Published in final edited form as:

Pigment Cell Melanoma Res. 2009 June ; 22(3): 251–252. doi:10.1111/j.1755-148X.2009.00563.x.

Eating thyself toward the dark side?

Michael S. Marks

Our current understanding of the molecular events governing pigmentation has been largely guided by identifying over 120 genes that are altered in human patients and animals with abnormal pigmentation. Most of these genes encode melanogenic enzymes, transporters, receptors or transcription factors that are expressed only in pigment cells or that function in only limited additional cell types. The fact that animals survive with mutations in these known genes indicates *a priori* that they are not required for viability. Undoubtedly, pigmentation must also require additional genes that regulate essential functions in many cell types, and which therefore may not be represented among viable animals with altered pigmentation. In a recent issue of PLoS Genetics, Ganesan and colleagues report a large number of candidate genes in this category that surprisingly drive pigment cells towards the dark side.

To identify genes involved in pigmentation in an unbiased way, the authors used a simple but novel high-throughput screen. They assayed for decreased melanin content in a human melanoma line that was transduced with a library consisting of small interfering RNA (siRNA) duplexes directed to each of over 21,000 unique human genes. The cell line, MNT-1, has been widely used as a model for eumelanin pigmentation and melanosome formation. Importantly, these cells constitutively release melanin into the medium, such that all of their melanosomes turn over in a matter of days. Thus, a block in the formation of new pigment - by interfering with either melanosome biogenesis or expression of melanogenic genes - would result in a decrease in melanin content within 4–5 days of culture, well within the time-frame of siRNA-mediated effects. By quantifying melanin content spectroscopically and controlling for loss of cell viability, Ganesan et al. identified at least 92 genes for which a siRNA-mediated reduction in expression consistently decreased melanin content by more than 2 standard deviations from the mean. A rigorous series of controls were used to ensure that the effects were reproducible, paralleled by loss of expression of the target gene, not due to off-target effects, and - for at least some of the siRNAs - reproducible in primary human melanocytes. As verification for the approach, the authors showed that pigmentation was substantially reduced by siRNAs to 13 of 68 genes previously shown to regulate melanogenesis at the cellular level, including enzymes (e.g. tyrosinase), transporters (e.g. OCA2/ pink-eyed dilute), trafficking regulators (e.g. subunits of Hermansky-Pudlak Syndrome-associated complexes BLOC-1 and BLOC-2), signaling proteins and receptors (e.g. endothelin receptor and BMP1), and transcription factors (e.g. ZIC2).

While the screen successfully identified known genes, the novel hits in the screen revealed several surprising features of the cellular and molecular control of pigmentation. The first surprising result was that half of the identified genes influenced expression of tyrosinase, the limiting enzyme in melanin synthesis. The target genes in this category spanned a broad array of protein classes, including nuclear transcription factors (e.g. nucleoplasmin 3), trafficking regulators (e.g. the small GTPases Rab4a and Arl4a), metabolic enzymes (e.g. methionine sulfoxide reductase), proteinase inhibitors (serpinB1 and serpinB2), and lipid binding proteins of unknown function (e.g. PLEKHA3). Depletion of half of these targets affected the level of tyrosinase mRNA expression, and depletion of some (e.g. Rab4a) resulted in lysosomal

degradation of the tyrosinase enzyme as judged by restoration of tyrosinase upon lysosome neutralization. Thus, this list provides a large group of potential regulators of tyrosinase trafficking, gene expression, and activity. Notably, most siRNAs that decreased tyrosinase protein levels also down-regulated mRNA levels for the transcription factor MITF. This suggests a previously unanticipated regulatory feedback loop in which tyrosinase activity might be required to maintain MITF-dependent melanocyte differentiation. Defining the mechanistic basis for this feedback loop might provide insights into potential regimens for treatment of pigimentary diseases.

Perhaps the most intriguing hits identified in this screen were a group of genes known to encode regulators of macroautophagy. Macroautophagy, or self-eating, is an essential cellular process in which cytosolic components are selectively entrapped within a unique double membrane structure called an autophagosome. The contents of the autophagosome are then degraded upon fusion with lysosomes. All cells undergo constitutive macroautophagy at a basal level, but macroautophagy is highly induced in response to certain stimuli such as starvation and is dysregulated in numerous diseases including cancer and neurodegeneration (Mizushima *et al.*, 2008). Autophagosome formation and fusion with lysosomes are mediated by a cascade of factors that were first discovered in the yeast, *Saccharomyces cerevisiae*, and that are conserved throughout metazoan evolution (Xie and Klionsky, 2007). Most of these factors are thought to be dedicated to autophagy alone, although some have additional known functions in cell signaling or trafficking.

In the siRNA screen or subsequent directed siRNA analyses, Ganesan *et al.* identified several genes that are thought to regulate nascent autophagosome formation. Four identified genes are homologues of core machinery components for nascent autophagosome formation in yeast, and include WIPI1 (homologous to yeast Atg18), Beclin1 (homologous to Atg6), and LC3-C and LC3-A (both homologues of yeast Atg8). A fifth identified gene, GPSM1/ ASG3, is thought to stimulate autophagy in colon carcinoma cells (Pattingre *et al.*, 2003). Ganesan *et al.* support a role for macroautophagy regulators in animal pigmentation by showing that mice engineered to lack beclin-1 have dilute coat color. Together, the data suggest the intriguing possibility that pigment cells employ a group of proteins involved in the early stages of autophagy to effect pigmentation.

