

# EXTRA CELLULAR MATRIX VS INTRACELLULAR PATHWAYS: WHAT IS THE CONTROLLING FACTOR

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## 1 INTRODUCTION

The focus on intracellular pathways has been a prime direction of research in the development of cancers. However there has from time to time been some focus on the extracellular matrix, the “ECM”, which relates in many ways to the stability of the cell, its localization. Cancer cells lose this sense of localization and begin to move.

The processes at play in the ECM have a significant impact on the processes that occur within a cell. Thus it is essential to have an understanding of the ECM. Recent work by Fisher and his people on MDA-9, a controller of certain ECM elements, demonstrates a control path that influences the internal pathways. We discuss the ECM in the context of the MDA-9 developments.

In this section we use a recent development in understanding the impact of Mda-9 and the nexus with the extra cellular matrix, ECM, and the control of metastatic melanoma.

We first review the Fisher Team efforts as recently presented and then we examine the standard intracellular pathways that have been examined and from that we provide an overview of the extra cellular matrix, ECM, which is the “glue” binding together cells and facilitating cell to cell communications.

We find this an interesting focus or research for several reasons:

1. It examines the ECM which has received limited focus.
2. It focuses on pathways as we have been also doing and specifically an interesting adjunct to the current B-RAF approach.
3. It establishes a clear path forward which is logically and experimentally based and verifiable.

There has been limited prior research on these issues. In Hearing and Leong, 380-386, there is a limited discussion regarding the ECM and melanoma with references. The work by Zent and Pozzi provides a broad and detailed perspective of the ECM with many cancers. However their work is not specific to melanoma. In Weinberg there are references but there does not appear to be any singular focus on the ECM as a standalone system element.

## 2 RECENT EVIDENCE

In a recent paper by Das et al, the authors (from Fisher’s Lab at Virginia Commonwealth) state<sup>1</sup>:

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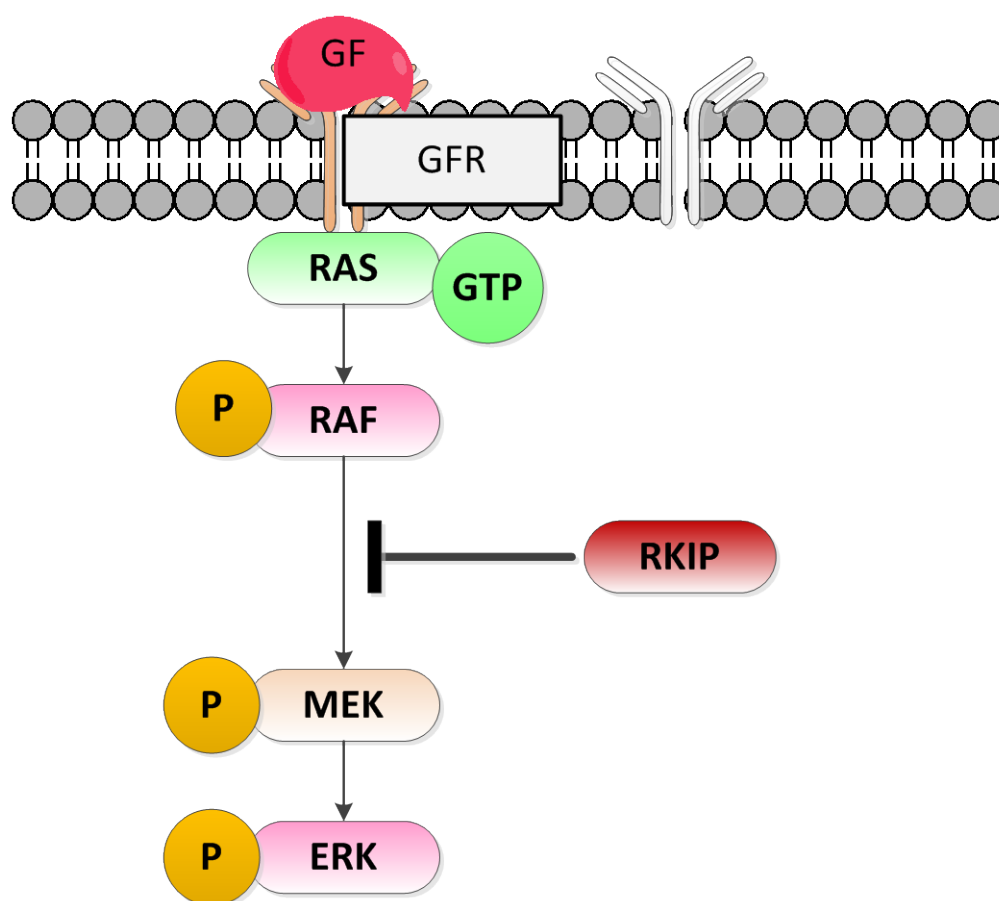
<sup>1</sup> <http://cancerres.aacrjournals.org/search?author1=Swadesh+K+Das&sortspec=date&submit=Submit> ; Therapeutics, Targets, and Chemical Biology Raf Kinase Inhibitor RKIP Inhibits MDA-9/Syntenin-Mediated Metastasis in Melanoma, Das, S., et al, *Cancer Res Published Online First October 11, 2012.*

*Melanoma differentiation associated gene-9 (MDA-9), also known as syntenin, functions as a positive regulator of melanoma progression and metastasis. In contrast, the Raf kinase inhibitor RKIP, a negative modulator of RAF-stimulated MEKK activation, is strongly downregulated in metastatic melanoma cells. In this study, we explored an hypothesized inverse relationship between MDA-9 and RKIP in melanoma. Tumor array and cell line analyses confirmed an inverse relationship between expression of MDA-9 and RKIP during melanoma progression.*

*We found that MDA-9 transcriptionally downregulated RKIP in support of a suggested crosstalk between these two proteins. Further, MDA-9 and RKIP physically interacted in a manner that correlated with a suppression of FAK and c-Src phosphorylation, crucial steps necessary for MDA-9 to promote FAK/c-Src complex formation and initiate signaling cascades that drive the MDA-9-mediated metastatic phenotype.*

*Lastly, ectopic RKIP expression in melanoma cells overrode MDA-9-mediated signaling, inhibiting cell invasion, anchorage-independent growth and in vivo dissemination of tumor cells. Taken together, these findings establish RKIP as an inhibitor of MDA-9-dependent melanoma metastasis, with potential implications for targeting this process therapeutically.*

From the paper by Houben et al we have the RKIP activation as shown below:



As Houben et al state:

*The Ras/Raf/MEK/ERK intracellular signalling cascade is a major determinant in the control of cell growth, differentiation, and survival and can be activated in response to a variety of extracellular stimuli. Stimulation of growth factor receptors results in the activation of the small G-protein Ras, which in turn interacts with the protein kinase Raf leading to its activation. MAP kinase kinase kinase (Raf) phosphorylates and activates MAP kinase kinase (MEK), and MEK phosphorylates and activates extracellular signal-regulated kinase (ERK) 1/2 (p42/p44 MAP kinases).*

*Although Raf and MEK appear largely restricted to only one class of substrates, ERK targets more than 70 substrates including membrane, cytoskeletal, cytoplasmic, nuclear, and even mitochondrial proteins. Recently, a negative regulator of this pathway has been described. The Raf Kinase Inhibitor Protein (RKIP) binds to either Raf or MEK and thereby interferes with the activation of MEK by Raf. The importance of the Ras/Raf/MEK/ERK signalling pathway for carcinogenesis is well established. Indeed, Ras genes (K-ras, H-ras, and N-ras) are the most frequently mutated oncogenes detected in human cancer.*

Houben et al further state about RKIP (12q24.23) as a target the following:

*To assess the relevance of the Ras/Raf/MEK/MAP kinase pathway, we analyzed for activating B-Raf mutations and we elucidated the presence of the Raf Kinase Inhibitor Protein (RKIP) and extracellular signal-regulated kinase (ERK) as well as the phosphorylation status of ERK. All MCC samples were negative for the B-Raf<sup>V600E</sup> mutation. Remarkably, RKIP, which was shown to interfere with the activation of MEK by Raf, was highly expressed in primary as well as in metastatic MCC. ... Western blot analysis of three MCC-derived cell lines revealed in one case the pattern present in situ (i.e. high RKIP expression and complete absence of phosphorylated ERK).*

Thus the Fisher team seems to seek out a RKIP inhibitor to slow the pathway. This is in addition to the B-RAF inhibitors which are currently in clinical use.

