ENDOSOMES AND MELANOMA

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Endosomes bring elements into a cell from its exterior and oftentimes these are used for cell proliferation. This paper examines a recent study of a small protein Rab7 which facilitates this process and when overexpressed may lead to metastatic melanoma.

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1 INTRODUCTION

As is well known, melanoma is a highly aggressive form of cancer and is tends to metastasize very rapidly. The analysis of various melanomas has demonstrated a wide variety of genetic alterations which have been alleged as cause for its initiation or factors in allowing for its aggressiveness. We have examined various elements from many internal pathway factors, extracellular factors, and epigenetic factors (including methylation and miRNA elements). In a recent paper by Alonso-Curbelo et al the authors examine endosomic elements, namely the interaction of RAB7 as a factor which brings in endosomes and apparently uses this process to add energy to the cell allowing it to enhance cell proliferation via Myc and other genes.

There is an almost continual determination of genes and their artifacts in the identification of specific cancer related presence. We have examined many of these before and each time there appears another target it is essential to ask; why this new target is chose and what does it do that results in a metastatic condition? The current analysis focuses on a small class of proteins which facilitate endocytosis, the bringing in of materials from the cell's surface.

2 THE RAB OBSERVATIONS

In a recent paper the authors have identified the small 200 nucleotide protein Ras7 as a significant factor in the metastatic behavior of melanoma. We first examine that express consideration, then examine the details behind it and consider some observations.

As Alonso-Curbelo et al state:

Although common cancer hallmarks are well established, lineage-restricted oncogenes remain less understood.

Here, we report an inherent dependency of melanoma cells on the small GTPase RAB7, identified within a lysosomal gene cluster that distinguishes this malignancy from over 35 tumor types.

Analyses in human cells, clinical specimens, and mouse models demonstrated that RAB7 is an early-induced melanoma driver whose levels can be tuned to favor tumor invasion, ultimately defining metastatic risk.

Importantly, RAB7 levels and function were independent of MITF, the best-characterized melanocyte lineage-specific transcription factor.

Instead, we describe the neuroectodermal master modulator SOX10 and the oncogene MYC as RAB7 regulators. These results reveal a unique wiring of the lysosomal pathway that melanomas exploit to foster tumor progression.

The above observation contains an interesting connection between several elements:

1. Extracellular Matrix elements (ECM) which we have examined before as factors in driving melanoma metastasis.

2. Lysosome activity which is the counter to exosomes, which we have examined as markers for melanoma.

3. Cell surface receptors and ligands which reflect cell cycle activation and control.

4. Transcription factor management through MITF and MYC which result in loss of cell cycle homeostasis.

Thus to a degree in this paper we seem to be seeing a multifaceted response modulated by the RAB gene products. Thus it is worth a brief review of endosomes and RAB and then a re-examination of the transcription factors in the context of cell cycle control.

The authors especially note the following:

- *Melanoma-restricted lysosomal gene cluster uncovers tumor-type-specific roles of RAB7*: RAB7 is one of several RAB genes and it has a specific functionality which at face value does not relate directly to the management of transcription factors. Thus it is necessary to examine that in some detail.

- *RAB7-controlled pathways selectively modulate melanoma cell phenotypes:* One does not look at all the RAB7 pathways and what controls them. We often think of MITF, SOX10 and MYC as having other control elements.

- *RAB7 is an early-induced melanoma driver that defines patient prognosis:* RAB7 modification by some form of upregulation which in turn is driven by oncogenes is a significant factor in metastatic behavior. The details will be examined somewhat herein.

- *MYC and SOX10 regulate RAB7 in an oncogene- and lineage-dependent manner, respectively:* The issue is that it is MYC and SOX10 that modulate RAB7 upwards and thus use it as a means to bring more elements into the cell which are then broken down via a lysosome and use by the cell in its proliferation. The question which seems unanswered is; if MYC and COX10 are transcription factors and also oncogenes, then how do they regulate RAB7 and if they up-regulate RAB7 what does that result in since RAB7 drives endosomic activity?

Now there is a commentary which adds to what is presented above. It states[1]:

The results of the study could help to determine the development of metastasis in patients suffering from the disease….

What is the function of these genes? Strangely, the factors that are increased in melanoma share a common mechanism: the formation of vesicles called endosomes.

Endosomes are machinery that tumour cells, via a process called endocytosis, can use to incorporate components into their environment and obtain energy by degrading them via autodigestion or autophagy. Autophagy is also used for self-cleaning to eliminate other proteins as well as damaged or unneeded cellular components.

Thus at the heart of this theory is that RAB7 is activated and results in increased processing of endosomes taking proteins from the surface of the cell and breaking them down and allowing the cells to proliferate more aggressively. We will try to demonstrate some of this activity but many of the details are still wanting. They continue:

Among all the genes that control endocytosis, the authors of the study focused specifically on one, called RAB7; this gene is highly expressed in melanoma cells. After more than six years of research, the research team …showed that RAB7 acts as an orchestra director, determining the fate of melanoma cells: at high concentrations of RAB7, cellular autodigestion is very active,

[1] http://www.news-medical.net/news/20140628/CNIO-researchers-identify-over-40-genes-that-predict-aggressiveness-of-melanoma.aspx

and this allows tumour cells to obtain energy, prevent the accumulation of toxic components and thus divide and proliferate; when RAB7 is reduced, cells use endosomes to recycle metastatic proteins, favouring their dispersal throughout the body.

To some degree this is a growth and proliferation issue. What specifically is driving RAB7 is open for debate and it does not appear to be any mutation in the gene or its product. The emphasis on RAB7 as the key factor may be begging the question in light of the many other factors known to take a role in melanoma development.

Defining "the key to the fate of the tumour cell", is just one of many new aspects of melanoma uncovered by this study. "Finding which mechanisms determine why melanoma is so aggressive is very complex because more than 80,000 mutations have been described for this tumour", …

This study is also relevant for clinical work. One application is the prognosis of the melanoma: the authors show in tumour biopsies that the amount of RAB7 in a cutaneous tumour defines the risk of developing metastasis. "This study opens avenues for the potential use of proteins that control vesicles and regulate autophagy as novel markers of patient survival", …

Furthermore, these results help to understand the mechanism of action of a compound that, as the group discovered in 2009, is lethal in melanoma cells as well as in other tumour cells. This RNA-based nanoparticle compound kills the cells by acting on the formation of vesicles.

