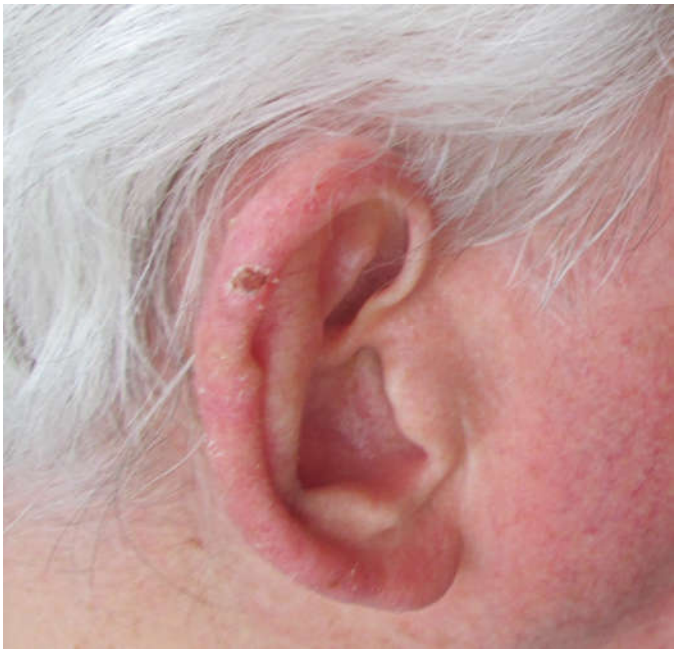

BASAL CELL CARCINOMA: A PARADIGM?

TGL 200

ABSTRACT
This Note examines Basal Cell Carcinomas, BCC, and their relationship to other more aggressive cancers especially prostate cancer, PCa. We examine the Hedgehog pathway and Gli transcription factor as a driver of cell proliferation in many malignancies.

Terrence McGarty

July 2023

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Communications relating to these documents and these should be sent to:
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Contents

1 INTRODUCTION

Basal Cell Carcinoma, BCC, is the most common malignancy in humans and the least likely to result in metastasis and death. In a sense this may afford an opportunity for a more simplistic model for the development of malignant states. What is currently viewed as a common driver in BCC is the Hedgehog pathway and this pathway also appears in more complex malignancies. We use BCC to examine this effect and then examine the Hedgehog pathway in prostate cancer, PCa.

1.1 OBJECTIVE

Our objective is twofold, First to examine basal cell carcinoma, BCC, as a relatively indolent cancer. Second, to use the BCC paradigm and extend it to other more aggressive cancers such as prostate cancer, PCa. Not surprisingly BCC pathways and related malignant support artifacts are common in many cancers, however their progression is often much more aggressive. It is anticipated that understanding BCC in relation to PCa, for example, may present addition insights to control. One are we find of particular interest is the loss of E cadherin and the activation of N cadherin. This happens in BCC as well as PCa. In PCa we see metastasis as a direct result of this change.

1.2 WHY BCC?

BCC is a common cancer; it has actions of significant pathways and is considered to be driven mostly by ultraviolet radiation exposure. Although considered a main driver BCC can also occur on unexposed areas of the skin. BCC on the head is generally considered as high risk since it demonstrates the results of UV exposure and may easily recure or result in additional separate lesions.

The skin exposed to UV shows the greatest number of mutations of any organ. Ironically given these multiple mutations we also have the most indolent cancer. It appears to be slow growing and has a low metastatic potential. Unlike many somatic cancers where just a few mutations can result in aggressive progression, BCC may have many such mutations but progresses in a more indolent manner. Thus BCC may present a paradigm for understanding many other cancers as well as therapeutic targets.

1.3 HEDGEHOG

BCC has been found to have the Hedgehog pathways as a main driver. It is this pathway that also results in the acceleration of the cell cycle and thus proliferation. As Suzman and Antonarakis have noted:

Activity in the Hedgehog pathway, which regulates GLI-mediated transcription, is important in organogenesis and stem cell regulation in self-renewing organs, but is pathologically elevated in many human malignancies. Mutations leading to constitutive activation of the pathway have been implicated in medulloblastoma and basal cell carcinoma, and inhibition of the pathway has

demonstrated clinical responses leading to the approval of the Smoothened inhibitor, vismodegib, for the treatment of advanced basal cell carcinoma.

Aberrant Hedgehog pathway signaling has also been noted in prostate cancer with evidence suggesting that it may render prostate epithelial cells tumorigenic, drive the epithelial-to-mesenchymal transition, and contribute towards the development of castration-resistance through autocrine and paracrine signaling within the tumor microenvironment and cross-talk with the androgen pathway.

In addition, there are emerging clinical data suggesting that inhibition of the Hedgehog pathway may be effective in the treatment of recurrent and metastatic prostate cancer. Here we will review these data and highlight areas of active clinical research as they relate to Hedgehog pathway inhibition in prostate cancer.

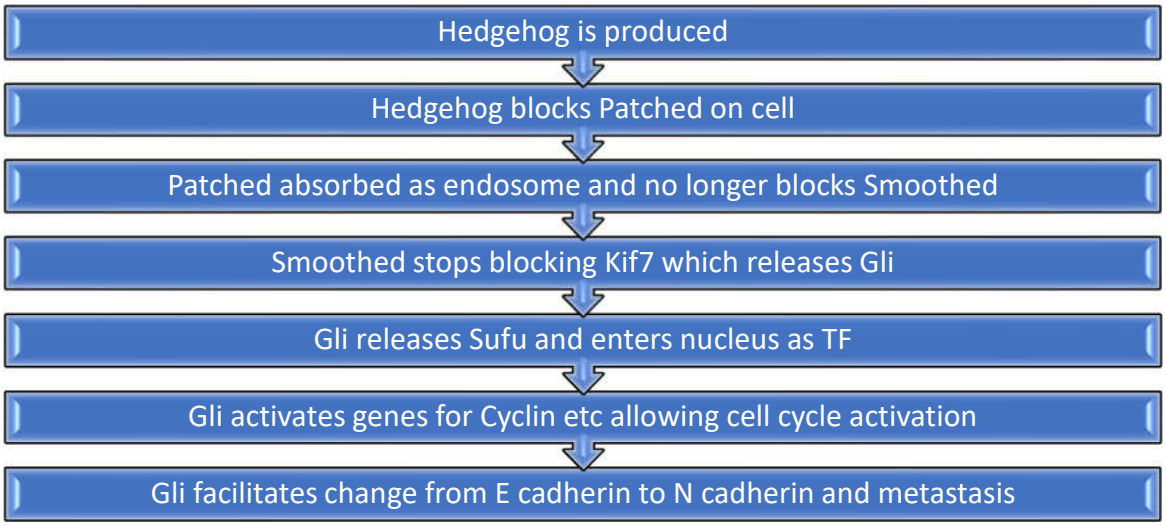
1.4 PARADIGM

The paradigm we will evoke is shown below for Hedgehog. It is only a partial one since it does not reflect two major issues:

1. It does not discuss the generation of Hh and activation of HH gene. We shall discuss this later but one may speculate that UV radiation may play a part.

2. It does not reflect the interaction or cross talk between multiple other genes. We shall examine this herein.

However this paradigm is an interesting example using BCC since it has been considered a major target for therapeutics.



The above is a simplistic structure for the items discussed herein. We consider this for the relatively indolent BCC and we also address it in the case of more aggressive prostate cancers. If

HH results in proliferation, as we also saw with the androgen receptor, then was may also find targets for therapeutics.

1.5 OUTLINE

The following is a brief outline of the issues discussed herein:

1. BCC: We first examine the skin and then the various types of BCC. For the most part BCCs are determined histologically and they are classified in various manners. Excision is the best option and Mohs surgery is suggested for high risk BCCs. Any BCC on the head is considered high risk.

2. Hedgehog: We then examine the hedgehog pathway in some detail. As we noted earlier we just assume that there is an excess release of hedgehog protein and that it then drives the path. The specifics on Hh release appears to be still an open question. Hedgehog seems to be the pathway of concern in BCC.

3. Phenotype vs Genotypes: In this section we address the phenotype and genotype issues. We have an extensive discussion of the related genes, pathways and aberrations. As we noted earlier BCC frequently has a multiplicity of mutant genes. The basal cells are always in some stage of mitosis and thus produce mutated offspring. No specific gene is dispositive. However we examine some specific genes such as TP53, Hippo-Yap, TERT, MYCN, DPH3-OXNAD1. The question is; are these causes or effects. p53, the protein of TP53 has been found in a majority of BCC. Yet as we will note the genomic studies of BCC are limited with a strong focus on murine models.

4. Prostate Cancer: We examine the impact of HH on PCa. This has just recently become a factor and it is most likely to become more relevant. PCa is a complex cancer and focus has been on androgens and the androgen receptor. However we know that at some point AR no longer functions and HH mat take a role.

5. Therapeutics: We examine various therapeutics, natural and recent one acting on HH.

2 BASAL CELL CARCINOMA

We now examine BCC and its variants.

2.1 OVERVIEW

As Crowson notes:

Basal cell carcinoma (BCC), first described by Jacob in 1827, is the most common malignant neoplasm of humans.

After a rise in incidence of roughly 20% between 1971 and 1977, by 1998 roughly one million new cases were being diagnosed annually in the United States. In consequence of its high incidence, BCC, although eminently curable when the diagnosis is made promptly and the lesion treated in its early phase, constitutes an enormous financial burden on the health care system. Lesions occur on both sun-protected and sunexposed skin, but often have a different biology and morphology in these locations. Typically, BCCs occur in the fourth decade of life and beyond although exceptions to this occur, in particular in the setting of specific genodermatoses or in patients with immune compromise.

As sun exposure plays a role in the development and transformation of BCC, patients with light skin phenotypes are particularly predisposed as expected; this includes in the context of blue eyes, red hair and easy freckling as well as those whose occupational or leisure activities lead them to pronounced and prolonged sun exposure. Additional risk factors for BCC include the exposure to arsenic, coal tar derivatives and irradiation, although by far ultraviolet (UV) light is the most important factor in lesion development and progression. BCC may, like squamous cell carcinoma, arise in the setting of scars, draining sinuses, ulcers, burn sites and foci of chronic inflammation.

The role of immune compromise in provoking an increased risk of BCC may be due to impairment of the immune surveillance of oncogenic viruses. The genodermatoses that enhance the risk of BCC include the prototype xeroderma pigmentosa, Rasmussen syndrome, Rombo syndrome, Bazex–Christol–Dupre syndrome, albinism and Darier's disease. These syndromes variably either decrease epidermal pigmentation and thus increase the risk of UV light-induced oncogenic transformation or promote genotypic instability in the epidermis. BCC has been associated with a variety of other lesions and/or neoplasms in the same or a nearby anatomic location.

For example, desmoplastic trichilemmoma is said to be associated with coexistent atypical basaloid neoplasms including BCC in up to 19% of cases; the aforementioned study shows the impact of selection bias as it was based first on the detection of the coincidence of BCC and desmoplastic trichilemmoma followed by a retrospective analysis of all accessioned desmoplastic trichilemmomas over a set time interval.

2.2 SKIN ANATOMY AN HISTOLOGY

The skin is the largest organ of the body. **A skin cell is about 30 µm in diameter and the top layer of the skin, the epidermis, may be 5 to 15 cells thick and this 150 to 450 µm in thickness, about 0.5 mm at the deepest.**

The dermatopathologist spends years of training to recognize and identify the multiplicity of skin disorders. This can be a very difficult process as exemplified by the ongoing debate on dysplastic nevi, are they pre-malignant or merely a condition unto themselves. One just needs to examine the years of work by Ackerman and colleagues in identifying melanocytic lesions. It is a complex and challenging process. It is a process which we will not even attempt to address herein.

However, our focus is on the specific intra and intercellular pathways. Examining the skin cellular structure provides a certain amount of insight and may be able to elucidate some of the extracellular flow issues including metastasis. However our goal in the chapter is not to provide details on dermatopathology but to provide a reasonable overview of its issues. Namely we want to be able to identify the melanocyte and to see what happens when a malignant condition is observed.

The challenge for the dermatopathologist in the coming years will be to not only use the visual clues, nor even the staining and immunohistological techniques, but to examine cells from a pathway distortion perspective both externally and internally. Namely do the cells express the desired or aberrant receptors and/or ligands and/or are the pathway elements normal or aberrant. Is the V600 BRAF present, for example? Currently there is a bifurcation of expertise in these areas and we may see an increase as time goes by and as we understand the pathway dynamics in a more complete manner.

The objectives of the Chapter are as follows:

1. Provide a general overview of the structure of the skin and the specific cells which compose this structure. The size of the cells and their “geographic” layout are essential to understanding the skin as an organ.

2. Provide a basic understanding of how the melanocyte integrates within that structure and what it appears to be in a health benign environment. This should provide a basis for understanding the importance of inter-cellular communications.

3. Identify the surrounding parts of the skin which interact with the melanocyte.

4. Identify abnormalities of the skin with emphasis on melanocytic disorders but also malignancies of other than melanocytes.

5. Identify and understand the simple melanocytic malignancy of melanoma and identifying and recognizing the typical morphological structures that are often found.

These objections, if met, will allow for a simple basis of understanding on how cells in a benign and malignant environment interact.

2.3 Skin Structure and Microscopy

The skin is composed of multiple layers. Simply, there is the epidermis in the top layer and the dermis the bottom, with a basal layer in between.

In the top epidermal layer we have the following type of cells:

1. Keratinocytes (92+% of total)

These are the most abundant cells, which are always growing and migrating upward where they die off and fall off the top layer. The very top layers of the akin are the stratum corneum which is at the very outermost surface. Just below that layer is the stratum granulosom, the layer of dying keratinocytes.

2. Langerhans cells (4% of total)

They are dendritic in shape and are exclusively in the epidermis. They function as the macrophage in the epidermis by processing contact antigens which they present to specific T cells. The Langerhans cells are thus a part of the immune system. They also provide a transport and contact mechanism to the lymphatic system.

3. Merkel cells (<1% of total)

These cells are considered to be touch receptors and reside in the basal layer and are generally unseen in normal microscopic observation.

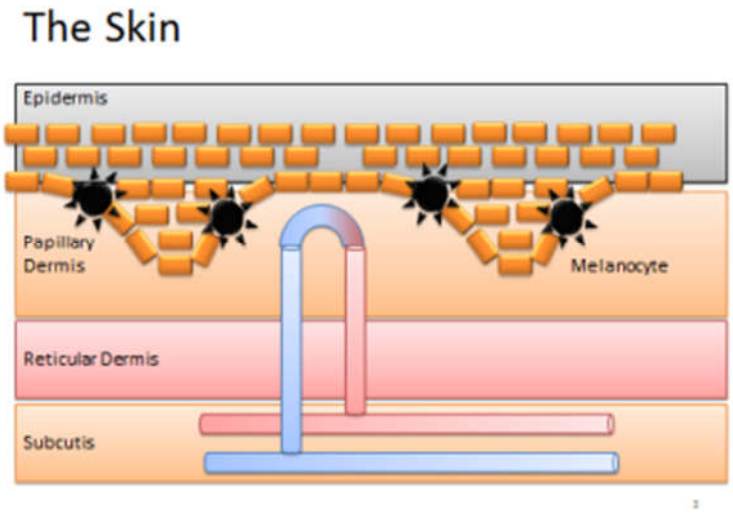
4. Melanocytes (3% of the total)

The melanocytes remain at the basal layer of the epidermis and have long tentacles which spread upward to the upper layers and from these tentacles they emit the melanocytes, the pigment of the skin and the general pigment of a nevus. Any movement, up or down, from the basal layer, of the melanocytes is pathognomonic of a malignancy of some form. Stability of the melanocyte is the *sine qua non* of a benign cell. Unlike the keratinocytes, which are reproducing and dying, the melanocytes are generally non-reproductive and stable. Their major function is to produce melanosomes. A single melanocyte provides about 30 keratinocytes with melanosomes.

The melanocytes are seen as clear cells in normal staining and appear as wedged between the keratinocytes.

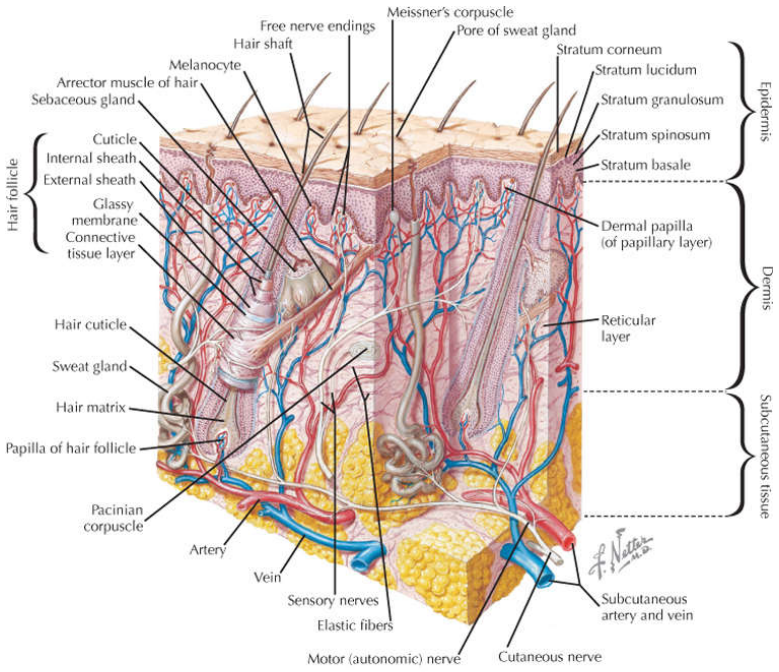
The figure below depicts the characteristics of the skin. The papillary dermis is about 0.4 to 0.6 mm in thickness and contains blood flow both from below and within the layer itself. It abuts the epidermis. It is composed of many collagen fibers and blood and never fibers. The blood flow to the basal layer and epidermal cells is via small capillaries that come up from the subcutis into

this top layer. The blood flow provides for oxygen and other nutrients and also eliminates any internal waste products and provides a pathway for the immune system.



The details from Netter are below. This shows a considerable amount of anatomical detail as compared to the above. Quoting Ovalle and Nahirney:

The skin *"... consists of **stratified squamous keratinized epithelium** on its outer part, called the **epidermis,** and an inner layer of **fibrous connective tissue,** called the **dermis.** A loose layer of **subcutaneous connective tissue,** the **hypodermis,** attaches skin to underlying structures and permits movement over most body parts. Skin has a dual embryologic origin: epidermis and its appendages derive mostly from surface ectoderm; dermis originates from mesoderm. The epidermis consists primarily of cells called keratinocytes, which make up more than 90% of the cell population. Other epidermal cells are **melanocytes** and Merkel cells, which derive from neural crest, and Langerhans cells, which have a monocytic origin. During embryonic development, skin appendages deriving from the epidermis grow down into the dermis*."



▲ **Schematic of skin and its appendages that shows the epidermis, dermis, and subcutaneous tissue.**

© Elsevier. Ovalle & Nahirney: Netter's Essential Histology - www.studentconsult.com

The skin is generally considered to be composed of the following layers. The first four are effectively in the epidermis and the last two in the dermis.

1. Stratum corneum: The top layer of the epidermis where the dead keratinocytes are lost.

2. Granular layer: Also called the Stratum Granulosum is below the top layer.

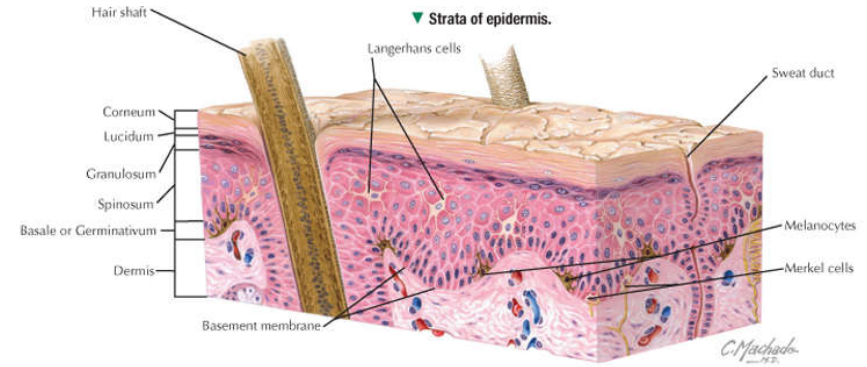
3. Spinous layer: Also called the Stratum Spinosum is just above the basal layer.

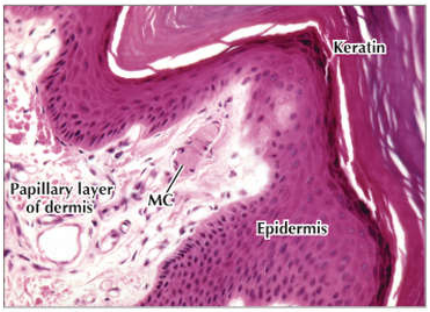
4. Basal Layer:

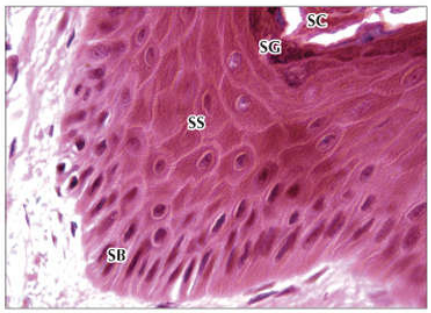
5. Papillary layer: Part of the dermis which is composed of thin, haphazardly arranged collagen fibers.

6. Reticular layer: Part of the dermis which is the thicker lower layer and extends from the base of the papillary layer to the subcutaneous tissue and is composed of thick collagen fibers that are arranged parallel to the surface of the skin.

From Ovalle and Nahirney we also have the graphic and specific cellular slides presenting actual views of the details:







▲ **LM of thick skin at the dermoepidermal junction.** A thick keratin layer characterizes the outermost stratum corneum. A dermal papilla that projects superficially into the epidermal region consists of loose connective tissue of the papillary layer. This layer contains many small blood vessels and a Meissner corpuscle (**MC**), which is an encapsulated touch receptor. 240×. *H&E.*

▲ **Higher magnification LM of the epidermis of thick skin.** The epidermis, a continually renewing epithelium, shows progressive differentiation and keratinization in a basal to superficial direction. Main features of its layers—strata basale (**SB**), spinosum (**SS**) (note prickle cells), granulosum (**SG**), and a small part of the corneum (**SC**)—are seen here. Part of the underlying dermis appears at the bottom. 575×. *H&E.*

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The Figure above shows the detail of each of the respective layers.

We can now be more specific by each layer. Gartner and Hiatt have the following Table which gives detail on the cells from a histological perspective[1]:

[1] Gartner, L:., J. Hiatt, Color Textbook of Histology, Saunders (New York) 2007.

Layer	*Histological Features*
Epidermis	Derived from ectoderm; composed of stratified squamous keratinized epithelium (keratinocytes)
Stratum corneum	Numerous layers of dead flattened keratinized cells, keratinocytes, without nuclei and organelles (squames, or horny cells) that will be sloughed off.
Stratum lucidum*	Lightly stained thin layer of keratinocytes without nuclei and organelles; cells contain densely packed keratin filaments and eleidin.
Stratum granulosum*	A layer three to five cell layers thick; these keratinocytes still retain nuclei; cells contain large, coarse keratohyalin granules as well as membrane-coating granules.
Stratum spinosum	Thickest layer of epidermis, whose keratinocytes, known as prickle cells, interdigitate with one another by forming intercellular bridges and a large number of desmosomes; prickle cells have numerous tonofilaments and membrane-coating granules and are mitotically active; this layer also houses Langerhans cells.
Stratum basale (germinativum)	This single layer of cuboidal to low columnar, mitotically active cells is separated from the papillary layer of the dermis by a well-developed basement membrane; Merkel cells and melanocytes are also present in this layer.
Dermis	Derived from mesoderm; composed mostly of type I collagen and elastic fibers, the dermis is subdivided into two regions: the papillary layer and the reticular layer, a dense, irregular collagenous connective tissue.
Papillary layer	Interdigitates with epidermis, forming the dermal papilla component of the rete apparatus; type III collagen and elastic fibers in loose arrangement and anchoring fibrils (type VII collagen); abundant capillary beds, connective tissue cells, and mechanoreceptors are located in this layer; occasionally, melanocytes are also present in the papillary layer.
Reticular layer	Deepest layer of skin; type I collagen, thick elastic fibers, and connective tissue cells; contains sweat glands and their ducts, hair follicles and arrector pili muscles, and sebaceous glands as well as mechanoreceptors (such as pacinian corpuscles).

Let us now examine each layer more specifically. Our objective is first to understand what a normal skin looks like and then a malignancy.

2.3.1 Epidermis

The epidermis is the top layer of the skin.

It is composed of the following is composed of the following four layers (deep to superficial or from the bottom to the surface)[2]:

1. Basal layer: source of replacement cells and epidermal stem cells. The cells are generated here and the keratinocytes then migrate upward. Also the melanocytes are located here and send their tentacles and melanosomes upward from this layer. Normally melanocytes do not move from this point.

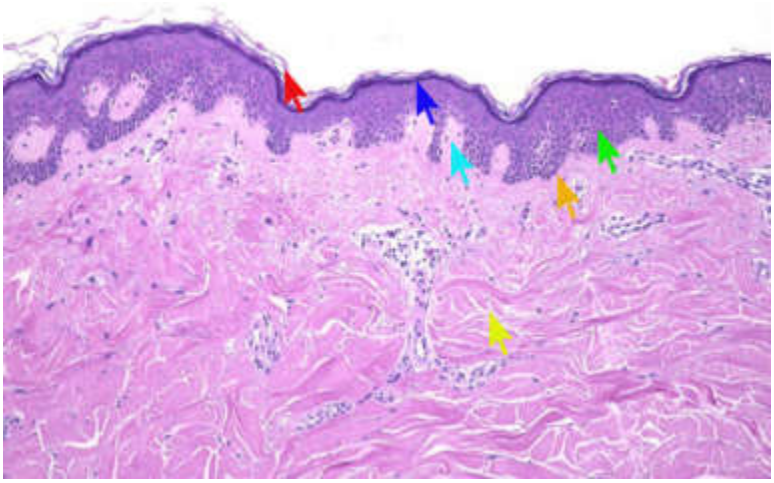
2. Spinous layer: center of epidermis that has a spiny appearance due to desmosomal junctions, area where keratinocytes produce keratin;

3. Granular cell layer: site of the epidermis' water barrier;

4. Stratum corneum: thick outer layers of flattened keratinized non-nucleated cells that provide a barrier against trauma and infection. This layer shows dying or deal keratinocytes flaking off.

[2] http://missinglink.ucsf.edu/lm/DermatologyGlossary/index.html

The details are below. Here we have the epidermis, composed of keratinocytes and the basal layer where we have melanocytes. The melanocytes have tentacles which grow through the keratinocyte layer and exude melanosomes, the dark pigmentation we see in colored skin patches. Below are the dermis and then the subcutis. The blood flow goes from arteries to veins and provides nutrients to the adjacent layers. A nevus will be seen as an agglomeration of melanocytes at the basal layer.



Habif defines the epidermis using a slightly different level of detail:

The epidermis is the outermost part of the skin; it is stratified squamous epithelium. The thickness of the epidermis ranges from 0.05 mm on the eyelids to 1.5 mm on the palms and soles. The microscopic anatomy of the epidermal-dermal junction is complex….

The innermost layer of the epidermis consists of a single row of columnar cells called basal cells. Basal cells divide to form keratinocytes, which comprise the spinous layer.

The cells of the spinous layer are connected to each other by intercellular bridges or spines, which appear histologically as lines between cells.

The keratinocytes synthesize insoluble protein, which remains in the cell and eventually becomes a major component of the outer layer (the stratum and corneum). The cells continue to flatten, and their cytoplasm appears granular (stratum granulosum); they finally die as they reach the surface to form the stratum corneum.

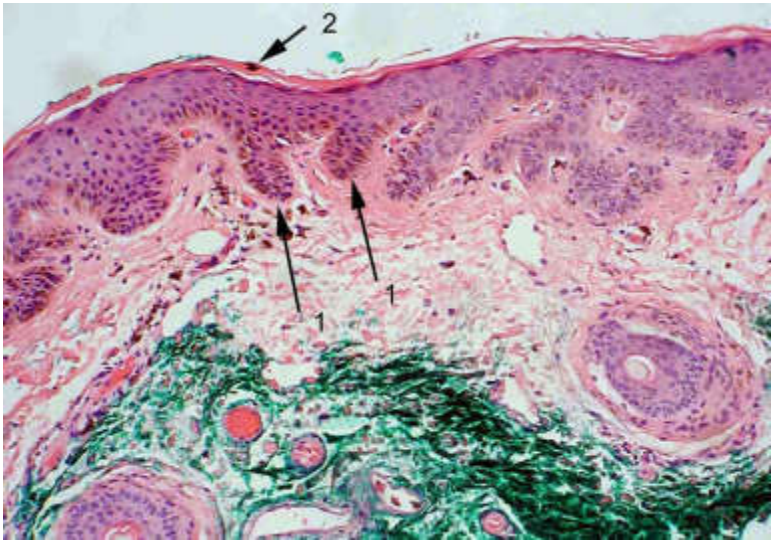
There are three types of branched cells in the epidermis: the melanocyte, which synthesizes pigment (melanin); the Langerhans cell, which serves as a frontline element in immune reactions of the skin; and the Merkel cell, the function of which is not clearly defined.

2.3.2 Dermis

In contrast the dermis is described as follows. The dermis is composed of cells, connective tissue, and ground substance and can contain blood and lymphatic vessels, nerves, glands, and hair follicles. It ranges from 1-4mm in thickness, making it much thicker than the epidermis. The dermis is divided into two layers: the papillary dermis and the reticular dermis. The papillary dermis is the region closest to the epidermis with papillae interdigitating with the epidermis; here, collagen fibers are thinner and loosely packed. In the deeper reticular dermis, collagen fibers are thicker and more densely and irregularly arranged. Elastin fibers are found in both the papillary and the reticular dermis, but they are more numerous within the latter.

Cutaneous appendages, like hair follicles, originate from the dermis. Blood vessels and nerves course through the dermis which supplies strength to the skin by its collagen and elastic fiber network. The vasculature to the skin is arranged in two plexi, the superficial plexus, located within the papillary dermis, and the deep plexus, located within the reticular dermis.

The basal layer is often seen as flowing in a wave like manner creating what are called Rete Ridges. We show them below:



Again quoting Habif we have:

The dermis varies in thickness from 0.3 mm on the eyelid to 3.0 mm on the back; it is composed of three types of connective tissue: collagen, elastic tissue, and reticular fibers.

The dermis is divided into two layers: the thin upper layer, called the **papillary layer**, is composed of thin, haphazardly arranged collagen fibers; the thicker lower layer, called the **reticular layer**, extends from the base of the papillary layer to the subcutaneous tissue and is composed of thick collagen fibers that are arranged parallel to the surface of the skin.

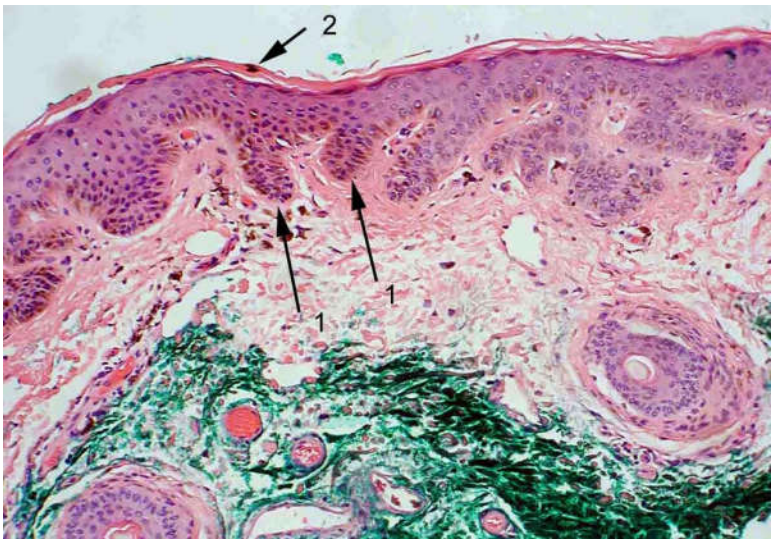
Histiocytes are wandering macrophages that accumulate hemosiderin, melanin, and debris created by inflammation. Mast cells, located primarily around blood vessels, manufacture and release histamine and heparin.

2.3.3 Some Basic Skin Architectural Elements

We now present some important specifics that will be incorporated in the description of melanoma.

2.3.3.1 Rete Ridges

Rete are a net or mesh of cells often at the bottom of the basal layer. As seen below they tend to extend downward and from time to time they may connect, anastamatose, but generally they are finger like extensions.



A microscopic description is as follows:

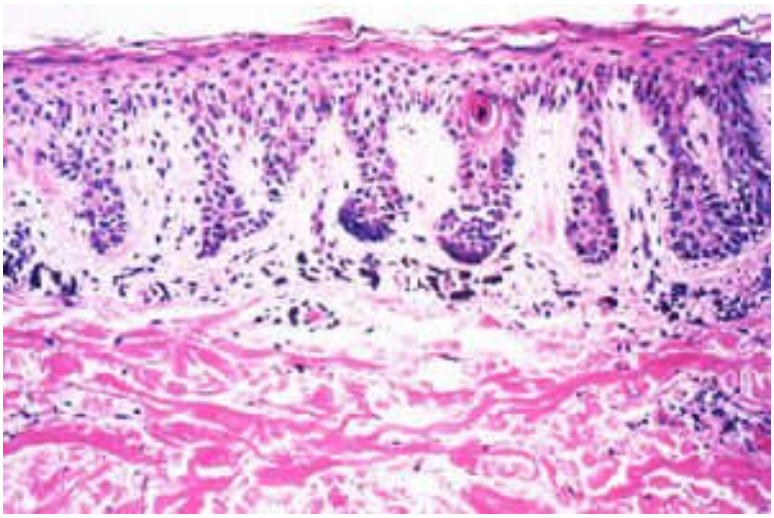
A. There are plump fibroblasts within the upper part of the dermis of this dome-shaped papule.