Now in an industry piece on the same article the author Ho states<sup>2</sup>:

*.... the scientist believes that they have the ability to eliminate melanoma differentiation associated gene-9 (mda-9)/syntenin, a specific protein. In the experiment, the researchers discovered that Raf kinase inhibitor protein (RKIP) was able to interact and suppress with mda-9/syntenin. The protein was originally cloned in a laboratory and past studies showed how it interacted with c-Src, another protein, to produce a set of chemical reactions that later boosted metastasis.*

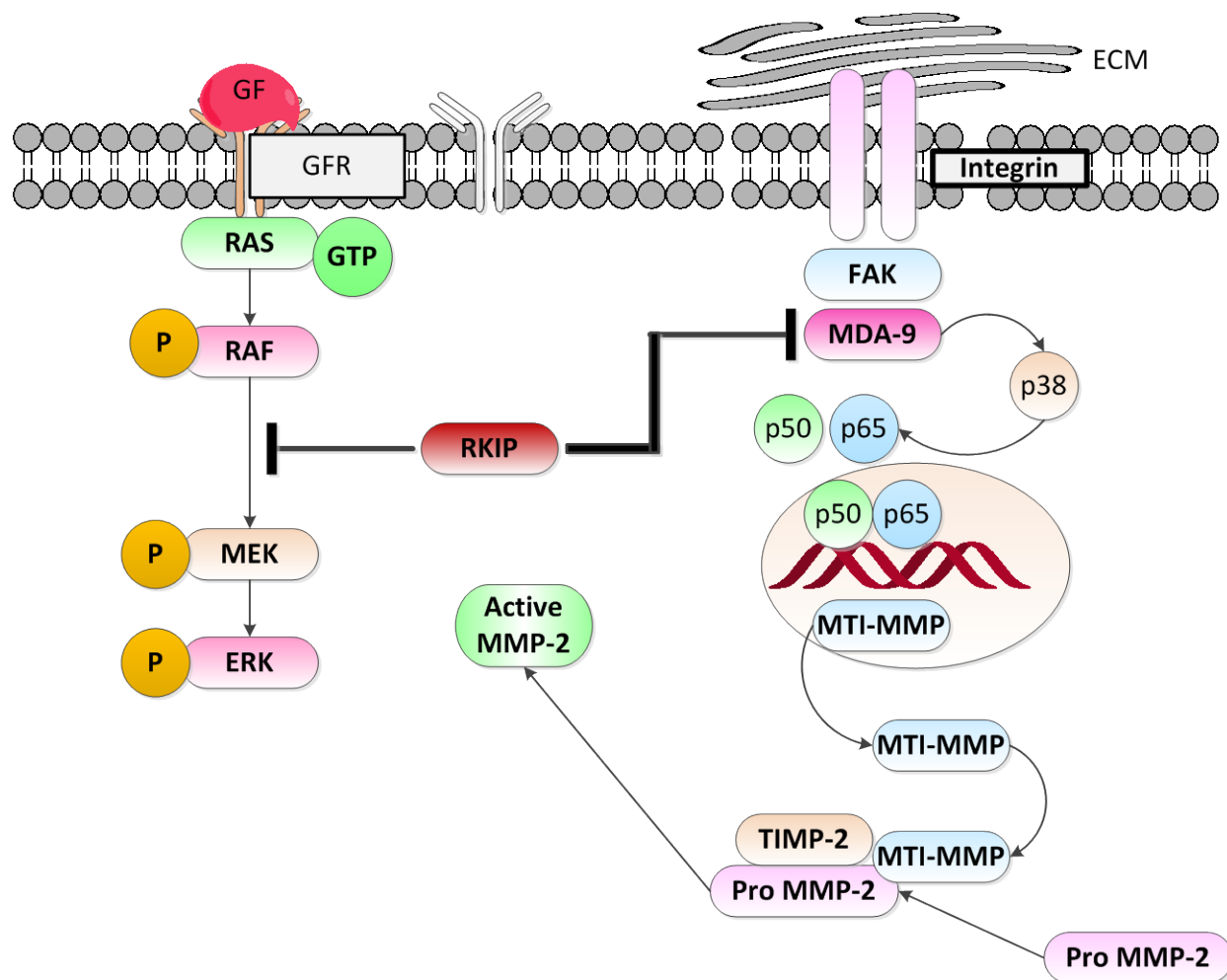
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<sup>2</sup> <http://www.redorbit.com/news/health/1112732493/stopping-the-spread-of-melanoma-by-removing-protein-affecting-metastasis/>; Ho, C., Stopping The Spread Of Melanoma By Removing Protein Affecting Metastasis, RedOrbit, November 15, 2012

*“Prior research suggests that RKIP plays a seminal role in inhibiting cancer metastasis, but, until now, the mechanisms underlying this activity were not clear,” explained Paul Fisher, the program co-leader of Cancer Molecular Genetics at Virginia Commonwealth University Massey Cancer Center, in a prepared statement. “In addition to providing a new target for future therapies, there is potential for using these two genes as biomarkers for monitoring melanoma development and progression.”*

*The team of investigators discovered that RKIP become attached to mda-9/syntenin, which resulted in limiting the expression of mda-9/syntenin. With the finding of this physical interaction, the scientists believe that they could possibly create small molecules that are similar to RKIP and the molecules could be used as drugs to treated metastasis in cancers like melanoma.*

We depict this pathway below:



The article continues:



*There was also a difference in terms of the level of mda-9/syntenin and RKIP. While malignant and metastasis melanoma cells had higher levels of mda-9/syntenin compared to RKIP, the healthy melanocyte cells that create pigment in eyes, hair, and skin had higher levels of RKIP than mda-9/syntenin. The researchers believe that different levels in the proteins could be used in diagnosis, particularly in following the progression of a disease or tracking a patient's response to a particular treatment.*

*“Our findings represent a major breakthrough in understanding the genetic mechanisms that lead to metastasis in melanoma. Prior studies have shown that levels of mda-9/syntenin are elevated in a majority of cancers, including melanoma, suggesting that our findings could be applicable for a wide range of diseases,” continued Fisher, who also serves as chairman of VCU's Department of Human and Molecular Genetics and director of the VCU Institutes of Molecular Medicine, in the statement.*

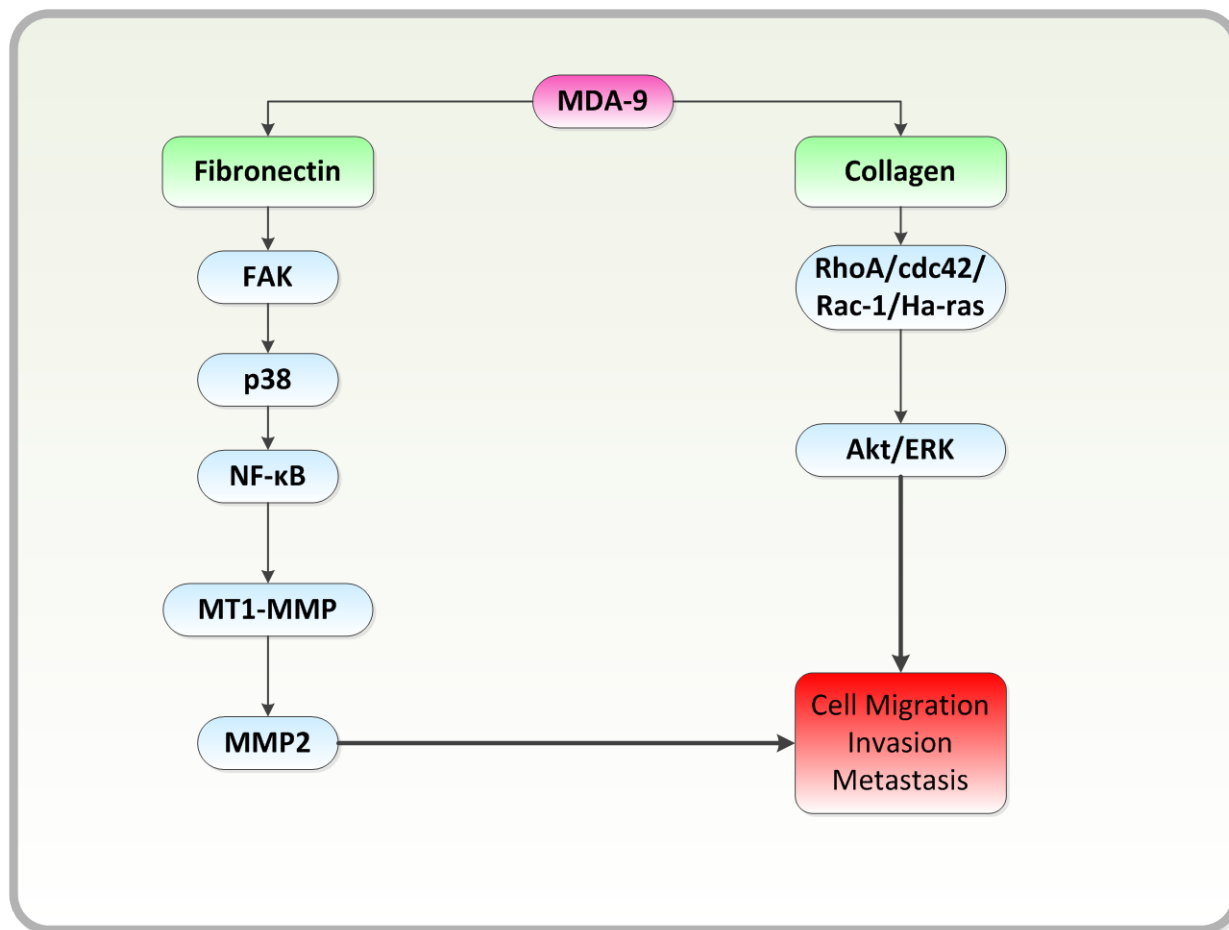
*Moving forward, the scientists plan to determine how they can develop small molecules that mimic RKIP. These molecules could potentially be utilized in new treatments for melanoma.*

This is a fundamental result. It demonstrates another pathway element and at the same time connects the intracellular pathways with the extra cellular matrix and their pathways. Potentially this is diagnostic, prognostic and a treatment as well.

