The Role of Subcellular Distribution upon Signalling by Polyomavirus Middle Tumour-antigen

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ABSTRACT

The middle T-antigen (MT) encoded by polyomavirus induces tumours by mimicking an activated cell surface receptor. A hydrophobic region close to the C-terminus locates MT to the plasma membrane where it initiates oncogenic signalling. HA or FLAG tags added to the C-terminus of MT could be detected on the outside of cells, demonstrating that MT is a transmembrane protein. Addition of a KDEL sequence retained MT in the endoplasmic reticulum, where it failed to transform cells as a consequence of its lack of binding to ShcA, PI3K, and PLC-γ1 despite the phosphorylation of the appropriate binding sites. Additional studies show that MT binding to PP2A is probably required in addition to the TMD for MT to exit the ER and migrate to the plasma membrane. MT on the cell surface is present in discrete clusters that contain phosphorylated ShcA and so represent active signalling complexes. New mutations in the hydrophobic region prevented MT clustering, and also inhibited cell transformation without altering the association of MT with its known binding proteins. Overall, these data, together with previous publications, illustrate that MT associates with signalling proteins at discrete subcellular membrane sites in its maturation pathway. MT binds to PP2A in the cytoplasm, to c-Src at the ER, and to ShcA, PI3K, and PLC-γ1 at subsequent locations en route to the plasma membrane. Formation of a large macromolecular complex in the plasma membrane is probably required for MT signalling. Similar cell surface complexes are observed with activated growth factor receptors, so it is possible that normal and oncogenic signalling from receptors is also dependent upon the assembly of large macromolecular complexes at the cell surface membrane. There was no obvious association of MT clusters with lipid rafts but there was colocalisation with the actin cytoskeleton.
DECLARATION OF ORIGINALITY

Unless otherwise stated, all experiments were performed by me, Yunsi Zhou, the author of this thesis.

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ABBREVIATIONS

BCA  bicinchoninic acid assay
bp  base pair
BPV  bovine papillomavirus
CCD  cytochalasin D
cDNA  complementary deoxyribonucleic acid
CHX  cycloheximide
CT-B  cholera toxin subunit-B
Cytb5  cytochrome b5
DIF  detergent-insoluble faction
DMEM  Dulbecco’s modified Eagle’s medium
DMSO  dimethylsulfoxide
DNA  deoxyribonucleic acid
DPBS  Dulbecco’s phosphate-buffered saline, PBS supplemented with CaCl2 and MgCl2
dsDNA  double-stranded DNA
DSF  detergent-soluble faction
EGFR  epidermal growth factor receptor
EphA  Ephrin type-A receptor
ER  endoplasmic reticulum
FAK  focal adhesion kinase
FBS  foetal bovine serum
FCS  fetal calf serum
FGFR  fibroblast growth factor receptor
c-FMS  colony stimulating factor 1 or macrophage colony-stimulating factor receptor
GFR  Growth Factor Receptor
GM1  monosialotetrahexosyl ganglioside
HaPyV  hamster polyomavirus
HER2/Neu/ErbB2  human epidermal growth factor receptor 2
HPV  human papillomavirus
IB  immuno-binding
IEM  immuno-electron microscopy
IF  immunofluorescence staining/labelling
IGF-IR Insulin-like Growth Factor I Receptor
IRβ insulin receptor subunit β
IP immunoprecipitation
kDa Kilo Daltons
LB lysogeny broth
LT Large T-antigen
MβCD Methyl-β-cyclodextrin
MC PyV Merkel Cell polyomavirus
MF membrane fraction
MMTV mouse mammary tumour virus
MPyV murine Polyomavirus
MSC mesenchymal stem cell
MT Middle T-antigen
NMF non-membrane fraction
NSCLC non-small cell lung cancer
OA okadaic acid
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffer saline
PCR polymerase chain reaction
PDGFRβ platelet-derived growth factor receptor β isoform
PLC-γ1 phospholipase Cγ1
PM plasma membrane
PP2A protein phosphatase 2A
Pr Pearson’s coefficient
PTK protein tyrosine kinase
PyV polyomavirus
Rb retinoblastoma protein
RNA ribonucleic acid
RT room temperature
RTK Receptor Tyrosine Kinase
SDS sodium dodecyl sulphate
SFK Src Family Tyrosine Kinase
SH Src-homology domains
siRNA small interfering RNA
SRP signal recognition particles
ST Small T-antigen
SV40 simian virus 40
TA tail-anchored
TBE Tri-borate/EDTA
TBS-T Tris-Buffered Saline and Tween-20
TMD transmembrane domain
TRC transmembrane-domain recognition complex
VSV G vesicular stomatitis virus G protein
WB Western blotting
WCL whole cell lysates
wt wild type
w/w weight to weight
Φ diameter
## LIST OF MT MUTANTS

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<td>MTGGDV</td>
<td>Sequence GGDV added to the C-terminus, a control for MTKDEL. Strongly transforms</td>
<td>Zhou et al. 2011; Figure 3.2-3.5</td>
</tr>
<tr>
<td>MTSec61β</td>
<td>Hydrophobic sequence substituted with that of ER-resident tail-anchored protein Sec61β. Weakly transforming</td>
<td>Zhou et al. 2011; Figure 3.2 and 3.5</td>
</tr>
<tr>
<td>180Δ10</td>
<td>Lacks both PP2A and Src binding sites. Non-transforming</td>
<td>Brewster et al. 1997; Figure 3.7</td>
</tr>
<tr>
<td>200Δ10</td>
<td>Lacks Src binding site only. PP2A binding site intact. Non-transforming</td>
<td>Brewster et al. 1997; Figure 3.7</td>
</tr>
<tr>
<td>Δ419-421</td>
<td>Deletion of final 3 amino acids at the C-terminus. Strongly transforms</td>
<td>Figure 3.16</td>
</tr>
<tr>
<td>Δ416-421</td>
<td>Deletion of final 6 amino acids at the C-terminus. weakly transforms</td>
<td>Figure 3.18</td>
</tr>
<tr>
<td>416G6</td>
<td>Glycine substitution of the final 6 amino acids at the C-terminus. Very weakly transforms</td>
<td>Figure 3.19</td>
</tr>
<tr>
<td>L405E</td>
<td>Mutation of Leucine 405 to Glutamate in the hydrophobic sequence. Binds to all known MT-binding proteins associated with transformation but cannot transform. C-terminus is not exposed on cell surface</td>
<td>Figure 4.10 – 4.13</td>
</tr>
<tr>
<td>L405E KDEL</td>
<td>L405E mutation with ER-retention sequence KDEL added to the C-terminus. Escaped entrapment in the ER but unstable. Non-transforming</td>
<td>Figure 4.16</td>
</tr>
<tr>
<td>G406L</td>
<td>Mutation of Glycine 406 to Leucine in the hydrophobic sequence. Transforms</td>
<td>Figure 4.10 – 4.13</td>
</tr>
<tr>
<td><em>neu</em>-wt MT</td>
<td>Hydrophobic sequence substituted with that of wild type ErbB2/<em>neu</em> receptor. Strongly transforms</td>
<td>Figure 4.10 and 4.11</td>
</tr>
<tr>
<td><em>neu</em>-mutant MT</td>
<td>Hydrophobic sequence substituted with that of</td>
<td>Hynes and Stern.</td>
</tr>
<tr>
<td>Protein</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>ErbB2/neu receptor</td>
<td>carrying the activating mutation of Valine to Glutamate (V664E). Binds to all known MT-binding proteins associated with transformation but cannot transform. C-terminus not exposed on cell surface</td>
<td>1994; Figure 4.10 and 4.11</td>
</tr>
<tr>
<td>FGFR3-wt MT</td>
<td>Hydrophobic sequence substituted with that of FGFR3 receptor. Trapped in the ER and Golgi. Non-transforming</td>
<td>Figure 4.10 and 4.20</td>
</tr>
<tr>
<td>FGFR3-mutant MT</td>
<td>Hydrophobic sequence substituted with that of FGFR3 receptor with the activating mutation of Glycine to Arginine (G380R). Trapped in the ER and Golgi. Non-transforming</td>
<td>Figure 4.10 and 4.20</td>
</tr>
<tr>
<td>Δ402-406</td>
<td>Deletion of 5 amino acids in the middle of the hydrophobic region. Trapped in the Golgi. Non-transforming</td>
<td>Figure 4.21</td>
</tr>
<tr>
<td>dl1015</td>
<td>Deletion between amino acid residues 339-347. Intact binding sites for PP2A, Src, Shc, PI3K and PLC-γ1 but unable to transform. Defect unknown.</td>
<td>Magnusson and Berg. 1979; Magnusson et al, 1981; Figure 4.17</td>
</tr>
<tr>
<td>NS2/DC1/DC2</td>
<td>PP2A binding disrupted. Non-transforming</td>
<td>Dilworth et al. 1994; Zhou et al. 2011; Figure 3.8, 3.9, Table T3.1</td>
</tr>
<tr>
<td>Δj-DC1</td>
<td>DC1 mutation combined with the deletion in the dnaJ domain. Lacks binding to Hsc70 and PP2A. Non-transforming</td>
<td>Dilworth et al. 1994; Whalen et al. 2005; Zhou et al. 2011; Figure 3.8, 3.9, Table T3.1</td>
</tr>
</tbody>
</table>
CHAPTER 1 – INTRODUCTION