B. There is a proliferation of enlarged melanocytes arranged as solitary units and as nests within the epidermis, at the dermo-epidermal junction and down epithelial structures of adnexa. There is marked solar elastosis.

If this biopsy captured the entire pigmented lesion this could be a junctional melanocytic nevus

2.3.3.2 Lentigenes

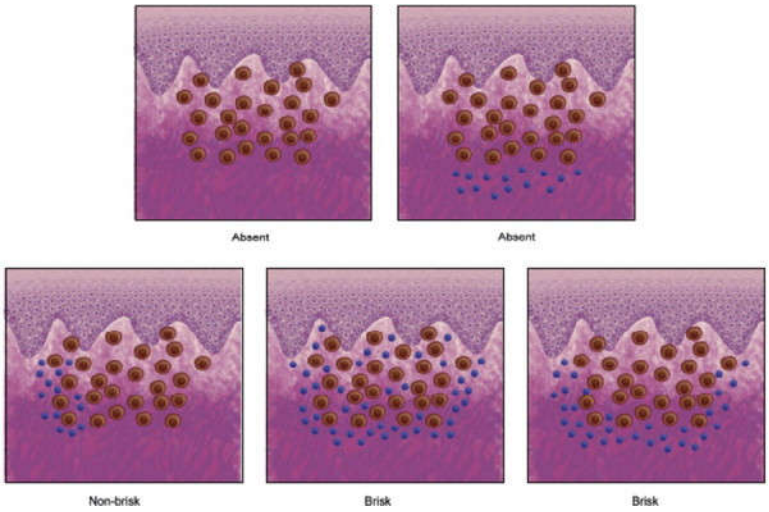
Lentigenes have a distinct histologic pattern of elongated, club-shaped rete ridges which often anastomose.



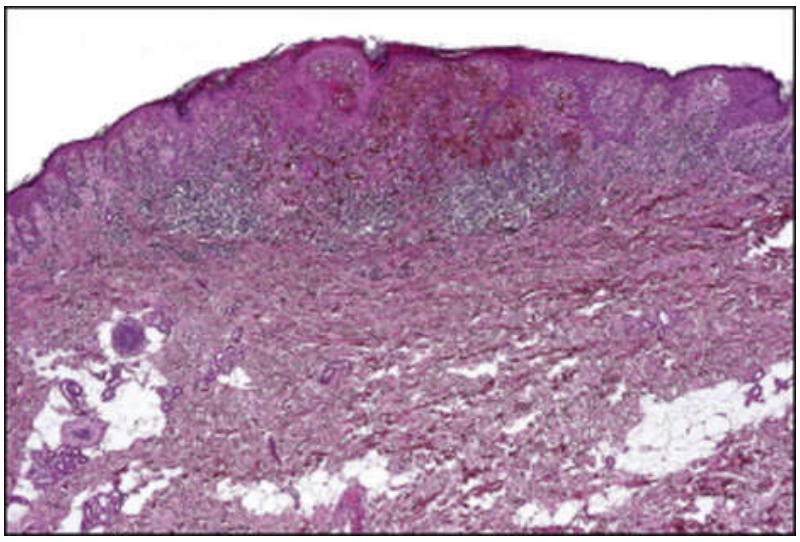
Lentigenes (which is the plural of lentigo) is a flat brownish spot on the skin resulting from excess melanin. Lentigo maligna is a macular (raised) patch. One can see the melanocytes in the basal layer as he clear cells.

2.3.3.3 Tumor Infiltrating Lymphocytes

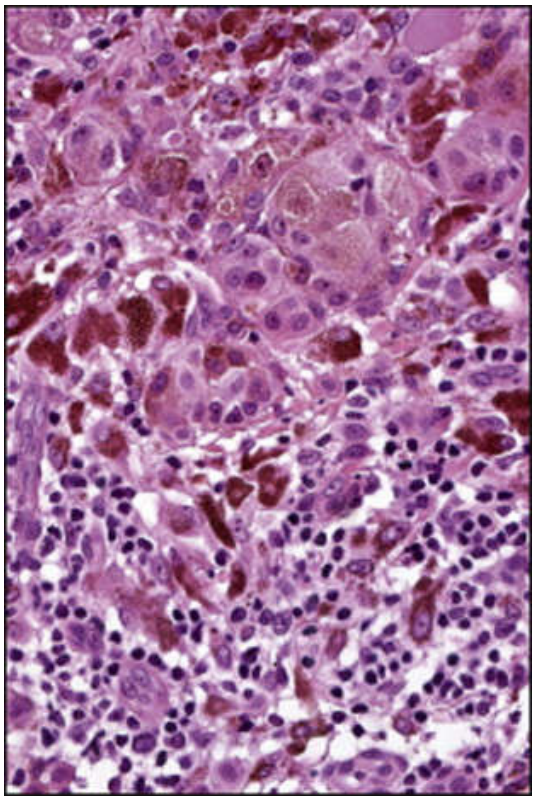
Tumor infiltrating lymphocytes are lymphocytes which have left the blood stream and have infiltrated the cellular area. Melanomas frequently have TILs which is a good prognostic factor. We show from McKee and Calonje the examples of TILs.



We show a cell below demonstrating TILs. They are the dark spotted areas.



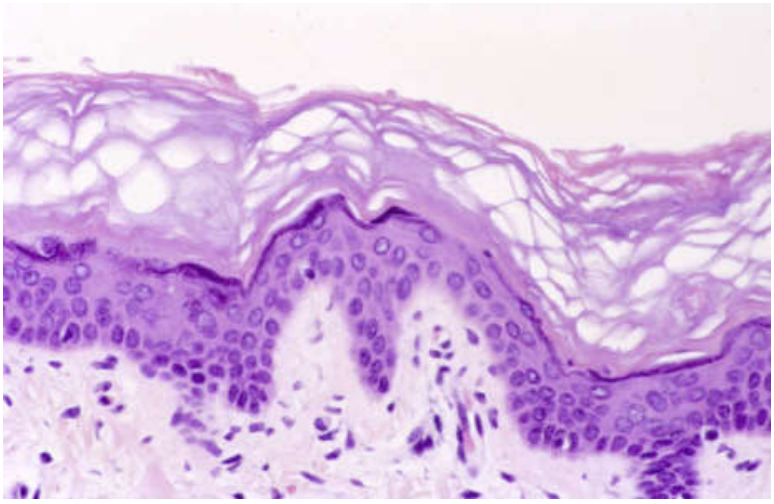
The following is the high power description.



2.3.3.4 Melanocytes

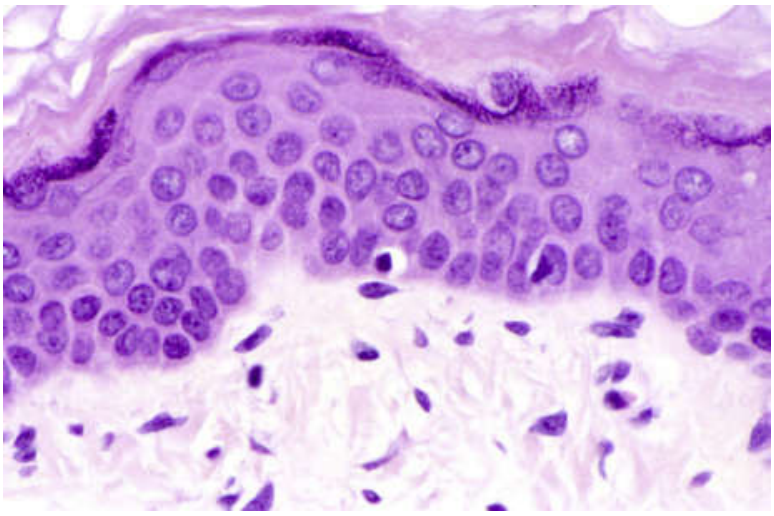
Melanoma is a cancer of the melanocyte. The normal condition of the melanocyte is to stay at the basal layer of the epidermis, with its dendritic arms moving upward and distributing melanosomes to the keratinocytes in response to ultra violet radiation. It is a protective function. The trouble starts when the melanocyte fails to stay put and to migrate and then to multiply.

We show a typical histological section below[3]:



This shows the typical structure comparable to what we have shown above.

The following is an example showing a prominent melanocyte[4]. Note at the basal layer, namely the bottom layer, we see a dark nucleolus with a clear cytoplasm. That is a single melanocyte, and there is another to its right. The melanocytes normally have a clear appearance, are at the basal layer and often have a prominent nucleus.



The above is a 200X magnification. One can see the keratinocytes building from the bottom of the basal layer and moving upward where they get squashed down and then slough off the surface. The melanocytes have a generally longer life cycle and they remain fixed.

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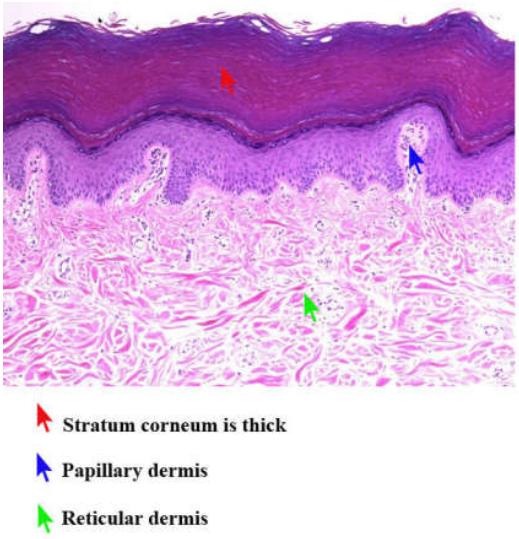
[3] http://pathology.mc.duke.edu/research/Histo_course/epi1.jpg

[4] http://pathology.mc.duke.edu/research/Histo_course/melanocyte.jpg

In this chapter we look at the histology of the melanocyte. In many ways it differs from other cancers in that we can focus on the aberrant behavior of a single cell type, the melanocyte. In a benign state it belongs to the basal layer and should remain there. The melanocyte produces melanosomes for pigmentation. When it loses its normal control mechanism and starts to become a pre-malignant cell the melanocytes lose their location sense. They move from the basal layer to the epidermis and then can be called melanoma in situ. Then they spread outward from the basal layer and become superficial spreading melanoma. Then they start the vertical stage and that is when we see metastatic potential. We examine those stages herein.

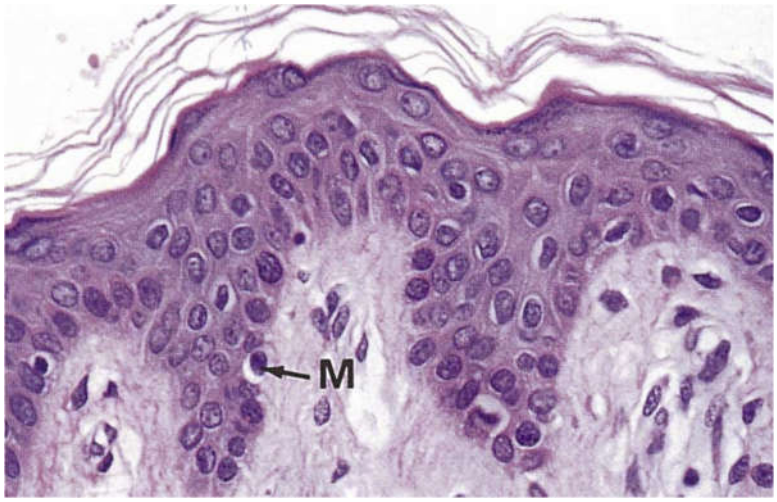
We continue to examine the normal skin histology. The following slide details more structure of the normal skin[5]. It demonstrates the dermis structure as well, specifically the papillary and reticular dermis.



The following shows another example of a melanocyte in normal skin[6]:

5 http://missinglink.ucsf.edu/lm/DermatologyGlossary/normal_skin.html

6 http://www.oucom.ohiou.edu/dbms-witmer/Downloads/Basic%20Skin%20Histology2-21-01.pdf



Again, in the above, we see the melanocyte in the basal layer, it has a clear cytoplasm and it has a prominent and round nucleolus.

2.3.4 Basal Cell

As Rubin et al state[7]:

Basal-cell carcinoma characteristically arises in body areas exposed to the sun and is most common on the head and neck (80 percent of cases), followed by the trunk (15 percent of cases) and arms and legs. Basal-cell carcinomas have also been reported in unusual sites, including the axillae, breasts, perianal area, genitalia, palms, and soles.

Nodular basal-cell carcinoma is the classic form, which most often presents as a pearly papule or nodule with overlying telangiectases and a rolled border, at times exhibiting central crusting or ulceration.

Occasionally, nodular basal-cell carcinoma may resemble enlarged pores or pits on the sebaceous skin of the central portion of the face. Superficial basal-cell carcinoma presents as a scaly erythematous patch or plaque. Both nodular and superficial forms may contain melanin, imparting a brown, blue, or black color to these lesions.

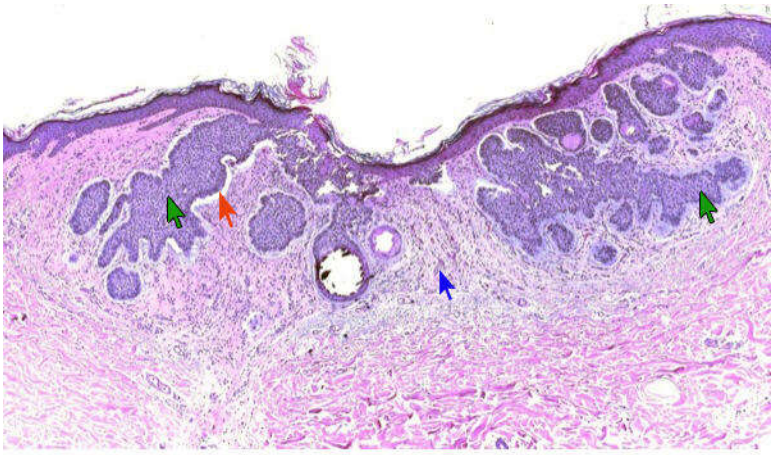
The morpheaform type, also known as sclerosing, fibrosing, or infiltrative basal-cell carcinoma, typically appears as an indurated, whitish, scar-like plaque with indistinct margins.

Suspicious lesions occurring in high-risk areas, such as the central portion of the face, should undergo prompt biopsy to obtain a timely diagnosis and to expedite definitive treatment. Skin biopsy will also identify amelanotic (nonpigmented) or minimally pigmented melanomas, which can sometimes mimic basal-cell carcinoma.

[7] Rubin, A., et al, Basal Cell Carcinoma, NEJM, 353;21, November 24, 2005

Rubin et al also state:

Inappropriate activation of the hedgehog (HH) signaling pathway is found in sporadic and familial cases of basal-cell carcinoma, medulloblastoma, rhabdomyosarcoma, and other tumors

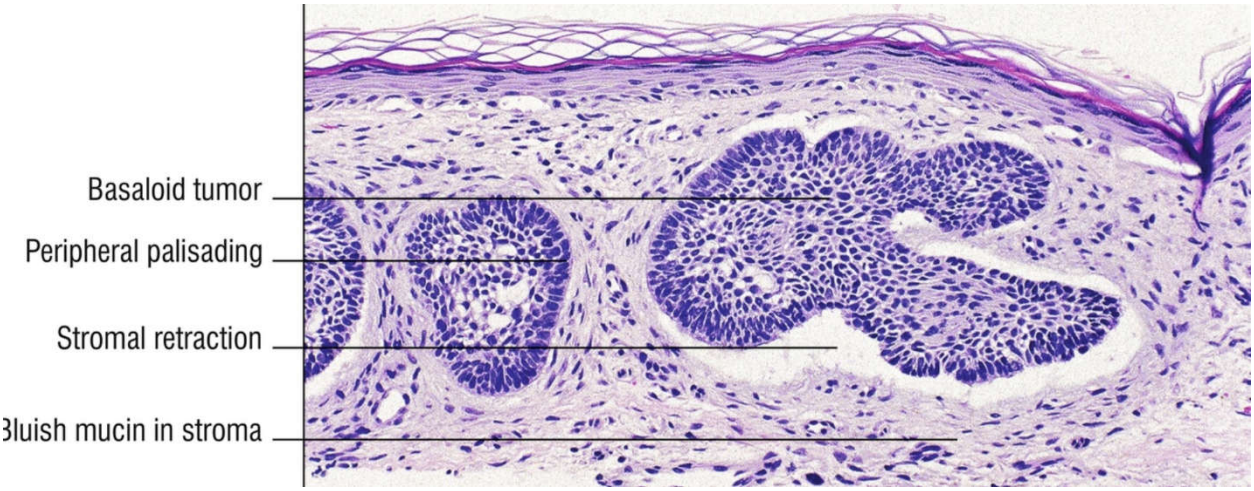


A cleft often separates the basaloid cells from the stroma

Basaloid cells

Blue (myxoid) stroma often contains mucin

Although BCC is common, we shall not be focusing on it in any manner but the paper by Rubin et al is useful so as to understand the pathway issues which have some parallels.



As Trieu et al have noted:

- Upstream Hh pathway activation is necessary, but not sufficient, for BCCs to progress
- Failed nascent tumors become dormant and may spontaneously regress over time
- Secondary mutations enable rare tumors to overcome barriers to progression
- Increased Mycn promotes BCC progression

2.4 TYPES OF BCC

We now examine the types of BCC based upon a histological process. Crowsen notes:

Traditionally, BCCs have been classified as solid (or undifferentiated) vs those tumors that manifest specific differentiation features (ie to eccrine, sebaceous or other cell lines). However, the only proven histologic prognosticator of biologic behavior, and therefore a major determinant of what constitutes an appropriate therapeutic approach, is the architectural growth pattern. Thus, the architecture is a critical issue, while the differentiation patterns need to be considered only insofar as they must be recognized as part of the histologic spectrum of BCC, and as they impact differential diagnosis.

Their misidentification as Merkel cell carcinoma or as sebaceous, eccrine, or follicular neoplasia is a diagnostic pitfall that carries with it a risk of over- or undertreatment. ***One needs to be aware that shave and punch biopsy specimens have an intrinsic error rate, due to the nature of the tissue sample, of roughly 20% in predicting classification of BCC subtypes when compared to completion excisions at the same anatomic location****. The reason for this may be explained by the fact that biological transformation of BCC tends to occur at the base and edges of the growing neoplasm. The Undifferentiated BCCs*

We classify BCCs as belonging to indolent-growth or aggressive-growth subsets.

The indolent-growth variants include superficial and nodular BCC.

The aggressive growth tumors are infiltrative BCC, metatypical BCC, and morpheiform or sclerosing BCC.

In one large retrospective series of 1039 consecutive neoplasms, and using a slightly dissimilar classification scheme, 21% were nodular, 17.4% were superficial, 14.5% were micronodular, 7.4% were infiltrative and 1.1% were morpheiform.32 Roughly one-third of all tumors showed an admixture of patterns.32

2.4.1 Superficial BCC

Superficial BCC is characterized by a proliferation of atypical basaloid cells that form an axis parallel to the epidermal surface and demonstrate slit-like retraction of the palisaded basal cells from the subjacent stroma (The resulting cleft-like spaces often contain alcian blue-positive mesenchymal mucoid material, a presumed product of the stromal cells. Tumor cells may colonize the hair follicle and rarely the eccrine adnexal structures and, as mentioned above, often take origin from the follicular bulges.

Mitoses are infrequent and apoptic cells rare in the atypical basaloid buds for reasons reflecting their biologic derivation as described below. Some cases manifest melanin pigmentation of the epithelium and in the histiocytes in the subjacent stroma; of pigmented BCCs, most are held to reflect superficial tumors in some series, although in our experience nodular BCCs constitute by far the most frequent form of pigmented BCC. A band like, often heavy, lymphoid infiltrate may be present. When seen in the setting of a biopsy for suspect superficial or superficial multifocal BCC, a band-like lymphoid infiltrate should prompt a careful search through multiple levels looking for foci of superficial BCC.

2.4.2 Nodular BCC

Nodular BCC represents the most common form of the neoplasm in our experience. Nodular BCC is also referred to as nodulocystic BCC by some observers, although this term is not employed by us. This is the type of BCC that clinically shows a translucent pearly papule or nodule with a rolled border and telangiectasia. The nodular form of BCC is characterized by discrete large or small nests of basaloid cells in either the papillary or reticular dermis accompanied by slit-like retraction from a stroma in which the fibroblasts do not appear to be plump or proplastic. Any of the differentiated elements (eccrine, sebaceous, etc) may be seen in nodular tumors and roughly one-third of cases will show a coexistent superficial component.

As both superficial and nodular BCC can be seen in sun-exposed or sun-protected skin, the dermis may show solar elastosis and this may be pronounced.

The surrounding stroma shows myxoid change, is rarely fibrotic and may show calcification in discrete islands of tumor or in adjacent stroma. Mitoses and individual cell necrosis are uncommon. The presence of abundant slit-like retraction may cause tumor nests to drop out from the stroma during processing yielding empty spaces with a rounded contour in the mid or deep dermis. This is an important clue to the diagnosis in the setting of the nodular and/or infiltrative growth patterns. A significant proportion of BCCs with a nodular component manifests a variable admixture of superficial and/or micronodular morphologies.

Melanin pigmentation of tumor cells and adjacent stromal histiocytes may be seen. Transition to micronodular and other aggressive growth forms of BCC may be seen.

2.4.3 Micronodular BCC

Micronodular BCC manifests a plaque-like indurated lesion with a poorly demarcated contour. As mentioned above, lesions may be difficult to remove and so have an increased incidence of recurrence.

Micronodular BCC manifests tumor nests with roughly the same shape and contour as nodular BCC, but which are nonetheless smaller and widely dispersed in an often asymmetric distribution extending deeper into the dermis and/or subcutis. These monotonous, small round tumor nests are accompanied by stromal proliferation cognate to the infiltrative growth BCC. Retraction spaces are not common and the surrounding stroma shows either a myxoid or

collagenized morphology suggesting that these lesions may be an intermediate step between nodular and aggressive growth subtypes.

The micronodular BCC has been reported to have a higher incidence of local recurrence and may penetrate more deeply into the reticular dermis and/or subcutis.

2.4.4 Aggressive growth BCC

The aggressive growth BCCs include the ***prototypic morpheaform BCC, infiltrative growth BCC and metatypical BCC****.*

2.4.5 Morpheaform BCC

Morpheaform or sclerosing BCC is characterized by columns of basaloid cells one to two cells thick enmeshed in a densely collagenized stroma containing proplastic fibroblasts.

Individual cell necrosis and mitotic activity is brisk considering the relative tumor volume and the neoplasms themselves are poorly demarcated, showing widespread invasion of the reticular dermis and penetration into the subcutaneous tissue. Slit-like retraction from the stroma is less common than for the nodular and superficial variants but is still often demonstrable. These neoplasms may coexist with other aggressive growth morphologic variants.

Morpheaform BCCs represent roughly 1–5% of all BCCs and clinically present as white or yellow depressed fibrotic scars that rarely ulcerate or bleed.

In our experience, these cancers occur mainly in a sun-exposed distribution. Although typically one to two cells in thickness, cords up to five cells in thickness may be present; the architecture comprises sharp angulation of such cords. Pronounced stromal fibroplasia and fibrosis surrounds the tumor tongues. By electron microscopy, a basal lamina is absent.

2.4.6 Infiltrative growth BCC

Infiltrative growth BCC comprises, at the light microscopic level, irregularly sized and shaped nests of tumor cells; the nests show sharp angulation of their peripheral contours, occasional foci of slit-like retraction, and frequent mitotic activity and individual cell necrosis of the neoplastic cells.

The stroma is frequently fibrotic with plump proplastic stromal fibroblasts. The nests are variable in size and shape with jagged contours. Typically the elongated tumor cell strands of the infiltrative growth component are 5– 8 cells in thickness. Roughly one-third of such cases show an admixed nodular component from which the lesions are held to derive following UV irradiation Like the morpheaform variant, these tumors are poorly circumscribed and may show invasion of subcutis and adjacent muscular and other structures.

Perineural infiltration is a distinct risk in this variant as in the morpheaform BCC, and, like the morpheaform variant, the clinical correlate is a depressed yellowish or fibrotic plaque that

typically lacks a rolled border or an elevated pearly nodule unless a nodular BCC component coexists.

2.4.7 Metatypical BCC

The metatypical BCC (equated by some authors with 'basosquamous carcinoma'39) is, in our view, a form of aggressive growth BCC with infiltrating jagged tongues of tumor cells, some of which manifest an abortive peripheral palisade and clearcut basaloid morphology, admixed with other areas that show intercellular bridge formation and/or cytoplasmic keratinization (Figure 11). Clinching the diagnosis is the presence of a coexistent classic nodular or superficial component.

We distinguish these neoplasms from keratotic BCC, which is most often a nodular BCC with central squamous differentiation, and from the mixed basal cell–squamous cell carcinomas that represent a collision between two clonally distinctive and geographically separate neoplasms in the same biopsy or excision specimen.

Metatypical BCC is the subtype of BCC that can be confused with squamous cell carcinoma and which promotes controversy considering its precise histomorphologic classification, as it shows both basal cell and squamous cell carcinoma differentiation in a continuous fashion.

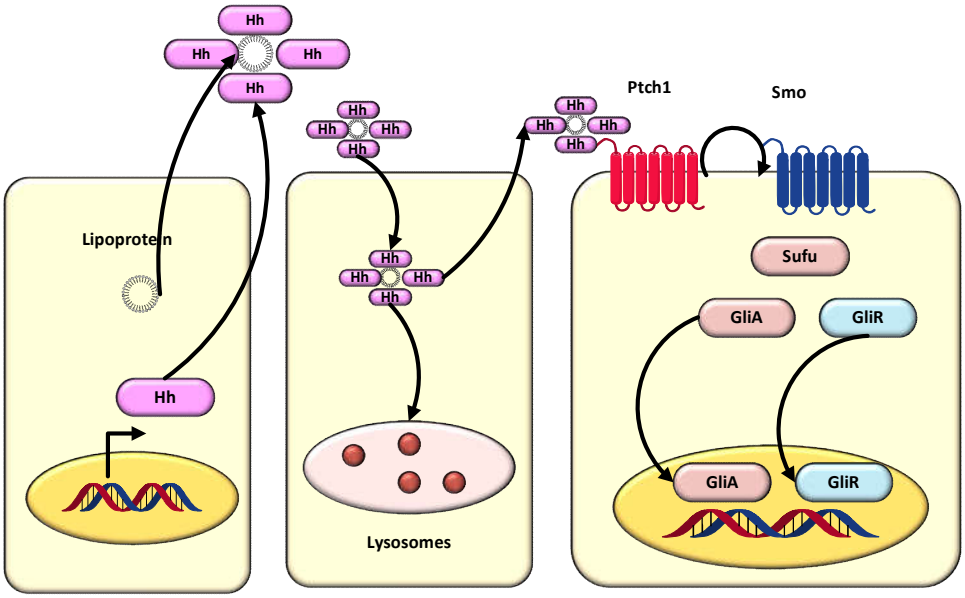
3 HEDGEHOG PATHWAYS

We now examine the Hedgehog pathways. As Wang et al had noted:

Hedgehog (Hh) signaling proteins regulate multiple developmental and adult homeostatic processes.

A defining feature of Hh signaling is that relatively small changes in the concentration of Hh ligand elicit dramatically different cellular responses. As a result, the processing, release and trafficking of Hh ligands must be tightly regulated to ensure proper signaling. In addition, sensitive and specific intracellular signaling cascades are needed to interpret subtle differences in the level of Hh signal to execute an appropriate response. A detailed understanding of the mechanisms that regulate these responses is critical to shaping our view of this key regulatory system.

We present below a generalized view of the HH pathway from source to action.



See Wang et al

Wang et al continue to discuss this model:

Current model of the vertebrate Hedgehog signaling pathway.

Hedgehog (Hh) proteins are synthesized as 45 kDa precursor proteins that are palmitoylated at the N-terminus by Skinny Hedgehog (Skn), and concomitantly auto-catalytically cleaved and cholesterol-modified (mediated by the C terminus) to yield a 19 kDa, dually lipidated, N-terminal signaling protein.

It is not totally understood what initiates and drives this process. They continue:

Hh proteins are then trafficked to the cell surface and released from cells as lipoprotein (LP)-associated oligomers in a process mediated by the 12-transmembrane-pass protein Dispatched (Disp).

These Hh oligomers travel from Hh-producing cells to Hh-responding cells via interactions with both glypicans and megalin, the latter of which also regulates Hh protein turnover. For simplicity, in this model Hh is depicted as trafficking only across a single cell via the actions of megalin, while, in vivo, Hh travels over many cell diameters by a process that is likely to be regulated by multiple molecules and mechanisms.

On responding cells, Hh interacts with a complex of Ptch1 and Cdo/Boc, resulting in de-repression of Smo and activation of the Hh signaling cascade.

In this model, Ptch1 regulates Smo activity via either Vitamin D3 (inhibits Smo) in the absence of Hh ligand, or oxysterols (activates Smo) in the presence of Hh ligand. Hh ligands also interact with Hip1, Ptch2 and Gas1 to regulate the range and level of Hh signaling. De-repression of Smo activates downstream pathways that culminate in activation of Gli activators and repression of Gli repressors.

Gli-dependent transcriptional read-out results in activation of positive targets, including Class II transcription factors (TFs, e.g Nkx2.2), and repression of negative targets, including Class I TFs (e.g. Pax6, Pax7), both directly via Gli binding, and indirectly, possibly via transcriptional regulation of other factors.

Importantly, Smo, Gli activators and Sufu all localize to the tips of cilia.

Sufu plays an essential role as a negative regulator between Smo and Gli. Rab23 negatively regulates Hh signaling by blocking the formation of Gli activators and promoting formation of Gli repressors, in a process possibly mediated by Tectonic.

By contrast, Talpid3 both positively and negatively regulates Hh signaling, presumably by affecting both Gli activator function and processing. Iguana regulates nuclear translocation of Gli proteins.

From Meng et al:

The Hh signaling pathway is one of the important signaling pathways that play key roles in the processes of embryonic development, carcinogenesis, maintenance of cancer stem cells (CSCs), and the acquisition of epithelial-to-mesenchymal transition (EMT) leading to metastasis.

The Hh signaling pathway is highly conserved from flies to humans and is essential for the normal development of an embryo. It is initiated by binding of one of the three Hh ligands

(Sonic, Indian, or Desert Hh) to the 12-pass transmembrane receptor Patched (PTCH). In the absence of ligand, PTCH represses Smoothened (SMO). In presence of ligand, this inhibition is relieved, enabling SMO to modulate a cytoplasmic complex containing Suppressor of Fused (SUFU) that modifies the three glioma-associated (GLI) transcriptional regulators through phosphorylation, sumoylation, and selective proteolysis.

GLI1 induces Hh target genes; GLI2 can act as an inducer or repressor depending on post-transcriptional and post-translational processing events in a cell contextual manner; GLI3 mainly serves as a repressor.

Vertebrate Hh signaling is further regulated by the translocation of signaling components through the cytoplasm, plasma membrane, nucleus, and the primary cilium. PTCH1 is initially located in the primary cilium and SMO is within cytoplasmic vesicles. Following classical Hh ligand binding to PTCH1, SMO moves to the primary cilium, where it interacts with the GLI processing complex, eventually resulting in the nuclear translocation of the GLI transcription factors. Transcription mediated by the GLI proteins is typically referred to as the canonical modulation of Hh signaling.

Notably, the activation of GLI transcription (canonical) can also be initiated by other secreted proteins including osteopontin, transforming growth factor-β (TGF-β), chemokines etc. This type of activation of GLI by stimuli other than Hh ligands is referred to as non-classical signaling. Studies of the Hh pathway, rather its functioning, have been facilitated by the availability of small molecule inhibitors that target distinct aspects of Hh signaling. The most commonly used has been cyclopamine which inhibits the activity of SMO, thereby impeding the transduction of the activation signal intracellularly and arresting the nuclear translocation of the GLI transcription factors.

