Studies on Polyoma Middle T Antigen Signaling

A thesis

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Abstract

The development of tumors in mice expressing transgenic polyomavirus middle T (MT) antigen in mammary cells has been intensively studied because it closely matches the progression of human breast cancer. Here I have examined the regulation of ERK by MT in mouse mammary gland epithelial cells. Stress responses are important in both the development and treatment of cancer. MT suppresses the strong activation of ERK by many stress conditions (serum starvation, oxidative stress, UV radiation, hypoxia or drugs causing ER stress). This is an unexpected result, since MT activates RAS, an upstream activator of ERK. This suppression is specific in that MT does not affect all aspects of response to a particular stress. MT affects ERK activation at multiple points: involvement of the ERK kinase MEK1/2 and ERK phosphatases. MT genetic analysis and gain of function assays indicated PI3K/AKT is required but not sufficient for ERK suppression by MT. In addition, based on human mammary epithelial cells MCF10A experiments, MT also uses Y322 to regulate ERK dephosphorylation.

ERK suppression by MT subjected to stress is involved in multiple cell phenotypes. Suppression of ERK improves cell survival exposed to peroxide and reduces COX2 expression caused by hypoxia. Suppression of ERK caused NMuMG cells accumulated in G1 cycle while MT-NMuMG cells can overcome ERK suppression via constitutively activated PI3K pathway. More importantly, ERK suppression also contributes to MT regulation of cytokine expression and cytokine responses.

Because ERK, which can regulate cytokine response, is affected by MT, I looked more generally at the role of MT in regulating cytokine. I found MT induces cytokines such as IL-6, CCL2 and CCL5 production. MT attenuates ERK activation induced by TNFα and sensitizes TNFα-induced apoptosis. MT enhances IL-6, CCL2 and CCL5 while reduces COX2 induction by TNFα. Gene Array study showed MT regulates cytokine expression at least at RNA level both under basal activities and after TNFα treatment.

Together, this work gives a new insight of polyomavirus MT signaling in regulation of ERK in response to stress and emphasizes the importance of MT regulating cytokine expression and cytokine response.

In chapter IV, I described some experiments directed at the possibilities that there is a second target for the MT Y315 site. These experiments did not reach any final conclusions.

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Chapter I. Overview of polyomavirus Middle T antigen related signal pathways

Murine polyomavirus (PyV) can induce transformation in a variety of cell types and tumors in animals (Dawe et al., 1987; Fluck and Haslam, 1996). Transformation is carried out by the early T antigens of the virus (Schaffhausen, 1982). They are large T (LT), middle T (MT), small T (ST) and tiny T which are produced by differential splicing. MT is the principle protein responsible for polyomavirus transformation and tumorigenesis (Freund et al., 1992a). In many cases, MT can be sufficient for transformation in vitro and induction of tumors in animals. As a transgene, MT causes tumors in a variety of tissues (Bautch et al., 1987; Rassoulzadegan et al., 1990). For example, when expressed in mouse mammary glands, MT efficiently induces metastatic carcinomas (Guy et al., 1992). When MT is not sufficient, other oncogenes such as E1A (Ruley, 1983), Myc (Land et al., 1983), mutant p53 (Utermark et al., 2007) as well as PyV LT and ST can complement MT to cause transformation. MT is also important for viral infection (Freund et al., 1992b). MT is the major regulator of viral DNA replication and early and late RNA transcription (Chen et al., 2006).

1.1. The identified polyomavirus Middle T antigen signal pathways

1.1.1. A brief introduction

MT is a membrane bound protein with 421 amino acids. At N-terminal, MT shares a DNA J domain (79 amino acids) with both LT and ST and an additional 112 amino acids with ST. At C-terminal, there is a hydrophobic membrane binding domain which is

required for MT association with membrane, formation of kinase active complexes and transformation (Carmichael et al., 1982).

Kinase activity was first found associated with MT immunoprecipitates by three groups at late 1970s (Eckhart et al., 1979; Schaffhausen and Benjamin, 1979; Smith et al., 1979). Further study showed MT does not have intrinsic tyrosine kinase activity (Schaffhausen et al., 1982) but instead functions as a scaffold and forms a complex with cellular signaling proteins including protein phosphatase 2A (PP2A) (Pallas et al., 1990) and Src family of protein tyrosine kinases (Courtneidge and Smith, 1983; Horak et al., 1989; Kornbluth et al., 1987). Interestingly, only a small fraction (about 5-20%) of MT forms the kinase active complex (Bolen et al., 1987). In complex with Src family of protein tyrosine kinases, MT is phosphorylated primarily on Y315 site (Schaffhausen and Benjamin, 1981), and to a lesser extent on Y250 (Harvey et al., 1984) and Y322 (Hunter et al., 1984).

The phosphorylation of Y315, Y250 and Y322 leads to association and activation of signal molecules like phosphatidylinositol 3-kinase (PI3K) (Whitman et al., 1985), ShcA (Campbell et al., 1994; Dilworth et al., 1994) and phospholipase C-γ (PLCγ) (Su et al., 1995) respectively (Fig.1). PI3K is a very important lipid kinase both in tumorigenesis and regulating normal cell function (Engelman et al., 2006) . Downstream signaling of PI3K includes both activation of AKT (Franke et al., 1995), a kinase important in regulating apoptosis, and activation of RAC1 (Connolly et al., 2000), a G-protein involved in cytoskeletal organization. Tyrosine phosphorylation of ShcA binding sites is responsible for the association of Grb2 (Nicholson et al., 2001; Ong et al., 2001) which leads to the recruitment of SOS, a RAS exchange factor, then leads to RAS activation

(Srinivas et al., 1994). Association of PLCγ1 with Y322 has effects on regulating inositol trisphosphate (IP3) (Gorga et al., 1990) levels and protein kinase C (PKC) (Delage et al., 1993) in MT transformed cells.

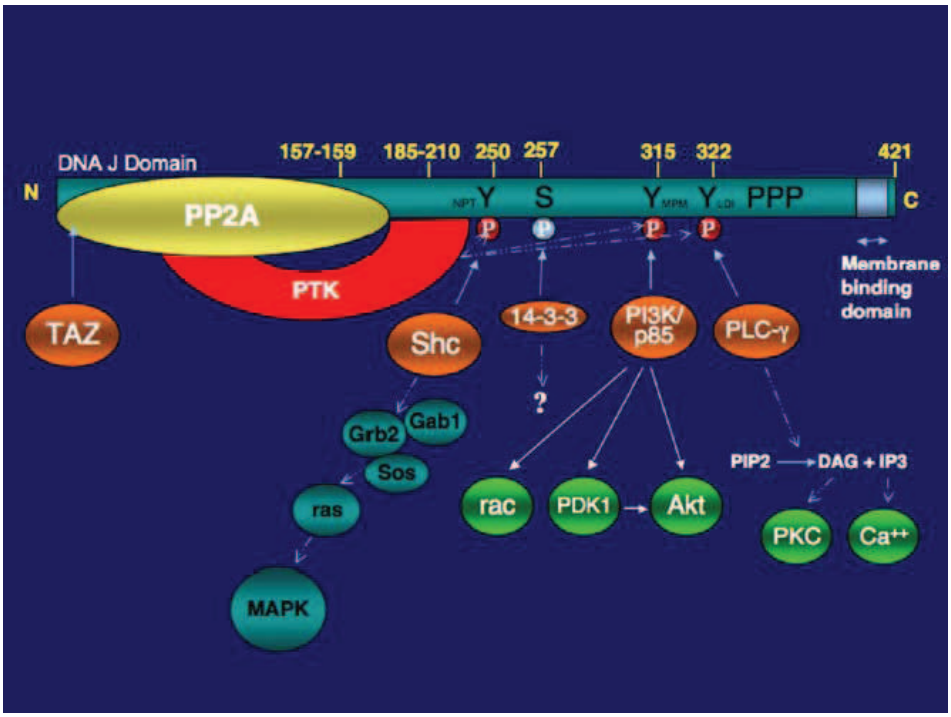

Fig. 1. Simplified diagram of major MT signalling pathways (Schaffhausen and Roberts, 2009).

1.1.2. PP2A/MT/Src complex

PP2A is a serine/threonine protein phosphatase involved in many aspects of cellular function (Janssens et al., 2005). PP2A forms a heterotrimeric ABC complex. The A subunit functions as scaffold and C subunit is the catalytic subunit. Both A and C subunits have two isoforms: α and β. The B subunit is a regulatory subunit and has many family members. MT binds the A and C subunits of PP2A in place of B subunit. Structural study

of SV40 ST binding with PP2A Aα shows the binding area in A subunit overlaps the binding sites for B subunit (Chen et al., 2007) which gives an reasonable explanation how MT displaces B subunit in MT/PP2A complex. In contrast to SV40 ST, which binds only Aα form of PP2A, MT binds both Aα and Aβ (Zhou et al., 2003). The significance of this difference is not clear yet.

Genetic analysis of MT mutants indicates the N-terminal sequences through amino acid 190 are important for MT association with PP2A (Campbell et al., 1995). MT with deletion at 2-7 or 13-25 amino acids can not bind to PP2A (Cook and Hassell, 1990). This suggests part of the J domain is also involved in the PP2A interaction. Beyond the J domain, the region between 120-153 amino acids which contains the two cysteine rich motifs (CXCXXC) has been shown to be important for binding (Glenn and Eckhart, 1993).

Association with PP2A is required for MT binding to Src family PTKs and transformation. PP2A seems act as a scaffold in the PP2A/MT/Src complex because the mutation of the catalytic subunit does not affect MT recruiting PTKs (Ogris et al., 1997). In addition to scaffold function of PP2A in assembling Src complex with MT, PP2A binding may also affect MT cellular localization since PP2A binding mutants are localized differently than wild type MT (Brewster et al., 1997). How MT affects PP2A catalytic activity and substrates specificity has not been well studied. There are some unpublished data from our lab showing that MT as well as ST both binds PP2A and dephosphorylates LIPIN.

MT associates with and activates Src family PTKs such as c-Src, c-Yes (Kornbluth et al., 1987), and Fyn (Horak et al., 1989). Deletion analysis showed MT interaction with

$pp60^{c\text{-}src}$ requires amino acids from 185 to 210 of MT, and the 185-210 deletion mutant does not bind to $pp60^{c\text{-}src}$ but has no effect on PP2A binding (Brewster et al., 1997). C-Src has multiple domains. The catalytic domain and C-terminal of Src are essential for MT interaction (Dunant et al., 1996). The regulatory phosphorylation site Tyr527 at C-terminal of Src is involved in interaction with SH2 domain to form an intramolecular inhibition and negative regulation of kinase activity (Cooper et al., 1986). Association with MT leads to reduced phosphorylation of Tyr527 of $pp60^{c\text{-}src}$ therefore significantly increases Src kinase activity (Cartwright et al., 1986).

In contrast to V-Src (Sefton et al., 1980), transformation by C-Src does not drive a general increase of tyrosine phosphorylation in MT transformed cells, though MT itself is a very important substrate. The association with Src family of PTKs is only a small fraction of MT in cells (Bolen et al., 1987). More strikingly, overexpression of either Src or MT does not increase complex formation effectively (Schaffhausen et al., 1987), and the process of complex formation takes hours (Cohen et al., 1990).

1.1.3. Y250 of MT

MT is phosphorylated at Y250 in the MT/PTK complex (Hunter et al., 1984). Phosphorylation of Y250 provides a docking site for ShcA family of adaptors. Shc family has three isoforms: p46, p52 and p66. MT binds all of them. All three members of Shc family have an N-terminal PTB domain and a C-terminal SH2 domain, as well as a proline-rich CH1 region at center. Both PTB and SH2 domain have potential to bind phosphotyrosine sequences (Schlessinger and Lemmon, 2003). PTB recognizes NPXpY motif, in which the specificity-defining residues occur to the N-terminal side of pY (Trub

et al., 1995). In contrast, SH2 domain recognizes pYXXM motif in which the specificity related to C-terminal to pY (van der Geer et al., 1996a). It is clear that PTB domain of Shc is involved in binding MT (Campbell et al., 1994). It is not clear whether SH2 domain of Shc contributes binding MT since there is an SH2 domain binding site YSVM at C-terminal of 250Y. In vitro experiments reported that Shc SH2 domain can bind MT (Dilworth et al., 1994). The way how Shc binds MT might affect differential downstream signaling.

After binding MT, Shc is phosphorylated on at least three sites: Y317 (Y313 in mouse) and Y239/Y240 (van der Geer et al., 1996b). It has been suggested that distinct functions exist in Y317 vs. Y239/Y240 (Gotoh et al., 1996). Expression of Y239F/Y240F but not Y317F Shc affects MT transformation in NIH/3T3 cells (Blaikie et al., 1997). Both of Y317 and Y239/Y240 sites have been reported to recruit Grb2 adaptor protein through its SH2 domain. The binding of Grb2 allows binding additional proteins such as SOS (Nicholson et al., 2001). SOS is a RAS exchange factor which leads to RAS activation and therefore RAF, MEK and ERK activation would be expected. Surprisingly, there has been little study of MT activation of ERK.

Mutation at Y250 or its N-terminal NPXY motif has significant effects on MT transformation has been shown in many cell systems (Bronson et al., 1997; Yi et al., 1997). However in others such as human mammary epithelial cells or fibroblasts, Y250F has no effect on transformation (Utermark et al., 2007). This suggests different signaling pathways are involved in MT transformation in different cellular contexts (for exp. human cell vs. mouse cell). Mammary epithelial cell-specific expression of MT Y250F by transgenic mice resulted in the induction of extensive mammary epithelial

hyperplasias, and significant delay in tumor induction (Webster et al., 1998). Different than the transgenic model, MT Y250S mutant polyomavirus (wt LT/ST) had almost wild-type ability to induce mammary tumors (Bronson et al., 1997). The possible explaination for the difference between polyoma MT Y250F transgene and polyomavirus MT Y250S infected animal might be from two aspects. First, LT and ST complement MT in the virus system but not in transgene. Secondly, transgene could cause defective mammary development during embryo stage while virus infection is after mice born. In addition, the MT250 mutant virus enhances tumors in certain tissues and reduces tumors in others. This further supports the idea that MT signaling is cell context dependent.

1.1.4. Y315 of MT

Y315 is one of the major sites phosphorylated by Src family of protein tyrosine kinase (Schaffhausen and Benjamin, 1981). p315YMPM motif is required for binding p85 subunit of PI3K through its SH2 domain (Kaplan et al., 1987). Interaction of p85 with MT recruits PI3K to the membrane and activation (Carpenter et al., 1993). PI3K is a family of lipid kinases which will be discussed in detail in a separate section later. MT directly binds class IA of PI3Ks. Activation of PI3K class I A enzyme phosphorylates the 3' position of the inositol ring to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3) which recruits proteins containing certain PH domains to the membrane such as Ser/Thr kinase AKT (Franke et al., 1995). MT activates AKT which prevents apoptosis (Dahl et al., 1998; Meili et al., 1998). MT was also found to activate the small GTPase RAC as a result of PI3K activation. In both fibroblasts (Urich et al., 1997) and endothelial cells (Connolly et al., 2000), dominant negative RAC were shown to block

MT transformation.

There is a difference for transformation using Y315F virus (Py1178T (Y315F mutant of MT, wt LT/ST)) versus Y315F MT cDNA in different cell lines. In F111 cells, Py1178T virus is highly defective in transformation compared to wild type virus (Carmichael et al., 1984). In Rat-1 cells, Y315F MT cDNA is capable transform cells as wild type MT (Oostra et al., 1983). Again, this indicates the signaling required for transformation is cellular context dependent.

Animal experiments further showed the importance of Y315. By using a mutant polyomavirus Py1178T researchers found lacking of phosphorylation of Y315 significantly affects tumorigenesis (Talmage et al., 1989). In bone and lung, more tumors were seen in mutant virus than wild type, while in other tissues, such as kidney, mammary gland tumors were significantly reduced (Freund et al., 1992a).

1.1.5. Y322 of MT

Y322 is also phosphorylated in MT/Src complex. PLCγ is shown to bind MT at Y322 and important for transformation (Su et al., 1995). There are not many studies reported on the downstream effectors of PLCγ activated by MT. In vivo study using MMTV (Mouse Mammary Tumor Virus promoter)-MT mammary model showed dominant negative allele of PLCγ significantly decreases lung metastases (Shepard et al., 2007).

1.1.6. Others

Minor tyrosine sites: Y258, Y288 and Y297

In addition to the three major tyrosine sites Y315, Y250 and Y322, there are evidence for additional tyrosine phosphorylation of MT (Y258, Y288 and Y297?) (Hunter et al., 1984). Study of three major tyrosine mutant (Y3F) and six tyrosine mutant (Y6F) of MT found that Y3F retained some activity while Y6F was inactive in induction of viral transcription and replication. This suggests minor tyrosine sites have redundant function as the three major tyrosine sites (Chen et al., 2006).

TAZ (transcriptional co-activator with PDZ-binding motif)

MT binds the WW domain of TAZ with amino acid residues from 2 to 4 (Tian et al., 2004). TAZ is a transcriptional coactivator and functions have been found in mesenchymal stem cell differentiation (Hong et al., 2005), cell proliferation (Lei et al., 2008) and breast tumorigenesis (Chan et al., 2008). All the three T antigens bind TAZ and TAZ binding defective polyomavirus is defective in viral DNA replication (Tian et al., 2004). It is not clear what particular contribution TAZ may make to MT function.

Heat shock 70 family proteins (Hsp70/Hsc70)

All the three T antigens share DnaJ domain at N-terminal, therefore they are all expected to bind Hsp70. The significance of binding Hsp70 among different T antigens is different. It is well known that binding of Hsp70 is important for LT to activate E2F-containing promoters (Sheng et al., 1997). As discussed previously, the DnaJ domain is important for recruiting PP2A and Src tyrosine kinase family members to form MT/PP2A/Src complex, it would not be unreasonable if there is differential function of Hsp 70 in MT and ST. In the case of MT and ST, binding of Hsp70 and PP2A seems

mostly mutually exclusive (Walter et al., 1987). The particular function of heat shock protein in MT transformation and tumorigenesis is not known. Deletion of the HPDK loop amino acid indicates it is necessary for DnaJ interaction with Hsp70 but is not needed for MT transformation (Campbell et al., 1995).

14-3-3 proteins

MT is associated with 14-3-3 proteins through phosphoserine at 257 (Cullere et al., 1998; Pallas et al., 1994). Mutagenesis showed S257 was not required for transformation of fibroblasts. In vivo studies using polyomavirus suggests the binding of 14-3-3 is important for the induction of salivary gland tumors and fibroscarcomas (Cullere et al., 1998) but not for other types of tumor. It is not known what signaling pathway downstream of 14-3-3 interaction with MT.

The proline-rich region

The proline-rich region (aa 332-347) at the C-terminal of MT has an important role in transformation and tumorigenesis. The polyomavirus dl1015 mutant (deletion from aa 339 to 347) has a normal tyrosine kinanase acitivty associated with it but is defective in transformation (Magnusson and Berg, 1979). Polyomavirus with deletion of three core prolines (aa 336-338) in MT is defective in tumor induction in animals (Yi and Freund, 1998). The mechanism of this defective transformation and tumorigenesis is unclear. Unpublished data from our lab showed the point mutation E349K which mimics d1015 defect is also defective in PI3K signaling.

1.2. RAS/RAF/MEK/ERK cascade and its regulation

The phosphorylation of Y250 of MT leads to association and activation ShcA, which is responsible for the association of Grb2 and leads to the recruitment of SOS, a RAS exchange factor, then leads to RAS activation. Y250 has been shown very important for MT transformation and turmorigenesis. The topic I am going to discuss later is mostly about how MT regulates ERK-the downstream effector of RAS/RAF/MEK cascade. So I would like to give some background introduction of RAS/RAF/MEK/ERK pathway.

1.2.1. Overview of the RAS/RAF/MEK/ERK signaling

1.2.1.1. RAS

RAS, a small GTP-binding protein, is a common upstream molecule of several signaling pathways including RAF/MEK/ERK, PI3K/AKT and RAL (Peyssonnaux et al., 2000). RAS mutations are frequently found in tumors. There are three ubiquitously expressed major RAS isoforms: H-, K-, and N-RAS. They are highly conserved even though they may have a different biological output. K-RAS has been found more efficient to recruit RAF to the cellular membrane and activation than H-RAS, while H-RAS is more potent in activating PI3K than K-RAS (Yan et al., 1998). In different cancer cell lines, the contributions of different isoforms are varied (Omerovic et al., 2008).

The best characterized route of RAS activation occurs at the plasma membrane: following growth factors or mitogens binding to their receptors and activating the coupling complex Shc/Grb2/SOS, RAS is activated to GTP-bound form and undergoes a conformational change (Sondermann et al., 2004). The GTP-bound active RAS can recruit its target effectors such as RAF, PI3K and RalGDS to the membrane (Fig.2) (Schubbert et al., 2007). RAF, MEK, and ERK are cytosolic protein kinases and form a

signal cascade by sequential activation. RAF activation is a complex process and required its recruitment to the plasma membrane by RAS.

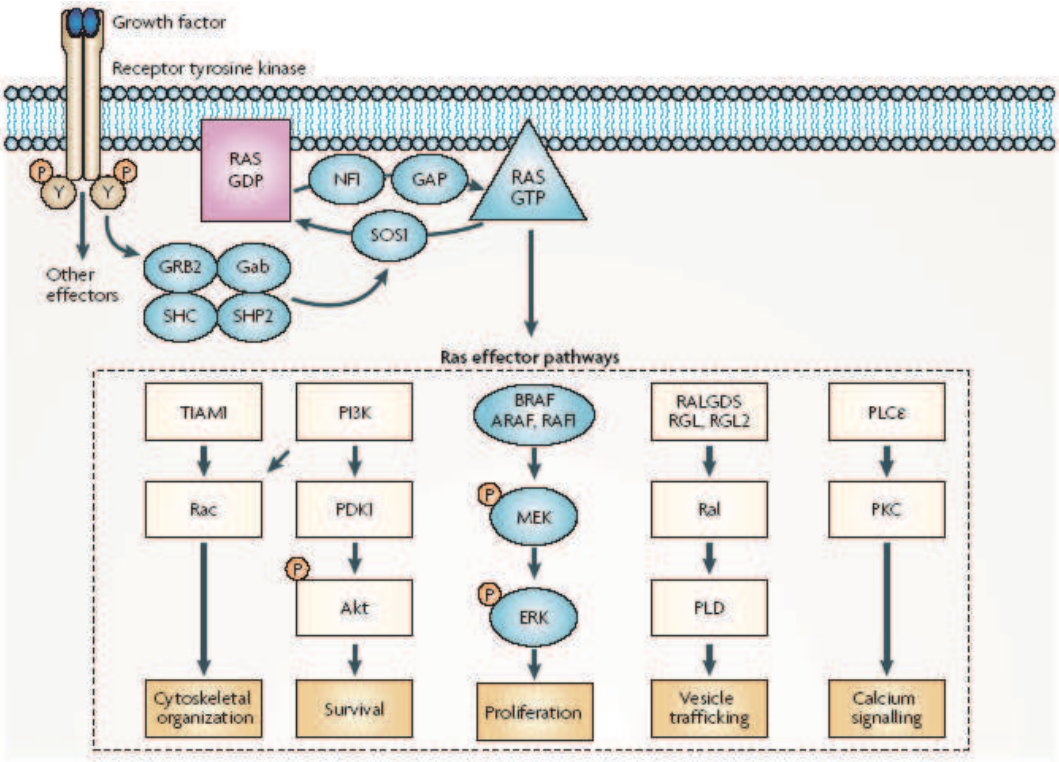

Fig. 2. The RAS signalling pathway (Schubbert et al., 2007)**.**

1.2.1.2. RAF

RAF is the main effector recruited by GTP-bound RAS to activate the MEK/ERK pathway. RAF is a serine/threonine (S/T) kinase. There are three RAF genes encoding RAF-1 (also called C-RAF), A-RAF and B-RAF (Marais and Marshall, 1996). RAF proteins share three conserved regions: CR1, CR2 and CR3. CR1 and CR2 are the N-terminal regulatory regions. CR1 comprises a RAS binding domain adjacent to a cysteine rich domain and serves to localize proteins to plasma membrane (Mott et al., 1996). CR2 is a Ser/Thr rich region which has an inhibitory 14-3-3 binding site. CR3 is in the C-terminal where the kinase domain is (Claperon and Therrien, 2007). In the inactive state,

the N-terminal domain acts as an autoinhibitor of the C-terminal kinase domain through binding of 14-3-3 proteins.

Activation of RAF is very complicated and usually includes a series of events: 1) recruitment to the cellular membrane (Yan et al., 1998); 2) dimerization of RAF proteins (Luo et al., 1996); 3) phosphorylation/dephosphorylation on different domains (Dhillon et al., 2002; Fabian et al., 1993; King et al., 1998); 4) association with scaffolding complexes (Kolch, 2005).

Activation of RAF-1 is the best understood among three RAF isoforms. There are at least thirteen regulatory phosphorylation sites on RAF-1. Inhibitory 14-3-3 binding sites Ser259 can be phosphorylated by AKT (Zimmermann and Moelling, 1999), which keep RAF-1 in an autoinhibited state and concealing RAS binding sites at N-terminal. Dephosphorylation of the inhibitory binding sites is the pivotal step for RAF-1 activation (Dhillon et al., 2002). Both PP1 and PP2A have been shown to positively regulate RAF activity through deposphorylating the inhibitory sites (Ory et al., 2003; Rodriguez-Viciana et al., 2006). Upon receptor tyrosine kinase (RTK) activation, RAF-1 is recruited to the cellular membrane via activated RAS and releases 14-3-3 which together promotes phosphorylation on the kinase domain activating sites.

For RAF-1 and A-RAF, activating sites are located in two regions of kinase domain: the negative-charge regulatory region (N region) and activation segment (Fig. 3). For B-RAF, activation requires only activation segment phosphorylation (Zhang and Guan, 2000). Differences in the N region lead to the differential regulation of the RAF isoforms (Garnett and Marais, 2004). S445 in B-RAF (equivalent to S338 in RAF-1 and S299 in A-RAF) is constitutively phosphorylated. In stead of Y340 and Y341in RAF-1 and A-

RAF, negative charged aspartic acids D447 and D448 in B-RAF, together let the N region of B-RAF carries a constitutive negative charge. Therefore unlike RAF-1 and A-RAF, B-RAF requires only activation segment phosphorylation to reach maximal activation by RAS (Marais et al., 1997). Not surprisingly, B-RAF is the primary target of oncogenic RAS among three RAF isoforms. B-RAF is mutated in about 7% of human cancers(Davies et al., 2002) and over 50 distinct point mutations most clustered in two regions of the kinase domain (Garnett and Marais, 2004). RAF-1 mutations are rare in cancer because the low basal kinase activity compared to B-RAF and single mutation probably would not be sufficient to convert RAF-1 active (Emuss et al., 2005).

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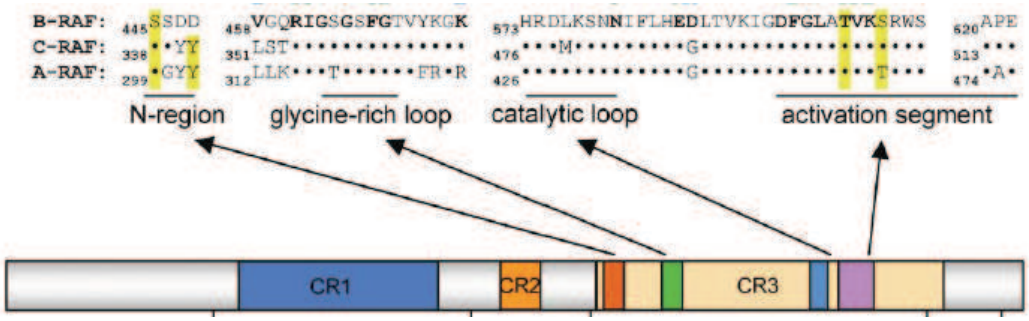


Fig.3. Alignment of three RAF isoforms in kinase domain. Yellow bars highlight the phosphorylation sites required for kinase activity. Residues mutated in the kinase domain of B-RAF in cancer are shown in bold. Dots represent the conserved sequences among B-RAF, C-RAF and A-RAF. Figure is adapted from (Garnett and Marais, 2004).

1.2.1.3. MEK

MEK1/2, also called mitogen-activated protein kinase (MAPK) kinase or ERK kinase, is a tyrosine (Y-) and S/T-dual specificity protein kinase. All three members of RAF family are able to phosphorylate and activate MEK. In addition to RAF, there are alternative ways to activate MEK/ERK signaling pathway. Other MEK kinases including

MEK kinase 1 (MEKK1) and tumor progression locus 2 gene (TPL2) also can be involved in activating MEK/ERK cascade according to the cellular context (Russell et al., 1995). Both PP2A and PP1 have been shown involved in dephosphorylation of MEK and negative regulation of MEK/ERK signaling (Manfroid et al., 2001; Mao et al., 2005). The predominant downstream target of MEK is ERK.

1.2.1.4. Extracellular signal-regulated kinases 1, 2 (ERK1/2)

The mammalian MAPK family includes extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK; also known as stress-activated protein kinase or SAPK). All the three MAPK family members are defined by their TXY motifs at the activation loop which require dual phosphorylation for full activation. ERK, also called MAPKs, are S/T kinases involved in many cellular programs including cell proliferation, differentiation and apoptosis. There are two classes of ERK isoforms: 1) classical MAPKs: ERK1 and ERK2 which consist a kinase domain; 2) big MAPKs: ERK3, ERK5 and ERK7/8 which have a kinase domain and a C-terminal domain. The classical ERKs require scaffold proteins for maximal activation while the big MAPKs do not because the C-terminal domain functions as a scaffold. ERK scaffold proteins play critical roles in determining the specificity, location, and duration of RAF/MEK/ERK signaling(Kolch, 2005). Here we only discuss classical ERK isoforms.

In contrast to the complexity of RAF activation, MEK and ERK can be fully activated simply by phosphorylation of the activation sites. MEK1 and MEK2 activate ERK1/p44 and ERK2/p42 through phosphorylation of activation loop residues Thr202/Tyr204 and Thr185/Tyr187, respectively. ERKs can directly phosphorylate many

transcription factors including Ets-1, c-Jun and c-Myc, which are thought to be important for polyomavirus replication. ERK can also phosphorylate the 90kDa ribosomal S6 kinase (p90Rsk) (Dalby et al., 1998), therefore leading to activation of transcription factor CREB. In addition to activate transcription factors and regulation of gene expression, ERK also regulates apoptosis through phosphorylation of apoptotic regulatory molecules such as Bad (Pucci et al., 2009), Bim (Luciano et al., 2003), caspase-9 (Allan et al., 2003). ERK activity can be negatively regulated through protein phosphatases, which will be discussed later in detail.

1.2.2. ERK signaling

ERK signaling has been well characterized in response to growth factor or mitogen and induces cell proliferation and differentiation (Cowley et al., 1994; Mansour et al., 1994). ERK activation has also been associated with pro- or anti-survival. The outcome of ERK signal depends on the strength, duration and spatial distribution. In the other words, it depends on ERK signal dynamics and the cellular context. For example, blockade of ERK activation reduces hydrogen peroxide-induced activation of caspase-3 and phosphorylation of histone H2B and protects renal epithelial cells from apoptosis (Zhuang et al., 2007). On the other side, there are even more evidence show that ERK is anti-apoptotic. Introduction of either MEK inhibitors or dominant negative MEK1 sensitize MCF-7 and H1299 cells to ER stress-induced cell death (Hu et al., 2004).

ERK activation has also been associated with both stimulation and inhibition of cell cycle progression. These different outcomes could be explained by the duration and intensity of ERK signal. Some studies showed that sustained ERK signals induced by

nerve growth factor (NGF) in NIH/3T3 cells caused cell cycle arrest which can be reversed by MEK specific inhibitor (PD98059). Upon this study, progression through G1 phase is mainly regulated by CDK4 and CDK2. Inhibition of MEK/ERK partially reversed the CDK4 and CDK2 activities through regulation of cyclin levels and p21cip1 (Pumiglia and Decker, 1997). The sensitivity to apoptotic stress can vary depending on the stage of cell cycle. ERK can affect stress sensitivity through cell cycle regulation. For example, a few studies showed evidence that G1 arrest caused by ERK activation provides resistance to different forms of apoptotic stress (Collins et al., 2005; Hama et al., 1998).

1.2.3. DUSPs and regulation of ERK dephosphorylation

The outcome of MAPK signaling is a balance between the upstream kinase activities and the activities of MAPK phosphatases. Interestingly, the activation of MAPK requires both threonine and tyrosine phosphorylation while dephosphorylation of either residue is sufficient to inactivate MAPK. In vivo studies from yeast to humans showed that all three major classes of protein phosphatase: serine/threonine phosphatases, tyrosine specific phosphatases and dual-specificity phosphatases can inactivate MAPK (Keyse, 2000; Saxena and Mustelin, 2000).

Dual-specificity protein phosphatases (DUSPs) belong to the protein tyrosine phosphatase (PTP) superfamily. The human genome seems to contain at least 61 genes for DUSPs (Alonso et al. 2003). It includes MAPK phosphatases (MKPs), which are also termed as classical DUSPs (Table.1), and atypical DUSPs (Table.2) based on phylogeny and structure similarity.

Table 1 Nomenclature, properties and physiological functions for dual-specificity MKPs

Gene/MKP	Trivial names	Chromosomal localisation	Subcellular localisation	Substrate specificity	Physiological function(s)
DUSP1/MKP-1	CL100, erp, 3CH134, hVH1	5q34	Nuclear	JNK, p38, ERK	Negative regulator of immune function. Protects mice from lethal endotoxic shock. Plays a key role in metabolic homeostasis and mediates resistance to cellular stress in mouse fibroblasts
DUSP4/MKP-2	Typ1, Sty8, hVH2	8p12-p11	Nuclear	JNK, p38, ERK	Unknown
DUSP2/None	PAC-1	2q11	Nuclear	ERK, p38	Positive regulator of inflammatory responses. Knockout mice are resistant to immune inflammation
DUSP5/None	hVH3, B23	10q25	Nuclear	ERK	Unknown
DUSP6/MKP-3	Pyst1, rVH6	12q22-q23	Cytoplasmic	ERK	Negative feedback regulator of ERK2 downstream of FGFR signalling
DUSP7/MKP-X	Pyst2, B59	3p21	Cytoplasmic	ERK	Unknown
DUSP9MKP-4	Pyst3	Xq28	Cytoplasmic	ERK>p38	Essential for placental development and function (labyrinth formation)
DUSP8/None	M3/6, hVH5, HB5	11p15.5	Cytoplasmic/ nuclear	JNK, p38	Unknown
DUSP10/MKP-5		1q41	Cytoplasmic/ nuclear	JNK, p38	Functions in innate and adaptive immunity
DUSP16/MKP-7		12p12	Cytoplasmic/ nuclear	JNK, p38	Unknown

Table.1. Classical DUSPs (Keyse, 2008)**.**

Human protein	DSP	Species ortholog	Chromosomal localisation	Expression	Subcellular localization	Substrates
Atypical DSPs						
VHR	DUSP3	T-DSP11 (m)	17q21	Wide	Nuclear/ Cytosolic	Erk, Jnk
MKPX/ JSP1/ VHX		LMW-DSP2 (m), TS-DSP2, JKAP	6p25.3	Wide	Cytosolic	Inhibits Erk, Jnk, p38 and activates Jnk
DUSP13	DUSP13	TMDP (m)/ TS-DSP6 (m)	10q22.2	Testis and skeletal muscle		Unknown
HSSH-1		SSH1 (fly)	12q24.12	Wide		ADF/Cofilin
HSSH2		SSH2 (fly)	17q11.2	Wide		ADF/Cofilin
HSSH3		SSH3 (fly)	11q13.1	Wide		ADF/Cofilin
HYVH1	DUSP12	GKAP (r), LMW_DSP4 (m)	1q21-q22	Wide	Cytosolic/ nuclear	Glucokinase
SKRP1	DUSP19	LDP-2 (m)/ TS-DSP1 (m)/ SKRP1 (m)	2q32.1	Wide	Cytosolic	Jnk
LMW-DSP20			22q12.1	Testis	Nuclear/ Cytosolic	Unknown
LMW-DSP21		BJ-HCC-26 tumor antigen	Xp11.4-p11.23	Testis	Nuclear/ Cytosolic	Unknown
MKP6/MKP-L	DUSP14	DSUP14 (m)	17q12	Wide	Nuclear/ Cytosolic	Erk and Jnk

Table.2. Atypical DUSPs (Alonso et al. 2003)**.**

1.2.3.1. Classical DUSPs: MKPs

There are 10 MKPs in mammalian cells and they are divided into three groups based on their substrate specificity, subcellular localization and regulation (Keyse, 2008). The first group contains DUSP1/MKP-1, DUSP2, DUSP4/MKP-2, and DUSP5 which are inducible phosphatases in the nucleus. The second group includes DUSP6/MKP-3, DUSP7/MKP-X and DUSP9/MKP-4 which are cytoplasmic MKPs and ERK-specific. The third group prefers p38 and JNK MAP kinases as substrates and consists of DUSP8, DUSP10/MKP-5 and DUSP16/MKP-7.