1.1 Overview

Over the past fifty years, research on polyomaviruses had contributed substantially to the understanding of basic cell biology, oncogenic signalling and transcription control. Studies of murine (mouse and rat) polyomavirus were particularly prolific from the late 1970s to early 1990s (Dilworth 2002). During that time, research on the mouse polyomavirus oncogene, Middle Tumour-antigen (MT), led to the discovery of cellular tyrosine kinases (Eckhart, Hutchinson et al. 1979) and PI3 Kinase activity was also first reported during the studies of MT associated oncogenic signalling (Whitman, Kaplan et al. 1985). Although many years have passed since MT was first discovered, it remains a useful tool for studying the events underlying cancer formation and metastasis in animal models.

1.2 Small DNA Tumour Viruses

Polyomaviruses (PyV) belong to the small DNA virus class, which are also known as DNA tumour viruses. These are non-enveloped, capsid-only viruses containing a circular double-stranded DNA (dsDNA) genome of about 5,000 bp in size. Murine polyomavirus (MPyV) was the first of its kind discovered by Ludvik Gross in 1953 (Gross 1953), when working on the cell free transmission of leukaemia in mice. Inoculation of filtered leukemic mice cell extracts into new-born mice induces tumour formation in a range of tissues. Other members of PyV family include simian virus 40 (SV40), murine polyomavirus (MPyV), avian polyomavirus, hamster polyomavirus (HaPyV), JC virus, BK virus, SA12 virus, lymphotropic polyomavirus, K virus, and the recently discovered, Human Merkel Cell polyomavirus (MC PyV) (Pipas 1992, Jiang and Imperiale 2012).
Other examples of small DNA virus family include adenoviruses, papillomaviruses, and parvoviruses. The genome of a small DNA virus is divided into early and late transcriptional regions where different sets of viral proteins are made using the host machinery, depending on the virus life cycle. The early transcription regions of polyomavirus, papillomavirus and adenovirus genomes all encodes viral proteins with oncogenic potential, termed tumour-antigens (T-antigens), probably developed through evolution as a means to promote the replication of both host and virus genomes (Dilworth 1995). Therefore these viruses are also known as small DNA tumour viruses (Jiang and Imperiale 2012).

A common strategy used by DNA tumour viruses is the inactivation of tumour suppressors such as p53 and the retinoblastoma protein (Rb) family members. In fact, many of the known tumour repressors were first discovered as cellular proteins associated with T-antigens (Lane and Crawford 1979, Linzer and Levine 1979). SV40 LT, adenovirus E1B and HPV E6 all target p53 tumour suppressor, although the mechanisms of p53 inactivation are different (reviewed by O'Shea and Fried 2005, Atkin, Griffin et al. 2009). Other tumour antigens, such as E6 and E7 from papillomavirus, bind and activate cyclins, cdks and other transcriptional factors that promote cell cycle entry. Some tumour antigens, such as MT, are able to activate multiple mitogenic signalling pathways by mimicking an activated growth factor receptor. Others bind and activate a growth factor receptor, as in the case of the E5 oncoprotein encoded by the bovine papillomavirus (BPV) which can bind and activate PDGFRβ (Sparkowski, Anders et al. 1995).
1.3 MPyV Genome

All polyomavirus genomes encode a large and a small size tumour-antigen, abbreviated as LT and ST, respectively. In addition, murine and hamster PyV genome also encoded an intermediate-sized tumour-antigen, MT (Pipas 1992). The recently discovered human MC PyV also has an early transcription region that encodes an additional polypeptide of a similar size to that of the murine PyV MT but has not yet been fully characterised.

Once inside the cell, the virus is transported into the nucleus (Kasamatsu and Nakanishi 1998), where the genome is uncoated and RNA transcription of the early region on the genome begins. The early transcription region encodes three tumour-antigens. These are large T (LT), middle T (MT) and small T (ST), with molecular weight of 100, 55 and 22 kDa, respectively (Ito, Spurr et al. 1977, Hutchinson, Hunter et al. 1978, Schaffhausen, Silver et al. 1978, Simmons, Chang et al. 1979). The tumorigenic effect of MPyV in vivo is attributed these three T-antigens working together. The late and early transcription regions are separated by a regulatory region that contains the viral origin of DNA replication (ori), the promoters for both early and late regions, transcriptional enhancers and binding sites for LT, which promotes virus DNA replication (Pipas 1992). LT binding to multiple sites around the origin of replication causes a switch of RNA transcription from the early region to the ‘late phase’, DNA strand, which encodes three viral capsid proteins, VP1, VP2 and VP3, for virion assembly during the lytic phase of the virus life cycle (Pipas 1992). Although virion proteins are essential for mature virion production, they are not required for transformation (Fried 1965).