3.1 HH SECRETION

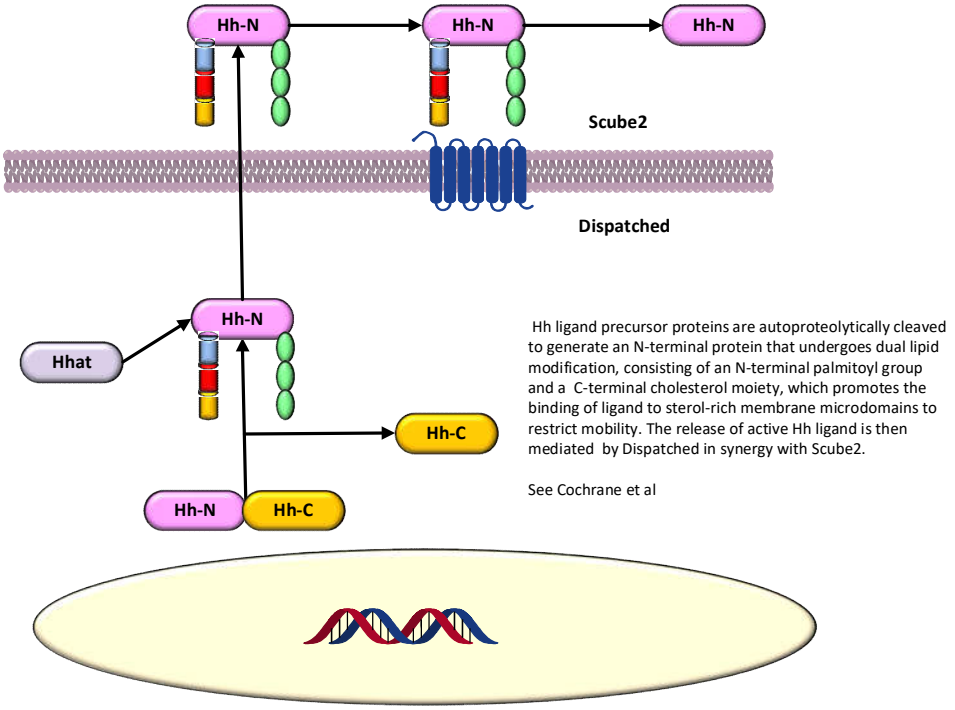
As Cochrane et al note:

The three mammalian Hh ligands, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh), are synthesized as precursor proteins that undergo autoproteolytic cleavage to produce an N-terminal signaling protein with dual lipid modifications.

Cleavage of the carboxyl-terminal peptide and subsequent transfer of a cholesterol moiety on the resulting C-terminus leads to Hh ligand retention at the plasma membrane. Hedgehog acyltransferase (Hhat) catalyses the addition of a palmitoyl group on the N-terminus, promoting the association of the ligand to sterol-rich membrane microdomains to restrict ligand mobility. Dispatched (Disp), a large multi-pass transmembrane protein, in synergy with Scube2, a secreted glycoprotein, bind to distinct components of the C-terminal cholesterol group to generate the release of Hh ligand from the plasma membrane and shelter lipidated Hh from the aqueous microenvironment.

Additionally, Hh contains the ability to form monomers and large multimers through their cholesterol linkages. Diffusion of Hh ligand is negatively regulated by the membrane protein Hh-

interacting protein 1 (Hhip1), which competes with the receptor Patched (see below) for ligand binding through association of the Zn2` containing pseudo-active site in Hh ligands. Similarly, the glycophosphatidylinositol (GPI)-linked heparan sulphate proteoglycan, Glypican-3, (Gpc3), is able to sequester Hh and prevent long-range ligand distribution



3.2 CLASSIC PATHWAYS

As Rimkus ey al note:

The canonical HH pathway contains several key components, including HH glycoproteins Shh, IHH, and DHH.

Upon secretion, Shh glycoproteins bind and inactivate the 12-transmembrane protein Patched1 (PTCH1), which normally inhibits the activity of the 7-transmembrane protein Smoothened (SMO).

In the presence of Shh ligand, PTCH1 inhibition of SMO at the primary cilium is abrogated resulting in the nuclear localization of glioma-associated (GLI) transcription factors, which are the terminal effectors of the Shh signaling. PTCH2 receptor shares approximately 54% homology with PTCH1, yet its expression pattern and signaling role in tissue vary significantly from PTCH1. PTCH2 is highly expressed in spermatocytes and helps mediate DHH activity in germ cell development. It has also been shown that in the absence of Shh ligand binding, PTCH2 has a decreased ability to inhibit SMO.

In the absence of ligand, Suppressor of Fused (SUFU) negatively regulates the pathway by directly binding to GLI transcription factors and anchoring them in the cytoplasm preventing the activation of GLI target genes. Cytoplasmic sequestration of GLI transcription factors by SUFU facilitates processing and degradation of GLI proteins, therefore inhibiting Shh pathway signaling.

SUFU has also been shown to form a repressor complex leading to interaction with DNA-bound GLI1 and suppression of GLI1-induced gene expression.

In vertebrates, there are three GLI transcription factors (GLI1, GLI12 and GLI3). GLI1 is the only full-length transcriptional activator whereas GLI2 and GLI3 act as either a positive or negative regulators as determined by posttranscriptional and posttranslational processing. In response to Shh ligand binding, GLI2 accumulates in the primary cilium and drives transcriptional activation, overcoming negative regulation by GLI3. In addition to regulation by SUFU, GLI1 is also regulated by the kinase Dyrk1. Dyrk1 can potentiate GLI1 activity by phosphorylation at multiple serine/threonine sites that has been shown to induce nuclear accumulation and GLI1-mediated transcription.

GLI transcription factors can activate target genes that includes targets involved in HH pathway feedback (e.g., GLI1, PTCH1), proliferation (e.g., Cyclin-D1, MYC), apoptosis (e.g., Bcl-2), angiogenesis (e.g., ANG1/2), epithelial-to-mesenchymal transition (e.g., SNAIL), and stem cell self-renewal (e.g., NANOG, SOX2). In addition to the classical (canonical) signaling axis, there are also non-classical (non-canonical) pathways related to Shh signaling.

Non-canonical Shh signaling refers to either:

(1) activation of signaling from PTCH1/SMO but independent of GLI transcription factors; or

(2) activation of GLI transcription factors independent of Shh ligand or PTCH1/SMO.

The latter is better studied and multiple pathways have been identified, mostly oncogenic, that can increase GLI activity. GLI transcription factors have been shown to be positively regulated by K-Ras, TGF-β, PI3K-AKT, and PKC-α.

K-Ras, in particular, seems to be a pathway capable of activating GLI1 independent of the Shh pathway as knockdown of SUFU does not affect K-Ras-induced GLI1.

Additionally, the GLI proteins have been shown to be negatively regulated by p53, PKA, and PKC-δ. GLI1 transcriptional activity has also been shown to be reduced with p53 overexpression and enhanced with p53 knockdown.

Furthermore, p53 has been shown to interact with TAF9 leading to suppression of GLI1 activity. PKA regulation of GLI1 is very specific as PKA directly phosphorylates Thr374 of GLI1, which promotes cytoplasmic localization and reduced activity of GLI1

As Carballo et al note:

Activation of Shh pathway can happen in two major ways: 1. canonical signaling: by ligand-dependent interaction or through receptor-induced signaling and 2. non-canonical signaling, when there's a mechanism of activation downstream of smoothened (Smo). The Shh canonical signaling occurs when the glycoprotein Shh binds and inactivates the 12-transmembrane protein Patched (Ptch1).

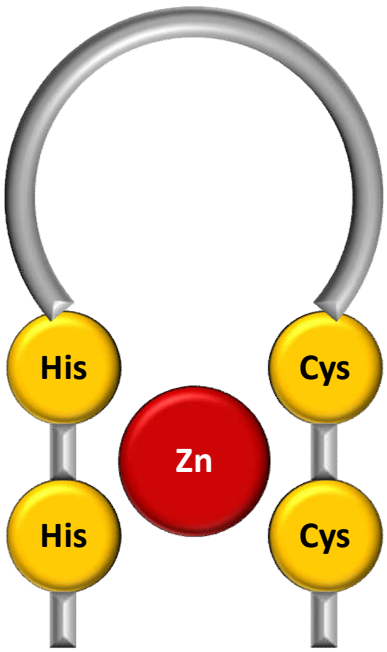
In the lack of the ligand Shh, the activity of the 7-transmembrane protein Smo is inhibited by Ptch1, so Shh protein binding Ptch1 regulates Smo activity. Smo is a GPCR-like (G protein–coupled receptor) protein, and the translocation into the cilia membrane is a requisite for Gli activation. In response to Shh signaling, Ptch1 inhibition of Smo at the PC is abolished, when Ptch1 is internalized and degraded. So, after Ptch1 degradation, Smo accumulates at the PC where is activated and stabilized by initiating the Shh downstream signaling cascade. This downstream signaling cascade results in the translocation of Gli family proteins to the nucleus that begins the transcription of target genes, including Ptch1 and Gli1, in a negative and positive feedback loop, respectively. Furthermore, Gli translocation to the nucleus also induces protein modulation of Wnt and Noggin.

Patched 2 (Ptch2) is another receptor for Shh that shares approximately 54% homology with Ptch1. However, the expression and signaling of Ptch2 is different from Ptch1, having decreased ability to inhibit Smo in absence of Shh ligand.

The Gli1 gene was initially cloned as an amplified oncogene of a malignant glioma and then characterized as a transcription factor of the hedgehog signaling pathway.

*Three Gli proteins (Gli1, Gli2 and Gli3) are **zinc-finger transcription factors** and are expressed in vertebrates, in overlapping and partially redundant domains.*

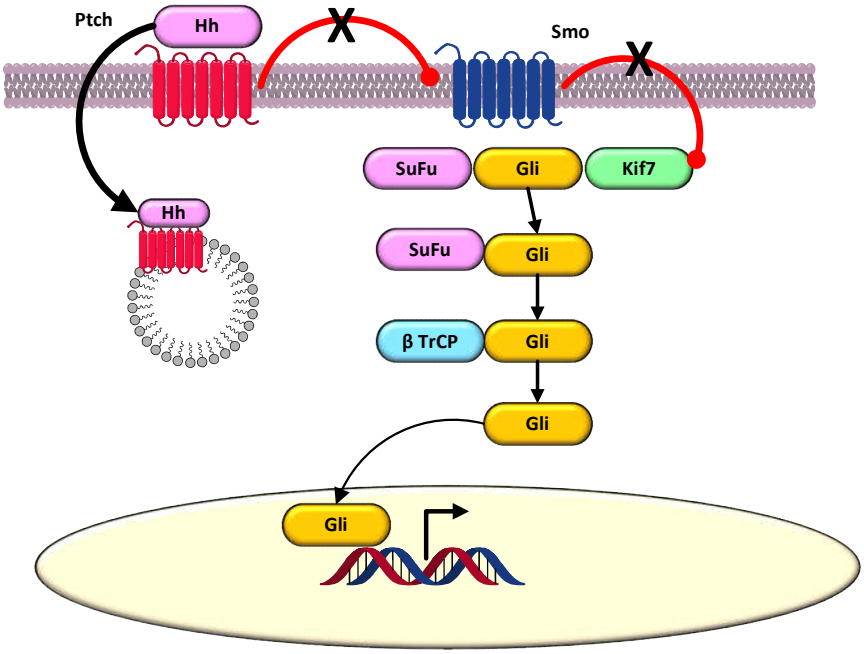
We show the typical zinc finger transcription factor below:



These three proteins are Shh-dependent, where only Gli1 occurs as a full-length transcriptional activator, while Gli2 and Gli3 act as either a negative or positive regulators (Gli2A - Gli2 activated or Gli2R - Gli2 repressor and Gli3A - Gli3 activated or Gli3R - Gli3 repressor, respectively) of the pathway which is determined by post-transcriptional and post-translational processing. Moreover, the change of Gli3A to Gli3R form is favored with respect to Gli2. Consequently, Gli2 has mainly an activator transcriptional behavior, while Gli3 acts as a repressor.

It has already been demonstrated that Gli2 can accumulate in the primary cilium and controls transcriptional activation, in response to Shh ligand binding, overcoming thereby the negative regulation of Gli3 .

The Gli3 has also a very important function in regulating Shh signaling. Without Shh, Gli3 has a repressor form (Gli3R). When Shh binds to Ptch and activates Smo, Smo converts Gli3R into an activated form (Gli3A). So, Gli3 works as a transcriptional factor with a dual function. The ratio of Gli3R/Gli3A is directly related to the control of several processes during organogenesis, such as digit types and number

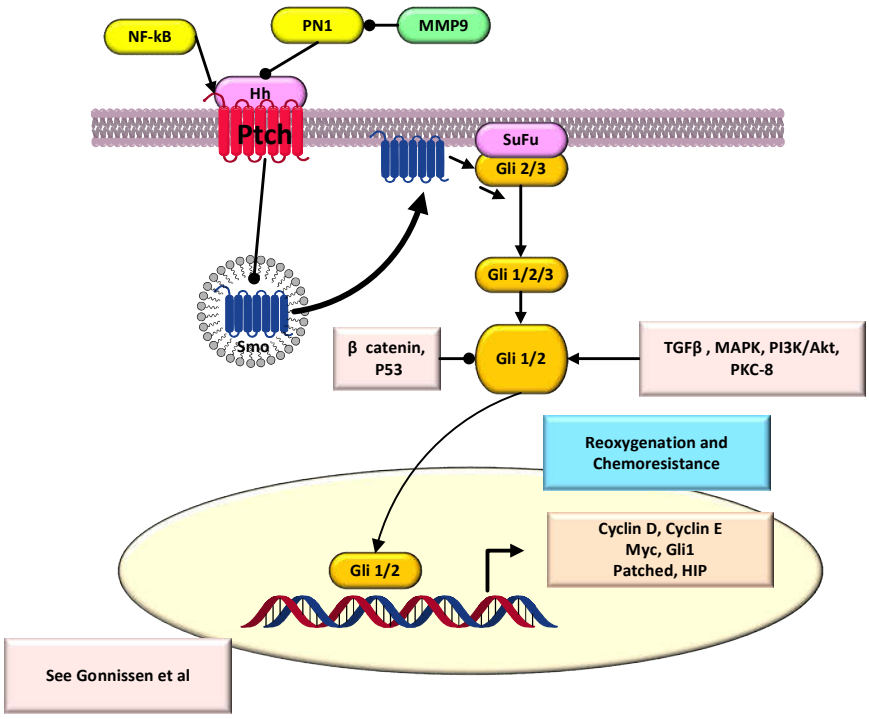


In contrast Gonnissen at al note:

Schematic overview of Hedgehog signaling and rationale for combination therapy with (chemo)radiotherapy. Upon Sonic Hedgehog (Shh) ligand binding to its receptor Patched (Ptch1) 1, the repression of Smoothened (Smo) is relieved, resulting in the movement of Smo from the intracellular vesicles to the primary cilium. Smo becomes activated and promotes the activation of the Gli proteins (Gli1/2) that enter the nucleus and promote transcription of the target genes (canonical pathway activation). The Gli transcription factors can also become activated by means of non-canonical pathway activation due to significant crosstalk with other important pathways such as the PI3K-Akt, KRAS, PKC-δ and TGFβ pathways.

The Hh signaling also has important interactions with Wnt pathway and P53.

The response to radiation therapy is determined by the four R's of radiobiology: repopulation, repair of sublethal DNA damage, redistribution and reoxygenation. Hh signaling can potentially interfere with all these processes and targeting Hh signaling could therefore increase radiosensitivity of tumor cells. Moreover, inhibition of Hh signaling could also improve the response to chemotherapy by targeting multidrug resistance and cancer stems cells in addition to its effects on tumor vasculature.



3.3 GLI

As Wang et al noted:

The epithelial-mesenchymal transition (EMT) is a process in which cells lose their cell-cell adhesive properties and gain migratory and invasive potential; it is essential for events in embryonic development, wound healing, fibrosis, cancer progression, and metastasis.

EMT involves multiple complex changes in the distribution and function of proteins, including E-cadherin, an adhesive protein inactivated in numerous cancers.

This process is regulated by a number of converging signaling cascades, including the Sonic Hedgehog (SHh) pathway, and confers critical traits required for seeding metastasis and developing stem cell properties that allow new cancer cell colonies to be launched

Acquisition of EMT features has been associated with poor prognosis and chemotherapeutic resistance—accordingly, further knowledge can improve our understanding of tumor recurrence and metastasis to identify potential therapeutic targets in EAC . Activation of SHh signaling has been implicated in the tumorigenesis and metastasis of various cancers.

The canonical cascade is initiated by Patched (Ptch) and Smoothened (Smo); Sonic Hedgehog ligand (Shh) binding to Ptch allows release of Smo, causing active fulllength Gli to enter the nucleus and activate transcription of target genes, including Gli1, Ptch1, and Cyclin D1, in a context- and cell-type dependent manner.

Among the Gli family of transcription factors (TFs), Gli1 and Gli2 are considered activators, while Gli3 serves as a repressor. Non-canonical Gli activation independent of Shh activation has also been noted in many cancer cells types, owing to stimulation by other oncogenic signaling pathways such as transforming growth factor β (TGF-β), epidermal growth factor receptor (EGFR), RAS, and AKT/ PI3K.

As Gli TFs constitute the final effectors of the SHh pathway, and are implicated in multiple other oncogenic signaling cascades, they represent an important downstream target for potential cancer therapeutics. Efforts have been made to develop inhibitors of the SHh pathway, including Vismodegib, a Smo inhibitor, as well as GANT-61, a Gli inhibitor that regulates Gli-dependent transcription, to promote anti-cancer activity.

In our lab, we have also developed a novel Gli inhibitor (Gli-i) that specifically blocks Gli1 and Gli2 transcriptional activity with significant efficacy. However, the relationship between EMT and SHh/Gli activation has not previously been studied in EAC, and existing data from other solid tumor types are controversial. While some studies have shown that SHh/Gli inhibition block EMT, the exact mechanisms have not been elucidated. In melanoma and pancreatic cancers, results suggest the role of Gli in facilitating cancer migration and invasion by regulation of E-cadherin.

On the other hand, another conflicting report proposes the inhibition of Gli in promoting the same EMT characteristics in pancreatic cancer. In lung squamous cell cancer (SCC), Gli expression is inversely correlated to that of E-cadherin. Studies in melanoma and hepatocellular carcinoma have linked Gli1 to vascular/ capsular invasion, advanced tumor stage, and upregulation of matrix metalloprotease (MMP)-2 and MMP-9, while siRNA silencing of Gli1 successfully reduced invasion and increased E-cadherin expression .

Our lab recently studied upregulated signaling in lung cancer, investigating Gli1's inverse correlation with E-cadherin; inhibition of the SHh pathway upregulates E-cadherin expression and suppresses lung cancer cell migration. In this study, aberrant Gli activation was studied in both EAC tissue samples and cell lines. Gli and EMT markers were found to be inversely correlated, and inhibition of the former minimized migration and invasion of EAC cells. Gli suppression induced upregulated E-cadherin expression and downregulated phosphorylated AKT, suggesting Gli may be critical for the metastasis and recurrence of esophageal adenocarcinomas.

As Sasai et al note:

All Hh polypeptides produced are transported into the endoplasmic reticulum (ER) and Golgi apparatus, where they undergo autoprocessing.

The polypeptides are cleaved into two parts, the amino-terminal domain, which functions as a signaling molecule, and the carboxyl-terminal domain, which functions in autoprocessing regulation. The amino-terminal part is further modified with palmitate and cholesterol. The carboxyl ends of the initial polypeptides act as cholesterol transferases, and palmitoylation is mediated by skinny Hh (Ski) acyltransferase (also known as Hh acyltransferase or HHAT), a

transmembrane acyltransferase located in the ER. The modified Shh protein was recently purified, and the fatty acids bound to the amino terminus were identified, which revealed that other unsaturated fatty acids in addition to palmitate are involved in the modification. These lipid modifications are essential for the stability of the protein in the extracellular matrix and during long-range transport.

The NiemannPick C (NPC1/2) proteins transport cholesterol from the endosome to the ER, and play an essential role in cholesterol modification of Hh. The membrane protein dispatched (Disp) and the secreted protein Scube2 bind to the cholesterol moiety of the modified Hh proteins and release them from the cell membrane. Some of the Hh proteins at the cell surface can be recycled. After recycling, the Hh proteins are released by lipid bilayer membrane vesicles called exosomes.

Exosomes are formed at the multivesicular body (MVB) in the cytosol, and bud from the plasma membrane with a size of 30–100 nm containing functional molecules. For exosome formation, membrane proteins collectively called endosomal sorting complexes required for transport (ESCRT) are essential. A specialized filopodial structure, the cytoneme, which forms in Hh-producing cells, is important for transmission of the Hh signal.

The cytoneme was initially identified in flies, and later reported in the chick embryonic limb bud. The presence of microvesicles and the cytoneme, together with the palmitoylation and cholesterol modification of Hh proteins, mediate the transport of the Hh signal to distant parts in the tissues.

Overall, the <u>efficient production and secretion of active Hh proteins</u> involves the following:

(i) processing of the polypeptides by autocleavage,

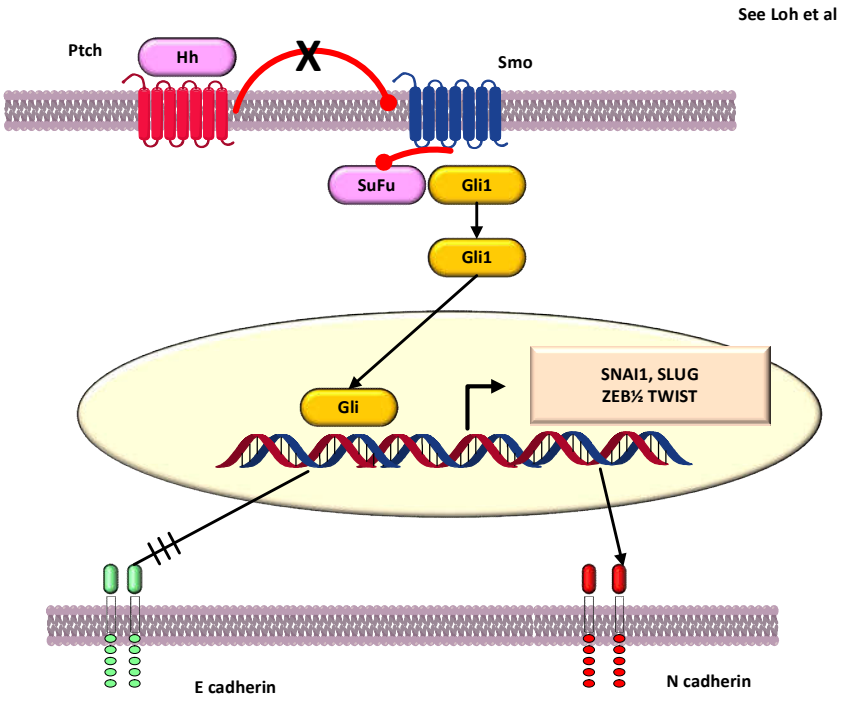
(ii) modification of the polypeptides by cholesterol and palmitate,

(iii) recycling and packaging of the proteins in the microvesicles, and (iv) the presence of cytosolic structures including cytonemes.

3.4 CADHERINS

As noted above, Cadherins change from E to N and thus lose their localization and establish an EMT change[8]. This initiates the basis for metastasis. The figure below from Loh et al describes this change.

[8] See https://www.researchgate.net/publication/330222973_EMT_and_Cancers



As Loh et al note regarding HH and the cadherins:

Aberrant activation of HH signaling mediates EMT in various cancers by altering the expression of E- and N-cadherin, as well as other mesenchymal markers. Li et al. discovered that a dominant negative of SNAI1 in GLI1 transformed epithelial cells increases E-cadherin levels and weakens their ability to grow independent of attachment. Xu et al. also stated that non-canonical GLI1 activation of the HH pathway and MAPK pathway could lead to the decrease of E-cadherin expression in thyroid cancer cell lines, which subsequently lead to BRAF mutation-induced SNAI1 expression.

Besides that, sonic hedgehog (SHH)/GLI1 expression induced a significant upregulation of expression level of S100A4, a member of the S100 gene family and a key EMT molecular marker in pancreatic cancer, while E-cadherin was markedly reduced [129]. S100A4 gene had been shown to promote the expression of TWIST and SNAIl along with other mesenchymal markers during EMT. As there is no evidence indicating direct activation of SNAI1 by GLI1, these data suggest a possible connection between SHH and S100A4 during EMT in pancreatic cancer cells.

Casticin or SMO-inhibitor (cyclopamine) treated ovarian cancer cell line showed notably increase in E-cadherin expression and reduction in N-cadherin expression. Resveratrol and cyclopamine treatment upregulated E-cadherin and downregulated GLI1, SNAI1 and N-cadherin in gastric cancer cell line. Similarly, a SMO knockdown human pancreatic cancer stem cell demonstrated a decrease in the expression of E-cadherin and increase in the expression of SNAI1 and N-cadherin. In esophageal adenocarcinoma (EAC), GLI-inhibitor (Gli-i) upregulated E-cadherin expression while downregulating N-cadherin and β-catenin expression by inhibiting GLI1 and GLI2 transcriptional activity. In addition, a combination of AKT-inhibitor (AKT-i) and N-SHH rescued the effect induced by AKT-i, which was observed when using Gli-i instead of

AKT-i. The study demonstrated that SHH/GLI1 signaling in EAC may potentially regulate EMT via AKT pathway. HH signaling has shown differing effects on E-cadherin as one study has highlighted that inhibition of HH signaling with cyclopamine caused the loss of E-cadherin and relocalization of ZO-1.

Another study has revealed that GLI1 promoted the redistribution of E-cadherin toward the cell membrane. Liao et al. found that abnormal GLI1 activation increased the expression of SNAI1 and E-cadherin in ovarian cancer cells, indicating that E-cadherin may be regulated via a different molecular network other than SNAI1

The change from E cadherin to N cadherin establishes the movement from localization to metastatic potential. Nodular BCC appears to have retained E cadherin whereas infiltrating and micronodular seems to have lost this.

3.5 Cell Cycle Basics

Cancer is basically uncontrolled cell growth, replication, and failure for cells to die off, normal apoptosis. It may also include loss of location stability and metabolic enhancement, but let us start with the key issue, replication. Then we examine two other major factors; apoptosis or cell death and cell to cell adhesion, or simply cells being where they should be. All of this examination is to be focused on the cell cycle. This section is a discussion of what is necessary to understand the importance of the cell cycle. The cycle is what often is broken in cancer cells, namely the cell reproduces again and again.

Cancer in many ways is a loss of the three factors:

1. Cell Replication: This is the normal or abnormal cell cycle.

2. Cell Death: This is normal cell death or apoptosis.

3. Cell Localization: The establishment and maintenance of a cells relative position and function.

We shall thus begin with the control of the cell cycle and then work upwards in terms of the cells control mechanism.

The following Figure presents a simple view of how cell signalling functions. There are six functions described, and not all must be present in any cell function. The steps are generally:

1. Ligand: There is some external activator that floats about and ultimately finds its home on the surface of a cell. Now the issue is not that there is one such protein floating about that eventually may find itself attached to the surface of a cell. The protein may be from afar or it may be from the very same cell. We could then consider the concentration of the protein as well, and its flow across cells themselves as well. This issue is a complex one and all too often it is treated like a simple one protein to one receptor issue. In reality it is a distributed random process.

2. Receptor: The ligand seeks and may ultimately find a receptor. The receptor is a protein on the cell surface. A cell produces the protein and the number of such receptors may be significant as well. Thus there exists a concentration in space of the ligands and they can attach to and activate receptors, proteins, on cell surfaces.

3. Adaptor: The Receptor when connected to a ligand effects a response and there may be an adaptor protein which then gets connected and starts the inter-cell communications process.

4. Transducer: The transducer, such as RAS or PI3K, converts the signal to the receptor as displayed by the adaptor into the beginning of a chain down through the cytoplasm. This is a highly controlled and redundant chain which can become unstable if certain genes are affected and the controlling proteins disabled.

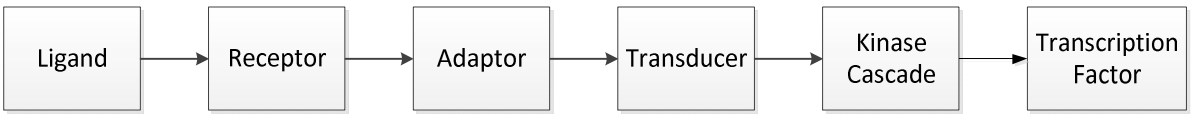
5. Kinase Cascade: This is the chain of protein communicating links and effectors from the Transducer to the cell nucleus and includes the initiation of the targeted transcription factor. As with the Transduce this kinase chain is controlled by redundant checks but if they become defective then the chain internal controls can be lost and the result become unstable.

6. Transcription Factor: This is the protein which has been activated within the nucleus which then commences transcription of the targeted sets of genes for the purpose of producing the resulting product.

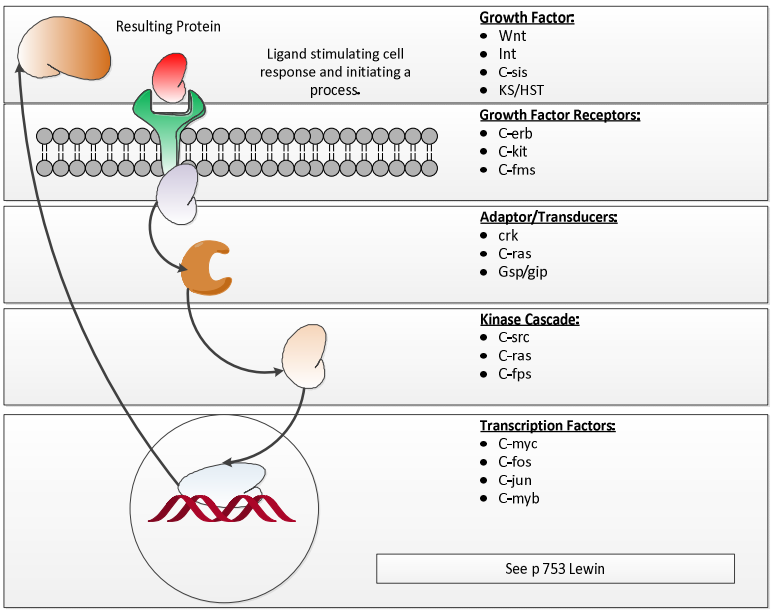
Note that this is a complex process.

Ligand	PDGF	Insulin	Growth Hormone	IL-1β	TGF-β
Receptor	PDGF Receptor	Insulin Receptor	GH Receptor	IL Receptor	TGF Receptor
Adaptor	SHP2/Grb2	IRS 1			
Transducer	SOS/Ras	PI3K	JAK	JAK	Type 1 Receptor
Kinase Cascade	MAPK	Akt			
Transcription Factor	Ternanry complex factors	FOXO	STATs	STATs	SMADs

See p 818 Lewin



The following depicts the process at several levels in a cell.



Now there are two major states a cell finds itself in; stasis and reproduction. A third, apoptosis, is natural cell death, we shall consider later. In stasis the cell is in G0 and producing proteins generally in response to external ligands or through normal internal processes. Unlike most standard biological models, we look at the proteins generally in terms of their concentrations and thus look at cell kinetics as well.

A cell in stasis is a little protein production factory, and each cell is pumping out the proteins and they then are in some extracellular balance. The cells in stasis communicate with one another via their respective ligands. In contrast when a cell reproduces it is standing out from the crowd if one will and looking out for itself.

We now examine first gene operations and then cell replication.

3.5.1 Cell Replication

We first address cell replication. First we examine the cell cycle from a generic perspective. We then examine the details on the pathways which may result in unstable cell reproduction.

The cell replication cycle goes through 4 stages. The dormant stage, G0, is not part of this process. The stages in cell reproduction are:

G0: This is the resting phase. It is during this phase that the cell is producing proteins via normal transcription processes. G0 may be resting related to the reproductive mitotic activities but the cell is quite active as a protein generating factory.

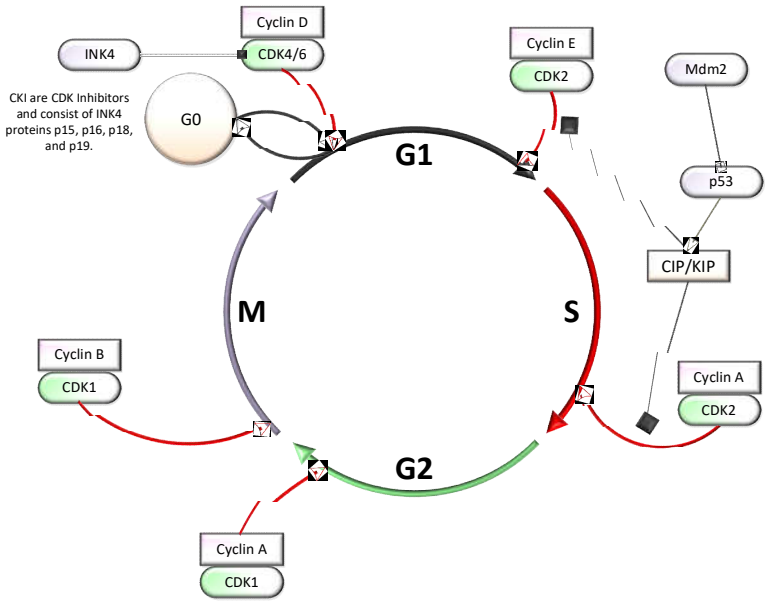
G1: Once the cell begins the G1 phase it is on its way to reproducing via mitosis.

S: The S phase is the phase where the DNA is duplicated. This is a sensitive stage; any error here can be propagated forward albeit there may still be checks available.

G2: This is the second gap phase.

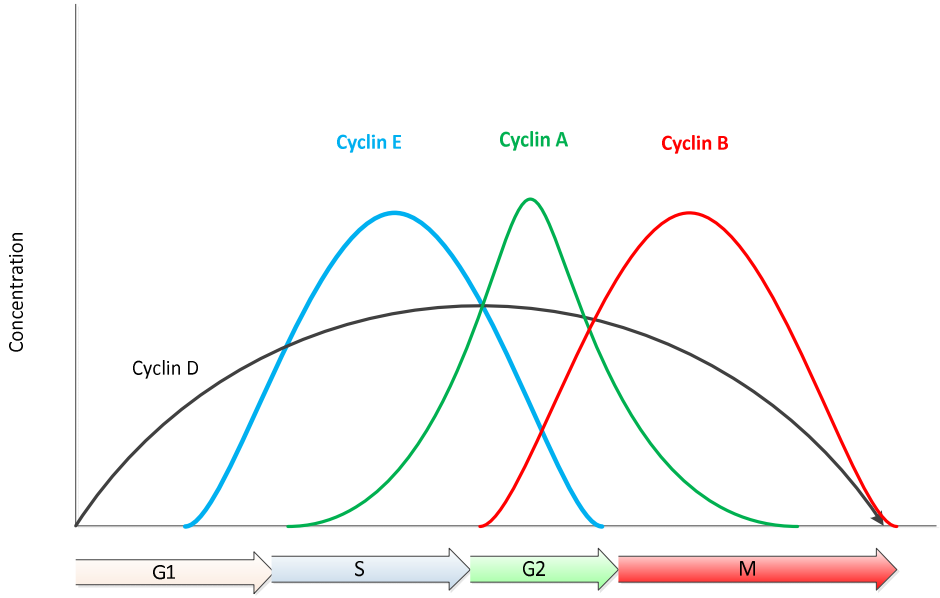
M: M phase includes mitosis and cytokinesis, namely the creation of two identical new cells.