The above seems to imply that if one can stop the formation of such vesicles that one then possibly "starves" the specific cells. The issue is targeting just these vesicles and not all vesicles. This observation is noted as follows:

"We knew how our nanoparticles act inside tumour cells, but not how they selectively incorporate inside the cells", …The size of these molecules requires cells to form endosomes in order to be able to trap the compound. This study demonstrates that this endosome formation (via RAB7) is very active in tumour cells but not in normal cells. Normal cells, therefore, do not incorporate RNA nanoparticles, reducing the risk of toxic effects.

However RAB7 proteins do function in all cells, taking in items and participating in the "digestion" of many such items, many of which if left undigested could be harmful to the cell. Thus it could be problematic to try such a direct approach. To some degree it may be akin to DNMT interference drugs used to block hypermethylation.

The work … could lead to the development of novel drugs that selectively target the mechanism of cell autodigestion as a potential therapeutic strategy.

The target of a specific drug then must block either the generation of Rab7 or the Rab7 directly. The sequellae of such blockage may be significant.

3 ENDOSOMES AND LYSOSOMES

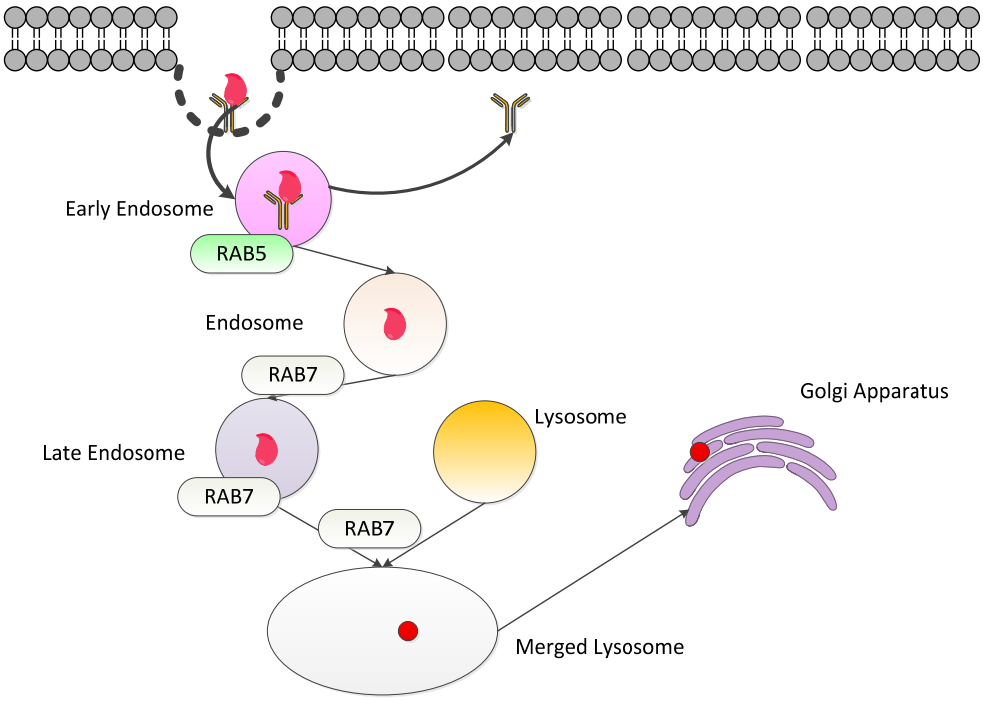
We have previously discussed exosomes and cancer prognosis. Exosomes are what cells eject. Endosomes are what cells ingest and process. They do this in a complex manner and utilize lysosomes which have digestive capabilities which allow for the extraction of cell nutrients.

Endocytosis has several key functions for a cell[2]:

1. It internalizes nutrients found outside the cell.
2. It facilitates and regulates the expression of cell surface proteins so that cells can control the up-take of certain ligands.
3. It facilitates the uptake of extracellular debris as well as other ECM items.
4. Recovery of membrane structure.

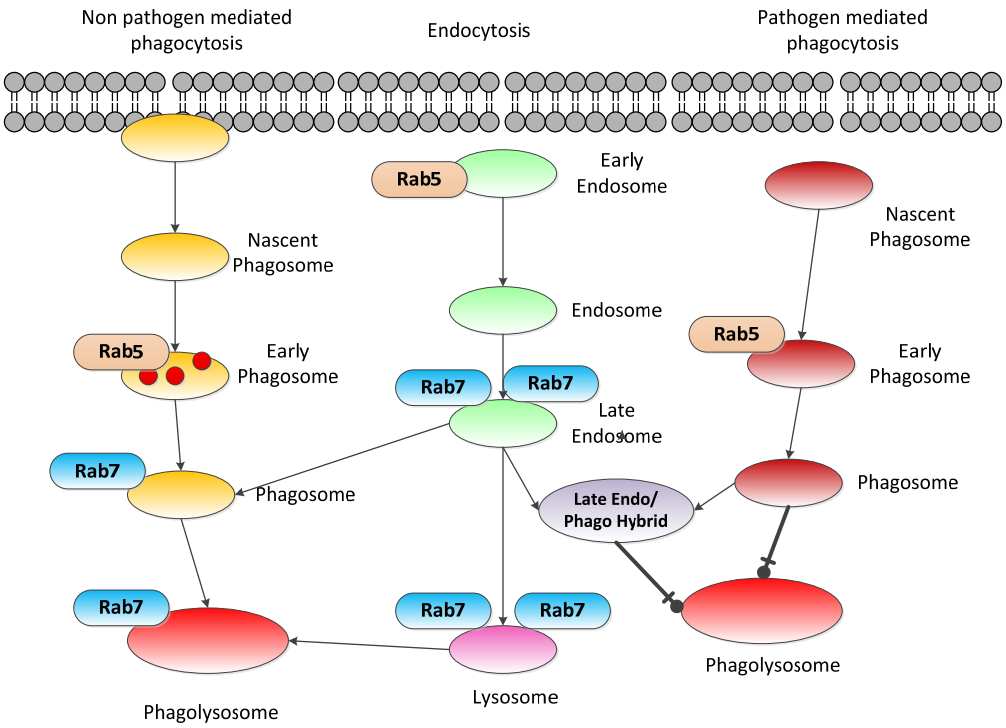
The endosomes are the transport vehicles and they function in concert with lysosomes which are enzymatically charged digestive vacuoles. Together they take in and process what is on the cell's surface and without the cell.