All three tumour antigens (LT, MT and ST) are encoded by the same stretch of viral DNA sequence. Using separate reading frames after the common region (Figure 1.1A), two immature mRNAs can be translated. One produces LT. The other is spliced to remove introns, resulting in two different mature mRNAs, which give rise to MT and ST. Therefore
two T-antigens with completely different C-terminal sequence are made, LT and MT, while
the bulk of ST is essentially the N-terminus of MT (Figure 1.1 B) (Smart and Ito 1978,
Dilworth 2002). LT, ST and MT all share the same J domain at their N-termini J domain,
which is highly conserved across all PyV T-antigens, including SV40. After this point, the C-
terminal coding sequence of the MPyV genome is unique and does not share any DNA
sequence homology with other polyomaviruses (unique region, Figure 1.1 A). And this
results in the production of three T-antigens, each with a unique C-terminal amino acid
sequence and function (Pipas 1992).

1.4 T-antigens

When expressed individually, LT can immortalise primary cells but cannot transform and is
therefore not required for tumorigenesis (Moore, Chowdhury et al. 1980, Rassoulzadegan,
Binetruy et al. 1982). ST can promote cell cycle progression but does not have any
transforming property (Mullane, Ratnofsky et al. 1998). Only MT can transform established
cell lines into a fully tumorigenic phenotype when expressed alone (Hassell, Topp et al.
antigens are responsible for the effective tumorigenic properties of the MPyV. The
expression of the three viral oncoproteins leads to dramatic changes in cell morphology such
as decreased cell-substratum adhesion, stress fibre remodelling with increased cell motility,
lack of contact inhibition and anchorage independent growth.
A. MPyV genome is divided into early and late regions and marked at 1 kb intervals. The coding regions in mature mRNA are indicated by solid coloured boxes outside of the circular genome. The non-coding mRNA regions are designated by solid lines, and the sequences removed by splicing in dotted lines. Unique region of MPyV has no sequence similarity to SV40 or other polyomavirus. The coding regions with base-pair numbers corresponding to their positions in the genome are indicated in each box. Adapted from a review by Dilworth, 1995.

B. Translated T-antigens share common sequence at the N-terminal region. All three T-antigens encoded by MPyV share the first 79 amino acid sequence that is the J domain (orange). MT and ST also share an additional 112 amino acids, which make up the PP2A binding region. The unique regions of LT, MT and ST are represented with lilac, red and green, respectively. Approximate positions of functional domains are indicated by boxes: Rb binding regions (Rb), DNA-binding domain (DBD), helicase domain (Helicase), Src-binding region (Src) and transmembrane domain (TMD). Adapted from a review by Cheng et al., 2009.
1.4.1 Large T-antigen

The 100 kDa LT was first identified as a major phosphorylated protein in vivo amongst the T antigens of MPyV (Schaffhausen and Benjamin 1979). LT is localised exclusively in the nucleus but not the nucleolus (Dilworth, Hansson et al. 1986). The J domain is a highly conserved structural domain found in dnaJ/Hsp40 family of molecular chaperones including Hsc70. Binding of PyV T-antigens to Hsc70 requires the HDPK residues in the viral protein N-terminus and subsequently activates the ATPase activity of the chaperone protein, which is thought to facilitate proper folding and maintaining structural integrity of the T-antigens (Cheng, DeCaprio et al. 2009).

Early knowledge in MPyV LT function was based on the studies of SV40 LT. The conserved LXCXE motif makes direct contact with a highly conserved ridge in the B domain of Rb family proteins, which inactivates the tumour suppressor (Cheng, DeCaprio et al. 2009). Like SV40 LT, MPyV LT contains a DNA binding domain (DBD), which allows LT to bind to the origin of replication on the viral genome, and a helicase domain in the C-terminal unique region, which plays an essential role in viral genome replication. However, unlike SV40 LT, MPyV LT does not target p53 directly and does not transform cells (reviewed by O'Shea and Fried 2005). Although PyV LT can immortalise primary cells, including human fibroblasts when the expression was driven by a heterologous promoter, it does not transform cells into a tumorigenic state (Strauss, Hering et al. 1990).

1.4.2 Middle T-antigen

Unlike LT and ST, which are cytoplasmic, MT is found in membranes, which can only be extracted in the presence of detergents (Ito, Brocklehurst et al. 1977). MPyV MT is a 55 kDa polypeptide comprised of 421 amino acid residues and associates with the plasma membrane
via a hydrophobic sequence very close to the C-terminus (Figure 1.2). Inducible MT expression in rat fibroblasts was accompanied with increase in transformed cell growth and kinase activity (Heiser and Eckhart 1985). By studying non-transforming strains of MPyV which express truncated MT, it was established that MT is tightly associated with the transforming properties of the virus and is associated with strong tyrosine kinase activity (Schaffhausen, Silver et al. 1978, Ito, Spurr et al. 1980, Dilworth 2002). However, like many viral oncogenic proteins, MT does not have any intrinsic enzymatic activity. MT translated in vitro or expressed in bacterial system does not possess any kinase activity. The kinase activity associate with MT come from the Src family tyrosine kinase bound to the virus protein (Courtneidge and Smith 1983). MT provides binding sites to bring cellular signalling molecules to the membrane and propagate mitogenic signals, which is similar to an activated Growth Factor Receptor (GFR) (Dilworth 1995, Dilworth 2002).

The N-terminal region of MT binds to the PP2A core dimer A and C (Glenn and Eckhart 1993). PP2A in complex with MT provides a structural platform for Src family tyrosine kinases to bind. These include pp60c-src, pp62c-yes and pp59c-fyn. Upon binding to MT, Src tyrosine kinase activity is activated, which phosphorylates MT at three major tyrosine residues: Y250, Y315 and Y322 (Figure 1.2). These phosphorylated tyrosine residues act as docking sites to recruit Src homology 2 (SH2) domain- or phospho-tyrosine binding (PTB) domain-containing proteins such as ShcA (to Y250), PI3K p85 subunit (to Y315) and PLC-γ1 (to Y322) (see Figure 1.2).
Figure 1.2 Mouse Polyomavirus MT Anatomy