### **3 STANDARD INTRA-CELLULAR PATHWAYS**

The following Figure is a repetition of the standard intra-cellular pathways. We have discussed these at length.





We shall be examining these in some detail. Let us first characterize some of the above identified elements controlled by the extracellular matrix path. The others we have examined in detail elsewhere.

### 3.1 FAK

FAK is also known as; PTK2, FADK; FAK1; FRNK; PPP1R71; p125FAK; pp125FAK. It is located at 8q24.3. It is a kinase.

NCBI states its function as follows:

*This gene encodes a cytoplasmic protein tyrosine kinase which is found concentrated in the focal adhesions that form between cells growing in the presence of extracellular matrix constituents. The encoded protein is a member of the FAK subfamily of protein tyrosine kinases but lacks significant sequence similarity to kinases from other subfamilies. Activation of this gene may be an important early step in cell growth and intracellular signal transduction pathways triggered in response to certain neural peptides or to cell interactions with the extracellular matrix. Several transcript variants encoding different isoforms have been found for this gene, but the full-length nature of only three of them have been determined.*

### 3.2 SRC

SRC is located at 20q12-q13. As noted in NCBI<sup>3</sup>:

*This gene is highly similar to the v-src gene of Rous sarcoma virus. This proto-oncogene may play a role in the regulation of embryonic development and cell growth. The protein encoded by this gene is a tyrosine-protein kinase whose activity can be inhibited by phosphorylation by c-SRC kinase. Mutations in this gene could be involved in the malignant progression of colon cancer. Two transcript variants encoding the same protein have been found for this gene.*

### 3.3 p38

The p38 gene has multiple names. It is MAPK14, RK; CSBP; EXIP; Mxi2; CSBP1; CSBP2; CSPB1; PRKM14; PRKM15; SAPK2A; p38ALPHA. It is located at 6p21.3-p21.2.

Its function described by NCBI is as follows<sup>4</sup>:

*The protein encoded by this gene is a member of the MAP kinase family. MAP kinases act as an integration point for multiple biochemical signals, and are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development.*

*This kinase is activated by various environmental stresses and proinflammatory cytokines.*

*The activation requires its phosphorylation by MAP kinase kinases (MKKs), or its autophosphorylation triggered by the interaction of MAP3K7IP1/TAB1 protein with this kinase. The substrates of this kinase include transcription regulator ATF2, MEF2C, and MAX, cell cycle regulator CDC25B, and tumor suppressor p53, which suggest the roles of this kinase in stress related transcription and cell cycle regulation, as well as in genotoxic stress response.*

*Four alternatively spliced transcript variants of this gene encoding distinct isoforms have been reported.*

### 3.4 NF-κB

We have discussed this before. We reiterate what that discussion contains.

NF-κB is a transcription factor that resides in the cytoplasm. It is called Nuclear Factor and was identified by David Baltimore as an enhancer factor for the κ chain of Ig light chain in B lymphocytes. When activated it moves to the nucleus and is a transcription factor in activating over 400 genes. It is activated by a large number of stimuli and its action of a large gene set causes significant DNA activity. NF-κB appears on 10q24 and is somatic and acts in a dominant manner.

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<sup>3</sup> <http://www.ncbi.nlm.nih.gov/gene/6714>

<sup>4</sup> <http://www.ncbi.nlm.nih.gov/gene/1432>

In a recent paper by Zhang et al they state:

*The majority of tumors progressing during androgen deprivation therapy (referred to here as androgen deprivation- resistant prostate cancer or ADRPC) express higher levels of AR transcript and protein suggesting that a marked increase in AR expression is a critical event in therapy resistance...*

*Recent studies also demonstrate that increased AR expression is both necessary and sufficient to convert prostate cancer growth from a hormone therapy-sensitive to a resistant state in xenograft models... Since AR mRNA levels are often increased in ADRPC without gene amplification , ...*

*it is likely mediated by transcription factors and transcription regulating signal transduction pathways that are altered during progression.*

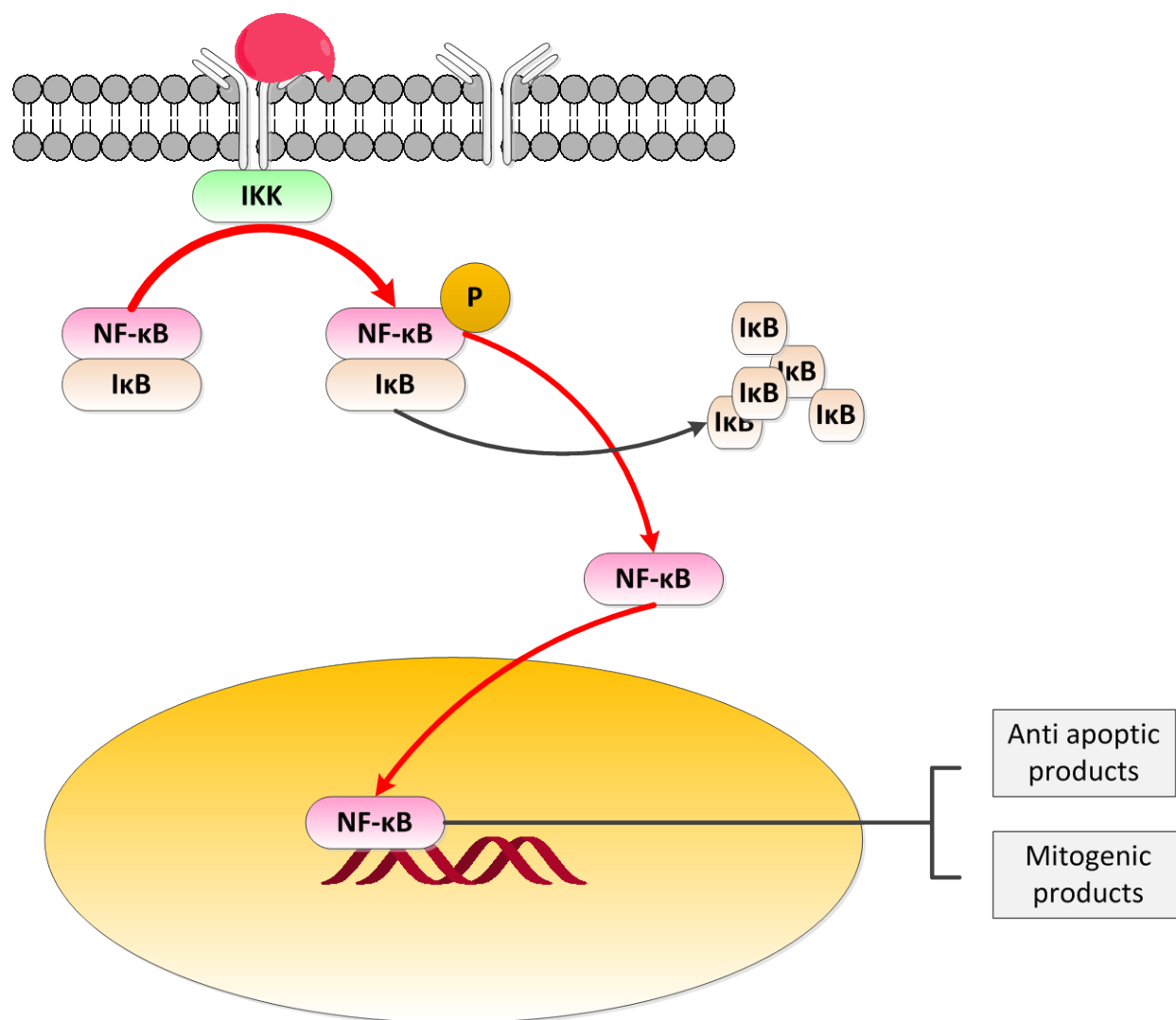
*Nuclear Factor (NF)- $\kappa$ B is a family of transcription factors composed of homo- and hetero-dimers initially identified as an enhancer binding protein for the immunoglobulin light chain in B lymphocytes...*

Zhang continues:

*Several studies have examined the expression of NF- $\kappa$ B in human prostate cancer and its relationship to clinical features of the disease. NF- $\kappa$ B/p65 is overexpressed in prostatic intraepithelial neoplasia and cancer compared with benign epithelium. Nuclear levels of NF- $\kappa$ B/p65 correlate with NF- $\kappa$ B-dependent expression of BclII, cyclin D1, matrix metalloproteinase-9, and vascular endothelial growth factor.*

*Recent work indicates that NF- $\kappa$ B/p65 expression is predictive of biochemical recurrence in patients with positive surgical margins after radical prostatectomy and nuclear localization of NF- $\kappa$ B is increased in prostate cancer lymph node metastasis and can be used to predict patient outcome. These results demonstrate that NF- $\kappa$ B/p65 is frequently activated in human prostate adenocarcinoma and expression may be related to progression.*

We now depict this putative pathway based upon the work of Kwang and Aggarwal. This is shown below. Activated NF- $\kappa$ B is clearly an activator of an anti-apoptosis process in the nucleus. The paper by Huang et al shows that blockade of NF- $\kappa$ B is an effective suppressor of angiogenesis, invasion and metastasis of prostate cancer.