We depict this process generally below:



Now we can examine this process alongside several others as we show below.

[2] See p 351 Cassimeris et al.



Note above we show both Rab7 and Rab5 actions.

As Tabata et al note:

Endocytosis involves the intracellular transport of extracellular and plasma membrane substrates to endosomes/lysosomes and is involved in many physiological processes. Autophagy is also a process in which cytoplasmic constituents, including organelles, are transported within double- membraned autophagosomes to lysosomes for degradation.

Autophagy has divergent physiological roles in cancer, infection, immunity, and other processes. Many reports suggest there is a common element between the endosomal and autophagic pathways, but these commonalities have not been fully elucidated….

In this study, we described a novel RH protein domain that associates directly with Rab7. Rubicon and PLEKHM1 negatively regulate endosomal transport by binding to Rab7 via their RH domains.

Furthermore, this study provides novel insight into the function of PLEKHM1. These two RH domain–containing proteins also have several significant differences. Rubicon must bind to the Beclin 1–PI3-kinase complex in addition to Rab7 for its function, whereas PLEKHM1 only requires Rab7 binding. Rubicon, unlike PLEKHM1, is involved in autophagosome maturation. Both of these proteins localize to endosomes but do not show complete colocalization.

Therefore, these two RH domain proteins seem to function through different mechanisms. PLEKHM1 bound not only to wild-type Rab7 and the Rab7QL mutant but also to the Rab7TN

mutant in a yeast two-hybrid assay and immunoprecipitation analyses in mammalian cells, suggesting that PLEKHM1 may bind to both the GTP-bound and GDP-bound forms of Rab7. However, the TN mutant is predicted to have reduced affinity for both GDP and GTP and may behave as a nucleotide-free Rab7 depending on the assay conditions.

Hence, in order to determine the precise nucleotide dependence, we performed an in vitro GST pulldown assay and found that recombinant PLEKHM1 strongly interacted with GTP $^{\gamma}$*S-loaded Rab7 but only minimally interacted with GDP-loaded Rab7. The data convincingly confirm that PLEKHM1 preferentially binds to the GTP-bound form of Rab7, corroborating our hypothesis that PLEKHM1, like Rubicon, is a Rab7 effector.*

The above analysis does reflect on a different binding domain and process from what was introduced earlier. We shall come back to this issue later.

4 RAB AND RAB7

We will now examine the RAB proteins in some detail. Vesicle targeting and fusion with acceptors on the membrane uses the collection of proteins at the vesicle membrane. These are managed by the Rab family of proteins which are small G proteins[3]. G proteins and Rab in particular are classified as amplifier, rectifier and organizing proteins. They can use GTP to act as a switch and these proteins are used by cells to make movement, transformations and activations. The Rab subclass of the Ras proteins plays a key role in the endocytosis and integration with lysosomes….

The following describes several of the Rab proteins and their functions[4]:

RAB Type	***Function***
Rab1 and Rab2	Control vesicular traffic from the endoplastic reticulum to the Golgi apparatus.
Rab6	Controls inter Golgi traffic.
Rab8	Controls transport from the Golgi apparatus to the plasma membrane.
Rab4, Rab5, Rab9	Regulate endocytosis.
Rab7	Specialized for recycling of down-modulated membrane receptors.
RAB7A 3q21.3 RAB7B 1q32	Specific sub-types of Rab7

From NCBI we have[5]:

RAB family members are small, RAS-related GTP-binding proteins that are important regulators of vesicular transport. Each RAB protein targets multiple proteins that act in exocytic / endocytic pathways. This gene encodes a RAB family member that regulates vesicle traffic in the late endosomes and also from late endosomes to lysosomes. This encoded protein is also involved in the cellular vacuolation of the VacA cytotoxin of Helicobacter pylori. Mutations at highly

[3] See Marks pp 32-37. "small G" proteins are monomers about 200 amino acids in length. They are in the Ras superfamily and thus Rab is also in that family.

[4] See Marks et al, p 383.

[5] http://www.ncbi.nlm.nih.gov/gene/7879 and http://www.ncbi.nlm.nih.gov/gene/338382

conserved amino acid residues in this gene have caused some forms of Charcot-Marie-Tooth (CMT) type 2 neuropathies.

As we had discussed above the Rab 5 and 7 facilitate the endocytic paths for bringing in materials from without the cell. Thus they are simply transport facilitators. It is alleged that they can be easily targeted for suppression but then again they appear to be active elements in many cells and the blockage of them in a systemic manner may potentially have significant negative effects.

Let us go back to a statement made by the authors:

Importantly, RAB7 levels and function were independent of MITF, the best-characterized melanocyte lineage-specific transcription factor. Instead, we describe the neuroectodermal master modulator SOX10 and the oncogene MYC as RAB7 regulators. These results reveal a unique wiring of the lysosomal pathway that melanomas exploit to foster tumor progression.

MITF has been examined before in the case of melanoma. MYC and SOX10 are transcription factors and MYC is a well-known oncogene. How they then regulate RAB7 is a key question. Are they transcription factors that up-regulate the gene? If so, then what has activated MYC and is this the better target. Is RAB7 then just an artifact of such up-regulation. The same can be asked about SOX10.

As regards to cancers, Rab7 has been studied extensively. Zhang et al have presented results where they state:

Aberrant endocytosis and altered lysosomal function result in defective growth-factor transport and unbalanced levels of surface proteins, such as integrins and E-cadherin, leading to tumorigenesis and cancer metastasis. Rab GTPases, as master regulators in membrane traffic, are proved to be involved in cancer development. Rab25 is a well-established tumorigenesis associated Rab and is highly homologous to Rab11, and endogenously overexpressed in most ovarian and breast cancer samples in a constitutively active form, which is unique among Rab proteins. ...provided data indicating that overexpression of Rab25 promotes cell transformation, inhibits apoptosis and induces tumour progression, probably through the PI3K/AKT signalling pathway. Rab25 may also be related to other cancer such as OC/PPC (ovarian/primary peritoneal serous carcinoma) and prostate cancer.