MT is represented linearly and outlined in black. Important phosphorylation sites in MT are written in bold together in line with numbers indicating the positions in MT amino acid sequence. Sequence 111 to 185 represents PP2A binding region. The CXCXXC motifs required for PP2A binding are depicted as C3. Sequence 185 to 210 represents the unique binding site for Src. PPP represents the Proline-rich region. PyV MT is bound to membranes via C-terminus hydrophobic region. It mimics an activated RTK by recruiting members of the Src family tyrosine kinases which in turn modify the viral molecule on tyrosine residues to create additional binding sites to recruit more signalling proteins as mentioned above. In the end, proliferative signals are propagated via the MAPK signalling pathway together with survival signals via the activation of AKT signalling pathway.
Activated Src can also phosphorylate these signalling molecules and adaptor proteins recruited to MT, which in turn alters their activities or creates further docking sites for signal transduction. A docking protein ShcA, recruited to the PP2A/MT/Src complex become phosphorylated on multiple tyrosine residues, which creates a binding site for the adaptor protein Grb2, which then in turn recruits guanine nucleotide exchange factor, Son of Sevenless 1 (Sos1), and adaptor, Gabl, to the MT signalling complex. Sos1 subsequently activates the Raf-MEK-ERK MAPK signalling cascade and leads to activation of transcription factors such as Elk, c-jun, that promote cell proliferation. PI3K p85 recruits the catalytic subunit of PI3K, p110, to the plasma membrane, where it converts phosphoinositide to PIP3. An increase in PIP3 activates PDPK1, a known activator of AKT serine/threonine kinase. AKT itself can also bind to PI3K p110 at the plasma membrane via its pleckstrin homology domain and becomes activated. Direct and indirect activation of AKT promotes DNA synthesis and inhibits apoptosis. PI3K p110 also activates the small GTP binding protein Rac, of which activation is necessary for MT to promote migratory signals and to activate c-fos. PLC-γ1 binds to MT p-Y322 via its SH2 domains (Su, Liu et al. 1995) where it catalyses the conversion of PIP2 to DAG and IP3. This leads to an increase of Ca^{2+} level inside the cell, which can activate the PKC family of phospholipid-dependent Ser/Thr kinases that regulate a wide range of protein targets involved in cell signalling. In the end, proliferative signals are propagated via the MAPK signalling cascade, together with pro-survival signals via activation of AKT. A comprehensive report on our current understanding on cancer-formation gained from the studies on MPyV MT is compiled by Fluck and Schaffhausen (Fluck and Schaffhausen 2009). The mechanism of MT interaction with binding partners and signal transduction will be discussed in detail in Section 1.10. Overall, the multi-complex MT signalosome depicted in Figure 1.2 resembles the events happening during GFR activation (Dilworth 1995). Therefore MT can be considered as a permanently
active analogue of a GFR (Ichaso and Dilworth 2001) and has been instrumental in our understanding of the molecular events governing GFR signalling.

### 1.4.3 Small T-antigen

Since the amino acid sequence of ST is mostly identical to the N-terminal region of MT in MPyV, both T-antigens are capable of binding to PP2A, a cellular serine/threonine-specific phosphatase (Pallas, Shahrik et al. 1990). The mechanism of PP2A – ST/MT interaction is discussed in more details on Section 1.10.1. While SV40 ST can transform cells, MPyV ST does not possess such a property. SV40 ST binds only PP2A Aα, while MPyV ST binds both Aα and Aβ isoform of PP2A scaffold subunits, therefore probably target different substrates (Andrabi, Hwang et al. 2011). Interestingly, PyV ST can block differentiation in multiple cell lines. MPyV ST seems to stabilise PP2A Aβ/AKT interaction, which leads to reduced phosphorylation of S473 on AKT therefore negatively regulates AKT (Hwang, Jiang et al. 2013). MPyV ST is likely to play a role in augmenting LT and MT in the immortalisation and transformation of primary cells (see 1.6).

### 1.5 Late Replication Cycle Proteins

At the late lytic cycle phase, three virion proteins, VP1, VP2 and VP3, are transcribed. VP1 is the major virion protein which makes up as much as three quarters of the total virion protein content (Atkin, Griffin et al. 2009). Immunofluorescence analysis of individually expressed MPyV virion proteins showed VP2 is localised to the perinuclear region of the cells and VP3 is found in the cytoplasm(Forstova, Krauzewicz et al. 1993).VP1 is localised to the nucleus (Stamatos, Chakrabarti et al. 1987) and only in the presence of VP1 can VP2 and VP3 be efficiently transported into the nucleus where new virus particles can be assembled (Delos,
Montross et al. 1993, Forstova, Krauzewicz et al. 1993). Recently it has been shown that VP2 and VP3 can activate caspases, which results in host cell destruction at the end of the virus replication cycle (Huerfano, Zila et al. 2010).

1.6 Cooperation of MPyV T-antigens induces tumour formation

MT is responsible for inducing cellular changes that leads to malignant transformation of established fibroblasts. These are post-crisis cell lines capable of indefinite growth. But expression of MT alone cannot immortalise primary rodent cells (Rassoulzadegan, Cowie et al. 1982). A second oncogene from the early transcription region is needed for the transformation of newly isolated primary cells (Schaffhausen and Benjamin 1981, Rassoulzadegan, Cowie et al. 1982). The inability of MT to immortalise primary cells is probably due to functional tumour suppressor p53 present in most primary tissues (Lomax and Fried 2001, Moule, Collins et al. 2004). It has been shown that mitogenic signals induced by MPyV MT activates ARF, an activator of p53, therefore induces p53-mediated blockage to cell division. In contrast, ST disrupts ARF-p53 signalling pathway, thus alleviating the p53-mediated cell arrest (Lomax and Fried 2001). The inability of MT to transform primary rodent cells can be overcome by co-expression of mutant p53 as well as the other two T-antigens (Rassoulzadegan, Cowie et al. 1982, Reihsaus, Kraiss et al. 1992, Lomax and Fried 2001). Therefore, transformation of primary cells requires cooperation of multiple oncogenes. The Ras oncogene and MPyV MT can only transform fibroblasts when cells are immortalised prior to transfection, for example, by cellular myc gene, PyV LT or adenovirus E1A (Land, Parada et al. 1983, Ruley 1983). Expression of PyV MT alone in most cultured cell lines is sufficient to induce full malignant transformation in a single step and does not require cooperating oncogenes (Mann, Stevenson et al. 1991). The resulting transformed cells are capable of forming invasive cancerous growth when injected into nude mice.
1.7 Transcription Factors in MT-induced Transformation

Early studies of lytically infected cells have demonstrated that transcription factor PEA1 (a homologue of AP-1 and c-Jun) and PEA3 (mouse homologous to Ets family transcription factors) are key targets for MPyV MT. These transcription factors bind to specific sequences in the MPyV enhancer region in the polyoma promoter. Both are highly active in fibroblast cell lines infected with MPyV and lead to altered transcription of transformation-related genes (Wasylyk, Imler et al. 1988). Both PEA1/AP-1and PEA3/ets are heavily implicated in breast cancer. Over-expression of the Est family members are associated with poor prognosis (Kurpios, Sabolic et al. 2003). In many breast tumour cells, expression of dominant negative AP-1 and Ets reverses the transformed phenotype (Galang, Garcia-Ramirez et al. 1996, Shepherd and Hassell 2001). Genes up-regulated by PEA1/AP-1 and PEA3/ets includes cyclin D1, matrix metalloproteases, plasminogen activator, EGF and VEGF (Fluck and Schaffhausen 2009). PEA1 can also be activated by other transforming oncogenes such as v-src and c-fos but not the immortalising oncogenes such as MPyV and SV40 LTs (Wasylyk, Imler et al. 1988). This suggests distinct targets and pathways exist between immortalising oncogenes and transforming oncogenes, and that PEA1/AP-1 mediates at least some of the events associated with transformation.