1.2.3.1.1. MKP3/ DUSP6

Among the cytoplasmic ERK specific phosphatases, MKP-3/ DUSP6 is the most studied and best characterized. Study in chick limb development showed MKP3 is induced in the mesenchyme by FGF8 signaling through the PI3K/AKT pathway (Kawakami et al., 2003). MKP3 activity prevents cell death in the limb mesenchyme and knockdown of MKP3 induces apoptosis, which suggests downregulation of ERK activity is essential in the mesenchyme during development. In addition to transcriptional upregulation by PI3K pathway, recent studies showed MKP3 can also be regulated at post-translational level. The Gimond group found MKP3 can be phosphorylated (Ser 159 and Ser 197) and degraded upon growth factor stimulation in MEK-dependent manner (Marchetti et al., 2005).

MKP-3 knock out mice show increased basal ERK1/2 phosphorylation in multiple tissues but no effect on ERK5, p38 or JNK activation (Maillet et al., 2008). Interestingly, loss of MKP3 did not increase or prolong ERK1/2 activity after α-adrenergic agonist phenylephrine (PE) stimulation which suggests one or more of DUSPs may compensate

for the loss of MKP3 and play a role following stimulation. Overexpression of MKP3 in heart showed absence of ERK1/2 phosphorylation at basal level or after phenylephrine (PE) stimulation (Purcell et al., 2007).

The expression of MKP-3 in cancer seems complicated. The majority of human pancreatic cancers have activating mutation of K-RAS, and MKP-3 was found significantly reduced at mRNA level in pancreatic cancer cell lines (Furukawa et al., 1998). On the other hand, MKP-3 is overexpressed in H-RAS expressing human breast epithelial MCF10A cells (Warmka et al., 2004) and melanoma cell lines with B-RAF activating mutations (Bloethner et al., 2005). The correlation of MKP3 with tumor initiation and maintenance is unclear.

1.2.3.1.2. Other MKPs

MKP-1/DUSP1 is upregulated in response to different stress conditions including DNA-damage agents, hypoxia (Laderoute et al., 1999), and oxidative stress (Keyse and Emslie, 1992). There are studies showing increased levels of MKP-1 expression in a range of human epithelial cancers including breast cancer (Wang et al., 2003). Studies in human prostate cancer showed MKP-1inversely correlated with JNK activity and apoptosis (Magi-Galluzzi et al., 1997; Srikanth et al., 1999). Other studies also indicate MKP-1 might mediate chemoresistance through JNK. MKP-1 knock out MEFs are sensitive to cisplatin which could be inhibited by blocking JNK but not p38 or ERK (Wang et al., 2006).

Fewer studies have been done in other cytoplasmic MKPs. There are studies that showed DUSP7/MKP-X (Pyst2) is overexpressed in acute leukemia in which activation of MAPK signaling influences Pyst2 expression (Levy-Nissenbaum et al., 2003). Studies

in MKP-4/DUSP9 showed it is down-regulated at initiation stage and lost at malignant conversion in an epidermal carcinogenesis model without RAS mutation. Reconstitution of MKP4 into tumor cells leads to G2-M associated cell death, microtubule disruption and tumor suppression which indicated it may have a tumour suppressor role (Liu et al., 2007).

1.2.3.2. VHR /DUSP3

Vaccinia virus VH1-related (VHR) is a small dual-specific protein phosphatase (encoded by the DUSP3 gene). It has been proposed that only ERK1 and ERK2 are authentic substrates for VHR based on in vitro study (Todd et al., 1999). Later studies in different cell systems showed both ERK and JNK can be dephosphorylated by VHR (Alonso et al., 2001; Rahmouni et al., 2006; Todd et al., 2002). Unlike many MKPs, VHR is not acutely upregulated in response to activation of MAP kinases. Instead, VHR is regulated during cell cycle. VHR is most abundant in mitotic cells and barely detected in cells synchronized in G1 cycle. It implies VHR is involved in regulation of cell cycle progression (Rahmouni et al., 2006). Less study has been devoted to investigating the role of VHR in cancer. One study by Rahmouni group found that cervical carcinoma is associated with an up-regulation and nuclear localization of VHR. The up-regulation is mainly due to its post-translational stabilization (Henkens et al., 2008). But whether the upregulation of VHR is a cause or a consequence of cervical carcinoma has not been studied.

1.2.4. Scaffold interaction and ERK signaling

Scaffold proteins provide a docking platform to assemble the multiple components

into a complex which has been believed to increase the efficiency of signal transfer. Study using engineered scaffold interaction shows scaffolds could reshape the system output (Bashor et al., 2008). A computational model on ERK cascade suggests scaffold protein could either amplify or attenuate the incoming signals based on different biological contexts (Locasale et al., 2007).

There are at least two well known families of ERK scaffold proteins involved in regulating plasma membrane activation of RAS signaling: kinase suppressor of RAS (KSR) and connector enhancer of KSR (CNK). The best studied MAPK scaffold is KSR. KSR has two isoforms KSR1 and KSR2 in mammalian cells. KSR1 is ubiquitously expressed while KSR2 expression pattern is less known. The evidence that KSR modulates RAS/ERK signaling was first derived from an assay by which it showed murine KSR1 cooperates with activated RAS to promote Xenopus oocyte maturation (Therrien et al., 1996). Later study using KSR1-/-MEFs and reintroduction of KSR1 shows KSR1 is required for maximal ERK activation in response to growth factors, but inhibits the response when the expression level is above its interacting components, which shows the biphasic scaffolding properties of KSR (Kortum and Lewis, 2004). Not only involved in mitogenic stimuli, KSR1 is also involved in ERK activation in response to stress stimuli such as TNFα and IL-1 (Fusello et al., 2006), and some DNA damaging agents (Razidlo et al., 2009).

Both Impedes Mitogenic signal Propagation (IMP) and (Cdc25C-associated kinase 1) C-TAK1 have been found involved in negative regulation of KSR in assembling of RAF/MEK/ERK complexes. RAS effector protein IMP is a RAS-responsive E3 ubiquitin ligase which undergoes auto-polyubiquitination and releases the inhibition of RAF/MEK

complex formation upon RAS activation (Matheny and White, 2009). C-TAK1 constitutively associates with KSR1. Phosphorylation of KSR1 on S392 by C-TAK1 confers its 14-3-3 binding and retains its cytoplasm localization. Dephosphorylation by PP2A (Ory et al., 2003) or mutation of S392 is sufficient for KSR1 localized to the plasma membrane (Muller et al., 2001).

There are at least three CNK (Connector enhancer of KSR) genes: CNK1, CNK2, and CNK3 in mammalian cells. Among them, the most ubiquitously expressed CNK is CNK1. By colocalizing Src family tyrosine kinase with membrane localized C-RAF, CNK1 augments C-RAF activation through increasing N-region phosphorylation (Ziogas et al., 2005). CNK1 can interact with other RAS family members such as Ral and Rho, therefore influences the signal output (Jaffe et al., 2004).

In addition to scaffolds regulating ERK signal at the plasma membrane, other ERK scaffolds such as MP1 (MEK1 partner) (Teis et al., 2002), Sef (similar expression to fgf genes) (Torii et al., 2004), and Paxillin (Ishibe et al., 2004) are involved in regulating ERK signaling in other intracellular compartments.

1.3. PI3K signaling pathway

As discussed previously, MT recruits PI3K to the membrane and activation, which is important for MT transformation and turmorigenesis. Next, I will discuss the PI3K signaling pathway briefly.

1.3.1. PI3K

Phosphatidylinositol 3-kinases (PI3Ks) are lipid kinases that phosphorylate the 3'-

hydroxyl group of phosphatidylinositol and phosphoinositides. The biological functions of PI3K are involved in many aspects such as cell metabolism, cell growth, protein synthesis, cell polarity and motility (see review (Vanhaesebroeck et al.)). There are three classes (I-III) of PI3K according to the substrate specificity and sequence homology. Class I PI3Ks are further divided into Class IA PI3Ks which have p85 family regulatory subunits and therefore are activated by receptor tyrosine kinases (RTKs) and Class IB PI3Ks which lack p85 family regulatory subunits and are not regulated by RTKs. Because MT directly binds and activates Class IA PI3K (both p110α and p110β isoforms), and the p110α isoform of PI3K has been shown essential for MT mediated transformation (Utermark et al., 2007), the following will focus on Class IA enzyme.

Class IA PI3K is a heterodimer enzyme which consists of a regulatory subunit (usually p85) and a p110 catalytic subunit (Fig. 4). There are several regulatory isoforms which share a core structure including a p110-binding domain (also called inter-SH2 domain) flanked with two SH2 domains. The binding of SH2 domain of p85 to phosphotyrosine residues in the context of pY(MVIE)XM (Songyang et al., 1993) on activated RTKs or adaptor protein results in both relieving the inhibition of p110 (Yu et al., 1998) and recruiting the enzyme to the substrate at plasma membrane. p85 has also been shown to stablize p110 subunit by forming heterodimer and increasing the half-life (Yu et al., 1998). On the other side, p85 protein stablility is also positively regulated by the p110 protein level (Brachmann et al., 2005). This indicates the ratio of p85 to p110 is tightly regulated in the cell.

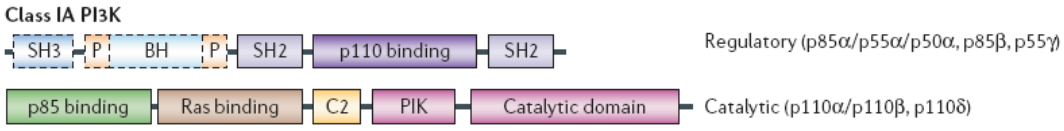


Fig. 4. Class IA PI3K consists of regulatory and catalytic subunits (adapted from (Engelman et al., 2006)).

There are three isoforms of catalytic subunit p110α, p110β and p110δ encoded by three genes PIK3CA, PIK3CB and PIK3CD respectively. They all have an N-terminal p85-binding domain, a RAS-binding domain (RBD), a C2 domain, a phosphatidylinositol kinase homology domain and a C-terminal catalytic domain.

In addition to binding phosphotyrosine containing protein through SH2 domain of p85 subunit, PI3K also can bind directly to RAS through RAS binding domain in catalytic subunit (Pacold et al., 2000; Rodriguez-Viciana et al., 1994). In vivo studies using p110α RAS binding domain mutant mice showed those mice were defective in development and highly resistant to RAS oncogene induced tumorigenesis. In vitro studies using MEFs showed loss of p110α binding to RAS significantly reduced PI3K activation induced by certain growth factors such as EGF and FGF-2, but not PDGF. It indicated the differential activation pathways of PI3K through p85 direct binding to RTK or indirectly through RAS. Both the in vivo and in vitro studies support the idea that RAS is involved in PI3K activation in a manner synergistic with binding of p85 to phosphotyrosine motif which allows membrane translocation of PI3K (Gupta et al., 2007).

Mutation of PI3KCA gene encoding p110α occurs with high frequency in human cancers including colon, breast, brain and lung cancer. The majority of mutations are clustered in hot-spot regions: helical (phosphatidylinositol kinase homology domain),

kinase and p85 binding domains (Samuels et al., 2004). For example, one hot-spot mutation H1047R localized in kinase domain showed increasing lipid kinase activity in vitro and oncogenic transformation of cells (Kang et al., 2005). Another hot-spot mutation E545K localized in helical domain activates PI3K through disruption of inhibitory charge-charge interaction with N-terminal SH2 domain of p85 (Miled et al., 2007). Further studies using PIK3CA mutated colorectal cell lines HCT116 (H1047R) and DLD1 (E545K) showed that the two different mutations confer identical phenotypes and AKT mediated resistance to apoptosis is essential for growth factor independent cell proliferation caused by PIK3CA mutation (Samuels et al., 2005). Using human mammary epithelial cell system, H1047R and E545K were also shown oncogenic ability in vitro and in vivo which correlates with the kinase activities (Zhao et al., 2005). Mutation of other class I PI3Ks is rarely found.

Phosphatase and tensin homologue (PTEN), a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase, involved in negatively regulating intracellular level of PIP3 and PDK/AKT pathway (Cantley and Neel, 1999). It was identified as a tumor suppressor gene and found mutated in a large number of cancers.

The major PI3K effectors include RAC, PDK1, AKT and TEC family of cytoplasmic tyrosine kinases such as BTK, ITK (Yang et al., 2001). Since TEC family members are mostly involved in immune system signaling, here I will only focus on other PI3K effectors.

1.3.2. RAC

Small GTPase RAC can be activated through PI3K activation of PH domain-

containing nucleotide exchange factor RAC-GEFs (Mertens et al., 2003). RAC has three isoforms: RAC1, RAC2, and RAC3. RAC1 is ubiquitously expressed and most studied among the three isoforms. RAC can coordinate diverse cellular functions and is especially known for actin cytoskeleton regulation (Burridge and Wennerberg, 2004). RAC has many effectors including kinase such as p21-activated kinase (PAK) (Bokoch, 2003), scaffold protein and transcriptional factors (review (Bustelo et al., 2007)). The relationship between RAC and PI3K is more than one way regulation, RAC can also bind and activate PI3K and form a positive feedback loop (Tolias et al., 1995).

1.3.3. AKT

AKT, also known as protein kinase B, was originally identified as the cellular homolog of the transforming oncogene of the AKT8 retrovirus (Staal et al., 1977). Somatic mutations of AKT have been observed in cancers (Carpten et al., 2007; Davies et al., 2008; Parsons et al., 2005).

The PIP3 product of PI3K reaction leads to recruit some proteins containing pleckstrin homology (PH) domains to the membrane by direct binding. Ser/Thr kinase PDK1 and Ser/Thr kinase PKB/AKT are the key targets. AKT is phosphorylated at two key sites: Thr308 and Ser473. It has been well documented that AKT Thr308 of activation loop is phosphorylated by PDK1 (Alessi et al., 1997; Stephens et al., 1998) and Ser473 of hydrophobic motif at the C-terminal is phosphorylated by mTOR complex 2 (mTOC2) (Sarbassov et al., 2005), and together result in full activation of AKT. Studies on MT have shown that MT activation of AKT blocks apoptosis (Dahl et al., 1998; Meili et al., 1998).

AKT has three isoforms: AKT1, AKT2, and AKT3. Increasing evidence shows non-redundant functions in different AKT isoforms, for example, AKT1 is linked to cell growth and survival while AKT2 is linked to invasiveness. Study in colorectal cancers found that AKT2 is expressed in late-stage and metastatic tumors. Suppression of AKT2 expression inhibited the metastatic ability which can not be rescued by overexpression of wild type AKT1 (Rychahou et al., 2008). Study in mammary epithelial cells found distinct roles for AKT1and AKT2 in growth factor stimulated phenotypes. Knockdown of AKT1 in insulin-like growth factor-I receptor (IGF-IR) stimulated cells promoted epithelial-mesenchymal transition (EMT) and cell migration which is accompanied by ERK activation. Further, knockdown of AKT2 suppressed EMT induced by AKT1 downregulation (Irie et al., 2005). Presumably, knockdown of AKT1 alone shifts the balance of the signaling through AKT2. These works gave evidence of distinct functions of AKT isoforms also the importance of AKT1 in cross talk with ERK signaling pathway. Furthermore, in vivo study using polyoma middle T (PyMT) and ErbB2/Neu-driven mammary tumor mouse model addressed the effects of ablation of AKT1, AKT2, and AKT3 on the induction and the biology of mammary tumors. In contrast to AKT3 which has less significant effect, ablation of AKT1 inhibits, while ablation of AKT2 accelerates tumor induction. Detailed study in AKT1 knock out mice suggested the delayed tumor induction is due to the inhibitory effects on cell proliferation and survival by AKT1 ablation (Maroulakou et al., 2007).

AKT has many targets such as GSK3, TSC1/2, MDM2, mTORC1, and Forkhead, which makes AKT involved in many aspects of cellular processes including metabolism, cell survival, and protein synthesis (Fig. 5).

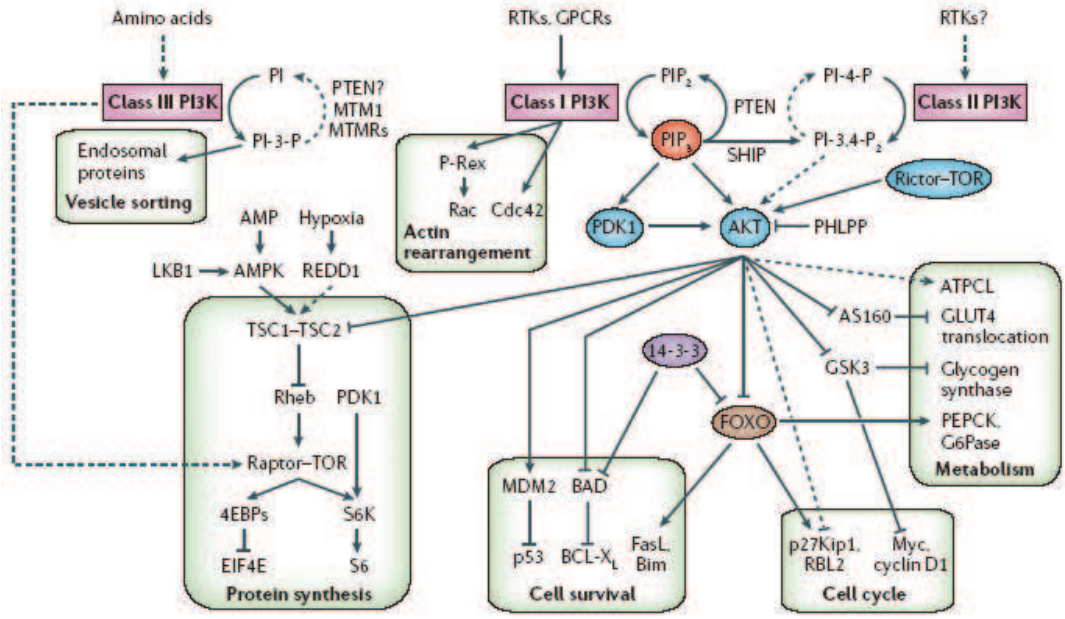


Fig. 5. PI3K/AKT signaling pathways (Engelman et al., 2006).

1.4. Cross talk between RAS/RAF/MEK/ERK and PI3K/AKT two signaling pathways

The cross talk between the two pathways occurs at more than one level. In response to certain growth factors, direct binding of RAS to PI3K enhances PI3K activation and is required for RAS induced tumorigenesis (Gupta et al., 2007). As mentioned earlier, PI3K/AKT can negatively regulate RAF-1 activity by phosphorylation on 14-3-3 protein binding sites (Zimmermann and Moelling, 1999). Also, study in Neuro2a cells showed suppression of MEK by inhibitor U0126 enhances AKT phosphorylation (Graham et al., 2006). More interestingly, study using prostate cancer cell lines found inhibition of ERK augments EGF induced PI3K/AKT activity through enhanced EGFR activation and

turnover (Gan et al.). Together, it indicates the reciprocal negative regulation of the two pathways.

PI3K and ERK pathways converge to regulating BH3-only family proteins in apoptosis and mTORC1 signaling in cell growth and protein translation. For example, both AKT and ERK can phosphorylate BH3-only family member Bim at different sites and regulate its pro-apoptosis function. AKT has been shown phosphorylation of Bim at Ser87 and association with 14-3-3 protein (Qi et al., 2006). ERK instead phosphorylates Bim at Ser69 and promotes its degradation (Luciano et al., 2003). The interaction between the PI3K/mTOR and RAS/ERK signaling has been also found in controlling translation initiation. It is well documented that PI3K/mTOR pathway stimulates two important translational regulators, the ribosomal S6 kinases and the eukaryotic initiation factor 4E. A recent study reported that RAS/ERK signaling promotes ribosomal protein S6 phosphorylation at Ser235/236 sites and stimulates cap-dependent translation (Roux et al., 2007), together with PI3K/mTOR1 signaling regulating protein translation.

Chapter II. Polyomavirus Middle T antigen suppresses the response of ERK to stress

Introduction

1. Stress and ERK activation

Cells are required to cope with a variety of stresses. Well-known stresses include physically damaging agents such as UV or γ irradiation, as well as chemicals that change ion concentrations or oxidation states. Infections with viruses also produce stress responses that can have a variety of effects on cell phenotype. Each kind of stress is associated both with responses that are specific to the kind of insult and responses that are relatively common to different kinds of stress. For example, the alterations in splicing of XBP1 seen in endoplasmic reticulum stress (Yoshida et al., 2001) or the activation of CHK1 kinase in response to UV irradiation (Walworth et al., 1993) are relatively specific responses. Inhibition of protein translation through the phosphorylation of eIF2alpha (Wek et al., 2006) or the activation of mitogen-activated protein kinases (MAPKs) would be more common outcomes of stress.

As mentioned earlier, the MAPKs are serine-threonine kinases that mediate intracellular signaling associated with cell proliferation, differentiation, survival, death, and transformation (Dhillon et al., 2007; Kim and Choi). The mammalian MAPK family includes extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase (JNK; also known as stress-activated protein kinase or SAPK). JNK and p38 have been particularly associated with stress responses, such as oxidative stress, endoplasmic

reticulum stress, calcium influx or UV (Nagai et al., 2007). ERK is more commonly considered in the context of cell proliferation (McCubrey et al., 2007), but can also be activated by stress (Nguyen et al., 2004).

ER stress and ERK activation

In eukaryotic cells, most membrane bound and secreted proteins fold and mature in the lumen of endoplasmic reticulum (ER). Cells can adjust the protein-folding capacity according to their physiological requirements. Under certain circumstances, failing to do this leads to the imbalance between the load of unfolded or misfolded proteins and the capacity of cellular machinery that copes with this load. This imbalance is called ER stress. The accumulation of unfolded proteins in the lumen of ER induces a set of signaling pathways called the unfolded protein response (UPR) (Ron and Walter, 2007).

The ER is highly sensitive to stresses that perturb cellular energy, Ca^{2+} concentration, and redox state (Gaut and Hendershot, 1993) which are required to allow optimum protein folding and modification. Physiological induction of ER stress and UPR occurs in differentiation and development of secretory cells such as plasma (Reimold et al., 2001) or pancreatic β cells, mutations in the genes encoding transmembrane proteins and infection by certain pathogens. The UPR also can be induced experimentally by inhibiting *N*-linked glycosylation in the ER or depleting ER calcium stores by drugs like tunicamycin and thapsigargin. Three sensors of ER stress have been discovered: Inositol-requiring enzyme-1 (IRE1)(Cox et al., 1993), protein kinase R-like ER kinase (PERK) (Harding et al., 1999) and the basic leucine-zipper transcription factor ATF6 (Haze et al., 1999). It has been reported that all three MAP kinase family members can be activated by ER stress through an IRE-1 dependent mechanism (Nguyen et al., 2004; Nishitoh et al.,

1998).

Oxidative stress and ERK activation

Reactive oxygen species (ROS) can be produced as a normal product of cellular metabolism, but also have the potential to cause cellular damage. One major contributor to oxidative damage is hydrogen peroxide (H_2O_2). Studies from ROS regulating T cell activation showed that protein tyrosine phosphatases (PTPs) are the targets of ROS, which inactivates catalytic cysteine residues of the enzymes. Peroxide stimulation of T cells induces phosphorylation of MAP kinase family members through direct inhibition of PTP activity of CD45, SHP-1, and HePTP. Inhibition of either phospholipase C (PLC) or protein kinase C (PKC) blocks peroxide induced ERK phosphorylation without affecting p38 and JNK (Lee and Esselman, 2002). In addition to affecting MAP kinase family phosphorylation, evidence showed that hydrogen peroxide also can induce ligand-independent activation of receptor tyrosine kinases (RTKs) through inhibiting receptor tyrosine dephosphorylation (Knebel et al., 1996).

UV irradiation and ERK activation

There are clear links between UV irradiation and p38 and JNK activation in the stress response pathway. Relative little study has been done to connect UV to ERK activation and evaluate consequence. Studies using human keratinocytes NCTC2544 suggested that the RAF-ERK pathway is required for UV-A induced AP-1 activation (Djavaheri-Mergny and Dubertret, 2001). Researchers also found that UV induced angiogenesis in human skin keratinocytes via ERK pathway (Kim et al., 2006).

Hypoxia and ERK activation

In the tumor microenviroment, hypoxia occurs and promotes angiogenesis,

metastasis and even resistance to therapy (Dewhirst et al., 2008). Responses to hypoxia through hypoxia-inducible factor family of transcription factors (HIFs) are best understood (Semenza, 2003). Study using human microvascular endothelial cells-1 (HMEC-1) has shown that ERK kinases are activated during hypoxia and HIF-1α is phosphorylated in hypoxia by an ERK-dependent pathway (Minet et al., 2000). Study in lung cancer cell lines A549 and NCI-H157 also showed hypoxia activation of ERK and AKT confers resistance to UV- mediated apoptosis (Lee et al., 2006). Research by using hypoxia-resistant T98G (HRT98G) malignant glioblastoma cells found that activation of ERK plays a pivotal role in resistance to death by chronic hypoxia (Kim et al., 2009).

2. Is ERK always activated in cancer cells?

Multiple studies have shown that constitutively activated MAPK signaling is related to malignancy (Dhillon et al., 2007; Mirzoeva et al., 2009). However, this is not always the case. A few studies including reverse phase protein array comparing normal prostate epithelium, prostate intraepithelial neoplasia (PIN), and invasive prostate cancer samples from patients showed cancer progression is associated with increased levels of AKT phosphorylation and decreased ERK phosphorylation. This pattern predicts poor clinical outcome in prostate cancer (Ghosh et al., 2005; Kreisberg et al., 2004; Paweletz et al., 2001). Further, in vitro studies using normal (PNT1A) and cancer (PC-3) prostate cell lines confirmed aberrant loss of ERK activity plays a pivotal role in the malignant phenotype (Moro et al., 2007). The interaction of PNT1A cells with extracellular matrix (ECM) leads to ERK activation that upregulates BRCA2, a tumor suppressor, and prevents aberrant cell proliferation. While in PC-3 cells, a defective ERK pathway leads

to unresponsive to adhesion to ECM. More strikingly, transfection of a constitutively active MEK1 that activates ERK is sufficient to reverse the PC-3 neoplastic phenotype through upregulation of BRCA2 and inhibition of DNA synthesis. The detailed mechanism of ERK suppression in PC-3 cells was not reported in that study.

Other studies showed the activation of ERK can affect the sensitivity of tumor cells to apoptosis. Evidence showed that activated ERK is required for drug-induced apoptosis or inhibition of cell proliferation in ovarian cancer cells (Kim et al., 2005). Study using RRR-α-tocopherol succinate (vitamin E succinate, VES), a potent apoptotic agent for cancer cells, found ERK and JNK, but not p38 are required for VES-induced apoptosis of human breast cancer MDA-MB-435 cells (Yu et al., 2001). Evidence from other studies showed both TGF-β and Fas signaling pathways could contribute VES-induced apoptosis (Simmons-Menchaca et al., 1995; Yu et al., 1999). TGF-β is known to be a potent growth inhibitor of breast cancer cells. In TGF-β sensitive breast cancer cells, ERK2 and JNK are involved in TGF-β-mediated negative cell growth control (Frey and Mulder, 1997). Using Fas-sensitive neuroblastoma cell line, Goillot *et al.* found ERK and JNK mediated Fas-induced apoptosis. More interestingly, ERK and JNK cooperated in different aspects of apoptosis process. ERK activation was transient and associated with Fas receptor induction, while JNK activation was sustained and correlated with the onset of apoptosis (Goillot et al., 1997).

Results

Part I: MT suppresses ERK activation subjected to stress

2.0. MT can activate ERK under normal growth condition in some cells

The conventional view of ERK activation pathway is: MT (Growth Factor) →Adaptor (Shc/Grb2)→SOS→RAS→RAF→MEK→ERK (Fig.6. left panel). Phosphorylation of MT of Y250 is known to activate the adaptor Shc that leads to the activation of RAS. This is expected to lead to the activation of RAS effectors including RAF. In turn this is expected to lead to the activation of ERK1 and ERK2. As expected from such a pathway, MT can activate RAS and its downstream target ERK in MT-transformed NIH/3T3 cells. As seen in Fig.6 (right panel), MT-NIH/3T3 cells activate RAS measured by RAS-GTP compared to NIH/3T3 cells in normal growth condition. As measured by ERK phospho-specific antibody, MT-NIH/3T3 cells activate ERK.

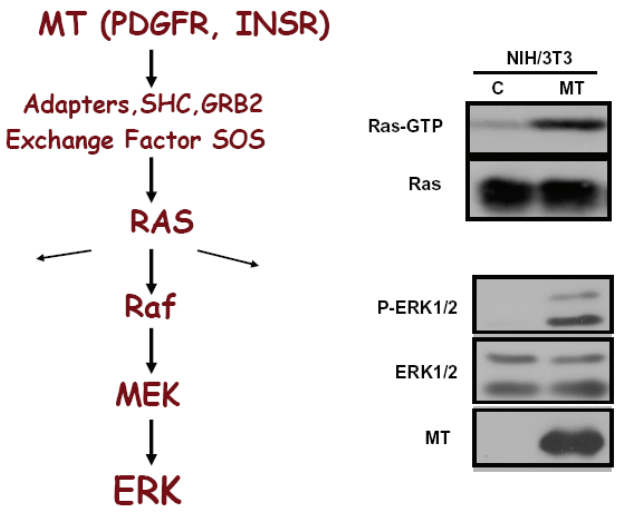


Fig. 6. MT activates RAS and its downstream ERK in NIH/3T3 cells.
Left: a simplified diagram of the signaling cascade through phosphorylation of Y250 of MT. **Right:** NIH/3T3 and MT-NIH/3T3 cells were tested for RAS activity by comparing levels of RAS-GTP measured by immunoprecipating GST-RAS binding domain of RAF to total RAS. Activation of ERK was measured by phospho-specific ERK antibody.

2.1. ERK activation is a common response to many stresses

To examine whether stress stimulates ERK activation, normal mouse mammary gland epithelial cells (NMuMGs) (Bradbury and Edwards, 1988) were subjected to different stresses. Those stress conditions included overnight starvation in serum-free medium, hypoxia, UV irradiation, drugs such as thapsigargin, which triggers the unfolded protein response (also called ER-stress), peroxide to produce oxidative stress and cycloheximide to block protein synthesis. All these stimuli caused ERK activation as measured by phosphospecific antibody recognizing phosphorylation of the activation loop residues Thr202/Tyr204 and Thr185/Tyr187 of ERK1 or ERK2 (Fig.7.A). Consistent with the activation of ERK, p90RSK kinase, a downstream target of ERK, is phosphorylated at its T573 ERK phosphorylation site after cycloheximide treatment (Fig.7.B).

How does ERK get activated? Ordinarily, an increase in MEK activation would be expected. To find out whether MEK is also activated by stress, cells were treated by two different kinds of stress, UV irradiation or cycloheximide. Compared to untreated cells, stress activated MEK as measured by phosphospecific antibody recognizing phosphorylation of MEK activation loop residue Ser221 (Fig.7.C). It is well documented that MEK can be activated by serum stimulation. Interestingly, though the phospho-MEK levels under both stress conditions were different and less than after a serum stimulation, there seems no significant difference in phospho-ERK signals. This result suggests different stimuli may affect ERK activation through different levels of regulation. These might include ERK kinase MEK, scaffold protein and/or ERK phosphatases. The outcome of ERK signal is the balance of activation signals and inactivation signals.

To further confirm that MEK is the kinase that phosphorylates ERK in cells subjected to stress, the specific MEK inhibitor U0126 was used to see if it would block stress induced ERK activation. Pretreated cells with specific MEK inhibitor U0126 completely blocked ERK phosphorylation occurring in response to cycloheximide treatment (Fig.7.D.). The same results have been obtained with U0126 after other stresses as well (not shown). Overall, these results show the activation of ERK in response to a wide variety of different stresses through a mechanism that requires MEK.

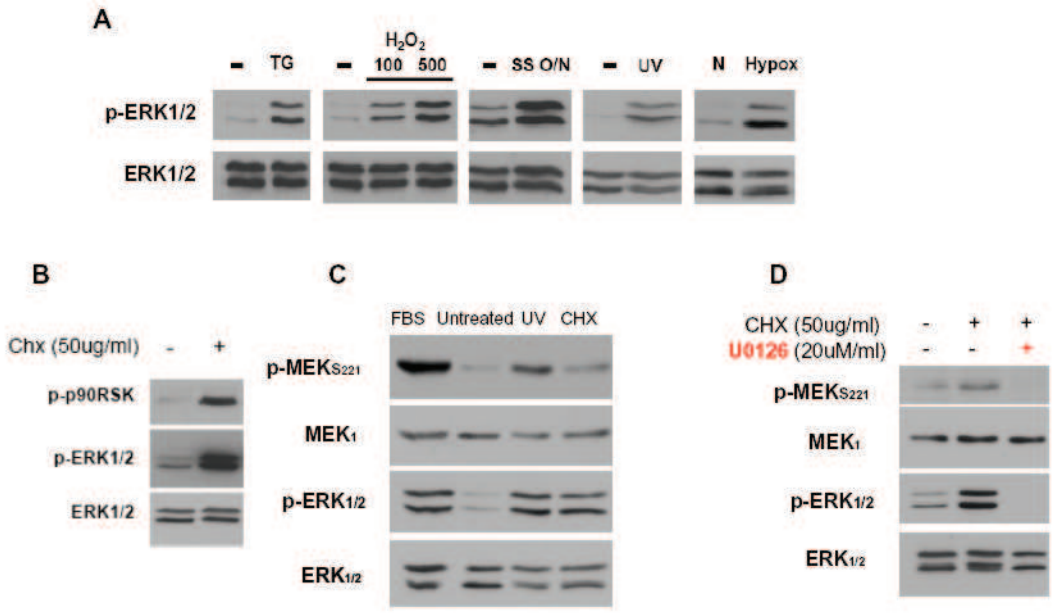


Fig. 7. ERK activation is a common response to many stresses.
A. Different kinds of stress activate ERK. NMuMG cells were untreated, treated with thapsigargin (TG) 500nM, peroxide (H_2O_2) 100 or 500 µM, UV (240J/m^2) irradiation, 0% serum overnight (SS O/N), or were transferred to 0.2% O_2 hypoxic conditions (Hypox) for 20h. Whole cell lysates were collected and western blots were performed, Results are representative of experiments performed at least three times.

B. Activation of ERK leads to activation of p90 RSK. NMuMG cells were untreated or treated with CHX (50µg/ ml) for 30 min. Whole cell lysates were blotted with indicated antibodies.

C. Different kinds of stress activate MEK to different levels. NMuMG cells were serum starved for 4 hours and then stimulated with 10% fetal bovine serum (FBS) for 10 min (as pos. ctl.), exposed to UV (240J/m^2) irradiation, or 50µg/ ml cycloheximide (CHX) for 30 min. Whole cell lysates were blotted with indicated antibodies.

D. Inhibition of MEK by U0126 blocks ERK. NMuMG cells were pretreated with DMSO or U0126(20µM) for 30 min, and then stimulated with CHX (50µg/ ml) for another 30 min. Whole cell lysates were blotted with indicated antibodies.

2.2. MT suppresses ERK phosphorylation response to stress

NMuMGs were transformed by wild type MT using a retroviral expression vector. As for many other cell lines, expression of MT is sufficient to cause NMuMGs to grow in soft agar without cooperation from any other polyoma oncogene (Fig.8A). MT-transformed NMuMGs strongly suppressed ERK activation resulting from six different kinds of stress: serum starvation, hypoxia, ER-stress (thapsigargin treatment), UV irradiation, peroxide stress, and cycloheximide treatment (Fig.8B&C.). The suppression of ERK was confirmed in the cases of peroxide and cycloheximide treatment by the concomitant suppression of p90 RSK phosphorylation (Fig.8C.).

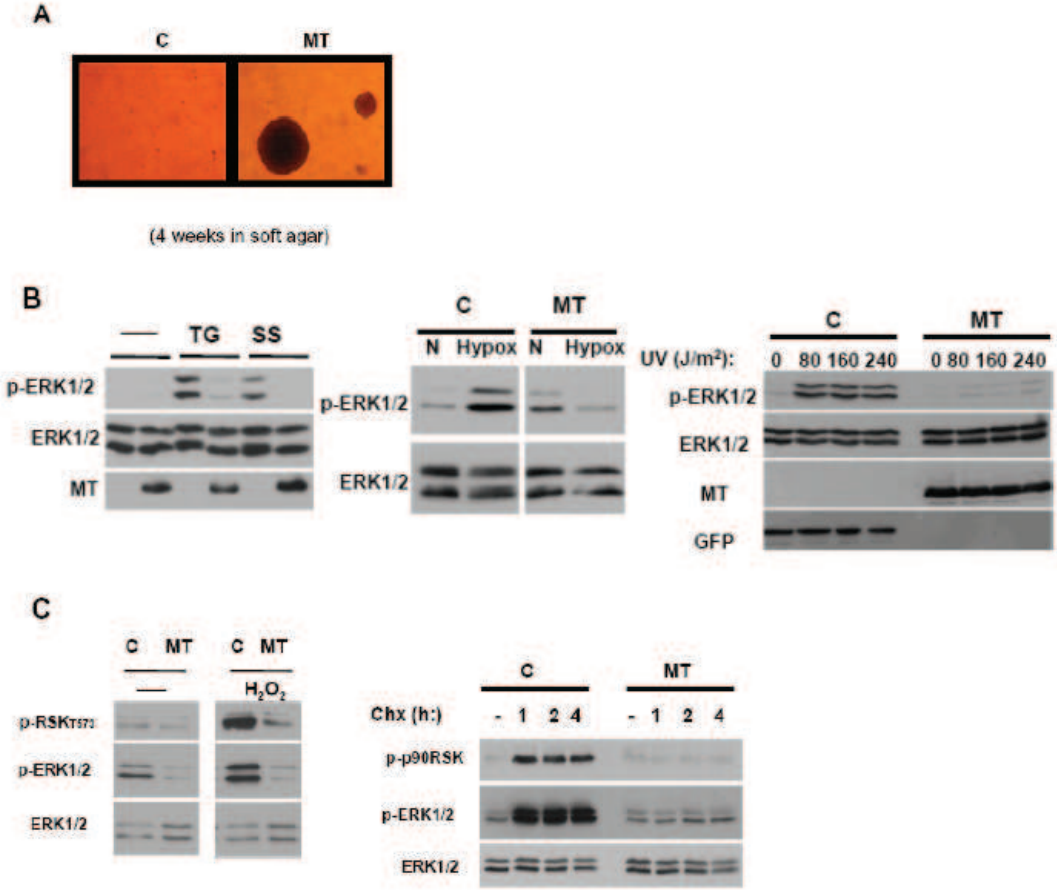


Fig. 8. MT suppresses ERK phosphorylation response to stress.