Zhang et al continue as follows:

The results ...showed that thyroid hormone production was regulated by Rab5a and Rab7. cAMP stimulation elevated the expression of Rab5a and Rab7 in adenomas, linking Rab7 to the formation of benign thyroid autonomous adenomas ...also found Rab7 is overexpressed in DMPM (diffuse peritoneal malignant mesothelioma).

In addition, v-Src induces activation of Rab7, which may be related to epithelial-to-mesenchymal transition during tumour progression ... indicate that Rab7 is involved in a cell survival pathway. Upon growth-factor depletion, Rab7 down-regulates surface nutrient transporters

through endocytic degradation, preventing growth-factor-independent survival, but inhibition of Rab7 sustains surface nutrient transporters, thus promoting long-term cell survival, which is dependent on the AKT survival signalling pathway. Furthermore, … inhibition of Rab7 co-operated with the adenoviral E1A protein to promote transformation of p53−/− MEFs (mouse embryonic fibroblasts), thus Rab7 was proposed to act as a potential tumour suppressor.

however, there is insufficient evidence to conclude that Rab7 functions as a tumour suppressor. As mentioned above, Rab7 is actually overexpressed in some cancer cells or tissues, as described previously, and the transformation effects of dominant-negative Rab7 required the crucial help of the E1A protein and the absence of p53 … and these studies were carried out under nutrient starvation condition which may differ slightly from the environmental conditions for tumorigenesis that are usually rich in growth factors.

…another view on the function of Rab7 in apoptosis. Inhibiting the upstream regulator RabGGT prominently induces apoptosis of germ cells in Caenorhabditis elegans and mammalian cancer cells. … examined the effects of knockdown of Rab5, Rab7 and components of the HOPS complex by RNA interference in C. elegans, and found that knockdown of both Rab proteins promoted germ cells apoptosis…

Taken together, the underlying mechanism for cancer, cell survival and apoptosis regulated by Rab7 is still not yet understood. Rab7 is also emerging as a regulator for the autophagic pathway, another mechanism for cell death and survival, which is related to many diseases, such as cancer and heart failure.

The autophagic process is initiated by engulfment of cytoplasmic materials into a unique membrane (phagophore) to form an autophagosome; the autophagosome then undergoes maturation through fusion with endosomal vesicles and lysosomes to form a lysoautophagosome, in which materials are degraded to provide nutrients and energy for cell survival under nutrient depletion.

It is thus fair to say that Rab7 functioning is still a work in progress.

5 MITF

We have written previously on MITF and melanoma and we review it again here. MITF is a transcription factor which has been linked to melanoma. It can be activated in the RAF/BRAF pathway and thus when activated results in the loss of apoptosis and the migration of cells.

5.1 WHAT IS MITF?

As noted by Carreira et al[6]:

It is widely held that cells with metastatic properties such as invasiveness and expression of matrix metalloproteinases arise through the stepwise accumulation of genetic lesions arising from genetic instability and "clonal evolution."

By contrast, we show here that in melanomas invasiveness can be regulated epigenetically by the microphthalmia-associated transcription factor, Mitf, via regulation of the DIAPH1 gene encoding the diaphanous-related formin Dia1 that promotes actin polymerization and coordinates the actin cytoskeleton and microtubule networks at the cell periphery.

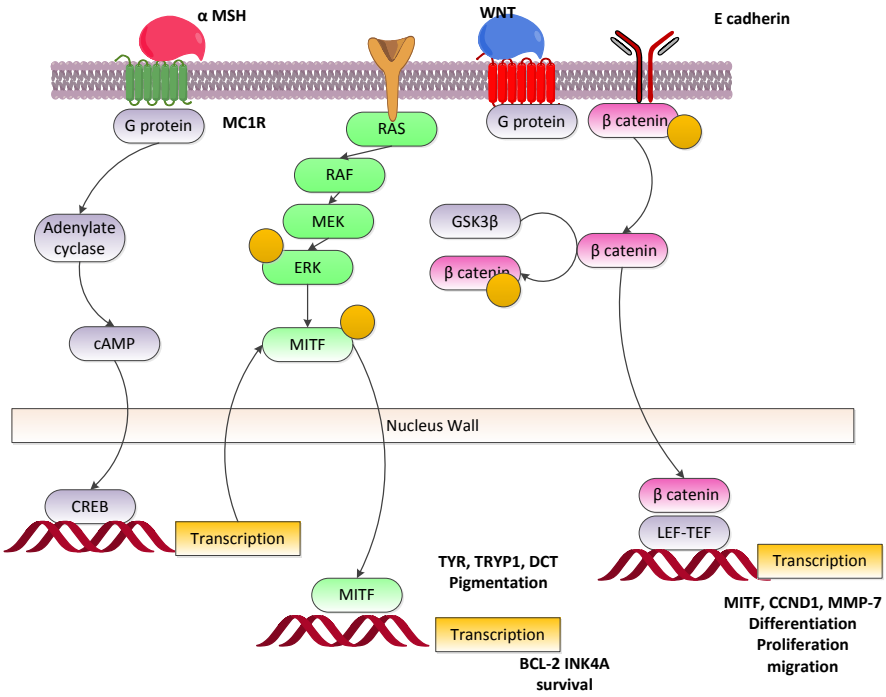
Low Mitf levels lead to down-regulation of Dia1, reorganization of the actin cytoskeleton, and increased ROCK-dependent invasiveness, whereas increased Mitf expression leads to decreased invasiveness.

Significantly the regulation of Dia1 by Mitf also controls $p27_{Kip1}$-degradation such that reduced Mitf levels lead to a $p27_{Kip1}$-dependent G1 arrest. Thus Mitf, via regulation of Dia1, can both inhibit invasiveness and promote proliferation.

The results imply variations in the repertoire of environmental cues that determine Mitf activity will dictate the differentiation, proliferative, and invasive/migratory potential of melanoma cells through a dynamic epigenetic mechanism.

Note that the discussion above shows that overexpression of MITF leads to less invasiveness. We show the details of the pathway dynamics below.

[6] Carreira, S., et al, Mitf regulation of Dia1 controls melanoma proliferation and invasiveness, Genes Dev. 2006 20: 3426-3439.