Now the cell starts G1 by being instigated by a bound pair of a cyclin and a CDK, a cyclin dependent kinase. In this specific case we start with a binding of cyclin D and CDK4/6. This is the initiating event moving into G1 from senescence in G0. We depict these processes below (from McKinnell et al p. 169.):



The cyclins in each stage grow in concentration and as such move the cell along in each of its reproductive stages.

The following shows the phases and the relevant concentrations of cyclin bound to CDKs. Note the increase in concentration activates a change or movement along the mitotic path.



Note in the above the concentration of a specific cyclin above a level of a previous cyclin initiates the next step in mitosis. The details as to how and why this happens are detailed in Morgan (Chapter 3).

Protein[9]	***Gene***	***Function***[10]
Cyclin A (also CCN1; CCNA, CCNA2, Cyclin A2)	4q25-q31	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. In contrast to cyclin A1, which is present only in germ cells, this cyclin is expressed in all tissues tested. This cyclin binds and activates CDC2 or CDK2 kinases, and thus promotes both cell cycle G1/S and G2/M transitions.
Cyclin B1 (CCNB1)	5q12	The protein encoded by this gene is a regulatory protein involved in mitosis. The gene product complexes with p34 (cdc2) to form the maturation-promoting factor (MPF). Two alternative transcripts have been found, a constitutively expressed transcript and a cell cycle-regulated transcript that is expressed predominantly during G2/M phase. The different transcripts result from the use of alternate transcription initiation sites.

[9] http://www.ncbi.nlm.nih.gov/gene/983

[10] From http://www.ncbi.nlm.nih.gov/gene/595 data bases as a source.

Protein[9]	***Gene***	***Function***[10]
Cyclin B2 (CCNB2)	15q22.2	Cyclin B2 is a member of the cyclin family, specifically the B-type cyclins. The B-type cyclins, B1 and B2, associate with p34cdc2 and are essential components of the cell cycle regulatory machinery. B1 and B2 differ in their subcellular localization. Cyclin B1 co-localizes with microtubules, whereas cyclin B2 is primarily associated with the Golgi region. Cyclin B2 also binds to transforming growth factor beta RII and thus cyclin B2/cdc2 may play a key role in transforming growth factor beta-mediated cell cycle control.
Cyclin C (CCNC)	6q21	The protein encoded by this gene is a member of the cyclin family of proteins. The encoded protein interacts with cyclin-dependent kinase 8 and induces the phophorylation of the carboxy-terminal domain of the large subunit of RNA polymerase II. The level of mRNAs for this gene peaks in the G1 phase of the cell cycle. Two transcript variants encoding different isoforms have been found for this gene.
Cyclin D (Cyclin D1)	11q13	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is, required for cell cycle G1/S transition. This protein has been shown to interact with tumor suppressor protein Rb and the expression of this gene is regulated positively by Rb. Mutations, amplification and overexpression of this gene, which alters cell cycle progression, is observed frequently in a variety of tumors and may contribute to tumorigenesis.

Protein[9]	*Gene*	*Function*[10]
Cyclin E (CCNE1)[11]	19q12	The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK2, whose activity is, required for cell cycle G1/S transition. This protein accumulates at the G1-S phase boundary and is degraded as cells progress through S phase. Overexpression of this gene has been observed in many tumors, which results in chromosome instability, and thus may contribute to tumorigenesis. This protein was found to associate with, and be involved in, the phosphorylation of NPAT protein (nuclear protein mapped to the ATM locus), which participates in cell-cycle regulated histone gene expression and plays a critical role in promoting cell-cycle progression in the absence of pRB. Two alternatively spliced transcript variants of this gene, which encode distinct isoforms, have been described.

The CDKs involved are:

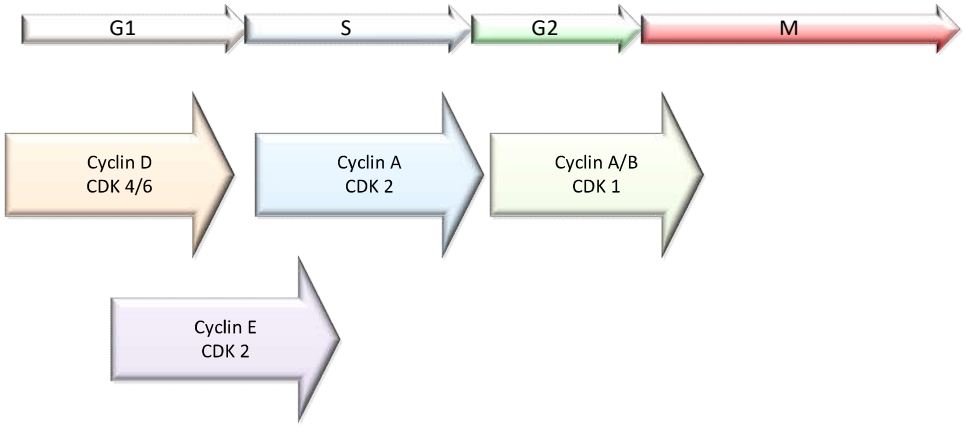
[11] http://www.ncbi.nlm.nih.gov/gene/898

Protein[12]	*Gene*	*Function*[13]
CDK 1 (also known as CDC2; CDC28A; P34CDC2)	10q21.1	This protein is a catalytic subunit of the highly conserved protein kinase complex known as M-phase promoting factor (MPF), which is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle. Mitotic cyclins stably associate with this protein and function as regulatory subunits. The kinase activity of this protein is controlled by cyclin accumulation and destruction through the cell cycle. The phosphorylation and dephosphorylation of this protein also play important regulatory roles in cell cycle control.
CDK 2 (also called p33)	12q13	It is a catalytic subunit of the cyclin-dependent protein kinase complex, whose activity is restricted to the G1-S phase, and essential for cell cycle G1/S phase transition. This protein associates with and regulated by the regulatory subunits of the complex including cyclin A or E, CDK inhibitor p21Cip1 (CDKN1A) and p27Kip1 (CDKN1B). Its activity is also regulated by its protein phosphorylation.
CDK 3	17q22	This gene encodes a member of the cyclin-dependent protein kinase family. The protein promotes entry into S phase, in part by activating members of the E2F family of transcription factors. The protein also associates with cyclin C and phosphorylates the retinoblastoma 1 protein to promote exit from G0.
CDK 4 (also CMM3; PSK-J3)	12q14	This protein is a catalytic subunit of the protein kinase complex that is important for cell cycle G1 phase progression. The activity of this kinase is restricted to the G1-S phase, which is controlled by the regulatory subunits D-type cyclins and CDK inhibitor p16 (INK4a). This kinase was shown to be responsible for the phosphorylation of retinoblastoma gene product (Rb). Mutations in this gene as well as in its related proteins including D-type cyclins, p16 (INK4a) and Rb were all found to be associated with tumorigenesis of a variety of cancers.
CDK 6 (also PLSTIRE)	7q21-22	The protein encoded by this gene is a member of the cyclin-dependent protein kinase (CDK) family. CDK family members are known to be important regulators of cell cycle progression. This kinase is a catalytic subunit of the protein kinase complex that is important for cell cycle G1 phase progression and G1/S transition. The activity of this k+inase first appears in mid-G1 phase, which is controlled by the regulatory subunits including D-type cyclins and members of INK4 family of CDK inhibitors. This kinase, as well as CDK4, has been shown to phosphorylate, and thus regulate the activity of, tumor suppressor protein Rb. Expression of this gene is up-regulated in some types of cancer.

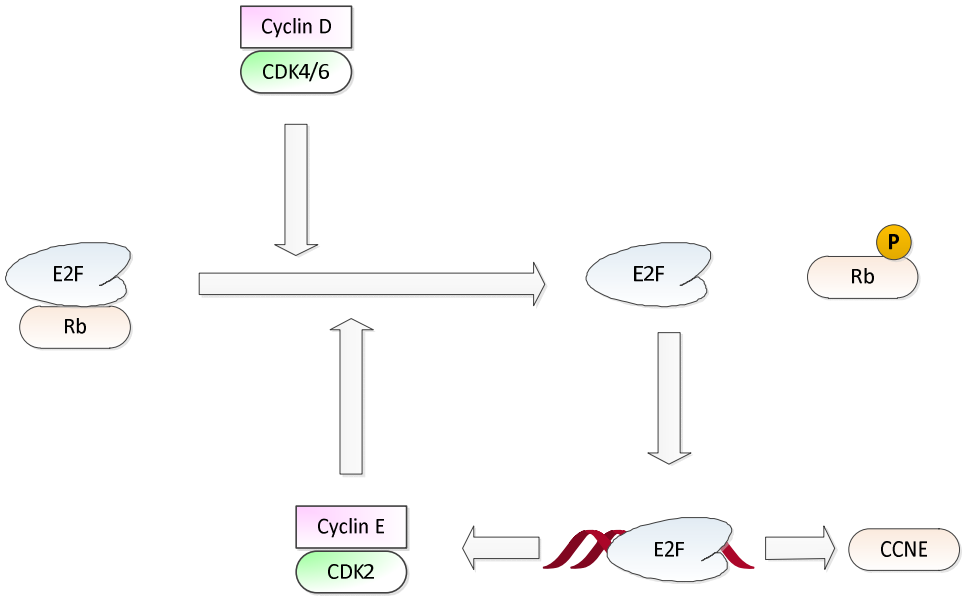
[12] http://www.ncbi.nlm.nih.gov/gene/983

[13] From http://www.ncbi.nlm.nih.gov/gene/595 data bases as a source.

Now the question is what activates these proteins, the cyclins and the CDKs, to make the cell cycle progress. This begins the creep upward in this pathway concern. We can redraw this process as follows and it will help to focus:

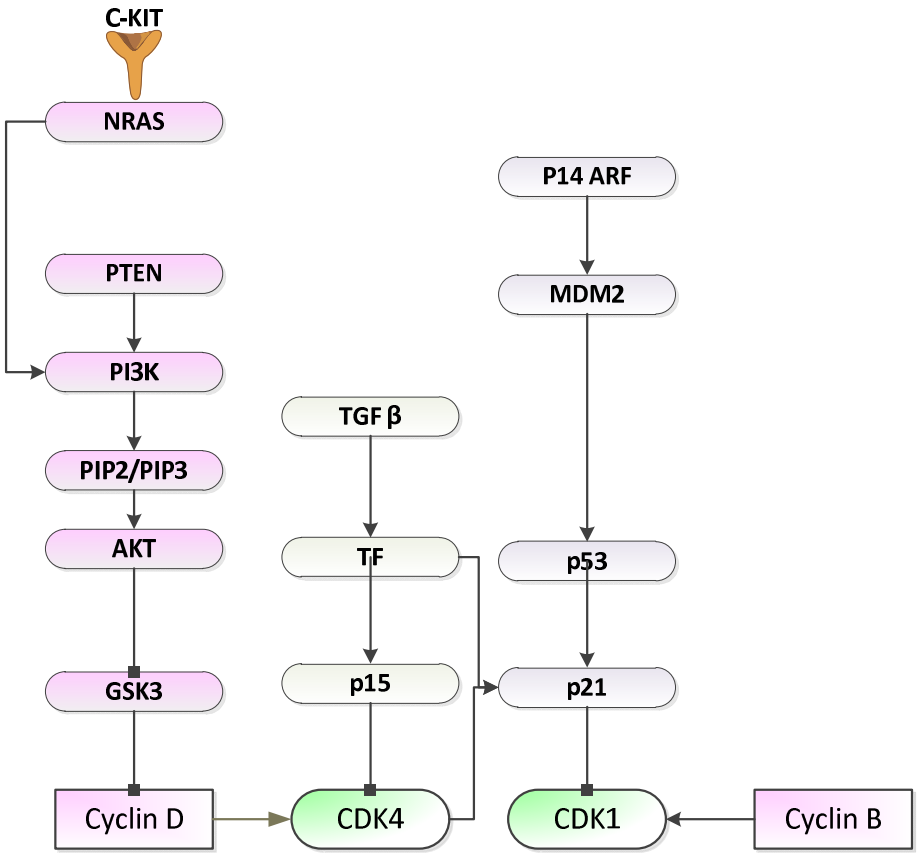


Now we ask what activates these proteins. We look at the activation of Cyclin E as shown by Bunz (p 219) below:

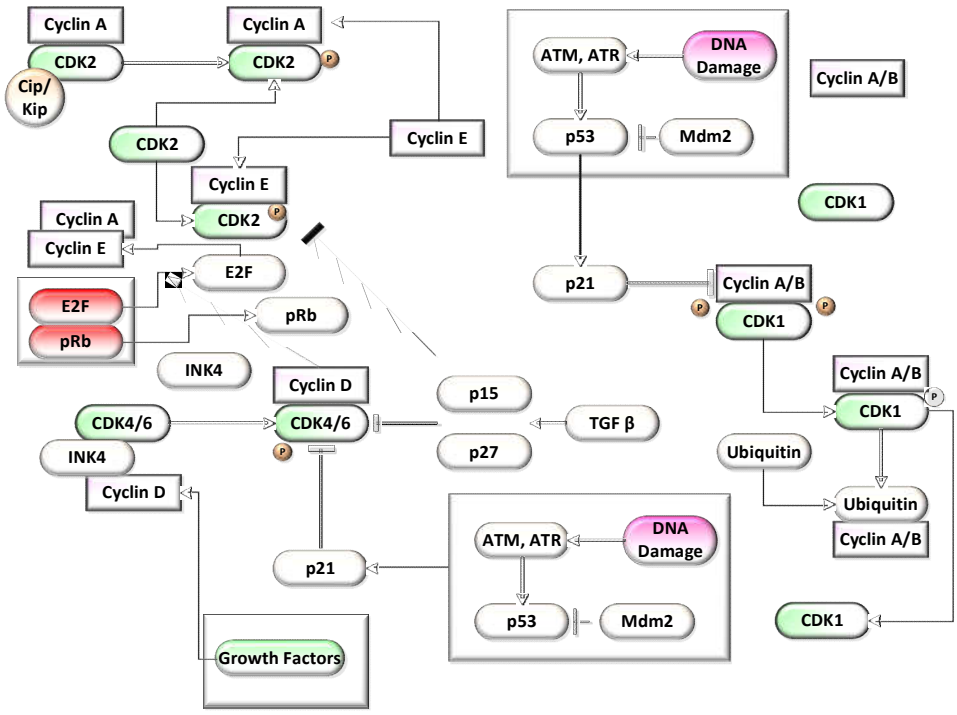


This is a feedback type reaction initiated by Rb the retinoblastoma gene protein. This feedback generates cyclin E which drives the cell through G1 and into the S cycle.

Now this establishes one base line for understanding cancer at the base of cell reproduction. Namely what can cause this process to continue unabated?



A more details analysis has been by Vermulen et al almost a decade ago. We shall use this as a baseline and then add to what we have learned in that period. The Vermulen network is shown as follows:



Now in the Vermulen configuration we have the following elements:

- CDKs
- Cyclins
- CDK Activating Enzymes
- CKI or CK Inhibitors

The following genes are elements of cell cycle control.

Gene	*Location*	*Function*
Jun[14]	1p32-p31	This gene is the putative transforming gene of avian sarcoma virus 17. It encodes a protein which is highly similar to the viral protein, and which interacts directly with specific target DNA sequences to regulate gene expression. This gene is intronless and is mapped to 1p32-p31, a chromosomal region involved in both translocations and deletions in human malignancies.

[14] http://www.ncbi.nlm.nih.gov/gene/3725

Gene	*Location*	*Function*
Fos[15]	14q24.3	The Fos gene family consists of 4 members: FOS, FOSB, FOSL1, and FOSL2. These genes encode leucine zipper proteins that can dimerize with proteins of the JUN family, thereby forming the transcription factor complex AP-1. As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation. In some cases, expression of the FOS gene has also been associated with apoptotic cell death.
Myc[16]	8q24.21	The protein encoded by this gene is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and cellular transformation. It functions as a transcription factor that regulates transcription of specific target genes. Mutations, overexpression, rearrangement and translocation of this gene have been associated with a variety of hematopoietic tumors, leukemias and lymphomas, including Burkitt lymphoma. There is evidence to show that alternative translation initiations from an upstream, in-frame non-AUG (CUG) and a downstream AUG start site result in the production of two isoforms with distinct N-termini. The synthesis of non-AUG initiated protein is suppressed in Burkitt's lymphomas, suggesting its importance in the normal function of this gene

3.5.2 Other Factors in the Cell Cycle

In a recent paper by Solimini et al the authors discuss the concepts of STOP and GO genes and carcinogenesis[17]. The paper reports on some extensive experimental results focusing on the issue of proliferation and the loss of certain sets of gene sites, the STP and GO sites.

The authors begin by discussing the current concepts of changes in oncogenes and tumor suppressor genes, some of the key pathway elements that we examine in analyzing intracellular pathway dynamics. They state:

Cancer progression is directed by alterations in oncogenes and tumor suppressor genes (TSGs) that provide a competitive advantage to increase proliferation, survival, and metastasis. The cancer genome is riddled with amplifications, deletions, rearrangements, point mutations, loss of heterozygosity (LOH), and epigenetic changes that collectively result in tumorigenesis.

[15] http://www.ncbi.nlm.nih.gov/gene/2353

[16] http://www.ncbi.nlm.nih.gov/gene/4609

[17] Solimini, N., et al, Recurrent Hemizygous Deletions in Cancers May Optimize Proliferative Potential, Science, 6 JULY 2012 VOL 337, p 104.

How these changes contribute to the disease is a central question in cancer biology. In his "two-hit hypothesis," Knudson proposed that two mutations in the same gene are required for tumorigenesis, indicating a recessive disease. In addition, there are now several examples of haploinsufficient TSGs.

Current models do not explain the recent observation that hemizygous recurrent deletions are found in most tumors. Whether multiple genes within such regions contribute to the tumorigenic phenotype remains to be elucidated…

The last sentence regarding the inability to explain the presence of hemizygous deletions under the current model is the main driver for this effort. Thus they argue and demonstrate experimentally that:

Tumors exhibit numerous recurrent hemizygous focal deletions that contain no known tumor suppressors and are poorly understood. To investigate whether these regions contribute to tumorigenesis, we searched genetically for genes with cancer-relevant properties within these hemizygous deletions.

We identified STOP and GO genes, which negatively and positively regulate proliferation, respectively.

STOP genes include many known tumor suppressors, whereas GO genes are enriched for essential genes.

Analysis of their chromosomal distribution revealed that recurring deletions preferentially over-represent STOP genes and under-represent GO genes.

*We propose a hypothesis called the **cancer gene island model**, whereby gene islands encompassing high densities of STOP genes and low densities of GO genes are hemizygously deleted to maximize proliferative fitness through cumulative haploinsufficiencies.*

Because hundreds to thousands of genes are hemizygously deleted per tumor, this mechanism may help to drive tumorigenesis across many cancer types.

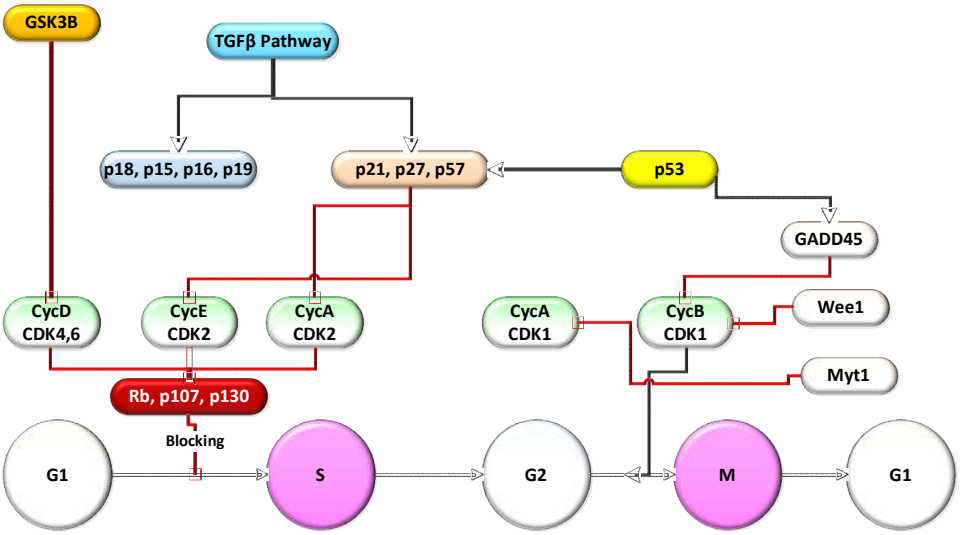
This is an intriguing hypothesis. It adds more pieces to an already complex puzzle. The Cancer Gene Island, CGI, hypothesis seems to indicate the complex changes in multiple gene sites. In particular there was a deletion of the STOP genes in preference to the GO genes. Unfortunately there did not seem to be a mechanism for these deletions, however the experimental evidence does indicate the phenomenon.

In their experimental analysis they have observed certain in vitro results which compel their hypothesis. They state:

This in silico analysis suggests that the loss of a single copy of GO genes has a negative impact on cellular fitness. To independently test this hypothesis, we turned to the other arm of our screen that identified candidate GO genes whose depletion limits proliferation and survival.

Because both normal and cancer cells are dependent on these essential GO genes, we analyzed data from proliferation screens on HMECs, one normal prostate epithelial cell line, and seven breast or prostate cancer cell lines

They provide an interesting pathway model as shown below (as modified, and also not that they have short hairpin RNAs (shRNAs)).



From Solimini et al: "*Known pathways with multiple shRNAs in the validation screen. Genes that validated in the secondary screen by a factor of ≥4 with multiple shRNAs are denoted with circles corresponding to three to five shRNAs (orange) and six or more shRNAs (red).*"

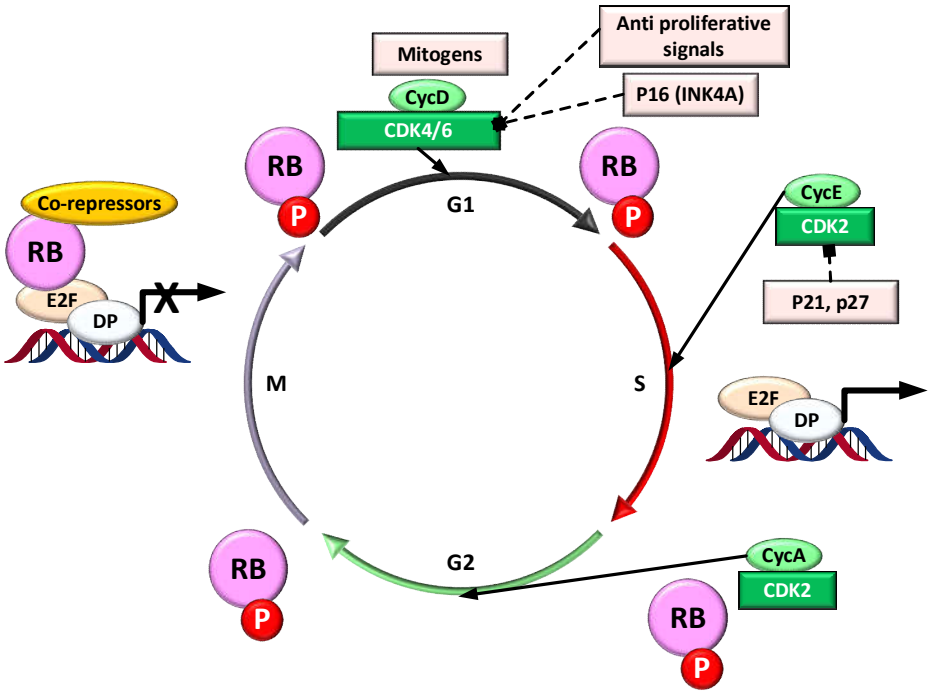
See McKinnell et al, Fig 5-6 p 169, The Biological Basis of Cancer, 2nd Ed, Cambridge, 2006

They conclude as follows:

The enrichment for genes localized to deletions suggests that we have identified dozens of new TSGs in recurrent deletions. We have also likely identified more TSGs outside of these regions because the STOP gene set is (i) enriched for known TSGs, many of which are not found in recurrent deletions, and (ii) enriched for genes that undergo somatic loss-of-function mutation.

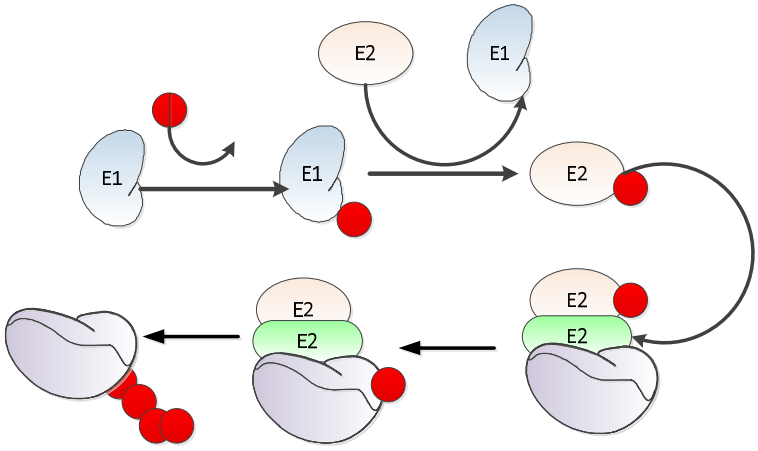
Finally, this work suggests that cells possess a substantial number of genes that restrain proliferation in vitro, which could be inactivated to promote clonal expansion during tumorigenesis in addition to the traditional driver genes currently known. Given the prevalence of multiple, large, recurring hemizygous deletions encompassing skewed distributions of growth control genes in tumors, we propose that the elimination of cancer gene islands that optimize fitness through cumulative haplo-insufficiencies may play an important role in driving tumorigenesis, with implications for the way in which we think about cancer evolution.

As with many such works this raises as many questions as it seems to answer. However the control or lack thereof of proliferation and the cell cycle is a critical issue in carcinogenesis.



3.5.3 Ubiquination

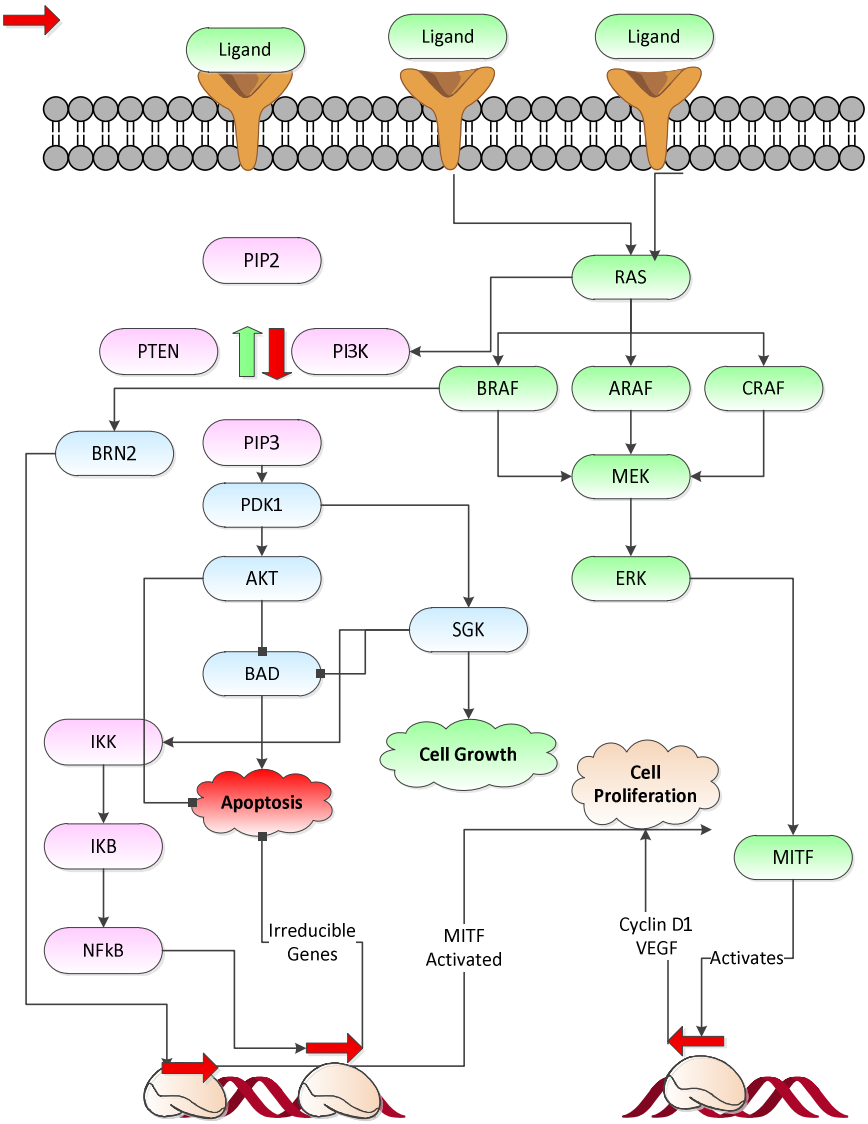
Ubiquitin is a small protein which acts with three related proteins; E1, E2, and E3. E1 is also called the ubiquitin activating enzyme, E2 the ubiquitin conjugating enzyme, and E3 ubiquitin ligase. Together they act to attach ubiquitin to a target protein and mark it for digestion and elimination. The process is shown below in general graphic form.



Ubiquination is an essential process within a cell to eliminate used or excess proteins. Although we will not discuss tis in detail, it is an essential process and the reader should refer to standard texts[18].

[18] See: Cassimeris et al p 688, Weinberg, p 242, Alberts et al, p 1065.

The following Figure depicts some of the mechanics in terms of genetic flow and control as to how Ubiquination occurs.



Simply there are three end states:

1. Cell Proliferation or Cell Cycle Mitosis

2. Cell Growth or the expansion and operations of a single cell outside of mitosis.

3. Apoptosis or cell death.

Now in the simplified model above we have several feedback loops, many driven by external ligands.

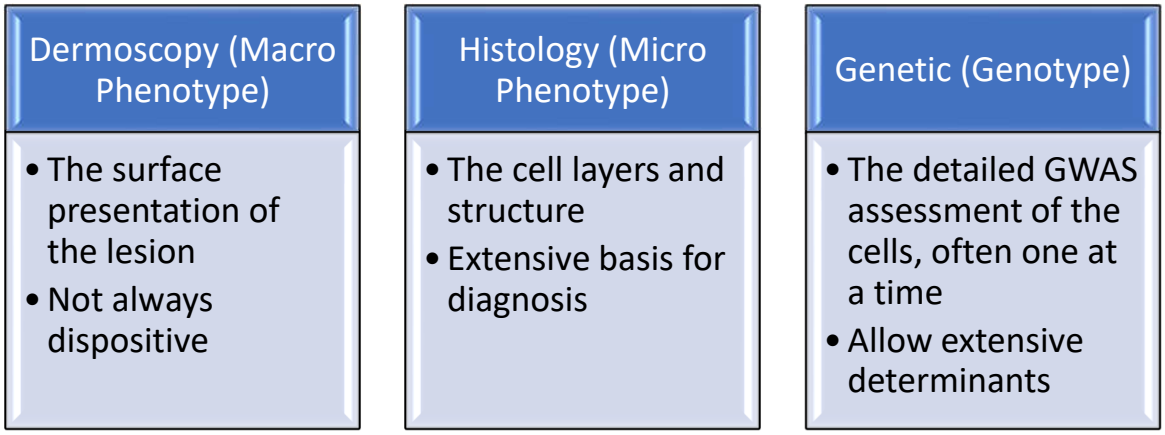
In this section we briefly review the issue of cellular growth. What makes cells reproduce? If we first examine skin cells, one of the many cells in the body which reproduce all the time, like blood cells, we can gain some insight.

Skin cells are reproducing all the time. Mostly the keratinocytes and getting sloughed off at the surface or rebuilding after a wound. The melanocytes frequently do not reproduce. They are neural crest derives and often just remain in the G0 state. They produce such products as melanosomes, and other proteins required for homeostasis. There are times when they may reproduce to a cluster state, such as found in lentigenes. This is a common response to excessive sun exposure. Namely we may see heavily pigmented areas of clustered melanocytes. Then we may have a nevus, the raised collection of melanocytes. In both cases the melanocytes tend to stay attached to the cluster, thus having functional E cadherin molecules.

Now what of prostate cells, they do not reproduce as quickly. The glands are generally stable and often reproduce after some nominal lifetime of the basal or luminal cell. However a cell is stressed, for example by some external driver as inflammation, or other external attack, and then the cells may regenerate and thus reproduce. Perhaps that is one of the mechanism which underlies indolent PCa. Melanoma for example is highly aggressive in any form, most likely driven by the aggressive growth medium. However, as is known, melanocytes alone are indolent. This is one of those “on the one hand, on the other hand” arguments.