Although Ganesan *et al.* did not test for effects of their siRNA hits on melanosome morphology, maturation or content, the most straightforward hypothesis based on their results is that pigment cells divert the machinery involved in autophagosome formation to facilitate melanosome formation. Consistent with this idea, the authors provide evidence that LC-3 and Apg5, two autophagy effectors, might accumulate on the membrane of Pmel17-containing stage II melanosomes. At least two potential roles for these effectors can be envisioned. First, they might facilitate membrane deposition required to segregate stage II melanosomes from the early endosomal system. Second, they might function at the level of multivesicular endosomes, which are intermediates in both stage II melanosome maturation and biosynthetic delivery of mature melanosome membrane proteins. Components of the machinery that regulates multivesicular body formation are also required for autophagy (Rusten and Simonsen, 2008); whether the autophagy machinery inversely regulates multivesicular body formation in metazoans is not yet known, but could potentially account for its requirement in pigmentation.

Another intriguing possibility is that the specific autophagy components identified in this screen also function in processes unrelated to autophagy. For example, Atg18 - the yeast orthologue of WIPI1 - is one of the few known proteins that bind to the doubly phosphorylated phospholipid, phosphatidylinositol(3,5)-bisphosphate (PtdIns(3,5)P₂), and has been shown to function in membrane retrieval from the vacuole - the yeast equivalent of the lysosome (Dove *et al.*, 2004). PtdIns(3,5)P₂ metabolism is directly altered in a form of Charcot-Marie Tooth

syndrome that is associated with hypopigmentation (Chow *et al.*, 2007), implying an important role for PtdIns(3,5) P_2 in membrane transport processes required for melanization (see (Marks, 2008)). If WIPI1 also binds PtdIns(3,5) P_2 , it might therefore regulate melanosome formation in an autophagy-independent manner. Another identified hit, GPSM1, is also indirectly linked to a previously known regulator of melanosome biogenesis, GIPC. GPSM1 stimulates autophagy by stabilizing the GDP-bound form of the heterotrimeric G protein component, G α i3 (Pattingre *et al.*, 2003). Levels of G α i3-GDP are also elevated by the activity of the GTPase activating protein, GAIP. GAIP directly interacts with GIPC, a PDZ binding protein that binds to the cytoplasmic domain of the melanosomal protein, Tyrp1, and that is thought to regulate Tyrp1 trafficking (Liu *et al.*, 2001). It is therefore possible that GPSM1, perhaps via stabilization of G α i3-GDP and subsequent sequestration of GAIP, regulates Tyrp1 trafficking. These data are consistent with the notion that GPSM1 and WIPI1 - and perhaps other autophagy components identified in the screen - influence pigmentation in a manner independent of their function in autophagy.

Regardless of the mechanisms of action that are sure to be elucidated in the future, Ganesan *et al* have provided us with provocative leads toward new players and pathways involved in melanization. The findings are sure to stimulate thought and new insight into the signaling and membrane trafficking pathways required for pigmentation.

Acknowledgments

I thank L. B. King, B. Watt, S. R. G. Setty and A. Sitaram for helpful comments and critical reading of the manuscript. This work is supported by grants R01 EY015625 and R01 AR048155 from the National Institutes of Health.

REFERENCES

- Chow CY, Zhang Y, Dowling JJ, Jin N, Adamska M, Shiga K, Szigeti K, Shy ME, Li J, Zhang X, Lupski JR, Weisman LS, Meisler MH. Mutation of FIG4 causes neurodegeneration in the pale tremor mouse and patients with CMT4J. *Nature* 2007;448:68–72. [PubMed: 17572665]
- Dove SK, Piper RC, McEwen RK, Yu JW, King MC, Hughes DC, Thuring J, Holmes AB, Cooke FT, Michell RH, Parker PJ, Lemmon MA. Svp1p defines a family of phosphatidylinositol 3,5-bisphosphate effectors. *EMBO J* 2004;23:1922–1933. [PubMed: 15103325]
- Liu TF, Kandala G, Setaluri V. PDZ-domain protein GIPC interacts with the cytoplasmic tail of melanosomal membrane protein gp75 (tyrosinase related protein-1). *J. Biol. Chem* 2001;276:35768–35777. [PubMed: 11441007]
- Marks MS. FIG4, Charcot-Marie-Tooth disease, and hypopigmentation: a role for phosphoinositides in melanosome biogenesis? *Pigment Cell Melanoma Res* 2008;21:11–14. [PubMed: 18353139]
- Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature* 2008;451:1069–1075. [PubMed: 18305538]
- Pattingre S, De Vries L, Bauvy C, Chantret I, Cluzeaud F, Ogier-Denis E, Vandewalle A, Codogno P. The G-protein regulator AGS3 controls an early event during macroautophagy in human intestinal HT-29 cells. *J. Biol. Chem* 2003;278:20995–21002. [PubMed: 12642577]
- Rusten TE, Simonsen A. ESCRT functions in autophagy and associated disease. *Cell Cycle* 2008;7:1166–1172. [PubMed: 18418046]
- Xie Z, Klionsky DJ. Autophagosome formation: core machinery and adaptations. *Nature Cell Biol* 2007;9:1102–1109. [PubMed: 17909521]