They continue:

Although Mitf is clearly required for melanoma proliferation, why it is necessary has not been previously established. To understand how Mitf depletion led to a block in G1/S transition, we examined a number of known markers of proliferation and the cell cycle. Western blotting of cells transfected with control or Mitfspecific siRNA revealed that depletion of Mitf led, as expected from our previous work, to decreased expression of $p21_{Cip1}$, but intriguingly also induced expression of the $p27_{Kip1}$ cyclin-dependent kinase inhibitor.

Note that similar results were obtained using a second Mitf siRNA directed against a different region of Mitf. We also observed reduced expression of cyclin E, and PCNA, most likely as an indirect effect of the cell cycle arrest, but no change in cyclin D1 or Cdk2 levels. Tubulin and lamin B were used as loading controls.

Finally they note:

In summary, Mitf appears to lie right at the heart of the melanocyte, coordinating survival, cell cycle entry and exit, cytoskeletal organization, melanosome assembly and transport, differentiation and migration/metastasis. As such, understanding Mitf regulation and function may well be the key to achieving one of the major aims of cancer research, an effective melanoma therapy.

Thus the MITF function, in their view, is critical.

5.2 MITF TARGETING AND CONTROL

Zoufal writes of work recently reported in Genes & Development by Pogenberg[7] et al[8] focused upon targeting and thus controlling MITF:

The results, published in the scientific journal "Genes & Development", throw new light on the workings of the so-called Microphthalmia-associated Transcription Factor MITF, that is not only connected to skin cancer, but also to a variety of hereditary diseases where the production of the skin pigment melanin is disturbed, and to certain aspects of ageing. "Our data could provide a rational basis for the development of tailor-made drugs targeting MITF", explains first author Vivian Pogenberg from the Hamburg branch of the European Molecular Biology Laboratory

But MITF also makes stem cells turn into melanocytes in the first place and controls cell proliferation and death in these cells. That's why MITF is called a master regulator. In fact, it also has functions in other cell types like mast cells of the immune system and bone eating osteoclasts...

Mutations in MITF not only play a role in the development of skin cancer, but also cause severe genetic diseases like the Tietz and Waardenburg syndromes that lead to deafness, skin and hair pigmentation defects, abnormal eye anatomy and altered vision. The transcription factor also plays a role in our hair turning grey with age and other age-related pigmentation alterations…

Crystals scatter X-rays in characteristic ways and produce diffraction patterns from which the structure of the crystal - and here MITF - can be reconstructed. The analysis revealed unexpected molecular insertions that give MITF a unique kink. MITF forms a dimer with a long coiled-coil protein "zipper", and the kink in this zipper limits MITF's ability to bind to other transcription factors.

This paper thus appears to provide a targeted means to control MITF and then perhaps melanoma metastasis. Identification of binding sites is essential to control[9].

5.3 MORE ON MITF

We can provide a bit more history on MITF and its functions. As NCBI states[10], MITF, microphthalmia-associated transcription factor (3p14.2-p14.1):

[7] See Pogenberg in http://genesdev.cshlp.org/

[8] Zoufal, T., X-ray analysis deciphers master regulator important for skin cancer, http://www.eurekalert.org/pub_releases/2012-12/ded-xad113012.php

[9] Pogenberg, V., Ogmundsdóttir, M., Bergsteinsdóttir, K., Schepsky, A., Phung. B., Deineko, V., Milewski, M., Steingrímsson, E., & Wilmanns, M. Restricted leucine zipper dimerization and specificity of DNA recognition of the melanocyte master regulator MITF. Genes & Development, 1 December 2012.

[10] http://www.ncbi.nlm.nih.gov/gene/4286

This gene encodes a transcription factor that contains both basic helix-loop-helix and leucine zipper structural features. It regulates the differentiation and development of melanocytes retinal pigment epithelium and is also responsible for pigment cell-specific transcription of the melanogenesis enzyme genes. Heterozygous mutations in the this gene cause auditory-pigmentary syndromes, such as Waardenburg syndrome type 2 and Tietz syndrome

MITF has been known to be a key player in melanoma metastasis. As Chin et al state in their review paper[11]:

MITF, a melanoma oncogene targeted by amplification

The promise of DNA-based structural alterations as the entry point for gene discovery has been illustrated by the recent identification of MITF as a melanoma oncogene. The discovery of MITF amplification in melanoma derived from an integrated analysis of genomic copy gains and losses, together with sample-matched mRNA expression data.

When clustering algorithms were applied to SNP array-derived chromosomal copy number data generated for the NCI-60 cancer cell line collection, some of these cell lines aggregated according to tissue of origin, including several melanoma cell lines. The bidimensionality of the hierarchical algorithm also enabled the identification of chromosomal alterations driving these lineage-restricted clustering patterns, and suggested that lineage-specific cancer genes might reside within the genomic regions implicated.

For the melanoma cell lines, the common genomic alteration was a region of copy gain at chromosome 3p14-3p13. To facilitate the identification of an oncogene targeted by this amplification event, the NCI- 60 collection was partitioned based on the presence or absence of copy gain at the relevant chromosome 3p locus. This partitioning served as a two-class distinction that drove a supervised analysis of sample-matched gene expression data. Although the gene expression signature that emerged was dominated by melanocyte lineage genes (as expected given that only melanoma cell lines comprised the 3p-amplified class), MITF was the only gene showing significantly increased expression in association with the 3p-amplified melanoma cell lines that also mapped to the common region of 3p copy gain.

MITF amplification was subsequently detected in 10% of primary cutaneous and 15%–20% of metastatic melanomas.

Although the majority of amplifications were low level (e.g., four to six copies per cell), high-level amplicons were also observed, including one sample that exhibited >100 copies per diploid genome. A Kaplan- Meier analysis performed on metastatic melanomas suggested that MITF amplification in this setting correlated with adverse 5-yr patient survival.

Finally, ectopic MITF overexpression complemented $BRAF_{V600E}$ in conferring soft agar colony growth to immortalized melanocytes engineered to express TERT, and to lack the pRB and p53

[11] Chin et al, Malignant melanoma: genetics and therapeutics in the genomic era, Genes Dev. 2006 20: 2149-2182

pathways. These functional studies thereby suggested a genetic context that might characterize a subset of human melanomas whose survival is dependent on MITF.