NF-κB is another transcription protein seen in melanoma. This protein is characterized by:

- NF-κB is a transcription factor that resides in the cytoplasm.
- It is called Nuclear Factor and was identified by David Baltimore as an enhancer factor for the  $\kappa$  chain of Ig light chain in B lymphocytes
- When activated it moves to the nucleus and is a transcription factor in activating over 400 genes
- It is activated by a large number of stimuli and its action on a large gene set causes significant DNA activity
- NF-κB appears on 10q24 and is somatic and acts in a dominant manner.

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls the transcription of DNA. NF-κB is widely used by eukaryotic cells as a regulator of genes that control cell proliferation and cell survival. As such, many different types of human tumors have mis-regulated NF-κB: that is, NF-κB is constitutively active. Active NF-κB turns on

the expression of genes that keep the cell proliferating and protect the cell from conditions that would otherwise cause it to die via apoptosis.

As Amiri and Richmond state:

*Nuclear Factor-kappa B (NF-κB) is an inducible transcription factor that regulates the expression of many genes involved in the immune response. Recently, NF-κB activity has been shown to be upregulated in many cancers, including melanoma. Data indicate that the enhanced activation of NF-κB may be due to deregulations in upstream signaling pathways such as Ras/Raf, PI3K/Akt, and NIK. Multiple studies have shown that NF-κB is involved in the regulation of apoptosis, angiogenesis, and tumor cell invasion, all of which indicate the important role of NF-κB in tumorigenesis. Thus, understanding the molecular mechanism of melanoma progression will aid in designing new therapeutic approaches for melanoma.*

They continue:

*Constitutive activation of NF-κB is an emerging hallmark of various types of tumors including breast, colon, pancreatic, ovarian, and melanoma [9–14]. In the healthy human, NF-κB regulates the expression of genes involved in normal immunologic reactions (e.g. generation of immunoregulatory molecules such as antibody light chains) in response to proinflammatory cytokines and by-products of microbial and viral infections [15–17]. NF-κB also modulates the expression of factors responsible for growth as well as apoptosis. However, increased activation of NF-κB results in enhanced expression of proinflammatory mediators, leading to acute inflammatory injury to lungs and other organs, and development of multiple organ dysfunctions as well as cancer.*

They then summarize NF-κB's role in melanoma as:

**3.1. Apoptosis resistance and cell proliferation:** *In processes such as tumor initiation and promotion where prolonged survival of cells is a crucial event, NF-κB plays an important role as a mediator of inhibition of apoptosis. In melanoma, NF-κB has been shown to activate expression of anti-apoptotic proteins such as tumor necrosis factor receptor-associated factor 1 (TRAF1), TRAF2, and the inhibitor-of apoptosis (IAP) proteins c-IAP1, c-IAP2, and melanoma inhibitor of apoptosis (ML-IAP), survivin as well as Bcl-2 like proteins...*

**3.2. Invasion and metastasis:** *In invasion and metastasis of melanoma, NF-κB may regulate the production of prostaglandins via cyclooxygenase-2 (COX-2), which has been shown to be overexpressed in melanoma [44,45]. It was shown that COX-2 is expressed in the majority of primary malignant melanoma, as well as in five human malignant melanoma cell lines....*

However as Liu et al (2006) state:

*Malignant melanoma is the most lethal skin cancer, whose ability to rapidly metastasize often prevents surgical cure.*

*Furthermore, the systemic treatment of melanoma is largely ineffective due to the intrinsic resistance of melanoma cells to numerous anticancer agents. Increased survival of melanoma cells is primarily attributed to the constitutive activation of the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B), which regulates the expression of many anti-apoptotic, pro-proliferative and pro-metastatic genes.*

*Canonical activation of the NF- $\kappa$ B pathway occurs when NF- $\kappa$ B switches its localization from the cytoplasm, where it is maintained inactive by assembly with the inhibitor I $\kappa$ B protein, to the nucleus, where NF- $\kappa$ B regulates gene expression. NF- $\kappa$ B activation relies upon the phosphorylation dependent ubiquitination and degradation of I $\kappa$ B mediated by the I $\kappa$ B kinase (IKK) complex and  $\beta$ -Trcp E3 ubiquitin ligases.*

*Consequently, both IKK activity and the levels of  $\beta$ -Trcp regulate the extent of I $\kappa$ B degradation and hence NF- $\kappa$ B activation. The genetic basis that underlies the elevated NF- $\kappa$ B activity in malignant melanoma largely remains elusive.*

*Constitutively active IKK has been demonstrated to sustain NF- $\kappa$ B activation in human melanoma cells, resulting in induction of the chemokine CXCL1. CXCL1, in turn, is capable of activating IKK and NF- $\kappa$ B and promoting cell survival and tumorigenesis .... However, the original genetic alterations that initiate this feed-forward mechanism in melanoma remain unclear.*

*One of the major oncogenic events described in the genesis of malignant melanoma is constitutive activation of the Ras-regulated RAF-MEK-ERK mitogen-activated protein kinase (MAPK) pathway. This is achieved most frequently by activating mutations in either BRAF (e.g. V600E substitution) or, less frequently, in N-RAS ... Recent evidence indicates that oncogenic BRAF activity is essential for human melanoma cell growth and survival ...*

*However, despite prior reports that RAF can activate NF- $\kappa$ B ..., the mechanism(s) by which BRAF<sub>V600E</sub> (BRAF<sub>VE</sub>) may elicit NF- $\kappa$ B signaling in melanoma cells have not yet been elucidated. Activation of the canonical NF- $\kappa$ B pathway depends on both IKK activity, which has been shown to be elevated in human melanomas....*

Liu et al conclusion is speculative but telling:

*Taken together, these data support a model in which mutational activation of BRAF in human melanomas contributes to constitutive induction of NF- $\kappa$ B activity and to increased survival of melanoma cells.*

Again we have the issue of speculation as to where and why the mutations occur. Here they speculate about the BRAF mutation resulting in the antiapoptotic control with NF- $\kappa$ B.

### 3.5 MMP-9

As NCBI states<sup>5</sup>:

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<sup>5</sup> <http://www.ncbi.nlm.nih.gov/gene/4318>



*Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases.*

*The enzyme encoded by this gene degrades type IV and V collagens. Studies in rhesus monkeys suggest that the enzyme is involved in IL-8-induced mobilization of hematopoietic progenitor cells from bone marrow, and murine studies suggest a role in tumor-associated tissue remodeling*

We shall discuss MMP in detail when we summarize the ECM.

### **3.6 CDC42**

As NCBI states<sup>6</sup>:

*The protein encoded by this gene is a small GTPase of the Rho-subfamily, which regulates signaling pathways that control diverse cellular functions including cell morphology, migration, endocytosis and cell cycle progression. This protein is highly similar to Saccharomyces cerevisiae Cdc 42, and is able to complement the yeast cdc42-1 mutant.*

*The product of oncogene Dbl was reported to specifically catalyze the dissociation of GDP from this protein. This protein could regulate actin polymerization through its direct binding to Neural Wiskott-Aldrich syndrome protein (N-WASP), which subsequently activates Arp2/3 complex. Alternative splicing of this gene results in multiple transcript variants.*

## **4 THE EXTRACELLULAR MATRIX**

The ECM has often been neglected when discussing cancer pathways. Weinberg has multiple references but does not seem to place it in any specific spotlight. In Lewin, Cell, the discussion is quite well focused but yet there is but passing reference to the impact on cancer pathways. Specifically in Lewin on p 850 there is reference to MMP-9, here a metalloproteinase, and melanoma<sup>7</sup>.