4 CLASSIFICATION: PHENOTYPE VS GENOTYPE

The challenge we often have in dermatological diagnosis is the alignment of what is visually seen such as in dermoscopy[19]. Then histologically and finally genetically. This is a set of measures between macro, micro phenotypes and the genotype. A challenge is to establish the connections between them. We can see these three dimensions as shown below:

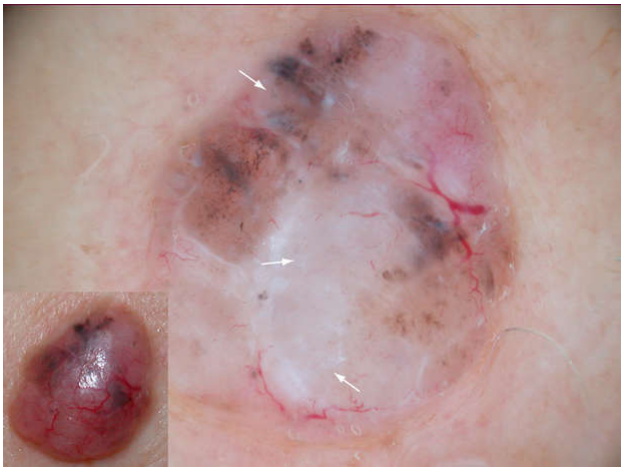


4.1 DERMOSCOPY

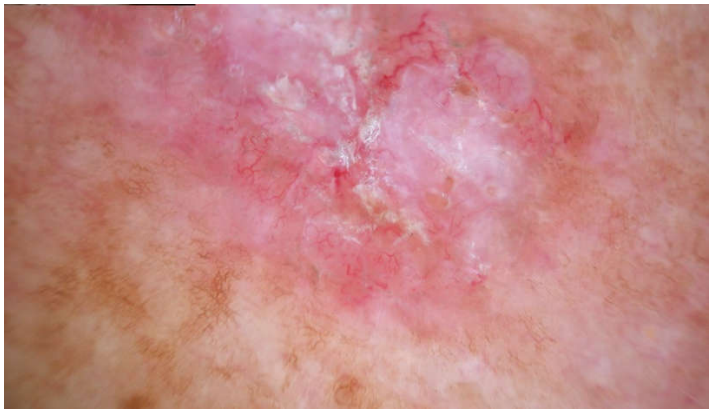
Dermoscopy is the procedure of using a scope and a solution to examine lesions to determine characteristics that may reveal malignant potential. It has been found useful for the identification of melanomas as well as basal and squamous cell carcinomas. It is a phenotypic approach whereby the examiner seeks to observe element that may be significant, especially for melanomas. It is also possible for the examiner to identify BCC potential although dermoscopy also is not dispositive.

Below we have one example of a typical BCC. It has white shiny surface with vagination being prominent.

[19] See Soyer et al Dermoscopy, Elsevier, 2012. Also see Pampena et al



A second example is shown below. Here there is a crusting like areas along with vagination.

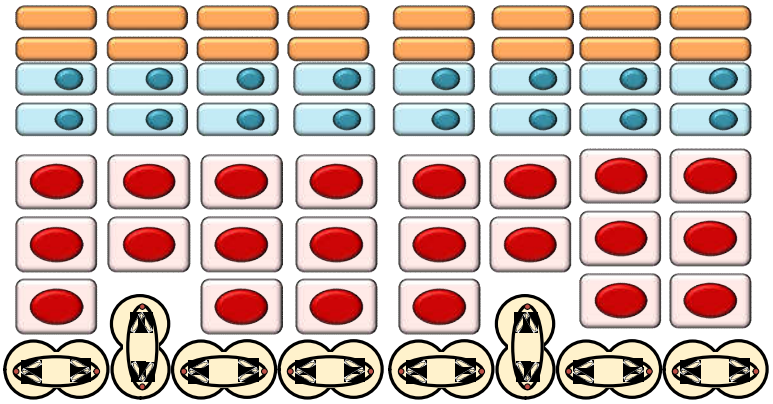


Generally with dermoscopy if the potential is positive for BCC then excision is performed and a histological examination made.

4.2 HISTOLOGY

We briefly examine the histology. This is part of the phenotype vs genotype argument that we all too often have to deal with. The simplistic view of the epidermis is shown below. Here the basal cells are active mitotic cells and then les so as we go towards the skin surface. This simplistic view can be useful for describing the phenotypes we see in classic pathology analyses.

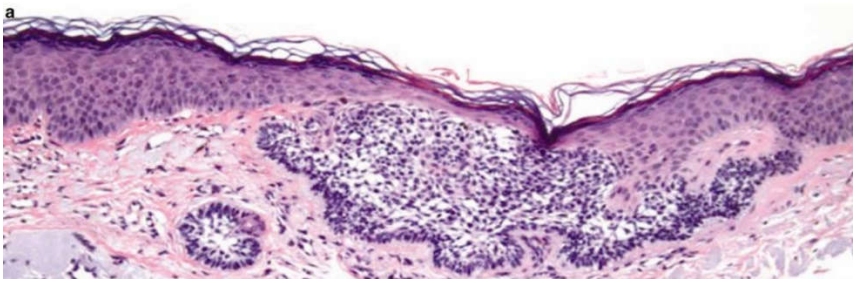
See Oro and Watt p 59



The basal cells with stain heavily due to the preponderance of nuclear material[20]. We now examine several basic phenotypes as discussed in Crowson.

4.2.1 Superficial

We start with superficial as shown below. This is a large cluster of mitotically active basal cells spreading below the epidermis. The cells align somewhat with the epidermis in a length manner.



As Crowson notes:

Superficial BCC is characterized by a proliferation of atypical basaloid cells that form an axis parallel to the epidermal surface and demonstrate slit-like retraction of the palisaded basal cells from the subjacent stroma

The resulting cleft-like spaces often contain alcian blue-positive mesenchymal mucoid material, a presumed product of the stromal cells. Tumor cells may colonize the hair follicle and rarely the eccrine adnexal structures and, as mentioned above, often take origin from the follicular bulges.

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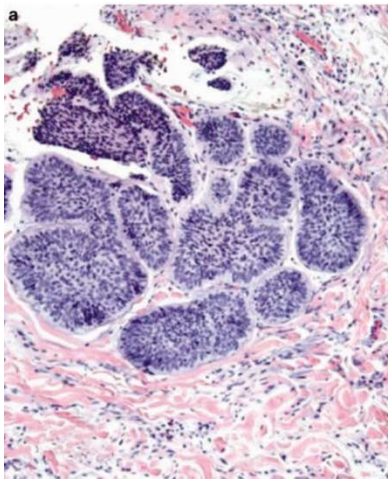
[20] Recall that with H&E stains we have Eosin is acidic, staining proteins pink since proteins are basic. Hemetox is basic and stains nucleic acids in DNA which are acidic, and the stain is a blueish. Thus the larger the DNA cover the bluer the cells and the less the DNA and just protein cytoplasm the pinker the cell.

Mitoses are infrequent and apoptic cells rare in the atypical basaloid buds for reasons reflecting their biologic derivation as described below. Some cases manifest melanin pigmentation of the epithelium and in the histiocytes in the subjacent stroma; of pigmented BCCs, most are held to reflect superficial tumors in some series, although in our experience nodular BCCs constitute by far the most frequent form of pigmented BCC. A band like, often heavy, lymphoid infiltrate may be present. When seen in the setting of a biopsy for suspect superficial or superficial multifocal BCC, a band-like lymphoid infiltrate should prompt a careful search through multiple levels looking for foci of superficial BCC.

4.2.2 Nodular

Nodular is the most common type. There are round clusters with the outer basal cells aligned as if in a palisade and a clear barriers around the clusters.



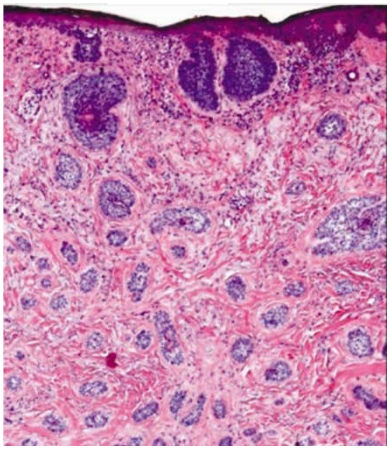
As Crowson notes:

Nodular BCC represents the most common form of the neoplasm in our experience. Nodular BCC is also referred to as nodulocystic BCC by some observers, although this term is not employed by us. This is the type of BCC that clinically shows a translucent pearly papule or nodule with a rolled border and telangiectasia. The nodular form of BCC is characterized by discrete large or small nests of basaloid cells in either the papillary or reticular dermis accompanied by slit-like retraction from a stroma in which the fibroblasts do not appear to be plump or proplastic. Any of the differentiated elements (eccrine, sebaceous, etc) may be seen in nodular tumors and roughly one-third of cases will show a coexistent superficial component. As both superficial and nodular BCC can be seen in sun-exposed or sun-protected skin, the dermis may show solar elastosis and this may be pronounced. The surrounding stroma shows myxoid change, is rarely fibrotic and may show calcification in discrete islands of tumor or in adjacent stroma. Mitoses and individual cell necrosis are uncommon.

The presence of abundant slit-like retraction may cause tumor nests to drop out from the stroma during processing yielding empty spaces with a rounded contour in the mid or deep dermis. This is an important clue to the diagnosis in the setting of the nodular and/or infiltrative growth patterns. A significant proportion of BCCs with a nodular component manifests a variable admixture of superficial and/or micronodular morphologies

4.2.3 Micronodular

Micronodular is a larger cluster of small clusters.



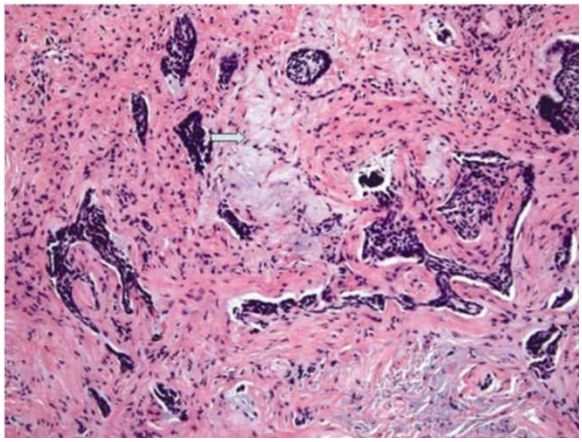
As Crowson notes:

Micronodular BCC manifests a plaque-like indurated lesion with a poorly demarcated contour. As mentioned above, lesions may be difficult to remove and so have an increased incidence of recurrence. Micronodular BCC manifests tumor nests with roughly the same shape and contour as nodular BCC, but which are nonetheless smaller and widely dispersed in an often asymmetric distribution extending deeper into the dermis and/or subcutis.

These monotonous, small round tumor nests are accompanied by stromal proliferation cognate to the infiltrative growth BCC. Retraction spaces are not common and the surrounding stroma shows either a myxoid or collagenized morphology suggesting that these lesions may be an intermediate step between nodular and aggressive growth subtypes. The micronodular BCC has been reported to have a higher incidence of local recurrence and may penetrate more deeply into the reticular dermis and/or subcutis.

4.2.4 Infiltrative

Infiltrative is a collection of somewhat disorganized groups taking no specific form.



As Crowson notes:

Infiltrative growth BCC comprises, at the light microscopic level, irregularly sized and shaped nests of tumor cells; the nests show sharp angulation of their peripheral contours, occasional foci of slit-like retraction, and frequent mitotic activity and individual cell necrosis of the neoplastic cells. The stroma is frequently fibrotic with plump proplastic stromal fibroblasts. The nests are variable in size and shape with jagged contours.

Typically the elongated tumor cell strands of the infiltrative growth component are 5– 8 cells in thickness. Roughly one-third of such cases show an admixed nodular component from which the lesions are held to derive following UV irradiation Like the morpheaform variant, these tumors are poorly circumscribed and may show invasion of subcutis and adjacent muscular and other structures.

Perineural infiltration is a distinct risk in this variant as in the morpheaform BCC, and, like the morpheaform variant, the clinical correlate is a depressed yellowish or fibrotic plaque that typically lacks a rolled border or an elevated pearly nodule unless a nodular BCC component coexists

Pyne et al also present a clinical study of a dermatoscope finding and a histology finding for the infiltrative class.

4.3 GENOMICS

Now we consider the genotypes. This will beg the question of how one aligns form and structure to genes and their expressions.

Pelligrini et al note:

Basal cell carcinoma (BCC) is the most common human cancer and represents a growing public health care problem.

Several tumor suppressor genes and proto-oncogenes have been implicated in BCC pathogenesis, including the key components of the Hedgehog pathway, PTCH1 and SMO, the TP53 tumor suppressor, and members of the RAS proto-oncogene family. Aberrant activation of the Hedgehog pathway represents the molecular driver in basal cell carcinoma pathogenesis, with the majority of BCCs carrying somatic point mutations, mainly ultraviolet (UV)-induced, and/or copy-loss of heterozygosis in the PTCH1 gene. Recent advances in sequencing technology allowed genome-scale approaches to mutation discovery, identifying new genes and pathways potentially involved in BCC carcinogenesis.

Mutational and functional analysis suggested PTPN14 and LATS1, both effectors of the Hippo–YAP pathway, and MYCN as new BCC-associated genes. In addition, emerging reports identified frequent non-coding mutations within the regulatory promoter sequences of the TERT and DPH3-OXNAD1 genes. Thus, it is clear that a more complex genetic network of cancer-associated genes than previously hypothesized is involved in BCC carcinogenesis, with a potential impact on the development of new molecular targeted therapies. This article reviews established knowledge and new hypotheses regarding the molecular genetics of BCC pathogenesis. …

Beyond HH signaling, other tumor suppressor genes and proto-oncogenes have been implicated in the pathogenesis of BCC, including the TP53 tumor suppressor gene and members of the RAS proto-oncogene family.

In a recent study including 293 BCC tumors, the driver pivotal role of PTCH1, TP53, and SMO has been confirmed; however, 85% of BCC also harbored additional driver mutations in other cancer-related genes, such as MYCN, PPP6C, PTPN14, STK19, and LATS1.

*Finally, emerging reports have identified somatic mutations within regulatory sequences as the promoters of the telomerase reverse transcriptase (**TERT**) gene and of the diphthamide biosynthesis 3 (DPH3) gene.*

Of note, the mutational pattern of genes involved in BCC tumorigenesis is consistent with UV-induced DNA damage, since genes harbor "UV signature" mutations.

Solar radiation (UVB and UVA) can mutagenize DNA, producing UV landmark C to T or CC to TT transversions via cyclobutane dimers and pyrimidine(6–4)pyrimidine photoproducts.

The transformation of the keratinocytes occurs when these mutations affect the function of multiple oncogenes, tumor-suppressor genes and important housekeeping genes, leading to an unregulated cell cycle …

4.3.1 End to End Pathways

As Trieu et al have noted:

- In the absence of Hh a cell-surface transmembrane protein called Patched (PTCH) acts to prevent high expression and activity of a 7 membrane spanning receptor called Smoothened (SMO).
- Patched has sequence similarity to known membrane transport proteins. When extracellular Hh is present , it binds to and inhibits Patched, allowing Smoothened to accumulate and inhibit the proteolytic cleavage of the Ci protein.
- In cells with Hh-activated Patched, the intact Ci protein accumulates in the cell cytoplasm and levels of CiR decrease, allowing transcription of some genes such as decapentaplegic (dpp, a member of the BMP growth factor family).
- For other Hh-regulated genes, expression requires not only loss of CiR but also the positive action of uncleaved Ci acting as a transcriptional activator.

Basal cell carcinomas (BCCs) frequently possess immense mutational burdens; however, the functional significance of most of these mutations remains unclear. Here, we report that loss of Ptch1, the most common mutation that activates upstream Hedgehog (Hh) signaling, initiates the formation of nascent BCC-like tumors that eventually enter into a dormant state.

However, rare tumors that overcome dormancy acquire the ability to hyperactivate downstream Hh signaling through a variety of mechanisms, including amplification of Gli1/2 and upregulation of Mycn.

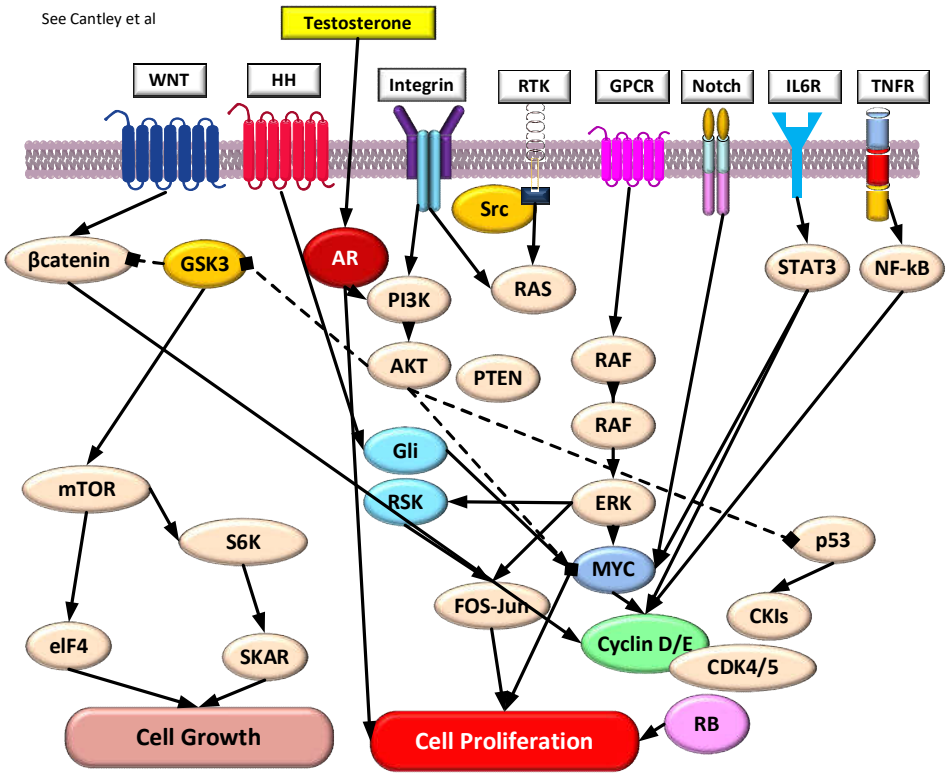
Furthermore, we demonstrate that MYCN overexpression promotes the progression of tumors induced by loss of Ptch1. These findings suggest that canonical mutations that activate upstream Hh signaling are necessary, but not sufficient, for BCC to fully progress. Rather, tumors likely acquire secondary mutations that further hyperactivate downstream Hh signaling in order to escape dormancy and enter a trajectory of uncontrolled expansion. …

A subset of macroscopic tumors acquire downstream Hh pathway hyperactivation What enables rare macroscopic tumors to ‘‘break through,’’ when millions of other Ptch1-deleted cells in the skin fail to progress? Since the inability to maintain high-level proliferation appears to be a common roadblock for nascent tumors arising in GP, LP, GPN1, GPP53, and SmoM2 mice, we reasoned that macroscopic tumors likely acquire mutations that confer sustained replicative ability. To identify these somatic changes, we performed whole exome sequencing (WES) on 16 macroscopic GPP53 tumors and 5 GPN1 tumors, along with matched normal control tissue. Although overall mutational burdens varied widely among tumors, the dominant genomic alterations were somatic DNA copy number changes, with far fewer single-nucleotide variations and insertion/deletions.

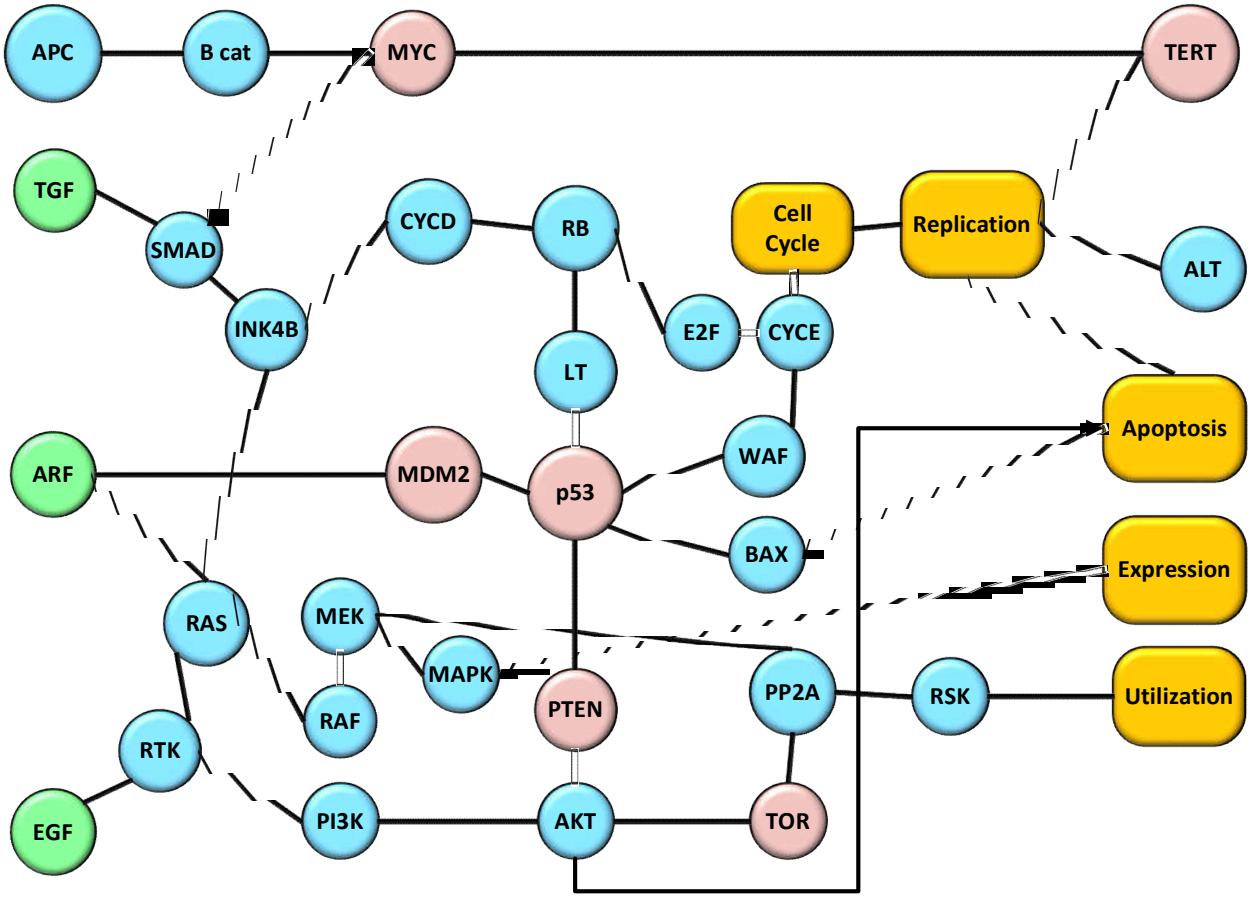
In particular, we detected two recurrent amplifications: 7/16 GPP53 tumors acquired copy number gains in regions of chromosome 1 encompassing Gli2, while 4/16 GPP53 tumors acquired gains in regions of chromosome 10 encompassing Gli1. These amplifications were often accompanied by smaller copy number changes on the same chromosome, and notably, no tumor exhibited amplification of both Gli1 and Gli2. Since these genes encode the key transcriptional mediators of Hh signaling, we next validated that GPP53 tumors with amplified chromosome 1 possessed increased Gli2 mRNA, whereas tumors with amplified chromosome 10

had increased Gli1 mRNA. As expected, regressed microscopic lesions presumably lacking these mutations had lower levels of both transcripts and displayed less Hh pathway activation.

Overall, our findings are consistent with previous studies showing that forced overexpression of either transcription factor induces BCC formation. Unlike these overexpression systems, however, a key distinction here is that GPP53 tumors spontaneously acquired Gli amplifications, which are similarly detected in 8% of human BCCs. These findings suggest that a subset of tumors initiated by loss of Ptch1 acquire secondary mutations to further hyperactivate downstream Hh signaling in order to drive progression.



Another view using KEGG pathways is shown below:



The above is a conceptualized interaction between many of the key genes involved in cancer processes. This also exemplifies the high level of complexity on assessing genes as well as the fundamental issue of gene cross-talk, a factor which may dominate a multiplicity of malignancies.

An excellent summary is also in DeVita. We first summarize this in the following Table using the materials:

Gene	Action
AKT	The AKT gene family consists of AKT1, AKT2, and AKT3, with phospho-AKT as the read out of their overall activation status. Elevated phospho-AKT level was reported to be adversely associated with patient survival.32 More recently, copy number gains of the AKT3 locus were detected in melanomas, suggesting that the AKT signaling point itself may be oncogenic. Interestingly, targeted depletion of AKT3 could trigger apoptosis, while AKT1 behaved as a tumor suppressor in melanoma cell lines, pointing to poorly understood distinct and overlapping functions of these related family members.
APAF-1	Allelic loss at 12q23 was exhibited by 10 of 24 (42%) melanomas in a study, with the common area of loss focused on the APAF-1 locus. LOH correlated tightly

Gene	Action
	with a reduction in Apaf-1 protein levels, as judged by immunohistochemistry. Although no mutations were detected, the loss of expression was determined to be due to silencing in a methylation-dependent manner.
ARF	Reciprocal to the *INK4A*-specific human mutations, *ARF*-specific insertions, deletions, and splice donor mutations have been described in human melanomas . However, in these cases, either the maintenance of INK4A function or true ARF inactivation was not shown, making it ambiguous whether the genetic disruption of ARF alone is sufficient for tumorigenesis.
BRAF	Somatic activating BRAF mutations are found at high frequency in human melanoma, dominated by a single species of point mutation (T →A nucleotide change), resulting in a valine to glutamate amino acid substitution (V600E). Although the T→A transversion is not classically associated with UV-induced damage, BRAF mutations appear to be more common in melanomas arising on sites with intermittent exposure to UV. However, melanomas from chronically sun-damaged skin are typically wild type for *BRAF*,
CDK4A	*CDK4* is a direct target of inhibition by p16INK4A and is a primary regulator of RB activation. If INK4A acts mainly through the RB pathway, it would be predicted that activating CDK4 mutations could functionally substitute for INK4A deletions. Indeed, rare germline mutations of *CDK4* that render the protein insensitive to inhibition by INK4A have been identified in melanoma-prone kindred. Somatically, these tumors retain wild-type INK4A function, suggesting that INK4A is epistatic to CDK4 and that Rb pathway deregulation is central to melanomagenesis.
CDKN2A	Its importance is explained in part by its unusual organization, which allows for two separate transcripts and corresponding tumor suppressor gene products to be produced: p16INK4A and p19ARF. Loss of p16INK4A results in the suppression of retinoblastoma (RB) activity via increased activation of the CDK4/6-cyclin D1 complex; loss of ARF (p14ARF in human and p19ARF in mouse) results in the suppression of p53 activity through increased activation of MDM2. Thus, deletion of the entire locus accomplishes the inactivation of two critical tumor suppressor pathways: RB and p53.
c-MET	The c-MET gene product and its ligand hepatocyte growth factor/scatter factor (HGF/SF) are known to activate the MAPK pathway, but have many additional functions. It has long been documented in the literature that overexpression of c-MET and HGF is correlated with melanoma progression, with nonfocal amplification of the c-MET locus at 7q33-qter being associated with invasive and metastatic cancers in humans and their high levels of expression in murine melanoma cell lines being similarly correlated with metastatic ability in explants.

Gene	Action
EGFR	Epidermal growth factor receptor (EGFR) is involved in a complex regulatory loop with the MAPK pathway, where there appears to be bidirectional signaling between EGFR and the RAS kinases. In melanomas, copy number gain of chromosome 7 is linked with overexpression of EGFR, despite the lack of focal amplifications. Functionally, although *in vitro* activation of EGFR does not affect melanoma growth, it increased the number of visceral metastases when implanted in severe combined immunodeficiency (SCID) mice. Confirmation of EGFR-MAPK cross-talk in melanoma was demonstrated in the inducible H-RAS-driven mouse model, where transcriptomic analysis revealed the up-regulation of EGF family ligands including amphiregulin and epiregulin.
INK4A	Human intragenic mutations of *INK4A* that do not affect the *ARF* coding region sensitize germline carriers to the development of melanomas.These aberrations can affect the coding region (e.g., exon 1α), either of the 5′ or 3′ untranslated regions (UTRs), the promoter, or splice donor/acceptor sites (reviewed in Sharpless7). This sufficiency of p16INK4A loss for the initiation of melanoma demonstrates that loss of the entire CDKN2A locus is not necessary. In a mouse model engineered to be deficient only for Ink4a (with intact ARF), melanomas formation was observed in cooperation with an oncogenic initiating event (e.g., activated H-RAS), albeit with a longer latency than in mice with deletions affecting the entire locus.
MAPK	The MAP kinase (MAPK) pathway contains some of the earliest elucidated human oncogenes, and subsequent analysis of their mechanisms of action unearthed a prevalence of activating mutations across a wide spectrum of tumor types. The focal point of MAPK activation is the ERK1/2 kinases, which classically mediate the transcription of genes involved in cell proliferation and survival, but which have also been shown to regulate differentiation and senescence. In addition, the RAS family of proteins has been shown to feed into the PI3K pathway.
MITF	MITF is a gene critical to the survival of normal melanocytes, and identification of MITF as a central modifier of melanoma created a novel class of oncogenes (along with androgen receptor) termed " lineage addiction" oncogenes. That is, a tumor may "hijack" extant lineage survival mechanisms in the presence of selective pressures to ensure its own propagation.
P53	The p53 pathway is critical in maintaining a cell with a normal genome via a multiplicity of mechanisms, including cell cycle checkpoints, DNA damage repair activation, and the appropriate induction of apoptosis. Its centrality in tumor suppression is evidenced by the high rate of its inactivation in solid tumors, with mutations in the TP53 gene well established to be present in over 50% of all tumors. By contrast, the TP53 locus is rarely mutated in human melanomas (reviewed in Chin12), although loss of p53 in mice does cooperate with activated H-Ras to induce melanomas. Similar to the LOH at *Cdkn2a* in mice heterozygous

Gene	Action
	for Ink4a/Arf knockout, mutant Tp53 heterozygotes also lose the wild-type allele somatically in H-RAS-driven melanomas. Thus, while p53 itself is spared in human melanomas, inactivation of its pathway is likely to be critical.
PTEN	Of the PI3K pathway mutations that do occur, loss of chromosome 10q encompassing PTEN tumor suppressor is the most frequent, the caveat being that there is likely additional tumor suppressor(s) resident in this region (see below). PTEN normally effects the down-regulation of phosphorylated AKT via suppression of levels of the second messenger PIP3. In various genetically engineered mice bearing solid tumors, PT EN loss can be analogous to p53 inactivation, in that one or the other can provide the " last straw" of oncogenesis. In melanoma, somatic point mutations and homozygous deletions of PTEN are rare. Although allelic loss of PTEN is observed only in about 20% of melanoma, loss of expression of PTEN is reported to be in the range of 40% of melanoma tumors.
RAS	Increasing evidence shows that the three different members of the RAS family are not functionally redundant, with separable roles not only among different tissue types, but even within the same tissue. Reflecting this is the differential mutation and genomic amplification rates of the RAS family members within melanomas: N-RAS is the most frequently targeted (33% of primary and 26% of metastatic melanoma samples17), followed by H-RAS (mainly in Spitz nevi). Despite its high incidence in other cancer types, K-RAS is rarely observed in melanocytic lesions.19 Interestingly, although N-RAS mutations are found in 54% of congenital nevi, they are rare in dysplastic nevi, implying a distinct evolutionary path from dysplastic nevi to melanoma.
RB	RB pathway is responsible for preventing cells from incorrectly entering into the cell cycle, and germline heterozygous loss of the *RB1* gene in humans results in the formation of retinoblastoma. The tumor modulating properties of the RB pathway are well established in many solid cancers, and its deregulation in melanoma is no exception, with demonstrable human mutations in *INK4A*, *CDK4*, or *RB1*.
RB1	Finally, germline mutations in RB1 itself have been found to predispose to melanoma in patients who have survived bilateral retinoblastoma. These melanomas show a somatic LOH of the remaining wild-type RB1 allele, strongly implying that an intact RB pathway was selected against in the preneoplastic melanocytes. In such patients, the estimates of increased lifetime risk of melanoma range from 4- to 80-fold.
TGF-β	TGF-β family members are active at various stages of human tumors, but its role in melanoma has only recently begun to become clarified. Studies in zebrafish embryos have translated into human data on the role of Nodal in melanomas. Secreted Nodal was shown to be the molecule responsible for zebrafish axial

Gene	Action
	duplications when human melanoma cells were implanted into the embryos. Subsequent immunohistochemical analysis of Nodal in human melanocytes and melanomas showed a significant correlation with tumor progression. Knockdown of Nodal in metastatic cell lines reduced their invasive capacity *in vi tro* and their growth in mouse xenografts.
WNT	WNT signaling has long been implicated in a wide variety of cancers including breast and colorectal. Its activation of downstream transcriptional events has been hypothesized to control lineage commitment and differentiation fates as well as self-renewal properties. Indeed, the WNT pathway has been linked to major developmental decisions in neural crest derivatives, with a differentiation bias toward the melanocytic lineage.

4.3.2 Hedgehog Gene

In addition to the abovementioned signaling pathway, there is a non-canonical HH cascade resulting in the activation of GLI transcription factors independent of HH ligands or PTCH1/SMO. GLI activity has been shown to be regulated positively by KRAS, TGF-β, PI3K-AKT, and PKC-α, and negatively by p53, PKA, and PKC-δ.