MITF also exemplifies a newly recognized "lineage survival" oncogenic mechanism, wherein tumor genetic alterations may target survival functions also operant in the relevant cellular lineages during development and differentiation.

Thus, while the discovery of MITF amplification began as a systematic genomics-based survey of many human cancer types, it provides a striking convergence of melanoma oncogene discovery and melanocyte development.

Next, as Genovese et al state[12]:

Histidine triad nucleotide-binding protein 1 (HINT1) is a haploinsufficient tumor suppressor gene that inhibits the Wnt/β-catenin pathway in colon cancer cells and Microphthalmia-associated transcription factor (MITF) activity in human mast cells. MITF and β-catenin play a central role in melanocyte and melanoma cell survival, and this study aimed to investigate the effects of HINT1 on the MITF and β-catenin pathways in malignant melanoma cells.

We found that HINT1 inhibits MITF and β-catenin transcriptional activity, and both proteins can be co-immunoprecipitated with an anti-HINT1-specific antibody in melanoma cell lines. Stable, constitutive overexpression of the HINT1 protein in human melanoma cells significantly impaired cell proliferation in vitro and tumorigenesis in vivo.

These effects were associated with a decreased expression of cyclin D1 and BCL2, well known MITF and β-catenin transcription targets, respectively. We also demonstrated that BCL2 and cyclin D1 can partially rescue the HINT1-driven phenotype. Moreover, we found in ChIP assays that HINT1 binds the chromatin at MITF and β-catenin sites in BCL2 and cyclin D1 promoters, respectively, and that mSIN3a and HDAC1, well known transcriptional repressors, can be co-immunoprecipitated with an anti-HINT1-specific antibody. These findings support the tumor suppressor activity of HINT1 gene in melanoma cells by promoting the formation of non-functional complexes with oncogenic transcription factors like MITF and β-catenin.

Finally as Larribere et al state[13]:

Microphthalmia-associated transcription factor (MITF) M-form is a melanocyte-specific transcription factor that plays a key role in melanocyte development, survival, and differentiation. Here, we identified MITF as a new substrate of caspases and we characterized the cleavage site after Asp 345 in the C-terminal domain.

[12] Genovese G, et al, The tumor suppressor HINT1 regulates MITF and β-catenin transcriptional activity in melanoma cells, Cell Cycle. 2012 Jun 1; 11(11):2206-15.

[13] Larribere, et al, The cleavage of microphthalmia-associated transcription factor, MITF, by caspases plays an essential role in melanocyte and melanoma cell apoptosis, Genes Dev. 2005 19: 1980-1985.

We show that expression of a non-cleavable form of MITF renders melanoma cells resistant to apoptotic stimuli, and we found that the C-terminal fragment generated upon caspase cleavage is endowed with a proapoptotic activity that sensitizes melanoma cells to death signals. The proapoptotic function gained by MITF following its processing by caspases provides a tissue-restricted means to modulate death in melanocyte and melanoma cells.

Their observation does show the impact of MITF in the control of melanoma.

5.4 OBSERVATIONS

The recent work demonstrates that we can through an understanding of the pathways then target specific pathway control proteins by understanding their structure. We can already control B RAF in certain circumstances by targeting its specificity and that controlling the path but allowing MITF control in a broad sense may actually be much more powerful if the results hold for clinical applications.

The ability to find, characterize, and design binding site specific blocking agents is an essential step in a broader control of multiple cancers.

From Eichoff we have:

In various cell types the MITF gene is transcribed from different promoters to generate cell type-specific isoforms. In the melanocytic-lineage, Wnt signaling is required for expression of the M-Mitf isoform essential for melanocyte development. M-Mitf has a role in the differentiation of neural crest cells to melanoblasts and melanocytes, as well as in their survival and maintenance, and is identified as the master regulator of melanocyte development .

M-Mitf regulates the expression of melanocyte lineage-specific pigment-producing factors such as DCT and tyrosinase (TYR). Both the proliferation of melanocytes and M-Mitf expression and melanocyte proliferation is Wnt signal driven and inhibited by the Wnt signal inhibitor dickkopf-related protein 1 (Dkk1). Dkk1 antagonises Wnt signaling by binding to the Wnt receptor complex and inducing its internalisation. On palmoplantar skin, fibroblast release of Dkk1 is an important regulator of the hypopigmentation which is characteristic of these tissues.

The importance of Wnt signalling is also a critical factor here. We have examined this in detail elsewhere.

6 MYC

MYC is a well know transcription factor whose effects are often strongly seen in many cancers. It is an oncogene. As Nillson and Cleveland note regarding Myc:

At first glance, the selection for Myc activation in cancer seemed obvious. First, it was quickly established that enforced Myc expression was sufficient to provoke the entry and continuous, mitogen-independent, proliferation of cells and that it effectively blocked terminal cell differentiation.

Subsequently, Myc was shown to be necessary for traverse into S phase of the cell cycle, a finding recently underscored by the conditional knockout of c-Myc. Thus, not surprisingly, both c-Myc and N-Myc are essential for vertebrate development.

In addition, numerous studies showed that Myc activation was sufficient to provoke diverse cancers and, more recently, that Myc is continuously required to maintain the transformed state.

Finally, to round out the story was the revelation that c-Myc functioned as an angiogenic switch, and that its expression was in fact essential for proper and coordinate regulation of angiogenic and anti-angiogenic factors in cancer and development. This was satisfying – now we know why Myc activation was so pervasive in cancer.

As Yamamura et al state:

Rho GTPases are small G proteins that regulate various cellular processes, including cytoskeletal dynamics, migration, vesicle trafficking, cell proliferation, apoptosis and transcription. Rho GTPases, their regulators and their effectors have been suggested to control tumor formation and progression. RhoA has been found to control cancer metastasis and progression. Recently, the c-Myc–Skp2–Miz1 complex was shown to activate the RhoA gene.

The Ras superfamily incorporates the Ras, Rho, Rab Art and Ran families[14]. Now Rab7 is in the Rho GTPase class it may be considered controlled via Myc in a similar manner and mentioned above.