A. MT transforms NMuMGs. Soft agar colony growth of NMuMG stable cell lines expressing GFP or MT. The assay was carried out as described in Materials and Methods.

B. MT transformed NMuMGs strongly suppressed ERK activation resulting from stress. NMuMG and MT-NMuMG cells were untreated, treated with TG (500 nM) for 30min, starved with 0% serum overnight, treated under hypoxic conditions (Hypox) for 48h, or exposed to different doses of UV irradiation. Whole cell lysates were collected and blotted with indicated antibodies. Results are representative of experiments performed at least three times.

C. Suppression of ERK is confirmed by blotting downstream targets. NMuMG and MT-NMuMG cells were untreated or treated with 100μM peroxide for 30min or cycloheximide (50μg/ ml) for the number of hours shown. Western blots were performed.

2.3. MT does not affect all the aspects of stress pathways

To examine whether MT affects ERK response specifically or suppresses the entire stress response, two kinds of well-characterized stress, ER stress and UV, were tested. ER stress induces IRE1 activation. IRE1 is a transmembrane kinase/endoribonuclease (RNAse). Upon activation, IRE1 initiates alternative splicing of Xbp-1 mRNA (Fig.9A.**Left**). RT-PCR shows that NMuMG cells responded to ER stress induced by thapsigargin by producing the alternatively spliced S-form of Xbp-1. This alternative splicing was not seen when cells are stressed by serum starvation. The expression of MT had no effect on this alternative splicing (Fig.9A.**Right**). ER stress also induces ATF6, BIP and the phosphorylation of PERK, a kinase that regulates translation. MT did not block any of these effects in thapsigargin-treated cells (not shown). Thus, although MT blocked ERK activation, it did not block other aspects of the stress response. UV irradiation activates ATM/ATR which leads to CHK1 phosphorylation (Fig.9B.**Left**). Although MT reduced activation of ERK in response to UV irradiation, it had no effect on the activation of CHK1 as shown by phosphospecific S345 antibody (Fig.9B.**Right**). This again indicated that effect of MT is specific to ERK and not general.

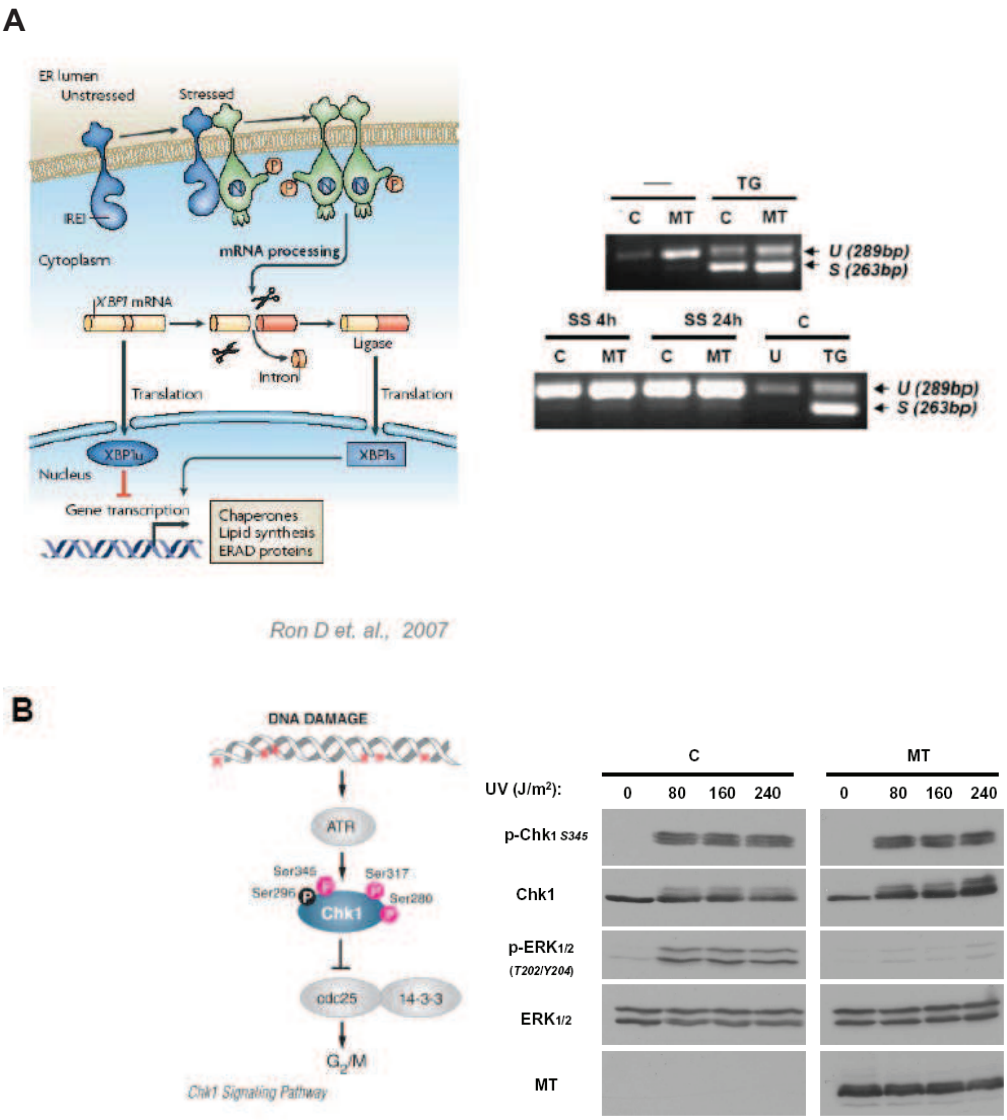


Fig. 9. MT does not affect all the aspects of stress pathways.

A. MT does not block Xbp-1 mRNA splicing induced by ER stress. NMuMG and MT-NMuMG cells were untreated, starved with 0% serum (SS) for the indicated times or treated with TG (500nM) for 1 hour. Xbp-1 mRNA splicing was determined by RT-PCR. Unspliced (U) and spliced (S) Xbp-1 mRNA products are indicated.

B. MT does not prevent CHK1 phosphorylation induced by UV irradiation. NMuMG and MT-NMuMG cells were exposed to UV irradiation at indicated doses, whole cell lysates were collected 30 min later and western blots were performed.

2.4. MT suppresses ERK specifically but not all the MAP kinase family members

Other members of MAP kinase superfamily, p38 and stress-activated protein kinase/Jun N-terminal kinase SAPK/JNK, are known preferentially activated by a variety of environmental stresses including UV and inflammatory stimuli. To see whether MT suppression entended to other MAP kinase family members, cells were exposed to UV irradiation at different doses. MT has no effect on the activation of p38 by UV. Results with JNK were intermediate (Fig.10.). MT can certainly modulate the JNK response, but its activity is clearly weaker than its effect on ERK. Taken together, these results show that MT strongly suppresses ERK activation resulting from stress, but does not affect all the aspects of stress pathways, not even all MAP kinase family responses.

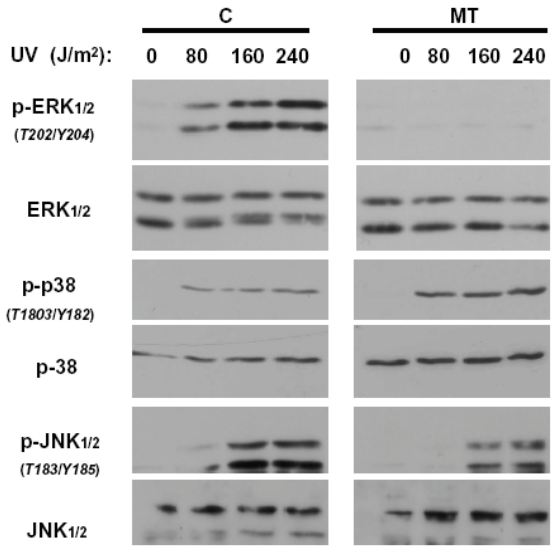


Fig.10. MT suppresses ERK specifically but not all other MAP kinase family members. NMuMG and MT-NMuMG cells were untreated or exposed to UV irradiation at indicated doses, whole cell lysates were collected 30 min later and three MAP kinase family members were detected by immunoblotting with total and phospho-specific antibodies. Results are representative of experiments performed at least three times.

2.5. MT affects ERK activation at multiple points: involvement of ERK Kinase and ERK phosphatases

The suppression of ERK exerted by MT was unexpected. As mentioned earlier, phosphorylation of Y250 is important for polyomavirus transformation. This phosphorylation has been thought to act in a conventional manner, setting off a cascade: MT (Growth Factor) → Adaptor (Shc/Grb2) → SOS→ RAS→ RAF→ MEK→ERK. To understand how MT might intervene to prevent ERK activation, the pathway upstream of ERK was examined.

2.5.1. MT activates RAS

First, we looked at RAS. RAS is a small GTP-binding protein. Upon activation, RAS becomes GTP-bound form and undergoes conformational change and recruits target effectors, for example RAF, to the cellular membrane. As expected from previous work, MT transformed cells showed a higher level of activated RAS compared to control cells. This is shown by the amount of GTP-RAS (Fig.11.). Unexpectedly, RAS activity was increased by 0% serum starvation overnight compared to normal growth conditions. It is unclear what mechanism underlies this observation, since it is well known that RAS is usually activated by growth factor and mitogens, though there are reports showing that some stress conditions such as oxidative stress can activate RAS (Aikawa et al., 1997). Also surprisingly, TG treatment did not cause RAS activation as measured by RAS-GTP though ERK is activated shown in Figure 2. This result suggests ERK activation under ER stress is not through RAS pathway. I will discuss this more in detail later. The key result is that in every case there is more RAS-GTP in the MT-NMuMG samples than in the NMuMGs, even though MT is suppressing ERK after stress.

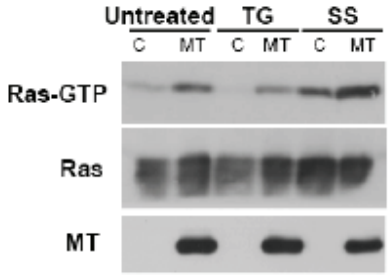


Fig. 11. MT activates RAS. NMuMG and MT-NMuMG cells were untreated, treated with TG (500nM) for 30 min, or starved with 0% serum medium (SS) overnight. RAS activation assays were performed as described in Materials and Methods. Results are representative of experiments performed at least three times.

2.5.2. MT activates RAF

RAF, a serine/threonine (S/T) kinase, is the main effector recruited by GTP-bound RAS to activate the MEK/ERK pathway. RAF activation is a complex process. As noted earlier, there are three isoforms of RAF family: A-RAF, B-RAF, C-RAF (RAF-1) and at least 10 regulatory sites of phosphorylation. Activation of C-RAF is the best understood and involves phosphorylation at multiple activating sites including Ser338, Tyr341, Thr491, Ser494, Ser497 and Ser499. p21-activated protein kinase (PAK) has been shown to phosphorylate C-RAF at Ser338 (King et al., 1998) and the Src family phosphorylates Tyr341 (Fabian et al., 1993) to induce C-RAF activity. First we chose to look at one RAF activation site: Serine 338, by phospho-Serine 338 specific antibody. We found MT activates C-RAF S338 under both normal and stress conditions (Fig.12.A). Since a single phosphorylation is not sufficient for C-RAF activation (Chong et al., 2001), to further determine whether MT activates C-RAF activity or not, a RAF kinase assay was performed. To get rid of background signal from serum caused RAF activation, 0.2% serum starvation overnight condition was used. It is well documented that some fibrolasts activate C-RAF in response to growth factor such as PDGF, therefore Balbc/3T3 clone A31 cells stimulated with PDGF were used as a positive control for C-RAF kinase assay (Fig.12.B, lane 1). Unlike fibroblast Balbc/3T3 clone A31 which easily shows C-RAF kinase activity stimulated by PDGF, C-RAF kinase signals were not seen until after longer exposure in NMuMGs under stress condtions (Fig.12.B). The kinase results are not very satisfying. However, in both A31 cells and NMuMG cells, MT activates C-RAF rather than suppressing it, where MT-NMuMGs showed increased activity after TG treatment compared to NMuMGs. Similar results were seen in a B-RAF kinase assay.

When normalized to the amount of protein immunoprecipitated, it is easier to see that MT still activates B-RAF (Fig.12.C). As expected, B-RAF has relative higher basal activity than C-RAF (need relative less exposure time to get B-RAF kinase signals than C-RAF kinase signals under same stress conditions) owing to the negative charges at N-region of kinase domain.

In consistent with RAS activation assay results, TG treatment (ER stress) did not activate RAF based on our data. It suggests other pathways rather than RAS/RAF are involved in activation ERK under ER stress. Since B-RAF kinase assay was done once, it needs further study to confirm whether TG enhances MT B-RAF activity.

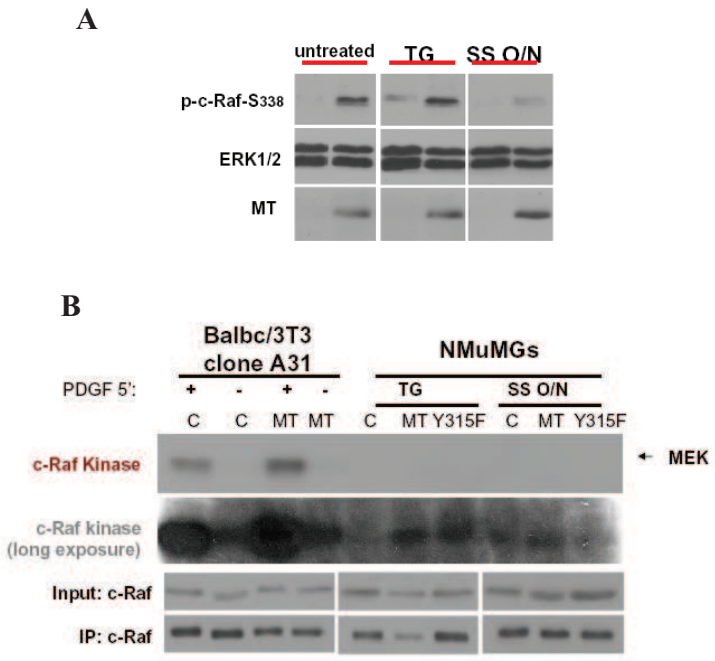


Fig. 12. MT activates RAF.
A. MT activates phospho-C-RAF (S338). NMuMG and MT-NMuMG cells were untreated, treated with TG (500nM) for 30 min, or starved with 0% serum (SS) overnight. Whole cell lysates were blotted with phospho-C-RAF (S338), ERK, and MT.

B. MT activates C-RAF kinase activity in NMuMGs. NMuMG, MT-NMuMG, and Y315F-NMuMG cells were treated with TG (500nM) for 30 min, or starved with 0.2% serum (SS) overnight. Cell extracts were immunoprecipitated using C-RAF antibody and a RAF kinase assay was performed as described in Materials and Methods. Balbc/3T3 clone A31 cells starved with 0.2% serum (SS) overnight and then treated with PDGF for 5min were used as a positive control for kinase assay. Results are representative of experiments performed at least twice.

C

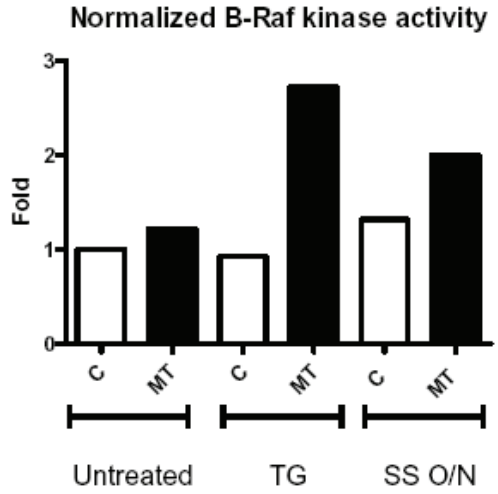


Fig. 12. MT activates RAF (continue).
C. MT activates B-RAF kinase activity. NMuMG and MT-NMuMG cells were untreated, treated with TG (500nM) for 30 min, or starved with 0.2% serum (SS) overnight. Cell extracts were immunoprecipitated using B-RAF antibody and a RAF kinase assay was performed as described in Materials and Methods. The bar represents B-RAF kinase activity normalized to immunopreciptated B-RAF in one experiment.

2.5.3. MT can affect MEK

MEK1/2, also called mitogen-activated protein kinase (MAPK) kinase or ERK kinase, is a tyrosine(Y-) and S/T-dual specificity protein kinase. All three members of RAF family are able to phosphorylate and activate MEK. Activation of MEK1/2 occurs through phosphorylation of two serine residues at positions 217 and 221 (in the activation loop of subdomainVIII) (Alessi et al., 1994). Specific phospho-MEK1/2 Serine221 antibody is used to test whether MEK is activated. Either the stress of serum-starvation or induction of ER stress with thapsigargin produced an increase in phospho-MEK in NMuMG cells. While there was more phospho MEK signal in untreated MT-NMuMG cells than the untreated NMuMGs, there appeared to be no change or even a decrease in MT-NMuMGs after stress (Fig.13.). This suggested that MT may have reduced MEK activity in response to stress, but the modest effect after TG, for example, did not seem to be sufficient to explain the striking effect in ERK.

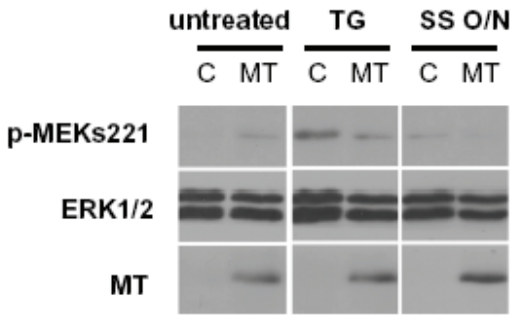


Fig. 13. MT can affect MEK. NMuMG and MT-NMuMG cells were untreated, treated with TG (500nM) for 30 min, or starved with 0% serum (SS) overnight. Phospho-MEK (S221), ERK and MT were determined by western blot. Results are representative of experiments performed at least three times.

2.5.4. MT activates ERK phosphatase activity

The level of ERK activity in cells is balanced by its phosphorylation by MEK and its dephosphorylation by phosphatases. As discussed previously, the activation of ERK requires both threonine and tyrosine phosphorylation while dephosphorylation of either residue is sufficient to inactivate ERK. In vivo studies from yeast to humans showed that all three major classes of protein phosphatase: serine/threonine phosphatases, tyrosine specific phosphatases and dual-specificity phosphatases can inactivate ERK as well as other MAP kinase family members.

To test whether phosphatase activity might be involved in the regulation of ERK activity, ERK phosphate turnover assays were performed. For these experiments, U0126 was added to prevent further ERK phosphorylation, and loss of ERK phosphate was assessed using a phosphospecific ERK antibody. In untreated NMuMG cells the half-life of ERK phosphate was a little over five minutes, and the signal was largely gone within twenty minutes. In MT cells, the signal was largely gone in five minutes (Fig.14.A). When cycloheximide was added to control cells, ERK phosphorylation appeared to be marginally more stable, but again the signal was largely gone in twenty minutes. To do a time course in MT cells, it was necessary to use more protein, because the amount of ERK phosphorylation was much lower. Nonetheless, the effect was quite striking. In MT cells, the half-life appeared to be on the order of one minute and the signal was largely gone in five minutes (Fig.14.B). This result showed MT substantially enhanced the rate of dephosphorylation of ERK.

To determine whether the enhanced rate of ERK dephosphorylation was unique to certain stress situations, the dephosphorylation rate was also examined after other stimuli.

Cells were serum starved for four hours, a treatment that does not activate ERK, and then stimulated with fetal calf serum (Fig.14.C) or treated with peroxide (Fig.14.D). As in the case of cycloheximide treatment, the loss of ERK phosphate in MT cells was more pronounced than in the controls, suggesting that MT activates dephosphorylation under all conditions. Since MT affects JNK activation partially, we wondered whether both JNK and ERK phosphate turnover were affected under stress. To test this, both phospho-JNK and phospho-ERK were blotted at different time point post exposure to UV irradiation (Fig.14.E). The phospho-JNK signal duration was similar in both control and MT cells. In contrast, phospho-ERK signal duration was very different. In control cells, phospho-ERK signal is persistent until at least 60 mins post UV exposure without MEK inhibitor. While in MT cells, the phospho-ERK signal was hardly detected after 20 mins even without MEK inhibitor.

Together, my data suggests a model in which MT may affect both ERK kinase and phosphatase activities under stress. ERK phosphate is a balance of ERK phosphorylation and dephosphorylation. In NMuMG cells, stress causes this balance to shift and ERK activation by activating MEK or inactivating ERK phosphatase (or both). In MT-NMuMG cells, even without stress, ERK phosphate turnover is significantly faster than NMuMGs (Fig.14.F, Left Panel). Under stress, MT reduces MEK activation and at the same time ERK phosphatase activity is constitutively activated by MT. MT uses both type of regulation: ERK kinase and ERK phosphatase to suppress ERK (Fig.14.F, Right Panel).

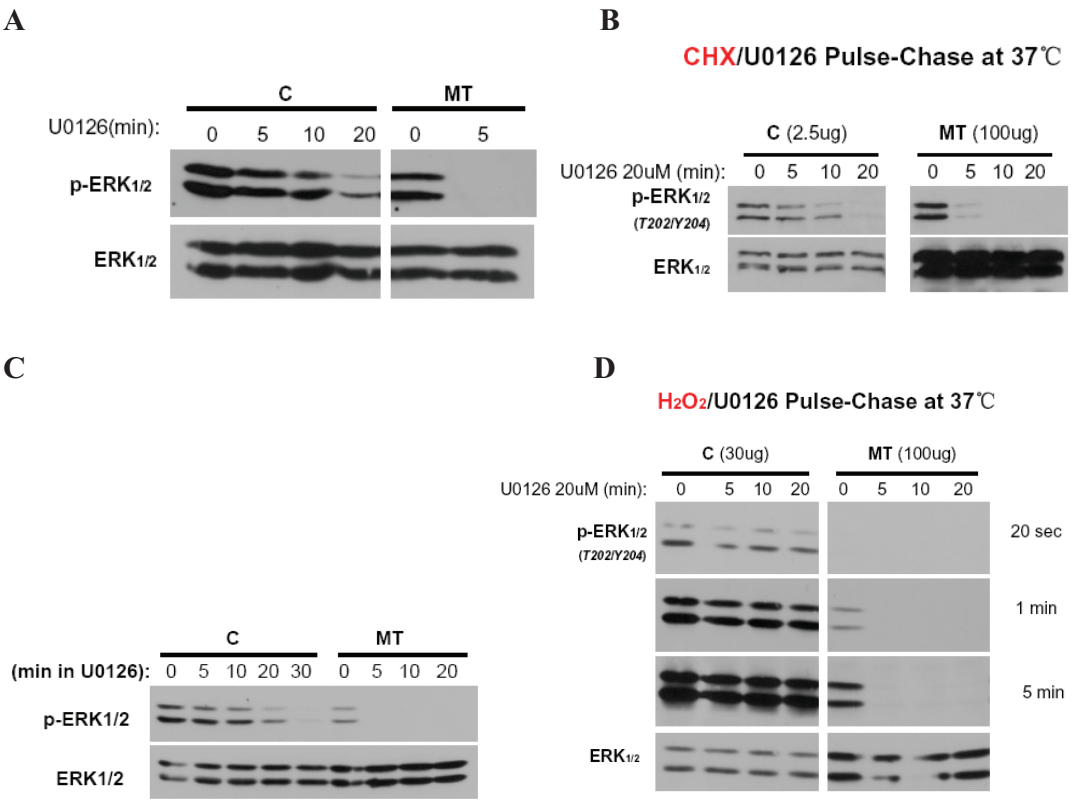
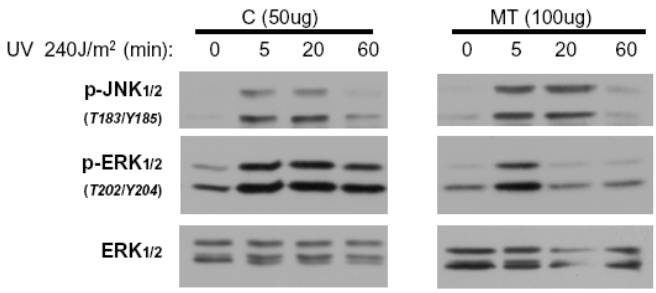


Fig. 14. MT activates ERK phosphatase activity.
Cells were untreated (**A**) or treated with CHX (50µg/ml) for 30 min (**B**), starved with 0%FCS for 5 hours then stimulated with FCS for 15min (**C**), or exposed to hydrogen peroxide (100µM) (**D**)and then exposed to MEK inhibitor U0126 (20µM). A chase assay was performed in which cells were extracted at the indicated time after U0126 treatment. Whole cell lysates were blotted with phospho-ERK and total ERK. Results are representative of experiments performed at least three times.

E



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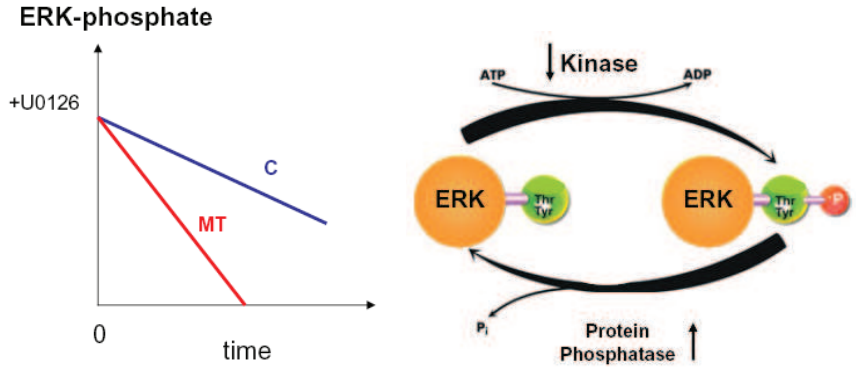


Fig. 14. MT activates ERK phosphatase activity (continue).
E. Cells were exposed to UV 240J/m^2. A "chase assay" was performed in which cells were extracted at the indicated time and blotted with phospho-JNK, phospho-ERK and total ERK.**F. Model of MT regulation of ERK activity. Left:** MT increases ERK-phosphate turnover. **Right:** MT can suppress ERK kinase (MEK) increases in response to stress and increase ERK phosphatase activity to regulate ERK.

2.6 DUSPs and ERK regulation

2.6.1. Okadaic Acid effects on ERK phosphorylation

Inactivation of ERK only requires dephosphorylation one of the threonine and tyrosine residues in the activation loop. Therefore, either a Thr/Ser phosphatase, a tyrosine phosphatase or a dual specific phosphatase (DUSP) could do the job (Fig.15A.). Since MT associates with PP2A, the possibility that the MT complex acts on ERK directly was considered. We first tested okadaic acid (OA) which has been shown to inhibit PP2A (and PP1 with a higher IC50) (Ishihara et al., 1989). Cells were treated with different doses of OA and blotted with phospho-MEK and phospho-ERK antibodies. With increasing of OA doses, both phospho-MEK and phospho-ERK were dramatically upregulated in NMuMG cells especially when OA is higher than 100nM. Surprisingly, when compared MT cells to control cells, phospho-MEK was upregulated the same way in both cells, but OA had much less effect on phospho-ERK in MT cells than in NMuMG cells (Fig.15B.). This result suggests MT still increases ERK phosphatase activity when OA is present. To confirm OA does not affect MT regulation of ERK through phosphatase, ERK phosphate turnover was measured after OA treatment (Fig.15C). The ERK phosphate turnover was slowed down in NMuMG cells by OA. In MT cells, it is still hard to see phospho-ERK signal at 5 min after adding U0126, indicating that ERK phosphate turnover was still fast. This result indicates that PP2A or PP1 is not likely to be involved in MT negatively regulation of ERK. As discussed below, the MT genetic analysis of the effect on ERK showed that PP2A binding was not sufficient.

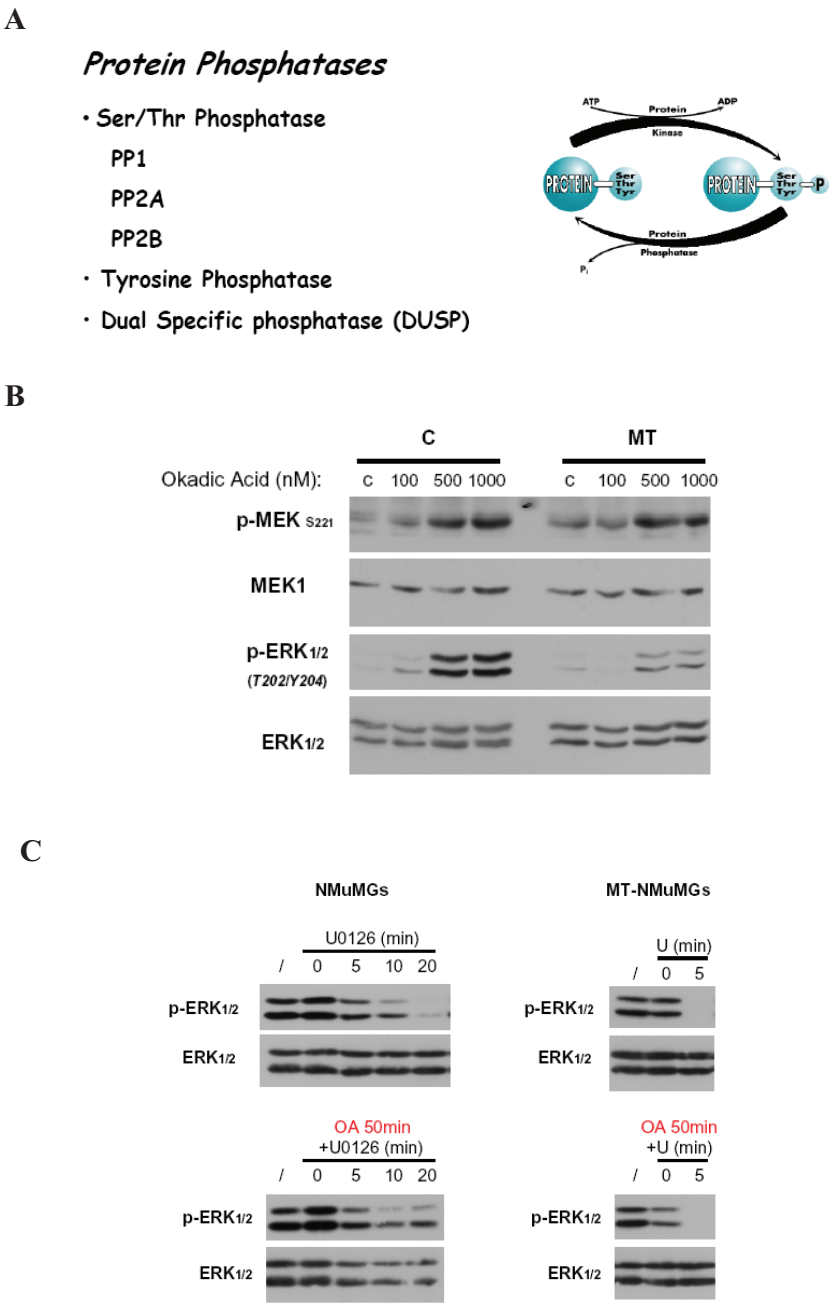


Fig. 15. Inhibition of PP2A & PP1 by okadaic acid (OA) has no significant effect on ERK suppression by MT. A. Classes of protein phosphatase. **B**. Okadic acid titration. NMuMG and MT-NMuMG cells were treated with different concentration of OA for 50 min, then cell exacts were blotted with indicated antibodies. **C**. Cells were untreated or treated with OA for 50 min then ERK phosphate turnover assay was performed. U (U0126).

2.6.2. VHR (DUSP3) and ERK regulation

There is a big family of dual specificity protein phosphatases (DUSPs) which dephosphorylate both tyrosine and serine/threonine residues within the TXY motif of the MAP Kinase family. Given the rather limited number of MAPK family kinases, it is surprising that the DUSP family is so large (Patterson et al., 2009). MAPK phosphatases vaccinia H1-related (VHR, also called DUSP3) and MKP1 (DUSP1) have been reported upregulated in cervical carcinoma cell (Henkens et al., 2008) and breast cancer respectively (Wang et al., 2003). This makes VHR and MKP1 interesting targets to examine. Interestingly, VHR was found increased in the MT-NMuMGs while MKP1 was not (Fig.16A.). To see if VHR was responsible for the MT phenotype, 5 shRNAs were used to knock it down. While three of these (e.g. shRNA1) had only modest effects, two, including shRNA2, were quite effective at knocking down the protein. A constitutive increase in phospho-ERK in the knockdown cells suggested that VHR did contribute to regulating ERK (Fig.16B). When cells were starved with 0% serum for 5 hours and then exposed to serum, knock down of VHR slightly prolonged ERK phosphorylation in MT cells, which was not due to prolonged MEK activation (not shown) (Fig.16C Upper panel). However, judged by the increase in ERK phosphorylation to CHX (Fig.16C Bottom panel), knocking down VHR alone is not sufficient to restore ERK phosphorylation in MT. This suggests that MT is coordinately regulating other DUSP family members as well.

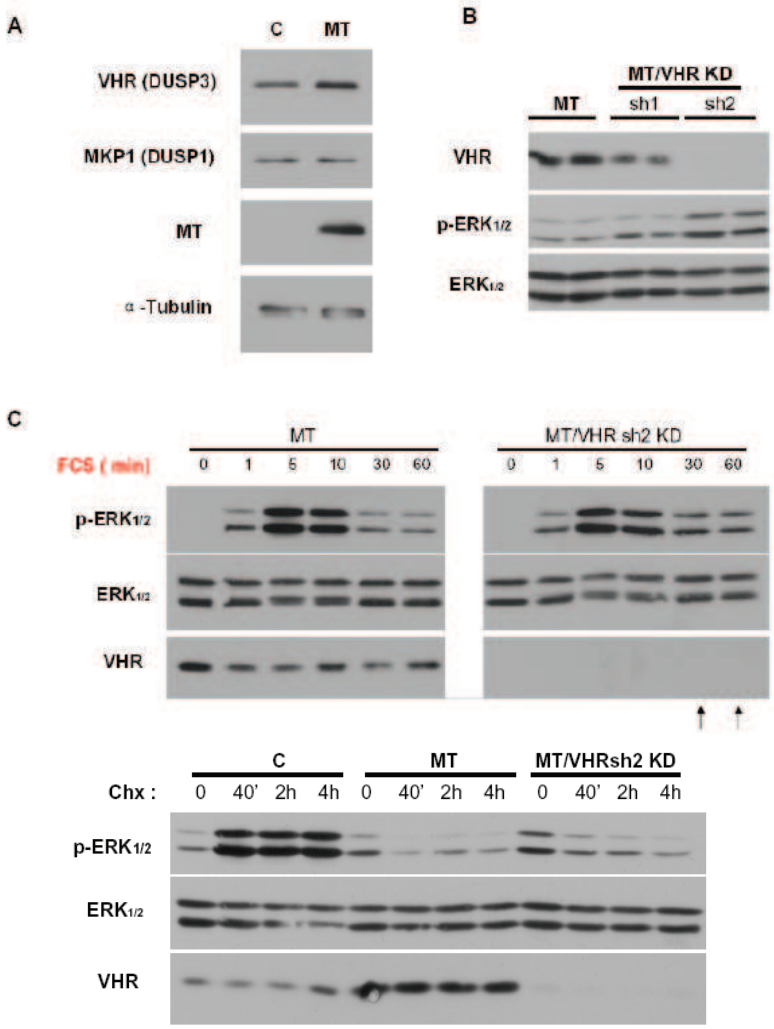


Fig.16. VHR (DUSP3) and ERK regulation.

A. VHR is upregulated in MT-NMuMGs. Cell lysates from NMuMGs (C), MT-NMuMGs were immunoblotted with antibodies for VHR, MKP1, MT and α-Tubulin.

B. Knock down of VHR by shRNA in MT-NMuMGs increases basal level of ERK phosphorylation. MT-NMuMGs and stable VHR KD MT-NMuMG cell lines were tested by blotting VHR and phospho-ERK. Samples were loaded as duplicates.

C. Knock down VHR alone is not sufficient to reverse ERK suppression in MT-NMuMGs. MT-NMuMGs and VHR KD MT-NMuMGs were starved with 0%FCS for 5 hours then stimulated with FCS for indicated time (upper panel). NMuMGs, MT-NMuMGs and VHR KD MT-NMuMGs were treated with CHX (50μg/ml) for indicated time (bottom panel). Whole cell lysates were collected and western blots were performed.