Other transcription factors whose activity is altered by MT-induced transformation include NF-κB (Nabel and Baltimore 1987) and PEA2/RUNX family core binding factor (Chiu, Imagawa et al. 1987, Gottlieb and Villarreal 2001). Transcriptional activation stimulated by MT is reviewed in detail by reviews by Flucks and Schaffhausen, Gottlieb and Villarreal (Gottlieb and Villarreal 2001, Fluck and Schaffhausen 2009).
1.8 MT in Other Polyomaviruses

1.8.1 Hamster Polyomavirus

The Hamster polyomavirus (HaPyV) is a virus closely related to murine polyomavirus and is the only other polyomavirus known to express MT (Delmas, Bastien et al. 1985). Naturally-occurring HaPyV was found to cause hair follicle epitheliomas. But the malignancies caused by inoculation of purified virus into new born hamsters were limited to lymphoma and leukaemia instead of the wide spectrum of tumours caused by inoculation with mouse polyomavirus (Delmas, Bastien et al. 1985) (Scherneck, Delmas et al. 1987). The HaPyV MT shares sequence homology with MPyV at the N-terminal region, required for PP2A and Src family tyrosine kinase binding, and at the C-terminal hydrophobic region required for membrane anchoring. The intervening sequence is unique to each MT (Delmas, Bastien et al. 1985) (Goutebroze, Dunant et al. 1997). Unlike MPyV MT, which binds and activates pp60c-src, pp62c-yes and pp59c-fyn, HaPyV MT only binds pp59c-fyn (Courtneidge, Goutebroze et al. 1991), a Src family tyrosine kinase expressed mostly in hematopoietic cells (Cooke, Abraham et al. 1991). This is likely to account for the ability of HaPyV MT to induce lymphoma and leukaemia (Courtneidge, Goutebroze et al. 1991, Brizuela, Ulug et al. 1995). The intervening sequence of HaPyV MT associate with PI3K and PLC-γ1 but not ShcA. The divergence at the intervening stretch may also modulate specificity activation of different SFK members (Goutebroze, Dunant et al. 1997).

1.8.2 Human Merkel Cell Polyomavirus

In recent years, the field of research on polyomavirus has been re-ignited following the discovery of a human polyomavirus in patients with Merkel Cell Carcinoma, an aggressive form of skin cancer associated with immuno-suppression and the elderly, hence the name
Merkel Cell Polyomavirus (MC PyV) (Engels, Frisch et al. 2002, Feng, Shuda et al. 2008). From 2007 to present, 9 different human polyomaviruses had been discovered (DeCaprio and Garcea 2013). The MC PyV and murine PyV genomes are very similar. Recently, Galloway and co-workers discovered an overprinting gene (ALTO, Alternate frame of the Large T Open reading frame) can be expressed as an alternative reading frame from the LT gene region of MC PyV (Carter, Daugherty et al. 2013). Intriguingly, ALTO is evolutionarily related to the MT of MPyV (Carter, Daugherty et al. 2013). A hydrophobic sequence was observed in the translated product of ALTO despite having almost no sequence similarity with MT and was found to associate with intracellular membranes. However, unlike MPyV MT, ALTO product seems to inhibit transformation by inducing apoptosis. Characterisation of this previously missed viral gene product is underway and will rely heavily on the knowledge gained from MPyV MT. DNA analysis by Galloway et al provided insight into de novo protein evolution as a means by which viruses overcome constraints of small genome size (Carter, Daugherty et al. 2013).

MC PyV has been detected in some non-small cell lung cancer patient samples, raising the possibility that MC PyV may be associated with the pathogenesis of NSCLC (Hashida, Imajoh et al. 2013). Human Polyomavirus may be more prevalent in pathogenesis than previously thought. Studies from MPyV will provide crucial insights into the cellular and molecular biology of the many recently discovered human PyVs (DeCaprio and Garcea 2013).

1.9 MT Oncogenic Mouse Models

In order to study the MT-driven tumorigenesis in specific tissues in vivo, Guy and co-workers constructed a PyV MT expression plasmid under the control of a mouse mammary tumour
virus (MMTV) promoter sequence (MMTV-PyV MT). Transgenic mice carrying the MMTV-PyV MT sequence express this oncoprotein in the mammary gland, which result in widespread spontaneous transformation of the mammary epithelium and rapid production of multifocal mammary adenocarcinomas (Guy, Cardiff et al. 1992). This is accompanied by strong metastasis potential to the lung of the majority of transgenic mice, which is a feature specific to breast cancer metastasis in human but rarely observed in other MMTV-oncogene based models, including MMTV-neu. Therefore MMTV-MT is a good model for human mammary cancer (Lin, Jones et al. 2003). Crossing MMTV-MT mice with transgenic mice lacking certain MT binding proteins allows the study of genetic requirement of MT-induced tumorigenesis. For example, crossing MMTV-MT mice with pp60c-src knockout mice demonstrated pp60c-src is an important target for mammary tumour formation in vivo. Mice lacking pp60c-src expression rarely developed mammary tumour while mice deficient in pp62c-yes developed tumours comparable rate to wild type when crossed with MMTV-MT mice (Guy, Muthuswamy et al. 1994). Transgenic mice with inducible MT expression using Cre-mediated recombination have also been achieved (Cecena, Wen et al. 2006). This allows localised, tissue-specific expressing of MT and has been a useful model for studying tumorigenesis in a broad range of tissues.

1.10 MT Binding Proteins

1.10.1 PP2A

The N-terminus of MT contains the binding region for protein phosphatase 2A core dimer, which is made up of a catalytic subunit C or PR35 and a regulatory subunit A or PR65 (Pallas, Shahrik et al. 1990). Cellular PP2A is a hetero-trimeric complex consist of a catalytic subunit C, a scaffolding subunit A and a regulatory subunit B (55 kDa). It is a major cellular
Ser/Thr protein phosphatase in eukaryotic cells and is involved in regulating a wide range of cellular function including transcription, replication, and can function as a tumour suppressor (Janssens and Goris 2001, Cheng, DeCaprio et al. 2009). The catalytic subunit C and regulatory subunit A each has two isoforms (α and β) while the other regulatory subunit come from a large family of B subunits (B, B’, B” and B”’ family) therefore resulting in at least 80 possible combinations.

Binding to PP2A core dimer requires MT N-terminal region 110 – 190 (Martens, Nilsson et al. 1989, Glenn and Eckhart 1993, Campbell, Auger et al. 1995, Brewster, Glover et al. 1997). The two repeats of highly conserved Cysteine-rich motif (CXCXXC) in the region 80–191 on MPyV ST and MT are important for the binding to PP2A (Campbell, Auger et al. 1995). PP2A regulatory subunit B requires regions of the scaffold subunit A and the highly conserved carboxyl terminus of the catalytic subunit C. MT binding to PP2A core dimers is mediated through subunit A only and does not require regions in the C subunit (Ogris, Gibson et al. 1997). It is still not clear whether phosphatase activity is required for MT function. The N-terminal region required for PP2A binding is also required for association with the Src family of tyrosine kinases and transformation (Friedmann, Doolittle et al. 1978, Markland and Smith 1987). Glover et al., have identified a Serine (S195) and Threonine (T203) residue preceded by basic Arginine residues in the region 191 – 210 of MT, which is required for the association of pp60c-src but not PP2A (Brewster, Glover et al. 1997). Both S195 and T203 are de-phosphorylated in the complex with pp60c-src. It was suggested that PP2A may play a role in maintaining these residues in a de-phosphorylated state (Glover, Brewster et al. 1999). To support this, an inhibitor of the phosphatase reduced the amount of pp60c-src and the PP2A bound to MT. However, the PP2A core dimer containing inactive catalytic subunits can still associate with MT and form stable MT-PP2A-
p60c-src complexes in cells (Ogris, Mudrak et al. 1999). Therefore PP2A is more likely to provide a structural change for the recruitment of pp60c-src.