As stated in NCBI for Skp2[15]:

This gene encodes a member of the F-box protein family which is characterized by an approximately 40 amino acid motif, the F-box. The F-box proteins constitute one of the four subunits of ubiquitin protein ligase complex called SCFs (SKP1-cullin-F-box), which function in phosphorylation-dependent ubiquitination. The F-box proteins are divided into 3 classes: Fbws containing WD-40 domains, Fbls containing leucine-rich repeats, and Fbxs containing either

[14] See Marks et al p 354.

[15] http://www.ncbi.nlm.nih.gov/gene/6502

different protein-protein interaction modules or no recognizable motifs. The protein encoded by this gene belongs to the Fbls class; in addition to an F-box, this protein contains 10 tandem leucine-rich repeats.

This protein is an essential element of the cyclin A-CDK2 S-phase kinase. It specifically recognizes phosphorylated cyclin-dependent kinase inhibitor 1B (CDKN1B, also referred to as p27 or KIP1) predominantly in S phase and interacts with S-phase kinase-associated protein 1 (SKP1 or p19).

In addition, this gene is established as a protooncogene causally involved in the pathogenesis of lymphomas. Alternative splicing of this gene generates three transcript variants encoding different isoforms.

And similarly for Miz1[16]:

This gene encodes a member of the protein inhibitor of activated STAT (PIAS) family. PIAS proteins function as SUMO E3 ligases and play important roles in many cellular processes by mediating the sumoylation of target proteins. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene.

Isoforms of the encoded protein enhance the sumoylation of specific target proteins including the p53 tumor suppressor protein, c-Jun, and the androgen receptor. A pseudogene of this gene is located on the short arm of chromosome 4. The symbol MIZ1 has also been associated with ZBTB17 which is a different gene located on chromosome 1.

Also note that Miz1 is also called PIAS2 protein inhibitor of activated STAT, 2.

As Qu et al state:

In addition to the PI3K/Akt pathway, c-Myc also plays a role during TGF-b-induced EMT. It has been demonstrated that high TGF-b levels are often associated with melanoma progression, and so does the Akt1, c-Myc, and SKP2 (S-phase kinase-associated protein 2) levels. However, it is not clear how these signals are interacted and integrated in melanoma metastasis.

The above observation specifically details c-Myc and Skp2 but also indicates the lack of specificity and clarity as to the roles of each, no less the specifics on the role of these in Rab7. Likewise Qu et al continue:

SKP2 is the substrate recognition subunit of SCF (SKP1- CUL1-F-box protein) ubiquitin ligase complex. Aberrant SKP2 expression plays an active role in tumorigenesis owing to its central role in degradation of a number of cyclin-dependent kinase inhibitors including p27kip1, p21cip1, and p57. SKP2 was overexpressed in melanoma and its levels were correlated with metastasis. SKP2 regulates c-Myc protein stability and activity at both transcriptional and post-

[16] http://www.ncbi.nlm.nih.gov/gene/9063

translational levels. Whether and how SKP2 is regulated during TGF-b-induced EMT remains to be elucidated.

Again we have a lingering level of uncertainty.

7 SOX10

SOX10 is a transcription factor and has been identified with melanoma by many authors[17]. They are characterized by the Sry-box binding site. Sry-box is a variant of the HMG domain. Sox10 has been identified with the performance of many functions. It is involved in maintaining pluripotency of neural crest cells, melanocytes are derived from neural crests, it remotes survival and proliferation, and in terminal differentiation. It requires other cell pathways to affect its results. In mice it has been shown that deletion of Sox10 results in the elimination of all melanocytes.

Sox10 also works directly with MITF and it is through this joint action that it supports proliferation and survival. Sox10 influences the M promoter as well as MITF.Sox10 expression precedes MITF expression.

From Eichoff we have:

In adult human skin, stem cells are found in the hair follicle where lineage-specific differentiation of neural crest cells to melanoblasts and melanocytes occurs in response to changes in signaling. Among the genes involved several are transcription factors that include the Wnt pathway target gene Microphthalmia-associated transcription factor (MITF), paired domain-and homeodomain-containing transcription factor 3 (PAX3), and Sry-related transcription factor 10 (SOX10).

Importantly, it has been shown that the loss of any of these factors results in the failure of melanoblasts to develop (White & Zon, 2008). The final fate determination for melanocytes occurs when migrating melanoblasts come into contact with epidermal keratinocytes, which regulate their rate of replication to establish a stable keratinocyte/melanocyte ratio (Fukunaga-Kalabis et al, 2006; Valyi-Nagy et al, 1993).

Differentiated human melanocytes remain strictly localized at the basement membrane and cannot survive within the upper epidermal layers unless transformed into nevi or melanoma cells.

The observations of melanocyte survival in the basement membrane are key. For example in melanoma in situ, as compared to superficial spreading melanoma, the melanocyte breaks loose from the basement membrane and wanders upward. The presence of melanocytes in the upper layers is often pathognomonic for MIS. However as we have discussed elsewhere the movement is also driven be E cadherin changes and β catenin expression.

From Shakhova et al we have:

Giant congenital naevi are pigmented childhood lesions that frequently lead to melanoma, the most aggressive skin cancer. The mechanisms underlying this malignancy are largely unknown,

[17] See Wegner pp 71-80 in Hearing and Leong.

and there are no effective therapies. Here we describe a mouse model for giant congenital naevi and show that naevi and melanoma prominently express Sox10, a transcription factor crucial for the formation of melanocytes from the neural crest. Strikingly, Sox10 haploinsufficiency counteracts NrasQ61K-driven congenital naevus and melanoma formation without affecting the physiological functions of neural crest derivatives in the skin.

Moreover, Sox10 is also crucial for the maintenance of neoplastic cells in vivo. In human patients, virtually all congenital naevi and melanomas are SOX10 positive. Furthermore, SOX10 silencing in human melanoma cells suppresses neural crest stem cell properties, counteracts proliferation and cell survival, and completely abolishes in vivo tumour formation. Thus, SOX10 represents a promising target for the treatment of congenital naevi and melanoma in human patients.

The above observation regarding Sox10 for survival begs the question; why? Silencing Sox10 may silence the melanocyte.