2.6.3. MKP3 (DUSP6) and ERK regulation

In support of the idea that multiple family members might be involved, another DUSP family member MKP3 (DUSP6) was found upregulated at the mRNA level by gene array and realtime-PCR (Fig.17A). Again 5 shRNAs were used to try to knock it down, but the best knock down at RNA level is about 50% (Fig.17B). To see whether the MKP3 knock down affects ERK suppression by MT, phospho-ERK signals were compared in NMuMG, MT- NMuMG and MKP3 knock down MT- NMuMG cells. No significant increase of phospho-ERK signals by MKP3 knock down was detected under stress (Fig.17C). Like the VHR knockdown, this suggests knock down single DUSP is not sufficient to reverse ERK suppression by MT. Knockdown both VHR and MKP3 in single cell should be tested to see whether it has bigger effect on ERK than knocking down single DUSP.

Together, those results suggested MT increases DUSP activity but could use multiple levels of regulation (kinase and phosphatase) to suppress ERK.

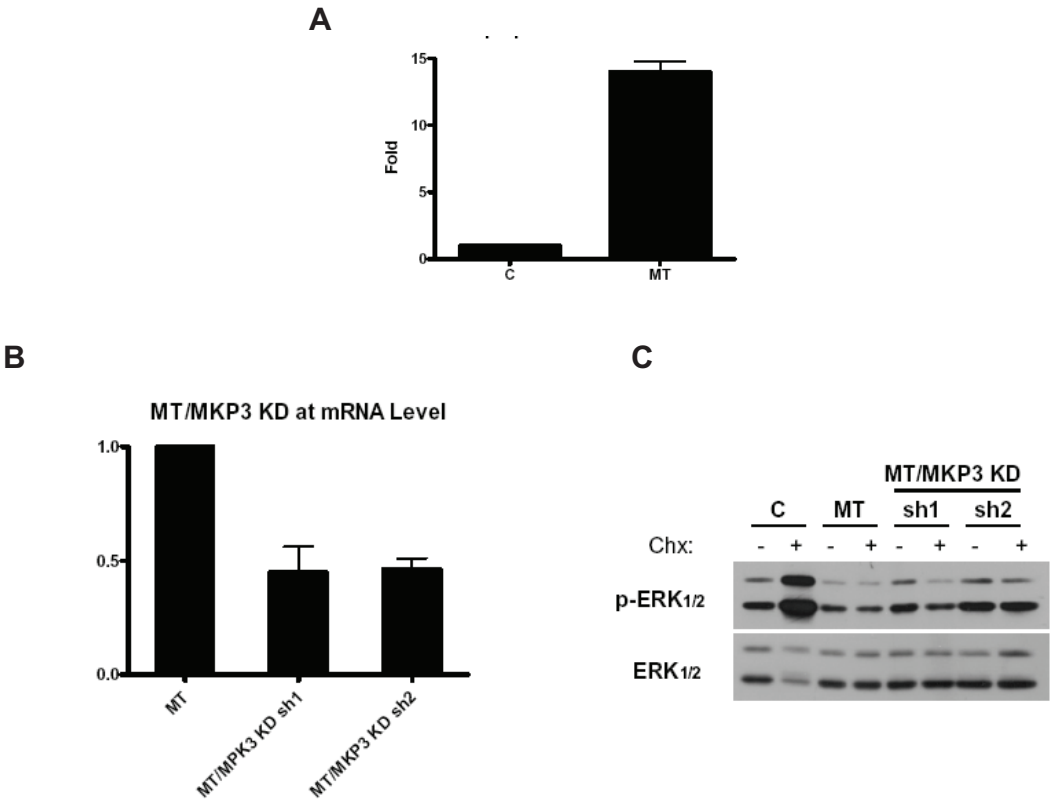


Fig. 17. MKP3 (DUSP6) and ERK regulation.

A. MKP3 is upregulated in MT-NMuMGs at RNA level. The level of MKP3 was quantified at mRNA level by Quantitative-PCR. Bars represent Mean+ SEM.

B. Knock down of MKP3 by shRNA in MT-NMuMGs at mRNA level. The level of stable MKP3 knock down in MT-NMuMG cells was quantified at mRNA level by Quantitative-PCR. Bars represent Mean+ SEM.

C. MKP3 knock down alone is not sufficient to reverse ERK suppression in MT-NMuMGs. Cells were untreated, or treated with CHX (50μg/ml) for 30 min. Cell lysates were collected and immunoblotted with phospho-ERK and total ERK.

2.7. MT genetic analysis

As discussed in Chapter I, MT forms a complex with PP2A and the Src family of tyrosine kinases which leads phosphorylation of tyrosine residues on MT including 315, 250 and 322 sites. The phosphorylated tyrosine motif provides a docking site for association and activation of different signaling pathways. So the next question I want to ask is which pathways are involved in regulation of ERK suppression by MT. To do this, I looked at MT genetics. The mutation of MT at Y315, Y250 or Y322 site has been shown defective in PI3K, RAS and PLCγ pathways respectively. To find out whether any of these pathways is involved in MT regulation of ERK, different MT mutant NMuMG stable cell lines were made and compared in terms of ERK phosphorylation.

2.7.1. Y315 and PI3K

2.7.1.1. PI3K defective MT mutant Y315F is defective in ERK suppression

To investigate how MT suppresses ERK, we compared ERK signaling in MT mutants and wild type MT. Y315F is a MT mutant defective in transformation in vitro and in tumorigenesis expressed either in a virus or a transgene. This mutant fails to activate PI3 kinase. PI3K p110α is known to be required for MT transformation. AKT is one of the direct downstream effectors of PI3K and its activation can be used as a marker of PI3K activation. As expected, Y315F is defective of PI3K activation measured by phosphorylation at both S473 and T308 sites required for full activation of AKT. Comparison of Y315F to wild type MT showed that the mutant was unable to suppress ERK activation after different kinds of stress treatment (Fig.18).

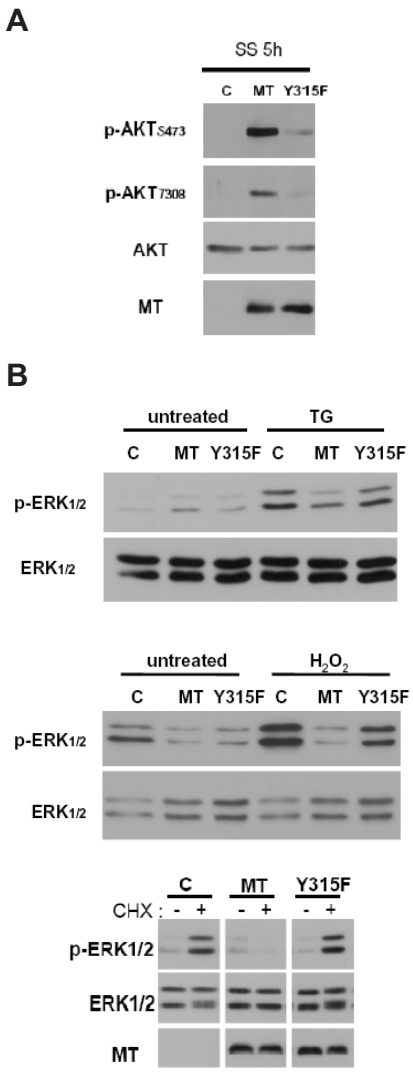


Fig. 18. PI3K defective MT mutant Y315F is defective in ERK suppression.

A. MT mutant Y315F is defective in activating PI3 Kinase as measured by AKT phosphorylation. NMuMG stable cell lines expressing GFP (C), MT or Y315F were 0% serum starved for 5 hours and cell lysates were collected for western blot. Results are representative of experiments performed at least three times.

B. MT mutant Y315F is defective in ERK suppression. Cells were untreated or treated with TG 500nM, peroxide (H_2O_2) 100 μM, or CHX 50μg/ml for 30 min. Whole cell lysates were blotted for phospho-ERK, ERK, and MT. Results are representative of experiments performed at least three times.

2.7.1.2. PI3K defective MT mutant Y315F is also defective in phospho-ERK turnover

To look at turnover of ERK phosphate, cells were treated with peroxide to induce stress. Turnover of ERK phosphate as a function of time was measured in the presence of U0126 that inhibits MEK to prevent new ERK phosphorylation. As before, the wild type MT cells showed very rapid loss of ERK phosphate, while Y315F cells looked like the controls (Fig.19). This result suggests that Y315F is defective in enhancing the turnover of ERK phosphate. Since Y315F binds PP2A same as wild type MT, this result also supports earlier data that binding of PP2A is not sufficient for ERK suppression by MT.

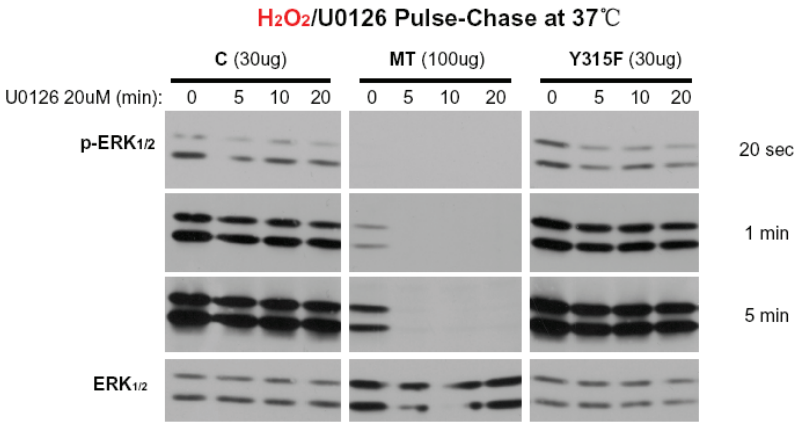


Fig. 19. PI3K defective MT mutant Y315F is also defective in phospho-ERK turnover. NMuMG stable cell lines expressing GFP (C), MT or Y315F were treated with peroxide (H_2O_2) for 30 min, and then exposed to MEK inhibitor U0126 (20μM). The chase assay was performed as described in Materials and Methods. Exposure time of phospho-ERK blots is indicated on the right.

2.7.1.3. PI3K effector on ERK regulation

2.7.1.3.1. RAC1

Small GTPase RAC can be activated through PI3K activation of PH domain-containing nucleotide exchange factor RAC-GEFs (Mertens et al., 2003). RAC has three isoforms: RAC1, RAC2, and RAC3. RAC1 is ubiquitously expressed among the three isoforms. Since MT activates PI3K, RAC1 is expected to be activated. Specific RAC1 inhibitor NSC23766 that prevents RAC1 activation by RAC-specific guanine nucleotide exchange factors (GEFs) TrioN and Tiam1 (Gao et al., 2004) was used to see whether blocking RAC1 could reverse MT suppression of ERK subjected to stress. Cells were pretreated with NSC23766 100μM for overnight and then exposed to CHX for 30 min (Fig.20). Both in NMuMG and MT-NMuMG cells, NSC23766 has no significant effect on ERK activation. This result suggests that RAC1 is not involved in MT negative regulation of ERK. Further experiments need to be done to confirm the efficiency of inhibition of RAC1 by NSC23766.

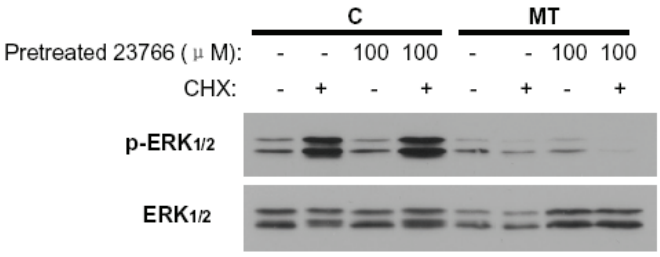


Fig.20. Inhibition of PI3K downstream effector RAC1 does not reverse ERK suppression by MT. NMuMGs and MT-NMuMGs were pretreated with or without RAC1 inhibitor NSC23766, and then exposed to CHX (50μg/ml) for 30 min. Levels of phospho-ERK, ERK were determined by immunoblotting. Results are representative of experiments performed at least three times.

2.7.1.3.2. AKT and ERK activation

Ser/Thr kinase PKB/AKT is the major target of PI3K. Earlier study showed AKT can negatively regulate RAF-1 activity by phosphorylation on 14-3-3 protein binding sites (Zimmermann and Moelling, 1999). Recent studies using prostate cancer samples from patients showed cancer progression is associated with increased levels of AKT phosphorylation and decreased ERK phosphorylation (Ghosh et al., 2005; Kreisberg et al., 2004; Paweletz et al., 2001). AKT has three isoforms: AKT1, AKT2, and AKT3. Increasing evidence shows non-redundant functions for different AKT isoforms (Rychahou et al., 2008). Study in mammary epithelial cells indicated distinct roles of AKT1 and AKT2 (Irie et al., 2005; Maroulakou et al., 2007). We wondered whether they have differential functions in regulating ERK activation.

To test this, we applied myristylation tagged AKT1/2, for which the Myr tag can target AKT to cellular membrane without PH domain and being constitutively activated by PDK. Stable cell lines with overexpression of Myr-AKT1, Myr-AKT2, Y315F+Myr-AKT1 or Y315F+Myr-AKT2 were established. As expected, Y315F+Myr-AKT1/2 reduced both MEK and ERK phosphorylation subjected to stress (Fig. 21Left). When ERK phosphate turnover was compared, no significant difference was observed among Y315F, Y315F+Myr-AKT1 and Y315F+Myr-AKT2 cells (Fig. 21 Right). This result indicates PI3K effector AKT both isoform 1 and isoform 2 can be involved in negative regulation of ERK. The level of regulation is mostly at ERK kinase (MEK or above) level rather than ERK phosphatase level.

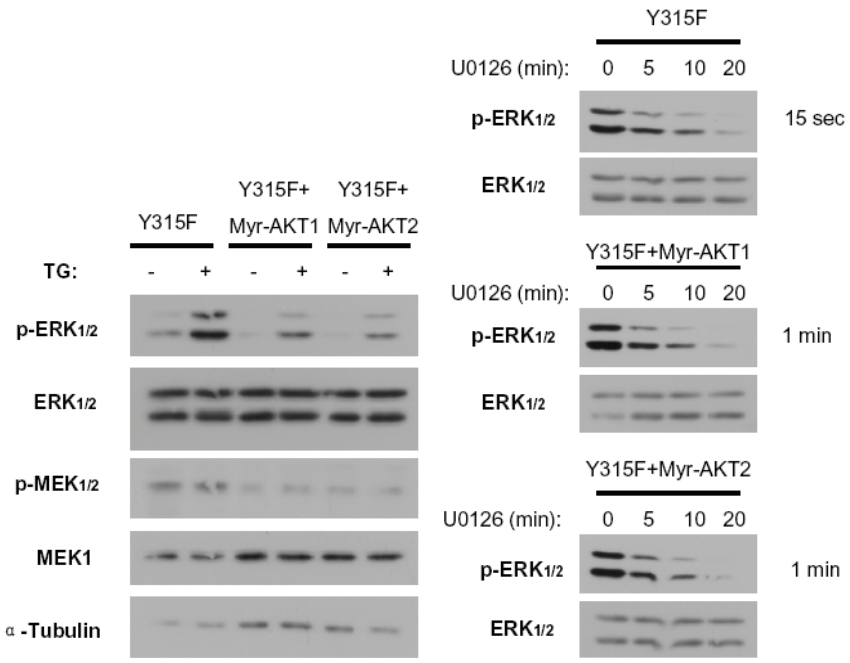


Fig. 21. Overexpression of constitutively activated AKT isoform 1 or 2 partially reduces phospho-ERK. NMuMG stable cell lines Y315F, Y315F+Myr-AKT1, Y315F+Myr-AKT2 were untreated or treated with TG (500 nM) for 30 min (Left panel); Or exposed to U0126 for indicated time after TG 30 min treatment (Right panel). Cell lysates were collected for western blot. Due to the reduced phospho-ERK in Y315F+Myr-AKT, prolonged exposure of phospho-ERK was used as indicated. Results are representative of experiments performed at least three times.

2.7.1.3.3. Inhibition of PI3K/AKT and its effect on ERK activation

If MT activation of PI3K/AKT is needed for suppression of ERK, then MT should be able to activate AKT in stressed cells as well as cells without stress. As expected, AKT is activated by MT under all the stress conditions. This was confirmed by examining phosphorylation of both Ser473 and Thr308 sites of AKT and one of AKT's downstream substrates, 4EBP1. Both phosphor-specific antibody at Thr37/46 and mobility shift of 4EBP1 confirmed that MT actives AKT even under stress (Fig.22 A).

Though overexpression of Myr-AKT in Y315F partially mimics wild type MT suppression of ERK under stress, it is still worth knowing whether blocking PI3K or AKT in MT cells can block ERK suppression. To test it, PI3K or AKT inhibitors were used. When PI3K inhibitor LY294002 was added 24 hours prior to stress (eg. CHX), it did result in a partial increase of ERK activation though it did not completely block phosphorylation of AKT in MT cells (Fig.22 B). Again this is consistent with a role for PI3K in suppression of ERK activation.There was no further increase of ERK activation when subjected to stress suggesting that LY was not blocking the MT suppression function. One possible explanation is PI3K only involved in regulating basal ERK activity, and there are other pathways independent of PI3K involved in regulating ERK activity subjected to stress. Another possible explanation is PI3K inhibitor did not completely block PI3K activity in MT cells which may affect the extent of the observed difference.

Since PI3K inhibitor LY294002 can only inhibit about 40% PI3K activity (measured by AKT Ser473 phosphorylation), AKT inhibitor VIII (PH domain dependent and AKT1 and AKT2 selective (Barnett et al., 2005)) was used. AKT activation was measured by

phosphorylation of AKT activation sites Thr308 and Ser473. As Fig.22 C showed (red box), under normal growth condition, block of AKT dramatically increases MEK1/2 phosphorylation in MT cells. Consistent with this, MEK1/2 substrate ERK phosphorylation was also increased accordingly. Though this phospho-MEK is higher than in NMuMG cells, difference in phospho-ERK is much less dramatic. It again supports the hypothesis that MT activates ERK phosphatases and it is AKT independent.

Interestingly, blocking AKT does not completely rescue MT suppresseing MEK phosphorylation subjected to stress condition (eg. CHX), which indicates in addition to AKT, MT may use other pathways regulating MEK phosphorylation under stress. The level of ERK phosphorylation results from a balance of its kinase and phosphatase activities. It is clear that some stress conditions activate ERK through both activation of kinase (MEK) and inactivation of ERK phosphotases. If AKT is not sufficient to interfere with both levels of regulation of ERK, as shown in Y315F+Myr-AKT, constitutively activated AKT has almost no effect on ERK phosphate turnover, block of AKT alone is not sufficient to reverse MT suppression of ERK in response to stress.

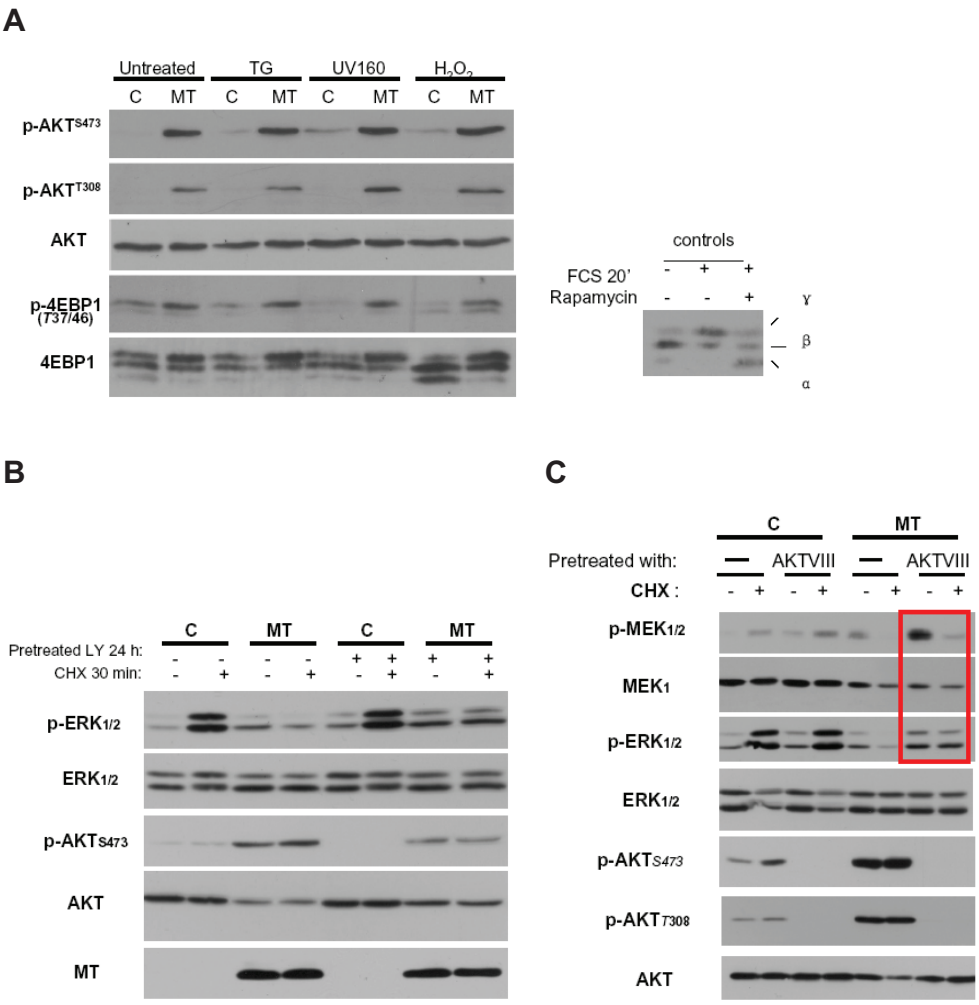


Fig. 22. MT constitutively actives PI3K/AKT under stress. Inhibition of PI3K or AKT by inhibitors partially reverses MT suppression of ERK.

A. MT constitutively activates PI3K downstream pathway AKT under all conditions. NMuMG and MT-NMuMG cells were untreated, treated with thapsigargin (TG) 500nM, UV (160J/m^2) irradiation or peroxide (H_2O_2) 100 μM. Whole cell lysates were collected and western blots were performed. 4EBP1 mobility shift in response to serum is shown in right panel.

B & C. Inhibition of PI3K or AKT by inhibitors partially reverses MT suppression of ERK. NMuMG and MT-NMuMG cells were pretreated with or without PI3K inhibitor LY294002 (**B**) or AKT inhibitor VIII (**C**), and then exposed to CHX (50μg/ml) for 30 min. Levels of phospho-ERK, ERK, phospho-AKT, AKT, and MT were determined by immunoblotting. Results are representative of experiments performed at least three times.

2.7.1.4. Rescue of PI3K in Y315F cells and effects on ERK

Overexpression of constitutively activated AKT isoform 1 or 2 in Y315F does not fully rescue Y315F defective in ERK suppression, and AKT inhibitor also only partially affects wild type MT suppression of ERK. To answer whether PI3K might be sufficient, NMuMG cells expressing the constitutively activated p110α PI3K mutant H1047R (Zhao et al., 2005) or Y315F+H1047R were prepared. H1047R-NMuMG cells showed reduced activation of ERK compared to control NMuMGs under CHX treatment (Fig. 23A). The small degree of reduction that is observed can be explained by a reduced activation of MEK as seen with phosphospecific antibody and no significant change in ERK phosphate turnover (Fig. 23B).

The Y315F+H1047R cells recovered PI3K level comparable to wild type MT measured by phosphorylation of AKT (Fig. 24A). When tested with different stress conditions, Y315F+H1047R showed a reduced response to TG and especially hydrogen peroxide than Y315F cells. This suggests a partial rescue by H1047R (Fig. 24B). When further we looked at ERK phosphate turnover, Y315F+H1047R did not strongly affect the turnover rate. It suggests either expression of H1047R in Y315F can not fully mimic wild type MT PI3K function or there are unknown pathways through MT Y315 which H1047R can not replace.

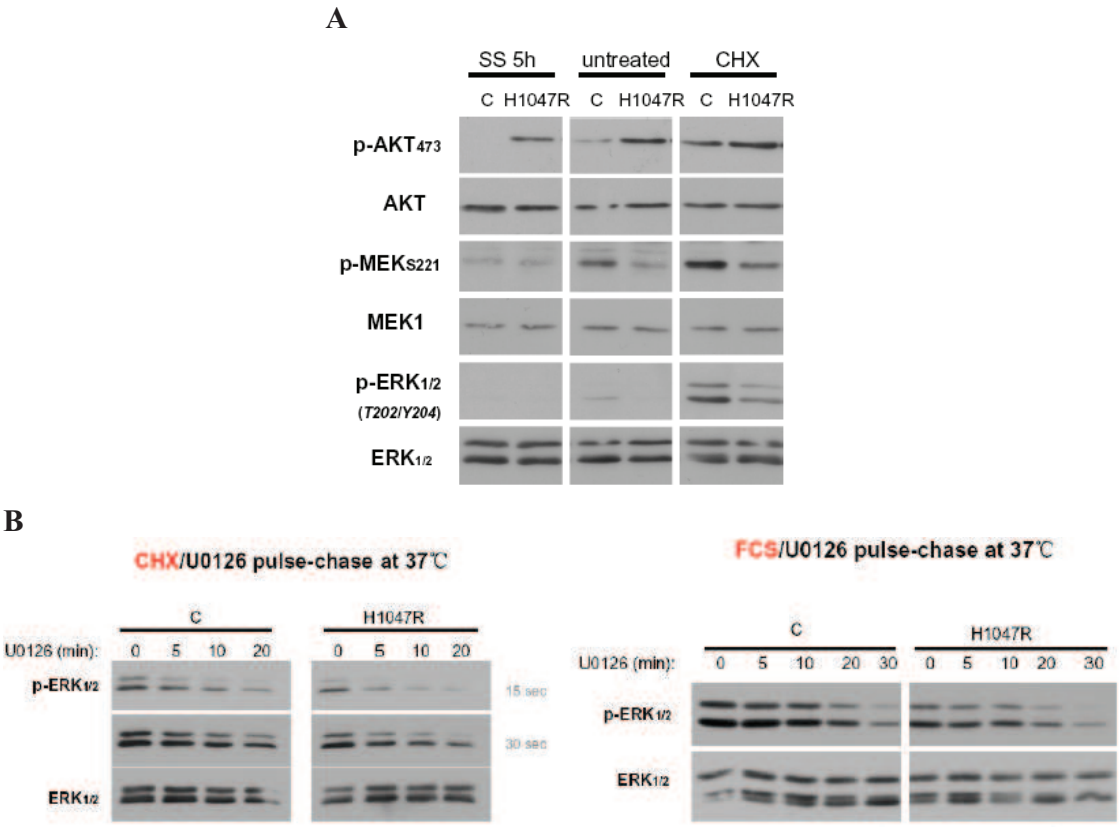


Fig. 23. Overexpression of constitutively activated PI3K p110α mutant H1047R partially suppresses MEK and ERK.

A. H1047R partially suppresses MEK/ERK. NMuMG stable cell lines expressing control vector (C) or PI3 kinase p110α constitutive active mutant H1047R were 0% serum starved for 5 hours, under growing condition or treated with CHX (50μg/ml) for 30 min. Whole cell lysates were blotted with antibodies as indicated. Results are representative of experiments performed at least three times.

B. H1047R does not significantly change ERK phosphate turnover. Cells were treated with CHX (50μg/ml) for 30 min (**left**), starved with 0%FCS for 5 hours then stimulated with FCS for 15min (**right**) and then exposed to MEK inhibitor U0126 (20μM). A chase assay was performed in which cells were extracted at the indicated time after U0126 treatment. Whole cell lysates were blotted with phospho-ERK and total ERK.

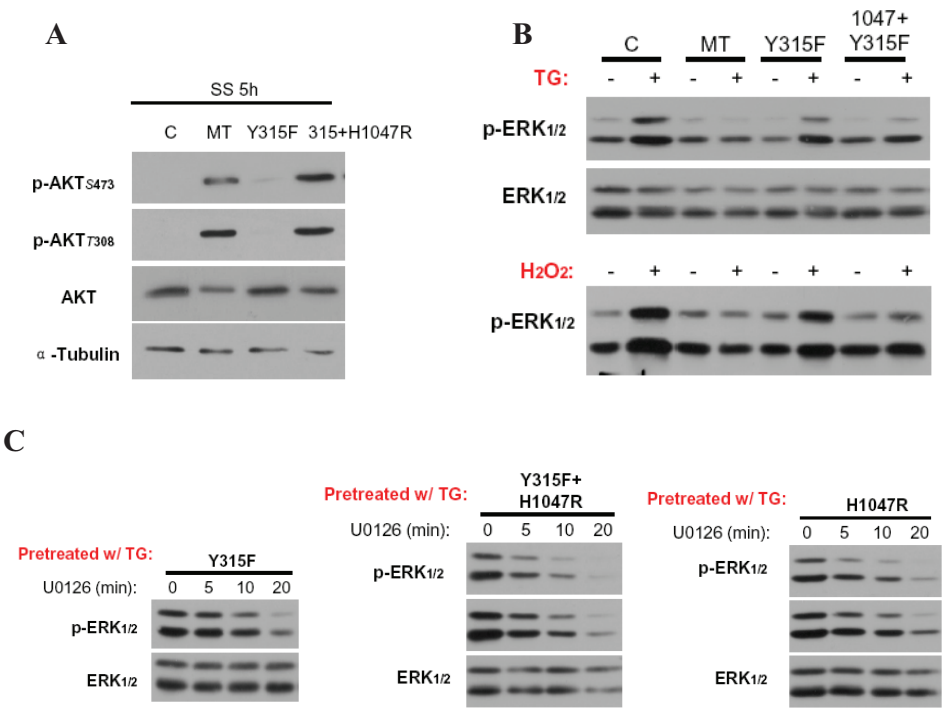


Fig. 24. Rescue of Y315F PI3K activation by p110α H1047R partially rescues lack of ERK suppression by Y315F.

A. Overexpression of constitutively activated PI3K p110α H1047R in Y315F. NMuMG stable cell lines expressing control vector (C), MT, Y315F or Y315F+H1047R were 0% serum starved for 5 hours. Cell lysates were collected for western blot. Results are representative of experiments performed at least three times.

B. Y315F+H1047R partially rescues lack of ERK suppression by Y315F. Cells were untreated or treated with TG 500nM, peroxide (H_2O_2) 100 μM for 30 min. Whole cell lysates were blotted with antibodies against the indicated proteins as indicated. Results are representative of experiments performed at least three times.

C. Comparing ERK phosphate turnover under stress in different cells. Cells were treated with TG 500nM for 30 min, and then exposed to MEK inhibitor U0126 (20μM). A chase assay was performed in which cells were extracted at the indicated time after U0126 treatment. Whole cell lysates were blotted with phospho-ERK and total ERK. Results are representative of experiments performed at least three times.

2.7.2. P248, Y250 and RAS

2.7.2.1. PI3K and RAS double defective MT mutants P248H and Y250F are defective in ERK suppression

Phosphorylation of MT at Y250 is known to activate the adaptor Shc that leads to the activation of RAS. As showed previously, MT activates RAS under different conditions. We wondered is RAS activation required or sufficient for ERK suppression? To answer this question, two Shc binding defective mutants P248H and Y250F were tested. Without binding Shc adaptor, RAS activation is compromised. As expected, P248H and Y250F were defective in binding and phosphorylation of Shc as shown in phospho specific antibody (Fig. 25A). To our surprise, PXYM motif mutants (P248H and Y250F) were also defective in PI3K by measuring AKT Ser473 phosphorylation. This is surprising based on that Y250 sites are not required for PI3K activation in human hTERT-HMEC and HMF cell lines (Utermark et al., 2007). It suggests NPXYXM motif or RAS activation may be also required for binding p85 subunit and activation of PI3K (directly or indirectly). Further, we tested P248H and Y250F under stress. Since both mutants were defective in PI3K, it was not surprising to see that both P248H and Y250F were also defective in suppression of ERK when treated with CHX for 30 min (Fig. 25B).

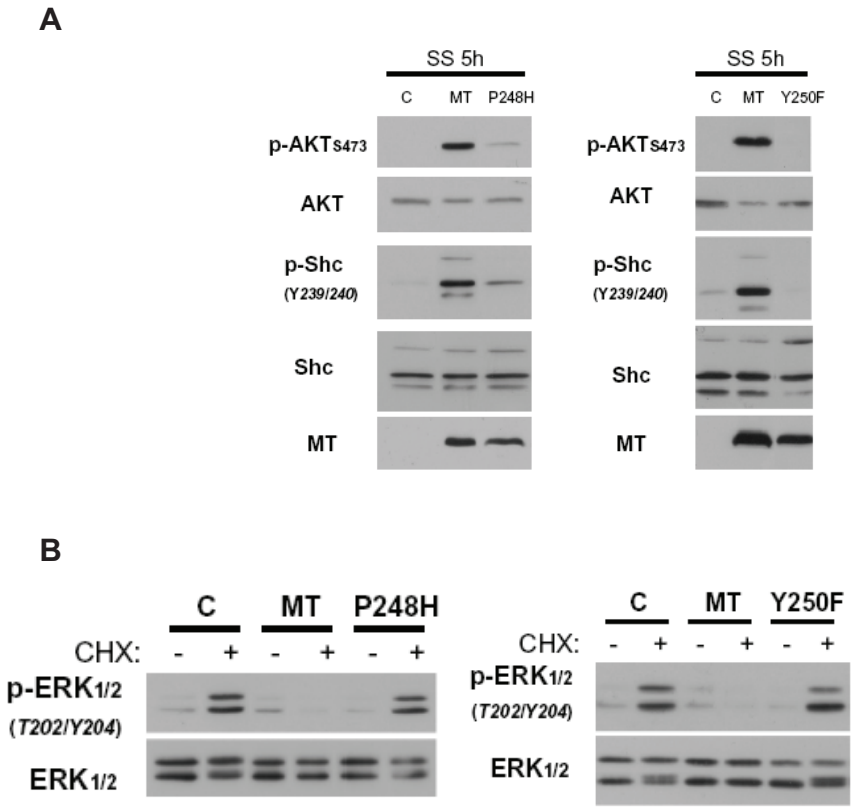


Fig.25. PI3K and RAS double defective MT mutants P248H and Y250F are defective in ERK suppression.

A. MT mutants P248H and Y250F are PI3K and RAS double defective. NMuMG cell lines expressing control vector, MT, P248H or Y250F were starved with 0% FCS for 5 hours and western blots were performed. Both AKT the downstream of PI3K and Shc the upstream of RAS were blotted. Results are representative of experiments performed at least three times.

B. PI3K and RAS double defective MT mutants P248H and Y250F are defective in ERK suppression. Cells were untreated or treated with CHX 50μg/ml for 30 min. Whole cell lysates were blotted with phospho-ERK and ERK. Results are representative of experiments performed at least three times.

2.7.2.2. Overexpression of constitutively activated H-RAS mutant G12V does NOT suppress ERK

Because RAS mutants (P248H and Y250F) are also PI3K defective, it is impossible to analyze RAS contribution in regulating ERK suppression by those mutants. Another way to look at how RAS contributes to ERK suppression is a gain of function assay. H-RAS mutant G12V is a constitutive active RAS mutant found in human cancer. By infecting with G12V retrovirus, we made a stable G12V RAS expressing NMuMG cell line. Exogenous expressed H-RAS (G12V) was detected by Flag tag and RAS activation status was confirmed by RAS activation assay (Fig. 26.Left). G12V was compared to NMuMG control and wild type MT. When cells were grown in 0% serum media for 5 hours, only G12V activated both MEK and ERK measured by phospho-specific antibodies. Compared to control cells, MT activated RAS which is consistent with previous results and the level of activated RAS seems no different than G12V. The level of phospho-MEK normalized to total MEK seemed not lower than G12V, while the phospho-ERK is much less. When subjected to stress, for example CHX, G12V cells were similar to control cells (Fig. 26.Right). This supports the conclusion that RAS activation alone is not sufficient to suppress ERK.

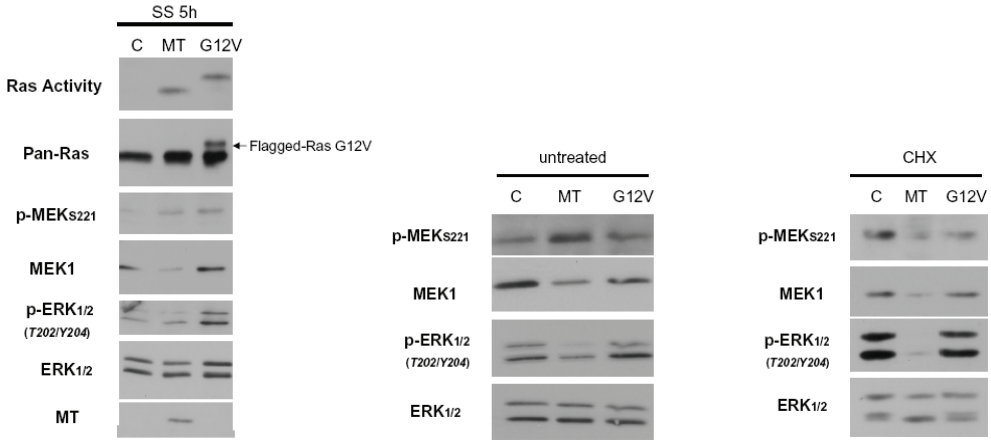


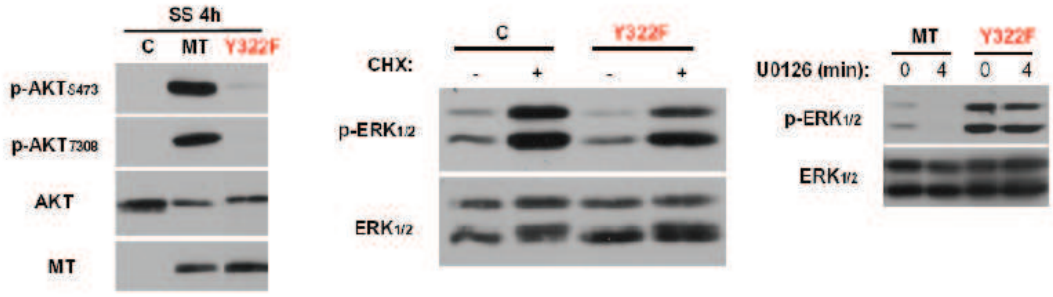
Fig.26. Overexpression of constitutively activated H-RAS mutant G12V does NOT suppress ERK. NMuMG cell lines expressing control vector, MT or G12V were starved with 0%FCS for 5 hours (**Left**), under normal growing condition (**Middle**) or treated with CHX for 30 min (**Right**), western blots were performed. Results are representative of experiments performed at least three times.