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<sup>6</sup> <http://www.ncbi.nlm.nih.gov/gene/998>

<sup>7</sup> As NCBI states: “Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. The enzyme encoded by this gene degrades type IV and V collagens. Studies in rhesus monkeys suggest that the enzyme is involved in IL-8-induced mobilization of hematopoietic progenitor cells from bone marrow, and murine studies suggest a role in tumor-associated tissue remodeling.” see <http://www.ncbi.nlm.nih.gov/gene/4318>

The ECM is the collection of molecules that lie between the cell walls. The ECM provides for structural integrity as well as facilitates and even participates in cell to cell communications. The ECM is a highly complex and quite active element in the ongoing life of the cells. In addition we all too often look to what happens in a cell, with at best a nod to ligands, and we do not look at the cell internals as well as the ECM as a holistic system totality. The work of the Fisher Team in a small way may help refocus this effort on the complex as a working whole.

We will follow Lewin and deal with the principal participants in the ECM. There are a wealth of books which focus on this area.

#### **4.1 COLLAGEN**

Collagens provide structure support. They are triple helical proteins wrapped to provide that supporting structure between the cells. There are many types of collagen and the actual assembly commences within the cell and the semi-finished product passes through the cell wall to the ECM. For our purposes the collagen complexes are at this time of limited interest.

#### **4.2 FIBRONECTIN**

Fibronectin facilitates the process of connecting cells to matrices of collagen. Fibronectin proteins have a six element structure. Cells bind to fibronectin via receptors called integrins. The fibronectin binding thus activates pathways within the cell, thereby establishing an intra and intercellular pathway complex. The pathways activated control growth, movement and cell differentiation.

We can now examine some of the relevant literature on fibronectin and melanomas. As Yi and Ruoslahti state:

*Fibronectin is a prototypic extracellular matrix (ECM) protein that is deposited by various types of cells into an adhesive fibrillar meshwork of protein (1). Fibronectin, and ECM in general, control many cellular functions, including growth, migration, differentiation, and survival. The signals that control these behaviors are transmitted from the ECM to the cell by integrins, a family of transmembrane receptors (2, 3). Malignant cells often bypass the ECM–integrin signaling system; they are not bound by the spatial constraints imposed by the ECM on normal cells, and they no longer require ECM contact for survival*

Liu et al state:

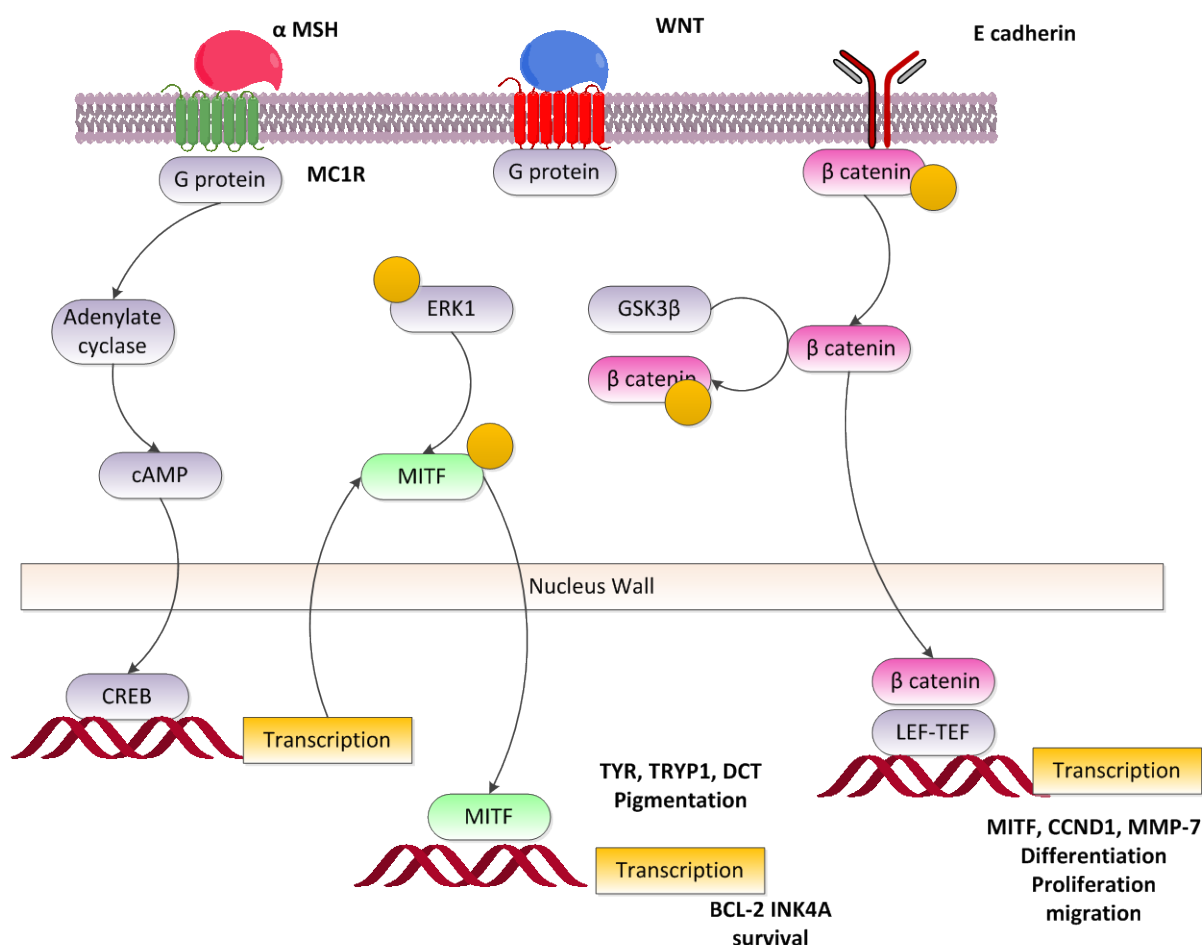
*Tumor cells frequently exhibit decreased adhesiveness due to failure to deposit stromal fibronectin (FN), permitting more rapid proliferation, migration, invasion, and metastasis. Although up-regulation of FN has been noted in gene profiles of carcinomas compared with normal tissue, reduced FN expression has been described at the peripheral margins of invading tumors. In this study, we investigate the role of FN in cancer behavior. ... Neoplastic transformation is often characterized by changes in the organization of the cytoskeleton, decreased cell adhesion, and aberrant adhesion–mediated signaling (2). Disruption of normal cell adhesion contributes to enhanced proliferation, migration, and invasion leading to*

*metastasis. Fibronectin (FN) is an extracellular matrix protein with putative roles in mediating these actions. Indeed, tumor cells with decreased adhesiveness frequently fail to deposit stromal FN (3). In particular, reduced FN expression has been noted in transformed cell lines and primary tumors (4), including thyroid cancer (3, 5, 6), where diminished FN has been identified at the periphery of invasive tumor margins. In this context, we found that down-regulation of FN stimulates thyroid cancer cell proliferation and tumor growth (7). Conversely, 1, 25-dihydroxy vitamin D3 treatment increases cell adhesiveness and inhibits cell proliferation and tumor growth through enhanced FN expression.*

We will come back to fibronectin in our later analysis.

### 4.3 E-CADHERIN

We have discussed E-cadherin at length in previous work. It plays a critical role in stabilizing cell adhesion and localization. Loss of E-cadherin results in loss of cell localization and thus cell movement. Specifically in melanocytes the cells begin to leave the basal layer and migrate upward as in melanoma in situ and downward as in superficial spreading melanoma.



As Swiatoniowski et al state:

*Integrins are molecules which play a significant role in cell-extracellular matrix (ECM) interactions. They interact with the RGD tripeptide of fibronectin (FN), one of the main components of ECM. Labile expression of FN has been proven to play an important role both in the normal developmental process (morphogenetic movements) and in the course of carcinogenesis ... Many authors have implicated loss or decrease of EC expression as an independent negative prognostic marker in breast cancer patients (6-9). There is increasing experimental evidence for a relationship between the EC level and different features of breast cancer, including histological grade (7, 16) and axillary lymph node involvement (13-16)... In conclusion, our experiment revealed no prognostic value for EC or FN expressions in a homogenous group of patients*

#### **4.4 PROTEOGLYCAN**

Proteoglycans are single polypeptide with multiple sugars attached. They provide for hydration in the ECM.

#### **4.5 PROTEASE**

The proteases are ECM proteins which function to degrade the refuse in the ECM. The metalloproteinases are a family of proteases. They are also called MMP. MMP-9 and MMP-2 are ones of the MMPs often associated with melanoma.