As Hoek et al state:

Upregulation of SOX10 by Mitf-transfection is an interesting finding as SOX10 has long been held to be a regulator of MITF …indicating the possibility that these transcription factors regulate each other's expression. It may be that the myelinating cell genes mentioned here are detected because they are directly regulated by SOX10 … while its gene is being regulated by MITF, rather than being directly regulated by MITF itself. This, nevertheless, suggests that MITF may have a role alongside SOX10 in regulating the processes of myelination.

The role of MITF and SOX10 are known to be aligned, and one preceding the other. Yet the complete details of the interactions appears to be yet determined.

As Saskia et al state[18]:

The transcription factor SOX10 (SRY (sex determining region Y)-box 10) has a key role in the embryonic development of melanocytes. Recently, it has been suggested that SOX10 is highly relevant for melanoma development and survival. However, the distinct functions and downstream targets of SOX10 in melanoma remain widely unknown. In this study, we inhibited SOX10 via RNA interference in different human melanoma cell lines and found a significantly reduced invasion capacity in vitro and in the chick embryo model.

This recent paper still reflects the uncertainty for the downstream control of SOX10. Just what it does as regards to RAB7 is yet to be determined.

At later time points, SOX10 inhibition reduced proliferation and induced cell death. We identified melanoma inhibitory activity (MIA) as a direct target gene of SOX10, which is an essential protein for melanoma cell migration and invasion. Expression levels of SOX10 and MIA strictly correlated in melanoma cell lines, and SOX10 inhibition reduced MIA expression

[18] http://www.nature.com/jid/journal/vaop/ncurrent/full/jid2014128a.html

and promoter activity. Direct binding of SOX10 to the MIA promoter was demonstrated by electrophoretic mobility shift assay and chromatin immunoprecipitation.

Ectopic expression of MIA in SOX10-inhibited melanoma cells restored the invasion capacity, supporting the hypothesis that MIA is responsible for SOX10-mediated melanoma cell invasion. Our data provide evidence for a critical role of SOX10 in melanoma cell invasion through the regulation of MIA and highlight its role as a therapeutic target in melanoma.

The last observation was also present in the comments by Wegner. The interrelationship between SOX10 and MIT and MITF and MIA are somewhat understood. The details again need clarification.

8 OBSERVATIONS

This analysis of the Rab7 protein as a driver of melanoma metastatic dissemination appears to be predicated on the observation that endosomes can transport into the cell items which foster growth and proliferation. Further it is alleged that Sox10 and Myc are regulators of Rab7, and unlike their normal roles as oncogene transcription factors that in this case they appear to up-regulate Rab7 which in turn provides the cell with added nutrients that enable growth and proliferation. We have tried to piece together the details of this assertion and there appears to be multiple missing steps.

1. Pathway Control by Myc and Sox10: What are the pathway control elements related to the RAB7 controls? We have a general understanding but many details are yet to be fully elucidated.

2. Actions precipitated by Rab7 that promote Growth and Proliferation: What does RAB7 do to drive proliferation? Admittedly it can bring in nutrients but specifically what and why?

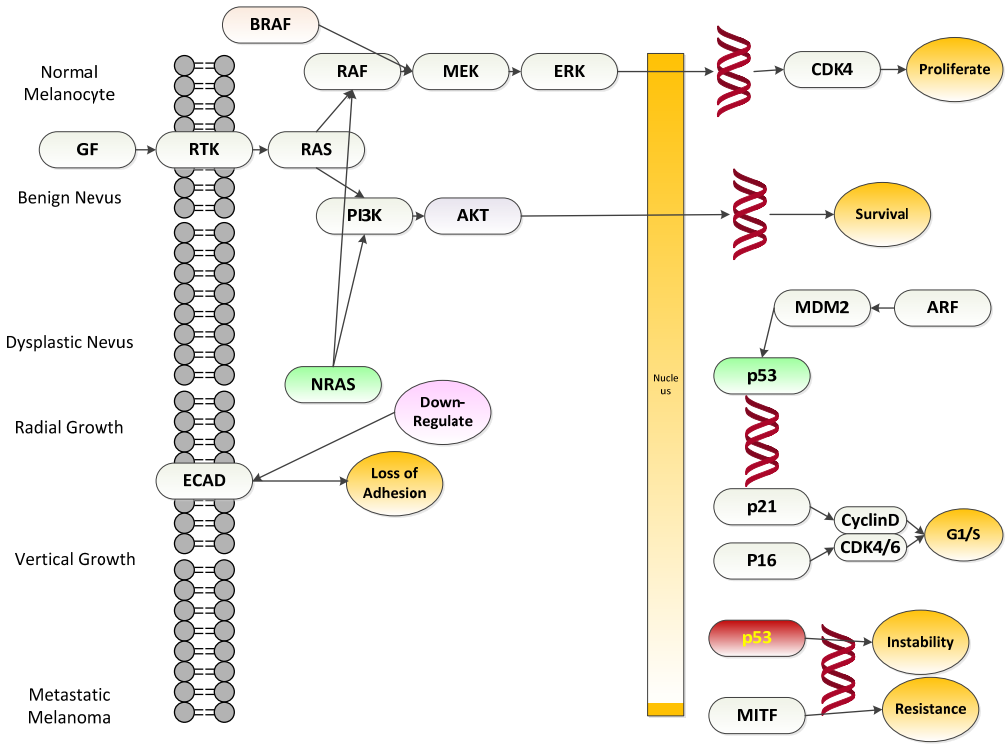
3. Mechanisms for the Control of Rab7: Just how do various sets of enzymes/proteins control RAB7? Is it just transcription control, or are there epigenetic factors as well?

4. Control of Myc and Sox10 versus Control of Rab7: What controls the two transcription factors which in turn activate RAB7?

5. It is known that there exists a melanoma stem cell. What is the relationship between the up-regulated RAB7 and the stem cell characteristics. In Girouard and Murphy we have an excellent overview of the melanoma stem cell. Thus if we accept the stem cell model is the RAB behavior an identifies of such a cell or is it a just an artifact?

Now from KEGG we have the following generic progression of melanoma and identified gene and pathway changes[19]:

[19] http://www.genome.jp/kegg-bin/show_pathway?hsa05218



Note that we do not see the targets discussed herein. In fact there are many alternative targets that have been discussed at length elsewhere.

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10 WHITE PAPERS REFERENCES

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http://www.telmarc.com/White%20Papers/default.html

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No. 111 CRISPR and Cancer, April 2014
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