2.7.3. Y322 and PLCγ

In addition to Y315 and Y250, Y322 is also phosphorylated in MT/Src complex. PLCγ is shown to bind MT at Y322 and important for transformation (Su et al., 1995). There are not many studies reported on the downstream effectors of PLCγ activated by MT. In order to understand whether MT Y322 is involved in regulating ERK activation, Y322F-NMuMG cell line was made the same way as wild type MT. Unexpectedly, Y322F was also defective in PI3K activation (same as Y315F and Y250F). Not surprisingly, Y322F is defective in ERK suppression (Fig.27A).

To address the question whether Y322 (PLCγ**)** is involved in ERK regulation by MT, other cell lines were tested. Human mammary epithelial cells MCF10A in which both Y250F and Y322F activate PI3K like wild type MT. However, when I looked at ERK phosphate turnover, MT and Y250F increased ERK phosphate turnover while Y322F behaved like MCF10A control cells (Fig.27B). These results suggested that in MCF10A cells, MT uses Y322 regulating ERK phosphatase activity (ERK phosphate turnover). Interestingly, MCF10A cells were not sensitive to stress in terms of ERK activation. Further experiments need to be done to learn how the PLCγ pathway is involved in regulating ERK phosphate turnover.

A



B

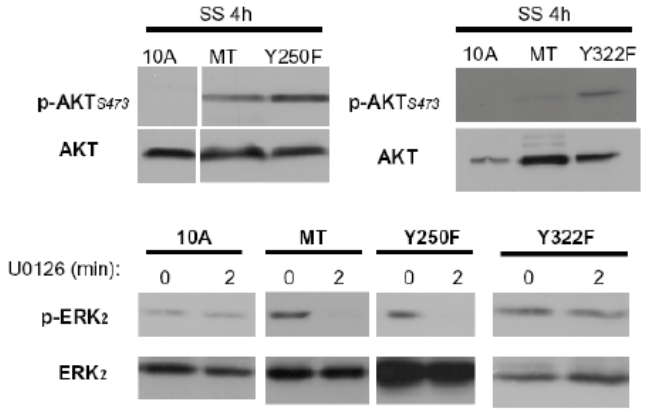


Fig.27. Y322F is defective in ERK suppression.

A. Y322F-NMuMG is PI3K defective. NMuMG cell lines expressing control vector, MT, or Y322F were starved with 0% FCS for 4 hours (**Left**), treated with CHX 50μg/ml for 30 min (**Middle**) or pretreated with CHX and then treated with U0126 (20μM) for indicated time (**Right**). Results are representative of experiments performed at least three times.

B. Y322F-MCF10A is defective in ERK phosphate turnover. MCF10A, MT-MCF10A, Y250F-MCF10A, Y322F-MCF10A cells were 0% serum starved for 4 hours (**upper panel**) or treated with H_2O_2 100μM for 30 min and then treated with U0126 (20μM) for indicated time (**lower panel**). Whole cell lysates were blotted with phospho-ERK and ERK. Results are representative of experiments performed at least three times.

2.7.4. Model of ERK regulation by MT

Based on the MT genetic analysis, we come to a model of ERK regulation by MT (Fig.28). ERK activity in cells is a balance of phosphorylation by MEK and dephosphorylation by protein phosphatases such as DUSPs. Stress causes the balance shift and ERK activation. In MT cells, MT uses PI3K/AKT pathways through Y315 to reduce MEK activity and Y322 pathways to enhance ERK dephosphorylation under stress.

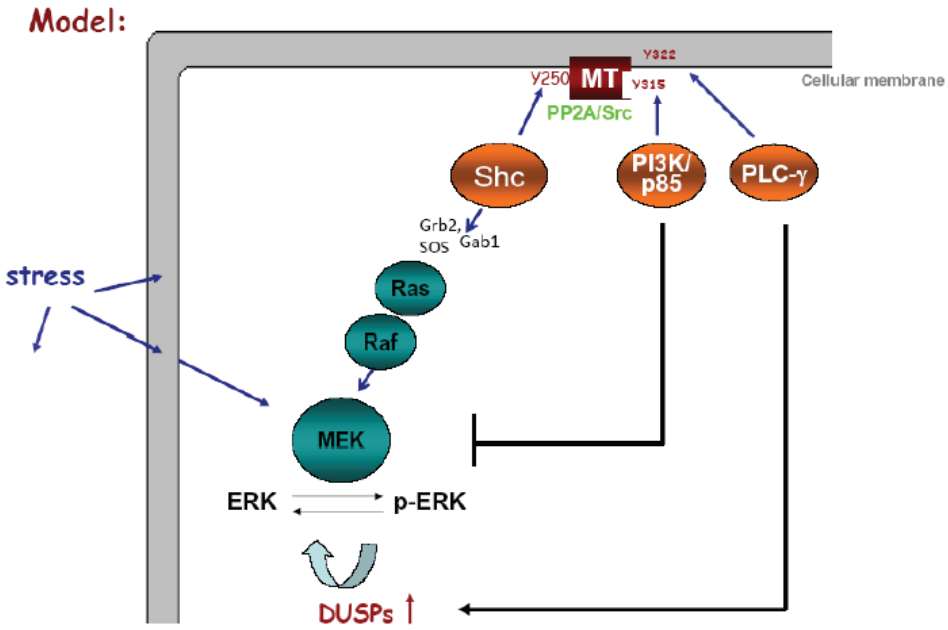


Fig.28. Model of ERK regulation by MT.

2.8. ERK regulation in MT transformed other cell lines

To investigate whether MT suppression of ERK under stress conditions is unique to NMuMG cells, or a general phenomenon, NIH/3T3 fibroblasts were also tested. The same MT revtrovirus were used to establish 3T3 stable cell lines as was used for NMuMGs. First, we wanted to know how fast ERK phosphate turns over in MT-NIH/3T3 cells. ERK phosphate turnover in NIH/3T3 and MT-NIH/3T3 were compared to NMuMGs under normal growth conditions. Surprisingly, ERK phosphate turns over in both NIH/3T3 and MT-NIH/3T3 in less than 5 min, which is significant faster than NMuMGs (Fig. 29A). This result suggests NIH/3T3s have much higher basal ERK phosphatase activity than NMuMGs, which makes it difficult to observe the difference of turnover and study MT regulation of ERK phosphatase in NIH/3T3 cells. Nevertheless, it was still worth to look at wild type MT and MT mutant in the regulation of ERK under stress conditions. Different kinds of stress were tested. Unlike NMuMGs, NIH/3T3s were only sensitive to certain stresses, such as peroxide. As shown previously, MT activated ERK in NIH/3T3 cells under normal growth conditions, but suppressed ERK activation under peroxide treatment compared to NIH/3T3 control cells. The PI3K defective mutant Y315F was not only defective in ERK suppression but seemed to enhance ERK activation (Fig. 29B). Similar results were seen in Balb/C A31 cells (Fig. 30). Those results suggest there is a common functional negative regulation of ERK by MT, though MT may use different mechanisms regulating ERK activity in different cellular contexts.

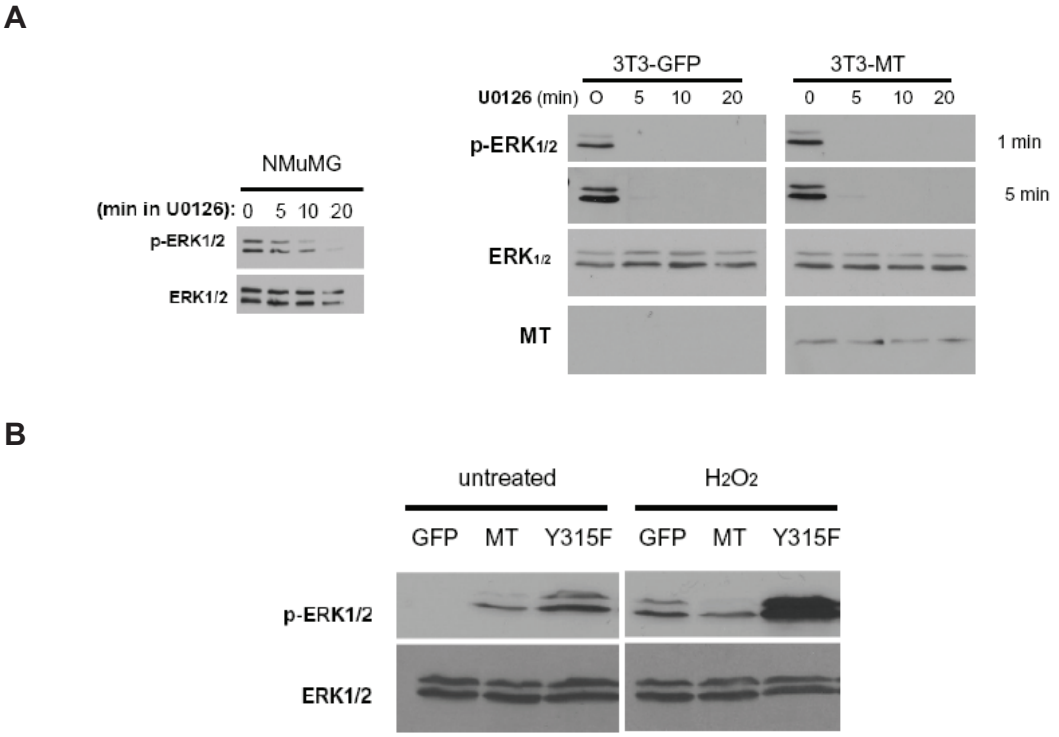


Fig. 29. ERK regulation in MT transformed fibroblast NIH/3T3.

A. NIH/3T3 has more basal ERK phosphatase activity compared to NMuMGs. NMuMGs on the left, stable 3T3 cell lines expressing GFP or MT were exposed to MEK inhibitor U0126 (20μM). A chase assay was performed in which cells were extracted at the indicated time after U0126 treatment. Exposure time of phospho-ERK was indicated on the right of blots. Results are representative of experiments performed at least twice.

B. PI3K defective mutant Y315F is also defective in suppression of ERK in NIH/3T3. NIH/3T3 stable cell lines expressing GFP, wild type MT and mutant Y315F were untreated or treated with 1mM peroxide for 30min and whole cell lysates were blotted. Results are representative of experiments performed at least three times.

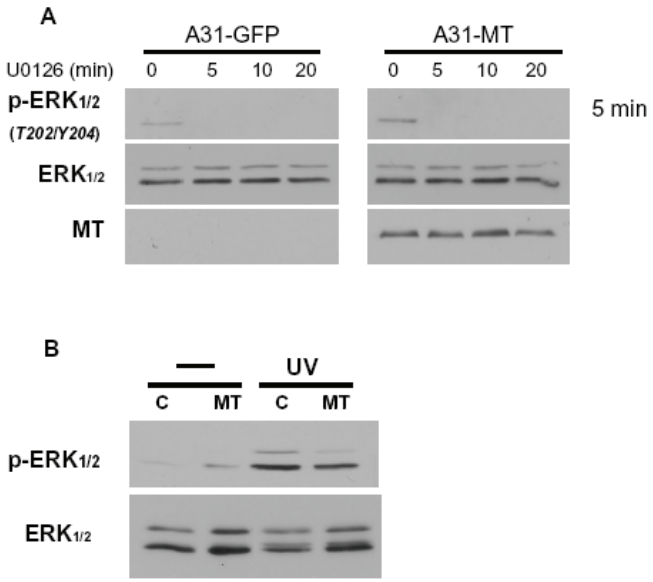


Fig. 30. ERK regulation in MT transformed fibroblast Balb/C A31.

A. A31 has more basal ERK phosphatase activity compared to NMuMGs. Stable A31 cell lines expressing GFP or MT were exposed to MEK inhibitor U0126 (20μM). A chase assay was performed in which cells were extracted at the indicated time after U0126 treatment. Exposure time of phospho-ERK signal was indicated on the right of blots. Results are representative of experiments performed at least twice.

B. MT suppresses ERK in A31 cells subjected to UV. Cells were untreated or exposed to UV (240J/m^2) irradiation. 30 min later the whole cell lysates were collected and blotted. Results are representative of experiments performed at least twice.

Part II: Suppression of ERK alters the stress phenotypes

ERK signaling has been found to be involved in many aspects of cellular functions including cell proliferation, cell survival, protein translation and cell cycle progression, etc. We looked at some cell phenotypes in NMuMGs and MT-NMuMGs.

2.9. ERK signaling is required for NMuMG, but not MT-NMuMG cell cycle progression

Both NMuMGs and MT-NMuMGs were analyzed to investigate whether ERK activation is required for cell cycle progression. As shown in Fig.31A, suppression of ERK by U0126 alone is sufficient to lead NMuMG cells to accumulate in G1 (G1 from 44% to 80%). In contrast to the control cells, MT cells did not show significant G1 cell accumulation when ERK is blocked by U0126 (G1 from 36% to 48%). MT mutant Y315F cells (PI3K-) were arrested in G1 similar to controls (G1 from 48% to 81%). This result suggests that ERK activation is required in both NMuMGs and Y315F for efficient cell cycle progression but not wild type MT cells. The known defecting signaling in Y315F is the PI3K pathway, suggesting MT may depend on PI3K for cell cycle progression. To answer whether MT cells cell cycle progression depends on PI3K activity, the same experiment was repeated by treating cells with PI3K inhibitor LY294002 for a comparision with MEK inhibitor U0126 and U0126 plus LY294002 (Fig.31B.). Consistent with earlier data, U0126 alone causes NMuMG cells, but not MT-NMuMG cells to accumulate in G1. LY294002 causes both NMuMG and MT-NMuMG cells arrest at G1 cycle. The effect on MT-NMuMGs is less than NMuMGs partially because LY294002 cannot completely block PI3K activity in MT-NMuMGs (not shown).

Interestingly, U0126 plus LY294002 has an additive effect on MT-NMuMGs but not NMuMGs.Together, this suggests both ERK and PI3K signalings are required for NMuMG cell cycle progression. Inhibition of either ERK or PI3K signaling blocks NMuMG cells progression into S phase. MT-NMuMG cells depend less on ERK than on PI3K for cell cycle progression. However, inhibition of both ERK and PI3K has a striking effect on MT cell cycle progression compared to blocking either individual pathway. This result is consistent with previous study in our lab that MT uses both Y250 and Y315 for cell proliferation (Mullane et al., 1998).

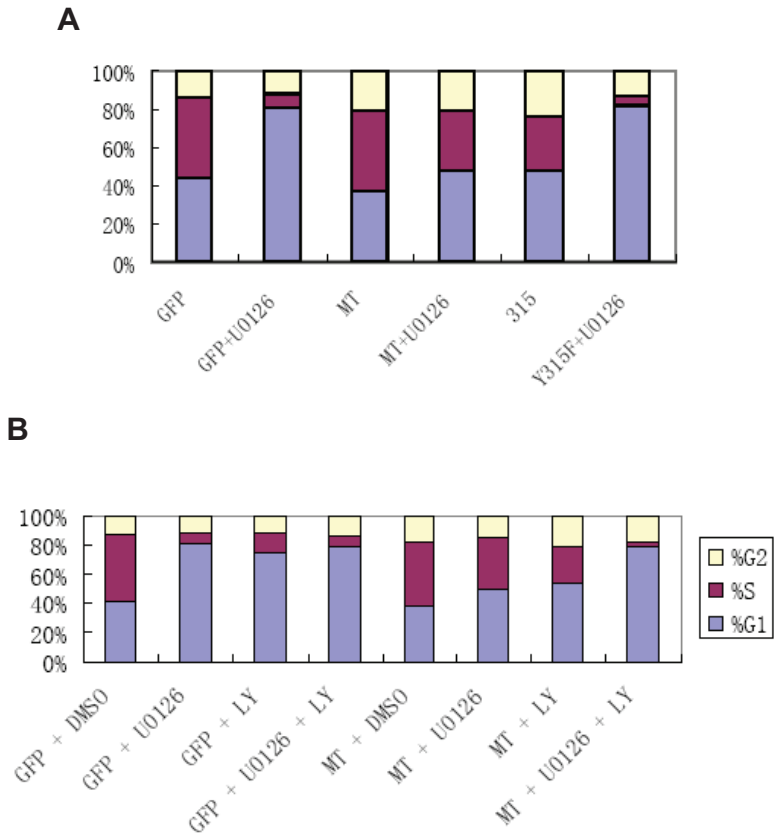


Fig. 31. ERK signaling is required for NMuMG cell cycle progression.

A. ERK signaling is required for NMuMG cell cycle progression. NMuMG stable cell lines expressing GFP, MT or Y315F were untreated or treated with U0126 (20μM) for 16 hours, then collected and analyzed by flow cytometry as described in Materials and Methods.

B. Block either ERK or PI3K signaling blocks NMuMG cell progression into S phase. Block both ERK and PI3K signaling blocks MT-NMuMG cell cycle. NMuMG stable cell lines expressing GFP or MT were untreated (DMSO), treated with U0126 (20μM), or/and LY294002 (20μM) for 16 hours, then collected and analyzed by flow cytometry as described in Materials and Methods.

2.10. MT dephosphorylates overexpressed intrinsically activated ERK

As discussed in introduction, aberrant loss of ERK activity plays a pivotal role in the malignant phenotype observed in certain types of cancer and predicts poor prognosis. Activation of ERK in some cancer cells can lead cell death. What is the consequence if constitutively activated ERK is overexpressed in MT cells? Based on my hypothesis, MT either dephosphorylates exogenous ERK or if not, cells will not be able to survive. To test this hypothesis, first we looked at whether MT cells also dephosphorylate exogenous wild type ERK. HA tagged ERK2 were transient transfected into both NMuMG-GFP and MT-NMuMG cells. After 24 hours cells were treated with stress such as TG, and HA-ERK2 was precipitated and phospho-ERK was blotted. Like endogenous ERK1/2, MT also suppressed exogenous ERK phosphorylation (Fig.32A.). Next we wanted to ask about the consequence of overexpression of intrinsically activated ERK (MEK-independent) or hyper activatable ERK that has reduced affinity for MKP (lack of phosphatase affinity) in NMuMG-MT cells. My hypothesis is MT would either dephosphorylate exogenous ERK or if not, the cells will not be able to survive. To test this hypothesis, different active ERK mutants have been transfected into MT cells. ERK1-R84S and ERK2-R65S mutants had been shown acquire an efficient autophosphorylation activity on both the Thr and the Tyr residues of the phosphorylation loop (Levin-Salomon et al., 2008). Sevenmaker mutation ERK2- D319N has no effect on the catalytic activity of ERK, but has been shown to reduce the affinity for DUSP phosphatases, thereby prolonging its activity (Bott et al., 1994). The double mutant R65S+D319N therefore has higher activity than ERK2-R65S (Levin-Salomon et al., 2008). Wild type and ERK mutants (R65S, R65S+D319N) can be dephosphorylated by

MT as endogenous ERK as our primary experiment suggested (Fig.32B.). Since MT dephosphorylated double mutant R65S+D319N as well, it suggests D319N site may not be sufficient to block ERK phosphatase binding ERK in MT cells. Because MT still inactivates these ERK mutants, we can not get constitutively activated ERK, so we can not answer if ERK acitivty would kill or arrest MT cells.

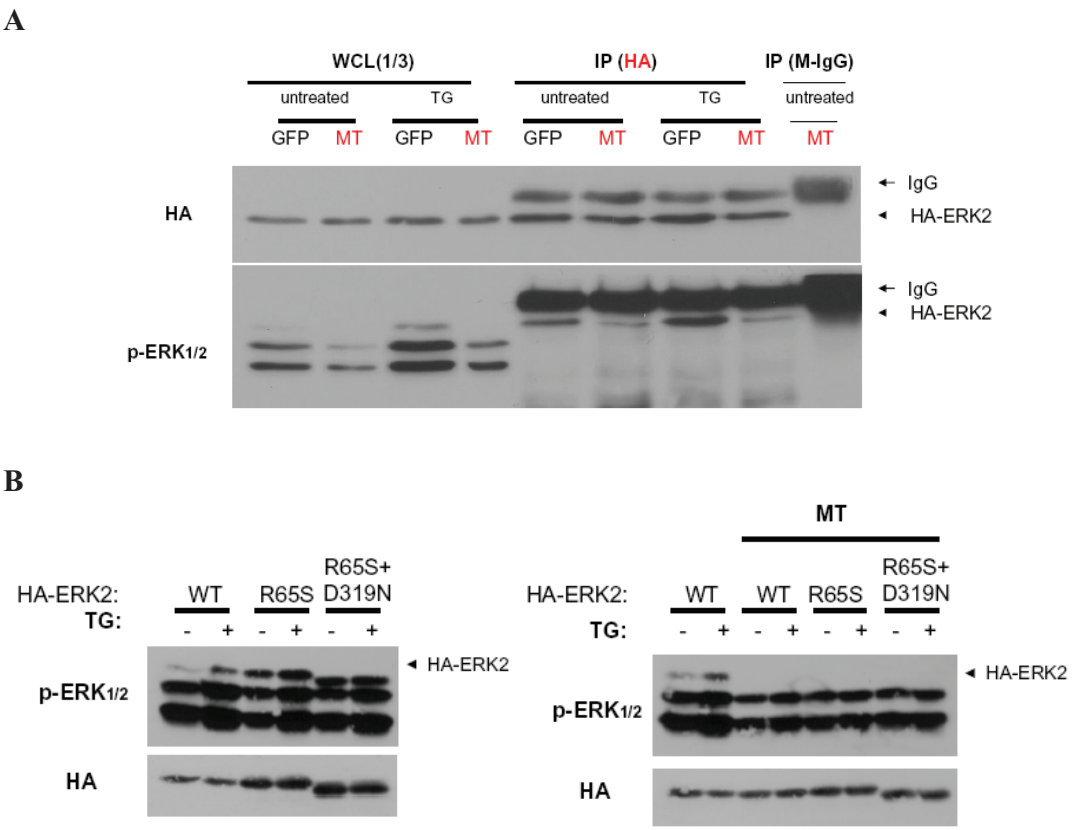


Fig.32. Overexpression of activated ERK mutants in MT cells.

A. MT dephosphorylates exogenous wild type ERK. NMuMG(GFP) and MT-NMuMG cells were transfected with HA-ERK2 by using Lipofectamine 2000 described as Material and Methods. 24 hours later cells were untreated or treated with TG (500nM) for 30 min and HA immunoprecipitation was performed for western blots.

B. MT dephosphorylates overexpressed intrinsically activated ERK. NMuMG and MT-NMuMG cells were transfected with indicated plasmids by using Lipofectamine 2000 described as Material and Methods. 24 hours later cells were untreated or treated with TG (500nM) for 30 min and western blots were performed.

2.11. Suppression of ERK improves cell survival exposed to peroxide

In extreme cases, cell survival can be compromised by the stress. The role of ERK activation in cell survival appears to vary. It can contribute to survival (Balmanno and Cook, 2009), such as after UV treatment of keratinocytes (He et al., 2004; Ming et al.). However, ERK can also function pro-apoptotically (Mebratu and Tesfaigzi, 2009). ERK is proposed to enhance apoptosis caused by UV in myeloblastic leukemia cells (Li et al., 2002), to promote apoptosis in fibroblasts treated with peroxide (Lee et al., 2003) or to promote apoptosis in NIH/3T3 cells treated with etoposide (Tang et al., 2002).

To understand what role ERK plays in survival, NMuMGs and MT-NMuMGs were subjected to oxidative stress with hydrogen peroxide. NMuMG cells die in response to this treatment as shown by phase contrast pictures at 24 hours after treatment. When cells are treated with U1026, there is a substantial improvement in cell survival. This suggests that ERK activation here contributes to cell death. The MT-NMuMGs cells are resistant to this stress treatment, consistent with the result of ERK suppression (Fig.33). The improved survival of MT-NMuMGs compared to the controls treated with U0126 suggests that MT contributes to survival using mechanisms in addition to ERK suppression.

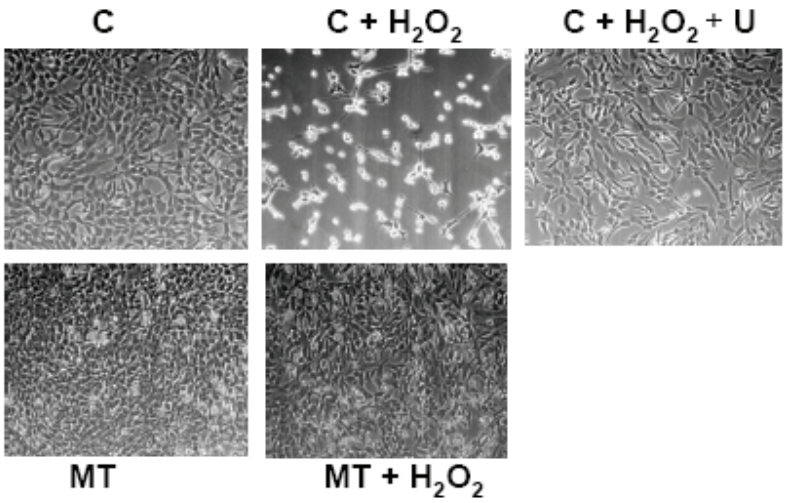


Fig. 33. Suppression of ERK improves cell survival exposed to peroxide. NMuMG and MT-NMuMG cells were untreated or pretreated with U0126 (5μM) (U) 30 min before treated with H_2O_2 (50μM) for 24h. Results are representative of experiments performed at least three times.

2.12. Suppression of ERK reduces COX2 expression caused by hypoxia

Inflammation is involved in response both to stress and to virus infection. COX-2 is induced by ER stress (Hung et al., 2004), UV (Buckman et al., 1998), or via reactive oxygen (Chiu et al.). Roles for inflammation and COX-2 in virus infection are well-known but varied (Steer and Corbett, 2003). COX-2 expression is increased after CMV infection and its product PGE2 s is known to promote replication (Hooks et al., 2006). On the other hand, EBV blocks both COX-2 and PGE2 production (Savard et al., 2000). Since COX-2 activation can be dependent on ERK (Chiu et al.), its responses to stress in NMuMGs and MT-NMuMGs were compared. The levels of both COX-2 mRNA determined by real time PCR and COX-2 protein measured by western blotting were increased after hypoxia. This elevation seems to involve activation of ERK, since inhibition with U0126 attenuated the response. The COX-2 response is also attenuated in the MT-NMuMGs, consistent with the effect of MT suppression on ERK (Fig.34).

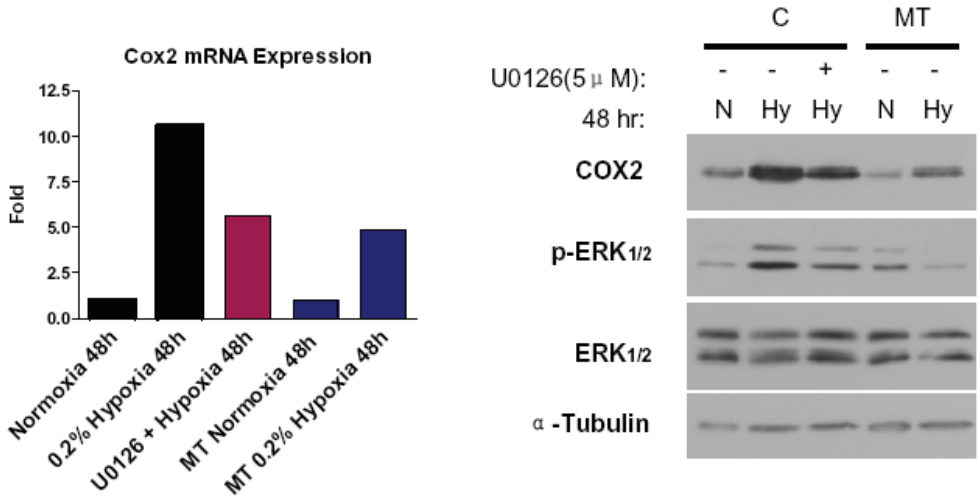


Fig. 34. Suppression of ERK reduces COX2 expression caused by hypoxia. NMuMG and MT-NMuMG cells were cultured in normal condition or transferred to hypoxic conditions as described in Materials and Methods. RNA (**Left**) and protein (**Right**) were collected at 48h. RT-quantitive PCR and western blots were performed.

Summary of results

Studies of both PI3K-AKT and RAS-MAPK pathways have provided important insights into mammalian growth control and cancer over years. It is known that constitutively activated mutation of RAS or PI3K has been found in many human cancers. Interestingly, the downstream substrate ERK of the RAS pathway is not always activated.

Here I used mouse mammary gland epithelial cells (NMuMGs) transformed by MT as a model system to study regulation of ERK. I found that instead of activating ERK, MT had little effect on the level of ERK activation under basal conditions. ERK is also activated by different kinds of stress in untransformed NMuMG cells. MT suppressed this stress activation. Both lack of basal activation and suppression of ERK were unexpected because previous studies showed ERK signaling is activated in MT-NIH/3T3 cells under normal growth conditions. The suppression of ERK by MT is specific because MT does not affect all the aspects of stress pathways, not even all MAP kinase family member responses.

To understand how MT prevents ERK activation, the RAS/RAF/MEK/ERK signaling cascade has been examined. MT activates RAS and RAF under stress conditions. MT transformed cells generally show higher levels of phosphoMEK, suggesting its activation. In response to stress, the amount of phosphoMEK does not rise like controls, but in fact can even go down. Most importantly, MT activates ERK phosphatase activity in NMuMGs. MT also increased ERK phosphate turnover in human mammary epithelial cell line MCF10A. This suggests negative regulation of ERK by MT is not unique to NMuMG cells but a more general phenomenon. There are at least two ERK phosphatases, VHR/DUSP3 and MKP3/DUSP6, found upregulated by MT.

Knockdown either one of the two phosphatases did not completely reverse the increase in ERK phosphate turnover by MT, which indicates MT regulates additional ERK phosphatases.

Genetic analysis of MT showed Y315F mutant (PI3K-) is defective in blocking ERK phosphorylation in response to stress. Unlike wild type, the mutant failed to increase the turnover of ERK phosphate. Inhibition of PI3 kinase or AKT by specific inhibitors in wild type cells partially mimics Y315F. To try to rescue the mutant, overexpression of constitutively active PI3K p110α mutant H1047R, AKT1 or AKT2 was carried out. When these were overexpressed, less increase in ERK phosphate than that with 315 alone or controls after stress was observed, so there appeared to be only a partial rescue.

More detailed MT genetic analysis in NMuMGs was not possible. Mutation of either Y250 (Shc) and Y322 (Plcγ1) abolished PI3K activation, making an independent analysis of those activities impossible. In MCF10A, both Y250F and Y322F could activate AKT. ERK phosphate turnover in Y250F looked like wild type MT, but Y322F looked like controls. This suggests a role for Y322 signaling in regulating ERK.

ERK suppression by MT subjected to stress is involved in multiple cell phenotypes. Suppression of ERK improves cell survival exposed to peroxide and reduces COX2 expression caused by hypoxia. Suppression of ERK caused NMuMG cells accumulated in G1 cycle while MT-NMuMG cells can overcome ERK suppression via constitutively activated PI3K pathway. More importantly, ERK suppression also contributes to MT regulation of cytokine expression and cytokine responses (see Chapter III).

Discussion

This work deals with regulation of ERK by MT. Initially, there are at least two general questions. The first is why would MT suppress ERK? Suppression seems unexpected. It has been generally assumed that activation of ERK is a part of the proliferative response of cells to growth factors. Indeed, in NMuMGs my data show that ERK activation is needed for cell cycle progression. Models of polyomavirus behavior postulate that this cell cycle response is required to create an environment that is supportive of viral replication. However, despite the effect of MT on ERK, the MT-NMuMGs progress through the cell cycle even when ERK activation is inhibited with U0126. This might mean that ERK activation is unnecessary to create an enviroment suitable for replication. It would be interesting to test this idea directly by measuring polyoma DNA replication in the presence and absence of U0126. There are cells such as NIH/3T3s where MT does activate ERK. Interestingly, in those cells mutations that prevent Shc (ERK) activation do reduce cell cycle progression (Mullane et al., 1998). In those cells the MEK inhibitor might be expected to have an effect on replication. The fact that there are cells or conditions where MT can either activate or repress ERK is not necessarily surprising. For example, ST activates AKT differently under cellular conditions (Andrabi et al., 2007) and MT signaling to PI3K, for example, is important for tumor induction in some tissues but seems to restrict tumor induction in others (Freund et al., 1992a).

The answer to why MT might repress ERK may be found in the conditions that I have been examining -- in response to a variety of stresses. Viruses and cells are always

in an attack/counterattack situation. Infections activate responses such as the activation of PKR in response to viral RNA infection or interferon responses to DNA virus infections. The stress responses potentially limit the extent of viral infection. For example phosphorylation of eIF2 alpha was seen in response to ER stress, UV irradiation and serum starvation in NMuMGs. This phosphorylation blocks global protein synthesis, a response that would restrict the virus. MT does, in fact, reduce eIF2 alpha phosphorylation in response to stress, but that appears to be unrelated to ERK effects, since U0126 does not block the phosphorylation (not shown). As I will discuss, below effects on cell survival and cytokine production and response were observed. It is easy to imagine that the interplay of the infected/transformed cell with the immune system would be of great importance to the virus.

Activation of MEK by RAS activation of RAF or RAS independent activation of other kinases

The second broad question is how MT carries out this regulation. In the most generic sense, the state of ERK activation is a balance of phosphorylation coming from upstream signals and dephosphorylation coming from cellular phosphatases. The canonical pathway of activation is through the activation of RAS leading to RAF activation of MEK, which can activate ERK. Since U0126, which is a specific MEK inhibitor, blocks that activation that I have studied, it appears that RAS targeting of MEK could be important.

Some stress conditions such as serum withdrawal (0% starvation overnight) caused RAS activation. RAS is well known to be activated by growth factors and mitogens, therefore, serum withdraw activation of RAS is very unexpected. Study using cultured

astrocytes had found that serum deprivation transiently enhanced both H-RAS expression and activity that led to activation of ERK1/2. And this change seemed a secondary response to enhanced formation of ROS to trophic deprivation (Messina et al., 2008). This study also showed that acute knock down of H-RAS protected astrocytes from serum deprivation. Usually 0.2-0.5% serum was used for long period (overnight) starvation or 0% serum was used for short period (4-6 hour) starvation reported in literature. So it is possible that with 0% serum overnight treatment, cells are under trophic deprivation stress that leads to activatation of a stress response rather than being quiescent.

However, some stress conditions such as ER stress (TG treatment) did not activate RAS measured by RAS-GTP, but still activated MEK. How could MEK be activated without RAS activation? There could be an effect directly on MEK, such as preventing its dephosphorylation. More likely, it arises from activation of kinase activity towards MEK. RAF would be the most likely candidate. It has been reported that oxidative stress can activate RAF through c-Src- PLCγ- PKC, a RAS-independent pathway (Zou et al., 1996) [or a RAS-dependent pathway (Aikawa et al., 1997)]. Other kinases might be considered. MEKK1 has been shown preferentially regulating JNK pathway (Yan et al., 1994). However, MEKK1 is also capable of activating RAF or MEK independent of RAS (Karandikar et al., 2000; Lange-Carter et al., 1993). Study using COS cells showed dominant negative MEKK1 but not MEKK2, 3, or 4 inhibits ERK activation in response to EGF (Fanger et al., 1997). I tried to see if MT affects the level of MEKK1, but the MEKK1 antibody I used was not good, so the result was not clear. Tumor progression locus 2 gene (TPL2), also known as COT (cancer osaka thyroid), encodes a serine and

threonine protein kinase and is a member of MAPK kinase kinase family. Overexpression of TPL2 has been shown to activate ERK, p38 and JNK pathways (Chiariello et al., 2000; Patriotis et al., 1994; Salmeron et al., 1996). TPL2 is required for MEK and ERK1/2 activation in response to lipopolysaccharide (LPS) (Dumitru et al., 2000). To see if TPL2 is involved in stress activation of ERK, I compared TPL2-/- MEFs to wild type MEFs. My result showed there was no significant difference in term of ERK activation in response to different kinds of stress (not shown). Therefore, TPL2 is unlikely involved in activation MEK/ERK in MEFs. It remains possible that it could function in NMuMGs. Since IRE1 induced by ER stress is a kinase that leads to phosphorylation of ASK1, another MAPKKK (Yang et al., 2009), perhaps it can reach MEK as well. Whether or not it is RAS independent is not clear. Further study would be need to prove that ERK activation is through IRE1 under ER stress, and dominant negative IRE1 could be used to block ERK activation.