Upregulation of HH signaling represents the most significant pathogenic event in BCC. More than 90% of BCCs show a loss of PTCH1 function by inactivating PTCH1 mutations, as well as by aberrant activation of SMO through activating SMO mutations.

The PTCH1 gene has been mapped to 9q22.3 and consists of 23 exons spanning approximately 74 kb encoding a 1447 transmembrane glycoprotein. The sequence suggests that PTCH1 is a transmembrane protein with 12-membrane spanning domains and 2 large extracellular loops. The inactivation of PTCH1 in BCCs may be a necessary, if not sufficient, event for carcinogenesis. Sporadic BCCs have been reported to carry inactivating point mutations, copy loss of heterozygosity (LOH), and copy-neutral LOH (due to uniparental disomy) in PTCH1. PTCH1 somatic mutations range between 11% and 75% and are represented by non-synonymous mutations with a predominance of nonsense and splice site mutations throughout the entire length of the PTCH1 gene, without evidence for a hot-spot region.

About half of these mutations contains the "UV-signature" C-T and tandem CC-TT transitions; however, the UV radiation origin of PTCH1 mutations is controversial, since other factors, such as oxidative stress, have been implicated in the mutagenesis of this gene. Besides point mutations, somatic copy number aberrations (SCNAs) of PTCH1 have been frequently reported in BCC. LOH of the PTCH1 allele is the most frequently identified SCNA in BCC and occurs in about 42–70% of the tumors due to loss of part of or the whole of 9q chromosome arm …

4.3.3 TP53

TP53 or p53 is a significant gene protein complex transcription factor that plays a major role in managing malignant transitions[21]. Focus on other pathway defects is continuing and there has been recent focus on MDM4, which is a control element of p53, the product of TP53 which is a key control element of proliferation and apoptosis. In a recent paper by Gembarska et al the authors state the following[22]:

The inactivation of the p53 tumor suppressor pathway, which often occurs through mutations in TP53 (encoding tumor protein 53) is a common step in human cancer. However, in melanoma—a highly chemotherapy-resistant disease—TP53 mutations are rare, raising the possibility that this cancer uses alternative ways to overcome p53-mediated tumor suppression. Here we show that Mdm4 p53 binding protein homolog (MDM4), a negative regulator of p53, is upregulated in a substantial proportion (~65%) of stage I–IV human melanomas and that melanocyte-specific Mdm4 overexpression enhanced tumorigenesis in a mouse model of melanoma induced by the oncogene Nras.

MDM4 promotes the survival of human metastatic melanoma by antagonizing p53 proapoptotic function. Notably, inhibition of the MDM4-p53 interaction restored p53 function in melanoma cells, resulting in increased sensitivity to cytotoxic chemotherapy and to inhibitors of the BRAF (V600E) oncogene. Our results identify MDM4 as a key determinant of impaired p53 function in human melanoma and designate MDM4 as a promising target for antimelanoma combination therapy.

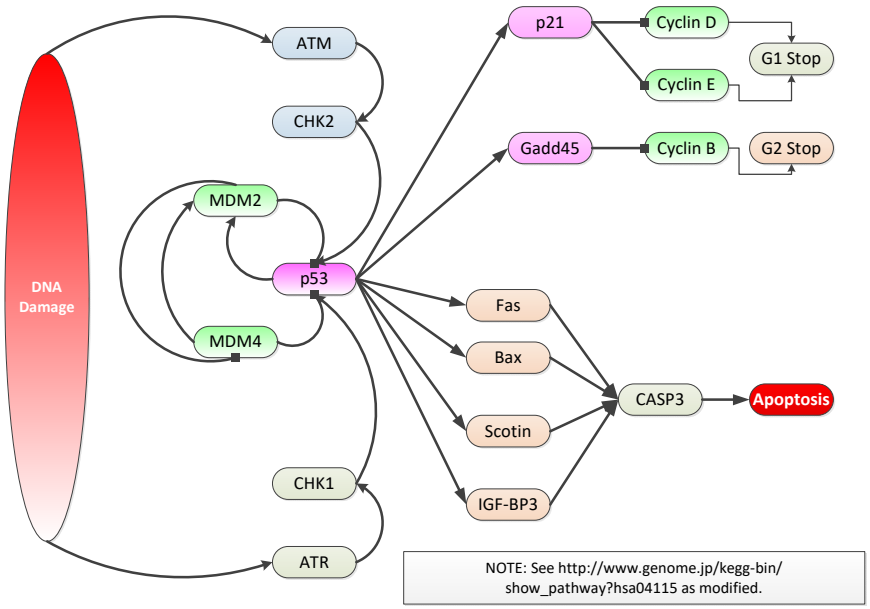
Now MDM4, also called Mdm4 p53 binding protein homolog, is located at 1q32. It acts in a somewhat complex manner to control p53 functions. From NCI we have the following description of the gene and its product.

This gene encodes a nuclear protein that contains a p53 binding domain at the N-terminus and a RING finger domain at the C-terminus, and shows structural similarity to p53-binding protein MDM2. Both proteins bind the p53 tumor suppressor protein and inhibit its activity, and have been shown to be overexpressed in a variety of human cancers. However, unlike MDM2 which degrades p53, this protein inhibits p53 by binding its transcriptional activation domain. This protein also interacts with MDM2 protein via the RING finger domain, and inhibits the latter's degradation. So this protein can reverse MDM2-targeted degradation of p53, while maintaining suppression of p53 transactivation and apoptotic functions.

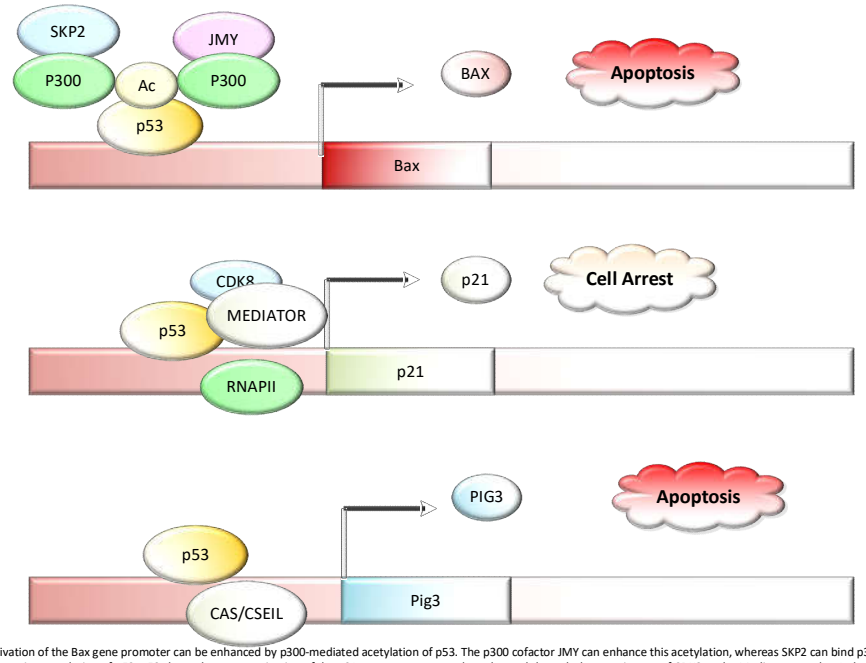
The sources for information on p53 pathway and its relation to MDM4 are extensive . Specific details of the p53 pathway are shown in the NCI data bases for pathways. However, we shall present a simplified description based upon KEEG pathway data. This we do below (We combine from the KEGG genome database) We can further examine some of the p53 functions in the Figure below:

[21] See Lozano and Levine

[22] http://www.nature.com/nm/journal/vaop/ncurrent/pdf/nm.2863.pdf



Note in the above that p53, when functioning properly, can detect DNA damage and correct it or lead the cell to apoptosis. We show some of these below.



Activation of the Bax gene promoter can be enhanced by p300-mediated acetylation of p53. The p300 cofactor JMY can enhance this acetylation, whereas SKP2 can bind p300 and antagonize acetylation of p53. p53-dependent transactivation of the p21 gene promoter can be enhanced through the recruitment of CDK8 to the Mediator complex. Independent binding of the long-range chromatin modifier CAS/CSE1L, which is the human orthologue of yeast Cse1, to promoters, such as the Pig3 gene promoter, can also enhance p53-dependent transcription.

Pelligrini et al continue:

The second most frequent event associated with BCC pathogenesis is the inactivation of the TP53 gene. T

he TP53 tumor suppressor gene is involved in cell cycle arrest and activation of programmed cell death . As a guardian of the genome, TP53 is stabilized upon stress by phosphorylation and alters the expression of different sets of downstream target genes including those that cause cell cycle arrest.

In a mouse model investigating BCC pathogenesis, loss of TP53 has been shown to upregulate the activity of the HH pathway by increasing SMO expression and rendering the mouse interfollicular keratinocytes receptive for X-ray induced BCCs.

Inactivating TP53 genetic alterations have been detected in 50% of human cancers, including all skin carcinomas, which are believed to be a very early if not initial event in carcinogenesis.

In skin cancers, the majority of TP53 missense substitutions are located in the central DNA-binding core domain (codons 102–292) and include codons 177, 196, 245, 248, 278, and 282, producing a full-length protein with altered function.

Regarding BCC, non-synonymous mutations in the TP53 gene have been reported in about half of sporadic cases whereas LOH has been described with a much lower frequency in BCC as compared to other tumors as colon, lung, and bladder cancers.

Hot spots occurring specifically in BCC have been found at codons 177, 196, and 245 .

Codon 177 seems to be specific for BCC since it is not frequently mutated in other malignancies.

Little is known about this codon but it is interesting to note that it includes a sequence slowly repaired after UV-irradiation. Both codons 196 and 245 have been found to be mutated in breast and colon cancers.

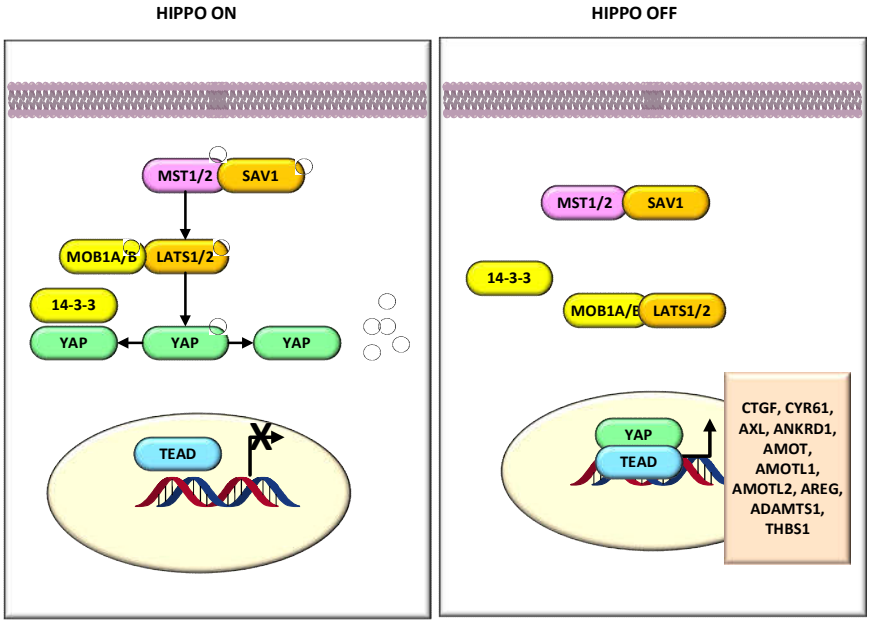
Codon 245 seems to play a major role in carcinogenesis being implicated in several tumor types, such as lung, head and neck, ovary, stomach, and esophagus malignancies. The majority of TP53 mutations in BCC are C to T transitions, with a high frequency of CC to TT double base changes, clearly indicative of UV-induced changes.

A lower level of TP53 mutations were indeed identified in BCCs from sunscreen users compared to that of non-sunscreen users

4.3.4 Hippo-YAP Signaling Genes

Hippo is a Ser/Thr directed protein kinase. Hippo inactivates the transcriptional actions of Yap[23]. The authors demonstrate this as shown below:

23 SEE MARKS ET AL, P 449



See Boopathy and Hong

Boopathy and Hong have noted:

Angiogenesis is a highly coordinated process of formation of new blood vessels from pre-existing blood vessels. The process of development of the proper vascular network is a complex process that is crucial for the vertebrate development. Several studies have defined essential roles of Hippo pathway-YAP/TAZ in organ size control, tissue regeneration, and self-renewal. Thus Hippo pathway is one of the central components in tissue homeostasis.

There are mounting evidences on the eminence of Hippo pathway-YAP/TAZ in angiogenesis in multiple model organisms. Hippo pathway YAP/TAZ is now demonstrated to regulate endothelial cell proliferation, migration and survival; subsequently regulating vascular sprouting, vascular barrier formation, and vascular remodeling.

Major intracellular signaling programs that regulate angiogenesis concomitantly activate YAP/TAZ to regulate key events in angiogenesis. In this review, we provide a brief overview of the recent findings in the Hippo pathway and YAP/TAZ signaling in angiogenesis …

In mammals, the core Hippo pathway is largely characterized by Serine/Threonine kinases; mammalian Sterile 20-related 1 and 2 kinases (MST1 and MST2; orthologs of Drosophila Hippo [Hpo]) and Large tumor suppressor 1 and 2 kinases (LATS1 and LATS2; orthologs of Drosophila Warts [Wts]).

MST kinases are known to undergo auto-activation through auto-phosphorylation on the activation loop of the MST dimer, MST1 at Thr183 and MST2 at Thr180. The carboxyl terminus

of MST kinases have a distinctive coiled-coil structure called SARAH domain [named after the three genes that contain the homologous structures – Salvador (Salvador 1/WW45), RASSF1-6 and Hippo (MST1/MST2)]. SARAH domain mediates homo- and heterodimerization of MST1/MST2. MST1/MST2 heterodimer forms a complex with SARAH domain containing protein, Salvador 1 (SAV1). Being 96% identical proteins, MOB kinase …

Large tumor suppressor kinases belong to AGC group of kinases (named after the protein kinase A, G, and C families) that recognize the substrate consensus sequence HXRXXS/T.

Key substrates of LATS kinases are transcription co-activators, YAP and TAZ. YAP is a key transcriptional regulator and the first protein identified with a WW domain (a motif comprising of two Tryptophan [W] residues) and TAZ is a YAP paralog (with 44% identity to YAP). YAP has five (Ser61, Ser109, Ser127, Ser164, and Ser381) and TAZ has four (Ser66, Ser89, Ser117, and Ser311) HXRXXS/T motifs phosphorylated by LATS1/LATS2 kinases. Phosphorylation of YAP and TAZ by LATS kinases either primes to their binding with 14-3-3 proteins leading to cytoplasmic sequestration of YAP/TAZ

Pelligrini et al continue:

The Hippo pathway is crucial in organ size control, and its deregulation contributes to tumorigenesis.

This pathway was initially investigated in Drosophila, in which mosaic mutations of Hippo-related genes resulted in tissue overgrowth. The Hippo axis includes a series of kinases that, through a cascade of phosphorylation events, inactivate the transcriptional co-activator Yes-associated protein (YAP), thus regulating cell proliferation and apoptosis.

The YAP1 protein is the major downstream effector of the Hippo pathway and the kinases MST1/2 and LATS1/2 represent the core component of the mammalian Hippo signaling. MST1/2 phosphorylate and activate LATS1/2 kinases, preventing the translocation of YAP1 and its family member TAZ (trascriptional co-activator with PDZ-binding motif) into the nucleus. YAP1 transactivation may be inhibited by the non-receptor tyrosine phosphatase 14 (PTPN14) that promotes its nucleus-to-cytoplasm translocation through LATS1 activation

4.3.5 MYCN/FBXW7 Signaling

MYCN is one of the MYC family of transcription factor genes and resulting proteins. It has been well established as a driver of a multitude of cancers (see Dang and Eisenman). MYCN had been identified with neuroblastoma in the 1980s.

Pelligrini et al continue:

MYCN is a member of the MYC family of transcriptional activators and a potential downstream effector of the HH pathway. Changes in the levels of MYC family transcription factors profoundly influence cell growth, proliferation, differentiation, and apoptosis. MYCN missense mutations have been identified in 30% of BCCs, with most of the mutations mapping in the

region encoding the MYC box 1 domain, which is involved in the interaction with FBXW7 tumor suppressor. FBXW7 is a component of the SCFFbw7 ubiquitin ligase that promotes proteasome-dependent MYC degradation through the ubiquitin pathway.

Functional studies demonstrated that four of the most frequent N-MYC substitutions found in BCC, T58A, P59L, P60L, and P63L impaired the binding with the FBXW7, resulting in excessive amounts of the N-MYC protein. Aberrant copy-gain rarely occurs in BCC, while gene amplification is the main mechanism of pathogenic up-regulation of MYCN in medulloblastoma and neuroblastoma.

Interestingly, deleterious mutations and LOH events in the FBXW7 gene occur in 5% and 8% of BCCs samples, respectively, suggesting a selective pressure for enhanced N-MYC stability in BCC

As Liu et al note:

MYCN, a member of MYC proto-oncogene family, encodes a basic helix-loop-helix transcription factor N-MYC. Abnormal expression of N-MYC is correlated with high-risk cancers and poor prognosis. Initially identified as an amplified oncogene in neuroblastoma in 1983, the oncogenic effect of N-MYC is expanded to multiple neuronal and nonneuronal tumors. Direct targeting N-MYC remains challenge due to its "undruggable" features. Therefore, alternative therapeutic approaches for targeting MYCN-driven tumors have been focused on the disruption of transcription, translation, protein stability as well as synthetic lethality of MYCN. In this review, we summarize the latest advances in understanding the molecular mechanisms of MYCN dysregulation in cancers …

Here we describe the regulatory network of MYCN expression. Multiple mechanisms can cause abnormal level of NMYC, including gene amplification, enhanced transcription, translation and protein stability. Various therapeutic targets have been found to address N-MYC overexpression based on knowledge of these regulatory mechanisms. However, strategies that globally inhibiting gene expression (such as inhibiting CDK7 and BDR4) has not yet convincingly demonstrated that these inhibitors specifically target tumors with high N-MYC level, nor have these inhibitors reached advanced stages in clinical trials.

Although directly and specifically targeting N-MYC has not yet been available, promise remains in developing new approaches to effectively treat MYCN-driven tumors. For examples, short interfering RNA (siRNA)-mediated silence of MYCN induces neurogenesis and inhibits proliferation in neuroblastoma models resistant to retinoic acid. Clinical applications of siRNA are developing and the first siRNA-based drug Patisiran (Onpattro) was approved for clinical use to treat transthyretin amyloidosis by the U.S. Food and Drug Administration (FDA) in 2018. In addition, Yoda et al. identify a pyrrole-imidazole polyamide, MYCN-A3, able to directly target MYCN amplicons, which specifically reduces copy number and suppresses gene expression of MYCN.

4.3.6 TERT-Promoter

Telomeres are those ends of DNA which have the tendency to be lost each time a cell reproduces leading eventually to a loss of function. Cancer on the other hand may have mastered the loss of sections of the telomeres and thus may have an ability to prolong their life to many reproductions, namely unlimited. There has been significant interest in targeting telomeres and especially the related enzyme, telomerase, to control cancer cells. In a recent pair of papers the authors have focused on this process in melanomas and especially on UV activation. The authors have discovered somatic mutations in TERT genes which are used to produce Tert and control the Telomeres during cell reproduction. In addition they authors argue that these mutations result from UV radiation.

The focus on telomeres and cancer has been an area of active interest for almost two decades. As Shay et al (2012) state:

To grow indefinitely, human cancer cells must counteract the progressive loss of telomeric DNA that universally accompanies cell division. To do this, about 85 to 90% of cancers use telomerase, an enzyme that synthesizes the tandem 52-TTAGGG-32 hexanucleotide repeats of telomeric DNA by reverse transcription using its own RNA subunit as a template. Because telomerase is not expressed in most normal human cells, telomerase inhibition is considered an almost universal oncology target, and several clinical trials are under way

The above focuses on the critical importance of telomerase. Before continuing it is worth reviewing the telomere. As Shay and Wright state:

Telomeres are tracts of repetitive DNA (TTAGGG/ AATCCC for human telomeres) that protect chromosomes from degradation and loss of essential genes, and allow the cell to distinguish between double-strand breaks and natural chromosome ends. Human telomeres at birth contain 15–20-kilobase pairs of the repetitive sequence TTAGGG followed by a 32 single-strand overhang on the G-rich strand, which is believed to be inserted within the double-stranded region to give a lariat-like structure called a t-loop.

Telomeres progressively shorten in most human cells with increased age, and telomere length in almost all middle-aged human tissues is approximately half that of the new born length. Telomere-specific proteins (such as protection of telomeres-1 (POT1), telomeric repeat-binding factor-1 (TRF1) and TRF2) bind directly to the single- and double-strand telomere regions to form a complex, providing a cap over the ends of the chromosomes that protects chromosome termini from degradation, recombination and end-joining reactions.

The authors further state that telomeres are somewhat maintained in humans via the use of telomerase as follows:

Telomere length is maintained by a balance between processes that lengthen telomeres, such as the activity of the cellular ribonucleoprotein enzyme complex telomerase, and processes that shorten telomeres, such as incomplete synthesis of the lagging DNA strand and end processing events. Telomerase stabilizes telomere length by adding TTAGGG repeats onto the telomeric

ends of the chromosomes, thereby compensating for the continued erosion of telomeres that occurs in its absence. Human telomerase contains two essential components, a telomerase reverse transcriptase *catalytic subunit (hTERT) and a functional* telomerase RNA *(hTR, also known as TERC…*

Other earlier authors such as Campisi et al state:

Telomeres are the repetitive DNA sequences and specialized proteins that form the distinctive structure that caps the ends of linear chromosomes. Telomeres allow cells to distinguish the chromosome ends from double strand DNA breaks. The telomeric structure prevents the degradation or fusion of chromosome ends, and thus is essential for maintaining the integrity and stability of eukaryotic genomes. In addition and perhaps less widely appreciated, telomeres may also indirectly influence gene expression.

The length, structure and organization of telomeres are regulated by a host of telomere-associated proteins, and can be influenced by basic cellular processes such as cell proliferation, differentiation, and DNA damage. In mammalian cells, telomere length and/or telomere structure have been linked to both cancer and aging. Here, we briefly review what is known about mammalian telomeres and the proteins that associate with them, and discuss the cellular and organismal consequences of telomere dysfunction and the evidence that cells with dysfunctional telomeres can contribute to cancer and aging phenotypes.

As reported in the Harvard Gazette we have[24]:

Two mutations that collectively occur in 71 percent of malignant melanoma tumors have been discovered in what scientists call the "dark matter" of the cancer genome, where cancer-related mutations haven't been previously found….

This non-coding DNA, much of which was previously dismissed as "junk," accounts for 99 percent of a cell's genome. A large number of oncogenic mutations in cancer have been identified in the past several decades, but all have been found within the actual genetic blueprints for proteins….

"In addition, this represents the discovery of two of the most prevalent melanoma gene mutations. Considered as a whole, these two TERT promoter mutations are even more common than BRAF mutations in melanoma. Altogether, this discovery could cause us to think more creatively about the possible benefits of targeting TERT in cancer treatment or prevention," Garraway said.

The mutations affect a promoter region — a stretch of DNA code that regulates the expression of a gene — adjacent to the TERT gene. TERT contains the recipe for making telomerase reverse transcriptase, an enzyme that can make cells virtually immortal, and is often found overexpressed in cancer cells. A promoter region of DNA controls the rate of a gene's

[24] http://news.harvard.edu/gazette/story/2013/01/mutations-drive-malignant-melanoma/

transcription — the copying of its DNA recipe into a message used by the cell to manufacture a protein….

The researchers said the same mutations are present in cell lines from some other malignancies, and that preliminary evidence showed they might be unusually common in bladder and liver cancers. They also noted that the discovery of these important mutations in DNA previously not linked to cancer-causing alterations highlights the value of whole-genome searches of tumor DNA.

Another report on Science 2.0 states[25]:

They analyzed the genomes of family members and found an identical mutation in the gene for telomerase, an enzyme often called 'immortality enzyme', in all persons studied. Telomerase protects the ends of chromosomes from being lost in the process of cell division and, thus, prevents that the cell ages and dies. The inherited gene mutation leads to the formation of a binding site for protein factors in the controlling region of the telomerase gene, causing it to become overactive. As a result, mutated cells overproduce telomerase and hence become virtually immortal.

This finding prompted the scientists to also look for mutated telomerase genes in non-inherited (sporadic) melanoma, which is much more common than the familial variant. In most of the tissue samples of melanomas of all stages they found alterations in the telomerase gene switch, which the researchers clearly identified as typical consequences of sun exposure. Even though these mutations were not identical to those found in the melanoma family, they had the same effect: overactive telomerase…

This is also confirmed by the surprising incidence of this alteration: The telomerase gene is the most frequently mutated gene in melanoma. "This is something we hadn't expected, because malignant melanoma has been genetically analyzed thoroughly. But this mutation always seems to have been overlooked," says Kumar.

It should be noted in the above the reference to sun exposure. The argument is that the telomerase change is a direct consequence of the UV exposure. We will focus on that observation later. The “overlooked” nature of this gene and its product is also of issue in that many researchers have examined telomerase extensively so frankly it is not truly new, even as a target for control.

Before continuing it is worth a quick summary of TERT, the telomerase that maintains the telomere. TERT is located at 5p15.33. From NCBI we have[26]:

Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG. The enzyme consists of a protein component with reverse

[25] http://www.science20.com/news_articles/familial_gene_mutation_immortalizes_malignant_melanoma-101871

[26] http://www.ncbi.nlm.nih.gov/gene/7015

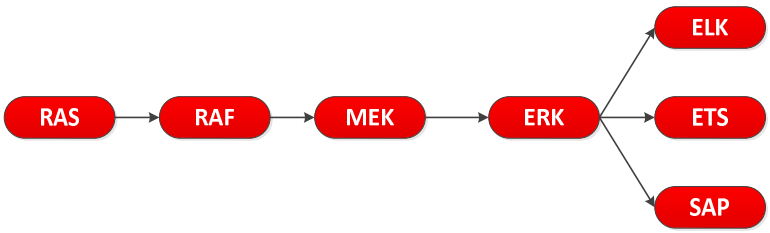
transcriptase activity, encoded by this gene, and an RNA component which serves as a template for the telomere repeat. Telomerase expression plays a role in cellular senescence, as it is normally repressed in postnatal somatic cells resulting in progressive shortening of telomeres. Deregulation of telomerase expression in somatic cells may be involved in oncogenesis.

Studies in mouse suggest that telomerase also participates in chromosomal repair, since de novo synthesis of telomere repeats may occur at double-stranded breaks. Alternatively spliced variants encoding different isoforms of telomerase reverse transcriptase have been identified; the full-length sequence of some variants has not been determined. Alternative splicing at this locus is thought to be one mechanism of regulation of telomerase activity.

The observation can be made that if we do not have adequate TERT then the Telomere ends decay and ultimately the cell line dies off. This is the typical case. Therefore take a malignant melanoma cell. If it has in its pathways and receptors been activated to mitotic duplication then if the TERT is inadequate then the Telomere ends get cut shorter each time it goes through mitosis and at some point it just stops. For example, and this is just for exemplar purposes only, we have a malignant melanocyte, then it goes through mitosis say 10,000 times but each time it would lose a piece of the Telomere until they are all gone, then th cell cannot go again. But if there is an overabundance of TERT, then the TERT resupplies what may be lost and this cell has no way of stopping, at least due to this factor.

The ETS family of genes is positive or negative regulators of gene expression. They can up or down regulate expression. They are named for the initial gene discovered, the E26 Transforming Sequence, where E26 was the oncogene v-ets characterized in 1986 of an avian transforming virus called E26. It is also called the erythroblast transforming specific family, as discussed by Zong et al.

The ERG gene was first presented in the paper by Shyam and Reddy et al in 1987. There the authors identified it and set it in the ETS family. From Weinberg, we see that the ETS are transcription factors driven by the RAS/RAF pathway along with other such factors.

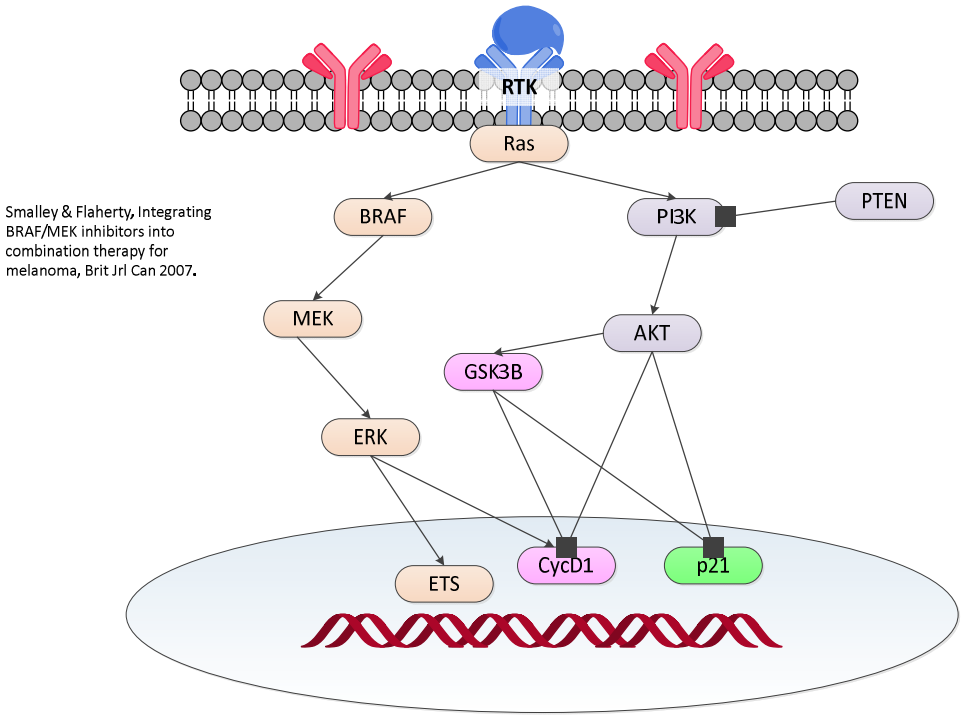


ETS also plays a significant role in the process. We briefly review that as well. ETS is located at 11q23.3. From NCBI we have[27]:

[27] http://www.ncbi.nlm.nih.gov/gene/2113

This gene encodes a member of the ETS family of transcription factors, which are defined by the presence of a conserved ETS DNA-binding domain that recognizes the core consensus DNA sequence GGAA/T in target genes. These proteins function either as transcriptional activators or repressors of numerous genes, and are involved in stem cell development, cell senescence and death, and tumorigenesis. Alternatively spliced transcript variants encoding different isoforms have been described for this gene

From Smalley and Flaherty we have the following pathway for ETS:



Smalley & Flaherty, Integrating BRAF/MEK inhibitors into combination therapy for melanoma, Brit Jrl Can 2007.

The mutations we discuss here are somewhat new and they are present in a relatively large number of samples, at least percentage wise. We know that ETS has transcription control and we can see from above the relationship to BRAF as well. Thus there are many points of loss of control in a melanoma cell. Specifically, as Chudnovsky et al note[28]:

Multiple genetic alterations occur in melanoma, a lethal skin malignancy of increasing incidence. These include mutations that activate Ras and two of its effector cascades, Raf and phosphoinositide 3-kinase (PI3K). Induction of Ras and Raf can be caused by active N-Ras and B-Raf mutants as well as by gene amplification. Activation of PI3K pathway components occurs by PTEN loss and by AKT3 amplification.

Melanomas also commonly show impairment of the p16(INK4A)-CDK4-Rb and ARF-HDM2-p53 tumor suppressor pathways. CDKN2A mutations can produce p16(INK4A) and ARF protein loss. Rb bypass can also occur through activating CDK4 mutations as well as by CDK4

[28] http://www.ncbi.nlm.nih.gov/pubmed/15951821?dopt=Abstract

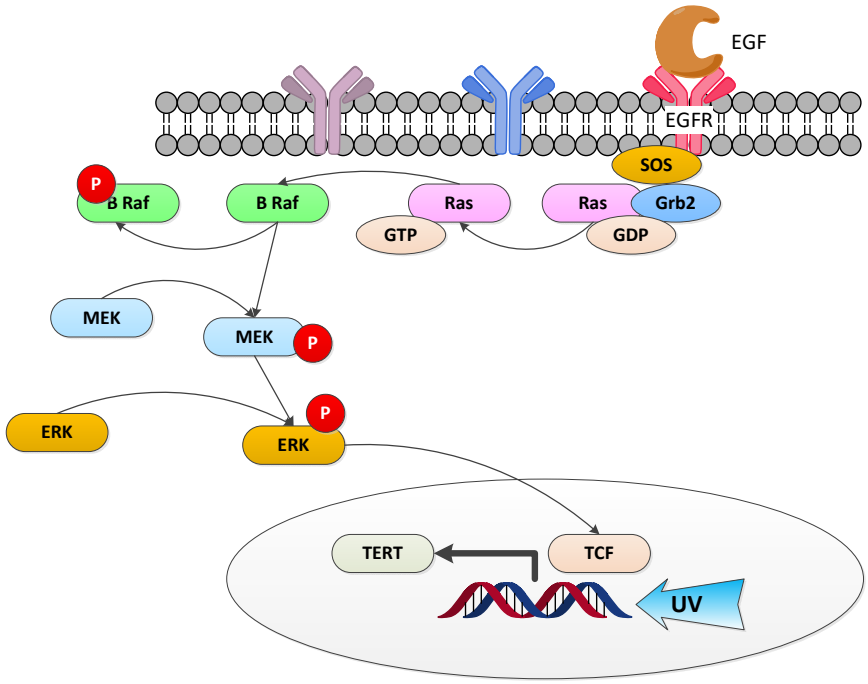
amplification. In addition to ARF deletion, p53 pathway disruption can result from dominant negative TP53 mutations. TERT amplification also occurs in melanoma. The extent to which these mutations can induce human melanocytic neoplasia is unknown. Here we characterize pathways sufficient to generate human melanocytic neoplasia and show that genetically altered human tissue facilitates functional analysis of mutations observed in human tumors.