There has been extensive work examining the MMPs and melanoma some dating back to the 1990s, see that of Luca et al. A recent result by Hoffman et al state:

*Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are involved in tumour progression and metastasis. In this study, we investigated the in vitro and in vivo expression patterns of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 mRNA and protein in a previously described human melanoma xenograft model. This model consists of eight human melanoma cell lines with different metastatic behaviour after subcutaneous (s.c.) injection into nude mice. MMP-1 mRNA was detectable in all cell lines by reverse transcription polymerase chain reaction (RT-PCR), but the expression was too low to be detected by Northern blot analysis. No MMP-1 protein could be found using Western blotting. MMP-2 mRNA and protein were present in all cell lines, with the highest expression of both latent and active MMP-2 in the highest metastatic cell lines MV3 and BLM. MMP-3 mRNA was expressed in MV3 and BLM, and in the non-metastatic cell line 530, whereas MMP-3 protein was detectable only in MV3 and BLM.*

*None of the melanoma cell lines expressed MMP-9. TIMP-1 and TIMP-2 mRNA and protein, finally, were present in all cell lines. A correlation between TIMP expression level and metastatic capacity of cell lines, however, was lacking. MMP and TIMP mRNA and protein expression levels were also studied in s.c. xenograft lesions derived from a selection of these cell lines. RT-PCR analysis revealed that MMP-1 mRNA was present in MV3 and BLM xenografts, and to a lesser extent in 530. Positive staining for MMP-1 protein was found in xenograft lesions derived from both low and high metastatic cell lines, indicating an in vivo up-regulation of*

*MMP-1. MMP-2 mRNA was detectable only in xenografts derived from the highly metastatic cell lines 1F6m, MV3 and BLM. In agreement with the in vitro results, the highest levels of both latent and activated MMP-2 protein were observed in MV3 and BLM xenografts.*

*With the exception of MMP-9 mRNA expression in 530 xenografts, MMP-3, MMP-9, and TIMP-1 mRNA and protein were not detectable in any xenograft, indicating a down-regulated expression of MMP-3 and TIMP-1 in vivo. TIMP-2 mRNA and protein were present in all xenografts; interestingly, the strongest immunoreactivity of tumour cells was found at the border of necrotic areas. Our study demonstrates that of all tested components of the matrix metalloproteinase system, only expression of activated MMP-2 correlates with increased malignancy in our melanoma xenograft model, corroborating an important role of MMP-2 in human melanoma invasion and metastasis.*

We shall see the impact of MMPs as we examine the pathways.

#### **4.6 INTEGRINS**

Integrins are for the most part the receptors for ECM proteins. They are one of many such cell surface receptors. The integrins play important roles in cell homeostasis and cell to cell communications.

#### **4.7 MDA-9**

Let us briefly examine the gene MDA-9 and its protein Mda-9 and what is known and how it has evolved. Now MDA-9 is located on (8q12). As the NIH data base states:

*The protein encoded by this gene was initially identified as a molecule linking syndecan-mediated signaling to the cytoskeleton. The syntenin protein contains tandemly repeated PDZ domains that bind the cytoplasmic, C-terminal domains of a variety of transmembrane proteins. This protein may also affect cytoskeletal-membrane organization, cell adhesion, protein trafficking, and the activation of transcription factors.*

*The protein is primarily localized to membrane-associated adherens junctions and focal adhesions but is also found at the endoplasmic reticulum and nucleus. Alternative splicing results in multiple transcript variants encoding different isoforms<sup>8</sup>.*

In the paper, Src kinase activation is mandatory for MDA-9/syntenin-mediated activation of nuclear factor-κB, by H Boukerche, H Aissaoui, C Prévost, H Hirbec, S K Das, Z-Z Su, D Sarkar and P B Fisher, the author's state:

*The scaffolding postsynaptic density-95/disk large/zonula occludens-1 (PDZ) domain-containing protein melanoma differentiation associated gene-9 (MDA-9)/syntenin is a tandem PDZ protein overexpressed in human melanoma, and breast and gastric cancer cells. MDA-*

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<sup>8</sup> <http://www.ncbi.nlm.nih.gov/gene/6386>

*9/syntenin affects cancer cell motility and invasion through distinct biochemical and signaling pathways, including focal adhesion kinase and p38 mitogen-activated protein kinase (MAPK), resulting in activation of the nuclear factor (NF)-κB pathway.*

*MDA-9/syntenin also promotes melanoma metastasis by activating c-Src, but how c-Src regulates NF-κB activation is unclear. Using a human melanoma model, we document that MDA-9/syntenin–c-Src interactions are positive regulators of NF-κB activation. Inhibition of c-Src by PP2 treatment, by blocking c-Src or mda-9/syntenin expression with small interfering RNA, or in c-Src (–/–) knockout cell lines, reduces NF-κB activation following overexpression of mda-9/syntenin or c-Src.*

*Deletion or point mutations of the PDZ binding motif preventing MDA-9/syntenin association with c-Src reveals that both PDZ domains, with PDZ2 being the dominant module, are required for activating downstream signaling pathways, including p38 MAPK and NF-κB. We also document that MDA-9/syntenin–c-Src complexes functionally cooperate with NF-κB to promote anchorage-independent growth, motility and invasion of melanoma cells. These findings underscore PDZ domains of MDA-9/syntenin as promising potential therapeutic targets for intervening in a decisive component of cancer progression, namely, metastatic tumor spread<sup>9</sup>....*

*(MDA-9 Acts as a PDZ domain-containing adapter protein. In adherens junctions, it couples syndecans to cytoskeletal proteins or signaling components. Seems to be required for the targeting of TGF-α to the cell surface in the secretory pathway. By virtue of its association with a large number of additional proteins, including class B ephrins, TGF-α, phosphotyrosine phosphatase, neuroligin, neuroligin, schwannomin/merlin, IL-5 receptor, various glutamate receptor subtypes, and the syndecan family of heparan sulfate proteoglycans, MDA9 has been implicated in diverse processes, including protein trafficking, activation of the transcription factor SOX4, cytoskeleton-membrane organization, and cell adhesion/migration....*

*(MDA-9) Its expression is induced by IFN-γ in melanoma cells. Is believed to be involved in cancer metastasis. In melanoma, it promotes the metastatic phenotype by activating NFκB and focal adhesion kinase (FAK), which promotes induction of matrix metalloproteinase (MMP) and then migration and extracellular matrix invasion of melanoma cells. Syntenin is overexpressed and promotes cell migration in metastatic human breast and gastric cancer cell lines.*

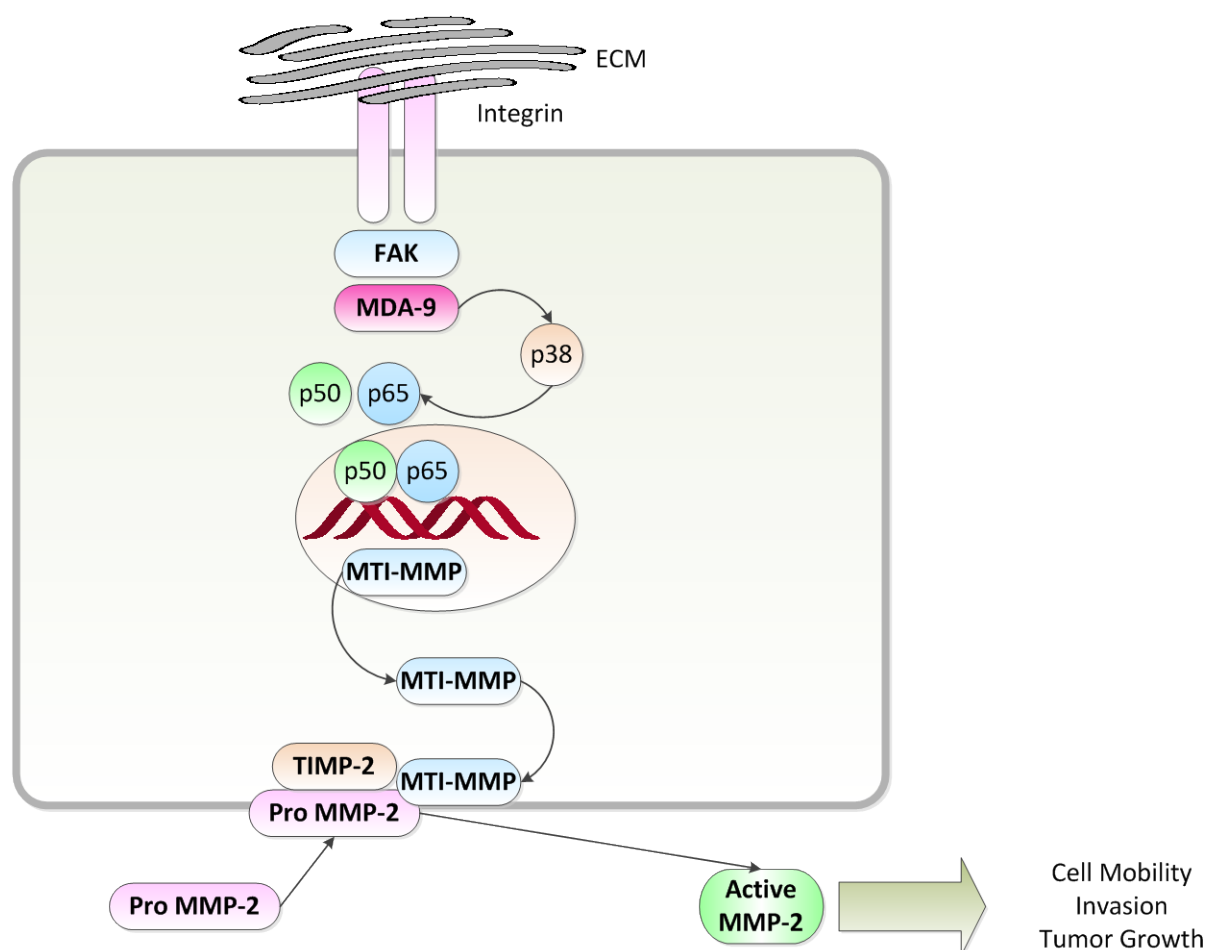
The gene product is also called by many other names, specifically:

