. Based on these results, internal localization of OPN3 suggests that it may have an internalized GPCR function.

- Localization studies showed that the absence of helix 8 (H8) and the c-terminus does not affect localization of OPN3 (Figure 3.6). This finding suggests that important domains for OPN3 localization are before H8. Taken into consideration that all n-terminal and intracellular loop 1 tagged OPN3 constructs were retained in endoplasmic reticulum, these areas might have residues and motifs crucial for trafficking of OPN3.
- To investigate OPN3 expression in different tissues, I used a commercially available human tissue qPCR array (Figure 3.9). Among all the tissues screened in the tissue array, the highest human OPN3 mRNA expression was found in placenta and the highest mouse OPN3 mRNA expression was found in ovary. Although we found high level of OPN3 mRNA in cultured primary human melanocytes and keratinocytes [21, 22], skin did not show high OPN3 mRNA expression compared to other tissues. In addition to that, mouse brain tissue was not one of the highly expressing tissues in the mouse tissue array although, previous studies showed OPN3 was highly expressed in certain regions of the brain [44, 47]. Detecting cell type specific expression of a gene of interest is difficult in tissue array because each qPCR well contains cDNA from all types of cells in that particular tissue. Abundance of other types of cells beside OPN3 expressing cells in human skin and mouse brain might be a reason why they do not show high OPN3 expression in the tissue array.

Functionality of Human OPN3

- Intriguingly, OPN3 is highly expressed in internal organs, which do not interact with external light stimuli. This suggests that OPN3 might have a light independent function. There are many examples of divergence of function in sequence-related groups of proteins [155-157]. More specifically, in the opsin

field, the Copenhagen group showed that melanopsin could trigger developmental changes in retinal neuron number and vasculature in response to oxygen demand [158]. OPN3, although classified as an opsin, might have a light-independent function.

- Another interesting point is that, if OPN3 functions in a Gai dependent manner, then what would be the role of the MC1R and OPN3 interaction. OPN3 might have more than one function as it is demonstrated for many GPCRs. In addition to that, co-immunoprecipitation of MC1R and OPN3 might be the result of higher order heterooligomeric complex or a scaffold-mediated protein complex, rather than heterodimerization.
- It is also important to point out that, the Ca^{2+} and cAMP indicators used in this study, Fluo-4 AM and Epac-H187, rely on excitation of fluorophore. This leads to light exposure of OPN3 before and during the experiment. This might alter or saturate the function of OPN3. Because of this reason a biochemical approach such as luciferase assay or enzyme-linked immunosorbent assay (ELISA) could be used to address possible light activation of OPN3 without previously exposing the protein to light.
- In addition to that, it should be kept in mind that dim red light, under which Ca^{2+} and cAMP imaging performed, could also affect the function of the protein of interest, especially such photosensitive protein as an opsin.

Future Directions

What is the absorption spectrum of human OPN3?

The previous attempts in human OPN3 transfected HEK 293 cells have failed to show light-activated function or light absorption of human OPN3 [44, 52]. One reason stated by the authors is that the expression level of the human OPN3-based pigment

was too low to determine its absorption maximum. The approach they took to solve this problem was the c-terminal truncation, which resulted in a 50-fold higher yield of purified OPN3-based pigment [52]. This allowed them to determine the absorption maximum of the mosquito, zebrafish, pufferfish and chicken OPN3-based pigment; however, human and mouse OPN3 expression was still too low to perform an absorption spectrum study [52]. At this point, it is worth it to mention that the absence of the unique c terminus would likely affect the function of OPN3. Moreover, OPN3 has a long c-terminus, which contains 13 possible phosphorylation sites (10 serine and 3 threonine) (Figure 3.2A). These sites might play a role in arrestin mediated internalization of OPN3. The c-terminal truncation might improve the purified OPN3-based pigment yield by inhibiting the internalization, which leads to accumulation of proteins on the membrane. However for studying the role of a novel protein, it is important to keep the structure of the protein as intact as possible to address its function. Once the basic function is determined thoroughly, further modulations of the protein would enhance our understanding of functional dynamics of the protein.

Localization studies showed that c-terminal tagged human OPN3 could be expressed successfully in HeLa cells (Figure 3.4). In addition, immunofluorescent and western blot studies of heterologously expressed human OPN3 also indicate that expression level of the human OPN3 was not low (Figure 3.7A,C). Based on these findings, c-terminal tagged human OPN3 pigments could potentially be purified for absorption spectrum studies. In the case of light absorption, K299G and W268F OPN3 mutants, the hypothetically light insensitive OPN3 constructs discussed in Chapter 4, could be used as negative control.

What part of OPN3 plays a role in the interaction with MC1R?

It has been shown that transmembrane 4 (TM4) and 5 (TM5) are important for

dimerization of serotonin and M3 muscarinic receptors [159, 160]. Furthermore, a recent study by the Palczewski group identified TM5 residues, that play a role in dimerization and spectral shift of red and green cone opsins, to be important faces for opsin dimerization [89]. Following that, the Yau group showed that rhodopsin dimerization relies on homotypic transmembrane 1 (TM1) and helix 8 (H8) interactions, rather than TM4 and TM5 [90]. There is a need for further studies to clarify the exact transmembrane regions and residues crucial for opsin heterodimerization.