As Horn et al state:

Cutaneous melanoma occurs in both familial and sporadic forms. We investigated a melanoma-prone family through linkage analysis and high-throughput sequencing and identified a disease-segregating germ line mutation in the promoter of the telomerase reverse transcriptase (TERT) gene, which encodes the catalytic subunit of telomerase. The mutation creates a new binding motif for Ets/TCF transcription factors near the transcription start and in reporter gene assays, caused up to 2-fold increase in transcription.

We then screened the TERT promoter in sporadic melanoma and observed recurrent UV signature somatic mutations in 125/168 (74%) of human cell lines derived from metastatic melanomas, corresponding metastatic tumor tissues (45/53, 85%) and in 25/77 (33%) primary melanomas. The majority of those mutations occurred at two positions in the TERT promoter and also generated binding motifs for ETS/TCF transcription factors.

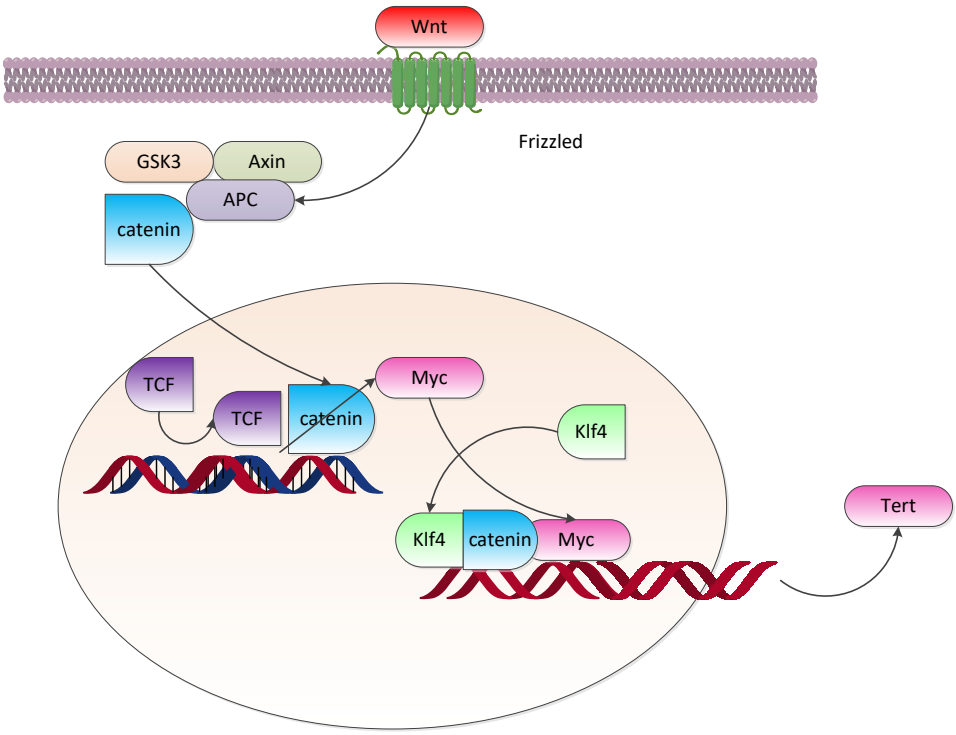
Horn et al conjecture the following pathway:



As Huang et al state:

Systematic sequencing of human cancer genomes has identified many recurrent mutations in the protein coding regions of genes but rarely in gene regulatory regions. Here we describe two independent mutations within the core promoter of TERT, the gene coding for the catalytic subunit of telomerase, which collectively occur in 50 of 70 (71%) of melanomas examined. These mutations generate de novo consensus binding motifs for ETS transcription factors, and in reporter assays the mutations increased transcriptional activity from the TERT promoter by 2 to 4-fold. Examination of 150 cancer cell lines derived from diverse tumor types revealed the same mutations in 24 cases (16%), with preliminary evidence of elevated frequency in bladder and hepatocellular cancer cells. Thus, somatic mutations in regulatory regions of the genome may represent an important tumorigenic mechanism.

We have discussed before the Wnt pathway connection to TERT as well. As shown below we have discussed this option as well.



This has been discussed by Hoffmeyer as well as by Greider. As Greider states:

Recent studies have proposed that the Wnt pathway is linked to TERT in a quite different way. Constitutive overexpression of TERT in mice activates the Wnt pathway, suggesting that TERT may also function as a transcription factor. Although one study did not observe Wnt pathway activation in response to TERT overexpression, other studies have raised questions about the physiological relevance of the constitutive overexpression of TERT. Deletion of TERT in mice does not affect expression of target genes in the Wnt pathway, nor give rise to the cellular phenotypes that loss of Wnt signaling induces, indicating that TERT regulation of Wnt signaling may be limited to situations where TERT is overexpressed.

It is reasonable to propose that Wnt regulates TERT given that Wnt signaling plays an essential role in stem cell self-renewal and that TERT is needed for the long-term growth of stem cells. TERT regulation seems to require not one, but two master transcriptional regulators to assure that there is neither too much, which may allow the growth of cancer cells, nor too little, which might lead to stem cell failure. The finding by Hoffmeyer et al. that both β-catenin and Klf4 are required to activate TERT expression puts the horse (Wnt) before the cart (TERT) and provides a foundation for linking telomerase levels and self-renewal.

Thus TERT regulation is truly a complex process. We have examined the impact of Wnt on melanoma previously. This recent work is on mutations on TERT genes yet we also must consider the influence of Wnt as well.

Pelligrini et al continue:

TERT gene encodes the catalytic reverse transcriptase subunit of telomerase that maintains telomere length. Increased telomerase activity is perceived to be one of the hallmarks of human cancers, and the transcriptional regulation of the TERT gene is the major cause of its cancer-specific activation

The TERT gene is located on chromosome 5p15.33 and the promoter region of this gene is considered the most important regulatory element for telomerase expression. The core promoter region consists of 260 base pairs with several binding sites for transcription-factor that regulate gene transcription.

TERT promoter mutations have been detected at a high frequency in many different cancers as melanoma, non-melanoma skin cancers, bladder cancer, and glioma. They have been related to increased TERT expression through de novo creation of binding sites for Ets/TCF transcription factors, higher telomere length, and with markers of poor outcome. The high recurrence, specificity, and gain of function of non-coding promoter TERT mutations support that they are driver rather than passenger events in cancer development

4.3.7 DPH3-OXNAD1 Bidirectional Promoter

Similar to TERT, recurrent mutations in noncoding positions close to the transcription start site have been reported in the bidirectional promoter of both DPH3 and oxidoreductase NAD-binding domain containing 1 (OXNAD1) genes. DPH3 is required for the synthesis of diphthamide, a modified histidine residue in eukaryotic translation elongation factor 2 that helps in the maintenance of translation fidelity. DPH3 silencing impairs in vivo metastasis in mouse melanoma cells, and its family member DPH1, which is also required for diphthamide synthesis, has been attributed a tumorsuppressor role

As Trieu et al note:

Nascent BCC-like tumors driven by hallmark mutations fail to progress We and others have previously demonstrated that microscopic BCC-like tumors form efficiently after Ptch1 deletion in hair follicle and surface mechanosensory touch dome (TD) epithelia. To further assess the

growth kinetics of these lesions, we analyzed mice expressing tamoxifen (TAM)-inducible Cre recombinase under the control of the Gli1 promoter (Gli1-CreERT2), coupled with homozygous Ptch1 floxed alleles (GP mice). As we previously reported, 5 weeks after TAM administration in GP mice, numerous microscopic lesions arose from Gli1+ stem cells in the hair follicle and TD. Hair follicle-associated tumors resembled nodular BCC, whereas TD-derived tumors possessed features reminiscent of infundibulocystic BCC and fibroepithelioma of Pinkus. To examine the long-term fates of these nascent tumors, we collected serial biopsies up to 17 weeks post-TAM.

Unexpectedly, we observed that hair follicle-associated lesions spontaneously regressed over time, leaving behind small residual tumor nests. In no instance did we observe macroscopic tumors in any GP mice. By contrast, TD-derived tumors neither progressed nor regressed between 12 and 17 weeks post-TAM, although we occasionally observed 1-mm-diameter papules that did not enlarge over time. Macroscopic tumors failed to appear, even when we followed GP mice up to 25 weeks post-TAM.

To determine whether the lack of tumor progression is generalizable to lesions originating from other stem cell populations, we also targeted Lrig1+ hair follicle stem cells for Ptch1 deletion (LP mice). Similar to above, we observed nascent microscopic lesions in LP mice but no macroscopic tumors (Figures 1E and 1F). Finally, we assessed tumor formation following overexpression of a constitutively active form of Smo (SmoM2), targeted to either Gli1+ or Lrig1+ stem cells (Mao et al., 2006). Again, abundant microscopic BCC-like tumors emerged but no macroscopic tumors (Figure 1G). Altogether, these findings demonstrate that nascent tumors initiated by either loss of Ptch1 or gain of Smo fail to progress in the most widely studied conditional models of BCC (Figure 1H).

Nascent BCCs become dormant despite constitutively elevated Hh signaling Sporadic BCCs often arise in aged skin, which undergoes epidermal and dermal changes over time. To better characterize our BCC model, we asked whether aging confers a permissive environment for GP tumors to progress. We therefore induced Ptch1 deletion in young or older mice at 4 or 25 weeks of age, respectively, and assessed tumor kinetics relative to animals induced at 8 weeks of age, our standard starting point In all cases, we observed abundant microscopic tumors at 5 weeks post-TAM, followed by spontaneous regression of hair follicle-associated tumors at 12–17 weeks post-TAM. As before, no macroscopic tumors emerged.

These findings indicate that the relative age of the tumor (time after initiation), rather than the absolute age of the animal, likely determines regression kinetics in our system. Since BCCs regress in response to pharmacological inhibition of Hh signaling, we next asked whether spontaneous tumor regression occurs due to the inability to maintain high-level Hh signaling. To measure downstream pathway activity, we incorporated a Gli1-responsive b-galactosidase (LacZ) allele into GP mice and assessed LacZ activity.

Alternatively, we quantitated mRNA in situ for the canonical Hh target gene, Ptch2. In both cases, we found that Hh pathway activity is maintained even in regressed residual tumors. Further characterization of regressing tumors revealed that proliferation is significantly reduced between 12 and 17 weeks post-TA, concordant with previous findings in Ptch1-deficient skin lesions. This reduction was detected in both hair follicle- and TD-derived tumors iI, S1B, and

S1C). Notably, regressed GP tumors were not apoptotic and did not express classic markers of senescence, such as p16 and p21 (Figures S2A and S2B). Interventions such as treating the skin with a phorbol ester tumor promoter failed to restore proliferation, while depilation caused hair follicles to enter the anagen growth phase without affecting neighboring regressed lesions.

These findings indicate that nascent tumors initiated by deletion of Ptch1 eventually become suspended in a dormant state where cells are neither highly proliferative, apoptotic, nor senescent—features that somewhat resemble those of dormant hair follicle stem cells.

Loss of p53 is not sufficient to drive BCC tumor progression TP53 is also commonly mutated in BCC, and loss of Trp53 promotes tumorigenesis in an irradiated model of BCC; however, the mechanism by which p53 modulates BCC progression remains unclear.

Indeed, our previous studies demonstrated that deleting Trp53 affects neither initial tumor formation nor drug-induced regression. To examine whether loss of p53 affects later stages of tumor progression, we first confirmed that p53 is highly expressed in basal layer cells in GP tumors, which again mimics the expression pattern seen in basal matrix progenitors in the normal-growing hair follicle.

We next generated GP mice harboring homozygous Trp53 conditional deletion alleles (GPP53 mice) and observed that nearly all microscopic GPP53 lesions still underwent spontaneous regression. As seen in GP and GPN1 mice, GPP53 tumors similarly exhibited reduced proliferation over time, and we confirmed that dormant regressed lesions deleted p53, as expected. Along with our previously published data, these findings suggest that losing p53 does not affect tumor initiation, persistence, dormancy, or drug response.

In contrast to GPN1 mice above, GPP53 animals harbored lower microscopic tumor burdens following spontaneous regression. Nonetheless, most GPP53 mice developed at least one macroscopic tumor between 12 and 17 weeks post-TAM.

MYCN overexpression promotes key features of tumor progression MYCN amplification occurs in 12% of human BCCs, while focal mutations that lead to protein stabilization have been detected in 30% of these tumors. As noted above, nascent GP lesions resemble growing hair follicles, and consistent with this theme, we observed enriched Mycn protein and RNA in the basal layer of both early tumors and hair follicle-matrix progenitors.

In contrast, expression of other Myc family members (Myc and Mycl) was not as highly enriched in these compartments.

In GP microscopic tumors, cells with high Mycn are more likely to be proliferative, suggesting a role in cell-cycle regulation. To directly test the role of Mycn in our system, we generated mice expressing Gli1-CreERT2, coupled with Cre-inducible reverse Tet transactivator (rtTA), and a bidirectional tetracycline-responsive element (TRE)-driven MYCN/luciferase (GT mice). In this system, GT mice are first injected with TAM to activate rtTA expression, which subsequently drives MYCN/luciferase overexpression in the presence of doxycycline (DOXY). Following 12–20 weeks of continuous DOXY treatment, GT mice formed dysmorphic anagen hair follicles but

no tumors. Having validated this system, we next incorporated these genetic elements into our Ptch1-deficient model (GPT mice). Tumors were initiated by TAM and allowed to grow for 5 weeks before mice were shifted onto DOXY-chow to activate MYCN expression for an additional 12 weeks.

We noted that transgene expression was localized primarily to the tumor suprabasal compartment, which may either reflect biased TRE promoter activity or inward movement of transgene-expressing basal layer tumor cells. Regardless, MYCN overexpression induced massive proliferation in nascent tumors. Strikingly, we also observed that Notch activation was re-localized away from the tumor suprabasal compartment to cells residing just inside of the basal layer, possibly reflecting early suprabasal cells that express lower levels of the transgene.

These results indicate that MYCN overexpression is sufficient to promote key features of tumor progression— increased proliferation and reduced Notch—that we observed in human BCC and mouse type 1 BCC-like tumors. Despite these findings, most GPT mice did not form macroscopic tumors, likely because MYCN overexpression caused increased apoptosis. In 4/16 GPT mice, however, we observed small palpable tumors, which contained transgene-expressing basaloid cells. We did not allow these lesions to continue growing due to frequent gastrointestinal-related morbidity in GPT mice. Similar to GPN1 and GPP53 macroscopic tumors, these rare GPT tumors likely also acquired additional somatic mutations that enabled them to progress.

Collectively, our findings argue that loss of Ptch1 by itself is not sufficient for full BCC progression and that secondary mutations—resulting in loss of Notch1 or p53, increased Gli, and/or gain of Mycn—contribute functionally to the critical transition from microscopic to macroscopic disease.

Our skin is the most highly mutated organ, and mutations in cancer-associated genes, such as NOTCH1/2, TP53, and RAS, are frequently detected in photoaged epithelia. Previously, we and others have observed that mutant cells with aberrant Notch1 or p53 can persist long term in the epidermis without forming tumors.

Targeted expression of oncogenic Kras in hair follicle stem cells also causes only temporary tissue disruption that normalizes over time.

Along a similar vein and consistent with previous findings, we report here that loss of Ptch1 or gain of Smo, both hallmark mutations in BCC, induces nascent tumors that do not progress. Given the high failure rate for tumor-initiated cells in the skin, it is likely that both cell-intrinsic and cell-extrinsic factors suppress tumor progression. Indeed, an idiosyncratic feature of the skin is the periodic phases of hair growth and regression, with the anagen growth phase favoring tumor formation and the telogen resting phase associated with tumor regression . In our GP model, nascent BCC-like tumors share some resemblance to growing hair follicles, including basal layer enrichment for multiple factors—high Hh pathway activation, proliferation, p53, and Mycn—as well as suprabasal activation of Notch signaling.

Thus, it is conceivable that early tumor growth and spontaneous regression are linked to the hair cycle. However, since regressed tumors in our model remain dormant even during subsequent anagen, other factors likely cause tumor exhaustion over time.

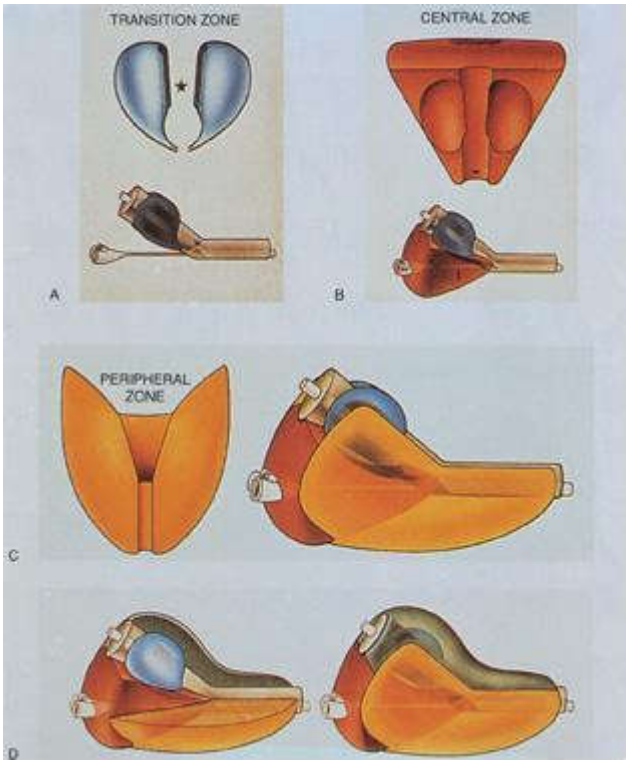
5 PROSTATE CANCER: ANOTHER TARGET

Prostate cancer is now the most common cancers in men in the US. The high incidence presents a significant burden yet mortality is still relatively low. However as has been noted by many PCa treatments and especially therapeutics lag decades behind what one sees in breast cancer, BCa. We have been examining PCa progress for more than a decade and the insight has been continuous but still slow paced[29].

5.1 THE NORMAL PROSTATE

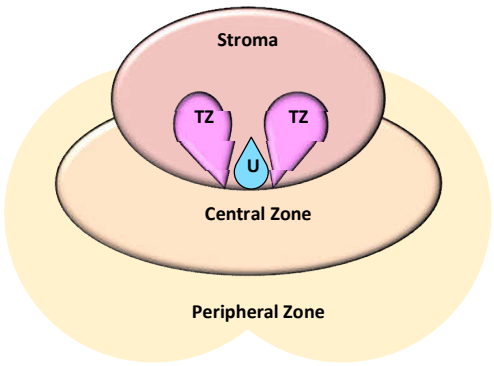
We first examine the normal prostate. The prostate is normally about 40 cc in dimension with the prostate surrounding the urethra below the bladder.

The basic structure of the prostate is shown below. It consists of three major zones; peripheral (dominant zone), central zone which is around the urethra), and the transition zone.



Another cross section view is below:

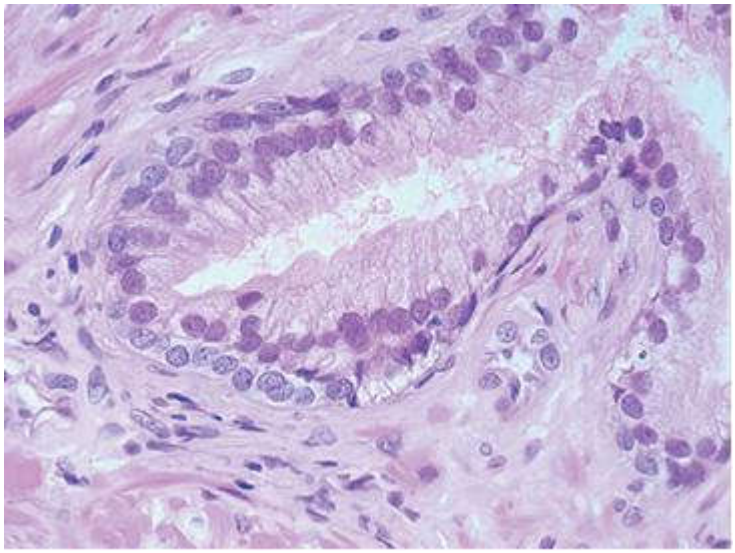
[29] See https://www.researchgate.net/publication/264960277_Prostate_Cancer_A_Systems_Approach and recently see https://www.researchgate.net/publication/370125480_Androgen_Receptor_Whither_Goest_Thou



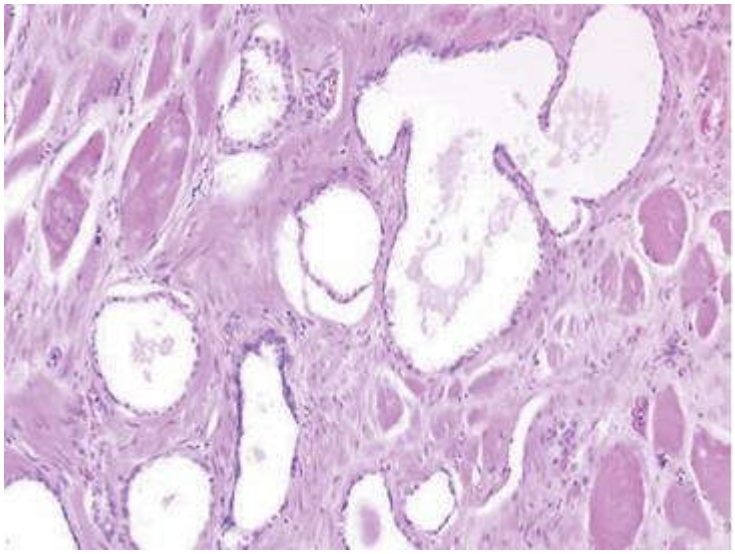
TZ Transition Zone
U Urethra

The cellular structure is depicted below. There are approximately 35-50 glands in the prostate, mostly in the peripheral zone and the glands have a lumen in which the prostatic secretions flow and the glands have basal cells and luminal cells as shown below. The basal cells are dark and the luminal cells are somewhat lighter.

Between the cells is the stroma which includes the blood flow from veins and arteries, the muscle and other stroma elements. Simply stated, the prostate is a collection of the basal/luminal glands scattered about veins, arteries, muscles and nerves.



The figure below depicts a second view of the prostate glands. Again this is with HE stain and under low magnification. The basal cells are clearly see with their dark stains and the luminal stand above them. The stroma is fairly well articulated in this slide.

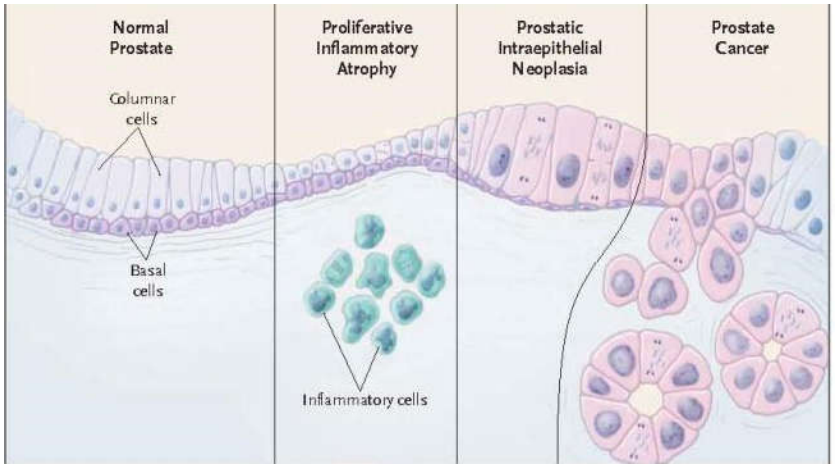


The normal prostate then is merely a collection of glands, glands composed of basal and luminal cells, with open glandular portions, the white areas above. As we noted before these glands emit various proteins and are an integral part of the male reproductive system.

5.2 Summary of Prostate States

We now provide a high level summary of the changes in the prostate histologically as PCa is developed. We do this to lay out the various changes we will examine and to better understand what we may be looking for when developing pathways. We believe that it is essential that we always go back and forth between abstractions of pathways, and the reality of the cell histology.

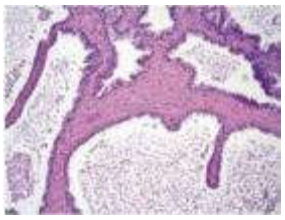
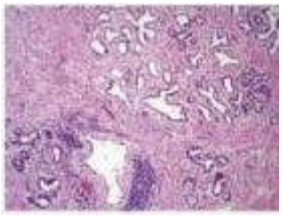
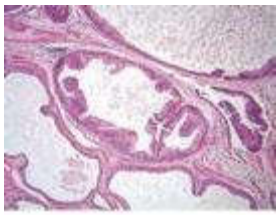
There is a general agreement, with of course many exceptions, as to the progression of prostate pathology and its related causes. A graphic from a recent NEJM article is shown below[30]:

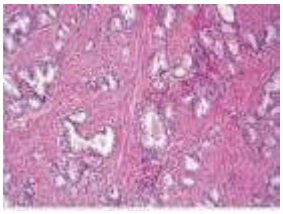
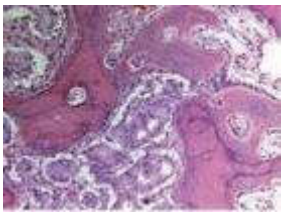


[30] See Nelson et al, Prostate Cancer, NEJM, July 24, 2003. p 376.

Not the progression from normal prostate with basal and luminal cells and then through PIA and then PIN and finally PCa. The PIN demonstrates a complex but contained development of cells. As one moves o PCa, that is when the cells move away from the existing gland, and they are for the most part luminal cells establishing de novo glandular like structures.

An excellent tabular summary from Taichman et al follows:

Disease State	*Histology*	*Details*
Normal Prostate		Large glands with papillary infoldings that are lined with a 2-cell layer consisting of basal and columnary secretory epithelial cells (luminal) with pale cytoplasm and uniform nuclei. Susceptibility genes or events related to hereditary PCa: RNASEL: regulates cell proliferation through the interferon regulated 2-5 oligoadenylate pathway ELAC2/HPC2: Loss of function of tRNA-3 processing endoribonuclease MSR1: Macrophage scavenger receptors process negatively charged macromolecules.
PIA		Atrophic glands have scant cytoplasm, hyperchromic nuclei and occasional nucleoli and are associated with inflammation Susceptibility genes or events: NKX3: Allelic loss of homeobox protein allowing growth of prostate epithelial cells PTEN: Allelic loss of phosphatase and tensin homolog allowing decreased apoptosis and increased cell proliferation. CDKN1B: Allelic loss of cyclin dependent kinase inhibitor p27 allowing increased cell proliferation
PIN		Intermediate to large size glands with proliferation changes contained within the gland and having nuclear abnormalities that resemble invasive carcinoma. Susceptibility genes or events: GSTP1: Hypermethylation of the upstream regulatory region inactivates the Pi class gluthionine S transferase enzyme which detoxifies carcinogens. Hepsin: Increased expression of this serine protease leads to increased invasiveness and disruption of the basement membrane. AMACR: Increased expression results in increased peroxisomal b-oxidation of branched chain fatty acids from red meat thereby increasing carcinogen exposure. TMPRSS2: Fusion of this androgen regulated gene with ETS family of transcription factors in late stages of PIN results in in increased breakdown of the extracellular matrix. Telomerase: Activation leads to maintenance of telomere length and immortalization of cells.

Disease State	*Histology*	*Details*
Prostate Cancer		Small irregular glands with cells having abnormal nuclei and nucleoli and lacking basal cells. Susceptibility genes or events: MYC: Overexpression leads to cell proliferation and transformation RB: Loss of expression leads to cell proliferation and transformation
Metastatic PCa		Nests of cancer cells within the bone Susceptibility genes or events: TP53: Mutation results in loss of multiple tumor suppressor functions E-cadherin: Aberrant expression leads to increased invasive and metastatic phenotype NM23: loss of this NDP kinase leads to increased metastasis EZH2: Histone methyltransferase PcG protein whose activation causes repression of genes that suppress invasion and metastasis
AR PCa		Cancer cells that grow in androgen depleted environment Susceptibility genes or events: AR: may remain active through amplification, phosphorylation by other steroids or non-androgen growth factors BCL2 Increased expression leads to protection from apoptosis Stem cells: potential repopulation by progenitor cells

Note in the above, Taichman et al make mention of the separate gene elements that are putatively assumed to have caused the subsequent event. These genetic changes then will become a key factor in how we view PIN transitions.

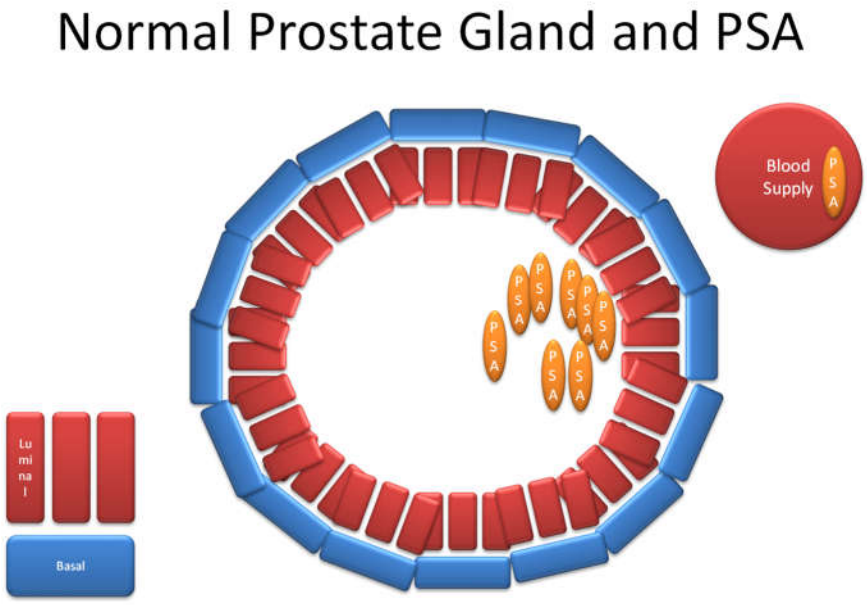
Also note in the above, it implies a set of sequences of genetic changes that moves from benign to malignant. The question then is; if a genetic change is necessary for a morphological change, then is the genetic change reversible or are the genetically changed cells killed off by some other process, and if so what process?

To understand this question, and hopefully set a path to answering it, we lay out the known elements in the path towards malignancy, look at the gene maps and dynamics, and then attempt to establish a model for examining the dynamic processes which move the cell forward to malignancy or backwards towards a benign state.

Before detailing the cellular level of the pathology it is worth while discussing the PSA issue and the controversies related thereto.

The normal prostate is a 40 cc globe like gland just below the bladder and surrounding the urethra. It is composed of 35-50 small glands and between the glands is a stroma composed of nerves, muscles, and blood supplies, with some other connective tissues. A typical gland is shown below along with an adjacent blood flow.

The following Figure graphically depicts the gland in the prostate and the PSA released mostly into the lumen of the gland but a small percent gets released into the blood supply.



PSA, prostate specific antigen, is a gene product of chromosome 19[31]. The PSA gene is androgen regulated. PSA is synthesized in the epithelial cells. It is secreted into the lumen of the prostate gland ducts and works its way into the serum most likely by diffusion. PSA tends to increase with hypertrophy and PCa. This most likely is due to cell proliferation and thus a larger base of excretion of PSA into the lumen. There does not however seem to be any studies relating serum PSA to prostate size, volume. A normal prostate is about 40 cc in volume and large prostates say of 60 cc may have more epithelial cells and thus putatively a larger PSA in the serum, however there does not appear to be evidence supporting this conjecture.

Most serum PSA is bound to proteins. Some is unbound and thus free. Thus, the Percent Free PSA is often also measured. PSA released from cancer cells however is often not processed by intracellular proteolytic chains and thus is not free. High percent free is often a sign of no malignancy[32].

PSA velocity is another measure of malignancy potential. The definition of PSA velocity is the three sample average of PSA change per year or percent change per year. That is, we take three time samples, and then calculate two velocities, from the second less first, and the third less

[31] See Kantoff, Prostate, p 213.

[32] Su, Prostate, p 5.

second, and annualize each and take the average. If the velocity exceed 0.75 we have a threshold which requires examination[33].

5.3 PROSTATE CANCER AND HEDGEHOG

As Sheng et al had noted:

Our data indicate that activation of the hedgehog pathway, through loss of Su(Fu) or overexpression of sonic hedgehog, may involve tumor progression and metastases of prostate cancer. Thus, targeted inhibition of hedgehog signaling may have significant implications of prostate cancer therapeutics. ...

Taken together, our findings suggest that activation of the hedgehog pathway involves prostate cancer progression.

There might be several mechanisms by which the hedgehog pathway is activated in advanced prostate cancers, including loss of Su(Fu) protein expression, over-expression of sonic hedgehog or other alterations. We demonstrate that activation of the hedgehog pathway is associated with DNA synthesis and cell invasiveness in prostate cancer cells. Inhibition of the hedgehog pathway, on the other hand, causes apoptosis possibly through down-regulation of Gli1.