It has been reported that MT-PP2A association changes the substrate specificity of the phosphatase, which become sensitive to phosphatase inhibitor, okadaic acid (OA), but insensitive to other PP2A-specific phosphatase inhibitor, inhibitor-1 and -2 (Cayla, Ballmer-Hofer et al. 1993). PP2A subunits can be regulated through post-translational modifications such as phosphorylation (Chen, Martin et al. 1992), methylation of the C subunit (Bryant, Westphal et al. 1999) and ubiquitination (Trockenbacher, Suckow et al. 2001). Recently, it was observed that PP2A scaffolding subunit A forms a complex with de-ubiquitination protein USP28 in normal rat fibroblasts. Down-regulation of USP28 in MT-transformed cells resulted in raised level of mono-ubiquitinated PP2A without affecting the stability of the phosphatase (unpublished data). There may be many ways in which MT interaction changes substrate specificity of the PP2A core dimer.

1.10.2 Src Family Tyrosine Kinase

The potent transforming ability of MT is attributed to its association with Src family non-receptor tyrosine kinases. Association with Src requires the N-terminal region of MT, which overlap largely with PP2A binding region. In addition to PP2A binding region, association with Src also requires residues 185 to 210 (Brewster, Glover et al. 1997, Glover, Brewster et al. 1999). In vitro translation of the first 220 amino acids of MT N-terminus was enough to form a complex containing PP2A and pp60c-src complex (unpublished data). However, the mechanism behind MT induced activation of Src tyrosine kinase is still not fully understood.

The src gene family has nine known members (blk, c-fgr, fyn, hck, lck, lyn, c-src, c-yes and yrk), each encoding a cytoplasmic protein-tyrosine kinase involved in signal
transduction (reviewed by Brickell 1992, Kefalas, Brown et al. 1995). Src is the most studied prototype of the family of 8 other closely related protein tyrosine kinases. The amino acid sequence of SFKs can be divided into four domains. The N-terminal sequence of pp60c-src contains a single myristoylation site that localise the protein tyrosine kinase to cell membranes. Some SFKs, like c pp62c-yes and pp59c-fyn are also palmitoylated in this region. Next to the N-terminal lipid modification domain is the unique domain, followed by a Src homology (SH) 3 domain, which recognises and binds specifically to Proline rich motifs with consensus sequence, PXXP; and then a SH2 domain that interacts with phospho-tyrosine motifs such as the consensus sequence pYEEI (Koch, Anderson et al. 1991). This is followed by the catalytic domain with C-terminal tail that contains a negative regulatory phosphorylation site at Y527. The SH2 domain of Src binding to its own phospho-tyrosine site prevents substrate access, thus locks the kinase in a closed conformation (Ingley 2008). The catalytic domain contains a kinase activation loop (A-loop) with an auto-activating Y416. Most of the Src tyrosine kinases in normal cells are phosphorylated on Y527, and are therefore inactive. pp60c-src association with MT requires the kinase domain and part of its C-terminal regulatory tail sequence around Y527, but the tyrosine kinase activity is not required (Cartwright, Eckhart et al. 1987, Dunant, Senften et al. 1996). As a result, v-Src, which lacks the regulatory tail region, cannot bind to MT (Cheng, Harvey et al. 1988). Despite being closely related, only selective members of the SFK associate with MT. MT encoded by MPyV preferentially associates with pp60c-src (Courtneidge and Smith 1983), pp62c-yes (Kornbluth, Sudol et al. 1987), and, to a lesser extent, pp59c-fyn (Cheng, Harvey et al. 1988, Kypta, Hemming et al. 1988) but not pp56c-1ck (Louie, 1998) nor pp59/61hck (Dunant, Senften et al. 1996). However, HaPyV MT can only bind pp59c-fyn despite a high level of sequence homology in the N-terminal regions of the two MTs (Courtneidge, Goutebroze et al. 1991). This suggests different mechanisms are employed by the mouse and
hamster PyVMTs for their association with particular SFKs. Indeed, HaPyV MT binds to
pp59c-fyn via the SH2 domain of the kinase and a phospho-tyrosine 324 in the unique region

MT itself is a major substrate for activated pp60c-src, which phosphorylates a number
of tyrosines on MT. Three of the most well characterised phospho-tyrosine residues act as
binding sites for adaptor protein Shc (Y250) (Campbell, Ogris et al. 1994, Dilworth, Brewster
et al. 1994), the SH2 or Phospho Tyrosine Binding (PTB) domains of PI3K (Y315)
(Whitman, Kaplan et al. 1985) and PLC-γ1 (Y322) (Su, Liu et al. 1995).

1.1.0.3 PI3 Kinase

The MT-associated PIK activity correlated well with transformation potential that was only
observed in transformation-competent MT species (Whitman, Kaplan et al. 1985, Kaplan,
Whitman et al. 1986). The PIK activity is associated with a prominent phosphorylated
polypeptide of 85 kDa in size that immunoprecipitated with transformation-competent MT
molecules. The polypeptide was later found to be the regulatory subunit of PI3K (Kaplan,
Whitman et al. 1986, Courtneidge and Heber 1987). PI3K p85 was found phosphorylated
upon PDGF stimulation, which provided the first link between MT transformation and RTK
signalling. Therefore MT can be considered a permanently activated GFR (Dilworth 1995).
PI3K p85 contains two SH2 domains, which bind to specific phospho-tyrosine on activated
RTK (Escobedo, Kaplan et al. 1991, Hu, Margolis et al. 1992). Both SH2 domains can bind
to phosphorylated Y315 on MT, which has a consensus sequence pYMPM (Kaplan, Whitman
et al. 1986, Talmage, Freund et al. 1989). The N-terminal SH2 domain of p85 was used as a
prototype for establishing specificity of the SH2 domain and have contributed to the

PI3K is made up of an adaptor regulatory subunit p85 and a catalytic subunit p110 (Carpenter, Auger et al. 1993). Binding of p85 to MT recruits p110 to the MT complex at the plasma membrane, which generates an increase in PIP3 level. Proteins with pleckstrin homology (PH) domain bind to PIP3 on the inner leaflet of the plasma membrane. Amongst these are phosphoinositide-dependent protein kinase 1 (PDPK1) and the Ser/Thr Kinase AKT. PDPK1 is a known activator of AKT by phosphorylating AKT on Thr308. Activated AKT in turn phosphorylates downstream targets regulating cell growth and DNA replication. One of these is the transcription factor E2F that promotes G1/S phase transition during cell cycle. Activated AKT can also block apoptosis by phosphorylating and inactivating the apoptosis regulator, BAD, hence promoting cell survival in cells expressing MT. Also activated is Rho GTPase Rac1, a PH domain-containing small GTP-binding protein involved in actin dynamics that promotes membrane ruffle formation upon GFR or H-Ras activation (Ridley and Hall 1992, Ridley, Paterson et al. 1992, Urich, Senften et al. 1997). Dominant negative mutants of Rac can block transformation by MT in fibroblasts and endothelial cells (Urich, Senften et al. 1997, Connolly, Soga et al. 2000). Rac activation is also required for MT to activate c-fos (Chen, Freund et al. 1999).