How does MT affect MEK (KSR, AKT, others)?

In the absence of stress, MT activates MEK measured by phosphospecific antibody in NMuMGs. When NMuMGs are stressed, the level of phospho-MEK increases. The MT-NMuMGs show little if any increase and even a reduction of phospho-MEK when cells are stressed. In no case after stress is the amount of phospho-MEK in MT cells as high as NMuMGs. In addition to changes in MEK kinases discussed above, activation of MEK also requires scaffold protein such as KSR that brings MEK to activated RAF. It has been suggested that if the expression level of scaffold protein such as KSR is above its interacting components, it may inhibit the cascade by keeping the kinase away from its substrates (Kortum and Lewis, 2004). When I compared the endogenous level of KSR1

in both NMuMGs and MT-NMuMGs, there was no significant difference (not shown). By using KSR1-/- MEFs, I also found that KSR1 is involved in mitogen induced ERK activation, but there was no significant effect on UV induced ERK activation (not shown). Because the MEF experiments might not apply to NMuMGs, I looked further at KSR phosporylation. Both KSR and RAF are negatively regulated by 14-3-3 protein binding (Muller et al., 2001; Zimmermann and Moelling, 1999). Phosphorylation of KSR1 on S392 by C-TAK1 confers its 14-3-3 binding and retains its cytoplasm localization. My data showed there was no significant difference in terms of KSR1 phosphorylation on S392 in MT-NMuMGs compared to NMuMGs (not shown). In the case of RAF, 14-3-3 protein binding is regulated by AKT phosphorylation on Ser259. Consistent with this, inhibition of AKT by AKT inhibitor VIII in MT-NMuMG cells dramatically increases MEK phosphorylation at Ser221 (Fig. 22C). Under stress conditions such as CHX or TG, the effect of inhibition of AKT on MEK phosphorylation is much less than under normal growth conditions. This may suggest that RAF may not be the kinase responsible for activation of MEK under certain stress condition or dephosphorylation of MEK is enhanced by stress.

Other scaffold proteins, such as CNK or MP1 might be also involved in assembling MEK/ERK to form the cascade complexes. Further study need to be done to get the whole picture of the scaffolds involved in different stress regulation of MEK activation.

MT genetics and rescue of MT genetic defects in suppression of ERK activation

Examination of the MT genetics of ERK suppression in NMuMGs was only partially successful. As we showed, compared to wild type MT, Y315F is unable to suppress ERK activation and is also unable to increase turnover of ERK phosphate.

Mutants in other pathways, Y250F and Y322F were as defective as Y315F in failing to suppress ERK. However, neither mutant was able to activate PI3K as measured by phospho-AKT, so it was impossible to decide whether the Shc or PLCγ pathway was making an independent contribution to suppression. The Y250F defect in PI3K was not surprising. It has been shown that in human cells, Y250F is able to activate PI3K and transform cells. In mouse cells, Y250F is defective in PI3K activation and transformation (Utermark et al., 2007). There could be several possibilities that explain Y250F is defective in PI3K activation in NMuMGs. The first is that RAS activation is required for optimal PI3K activation. It has been shown in response to certain growth factors that direct binding of RAS to PI3K enhances PI3K activity and required for RAS induced tumorigenesis (Gupta et al., 2007). Since Y250F is defective in activation of RAS, it is possible that PI3K activity is reduced too. The second possibility is that Y250 can be a binding site for both NPXY and SH2 domains. In vitro study showed Shc SH2 domain can bind MT at Y250 (Dilworth et al., 1994), which supports the idea that 250YXXM motif provides a binding site for SH2 domain containing protein. Perhaps Y250 may be involved in direct binding SH2 domain of p85 subunit of PI3K and enhancing the activation of PI3K. A third possibility, perhaps the most likely, is that Y250 is a recruitment site for an adaptor such as Gab-1 that can recruit PI3K to the MT complex (Ong et al., 2001). This mode of recruitment might be more important in some cells than in others.

The defect of Y322 was not expected. It has been shown that in Balb 3T3 cells Y322F associated with PI3K like wild type (Su et al., 1995). It is not required for PI3K activation in certain human cells and mouse cells (Utermark et al., 2007). My result may

be related to NMR data showing that the N-terminal SH2 domain of p85 subunit of PI3K showed a higher affinity (10 fold) for a peptide with two phosphotyrosines (315pY/322pY) than the same peptide only has one (315pY/322Y) (Weber et al., 2000). This observation supports the idea that there is a second binding site for N-terminal SH2 domain of p85 subunit of PI3K. Therefore, it is possible that phosphorylation of Y322 may enhance the binding of N-terminal SH2 domain of p85 to MT and activation of PI3K in NMuMGs. The importance of such a mechanism would depend on how much doubly phosphorylated MT there is. There is no data one way or the other on this, but there could certainly differences among cells in the amount of MT tyrosine phosphorylation.

Since all mutants defective in PI3K in NMuMGs were defective ERK suppression and since the only known defect in Y315F is in PI3K activation, I carried out experiments to test the role of PI3K activation in ERK regulation. These experiments gave mixed results. To understand whether PI3K is sufficient for ERK suppression by MT, H1047R retrovirus were used to make Y315F+H1047R NMuMG stable cell lines to see if rescue of Y315F PI3K activity can rescue lack of suppression of ERK by Y315F. H1047R can fully recover PI3K phosphorylation of AKT as wild type MT and partially rescue the lack of ERK suppression by Y315F (Fig. 21). However when look at ERK phosphate turnover, Y315F+H1047R did not significantly increase the rate of ERK phosphate turnover as MT.

If the signaling through Y315 is what MT uses to suppress ERK why would activated PI3K not complement the defect? First, there are three isoforms p110α, p110β and p110δ in class IA of PI3K. We only tested the H1047R, which is a p110α mutant. MT also binds and presumably activates 110β. Perhaps 110β is involved in suppression. Second, MT is a cellular membrane protein, and it is well known that MT activates PI3K

in cellular membrane. It is not known in terms of MT activation whether there is important submembrane localization, which might not be seen with H1047R. A third possibility is there may be other signaling pathways rather than PI3K through Y315. This has been suggested by Hong (Hong et al., 2003). If so, PI3K would not be able to rescue Y315F.

Other than PI3K, are any other pathways involved in MT regulation of ERK?

In general, I wanted to know if the effect of MT on the turnover of ERK phosphate could be seen in other cells. I also wanted to try to test other MT functions in a background where mutations such as Y250F and Y322F did not affect PI3K kinase activation. To do this, I turned to a series of MCF10A cell lines developed by Tao Jiang that expressed different MTs. MCF10As are human breast epithelial cells that are often used for breast cancer studies. In them, Y250F and Y322F both could acivate PI3K signaling as measured by AKT phosphorylation after brief serum withdrawal. By comparing wild type and mutants (Y250F and Y322F) in human mammary epithelial MCF10A cells, we found that wild type or Y250F MT also activated ERK phosphatases while Y322F was defective. This result suggested that signaling through Y322 is an additional pathway involved in ERK regulation.

The known downstream target of Y322 is PLCγ (Su et al., 1995). There are not many studies reported on the downstream effectors of PLCγ activated by MT (Shepard et al., 2007). Also most published studies showed activation of PLCγ and PKC pathways are involved in activating ERK under certain conditions rather than suppressing (Armstrong et al., 2009; Ueda et al., 1996). One way to confirm PLCγ pathway involved is to use activator of PKC such as TPA or inhibitor of PLCγ such as drug U-73122 to see

the effect on ERK. Transient treatment NMuMGs with TPA actually activates MEK/ERK under both normal and stress conditions (not shown). The other well-known effect of PLCγ activation is elevation of intracellular calcium. Experiments in MCF10A cells using ionomycin to elevate intracellular calcium did not show a restoration of ERK suppression by Y322F.

The results raise two questions. First, if the turnover of ERK phosphate depends on Y322, why is Y315F defective in NMuMGs? One could imagine that PI3K is somehow necessary for some PLCγ signaling event (Falasca et al., 1998). However, as discussed above, expression of activated PI3K or AKT did not restore the turnover of ERK phosphate in Y315F. This leaves us with the possibility that overexpressed PI3K or AKT signaling localization is different from the localization where MT activated PI3K and AKT signalings in NMuMGs. A second question is how can activators that mimic the activation of PLCγ fail to complement Y322F for ERK suppression? Of course, localization of the PLCγ signal could be important. It is known that the site of generation of a calcium signal can determine the output (Ghosh and Greenberg, 1995). A more interesting possibility is that there is another as yet uncharacterized function from Y322F. Certainly in the case of the PDGF receptor, Nck is known to bind to the same phosphotyrosine as PI3K (Nishimura et al., 1993).

How does MT regulate DUSPs?

Whatever pathways are used for MT activation of ERK phosphate turnover, the more direct question is how the activity of the ERK phosphatases are regulated. There are at least two DUSPs upregulated in MT-NMuMGs. Very interestingly, they seemed to be regulated at different levels. MKP3/DUSP6 is significantly upregulated at RNA level.

As discussed earlier, MT activates transcrption through Ets and AP1 sites. The promoter for DUSP6 has not been well-characterized. VHR/DUSP3 is increased most at protein level in MT. This is not likely to be at the level of turnover since cycloheximide chase experiments did not show a difference in the rate of turnover (not shown). If it is not degradation or RNA, then a translational effect seems likely. MT is known to affect 4EBP1, the cap binding protein. This would lead to global effects on protein translation. However, specific effects could also occur. It is known that translation can be regulated by signaling that regulated binding of translational regulators such as CPEB to mRNA (Richter, 2007).

Hydrgen peroxide stress provides an additional issue. All the PTP and DUSP family members have a cysteine at catalytic active site which is supposed to be inactivated by peroxide. When exposed to peroxide, NMuMGs did response and led to more ERK phosphorylation while MT-NMuMGs did not. This result can be explained by either the level of DUSP activity is really high in MT cells and therefore needs more peroxide to inactivate it, or MT regulates certain pathways which block peroxide effect.

A final question is why I could not see effects with knockout of single DUSPs. This is likely to be because multiple enzymes have redundant function. siRNA DUSP knockdowns suggest that at least 5 DUSP are involved in gonatdotropin releasing hormone regulation of ERK (Armstrong et al., 2009). Further experiments of knockdown both VHR and MKP3 should be tested to verify whether they have redundant function in regulation of ERK.

Chapter III. Polyomavirus Middle T antigen modulates cytokine expression and cytokine response

Introduction

When expressed as a transgene, MT can induce tumors in a variety of tissues (Aguzzi et al., 1990; Cecena et al., 2006). In the mouse mammary gland, expression of MT using the MMTV promoter can induce metastatic tumors (Guy et al., 1992). This MMTV-MT mammary tumor system has been widely used to understand the process of tumorignesis. It is known that mutations affecting particular pathways of MT have different effects on tumorigenesis in different tissues (Freund et al., 1992a). The strain of mice also makes a significant difference for the latency and metastatic potential (Lifsted et al., 1998). Stromal cells in the surrounding tumor microenviroment are also important for tumor iniation and metastasis. Macrophages, for instance, have been shown to be involved in tumor cell migration and metastasis in mammary tumors (Wyckoff et al., 2004). Study using $Csf1^{op/op}$ polyoma MT transgenic murine mammary model has shown the lack of macrophages decrease invasion and metastasis but not the incidence of the primary tumors (Lin et al., 2001a; Lin and Pollard, 2004). How MT expression regulates cytokine and macrophages infiltration at molecular level has not been reported and is worth investigation.

1. Inflammation and cancer

The link between inflammation and cancer has gotten more attention recently as

evidence appears from multiple studies including both epidemiology and molecular biology (Balkwill et al., 2005; Balkwill and Mantovani, 2001; Coussens and Werb, 2002). In a simplified model, the connection between inflammation and cancer can be understood as two pathways: an extrinsic pathway, driven by extrinsic inflammation or infection that increase the risk of cancer development, which has been seen in colorectal (Flossmann and Rothwell, 2007), and prostate cancer (Vasto et al., 2008); and an intrinsic pathway, activated by genetic lesions such as activation of oncogenes that cause inflammation and neoplasia (see review (Mantovani et al., 2008)). One example of the intrinsic pathway model is seen in human papillary thyroid carcinoma, in which activation of the oncogene RET/PTC induces inflammatory transcriptional program that is associated with malignant behavior (Borrello et al., 2005). The two pathways converge and produce a cancer-related inflammatory microenviroment which promotes malignant cells survival, proliferation, and metastasis.

The key factors involved in this process includes transcription factors (such as NF-κB (Karin, 2006), signal transducer and activator of transcription 3 (STAT3) (Yu et al., 2007)), and inflammatory cytokines, chemokines, and prostaglandins (such as TNF-α (Szlosarek and Balkwill, 2003), IL-6 (Grivennikov and Karin, 2008), COX2 (Koehne and Dubois, 2004)).

2. TNFα-a master switch for inflammation to cancer

The TNF super family includes at least 19 different ligands which share a C-terminal extracellular domain, also called TNF homology domain (Aggarwal, 2003). TNFα is a type II transmembrane protein like most members of the super family. TNFα acts through

TNF receptors. There are two TNF receptors. TNFα binds both TNF receptor 1(TNFR1) and TNF receptor 2 (TNFR2). Most normal and transformed cells express TNFR1 while TNFR2 is limited to certain cell types, for example, endothelial cells and immune cells. TNFR1 activation results in different outcomes depending on the cellular context. TNF can activate both apoptotic and anti-apoptotic signals in which anti-apoptotic signals require active protein synthesis mediated through NF-κB (Karin and Lin, 2002). Most TNF superfamily members activate NFκB through ubiqutin-mediated degradation of its inhibitor, IκBα. In an inactive state, the NFκB/Rel transcriptional factors are present in cytosol complexed with the inhibitory IκB proteins (Baeuerle and Baltimore, 1988). Activation of NFκB occurs when phosphorylation of IκBα at Ser32 and Ser36 followed by its proteasome-mediated degradation which leads to release and nuclear translocation of active NFκB (Brown et al., 1995). If NF-κB activation is inadequate, apoptosis will be a late response mediated through caspase 8 and 3 (Balkwill, 2009).

TNFα was first identified as its ability to induce rapid necrosis of experimental cancers (Balkwill et al., 1986; Brouckaert et al., 1986). Later studies using knock out mice found TNFα is more tumor-promoting than expected. TNFα null mice are more resistant to skin cancer than wild type mice (Moore et al., 1999). TNFR1 and TNFR2 knock out mice also showed reduced susceptibility to TPA (12-O-tetradecanoylphorbol 13-acetate) induced skin cancer (Arnott et al., 2004). Inhibition of TNFα production in liver stromal cells decreased the liver tumor incidence (Pikarsky et al., 2004). In the Mdr2 P-glycoprotein gene -/- liver tumor model (spontaneously develops cholestatic hepatitis followed by hepatocellular carcinoma) (Pikarsky et al., 2004), TNFα induced NFκB activation was identified as the critical mediator in promoting tumor development.

More generally, studies using deletion of IKKβ in different cell systems showed inactivation of IKKβ/ NFκB pathway attenuates inflammation associated tumors (Greten et al., 2004). Together, all this work indicates the importance of TNFα signaling in tumor development.

3. Overview of cytokines and chemokines during mammary tumorigenesis

IL-6 and breast cancer

Interleukin (IL)-6 is an inflammatory cytokine. Many cell types including macrophages, T-cells and stromal cells can produce IL-6 in response to TNFα and interleukin-1 (IL-1). The regulation of IL-6 is mainly at the transcriptional level. In addition to transcription factor regulatory sites (including NFκB, CREB, C/EBP and AP-1) around-2kb upstream of IL-6 promoter region (Xiao et al., 2004), a novel transcriptional active region was found located -5kb upstream of IL-6 transcription start site (Samuel et al., 2008). IL-6 has been found playing multiple roles in cellular and physiological responses including inflammation, hematopoiesis, and tummorigenesis (Hodge et al., 2005). IL-6 signaling starts with binding to IL-6 receptor and activation of JAK kinases (Stahl et al., 1994). JAK kinases therefore phosphorylate and activate STAT transcriptional factors, for example, STAT3 (Takeda et al., 1998). In different cellular contexts, IL-6 is also able to activate RAS pathway (Ogata et al., 1997).

IL-6 regulates chronic inflammation which creates a inflammatory microenviroment beneficial to cancer growth (Lu et al., 2006). There is growing evidence suggesting the importance of IL-6 signaling in tumorigenesis. Some clinical studies from breast cancer patients showed the extent of the increase of IL-6 in serum is correlated with poor disease

outcome (Bachelot et al., 2003; Knupfer and Preiss, 2007). A recent study found that IL-6 gene expression is upreguated in mammospheres obtained from aggressive ductal breast cancer carcinomas. More interestingly, they also found IL-6 triggers normal mammosphere and tumor mammosphere's self-renewal, survival under hypoxia condition and invasive potential (Sansone et al., 2007).

Chemokine

Chemokines are a superfamily of chemotactic cytokines. They are small (8-10kDa) proteins. The classification of chemokines is based on the position of the first two cysteines in the N-terminus which forms a conserved tetra-cysteine motif with other cysteines. CXC (separated by a non-conserved amino acid) and CC (next to each other) are the two major classes of chemokine. In addition to the major classes, there are also CX3C and XC. So far, there are at least 50 human chemokines identified (Ali et al., 2007).

Chemokines were originally characterized by stimulation of leukocytes chemotaxis during inflammatory response. It is now known that they also play important roles in homeostasis, proliferation, angiogenesis (Hwang et al., 2005; Strieter et al., 2005) and cancer metastasis (Izraely et al.). Not only leukocytes, fibroblasts, endothelial cells, and epithelial cells (both normal and malignant) are also able to produce chemokines. Local chemokine production and the chemokine gradient are important determinants of the types of cells that make up the immune-cell infiltrate in solid tumors. For instance, early studies had shown CC chemokines are important determinants of the macrophage and lymphocyte infiltrate in human breast cancer, cervical cancer, and sarcomas (Bottazzi et al., 1983). In human breast cancer, CCL5 (Azenshtein et al., 2002) (Zhang et al., 2009)

and CCL2 (Saji et al., 2001) have been shown in correlation with tumor progression. In support of this, the murine breast cancer model study has shown CCL5 receptor antagonist Met-CCL5 inhibits macrophage infiltrate and slows down tumor growth (Robinson et al., 2003).

Chemokine receptors belong to G-protein-coupled receptor family with seven transmembrane domains and can bind more than one type of chemokine. The profile of chemokine-receptor expression is determined not only by the lineage and differential stage of the individual cell, but also the microenvironmental factors, for example, the local chemokine and inflammatory cytokine concentration and hypoxia (Balkwill, 2004a). Malignant cells from different cancer types may express different profiles of chemokine receptors, while the most common one found in human and murine cancer cells is CXCR4 (Balkwill, 2004b). Chemokine receptors and their ligands direct cell movement during inflammation and cancer by affecting cell motility and invasiveness (see review (Balkwill, 2004a)).

Tumor associated macrophage (TAM)

Tumors are masses of tumor cells and stromal cells. The predominant stromal cells found in tumors are macrophages, lymphocytes, endothelial cells and fibroblasts. Tumor associated macrophage (TAM) infiltration has been shown correlated with metastasis and poor patient prognosis (review (Bingle et al., 2002)). Studies have shown that TAMs produce mediators that promote angiogenesis (Lewis et al., 2000), matrix degradation and tumor growth (Allavena et al., 2008). Macrophages are recruited through the local production of chemokines such as CSF-1, CCL2 and CCL5. It is known that macrophage plays an important role in MT tumorigeneis.

Results

3.1.1. MT attenuates ERK activation induced by TNFα

In chapter II, I had studied different kinds of stress induced ERK activation. TNFα, which is considered a biological stress, has gotten more attention recently as a possible switch from inflammation to tumorigenesis. Some work showed TNFα can activate MAPK pathways (Rivas et al., 2008; Yeh et al., 2008). In MT transgenic mice, it is known that MT recruit macrophages which secret different cytokines including TNFα. To get more insight of how MT affects response to TNFα, we compared NMuMGs and MT-NMuMGs. First, I tested NMuMGs treated with TNFα at different time courses. TNFα induced transient ERK activation at very early time points (peak at 5-15 min). MT reduced ERK activation similar as other stress conditions showed earlier (Fig.35.Left). It is known that TNFα also activates other MAPK family members such as JNK, p38. This was also confirmed in our system. Consistent with my previous data, MT specifically suppresses ERK, but not JNK or p38 after TNFα treatment (Fig.35.Right).

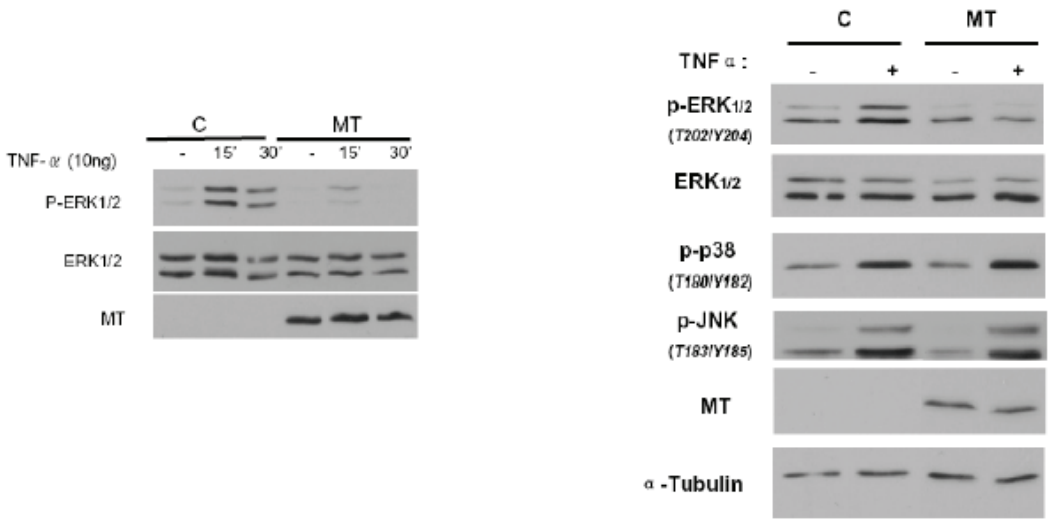


Fig. 35. MT attenuates ERK activation induced by TNFα.
NMuMG and MT-NMuMG cells were untreated or treated with TNFα (10ng/ml) for indicated time (**Left**) or 15 min (**Right**), whole cell lysates were collected and three MAP kinase family members were detected by immunoblot. Results are representative of experiments performed at least three times.

3.1.2. MT sensitizes TNFα-induced apoptosis

As shown in Figure 33, suppression of ERK seemed to improve survival of cells exposed to peroxide. To investigate the consequences of ERK activation subjected to TNFα, NMuMG cells were pretreated with DMSO or specific MEK inhibitor U0126 30 min before treated with TNFα. 24 or 48 hours later, protein samples were collected and blotted with different survival and apoptotic markers (Fig.36.A). PARP is a nuclear poly (ADP-ribose) polymerase and one of the main cleavage targets of caspase-3. Cleavage of PARP occurs during caspase-3, or -7 dependent apoptosis. Block of ERK activation by U0126 enhanced PARP cleavage and PAR. This indicates that ERK activation stimulated by TNFα protects cell from apoptosis. Combining the peroxide results (suppression of ERK protects) with TNFα results, it appears ERK functions in survival. If suppression of ERK has a pro-apoptotic function in cells subjected to TNFα, we might expect that MT sensitizes TNFα induced apoptosis. As expected, compared to control cells treated with TNFα, MT cells treated with TNFα induced more PARP cleavage (Fig.36.B) and sensitized cells to TNFα-induced apoptosis. The downstream effectors of ERK involved in TNFα-induced apoptosis need to be determined.

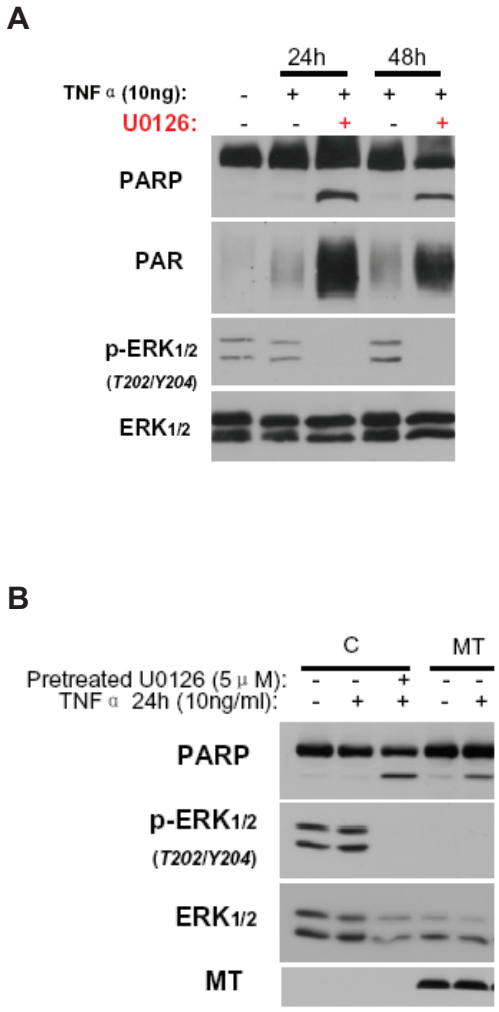


Fig. 36. MT sensitizes apoptosis in cells subjected to TNFα.

A. **Suppression of ERK sensitizes TNFα induced apoptosis.** NMuMG cells were pretreated with or without U0126 (20μM) 30 min before treated with 10ng/ml TNFα for the indicated times. Whole cell lysates were blotted with PARP, PAR, phospho-ERK and total ERK. Results are representative of experiments performed at least three times.

B. **MT sensitizes apoptosis subjected to TNFα,** NMuMG stable cell lines expressing GFP (C) or MT were untreated or pretreated with or without U0126 (5μM) for 30 min, and then exposed to 10ng/ml TNFα for 24 hours. Whole cell lysates were blotted with PARP, phospho-ERK, total ERK and MT. Results are representative of experiments performed at least three times.

3.2. MT enhances cytokine IL-6 induced by TNFα

MT sensitizes cells to apoptosis induced by TNFα. I wondered whether MT also affects cytokine response to TNFα. To answer this question, first we looked at IL-6 because IL-6 has been shown to play important roles during tumorigenesis. Very surprisingly, MT enhanced IL-6 at RNA level (7 fold) in the absence of TNFα,. After TNFα treatment, IL-6 was slightly induced (3.5 fold) in NMuMGs, while IL-6 was upregulated significantly (168 fold) at RNA level in MT-NMuMGs (Fig.37.A). Secreted IL-6 collected from the same experiment measured by ELISA assay showed a dramatic increase in protein as well. Again MT induced IL-6 even without TNFα treatment. MT enhanced IL-6 about 40 fold compared to NMuMGs after TNFα treatment (Fig.37.B). This result suggested that MT regulates cytokine IL-6 expression and responses to TNFα.

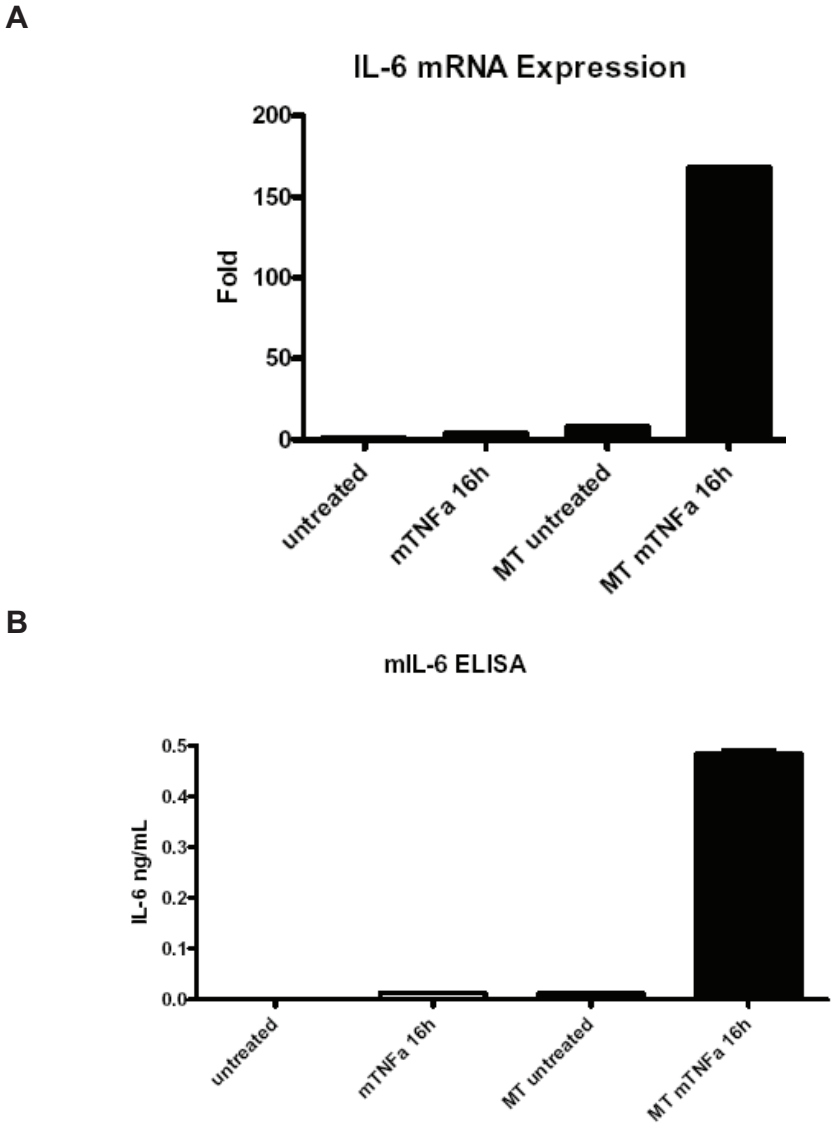


Fig. 37. MT enhances cytokine IL-6 subjected to TNFα.

A. NMuMG and MT-NMuMG cells were untreated or treated with TNFα (10ng/ml) for 16h and RNA was collected. Quantitative PCR was performed as described in Materials and Methods.

B. NMuMG and MT-NMuMG cells were untreated or treated with TNFα (10ng/ml) for 16h and supernatant was collected. ELISA was performed as described in Materials and Methods. Bars represent Mean+ SEM.

3.3. Suppression of ERK by U0126 increases IL-6 mRNA induced by TNFα

To investigate whether suppression of ERK by MT contributes to enhanced IL-6 expression induced by TNFα, NMuMG cells were pretreated with different doses of U0126 for 30 min and then exposed to TNFα for 16 hours. Quantitative PCR was performed. Cells treated with TNFα had about 3 fold increase of IL-6 compared to untreated. Pretreated cells with MEK inhibitor increased TNFα induced IL-6 to 10-20 fold at RNA level in a dose dependent manner (Fig.38.). This result suggested that suppression of ERK by MT sensitizes TNFα-induced apoptosis and favors IL-6 expression at the RNA level. IL-6 has been reported to have anti-apoptotic (Lin et al., 2001b; Moran et al., 2009) or pro-apoptotic (Regis et al., 2009; Wagley et al., 2007) effect in different cellular contexts. Whether induction of IL-6 in MT cells contributes to TNFα-induced apoptosis needs further study.

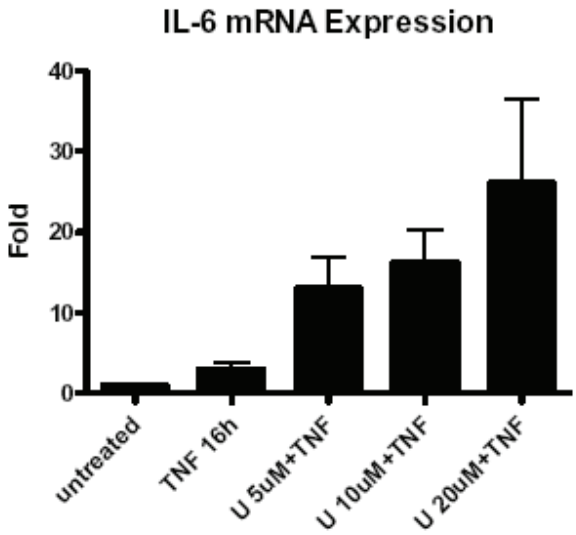


Fig. 38. Suppression of ERK by U0126 enhances cytokine IL-6 subjected to TNFα. NMuMG cells were untreated or pretreated with U0126 (U) and then treated with TNFα (10ng/ml). RNA was collected after 16 hours and Quantitative PCR was performed as described in Materials and Methods. Bars represent Mean+ SEM.

3.4. Suppression of ERK increases IL-6 mRNA induced by UV irradiation

Other kinds of stress have also been shown to induce IL-6. One example of this is increased production of IL-6 in response to UV irradiation (Schwarz and Luger, 1989). As shown in the left panel of Figure 3.4, examination of IL-6 mRNA eight hours after treatment of NMuMGs with 30J/m^2 UV showed an increase of two to three fold in IL-6 mRNA. Prevention of increased ERK activity with U0126 leads to an increase of nine-fold in IL-6 (Fig.39 left). In the MT-NMuMGs, UV treatment also results in an increase in IL-6 production compared to the NMuMG cells (Fig.39 right). Together, these data suggested suppression of ERK increases IL-6, and MT enhances IL-6 at RNA level subjected to UV.

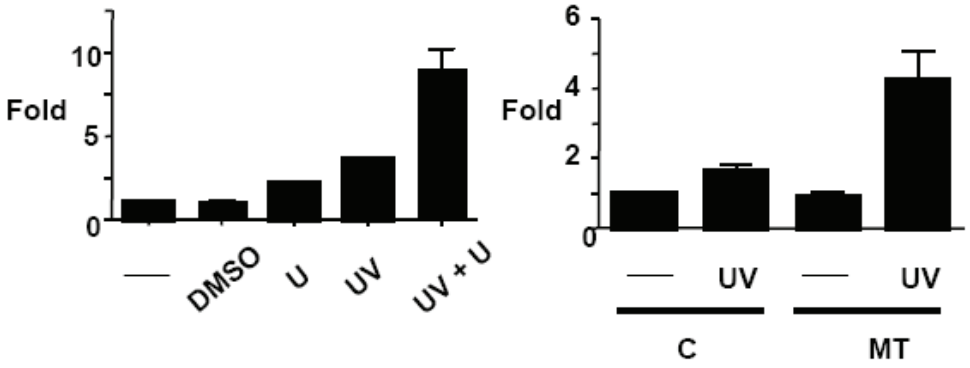


Fig. 39. Suppression of ERK increases IL-6 mRNA induced by UV irradiation. Left panel: NMuMGs were untreated or pretreated with DMSO, U0126 (5μM) (U) and then exposed to UV (30J/m^2). RNA was collected after 8 hours and Quantitative PCR was performed as described in Materials and Methods. Bars represent Mean+ SEM. **Right Panel:** NMuMGs or MT-NMuMGs were untreated or UV irradiated as in A. RNA was analyzed as above. Bars represent Mean+ SEM.

3.5. MT reduces induction of COX2 by TNFα.

As mentioned earlier COX2 expression also depends on ERK, and my data showed MT suppresses COX2 expression subjected to hypoxia. I wondered whether TNFα also activates COX2. As measured by realtime-PCR, COX2 was induced up to 4 to 5 fold after 17 hour TNFα treatment. In contrast to enhanced IL-6 induction by MT, MT reduced COX2 induced by TNFα (Fig.40) similar as hypoxia stress showed earlier. Further experiments need to verify whether MT suppressed COX2 induced by TNFα is also through ERK suppression.

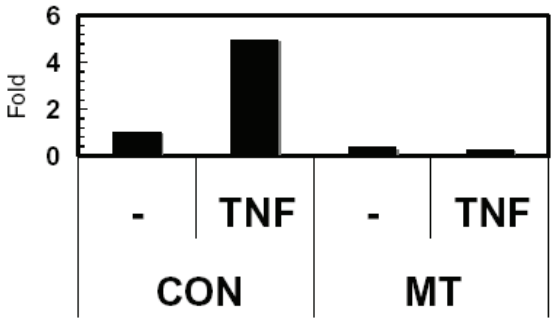


Fig. 40. TNFα stimulates COX2 RNA, but MT reduces it.
NMuMG and MT-NMuMG cells were untreated or treated with TNFα (10ng/ml) for 16 hours. RNA was collected and Quantitative PCR was performed as described in Materials and Methods. Results are representative of experiments performed at least three times.

3.6. MT modulates cytokine expression and cytokine response

So far, the data showed MT enhances IL-6, and reduces COX2 under certain stress conditions like UV, hypoxia, and TNFα. To figure out whether MT has broader effects on the spectrum of cytokines than I had tested, high throughput assays such as cytokine array and gene array were applied.