1. MDA9
2. MDA-9
3. TGF α cytoplasmic domain interacting protein18
4. TACIP18
5. SYCL
6. Syntenin-1
7. Syndecan binding protein 1
8. SDCBP

<sup>9</sup> <http://www.nature.com/onc/journal/v29/n21/pdf/onc201065a.pdf>

## 9. Melanoma differentiation associated protein 9

From Das et al. we have the following modified figure<sup>10</sup>:

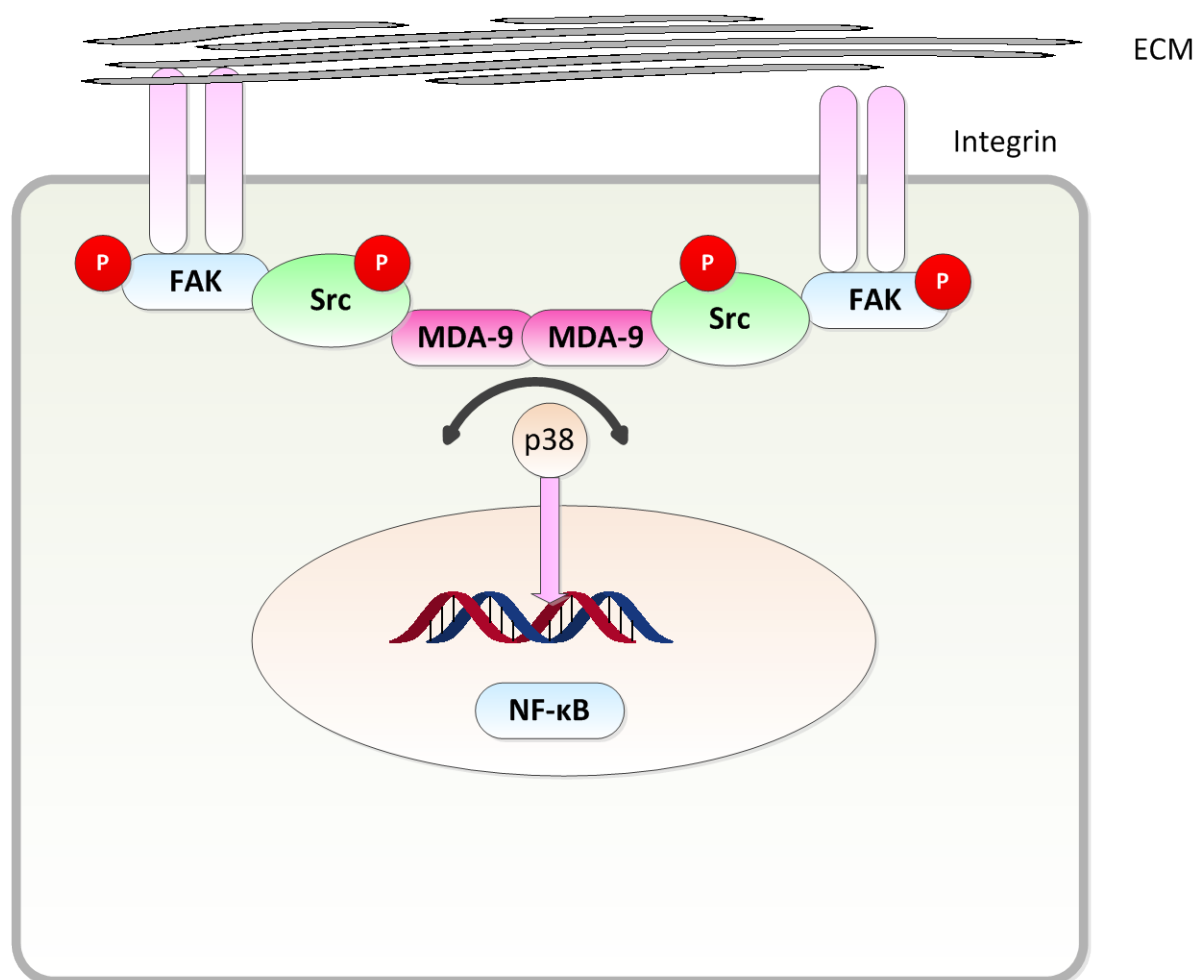


Das et al state regarding the above pathway model:

*Schematic diagram for mda-9/syntenin mediated NFκB activation. Upon interaction with ECM (fibronectin), MDA-9/syntenin activates the p38/MAPK by augmenting FAK phosphorylation. This results in degradation of IκBα and movement of p65 from the cytoplasm where interaction with p50 results in binding to target genes (MT1-MMP) resulting in enhanced production of MT1-MMP, which interacts with TIMP-2 activating pro-MMP-2 to produce active MMP-2. This product then enhances cell motility, invasion, and cancer cell growth. mda-9/Syntenin activates the NF-κB pathway.*

The original Figure appears to be from Boukerche et al as shown with some mods below:

<sup>10</sup> <http://www.bioscience.org/2012/v17/af/3911/fulltext.asp?bframe=figures.htm&doi=yes>



Note the differences. First the original shows multiple integrins and multiple FAK binding and in turn a binding of MDA-9 initiating the p38 pathway. Also note the explicit presence of NF-κB and its result of genes forcing mobility, invasion and metastasis. The authors state:

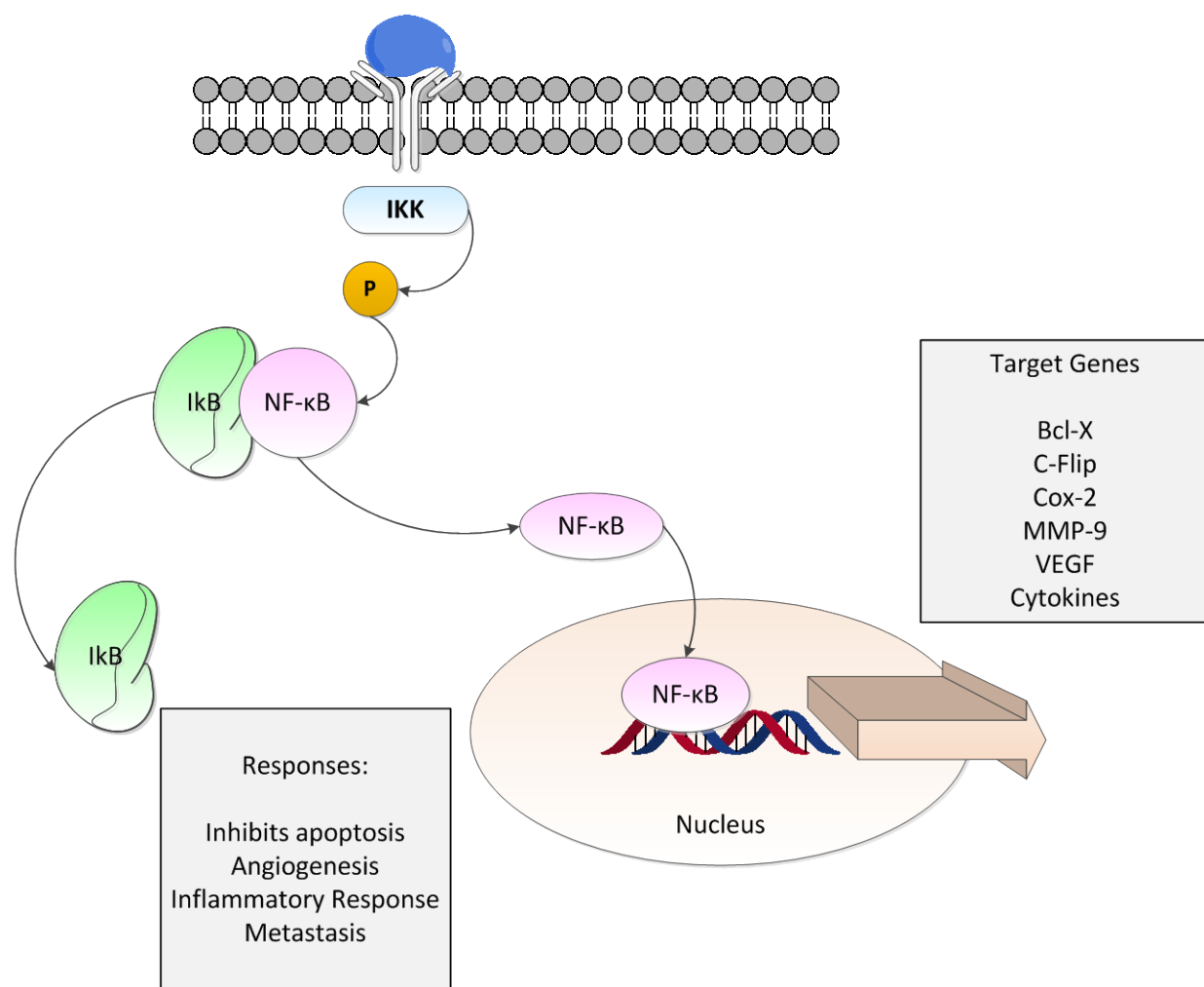
*Hypothetical model of signal transduction pathways coordinately regulated by MDA-9/syntenin through its interaction with c-Src. MDA-9/ syntenin interaction with c-Src results in clustering of c-Src/FAK signaling complexes at high concentrations on the plasma membrane. The activated c-Src/FAK complexes activate the p38 MAPK/NF-κB pathways that regulate expression of genes involved in migration and invasion and thus play a crucial role in MDA-9/syntenin-mediated tumor progression.*

The initiation of NF-κB is a significant factor since this transcription factor is what appears to be the instigator of the metastatic processes.