Our studies predict that targeted inhibition of the hedgehog pathway may be an effective way to prevent prostate cancer progression.

As Buttyan et al note:

Hedgehog (Hh) is an oncogenic cell signaling pathway causative of skin and brain cancers.

While long suspected to play a role in other solid tumors, including prostate cancer, the types of mutations in upstream Hh signaling elements, especially in Patched and Smoothened (Smo), that drive hyperactive Hedgehog in skin and brain cancers are not found in these other tumors.

When active, Smo suppresses a site specific proteolysis of Gli2 and Gli3 that removes their C-terminal transactivation domains rendering them into functional repressors.

Smo action maintains Gli2/ Gli3 in high molecular weight, transcriptionally active forms that mediate the genomic effects of Hedgehog signaling.

Recently, Li, et al described a novel Smo-independent means of Gli activation in prostate cancer cells that is based upon the direct binding of androgen receptor (AR) proteins to Gli proteins. ARs (full-length liganded and constitutive-active truncated variants) recognize and bind to the C-terminal Protein Processing Domains (PPD) of Gli2 and Gli3. Furthermore it was shown that

[33] Su, prostate. p 5.

ARs effectively competed for Gli-PPD binding with the E3 ubiquitin ligase, β-TrCP, which marks Gli2/Gli3 for degradation.

So AR binding to Gli2/ Gli3 effectively protects them from the constitutive degradation process that defines the Hedgehog inactive state and bypasses the need for upstream Smo activity.

This identifies a new non-canonical means for Hedgehog activation in tumors and explains the failure of Smo based therapeutics to affect progression of advanced prostate cancer. More important, it shows that androgen action in prostate cancer is inexorably linked to the activity of Gli transcription factors …

This finding recognizes a secondary function of the AR as a Gli co-activator in prostate cancer cells.

It also raises interesting questions about other steroid driven tumors, including breast cancer. Does the estrogen receptor (ER) share the Gli-activation potential of ARs? Furthermore, glucocorticoid receptor (GR) is thought to replace AR function in some hormone resistant prostate cancers. Does GR provide the Gli-activation functions of AR in these instances? Resolution of these questions may explain reports of Hh activity in breast and other solid tumors and may ultimately impact on our understanding of the evolution of steroid receptors as AR is the evolutionary youngest of the steroid receptors whereas ER is the oldest

As Suzman and Antonarakis note:

Normal prostate development involves Hh pathway activation in the epithelial and stromal compartments leading to ductal morphogenesis via concentrated SHH expression at sites of ductal bud formation.

In the adult prostate gland, the pathway is involved in regenerating prostate epithelial cells and regulating their differentiated state. Over-expression of Hh signaling, however, has been noted in prostate cancer in both animal models and human samples. Early reports exploring the role of inhibiting the pathway via cyclopamine or anti-SHH antibodies suggested a direct role for the Hh pathway in the development and progression of prostate cancer.

In this context, Hh signaling may promote tumor formation from prostatic epithelial cells, drive the epithelial-to-mesenchymal transition (EMT) which is associated with tumor invasion, and contribute towards the development of castration-resistance and chemotherapy-resistance.

The pathway appears complex and may reflect interactions within the tumor microenvironment between tumor cells and stromal cells. Within the microenvironment, the localization of expression of the Hh pathway and the nature of the interaction between epithelium and stromal components remains an area of controversy.

For example, in one study of xenografted tumors, GLI1 mRNA localized to the stromal compartment while SHH localized to the prostatic epithelium, indicating active paracrine Hh signaling from the tumor in the surrounding stroma. However, in a study evaluating human

prostate tissue, in situ hybridization of GLI1 mRNA localized to the epithelium but not to the surrounding stroma and was co-expressed with PTCH1 and SHH, suggesting autocrine Hh signaling. Tzelepi et al. found that epithelial expression of GLI1, SHH, SMO, and PTCH by immunohistochemistry was higher in primary prostate carcinomas compared with non-neoplastic peripheral zone tissue, but was lower in the surrounding stromal tissue. Higher-grade and higher-stage prostate cancers demonstrated even lower stromal localization of PTCH, with the lowest expression occurring in metastatic bone lesions. Thus, the Hh pathway components appear to be differentially expressed in the tumor microenvironment as compared to benign tissues.

The issue of whether clinically relevant Hh signaling in prostate cancer occurs via an autocrine or paracrine model remains an open question.

The Hh pathway may be particularly active in men with hormone-naïve localized prostate cancer at high risk for metastatic spread compared with low-risk tumors. Gene expression profiles from localized high-grade prostate tumors differed in men who either rapidly developed metastases within the first 5 years following radical prostatectomy versus those men who were metastasis-free for >5 years after surgery.

In men who developed early metastases, embryonic stem cell pathways, including the Hh and Notch pathways, were highly differentially expressed compared with the metastasis-free group as determined by gene expression profiling, and SHH was up-regulated 3.7-fold in the early-metastasis cohort, suggesting increased Hh signaling in localized prostate cancer with metastatic potential.

Similarly, Kim et al. evaluated 155 radical prostatectomy specimens from men with localized prostate cancers via immunohistochemistry and found increased expression of multiple components of the Hh pathway, including SHH, PTCH1, SMO, and GLI. In a multivariate model, increased SHH expression was an independent prognostic factor for biochemical recurrence beyond clinical factors that included Gleason score, stage, tumor volume, and pretreatment PSA.

Cross-talk between the Hh and androgen signaling pathways has been noted both in vitro and in human radical prostatectomy specimens

As Richards et al note:

The dominant cell type surrounding prostate epithelial (PrE) acini is a fibromuscular mesenchyme, also known as stroma, which exerts regulatory control over normal glandular differentiation and contributes to carcinogenesis. The stroma comprises the bulk of the prostate and contains fibroblasts, myofibroblasts, and smooth muscle cells The prostate mesenchyme influences gland formation during neonatal development, and stromal AR signaling is essential for normal gland morphogenesis.

Paracrine signaling between stroma and epithelium, which includes secreted WNTs, fibroblast growth factors (FGFs), sonic hedgehog, bone morphogenetic proteins (BMPs), and transforming

growth factor (TGF) b, has positive and negative regulatory roles in adult PrE maintenance, regeneration, and transformation…

Xia et al note:

Glioma-associated oncogene family zinc finger 2 (Gli2), a key component of the hedgehog signaling pathway, has been previously demonstrated to promote the malignant properties of prostate cancer in vitro. However, the role of Gli2 in the development of castration-resistant prostate cancer (CRPC) has yet to be fully elucidated. In the present study, Gli2 expression was knocked down in androgen-responsive prostate cancer cells using an inducible Gli2 short hairpin RNA.

Suppression of Gli2 expression resulted in significant reduction of cell viability, increased the proportion of cells in the G0/G1 phases of the cell cycle and reduced the expression of genes associated with cell cycle progression. Gli2 knockdown sensitized both androgen-dependent and -independent prostate cancer cells to the antiandrogen drug Casodex and prevented the outgrowth of LNCaP prostate cancer cells.

In addition, Gli2 knockdown significantly suppressed the development of CRPC in a LNCaP xenograft mouse model, which was reversed by the re-expression of Gli2. In conclusion, to the best of our knowledge, the present study was the first occasion in which the essential role of Gli2 in the development of CRPC was demonstrated, providing a potential therapeutic target for the intervention of CRPC

As Gonnissen et al note:

Furthermore, Hh pathway activation seems to be more pronounced in advanced PCa. Sheng et al. reported that high levels of Ptch1 and Hedgehog-interacting protein (Hhip) were more frequently detected in PCa with high Gleason score and metastatic PCa specimens. Moreover, Tzepeli et al. demonstrated that expression of Ptch in the tumor tissue correlated with tumor grade and stage. Epithelial Ptch expression was also found to be higher in metastatic tissue compared to primary PCa tissue.

Moreover, Hh signaling also correlated with Ki67 and vascular epithelial growth factor (VEGF), but not with CD31. The group of Azoulay et al. evaluated Hh ligand expression in 231 hormone-naïve (HNPC), 20 hormone-treated (HTPC) and 24 hormone-refractory (HRPC) prostate tumor samples. In HNPC, a significant correlation was found between Shh expression and Gleason score on the one hand and metastasis in the lymph nodes on the other hand.

Likewise, epithelial Dhh expression was significantly associated with Gleason score, tumor stage and seminal vesicle invasion. Multivariate analysis also presented the concomitant absence of Shh and Dhh in stromal cells as an independent prognostic parameter for biological recurrence in PCa . A study by Kim et al. showed that Hh signaling is associated with poor prognosis. In this retrospective study of 155 PCa samples, the protein expression of different Hh signaling components (Shh, Ptch, Smo, Gli and Sufu) was examined and correlated with clinicopathological parameters, including tumor size, Gleason score, pretreatment PSA and PSA

recurrence. All the investigated Hh components except Sufu were significantly correlated with Gleason score.

Furthermore, Shh expression was found to be a significant independent prognostic factor for PSA recurrence in multivariate analysis. Karhadkar et al. compared the gene expression of Hh ligands and Hh transcriptional targets PTCH1 and GLI1 between localized and metastatic prostate tissue. SHH and IHH were present in all samples, being either benign prostate tissue, localized or metastatic prostate tumor tissues.

However, while PTCH1 and GLI1 were expressed in all metastatic tumor samples, only 3 out of 12 localized tumor samples and none of the benign tissue samples expressed these genes. Moreover, PTCH1 mRNA levels were more than tenfold higher in metastatic tissue compared to localized PCa samples. Furthermore, the authors showed that transfection of a poorly metastatic cell line (AT2.1) with Gli1 increased the metastatic potential of this cell line remarkably, illustrating the role of Hh signaling in promoting metastasis.

In order to investigate the progression of PCa and the development of metastasis, proper representative animal models of PCa are necessary. For PCa, two different mouse models have been described; the LADY PCa mouse model and the TRAMP PCa model. An in vivo study in the LADY PCa mouse model by Gipp and colleagues has shown that the expression of Hh signaling components, Shh, Ptch1 and Gli1 are not increased during PCa development, whereas another study by Bragina et al. using a TRAMP PCa mouse model did show an age-dependent increase in Hh activity associated with tumor development.

These contradictory results could be linked to the differences between the two tumor models. The LADY mice develop rather low-grade prostatic intraepithelial neoplasia and invasive carcinoma, which generally fail to metastasize, whereas TRAMP mice are more advanced and able to metastasize primarily to lungs and lymph nodes.

In addition, a potential relationship between Hh signaling and androgen-independent PCa has been described by multiple independent groups. Long-term androgen deprivation has been shown to induce an up-regulation of Hh signaling both in human specimens and in PCa cell lines, suggesting an active role for Hh signaling in the progression to androgen-independent PCa.

For instance, Efstathiou et al. reported that different Hh signaling components (Shh, Smo, Gli1 and Gli2) were increased after androgen deprivation therapy (ADT) compared to untreated control samples, both in human as in mouse xenograft samples. Moreover, combination of ADT and chemotherapy also resulted in an increased epithelial Bcl2 and nuclear pAKT expression, emphasizing the role of Hh signaling activation in tumor progression.

A recent study by Ibuki and colleagues demonstrated that the Hh inhibitor, TAK-441 was able to delay the progression to castration-resistant PCa (CRPCa) in a PCa xenograft mouse model.

However, in this study, TAK-441 had no effect on cell viability of LNCaP cells in vitro after androgen withdrawal, indicating that the effect of Hh inhibition on tumor progression is probably due to paracrine Hh signaling in the surrounding tumor stroma.

This is in contrast with a study by Chen et al., which showed that inhibition of Hh signaling in the absence of androgens resulted in a decrease of LNCaP cell growth in vitro and this effect was rescued by the addition of androgens to the medium.

In addition, inhibition of Hh signaling led to down-regulation of androgen receptor (AR) signaling activity, at least partly due to direct binding of Gli2 and/or Gli1 to the AR.

Shaw et al. investigated the effect of concomitant inhibition of Hh signaling and ErbB signaling in CRPCa cells in vitro. The Hh and ErbB pathways seemed to have synergistic effects on the proliferation of CRPCa cells, resulting in a more pronounced inhibition of CRPCa cell growth. Nevertheless, more investigation is needed to gain more insight into the exact mechanisms behind the involvement of Hh signaling in the progression to CRPCa

6 THERAPEUTICS

6.1 OVERVIEW

As Rimkus at al note:

The sonic hedgehog (Shh) signaling pathway is a major regulator of cell differentiation, cell proliferation, and tissue polarity. Aberrant activation of the Shh pathway has been shown in a variety of human cancers, including, basal cell carcinoma, malignant gliomas, medulloblastoma, leukemias, and cancers of the breast, lung, pancreas, and prostate. Tumorigenesis, tumor progression and therapeutic response have all been shown to be impacted by the Shh signaling pathway. Downstream effectors of the Shh pathway include smoothened (SMO) and glioma-associated oncogene homolog (GLI) family of zinc finger transcription factors.

Both are regarded as important targets for cancer therapeutics. While most efforts have been devoted towards pharmacologically targeting SMO, developing GLI-targeted approach has its merit because of the fact that GLI proteins can be activated by both Shh ligand-dependent and -independent mechanisms. To date, two SMO inhibitors (LDE225/Sonidegib and GDC-0449/Vismodegib) have received FDA approval for treating basal cell carcinoma while many clinical trials are being conducted to evaluate the efficacy of this exciting class of targeted therapy in a variety of cancers. In this review, we provide an overview of the biology of the Shh pathway and then detail the current landscape of the Shh-SMO-GLI pathway inhibitors including those in preclinical studies and clinical trials

6.2 NATURAL THERAPEUTICS

As Ślusarz et al note:

Many botanical compounds have been proposed to prevent cancer. We investigated the cancer treatment and prevention abilities of apigenin, baicalein, curcumin, epigallocatechin 3-gallate (EGCG), genistein, quercetin, and resveratrol both in vivo in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice as well as in vitro in prostate cancer cell lines. In our experiments, these seven compounds act similarly to the Hedgehog antagonist cyclopamine, a teratogenic plant alkaloid, which had been previously shown to "cure" prostate cancer in a mouse xenograft model. With IC50 values ranging from <1 to 25 μmol/L, these compounds can inhibit Gli1 mRNA concentration by up to 95% and downregulate Gli reporter activity by 80%. We show that four compounds, genistein, curcumin, EGCG, and resveratrol, inhibit Hedgehog signaling as monitored by real-time reverse transcription-PCR analysis of Gli1 mRNA concentration or by Gli reporter activity. Three compounds, apigenin, baicalein, and quercetin, decreased Gli1 mRNA concentration but not Gli reporter activity.

Our results show that these compounds are also able to reduce or delay prostate cancer in vivo in TRAMP mice. All seven compounds, when fed in combination as pure compounds or as crude plant extracts, inhibit well-differentiated carcinoma of the prostate by 58% and 81%, respectively. In vitro, we show that all seven compounds also inhibit growth in human and mouse

prostate cancer cell lines. Mechanistically, we propose the Hedgehog signaling pathway to be a direct or indirect target of these compounds. These botanicals at pharmacologic concentrations are potentially safer and less expensive alternatives to cyclopamine and its pharmaceutical analogues for cancer therapy.

Introduction Prostate cancer remains the second most commonly diagnosed cancer in the United States. According to the Prostate Cancer Foundation, one of every three men diagnosed with cancer will be diagnosed with prostate cancer. It is also the second leading cause of cancer deaths of men in the United States. Because prostate cancer typically develops later in life, identifying botanical compounds that delay the progression of this disease will have a positive effect on quality of life and reduce healthcare costs of the aging population. It is well known that diet and other environmental factors can greatly reduce the risk of cancer incidence. In particular, dietary phytoestrogens and antioxidants have been implicated in protecting against cancer.

We have selected a group of seven botanical compounds that have been reported to have prostate cancer–protective activities and have been widely used in traditional medicine and in dietary supplements that are currently available in the United States.

Those compounds include apigenin from Matricaria recutita (chamomile), baicalein from Scutellaria baicalensis Georgi (Chinese skullcap), curcumin from Curcuma longa (turmeric), epigallocatechin 3-gallate (EGCG) from Camellia sinensis Kuntze (green tea), genistein from Glycine max (soy), quercetin from Ginkgo biloba, and resveratrol from Vitis vinifera (grape).

We have previously reported the cancer-preventive effect of each of these seven compounds in PC3 and LNCaP human prostate cancer cell lines (1). Here, we show that all seven can also individually inhibit growth of the mouse prostate cancer cell line TRAMP-C2. Pursuant to these results, we wanted to determine whether these seven botanical compounds would affect the Hedgehog signaling pathway, which, through its inhibitor cyclopamine, has been recently found to be important in prostate cancer and its treatment . ...

The four compounds, which inhibited Hedgehog signaling in both cell assays (genistein, curcumin, EGCG, and resveratrol), are potentially cheaper and safer alternatives to cyclopamine. All of these compounds have also been consumed in human diets for thousands of years and many are taken in dietary supplements today.

There has only been a limited number of reproductive safety studies conducted, which would shed light on the potential teratogenicity of those compounds that one might hypothesize because of their cyclopamine-like effects. Genistein did not cause any fetal malformations when fed to pregnant rats up to 1,000 mg/kg/d . Resveratrol has been reported to act as an antiteratogenic compound, as has curcumin.

Feeding pregnant rats diets supplemented with 14,000 ppm EGCG during organogenesis was nontoxic to dams or fetuses. Presumably, the fetus is protected from the Hedgehog-inhibitory effects of these compounds in some unknown manner. We also used tomatidine, which is related structurally to cyclopamine, as a classic negative control in our assays, but as reported by

others, tomatidine will begin to inhibit Shh signaling at doses above 10 µmol/L (see Fig. 1C in ref. 6), albeit the cyclopamine inhibition is much stronger and will work at ~100-fold lower concentration. We used cyclopamine as a recognized positive control for Hedgehog inhibition, and clearly, it works better than tomatidine…

Whereas three of our compounds Hedgehog-inhibitory activities (curcumin, EGCG, and genistein) match the potency of cyclopamine, four of the seven compounds show dose-response profiles more similar to the IC50 of tomatidine. This is not to say that these four botanical compounds are nonspecific but rather that they are just not as potent as cyclopamine. The ability of these botanical compounds to function, as low-cost, easily bioavailable substitutes for cyclopamine, is a major finding of this study.

Our findings that genistein, curcumin, EGCG, and resveratrol inhibit Hedgehog signaling provide prospective available, safer, and more affordable anticancer treatments for Hedgehog-driven cancers. Additionally, they help provide a better understanding of the mechanisms by which traditional herbal medicines and dietary supplements may be working to prevent and treat cancers.

6.3 TARGETED THERAPY

As Cowey has noted:

Basal-cell carcinoma is a commonly occurring skin malignancy that has the potential to progress into locally invasive or resistant disease, as well as spread distantly. Due to advances in the molecular understanding of the disease over the last two decades, it has been discovered that the Hedgehog pathway plays an important role in the pathogenesis of this disease and can be exploited as a treatment target.

Several agents that inhibit the Hedgehog pathway have reached clinical studies and one drug, vismodegib, has recently been US Food and Drug Administration (FDA) approved based on clinical activity and tolerability in patients with advanced basal-cell carcinoma. This review will describe the clinical development of vismodegib, as well as the proper application of the drug in clinical practice.

Other important clinical questions, such as mechanisms of resistance to vismodegib and the role of other Hedgehog pathway inhibitors currently in development will also be discussed….

The identification of the Hedgehog pathway's role in BCC, as well as drugs that are able to target this pathway, has led to a critical proof of-concept translation of these agents into the clinical management of advanced BCC. The first-in-class Smo inhibitor, vismodegib, has given the clinician an important tool in treating patients with this devastating disease. It is critically important that physicians understand when and how to use this novel agent in the management of these patients.

Other agents that work similarly to vismodegib are in development and are expected to expand the clinical options for these patients even further. Research into mechanisms of resistance of

Smo inhibitors, identification of other relevant molecular targets and an understanding of the use of Hedgehog pathway inhibitors in earlier stage disease remains a crucial next step to improving outcomes for patients BCC.

As Hoashi et al note:

6.3.1 FDA Approved Hedgehog Inhibitors

As mentioned above, the main driver of BCC carcinogenesis and progression is the constitutive activation and dysregulation of the hedgehog pathway. The hedgehog pathway is involved in many situations of fetal development, and is also strictly regulated after birth . Hedgehog pathway reactivation, caused by several associated mutations in factors of the hedgehog pathway, induces uncontrolled proliferation of the malignant cells leading to tumor formation. NBCCS patients have germline mutations in PTCH1 and show BCC growth at a very early age. Therefore, BCCs in NBCCS patients are currently mostly treated with hedgehog inhibitors (HHIs).

Moreover, HHIs have determined a paradigmatic shift toward laBCCs or mBCCs. As an HHI, SMO antagonist is promising and the first SMO antagonist is a naturally occurring alkaloid called cyclopamine, found in the corn lily. However, poor oral bioavailability, acid sensitivity and some degrees of specificity constrict the clinical usage of cyclopamine. Recently, several SMO inhibitors, including vismodegib (GDC-0449) and sonidegib (Erismodegib, NVP-LDE-225, LDE-225), have been engineered from cyclopamine and have gained success as targeted clinical cancer therapy

6.3.2 FDA Approved Immune Checkpoint Inhibitors

Immune checkpoints, including programmed death (PD)-1 and cytotoxic T cell lymphocyte associated protein (CTLA)-4 receptors, are expressed on activated T cells. PD-1 binds two ligands, the programmed death-ligand 1 (PD-L1) and programmed death-ligand 2 (PD-L2) on tumor cells.

CTLA-4 binds B7 (CD80/86) on antigen-presenting cells, which inhibits the immune cell activation. This may serve as a mechanism for inhibiting tumor infiltrating T cells, and has become a crucial therapeutic target for restoring immunity. Patients with advanced malignancies received significant benefit from studies of checkpoint inhibition with anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab and pembrolizumab) monoclonal antibodies.

In fact, melanoma, a disease that is more likely to metastasize and become life-threatening as compared to BCC, was the first cancer to demonstrate clinical benefit from cytokines and immune checkpoint blockade. Similar to melanoma, BCCs are generally characterized by UV damage, which translates into a high tumor mutational burden (TMB). Treatment with anti-PD-1 antibodies has shown a dramatic response in high TMB tumors. Neoantigens, the putative targets of immune cells that recognize and eradicate neoplastic cells, is deeply associated with high TMB. PD-L1 expression and TMB have been demonstrated to correlate with response to checkpoint inhibition.

BCCs have higher TMB with 47.3 median mutations/Mb than melanomas, which have TMB with 13.5 median mutations/Mb. BCC was defined as a 'cold tumor', meaning that few immune cells can infiltrate tumor cells in BCC. However, PD-L1 expression by tumor cells in BCCs ranges from 22% to 89.9%, while expression by tumor-infiltrating lymphocytes ranges from 82.0% to 94.9% Moreover, BCCs treated with checkpoint inhibition demonstrated greater PD-L1 expression in tumors (32% vs. 7%) and in TILs (47% vs. 18%), suggesting that treatment may induce PD-L1 expression, and that previously treated BCCs could possibly be more responsive to checkpoint inhibition

7 OBSERVATIONS

The issue of multi genomic damage in skin cells in BCC has received a considerable amount of attention of late. However there are lingering issues that we seek to address. We list them here.

7.1 NON CODING REGIONS

As Woo et al note:

Large-scale sequencing efforts of thousands of tumor samples have been undertaken to understand the mutational landscape of the coding genome. However, the vast majority of germline and somatic variants occur within non-coding portions of the genome. These genomic regions do not directly encode for specific proteins, but can play key roles in cancer progression, for example by driving aberrant gene expression control. Here, we designed an integrative computational and experimental framework to identify recurrently mutated noncoding regulatory regions that drive tumor progression.

Application of this approach to whole-genome sequencing (WGS) data from a large cohort of metastatic castration-resistant prostate cancer (mCRPC) revealed a large set of recurrently mutated regions. We used (i) in silico prioritization of functional non-coding mutations, (ii) massively parallel reporter assays, and (iii) in vivo CRISPR-interference (CRISPRi) screens in xenografted mice to systematically identify and validate driver regulatory regions that drive mCRPC.

We discovered that one of these enhancer regions, GH22I030351, acts on a bidirectional promoter to simultaneously modulate expression of U2-associated splicing factor SF3A1 and chromosomal protein CCDC157. We found that both SF3A1 and CCDC157 are promoters of tumor growth in xenograft models of prostate cancer.

We nominated a number of transcription factors, including SOX6, to be responsible for higher expression of SF3A1 and CCDC157.

Collectively, we have established and confirmed an integrative computational and experimental approach that enables the systematic detection of non-coding regulatory regions that drive the progression of human cancers

7.2 CAUSES

As Crowson notes:

BCCs express p53 protein and do so in a preferential fashion in aggressive growth variants. Mutations of p53 have been documented in up to 40% of studied BCCs; 72% of the mutations bear the signature of UV light induction.

The aggressive growth variants of sporadic BCC are associated with stromal fibroplasia and overexpression of what is likely mutant p53. It is this stromal response to tumor which makes the aggressive growth BCC less amenable to local therapy and control. The interaction between tumor and stroma, as demonstrated in many neoplastic systems, is critical to lesional pathogenesis. Loss of basement membrane material around individual tumor cell nests occurs with progression from indolent to aggressive growth neoplasms.

The working hypothesis is that activation of matrix metalloproteinases in the process of transformation results in the digestion of basal lamina around tumor nests, promoting the elaboration and/or release of proplastic cytokines and their subsequent availability to the rapidly proliferating aggressive growth neoplasms. Perhaps corroborating this hypothesis is the fact that the aggressive growth BCCs have been shown to selectively express syndecan-1 in the adjacent stroma, but not in the tumor cells.

Syndecan-1 is a member of the syndecan glycoprotein family, held to play an important role in inhibiting tumor growth and invasion through its role as a receptor for growth factors in the extracellular matrix. In our hands, the indolent growth variants are found widely distributed on both sun-exposed and sun-protected skin, while the aggressive growth variants such as infiltrative and morpheaform BCC are more frequent in sun-exposed skin. Australian observers have confirmed these findings in a large series using conventional histology.

Men and women are affected in roughly equal proportions with the majority of tumors occurring in the head and neck region. In our experience, male subjects tend to manifest more frequent localization to the left side of the face, which may reflect the fact that they were driving vehicles in North America prior to the advent of sun screens. Roughly one quarter of cases occur on the nose, the most frequently involved site. Less prevalent overall in more darkly pigmented races, the histologic types of BCC seen in Africans and Hispanics have a similar histomorphology

7.3 UV TARGETS

As D'Orazio et al have noted:

One of the greatest risk factors for the development of cutaneous melanoma is having a fair skin complexion, which is characterized by low levels of a UV-blocking dark pigment called eumelanin in the epidermis. Individuals with light skin pigmentation suffer comparatively more skin damage from UV because it is relatively easy for UV rays to penetrate the epidermis to damage both keratinocytes and melanocytes in the deeper layers of the epidermis.

Fair-skinned individuals are exposed to higher ―realized‖ doses of UV radiation in the skin and UV-induced mutations, which directly contribute to melanoma and other forms of skin cancer, accumulate over time. Much UV-induced pathology, including skin cancer, can be avoided by minimizing UV exposure. We and others are increasingly interested in heritable factors that determine melanoma risk to be able to intervene in the carcinogenic process.

One of the most important alleles that influences skin cancer risk is the melanocortin 1 receptor (MC1R), whose function is central to the adaptive pigmentation (tanning) response in the skin.

Besides mediating the tanning response, MC1R exerts a powerful influence on the ability of melanocytes to repair UV-induced DNA damage by the nucleotide excision DNA repair pathway. New insights into the ways in which MC1R and other genes function to protect the skin against the harmful consequences of UV may allow the rational development of pharmacologic strategies to reduce UV sensitivity and cancer risk. …

UV has many effects on skin physiology, with some consequences occurring acutely and others in a delayed manner. One of the most obvious acute effects of UV on the skin is the induction of inflammation. UVB induces a cascade of cytokines, vasoactive and neuroactive mediators in the skin that together result in an inflammatory response and causes ―sunburn‖

If the dose of UV exceeds a threshold damage response, keratinocytes activate apoptotic pathways and die. Such apoptotic keratinocytes can be identified by their pyknotic nuclei and are known as ―sunburn cells. UV also leads to an increase in epidermal thickness, termed hyperkeratosis.

By causing cell injury, UV induces damage response pathways in keratinocytes. Damage signals such as p53 activation profoundly alter keratinocyte physiology, mediating cell cycle arrest, activating DNA repair and inducing apoptosis if the damage is sufficiently great.

Several h after UV exposure, however, and damage response signals abate, epidermal keratinocytes proliferate robustly, mediated by a variety of epidermal growth factors. Increased keratinocyte cell division after UV exposure leads to accumulation of epidermal keratinocytes which increases epidermal thickness. Epidermal hyperplasia protects the skin better against UV penetration

As Narayanan et al note:

The damaging effects of UVR on the skin are thought to be caused by direct cellular damage and alterations in immunologic function. UVR produces DNA damage (formation of cyclobutane pyrimidine dimers), gene mutations, immunosuppression, oxidative stress and inflammatory responses, all of which have an important role in photoaging of the skin and skin cancer.

In addition to this, UVR creates mutations to p53 tumor suppressor genes; these are genes which are involved in DNA repair or the apoptosis of cells that have lots of DNA damage.

Therefore, if p53 genes are mutated, they will no longer be able to aid in the DNA repair process; as a result, there is ‘‘dysregulation of apoptosis, expansion of mutated keratinocytes, and initiation of skin cancer.’’ UVA radiation has an important role in the carcinogenesis of stem cells of the skin.

UVB radiation induces DNA damage, which causes inflammatory responses and Tumorigenesis

7.4 TEMPORAL EFFECTS

BCC is a slow growing malignancy on average. As compared to ovarian cancer, BCC is orders of magnitude slower. The question is: what drives the temporal factors of BCC. In turn, one wonders why metastasis is so rare, the slow growth?

7.5 TME Effects

The tumor microenvironment is known to have significant effects on malignancies. Details are lacking with BCC.

7.6 Monitoring Genomics of Individual Cells

BCC presents a unique malignancy for two reasons. First the high incidence. Second the fact that sample from the skin are generally minimally intrusive. Thus we have a great potential in sequencing the genes. It would be nice to have cell by cell sequences in a large body of BCC. With significant AI like processing one could gain a great deal as regards to the progression of the malignancy.

7.7 Genomic Classification

If we were able to collect massive single cell genomic markers one wonders how this would change classifications. We have seen in hematological cancers this effect dramatically. Here, not so much if at all. Phenotypes and genotypes can be dramatically different. Thus should biopsies be afforded sequencing and if so of what?

7.8 BCC Surface Markers

One wonders of BCC haver targetable cell surface markers.

7.9 Immunotherapeutic Options

Immunotherapy seems to be pervasive. If so how does it apply to BCC?

7.10 Adhesion and Loss of

There is the question of basal cell adhesion. Nodular BCC appears as highly adhesive whereas the more aggressive micronodular and infiltrative are not. It is known that E cadherin facilitates adhesion by N cadherin does not. This is common in melanoma and is the same a factor in BCC? As Loh et al note:

Epithelial-to-Mesenchymal Transition (EMT) has been shown to be crucial in tumorigenesis where the EMT program enhances metastasis, chemoresistance and tumor stemness. Due to its emerging role as a pivotal driver of tumorigenesis, targeting EMT is of great therapeutic interest in counteracting metastasis and chemoresistance in cancer patients. The hallmark of EMT is the upregulation of N-cadherin followed by the downregulation of E-cadherin, and this process is regulated by a complex network of signaling pathways and transcription factors. In this review,

we summarized the recent understanding of the roles of E- and N-cadherins in cancer invasion and metastasis as well as the crosstalk with other signaling pathways involved in EMT.

We also highlighted a few natural compounds with potential anti-EMT property and outlined the future directions in the development of novel intervention in human cancer treatments. We have reviewed 287 published papers related to this topic and identified some of the challenges faced in translating the discovery work from bench to bedside…

Loss of E-cadherin in cancer cells leads to metastatic dissemination and activation of several EMT transcription factors.

Ovarian cancer cell lines with higher E-cadherin expression showed poorer resistance to cell death, lower adhesion to extracellular matrices and weaker invasiveness in comparison to cell lines with higher N-cadherin expression.

E-cadherin is proven to promote nucleotide excision repair by increasing the expression of xeroderma pigmentosum complementation group C (XPC) and DNA damage-binding protein 1 (DDB1) in the event of ultraviolet-induced DNA damage. Uncontrolled growth due to dysfunctional contact inhibition of proliferation is one of the specific features of solid cancers.

Homophilic binding of E-cadherin inhibits cell proliferation by regulating expression levels of receptor tyrosine kinase (RTK) and tyrosine kinase Src in the absence of cell–cell interaction.

E-cadherin-expressing cell line with elevated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity and upregulated c-Myc expression was demonstrated to promote cell proliferation following the increase in adenosine triphosphate (ATP) production by glycolysis and mitochondrial oxidative phosphorylation (OXPHOS). Cancer cells commonly use aerobic glycolysis or mitochondrial OXPHOS, depending on the circumstances, to acquire ATP as energy to sustain their high rates of proliferation…

Fundamentally one may conjecture that HH pathway plays a key role in metastatic proliferation via the cadherin changes. One suspects this is the case for PCa as well thus establishing another target.

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