3.6.1. Cytokine Array

Traditionally, cytokines were detected by ELISA assay. Cytokine arrays allow simultaneously detection of multiple cytokines at same time. Also the detection range is much greater than traditional ELISA. To do the cytokine array, NMuMG cells and MT-NMuMG cells were untreated or treated with TNFα for 17 hours. Supernatants were collected and concentrated (4 times) by using Amicon Ultra 3KD filter and applied to Raybio mouse cytokine array I (Fig.41A). Unlike ELISA, the autoradiography is applied to get signals. The intensity of signals can be quantified by densitometry. The positive control signals at each corner of membranes can be used to normalize results from different membranes. In the untreated samples, similar intensity of positive control signals was detected for both NMuMGs and MT- NMuMGs. We found there was basal level of CCL2 (MCP-1) in NMuMG sample. Much higher CCL2 signal, IL-6 and CCL5 (MCP-5) signals were also detected in the MT- NMuMG sample. This suggests MT induced CCL2, IL-6 and CCL5 without TNFα stimulation. After 17 hour of TNFα treatment, CCL2 was upregulated in NMuMG sample. In MT- NMuMG sample, not only CCL2, but also IL-6 and CCL5 were significantly upregulated. Relative weak signals of GCSF, GM-CSF and IL-12p70 can also be detected (Fig.41B). Together the results showed MT regulates cytokine expression and cytokine response to TNFα. ELISA assay

needs to be used to quantify each cytokine in further study. As mentioned earlier, CC chemokines such as CCL2 and CCL5 are important for macrophage and lymphocyte infiltrate and tumor progression in breast cancer (Saji et al., 2001; Zhang et al., 2009). Therefore, it would be worth to study further each of these cytokines induced by MT transformed cells and to understand their function in MT mammary tumorigenesis.

Since the cytokine array we used does not include all the cytokines, it is possible MT may regulate other cytokines. Also due to the sensitivity limitation of the array, there could be cytokines induced but we could not observe.

A

	A	B	C	D	E	F	G	H
1	Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
2	Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13
4	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12p70	IL-13
5	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
6	IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
7	Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	Pos
8	Thrombopoietin	VEGF	Blank	Blank	Blank	Blank	Blank	Pos

B

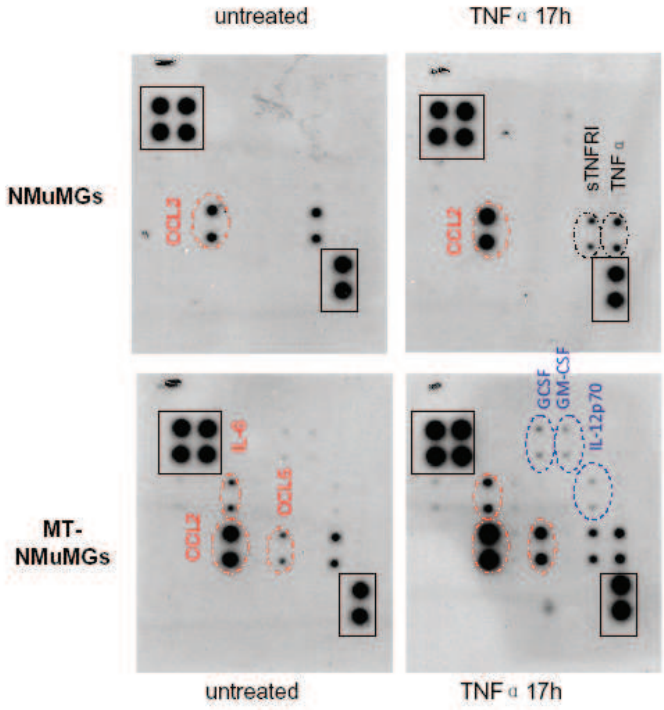


Fig. 41. MT modulates cytokine expression and cytokine response.
A. Raybio mouse cytokine array I. **B**. NMuMG and MT-NMuMG cells were untreated or treated with TNFα (10ng/ml) for 17h and cytokine array was performed as described in Materials and Methods. Positive controls are in square and rectangle at the corners of membrane.

3.6.2. Gene Array

Since most cytokines are regulated at RNA level. To get a better picture of cytokine regulation by MT, we applied gene array. NMuMGs and MT-NMuMG cells were treated the same way as cytokine array for TNFα 17 hours and mRNA was isolated and purified. Gene array (GeneChip® Mouse Genome 430A 2.0 Array (14,000 genes), Affymetrix) was applied. Consistent with the cytokine array data, MT upregulates CCL2 at RNA level about 12 fold even before TNFα treatment. In addition to CCL2, there are 34 genes upregulated more than 2 fold in MT cells (Fig.42.A). The downregulated genes by MT were all less than 3 fold (not shown). After TNFα treatment, there are 70 genes upregulated at least 2 fold. On the top of the list is CCL2, which is further upregulated by MT to almost 29 fold compared to NMuMG cells. More interestingly, other than chemokines, two matrix metalloproteinase (MMP) genes expression were significantly upregulated by MT after TNFα treatment (Fig.42.B). They are MMP13 and MMP3 (Sternlicht et al., 1999). MMPs are a family of zinc-dependent endopeptidases (Martin and Matrisian, 2007). The family is divided into different groups based on substrate specificity and cellular localization. They are involved in break down extracellular matrix and tumor invasion and metastasis (Zigrino et al., 2009). Other studies showed that CC chemokines such as CCL2 and CCL5 induce MMP9 in macrophages (Robinson et al., 2002). Whether CCL2 or CCL5 is responsible for induction of MMPs in MT cells needs investigation. There are 4 genes downregluated more than 3 fold by MT after TNFα treatment (Fig.42.D). Northern Blot or quantitative RT-PCR will be required to confirm gene array results. Whether MMP13 and MMP3 have any significant function during MT cell migration needs further study.

	MT vs. Ctl
Fold	**Gene Name**
12.7	chemokine (C-C motif) ligand 2
7.8	chemokine (C-X-C motif) ligand 1
6.7	chemokine (C-X-C motif) ligand 5
6.1	chemokine (C-C motif) ligand 7
4.9	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha
4.6	avian reticuloendotheliosis viral (v-rel) oncogene related B
4.3	unknown
3.4	RIKEN cDNA E430024C06 gene
3.4	ectonucleotide pyrophosphatase/phosphodiesterase 2
3.2	unknown
3.2	vascular endothelial growth factor C
2.8	vascular cell adhesion molecule 1
2.8	tumor necrosis factor receptor superfamily, member 9
2.7	mannose receptor, C type 1
2.7	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha (Nfkbia), mRNA
2.7	vascular cell adhesion molecule 1
2.6	Rho guanine nucleotide exchange factor (GEF) 3
2.6	Ras-related associated with diabetes
2.5	interleukin 2 receptor, gamma chain
2.5	RIKEN cDNA 1200016E24 gene
2.5	procollagen, type V, alpha 3
2.4	tumor necrosis factor receptor superfamily, member 9
2.2	laminin, beta 3
2.2	histocompatibility 2, D region locus 1
2.1	interleukin 2 receptor, gamma chain
2.1	RIKEN cDNA 1200003I10 gene
2.1	neural precursor cell expressed, developmentally down-regulated gene 9
2.1	TNFAIP3 interacting protein 1
2.1	heparan sulfate 6-O-sulfotransferase 2
2.0	platelet derived growth factor, B polypeptide
2.0	sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A
2.0	uridine phosphorylase 1
2.0	RIKEN cDNA 0610025L06 gene
2.0	LIM domain only 7

Fig. 42. MT modulates cytokine expression and cytokine response at RNA level. A. Genes upregulated more than 2 fold in MT-NMuMGs vs. NMuMGs without TNFα treatment. Total RNA was isolated in NMuMGs and MT-NMuMGs. Purified RNA were submitted to the DFCI Microarray Core Facility and DNA arrays were conducted on GeneChip® Mouse Genome 430A 2.0 Array, Affymetrix.

	MT vs. Ctl (+TNFα 17h)
Fold	**Gene Name**
28.9	chemokine (C-C motif) ligand 2
16.2	matrix metallopeptidase 13
14.1	chemokine (C-X-C motif) ligand 5
10.4	guanylate nucleotide binding protein 2
9.3	chemokine (C-X-C motif) ligand 1
8.4	ectonucleotide pyrophosphatase/phosphodiesterase 2
8.3	guanylate nucleotide binding protein 2
8.0	chemokine (C-C motif) ligand 7
7.9	serum amyloid A 3
6.2	matrix metallopeptidase 3
6.0	RIKEN cDNA 1200016E24 gene
5.9	RIKEN cDNA E430024C06 gene
5.9	guanylate nucleotide binding protein 1
5.2	Fas (TNF receptor superfamily member)
4.9	mannose receptor, C type 1
4.8	serine (or cysteine) peptidase inhibitor, clade B, member 2
4.6	RIKEN cDNA 1200016E24 gene
4.4	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha
4.3	RIKEN cDNA 1200003I10 gene
4.0	Tnf receptor-associated factor 1
3.9	unknown
3.8	proliferin
3.6	glycoprotein 49 A
3.3	guanylate nucleotide binding protein 4
3.3	uridine phosphorylase 1
3.2	tumor necrosis factor receptor superfamily, member 9
3.1	unknown
3.1	Nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha (Nfkbia), mRNA
3.0	tumor necrosis factor, alpha-induced protein 2
3.0	matrix metallopeptidase 10
2.8	vascular cell adhesion molecule 1
2.8	RIKEN cDNA 1810009M01 gene
2.7	ceruloplasmin
2.6	vascular cell adhesion molecule 1
2.6	toll-like receptor 2
2.6	vascular cell adhesion molecule 1
2.5	avian reticuloendotheliosis viral (v-rel) oncogene related B
2.5	syndecan 4

MT vs. Ctl (+TNFα 17h)	
Fold	**Gene Name**
2.4	mannosidase 2, alpha B2
2.4	TNFAIP3 interacting protein 1
2.3	ribosomal protein L30
2.3	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
2.3	chemokine orphan receptor 1
2.2	chemokine (C-C motif) ligand 9
2.2	DTW domain containing 1
2.2	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1
2.2	tumor necrosis factor, alpha-induced protein 2
2.2	solute carrier family 20, member 1
2.1	poly A binding protein, cytoplasmic 1
2.1	RNA, U22 small nucleolar
2.1	chemokine (C-C motif) ligand 9
2.1	lung carcinoma myc related oncogene 1
2.1	phosphatidic acid phosphatase type 2B
2.1	RIKEN cDNA 2600011C06 gene
2.1	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
2.1	RIKEN cDNA 2310003F16 gene
2.0	proteasome (prosome, macropain) 28 subunit, beta
2.0	phosphatidic acid phosphatase type 2B
2.0	RAB30, member RAS oncogene family
2.0	RIKEN cDNA 4930553M18 gene
2.0	RAS related protein 1b
2.0	interleukin 2 receptor, gamma chain
2.0	RIKEN cDNA 0610011I04 gene
2.0	RIKEN cDNA 2610019E17 gene
2.0	acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
2.0	tumor necrosis factor receptor superfamily, member 9
2.0	expressed sequence C79248
2.0	ribosomal protein S9 /// similar to 40S ribosomal protein S9

Fig. 42. MT modulates cytokine expression and cytokine response at RNA level (continue). B. Genes upregulated more than 2 fold in MT-NMuMGs vs. NMuMGs after TNFα treatment. Total RNA was isolated in NMuMGs, MT-NMuMGs 17 hour TNFα (10ng/ml) treatment. Purified RNA were submitted to the DFCI Microarray Core Facility and DNA arrays were conducted on GeneChip® Mouse Genome 430A 2.0 Array, Affymetrix.

MT vs. Ctl (downregulated)

Fold	Gene name
2.0	semaphorin 3E
2.0	inhibitor of DNA binding 2
2.0	paired related homeobox 2
2.0	inhibitor of DNA binding 2
2.0	component of Sp100-rs
2.0	cadherin 9
2.0	small proline-rich protein 2A
2.0	ceruloplasmin
2.5	small proline-rich protein 2A

Fig. 42. MT modulates cytokine expression and cytokine response at RNA level (continue). C. Genes downregulated more than 2 fold in MT-NMuMGs vs. NMuMGs. Total RNA was isolated in NMuMGs and MT-NMuMGs under growth condition. Purified RNA were submitted to the DFCI Microarray Core Facility and DNA arrays were conducted on GeneChip® Mouse Genome 430A 2.0 Array, Affymetrix.

MT vs. Ctl +TNFα(downregulated)

Fold	Gene name
2.0	development and differentiation enhancing
2.0	Angiopoietin-like 2 (Angptl2), mRNA
2.0	heat shock protein family, member 7 (cardiovascular)
2.0	procollagen, type IV, alpha 2
2.0	glucosaminyl (N-acetyl) transferase 1, core 2
2.0	DNA segment, Chr 8, ERATO Doi 594, expressed
2.0	myosin XVIIIa
2.0	aquaporin 1
2.0	MYST histone acetyltransferase monocytic leukemia 4
2.0	pantothenate kinase 1
2.0	vav 3 oncogene
2.0	trichorhinophalangeal syndrome I (human)
2.0	prostaglandin E receptor 4 (subtype EP4)
2.0	angiopoietin 2
2.0	expressed sequence AW061234
2.0	2,3-bisphosphoglycerate mutase
2.0	keratin complex 2, basic, gene 8
2.0	RIKEN cDNA 2610001E17 gene
2.0	vinculin
2.0	RIKEN cDNA 2310039E09 gene
2.0	RIKEN cDNA 1110067D22 gene
2.0	midline 2
2.0	praja 2, RING-H2 motif containing
2.0	thrombospondin 1
2.0	procollagen, type III, alpha 1
2.0	2,3-bisphosphoglycerate mutase
2.0	tetraspanin 8
2.0	deubiquitinating enzyme 1
2.0	vinculin
2.0	RAB27b, member RAS oncogene family
2.0	RIKEN cDNA 1110067D22 gene
2.0	keratin complex 2, basic, gene 8
2.0	tubulointerstitial nephritis antigen-like
2.0	ets variant gene 5
2.0	pyruvate dehydrogenase kinase, isoenzyme 4
2.0	MAS-related GPR, member F
2.0	thrombospondin 1
2.0	thrombospondin 1
2.0	keratin complex 2, basic, gene 8
2.0	2-cell-stage, variable group, member 1
2.0	myocyte enhancer factor 2C
2.0	Unc-51 like kinase 2 (C. elegans)
2.0	2,3-bisphosphoglycerate mutase

MT vs. Ctl +TNFα(downregulated)	
Fold	**Gene name**
2.0	PDZ and LIM domain 5
2.0	FBJ osteosarcoma oncogene B
2.0	peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
2.0	cDNA sequence BC011467
2.5	transforming growth factor, beta induced
2.5	CEA-related cell adhesion molecule 1
2.5	tribbles homolog 2 (Drosophila)
2.5	myocyte enhancer factor 2C
2.5	cytochrome b-561
2.5	RIKEN cDNA 4631426J05 gene
2.5	transforming growth factor, beta induced
2.5	CEA-related cell adhesion molecule 2
2.5	epoxide hydrolase 1, microsomal
2.5	myocyte enhancer factor 2C
2.5	keratin complex 1, acidic, gene 18
2.5	PDZ and LIM domain 2
2.5	inhibitor of DNA binding 4
2.5	myocyte enhancer factor 2C
2.5	inhibitor of DNA binding 4
2.5	CEA-related cell adhesion molecule 1
2.5	chemokine (C-X-C motif) receptor 6
2.5	tripartite motif-containing 54
3.3	procollagen, type III, alpha 1
3.3	tribbles homolog 2 (Drosophila)
5.0	CEA-related cell adhesion molecule 1
5.0	RIKEN cDNA 9030611N15 gene

Fig. 42. MT modulates cytokine expression and cytokine response at RNA level (continue). D. Genes downregulated more than 2 fold in MT-NMuMGs vs. NMuMGs after TNFα treatment. Total RNA was isolated in NMuMGs, MT-NMuMGs 17 hour TNFα (10ng/ml) treatment. Purified RNA were submitted to the DFCI Microarray Core Facility and DNA arrays were conducted on GeneChip® Mouse Genome 430A 2.0 Array, Affymetrix.

3.7. MT and NFκB regulation

3.7.1. MT does not affect IKB degradation subjected to TNFα.

Transcriptional activation is usually required for inflammatory gene expression. NFκB has been shown playing a critical role in inflammation associated tumor development (Greten et al., 2004; Pikarsky et al., 2004). Since my data showed MT regulates cytokine responses, I wondered whether this regulation is through transcription factor NFκB. NFκB-induced transcriptional activity is regulated at multiple levels including release from binding to its inhibitory IκB proteins (Baeuerle and Baltimore, 1988), translocation to the nucleus, phosphorylation (Schmitz et al., 2001) or/and acetylation (Chen et al., 2001) of subunits, and binding of co-activators (Gerritsen et al., 1997) etc.

Various stimuli including stress and pathogens can induce rapid inactivation of IκB and liberate NFκB. As mentioned earlier, phosphorylation of IκBα at Ser32 and Ser36 followed by proteasome regulated degradation which leads to release and nuclear translocation of active NFκB (Brown et al., 1995). To see whether MT can affect this step of regulation of NFκB pathway, cells were treated with TNFα for 15 min and both phosphorylation of IκBα at Ser32/36 (not shown) and total IκBα were compared in NMuMG and MT-NMuMG cells. TNFα treatment triggers a dramatic decrease of IκBα protein in both NMuMG and MT- NMuMG cells (presumably through proteasome-regulated degradation) (Fig.43). This result suggests that MT does not interfere at the IκBα level of NFκB regulation.

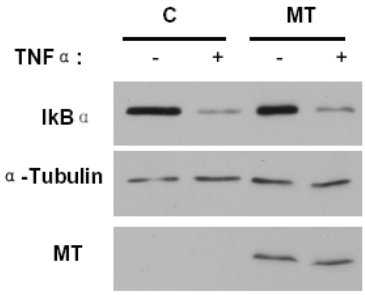


Fig. 43. MT does not affect IKB degradation subjected to TNFα.
NMuMG and MT- NMuMG cells were untreated or treated with TNFα (10ng/ml) for 15 min, whole cell lysates were collected and western blots were performed. Results are representative of experiments performed at least three times.

3.7.2. MT attenuates NFκB activity induced by TNFα

To further look at whether MT affects NFκB activity, NFκB luciferease assays were applied. Both NMuMGS and MT-NMuMGs were co-transfected with the 3κB-ConA-Luc reporter (Wang et al., 2007) and control CMV-β-gal constructs. 24 hours later cells were untreated or pretreated with MEK inhibitor U0126 for 30 min, and then treated with TNFα for different time periods. Luciferase assays were performed and normalized to β-gal activity (Fig.44). Compared to cells without any treatment, TNFα induced NFκB activity about 2-3 fold at both 6h and 17h time points. Although treatment with U0126 alone did not affect NFκB activity, pretreatment with U0126 enhanced TNFα induced NFκB activity about 1.5 fold. Surprisingly, MT-NMuMG did activate NFκB activity with a level similar as NMuMGs at early (6h) time point, but attenuated NFκB activity at late (17h) time point. Together, these results indicate that suppression of ERK may favor NFκB activity induced by TNFα. MT attenuates NFκB activity in cells subjected to TNFα which may partially explain MT sensitizes TNFα-induced apoptosis. It would be worth to understand how MT attenuates the NFκB pathway and its relation with TNFα-induced apoptosis. Further experiments such as measurements of NFκB target activities could by used to confirm the NFκB luciferase assay results. MT regulation of cytokine response to TNFα may be through NFκB independent pathways. Other transcriptional factors such as C/EBP, AP-1, ETS which have been also found involved in transcriptional regulation of inflammatory genes could be investigated.

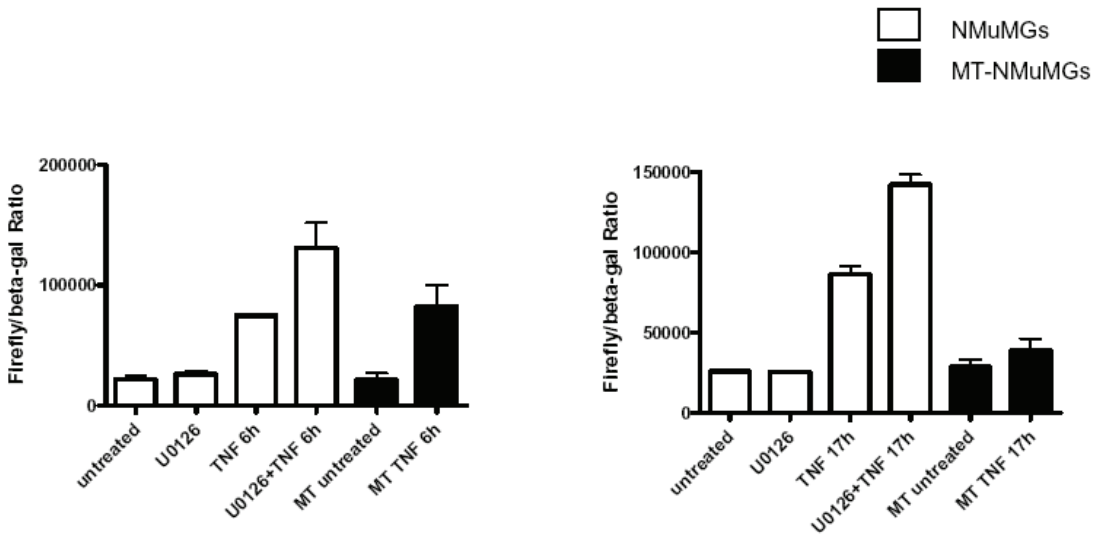


Fig. 44. ERK suppression affects NFκB activity induced by TNFα. MT effect on NFκB activity induced by TNFα. NMuMG and MT-NMuMG cells were co-transfected with NFkB-3X-luc reporter and CMV-β-gal vector, 24 hours later cells were pretreated with DMSO,U0126 for 30 min, and then treated with TNFα (10ng/ml) for 6h (left) and 17h (right). Luciferase activities were performed and normalized to β-galactosidase activity as described in Materials and Methods. Bars represent Mean+ SEM.

Discussion

MT sensitizes cells to TNFα induced apoptosis

MT potentiates TNFα induced apoptosis. This result is unexpected because MT has been reported to protect cells from apoptosis under other conditions such as serum starvation in F111 and Rat-1 cells (Dahl et al., 1998). This is yet another example of the contextual nature of signaling. Blocking ERK with U0126 also potentiates apoptosis. In both NMuMG and Y315F-NMuMG cells (not shown), pretreated with MEK inhibitor to block ERK activation, exposure to TNFα caused more apoptosis as observed by measuring PARP cleavage. This result suggested that ERK activation is anti-apoptotic with respect to TNFα stimulation. Since MT blocks ERK activation and sensitizes like U0126, the simplest explanation is that ERK inhibition is responsible for the MT effect. The downstream pathways coming from ERK involved in this protection specific to TNFα-induced apoptosis need to be determined. According to the published literature, there are a few possible pathways we could investigate further. For example, some evidence shows that ERK1/2 can regulate cell survival through phosphorylation BCL-2 family members (Luciano et al., 2003). ERK1 prevents TNFα-induced apoptosis through phsoporylation of BAD and inhibition of BAX translocation in Hela cells (Pucci et al., 2009). On the other hand, NFκB signaling also plays an important role in TNFα-induced apoptosis. As discussed previously, if NFκB activation is not adequate, apoptosis will be a late response to TNFα signaling. Therefore, to understand better how MT sensitizes TNFα-induced apoptosis, both BCL-2 family members and NFκB pathways need further study.

Suppression of ERK by MT sensitizes TNFα-induced apoptosis and favors IL-6

expression at the RNA level. IL-6 has been reported to have anti-apoptotic (Lin et al., 2001b; Moran et al., 2009) or pro-apoptotic (Regis et al., 2009; Wagley et al., 2007) effect in different cellular contexts. Whether induction of IL-6 in MT cells contributes to TNFα-induced apoptosis needs further study.

How does MT enhance some cytokines (eg. IL-6, CCL2, CCL5) and reduce others (eg. COX2)?

To the extent that we have measured RNA levels of the cytokines, it seems likely that MT is affecting transcription. This would not be surprising because MT is known to regulate AP1 and ETS activities, for example, the promoters of the cytokines shown here all have AP1 site. The following will focus discussing the regulation of IL-6, CCL2, CCL5 and COX2.

IL-6

IL-6 regulation is mostly at the transcriptional level. There are at least four transcriptional binding sites identified in IL-6 promoter region including NFκB, CREB, AP-1, and C/EBP. The role of each transcription factor in regulation of IL-6 expression has been shown depending on stimuli and cellular contexts (Beetz et al., 2000; Webb et al., 2002). To understand how MT regulate IL-6 at transcriptional level, the luciferase reporter vector with 2,120bp wild type or mutant IL-6 promoter region (-41bp to -2161bp) (Xiao et al., 2004) was transfected into NMuMGs or MT-NMuMGs. Luciferase activities were analyzed before and after TNFα treatment. Our efforts were not successful. Surprisingly, there is no significant IL-6 luciferase activities were observed in either condition. According to a study from a different group, a region 5kb upstream of start transcription has been identified also involved in regulating IL-6 expression (Samuel et

al., 2008). They suggested that IL-6 is regulated by a complex mechanism through different cis-regulatory elements. Whether the novel region is responsible for MT regulation of IL-6 at transcriptional level needs to be determined.

CCL2

Study in rat vascular smooth muscle cells showed TNFα induced CCL2 production is regulated by both AP-1 and NFκB pathways (Chen et al., 2004). My data showed while NMuMGs and MT-NMuMGs produce basal CCL2, MT cells produce significantly much more CCL2. It would be important to test whether MT regulates CCL2 through AP1.

CCL5

The study in liver sinusoidal endothelial cells derived from ethanol-fed rats (E-rLSECs) showed ethanol induced CCL5 expression is via activation of NFκB, HIF-1α, and AP-1. CCL5 promoter analysis showed that cis elements including HRE-1 (nt -22 to -19), HRE-2 (nt -32 to -29) and AP-1 (nt -250 to -244) were required for ethanol-induced CCL5 expression (Yeligar et al., 2009). Again, AP-1 is also important for CCL5 transcriptional regulation.

According to my data, different than CCL2, CCL5 was not seen in NMuMGs (measured by cytokine array) before and after TNFα treatment. CCL5 is constitutively secreted in MT-NMuMGs and upregulated after TNFα treatment. The lack of CCL5 in NMuMGs indicates CCL5 maybe more important than CCL2 in response to TNFα for MT cells. Whether MT uses same or different signaling pathways regulating CCL2 and CCL5 need more detail study.

Cyclooxygenase-2 (COX-2)

COX-2 normally is absent in most cells but could be induced in response to

inflammation stimuli and hypoxic environment (Kaidi et al., 2006). Evidence showed that COX-2 is overexpressed in adenocarcinomas and other tumors. Studies using COX-2 transgenic mice in mouse mammary glands showed it is sufficient to induce mammary gland tumorigenesis (Liu et al., 2001). Regulation of COX-2 expression has been found in both transcriptional level and post-transcriptional level. Like IL-6, the transcriptional regulation of COX-2 has been well established and includes many transcriptional factors: NFκB, CREB, AP-1, NFAT, PPAR, and C/EBP. Post-transcriptional regulation includes stabilization of COX-2 mRNA by RNA-binding protein (Young et al., 2009) or repression COX-2 mRNA by microRNAs (Strillacci et al., 2009).

Based on the known function of COX-2 in inflammation and tumorigenesis, it might be surprising that MT suppresses COX2 in response to both hypoxia and TNFα. However, in the context of virus (plus LT/ST), suppression of inflammation response favors viral escape of host immune surveillance and spread. One example is EBV blocks both COX-2 and prostaglandin E2 (PGE2) production in human monocytes (Savard et al., 2000). I had tested that suppression of ERK activation may contribute MT suppression of COX2 in response to hypoxia, whether the same mechanism is applied to TNFα is yet unclear. To indentify the level of regulation and pathways involved, quantitative RT-PCR and western blots could be used to measure relative mRNA and protein levels of COX2 before and after TNFα treatment with different pathway inhibitors. Luciferase assays using wild type and mutant COX2 promoter vectors can be used to test the transcriptional factors involved in regulating COX2 expression by MT.

MT and NFκB regulation

The regulation of cytokine expression has been shown most through transcriptional

level, and NFκB plays a major role in the cytokine regulation. As mentioned earlier, NFκB/Rel transcriptional factors are present in cytosol and kept in an inactive state by inhibitory IκB proteins. Upon stimuli, IκB is phosphorylated at Ser32 and undergoes proteasome-regulated degradation which releases NFκB and translocation to the nuclear and binding to the target genes. MT seems not affect the IκB degradation triggered by TNFα. It is unexpected that MT increases the cytokine response, but attenuates NFκB activation subjected to TNFα measured by luciferase assay. Since luciferase assay is not adequate to answer MT regulation of NFκB, further experiments such as measurements of NFκB binding and NFκB target activities need to be done to confirm attenuation of NFκB activaty by MT.

Other than IκB inhibitory regulation of NFκB, it is known that phosphorylation (Schmitz et al., 2001) and acetylation (Chen et al., 2001) modification can affect NFκB DNA-binding, transactivation, and duration properties. Whether MT affects the regulation of NFκB modification needs more detailed study.

Chapter IV. MT with a 315YAAA motif

This chapter described some data that did not look promising to contitune during the time. But it might be a useful record for future reference.

Introduction

Is PI3K the only signal coming from Y315?

A very unexpected result was obtained when the PI3K binding site at Y315 was mutated from YMPM to 315YAAA. MT lost PI3K binding and PI3K activity but still maintained significant transformation ability in Balb/C A31 fibroblasts (Hong et al., 2003). This transformation depended on Y315 because that 315FAAA was highly defective in transformation. Phosphopeptide mapping demonstrates that Y315 in YAAA is efficiently phosphorylated, which excludes the possibility that the failure of the YAAA mutant to bind PI3Kwas due to the lack of phosphorylation of Y315 in the context of YAAA. Furthermore, when a YAAA sequence was inserted into MT at 282, a non-essential site, WT-282YAAA formed foci considerably larger than those from WT MT. Therefore, there must be some protein else (protein X) which prefers to associate with Y315 in 315YAAA mutant and has function in transformation too. These data suggests a model in which PI3K and protein X compete for binding with Y315, when adding an additional YAAA motif enhances transformation, presumably by enhanced binding of protein X.

In order to find out protein X, we used two different strategies: one is using PTB and SH2 domain microarrays to screen the possible binding partners through pYMPM and pYAAA motif. Another one is using small peptides bearing pYMPM, YMPM, pYAAA or YAAA motif to concentrate binding proteins by peptide affinity purification.

Results

4.1. PTB and SH2 domain microarrays

As mentioned earlier, both PTB and SH2 domain containing proteins can bind pY residues. The specificity of recognition occurs to either N-terminal (PTB) or C-terminal (SH2) of the pY residue. PTB and SH2 domain microarrays have been successfully used to quantitatively analyze protein interaction networks on human ErbB receptors (Jones et al., 2006). By apply this high throughput screen we expected to see some protein X that might bind both pYAAA and pYMPM. It would be also expected to have a higher binding affinity to pYAAA than pYMPM based on published data (Hong et al., 2003).

Two peptides labeled with tetramethylrhodamine (Table.3) were synthesized and sent for microarray screen at Macbeath Lab (Harvard). At 1μM and 5μM of peptide concentration, quantitative binding was measured in terms of pYMPM versus pYAAA**.** The preliminary microarray data had high background owing to the presence of 6 acidic glutamates (E) in the peptide sequence. Therefore, relatively binding ratio of pYMPM/pYAAA was obtained (Table. 4). Peptides with few glutamates (4) were sent to repeat this experiment, but we haven't got the new microarray data.

At 1μM of peptide concentration, B Lymphoid Kinase (BLK) was found showing the lowest ratio 0.283 of binding pYMPM vs. pYAAA**.** In the other word, BLK has approximately 4 times more binding affinity to pYAAA than pYMPM.

The tyrosine kinase BLK is expressed preferentially in the B lineage. Malignant transformation of early lymphoid progenitors in mice can be generated by expressing an activated BLK (Tretter et al., 2003). To verify whether BLK is present in MT transformed cells and binds MT, BLK in vitro kinase assay was tested. B lineage cell line, J2-44,

which is known expressing BLK was used as a positive control. No BLK kinase activity was found in either A31 cells or MT transformed A31 cells (Fig.45.). This result suggested that BLK expression is tissue specific and not involved in binding MT in A31 cell context.

Table 3. Peptide sequences for protein domain microarry

WT	EPLEEEEEE**pYMPM**EDLY
YAAA	EPLEEEEEE**pYAAA**EDLY

Table 4. Significant binding ratio of pYMPM/pYAAA

Peptide Conc. (μM)	Protein Names	pYMPM/pYAAA
1	**BLK(B Lymphoid Kinase)**	**0.2833**
1	**SYK-N (Spleen Tyrosine Kinase)**	**2.1729**
1	**FGR (Src family kinase)**	**3.9045**
1	**RASA1-N (RasGAP)**	**3.9741**

Binding ratios with the PI3K regulatory subunit isoforms range from 0.9 to 1.5.

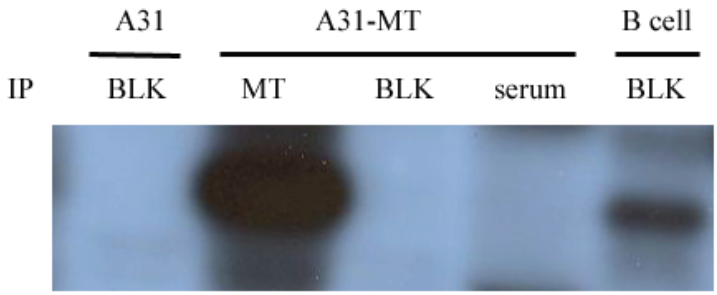


Fig 45. BLK in vitro kinase assay
Whole cell lysates from A31, MT-A31, and B cells were immunoprecipated with indicated antibodies or control serum, and in vitro kinase assay was performed as described in Material and Methods. Results are representative of experiments performed at least twice.

4.2. pYAAA and pYMPM peptide affinity purification

Since kinases were detected in the protein domain microarray data, it seemed reasonable to think that a binding partner of pYAAA and pYMPM might be a kinase. Therefore, in vitro kinase assays were carried out to see if any kinase activity associated with pYAAA and pYMPM motif. To do this, small peptides bearing pYMPM, YMPM, pYAAA or YAAA motif were synthesized (Fig. 46) and coupled to gel beads to form peptide affinity columns. Concentrated binding proteins were purified. To prove the peptide affinity purification system works, proteins purified by pYMPM and YMPM peptides were sent for sequencing. As expected, PI3K were purified by pYMPM but not YMPM peptide (not shown). Then in vitro kinase assays were carried out after peptide affinity purification.

There are a few observations (Fig.47). 1) Doublet bands around 50-55KD were affinity purified by pYAAA in both A31 and MT-A31cells. 2) Doublet bands were not observed in YAAA which indicated the binding depends on phospho-tyrosine. 3) The doublet bands observed in A31 cells were not clear in NMuMGs. 4) The doublet bands were around similar size as MT, it is hard to know whether this unknown partner of pYAAA also immunoprecipitated by MT. 5) Because the doublet bands in pYAAA were also seen in pYMPM not YMPM, it is consitent with the idea that it also binds MT. 6) The 85KD band in pYMPM presumely is p85 subunit of PI3K.

One way to confirm the doublet binds MT or not is to do proteolysis mapping in which proteins are digested into smaller peptides with different patterns. Chymotrypsin and V8 proteolysis mapping were used to compare the immunoprecipitation by MT and affinity purification by pYAAA and pYMPM peptides. In summary of partial proteolytic

digestion (see Material and Methods), immunoprecipitated protein samples were separated by cylinder gel first. Then cylinder gels were digested with proteases and separated by regular SDS-PAGE gel (Fig.48). The partial proteolytic digestion confirmed that p85 subunit of PI3K binds both MT and pYMPM (Fig.48), but not pYAAA (not shown). The MT proteolysis map did not give good enough resolution to answer whether the 50-55KD doublet bands were present in the immunoprecipitaed MT sample or not.

(N) KGSGSEEEEEpYMPMEDLYLD-NH2 (C)

(N) KGSGSEEEEE**YMPM**EDLYLD-NH2 (C)

(N) KGSGSEEEEEpY**AAA**EDLYLD-NH2 (C)

(N) KGSGSEEEEE**YAAA**EDLYLD-NH2 (C)

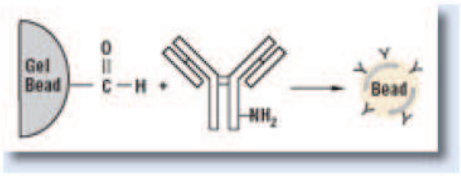


Fig. 46. Peptide sequences for affinity purification

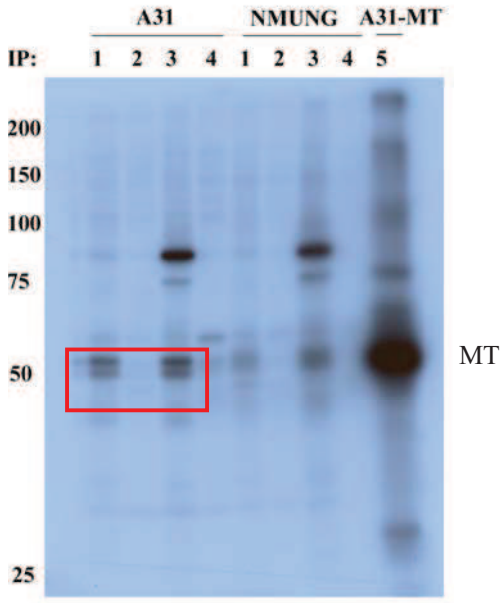


Fig.47. Tyrosine kinase activities present in pYAAA and pYMPM peptide affinity purification. Whole cell lysates were affinity purified or immunoprecipited with different peptides and antibodies: 1: pYAAA; 2: YAAA; 3: pYMPM; 4: YMPM; 5: MT. Kinase assay was performed and separated by SDS-PAGE. Gel was treated with 1N NaOH to get rid of Ser/Thr kinase signals. Results are representative of experiments performed at least twice.