It is not clear how PI3K is activated but there is evidence that the interaction between the p85 SH2 domains and phosphotyrosine can stimulate p110 PI3K activity (Carpenter, Auger et al. 1993). The PI3K regulatory subunit is encoded by two genes, resulting in two forms of p85, α and β. Both polypeptides can bind MT without any differences in MT function (Cohen, Liu et al. 1990, Cohen, Yoakim et al. 1990). PI3K catalytic subunit p110 has three isoforms, α, β and δ. Only p110α is involved in MT transformation (Utermark, Schaffhausen et al. 2007). The PIK3CA gene encoding p110α is frequently mutated in many
types of cancer (Samuels and Velculescu 2004) whereas the PIK3CB gene, encoding p110β, is only amplified in some tumour type (Fluck and Schaffhausen 2009). PI3K p110α plays an important role in RTK signalling, while loss of p110β has little impact on RTK activation (Fritsch and Downward 2013). Instead p110β is essential for G protein coupled receptor (GPCR) signalling (Guillermet-Guibert, Bjorklof et al. 2008). Studies on transgenic mice with a tissue specific deletion of p110α gene found that the loss of p110α alone was enough to block tumour growth induced by MT, indicating that PI3K binding is essential for MT transformation (Utermark, Schaffhausen et al. 2007). MT as a strong and selective activator of p110α has attracted attention from the drug industry, in the light of developing specific inhibitors for this PI3K, which is heavily implicated in cancer formation (communication with Tom Roberts, DNA Tumour Virus Meeting 2013).

1.10.4 Shc

Phosphorylation of Y250 is important for MT transformation (Hunter, Hutchinson et al. 1984). Mutation of Y250 and residues in the NPTY motif inhibited transformation without affecting binding to PP2A, Src and PI3K (Druker, Ling et al. 1990, Druker, Sibert et al. 1992). Dilworth and others later demonstrated that Y250 is a binding site for adaptor protein Shc (Campbell, Ogris et al. 1994, Dilworth, Brewster et al. 1994), an SH-domain containing transforming protein coupled to activated GFR and become phosphorylated, which provides binding sites to recruit other adaptor proteins to activate mitogenic signalling (Pelicci, Lanfrancone et al. 1992, Yokote, Mori et al. 1994). Three shc genes have been identified. ShcA is ubiquitously expressed in mammalian cells, while ShcB and ShcC expression appear limited to neuronal cells (Ravichandran 2001). Shc family proteins have three isoforms, p46, p52 and p66 (Pelicci, Lanfrancone et al. 1992). MT binds to all three of them, with p52 as the most prominent MT binding partner (Campbell, Ogris et al. 1994, Dilworth, Brewster et al.
She isoforms share common domain structure, an N-terminal PTB domain, a C-terminal SH2 domain and a central collagen homology 1 (CH1) domain (Schlessinger and Lemmon 2003). Both PTB and SH2 domains can bind to phospho-tyrosine. Dilworth and co-workers have demonstrated that both the p66 and p52 Shc associated with MT via SH2 domain and become phosphorylated, probably by pp60c-src (Dilworth, Brewster et al. 1994). Shc can be phosphorylated on at least 3 tyrosine residues, Y317, Y239 and Y240, all of which are phosphorylated in complex with MT. These phospho-tyrosine residues are located at the central CH1 domain, which interact with the SH2 domain of adapter protein Grb2, which is recruited to the MT signalling complex (Nicholson, Empereur et al. 2001). Association with Grb2 is important for MT transformation as it provides the means by which MT stimulates the MAPK pathway (Rozakis-Adcock, McGlade et al. 1992, Dilworth 2002). Although replacement of sequence surrounding Y250 on MT with that of Shc Y317 or Y239/Y240 allows direct binding between MT and Grb2, it requires the presence of both Shc tyrosine motifs on the same polypeptide in order to fully reconstitute the transforming potential of MT (Nicholson, Empereur et al. 2001). Therefore, association with ShcA is indispensable for MT to propagate mitogenic signals.

Grb2 bound to MT is phosphorylated on multiple sites and allows more signalling proteins to bind. With its SH3 domains, Grb2 can associate with Sos1 guanine nucleotide exchange factor as well as the docking molecule Gab1 and 2. Sos1 mainly associates with the N-terminal SH3 while the C-terminal SH3 domain of Grb2 interacts with Gab1, an insulin-receptor substrate-1–like docking protein (Nicholson, Empereur et al. 2001).

In MT transformed cells, Gab1 is constitutively tyrosine phosphorylated while it is barely detectable in normal non-stimulated cells (Ong, Dilworth et al. 2001). Gab1 associated with MT signalling complex is tyrosine phosphorylated, which provides binding sites for other SH2/PTB domain-containing proteins such as PI3K and SHP2, a protein tyrosine
phosphatase (Nicholson, Empereur et al. 2001, Ong, Dilworth et al. 2001). Loss of ShcA binding resulted in a marked decrease in MT-associated PI3K activity (Ong, Dilworth et al. 2001). Therefore MT can recruit PI3K directly or indirectly through ShcA-Grb2-Gab1 complex (Ong, Dilworth et al. 2001). A recent report has shown protein tyrosine phosphatase SHP2 can inhibit MT induced transformation by inhibiting Stat3 activity in endothelial cells (Yang, Jiang et al.). The presence of SHP2 in MT signalling complex also raises the question of whether this tyrosine phosphatase play a role in the removal of regulatory pY527 on C-terminal tail of c-Src thus priming the tyrosine kinase for MT binding (Yang, Jiang et al.).

1.10.5 PLC-γ1

Phospholipase C-γ1 (PLC-γ1) binds to phosphorylated Y322 on MT via an SH2 domains, which recognises the motif YLDI at the third major phosphor-tyrosine site (Y322) (Su, Liu et al. 1995). MT transfected cells show elevated level of tyrosine phosphorylated PLC-γ1. Activated PLC-γ1 catalyses the conversion of PIP2 to DAG and IP3. IP3 stimulates an increase of Ca²⁺ level inside the cell and subsequent calcium dependent signalling pathways. DAG activates PKC family of protein kinase. The role of PLC-γ1 in MT signalling is not yet clear. Loss of Y322 in MT has limited effect on MT transformation of adherent cell. However, in immune cells, PLC-γ1 association and activation alone is sufficient for the activation of IL-2 promoter and NEAT transcription factor, which is normally stimulated by T-cell receptor activation (Kennedy, Sekulic et al. 1998). In MMTV-MT breast cancer model, dominant negative PLC-γ1 expression reduced lung metastasis in mice (Shepard, Kassis et al. 2007). Therefore the contribution of PLC-γ1 activation to MT transformation is probably cell type dependent.
1.10.6 Regulatory Protein 14-3-3