From Pecorino, p 220, we have again presented the details (as modified)<sup>11</sup>:

<sup>11</sup> Pecorino, Molecular Biology of Cancer, Oxford (New York) 2<sup>nd</sup> Ed, 2005.





The above graphic clearly demonstrates the movement of the transcription factor into the nucleus, from a bound state with IκB to an unbound and active state. The target genes indicated includes an MMP gene which again goes into the ECM.

As Sarkar et al state:

*Melanoma differentiation associated gene-9 (mda-9), also known as syntenin, is a PDZ domain-containing adapter protein that is involved in organization of protein complexes in the plasma membranes, regulation of B-cell development, intracellular trafficking and cell-surface targeting, synaptic transmission, and axonal outgrowth. Recent studies now define a seminal role for mda-9/syntenin in cancer metastasis.*

Thus, Sarkar who is part of Fisher's Lab at Virginia, have had a focus on Mda-9. They continue:

*Adapter proteins play an essential role in modulating signal transduction from the extracellular environment to the intracellular milieu by virtue of their association with key regulatory molecules ... mda-9 was originally cloned as a gene differentially expressed in human melanoma*

cells reprogrammed to terminally differentiate by combination treatment with IFN- $\beta$  and the protein kinase C activator mezerein ... Analysis of the subcellular distribution of mda-9/syntenin revealed its localization at the areas of cell-cell contact in cells of epithelial origin in colocalization with F-actin, syndecan-1, E-cadherin,  $\beta$ -catenin, and  $\alpha$ -catenin (12). In fibroblasts, mda-9/syntenin localizes to focal adhesions and in stress fibers. Overexpression of mda-9/syntenin in different cells induces the formation of plasma membrane structures, including ruffles, lamellipodia, fine extensions, and neurite-like structures, showing its role in regulating the structure and function of the plasma membrane...

They continue:

*The major characteristic of malignant tumor cells is their ability to invade foreign tissues and form metastatic foci at distant locations in the body. Such a process requires tumor cell attachment to various matrix proteins, degradation of the extracellular matrix (ECM) mainly by matrix metalloproteinases (MMP), followed by migration into the surrounding stroma by tumor cells...A model of progression of melanoma suggests that it begins by conversion of a normal melanocyte into a benign nevi, subsequent transformation into a radial and then a vertical growth phase primary melanoma, and finally evolution into a metastatic melanoma.*

Finally Sarkar et al outline the overall set of functions which MDA-9 is involved in. Specifically they state:

1. **Interleukin-5 signaling.** mda-9/syntenin interacts with interleukin- 5 (IL-5) receptor  $\alpha$  and the transcription factor Sox4, thus mediating IL-5–induced Sox4 activation ...
2. **Cell-surface trafficking.** Although mda-9/syntenin is located predominantly in the plasma membrane, it is also identified in the early secretory pathway such as the endoplasmic reticulum, intermediate compartment, and cis-Golgi, thus facilitating cell surface trafficking of secreted molecules such as proTGF- $\alpha$ , an epidermal growth factor receptor ligand...
3. **mda-9/syntenin and ephrin signaling.** Ephrins and their cell surface tyrosine kinase receptors are implicated in controlling axon guidance and fasciculation ...
4. **Mediation of cohesiveness of epidermal stem cells.** In the basal layer of interfollicular epidermis the stem cells are clustered, a feature known as cohesiveness. These cells express high levels of Notch ligand D1, which is important for maintaining cohesiveness ...
5. **Regulation of glutamate signaling.** The excitatory neurotransmitter glutamate interacts with its cognate receptors and regulates postsynaptic excitatory currents. Glutamate receptors interact with mda-9/syntenin, ...
6. **Regulation of axon outgrowth.** Unc51.1 is a serine/threonine kinase that is important for neurite extension/parallel fiber formation in cerebellar granule neurons. mda-9/syntenin interacts with Unc51.1 and Rab5, a member of the Ras-like small GTPases that is a marker of early endosomes and is essential for endocytic membrane fusion and trafficking. ...

Boukerche et al in 2005 stated:

*Studies using an enhanced green fluorescent protein mda-9/ syntenin fusion protein showed that endogenous mda-9/syntenin colocalized with the E-cadherin complex and syndecan-1 at adherens junctions as well as with focal adhesions and stress fibers at cell-substratum contact in fibroblastic and epithelial cells. These findings suggest that Mda-9/syntenin might promote cytoskeletal organizational changes and intracellular signaling.*

*The organization of these dissimilar focal contacts is complex but was shown not only to contain the appropriate integrin but also cytoskeletal proteins (vinculin, talin, and  $\alpha$ -actinin) as well as several cytoplasmic protein tyrosine kinases, including members of the src family and focal adhesion kinase (FAK). Despite extensive research documenting an ability of mda-9/syntenin to form multivalent interactions, little is known about the role of Mda-9/syntenin in cancer development.*

Boukerche et al (2008) state:

*Prior studies confirm that Mda-9/syntenin stimulates motility through pathways involving FAK, p38MAPK, and NF- $\kappa$ B, leading to secretion of MMP-2 (4, 9). However, despite these intriguing observations, it is not fully understood how Mda-9/syntenin orchestrates these signaling molecules to enhance cancer cell motility and metastasis. A complex network of protein-protein interactions characterizes the structural organization of focal adhesions, involving known signaling molecules that play functional roles in various cellular activities and other less well-defined pathways.*

*We presently show that Mda-9/syntenin interacts with c-Src through its PDZ domain and activates the c-Src/FAK signaling pathway to maximize tumor cell motility and anchorage-independent growth of melanoma cells. Mda-9/Syntenin levels directly correlate with increased c-Src activity in a human melanoma model that closely mimics the early events of metastasis in humans.*

In 2010 Boukerche et al report (also in Fisher's Lab):

*MDA-9/syntenin affects cancer cell motility and invasion through distinct biochemical and signaling pathways, including focal adhesion kinase and p38 mitogen-activated protein kinase (MAPK), resulting in activation of the nuclear factor (NF)- $\kappa$ B pathway.*

*MDA-9/syntenin also promotes melanoma metastasis by activating c-Src, but how c-Src regulates NF- $\kappa$ B activation is unclear. Using a human melanoma model, we document that MDA-9/syntenin-c-Src interactions are positive regulators of NF- $\kappa$ B activation. Inhibition of c-Src by PP2 treatment, by blocking c-Src or mda-9/syntenin expression with small interfering RNA, or in c-Src (-/-) knockout cell lines, reduces NF- $\kappa$ B activation following overexpression of mda-9/syntenin or c-Src.*

*Deletion or point mutations of the PDZ binding motif preventing MDA-9/syntenin association with c-Src reveals that both PDZ domains, with PDZ2 being the dominant module, are required*

*for activating downstream signaling pathways, including p38 MAPK and NF-kappaB. We also document that MDA-9/syntenin-c-Src complexes functionally cooperate with NF-kappaB to promote anchorage-independent growth, motility and invasion of melanoma cells.*

*These findings underscore PDZ domains of MDA-9/syntenin as promising potential therapeutic targets for intervening in a decisive component of cancer progression, namely, metastatic tumor spread.*

## **5 OBSERVATIONS**

This set of papers from the Fisher Lab present several interesting connections between the ECM and the intra-cellular signaling paths. We have had prior arguments that one can develop models for metastasis by examining the cell as a target entity and then by modeling the environment, both the ECM and surrounding cells as influences on the target cell. In this work we can expand it to include ECM factors in some detail.

The suggested control of other pathway elements, beyond just the B-RAF control that we now have may be proven productive. Notwithstanding it does establish a research path that is based upon established cell dynamics.

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