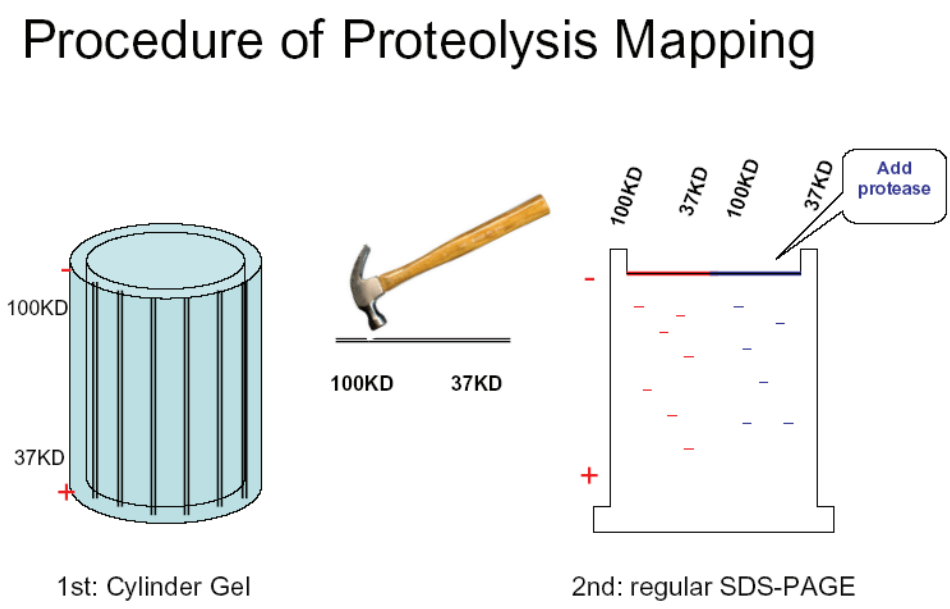


Fig. 48. Diagram of proteolysis mapping procedure

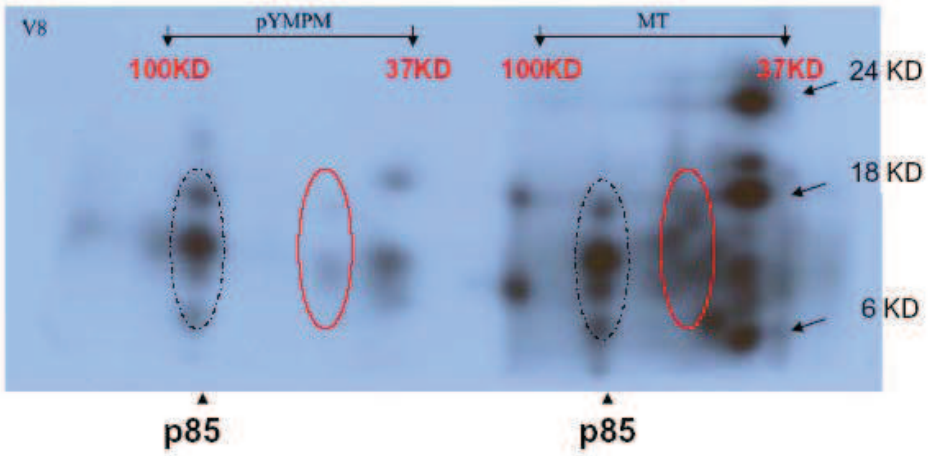


Fig. 49. V8 (20µg/ml) proteolysis mapping
MT immunoprecipitation is mapped as 24KD, 18KD and 6KD. Both MT and pYMPM precipitaed p85 subunit of PI3K are mapped as shown in dash circles. Unknown 55KD kinase present in both pYMPM and MT are mapped as shown in solid circles. Results are representative of experiments performed at least twice.

In kinase assay using the whole cell lysates, the 50-55KD kinase signals were very weak. When we coomassie stained the gel, there was no obvious band around 50-55KD in pYAAA or pYMPM affinity purification. There could be a few reasons: the 50-55KD kinase is low abundant in the cell lines we used; the binding is transient. To amplify the signal, mouse liver and brain tissues which contain more abundant proteins than cell lines were tested. Since the 50-55KD bands were present in both pYMPM and pYAAA, we chose to use pYAAA and YAAA (negative control). We did find the specific bands appeared in pYAAA not YAAA, also the kinase signal in brain was much stronger than liver and cell lines (Fig.50). It suggested that brain tissue might be a good source for looking for the binding partners of pYAAA motif.

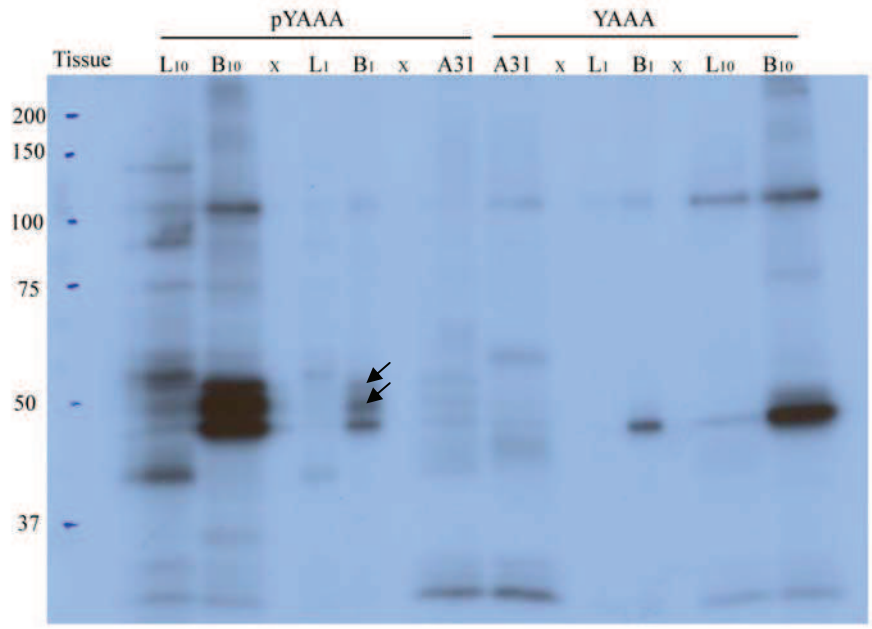


Fig.50. Tyrosine kinase activities in brain, liver and cell lines
Whole cell lysates from mouse tissue or A31 cells were affinity purified with pYAAA or YAAA peptides. Kinase assay was performed and separated by SDS-PAGE. Gel was treated with 1N NaOH to get rid of Ser/Thr kinase signals. (B: brain, L: liver; B10 and L10 used ten times of protein as B1 and L1 respectively)

4.3. 315YAAA transformation assays

To confirm the observation of 315YAAA enhances transformation, two different cell lines NMuMGs and mouse embryonic fibroblasts Balb/C A31 cells were tested. Stable cell lines made with wild type MT, Y315F, 315YAAA, and 315FAAA were set up for soft agar assay. Surprisingly, only wild type MT fully transformed cells, and all the mutants failed (Fig.51; Fig52). Though there are small colonies in NMuMGs for MT mutants, they are significantly smaller than wild type MT. For Balb/C A31, the colonies are much smaller than NMuMGs though the colonies grew for an extra week. 315YAAA did not show transformation ability in both NMuMGs and Balb/C A31 cells. Our results contradicted with Hong's discovery. There could be a couple of reason for the difference. First, the cell systems we used in this assay were different from theirs as we know that MT signaling is cell context dependent which may contribute to the difference in results. Second, the level of 315YAAA and 315FAAA protein expression was lower than wild type MT which may affect transformation ability.

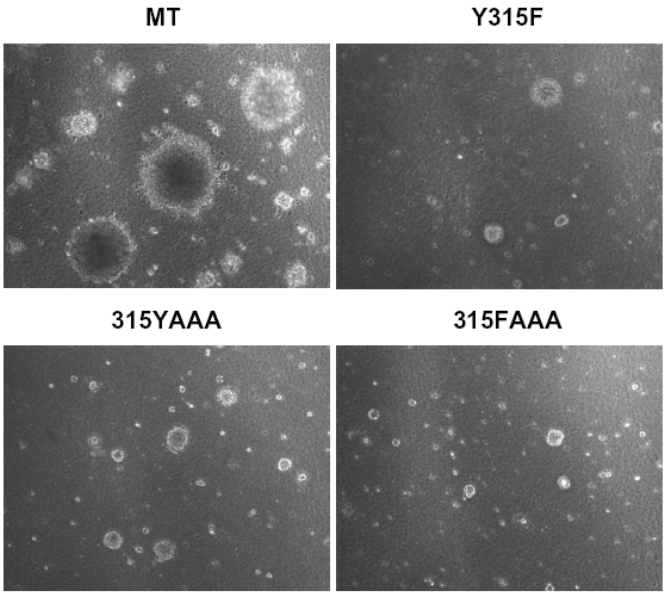


Fig.51. Transformation assay in NMuMGs (4 weeks)
Colony formation in soft agar was compared in wild type MT, Y315F, 315YAAA, and 315FAAA NMuMG stable cell lines.

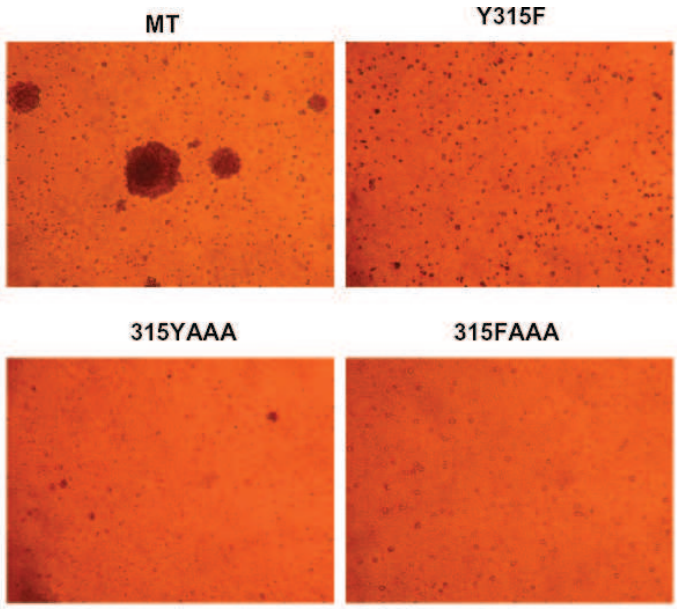


Fig.52. Transformation assay in Balb/C A31 (5 weeks)
Colony formation in soft agar was compared in wild type MT, Y315F, 315YAAA, and 315FAAA Balb/C A31 stable cell lines.

Summary:

Based on literature, there are possibilities that different signal molecules bind the same pTyr and initiate different pathways. One example is both p85 subunit of PI3K and NCK can share the same pY751 residue of PDGFR (Nishimura et al., 1993). The report that MT mutant 315YAAA is defective in PI3K but still can transform cells suggests a different signaling pathway than PI3K is regulated through 315YAAA (Hong et al., 2003). In general, it will be important to look at for situations when 315 defects can not be connected to PI3K. To look for this binding partner of 315YAAA, both mouse embryonic fibroblast cell line Balb/C A31 and normal mouse mammary epithelial cell line NMuMGs were tested. Unlike published results, MT 315YAAA mutant was not able to transform as wild type MT in those cell systems. This suggests Hong's observation could be cell dependent. By using peptide affinity purification, we were able to see specific tyrosine kinase signals binding to pYAAA peptide (around 50-55Kd) in protein lysate from brain tissue than liver tissue and cell lines. It again indicates pYAAA binding protein has different abundance in different tissues and cell types. In the future, to investigate the pYAAA binding partner in brain, peptide affinity assay should be scaled up in mouse brain tissue and silver stained gel bands should be sequenced by mass spectrometry for identification.

Future Directions:

The effect of MT on ERK in response to stress comes from two effects: decreased activation of MEK in response to stress and increased turn-over of ERK phosphate. We need to look in detail into how MT reduces MEK phosphorylation under stress. In principle, MT could suppress MEK kinase or/and enhance MEK dephosphorylation. As discussed earlier, different MAPKKKs could be involved in activating MEK in response to different stimuli. It would be helpful to identify a particular MAPKKK responsible for MEK activation in response to some particular stress. ER stress might be a good place to start. In ER stress, RAF family (RAF-1 and B-RAF) members were not activated, which indicates some other MAPKKK were involved in activating MEK. We should look at the known MEK kinases such as MEKK1, TPL2 and Mos by measuring in vitro kinase activity (using MEK as substrate) in response to ER stress. If any of these MEK kinases is involved in activating MEK under ER stress, either a specific inhibitor or shRNA could be used to further confirm the regulation. We would then ask whether MT regulates the activity or abundance of the MAPKKK. The same rationale would be applied to identify the MEK kinase involved in other stress conditions.

It is possible that MT also regulates MEK dephosphorylation. If we can identify a specific MAPKKK, then an inhibitor assay such as we have used to measure ERK turnover could be used to measure MEK phosphate turn-over. As the okadaic acid titration assay showed, when PP2A and PP1 were inhibited with increased doses of okadic acid, MEK phosphorylation is increased in both MT and NMuMG cells. This suggests MT does not use PP2A and PP1 to suppress MEK phosphorylation. Because the assay is done without stress stimuli, we need to make sure the same result is obtained

after stress. Assuming the result holds, then other phosphatases would need to be considered. PP5, for example, has been proposed to regulate the ERK pathway, although that is supposed to act at the level of RAF. Another alternative is that turnover of MEK phosphate is regulated at the level of localization. MEK is translocated to the nucleus when activated in response to growth factors (Jaaro et al., 1997); it could also be similarly translocated in response to stress. MT might suppress this. Turn-over would then be secondary to differences in localization.

My data showed that MTY315F was defective in suppressing MEK activation. Both overexpression of p110α H1047R and Myr-AKT1/2 in Y315F significantly rescue this defect. This PI3K/AKT negative regulation of MEK was also confirmed by inhibiting MT activation of AKT using AKT inhibitor. A remaining puzzle is that AKT inhibition increased MEK phosphorylation dramatically in MT cells under growth conditions, but it does not fully reverse MT suppression of MEK under stress. My explanation for this different effect on MEK is that under growth conditions, MEK activation is a balance of the positive signal from RAF activity and a negative signal from PI3K/AKT (negative regulation of RAF) in MT cells. Inhibition of AKT decreases the negative regulation and causes dramatically increase of MEK phosphorylation. Since RAF is not involved in all the stress conditions as mentioned before, that the inhibition of AKT has less effect is reasonable. To prove this hypothesis, it would be necessary to know there is some stress condition where RAF is the MAPKKK fully responsible for MEK activation. If we can find this stress condition, we should do the AKT inhibitor assay again and compare MEK phosphorylation before and after stress treatment in MT cells. It is very possible that other MAPKKK in addition to RAF family members are involved in activating MEK at

different stress conditions and AKT may not be involved in negative regulating all of them.

There is also more work to be done on the dephosphorylation of ERK. MT upregulates at least two DUSPs: VHR and MKP3. Single KD of DUSP in MT cells does not fully reverse MT suppression of ERK. This suggests in addition to VHR and MKP3, there maybe other DUSPs also targeted by MT. There are at least 60 human DUSP genes. As we know MT does not affect p38 phosphorylation, so those DUSPs that prefer p38 than ERK as substrates would be excluded from further screening. Ideally, we want to find out all the DUSPs regulated by MT. Eventually we would like to test whether knockdown of multiple DUSPs in a single cell will reverse MT suppression of ERK. My data suggests MT regulates DUSPs at different levels, both transcriptional and post-transcriptional. However, how MT regulates DUSPs expression level and activity is not clear and worth further study.

MT mutant Y322F is defective in both PI3K and PLC ɣ signaling in NMuMGs. We would like to test whether constitutively active PLC ɣ plus PI3K will fully rescue Y322F defect in NMuMGs. More importantly, we need to verify whether MT affects ERK dephosphorylation through the PLC ɣ pathway. Most published literature show both PLC ɣ and PKC signaling are involved in positive regulation of ERK. Based on previous published study on MT, MT activates PLC ɣ pathway. Our primary data also confirmed that MT constitutively activated PLC ɣ Y783 phosphorylation (not shown). To test the role of PLC ɣ in ERK regulation by MT, we would use a PLC ɣ specific inhibitor to pretreat cells before exposed to stress. Then the ERK phosphate turnover assay will be performed to compare to the cells without PLC ɣ inhibitor treatment. Since the inhibitor

assay looks at transient effects, it may not reflect the persistent effect in MT cells. We could also use stable PLC ɣ knockdown in both NMuMG and MT-NMuMG cells and compare the effects on ERK dephosphorylation.

As for MT modulates cytokine expression and cytokine response, we need to find out what other cytokines, besides those already uncovered, are also regulated by MT. To do this, we could carry out a bigger screen of cytokine arrays. Also we will confirm those cytokines significantly changed at RNA level measured by gene array. Among those interesting candidates, CC and CXC family chemokines and MMPs seem our priority targets to investigate based on their known functions. To further understand the role of cytokine and chemokines in MT transformation and tumorigenesis, both in vitro and in vivo assays would be considered.

It is known that cytokines are mainly regulated at transcriptional level. MT upregulates cytokine IL-6, CCL2 and CCL5 expression. Common transcriptional factor binding sites including NFκB and AP-1 are all present in IL-6, CCL2 and CCL5 promoter region. Our primary data suggests that MT attenuates NFκB activation measured by luciferase assay. Previous published data showed that MT activates AP-1. So next we would look at whether MT activates AP-1 in NMuMGs and measure AP-1 activity by luciferase assay. If MT activates AP-1, we will test wild type and AP-1 binding site mutant IL-6, CCL2 or CCL5 promoter luciferase activity. In the extreme case, if none of those known transcriptional binding sites are regulated by MT, then we have to identify the unknown regulatory regions in those cytokines.

We want to know how MT signaling pathways contribute to the regulation of individual cytokines. Because MT mutants Y250F (Ras-/PI3K-), Y315F (PI3K-/?),

Y322F (PLC ɣ -/PI3K-) are all defective in PI3K in NMuMGs, it would be impossible to interpret data using mutants in any simple way. Specific pathway inhibitors would be used to test which pathway contributes MT regulation of cytokine expression and response.

MT enhances both TNFα induced IL-6 and TNFα induced apoptosis. Studies show IL-6 could be either anti-apoptotic or pro-apoptotic according to cellular contexts. To find out what role IL-6 plays in MT enhanced TNFα induced apoptosis, IL-6 could be added directly to cells for certain time before treated with TNFα and compare apoptosis with cells without IL-6 treatment. Alternatively, IL-6 could be knockdown in MT cells and look at the effect on TNFα induced apoptosis.

Tumor-associated macrophages have been shown to play an important role in tumor microenvironment (Balkwill, 2004a). In MMTV-MT transgenic mouse model, macrophage infiltration has been shown required for MT mammary tumor invasion and metastasis (Lin and Pollard, 2004). In literature, the major cytokines shown to recruit macrophages are CSF-1, CCL2 and CCL5. My data showed that MT can regulate all of the three cytokines (cytokine array). Whether the three cytokines have the same or different functions in MT recruitment of stromal cells (including macrophages) would be worth studying. Previous study in our lab showed that MT secreted Osteopontin and Osteopontin was required for MT fibroblasts movement, such as in wound healing. To test any of the three cytokines CSF-1, CCL2 and CCL5 are required for the recruitment of stromal cells by MT-NMuMGs, cell migration assay would be performed with the presence of single or multiple cytokine combinations. Further knockdown of single or multiple cytokines will be used to confirm the results.

Previous studies found different strains of mice have different effects on MT tumorigenesis including both tumor latency and metastasis ability (Lifsted et al., 1998). To find out how macrophages from different mouse genetic background affect MT cytokine regulation and which cytokines regulated by MT are more important for MT tumorigenesis would help understand such differences observed in mice. To do this, primary macrophages collected from different strains of mice will be co-cultured with MT-NMuMG cells. Both RNA and supernatant from cell culture will be collected and compared for cytokine profile. Also a migration assay will be performed to compare how differently MT recruits different strains of macrophages in vitro. Since it is becoming clear that stromal cells such as cancer associated fibroblasts (CIF) also play similar roles as macrophages during tumorigenesis, fibroblasts from different strains of mice should also be tested.

Chapter V. Materials and Methods

Cells

Normal mouse mammary gland epithelial cells (NMuMGs) a gift of Dr. Michele M. Fluck (MSU), MEFs (KSR-/- and KSR add back) a gift of Dr. Robert Lewis (UNMC), MEFs (TPL2-/- and TPL2 add back) a gift of Dr. Philip Tsichlis (Tufts) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100unit/ml penicillin, 2mM glutamine and 100μg/ml streptomycin at 37℃ in 5% CO2. NIH/3T3 and Balb /C A31 cells were cultured in DMEM supplemented with 10% calf serum, 100unit/ml penicillin, 2 mM glutamine and 100μg/ml streptomycin. MCF-10A cells (from Dr. Larry Feig, Tufts) were cultured in Dulbecco's modified Eagle's medium-F-12 (Invitrogen) supplemented with 5% horse serum (Gibco), 0.5 μg/ml hydrocortisone (Sigma), 10 μg/ml insulin(Sigma), 20 ng/ml EGF (Peprotech), and 0.5 μg/ml amphotericin B (Fungizone). J2-44 (CD22-/-) B cells (Dr. Paul Mclean, Tufts) were maintained in RPMI1640 supplemented with 10% fetal bovine serum (FBS), 100unit/ml penicillin, 2mM glutamine, 100ug/ml streptomycin, 1% pyruvate, 50 μM β-mercaptoethanol and 20 μM HEPES. For experiments on hypoxia, cells were transferred to a hypoxic culture chamber (INVIVO2 Hypoxia Workstation, Ruskinn Technology). The composition of atmosphere in the chamber consisted of 0.2% O2, 5% CO2, and residual N2. Phoenix 293T packaging cells (DMEM, 10% FBS) were used to generate retrovirus for infection. Retrovirus supernatant were harvested 48 hours post-transfection of packaging cell lines. All infections were carried out in the presence of 4 μg/ml Polybrene (Sigma). After infection, cells were selected with antibiotic for 10-14 days to get stable cell pools. In most cases, single cell cloning was performed to obtain cell lines.

In all cases results obtained with cell pools could be recapitulated in the lines. Transient transfection was performed by using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions.

Plasmids

Retroviral vector pWZL-blasticidin wild-type MT and MT mutants were kindly given by Dr. Jean Zhao (Dana-Farber Cancer Institute, Boston, MA). pWZL-Neo-p110α (H1047R) was provided by Dr. Thomas M. Roberts (Dana-Farber Cancer Institute, Boston, MA). pWZL-Hygro-H-Ras (G12V) was provided by Dr. Teeru Bihani (Tufts). pMT3-HA-ERK1 was provided by Dr. Geoffrey Kilili (Tufts). pCGN-HA-ERK1(D318N) was provided by Dr. Yvona Ward (NIH). pMSCD-Myr-AKT1-PH$^-$(Flag) was provided by Dr. Alexei Degterev (Tufts). Migr1-Myr-AKT2-GFP was provided by Dr. Philip Tsichlis (Tufts). His-ERK1-R84S, pCEFL-ERK2-R65S (HA), pCEFL-ERK2-D319N (HA), pCEFL-R65S+D319N (HA) were provided by Dr. David Engelberg (Hebrew University, Israel). Luciferase reporters, 3κB-conA-Luc and conA-Luc were from Dr. Amy Yee (Tufts).

Antibodies and Reagents

PN116, a mouse monoclonal antibody recognizing all three polyoma T antigens prepared by MonoClonal Core Facility associated with J. DeCaprio's laboratory at Dana Farber Cancer Institute was used for middle T immunoblot. Antibodies against phospho-ERK1/2 (Thr202/Tyr204), phospho-MEK1/2 (Ser221), phospho-c-Raf (Ser338), phospho-AKT(Ser473 and Thr308), AKT, phospho-p90RSK (Thr573), phospho-

Chk1(Ser345), phospho-JNK(Thr183/Tyr185), JNK, phospho-p38(Thr180/Tyr182), 4EBP1 were from Cell Signaling Technology. Antibodies against ERK1/2, Chk1, COX2, MKP1/DUSP1 and p38 were from Santa Cruz. Anti- MEK1 was from BD Transduction Laboratories. Anti-α-tubulin was from Sigma. Anti-VHR was from R& D system. Peroxidase labeled goat anti-rabbit and goat anti-mouse IgG were purchased from Jackson ImmunoResearch. U0126 ethanolate, LY294002, thapsigargin, cycloheximide, Endoproteinase Glu-C (V8), α-Chymotrypsin, and puromycin dihydrochloride were purchased from Sigma. Hydrogen peroxide, blasticidin, and G418 sulfate were purchased from Fisher Scientific. Recombinant mouse tumor necrosis factor alpha was purchased from Cell Sciences. RAC inhibitor NSC23766 was from TOCRIS bioscience. AKT inhibitor VIII isozyme-selective, AKT1/2, was from Calbiochem.

Western Blots

Cells were washed twice with cold PBS, then lysed with 20 mM Tris-HCl pH8.0, 137mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA plus protease inhibitors (aprotinin 1mM; leupeptin 20μM; PMSF 1mM).and phosphatase inhibitors (Phosphatase Inhibitor Cocktail I and Phosphatase Inhibitor Cocktail II from Sigma-Aldrich) at 4℃. Cell lysates were clarified by centrifugation at 16,000xg for 10 minutes, and the supernatant protein concentrations were determined by using a Bio-Rad protein assay kit. 6X SDS-sample buffer (Boston BioProducts) was added to protein samples and heated at 100℃ for 3 minutes. 50-100ug of protein were resolved on SDS-PAGE gel and transferred to nitrocellulose membrane, and probed with primary antibodies.

Soft Agar Assay

Cells were trypsinized and diluted to $2X10^5$/ml. 1.25% stock agar was diluted with 2X DMEM medium to make 0.5-0.6% bottom agar in p60 plate. 1ml bottom agar mixed with $1X10^5$ cells (0.5ml) were plated on top and incubated at 37℃ in 5% CO_2. 0.3ml growth media was added once a week. Colonies were counted under microscope after two to three weeks.

In Vitro Kinase Assay of MT/Src Complex

Cells were lysated and immunoprecipitated by MT-1 polycolonal antibody, and then washed with PBS for three times and kinase buffer (20 mM Tris-HCl pH7.5, 5mM $MnCl_2$,) once. The precipitated MT/Src complex was incubated with 20-100μCi of [γ-^{32}P]ATP in 200μl kinase buffer for 15 min at room temperature, and then washed with PBS twice, 0.5M $LiCl_2$ once and water. 2X SDS-sample buffer were added to the washed samples and boiled for 3 minutes. Samples were resolved by SDS-PAGE gel. To remove Ser/Thr phosphorylation background, gels were treated with 1N NaOH at 55℃ for 1 hour and rinsed in solution with 10% (v/v) acetic acid, 30% (v/v) methanol and dried. MT/Src complex kinase activity was analyzed by autoradiography or phosphorimager.

RAS Activation Assay

Ras activation assay was performed by using RAS Activation Assay Kit (Millipore,CA) following the manufacturer's instructions. The assay is based on the binding of activated GTP-RAS to the RAS binding domain of RAF.

RAF Kinase Assay

Cells were serum starved (0.2%FCS) for 18-20 hours. A positive control was stimulated with PDGF for 5 minutes and washed with cold PBS twice, then lysed with cold RIPA buffer with protease and phosphatase inhibitors (20 mM Tris-HCl pH8.0, 137mM NaCl, 10% glycerol, 1% NP-40, 0.1% SDS, 0.5% Na-deoxycholate, 2mM EDTA pH8.0, aprotinin 1mM, leupeptin 20μM, PMSF 1mM, Vanadate, 5mM) directly in the plates. Cells were scraped into 1.5ml microtubes and centrifuged at 4℃, 16,000g for 10 minutes. Supernatants were used for RAF-1 or B-RAF immunoprecipitation. Protein lysates were immunoprecipitated for 4 hours at 4℃, then resuspended in 40μl of kinase buffer (30mM HEPES pH7.4, 7mM $Mncl_2$, 5mM $Mgcl_2$, 1mM DTT, 15μM ATP (cold)) containing 20μCi of [γ-^{32}P] ATP and 0.1μg of kinase-inactive MEK1 (K97R) (Millipore). The kinase reaction was proceeded for 30 minutes at room temperature. To terminate the kinase reaction, 6X SDS-sample buffer were added directly to the samples and boiled for 3 minutes. Samples were resolved by SDS-PAGE gel and dried. RAF kinase activity was analyzed by autoradiography or phosphorimager.

ERK Phosphate Turnover Assay

Cells were untreated or subjected to stimuli first, then specific MEK inhibitor U0126 (20μM) was added into media and cell extracts were prepared at indicated times. Western blots were performed.

Reverse Transcription (RT)-PCR

Total RNA was isolated by using RNeasy Plus Mini Kit (Qiagen) following the

manufacturer's instructions. Total RNA (1μg) was used for first-strand cDNA synthesis using the Iscript cDNA synthesis kit (Bio-Rad). Murine *Xbp-1* was amplified by using a sense primer 5'-TTACGGGAGAAAACTCACGGC-3' and an antisense primer 5'-GGGTCCAACTTGTCCAGAATGC-3'. A 289 bp and 263 bp amplicon were generated from unspliced *Xbp1-* and spliced *Xbp-1* respectively (Lin JH et al, 2007). Quantitative real-time PCR was carried out on MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). iQ^{TM} SYBR Green Supermix (Bio-Rad) was used as a fluorescent dye to detect the presence of double-stranded DNA. The mRNA levels of VHR, IL-6, COX2, CCL2 and CCL5 were quantified by using the following primers: VHR forward primer 5' -TCTGTGGCTCAGGACATCAC-3'; VHR reverse primer 5'-GGCCCTTTCAAAGTAAGCACTG-3'. MKP3 forward primer 5'-ATAGATACGCTCAGACCCGTG-3'; MKP3 reverse primer 5'-ATCAGCAGAAGCCGTTCGTT-3'. IL-6 forward primer 5'-GAGGATACCACTCCCAACAGACC-3'; IL-6 reverse primer 5'-AAGTGCATCATCGTTGTTCATACA-3'. Cox2 forward primer 5'-TGAGCAACTATTCCAAACCAGC-3'; Cox2 reverse primer 5'-GCACGTAGTCTTCGATCACTATC-3'. CCL5 forward primer 5'-GCTGCTTTGCCTACCTCTCC-3'; CCL5 reverse primer 5'-TCGAGTGACAAACACGACTGC-3'.β-actin forward primer 5'-ACCACACCTTCTACAATGAG-3'; β-actin reverse primer 5'-ACGACCAGAGGCATACAG-3'. The mRNA values were normalized to internal control β-actin mRNA.

shRNA Knock Down

Murine VHR (DUSP3) or MKP3/DUSP6 shRNA lentiviral plasmid vectors pLKO.1-puro (Sigma) were co-transfected with compatible packaging plasmids into HEK293T packaging cells. Lentiviral supernatants were collected 48 hours post-transfection, and lentivirus titer was measured. Cells were transduced with shRNA lentiviral stock according to the virus titer. 48 hours after transduction puromycin was added to cells to select stable knock down cell lines. The target mRNA level of knock down cells was quantified by using the primers as showed in RT-PCR.

Cytokine Array

Cells were untreated or treated with TNFα (10ng/ml) for 16-20h, supernatants were collected and concentrated with Amicon Ultra-15 3KD filter (Millipore) at 4℃ and incubated with RayBio Mouse Cytokine Antibody Array I following the manufacturer's instructions. Briefly, array membrane was blocked with blocking buffer at room temperature for 30 min. Concentrated supernatants (4x) were incubated with array membrane at room temperature for 2 hours and washed with wash buffers. Array membrane was incubated with Biotin-Conjugated Anti-Cytokines at room temperature for 1 hour and washed with wash buffers. Array membrane was incubated with HRP-conjugated Streptavidin at room temperature for 2 hours, washed and detected the way as regular immunoblots.

NFκB Luciferase Assay

Cells co-transfected with NFκB luciferase reporter and CMV-β-gal were washed

twice with cold PBS and spun down for two minutes at 10,000x g. Cell pellets were suspended in 100µl of CAT-Tris buffer (25mM Tris Ph7.5, 1mM EDTA) and lysed by three cycles of freezing (dry ice ethanol) and thaw (37℃). The lysate were spun at 10,000x g for 2 minutes and supernatant were assayed for luciferase activity using a luminometer. 30µl of exact were mixed with 270µl of color mixture (20X color mixture: 60µl of 100X Mg [0.1M $MgCl_2$, 4.5M β-Mercaptoethanol] 1.32 ml of 1x ONPG {4mg/ml of o-nitrophenyl-β-D-galactopyranoside dissolved in 0.1M sodium phosphate pH7.5 and 4.02ml of 0.1M sodium phosphate pH7.5}) and incubated at 37℃ until yellow color developed and then stopped by adding 500µl of 1M Na_2CO_3. The reacations were read at an optical density at 420nm. NFκB luciferase activity was normalized to CMV-β-gal activity to get the relative NFκB activity.

ELISA

IL-6 ELISA is performed by using mouse IL-6 DuoSet development kit (R&D System) following the manufacturer's instructions.

Cell Cycle Analysis

Attached cells were washed twice with PBS, then incubated with 1ml 0.1% EDTA/PBS at 37℃ for 5 min. Cells were blown off with pasteur pipette and spun at 2600rpm for 5 min at 4℃. Cells were resuspended in cold 1% FCS/PBS and spun again. Cells were resuspended in 200µl cold PBS with dropwise addition of 800ul ethanol and vortexing. After permeablization at 4℃ overnight, cells were washed with cold 1% FCS/PBS once and added Propidium Iodide staining solution (Propidium Iodide 50µg/ml,

10 mM Tris-HCl PH7.5, 5mM $MgCl_2$, RNase 20μg/ml) incubated at 37℃ for 30 min protected from light (vortex every 10 min). Cells were then ready for cell cycle analysis.

For FACS analysis, the PI/cell mixture was transferred to 5ml tubes (Falcon 2054). The cytometer used in these experiments is a FACSCalibur which operates using CellQuest software. A dot plot was created to have forward scatter (FSC) and side scatter (SSC) on each of the axes. FSC measures cell size and SSC measures cell density. Cell debris and non-cell particles are filtered out by gating appropriately on this plot. To assay PI staining, the FL3 channel was used. There are different types of FL3 channels which measure different aspects of the peak, such as area (FL3-A), width (FL3-W), and height (FL3-H). Typically about 10,000 total events were acquired. These results were analyzed using Modfit 3.0 software, which quantitated the peaks and used various algorithms to calculate the percentage of cells in G_0/G_1, S, and G_2/M phases.

Gene Arrays

Total RNA was isolated by using RNeasy Plus Mini Kit (Qiagen) following the manufacturer's instructions. RNA samples were submitted to the Dana Farber Cancer Institute Microarray Core Facility. Gene array experiments were conducted on GeneChip® Mouse Genome 430A 2.0 Array (Affymetrix). Results were analyzed by using BRB Array Tools (developed by Dr. Richard Simon and BRB-ArrayTools Development Team) and Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) by Dr. Sang Hyun Lee at DFCI.

PTB and SH2 Domain Microarrays

Two peptides labeled with tetramethylrhodamine (Table.3) were synthesized and sent for PTB and SH2 domain microarry screen at Macbeath Lab (Harvard) (Jones et al., 2006). At 1μM and 5μM of peptide concentration, quantitative binding was measured in terms of **pYMPM** versus **pYAAA.**

Peptide Affinity Purification

The four peptides pYMPM, YMPM, pYAAA and YAAA (Fig.48) were synthesized by Tufts Core Facility. Peptides coupling and affinity purification were performed by using Aminolink Plus Immobilization Kits (Pierce) following the manufacturer's instructions. Briefly, peptides were coupled to gel beads to form peptide affinity columns. Cells or tissues were extracted with 50 mM Tris-HCl pH7.4, 150mM NaCl, 1% Triton X-100, 1 mM EDTA plus protease inhibitors (aprotinin 1mM; leupeptin 20μM; PMSF 1mM).and phosphatase inhibitors (Phosphatase Inhibitor Cocktail I and Phosphatase Inhibitor Cocktail II from Sigma-Aldrich) at 4℃. Cell lysates were clarified by centrifugation at 16,000xg for 10 minutes, and the supernatant protein concentrations were determined by using a Bio-Rad protein assay kit. Peptide affinity purification was performed (gently rocking) at 4℃ for 4 hours. The peptide affinity purified proteins were washed with PBS for twice and in vitro kinase assays were performed

Partial Proteolytic Digestion

[γ-^{32}P]ATP labeled proteins are first resolved on cylindrical SDS gels (4mm O.D. Pyrex tubing, VWR) overnight. After electrophoresis, cylinder was broken with a hammer to take the gel out. For the second dimension gel, lay the cylinder gel on the top

of stacking gel and fill the upper tray with running buffer. Overlay with 2ml V8 enzyme (20-40μg/ml) solution and separate digested peptides according to the size (Fig.48).

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