Co-immunoprecipitation studies showed that OPN3 and MC1R proteins could be pulled-down together in HeLa and HEM cells (Figure 4.4). To examine the hypothetical heterodimer contact regions of OPN3 and MC1R, peptides of certain transmembrane regions of OPN3 could be used in peptide-competition experiments. After narrowing down the target transmembrane regions, specific site-directed mutagenesis could be utilized to find the exact location and function of residues crucial for heterodimerization. To quantify the disruption of heterodimerization, FRET or BRET (Förster or bioluminescence resonance energy transfer, respectively) could be used.

Would OPN3 functionally interact with MC3R and MC4R in the brain?

As mentioned in the Introduction Chapter, high expression of OPN3 is found in certain regions of the brain, such as the preoptic area, paraventricular nucleus (PVN) of hypothalamus, cerebral cortex, ventral horn of the spinal cord and the cerebellum [44]. Interestingly, the melanocortin 3 receptor (MC3R) and the melanocortin 4 receptor (MC4R) are expressed in the PVN (MC3R is also expressed in the arcuate nucleus (ARC) and MC4R in the dorsal motor nucleus of the vagus (DMV)) [161-163]. The PVN, ARC and DMV are brain areas that play a role in energy metabolism. In

humans, the most common cause of monogenic obesity is mutation of the MC4R gene [164]. While alteration of MC4R function leads to such dramatic measures, MC3R plays much subtler role in energy homeostasis. MC3R knock out (KO) mice models exhibited a mild obesity phenotype, whereas, as expected, MC4R KO mouse displayed obesity [165-167]. Furthermore, a recent study demonstrated heterodimerization of MC3R and the growth hormone secretagogue receptor (GHSR) which resulted in the alteration of function in both receptors [107]; however, the physiological role of this dimerization in the hypothalamus is waiting for further clarification.

Co-immunoprecipitation studies showed that human OPN3 and MC3R or OPN3 and MC4R proteins could be pulled-down together in HeLa cell expression system (Figure 4.5A,B). This raises an interesting question of whether OPN3 and MC3R or MC4R play a functional role together. For functional interaction of two proteins occur, first they need to localize in the same cell type. OPN3 wild-type (WT) and OPN3 KO mice, which we currently have in our lab, could be used to immunofluorescent label OPN3, MC3R, MC4R and biomarkers for specific cell types in hypothalamic brain tissue sections. In the case of co-expression of OPN3 and MC3R or MC4R in the same cell type of the PVN, this would lead to the following question: would they interact functionally? For addressing this question, a high-fat diet could be used to challenge energy metabolism of OPN3 KO mice, compared to OPN3 WT mice. If OPN3 modulates the function of MC3R or MC4R this would lead to alteration of handling of energy expenditure and lead to phenotypic changes such as body weight, cholesterol level and lean body mass. Furthermore, co-immunoprecipitation studies need to be repeated for mouse OPN3, MC3R and MC4R to address their hypothetical dimerization.

Conclusion

As the largest organ of human body, skin is exposed to light through the day. Besides protecting internal body organs from harmful effects of UVR, skin might have a light sensing functionality, which is yet to be found. Recently, our lab found that epidermal skin cells express light sensitive opsins, a member of the rhodopsin GPCR family. Moreover, these cells also express other GPCRs belonging to the rhodopsin GPCR family. These findings raise intriguing questions of whether opsins play a role in light sensitivity of skin and whether opsins functionally interact with other GPCRs expressed in skin cells. In this dissertation, I investigated the cellular localization and function of human OPN3 in human skin cells. In Chapter 2, we found that four light sensitive opsins, OPN1-SW, OPN2, OPN3 and OPN5, are expressed in epidermal skin cells. In addition to that, analysis of these opsins' relative mRNA levels revealed that OPN3 is the most highly expressed compared to other opsins [22]. In Chapter 3, I examined the localization of OPN3 in heterologously and endogenously expressing cells. This study showed that full-length human OPN3 could be expressed successfully in a heterologous system. In Chapter 4, I studied the function of OPN3 in heterologously and endogenously expressing cells. Findings of this study revealed that decreased OPN3 levels lead to increased melanin levels in epidermal melanocytes. Furthermore, OPN3 co-immunoprecipitates with MC1R in heterologously and endogenously expressing cells. Lastly, OPN3 modulates the α MSH induced cAMP response of MC1R via G α i activation.

The findings presented here expand our understanding of OPN3 function and its role as an extra-ocular opsin in the skin cells. Although opsins are mainly examined in the eye, the non-image forming phototransduction field is constantly advancing and finding new functions of opsins in numerous organisms as regulator of various biological processes, such as dermal phototransduction. The work demonstrated in this dissertation contributes to this advancement by addressing a non-image forming

function of OPN3 in human skin, highlighting its key aspects and setting the stage for further studies in the field.

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