Serine 257 is another major phosphorylation site on wild type MT and is a binding site for cytoplasmic regulatory protein 14-3-3 (Pallas, Fu et al. 1994, Cullere, Rose et al. 1998). However it is not clear which kinase phosphorylates this residue. Lack of 14-3-3 binding had little effect on MT transformation of cells in culture and only had tissue-specific reduction in MT-induced tumour formation in vivo (Cullere, Rose et al. 1998). S257C MT is defective in inducing salivary gland tumours when inoculated into new-born mice (Cullere, Rose et al. 1998). 14-3-3 family proteins are highly conserved ubiquitously expressed regulatory proteins that play a functional role in subcellular localisation of target proteins. They form homo- and hetero-dimeric cup-shape structures to bind to phospho-serine on target proteins, often transcription activators such as TAZ (transcriptional co-activation with PDZ-binding motif) and regulate their functions by sequestering in cytoplasm, often by masking the nuclear import/export sequence (Kanai, Marignani et al. 2000). Because each monomer in dimeric 14-3-3 can bind phospho-serine at the same time, it was thought to induce dimerisation of MT molecules. Using cell lines co-expressing mutant MT, Senften and co-workers showed that MT capable of membrane association and 14-3-3 binding can be co-immunoprecipitated. However, the ability to co-immunoprecipitated with one another is essential for MT transformation (Senften, Dilworth et al. 1997). MT mutant, dl8, lacking 14-3-3 binding site transforms fibroblasts with efficacy even greater than wild type and are known for forming unusually large colonies in soft agar (communication with Dilworth and Schaffhausen).
1.11 Proline –rich Region

Several reviews have noted a Proline-rich region from residue 332-347, C-terminal to PLC-γ1 binding region and N-terminal the TMD. This region contains three consecutive Proline residues surrounded by very hydrophobic amino acids (Fluck and Schaffhausen 2009, Schaffhausen and Roberts 2009). A deletion mutant, dl1015, in this region (339-347) is defective in transforming cells in culture despite its association with normal tyrosine kinase activity and binds to all known MT-associating proteins (Magnusson and Berg 1979, Magnusson, Nilsson et al. 1981) (unpublished data). It was suggested that signalling downstream of PI3K may have been affected by mutation in the Proline-rich region (Fluck and Schaffhausen 2009).

1.12 Additional Tyrosine Phosphorylation

Additional phospho-tyrosine sites (Y258, Y288 and Y297) have been identified in MT. MT-induced transcriptional activities were only completely lost when all six tyrosine sites were abolished. This suggests some unknown functions associating with these minor phospho-tyrosines, which are likely to overlap with the pathways from the three major phospho-tyrosine sites. All three relatively minor tyrosines are located between the Y250 Shc binding site and Y315 PI3K p85 binding site. It has been shown previously that this region on MT can be mutated without affecting transformation (Nicholson, Empereur et al. 2001, Ong, Dilworth et al. 2001).

Despite such long history of research on MT, not all MT associated proteins and downstream effector proteins have been found. Additional knowledge on how MT mediates oncogenic signalling will continue to benefit our understanding of normal and aberrant signalling events induced by growth factor activation.
1.13 Subcellular Localisation of MT

Early sucrose gradient fractionation experiment showed that [S\(^{35}\)] methionine-labelled MT and associated Src tyrosine kinase activity are mostly found in the plasma membrane. Some were observed in the ER fraction but little was associated with the mitochondrial or heavy membrane fractions or with the soluble proteins (Schaffhausen 1982). Using immuno-electron microscopy method, it was shown that a fraction of the MT was found in the plasma membrane with majority of the protein found in ER membranes (Dilworth, Hansson et al. 1986). These contradictory observations may be attributed to the use of virally infected cells in the later experiment, which is more representative of the location of newly synthesised MT. Therefore, the ER probably represents the location where MT was first synthesised. Migration of newly synthesised MT to the plasma membrane is a slow process. In virally infected fibroblasts, MT was observed in the ER as early 10 hrs post-infection. After 20 hrs, large amount of MT was found in the ER of most cells. Only after 30 – 40 hrs after infection, can the viral protein reach the plasma membrane in high amount (Dilworth, Hansson et al. 1986). It is still not clear whether MT-induced transformation signals are generated from the plasma membrane, like the classical model of growth factor signalling (Hoeller, Volarevic et al. 2005), or intracellular membrane compartments, such as the strategy used by bovine papilloma virus (BPV) oncoprotein E5 (Sparkowski, Anders et al. 1995). BPV E5 is another viral oncoprotein that binds to membrane and transforms human and rodent fibroblasts in culture (DiMaio and Mattoon 2001). It exists as a homodimer via a disulphide linkage between two Cysteine residues on the short C-terminal tail orientated towards the Golgi lumen and this dimer formation is important for transformation (Burkhardt, DiMaio et al. 1987, Zhang, Lewis et al. 1987). BPV E5 is localised to the ER and Golgi membranes, where it binds mainly to the immature form of the PDGFR\(\beta\) and promotes ligand-independent
activation of the receptor (Goldstein, et al., 1992a, Goldstein, Kulke et al. 1992, Sparkowski, Anders et al. 1995). Mutants of E5 that localise in the ER can interact with the immature PDGFRβ and induce autophosphorylation but this does not cause transformation. The transformation signals induced by BPV E5 therefore are generated in the Golgi apparatus (Sparkowski, Anders et al. 1995).

In normal cells, the classical model of growth factor signalling involves the binding of ligand to growth factor receptor monomers, dimers or multimers on the surface of the plasma membrane, which induces a conformational change of the intracellular domain of the receptor that leads to tyrosine kinase activation either on the RTK or through the recruitment of protein tyrosine kinases. This in turn leads to the recruitment of effector proteins to the activated receptor complexes at the plasma membrane. This assembly of multi-component active signalling complexes, termed ‘signalosomes,’ in the plasma membrane is thought to be where downstream signalling cascades are initiated (Hoeller, Volarevic et al. 2005). Activation of growth factor signalling is a tightly regulated and often transient. Growth factor receptors such as EGFR1 and PDGFRβ are rapidly internalised following their ligand-stimulated activation. The internalised receptors and ligand may be degraded by trafficking to lysosomes or recycled back to the cell surface after dissociation and degradation of the ligands via a subset of intracellular vesicles. Receptors can be internalised through clathrin-coated pits into EEA-1 positive endosomes, or caveolae, which are clathrin-independent membrane invaginations formed by caveolin-1 coated membrane domains. Receptors may continue to signal in endosomal compartments. Some activated EGFR are internalised into early endosomes carrying APPL-1, an effector of GTPase Rab5. The EGF stimulated APPL-1 subsequently translocate to the nuclei where it regulates cell proliferation (Miaczynska, Christoforidis et al. 2004). Other cell surface receptors such as transferrin receptor do not enter APPL-positive endosomes (Miaczynska, Christoforidis et al. 2004). There is some
evidence to suggest that receptors such as EGFR and PDGFRβ located in the endosomes can activate MAPK and AKT signalling pathways albeit at much lower magnitude compared to those at the cell surface (Wang, Pennock et al. 2002, Wang, Pennock et al. 2004). MT is located in most intracellular membranes except the mitochondria. It is not clear whether MT molecules in the intracellular membrane compartments are actively generating mitogenic signals.

### 1.14 Cytoskeletons and Focal Adhesion Complexes

Cells expressing MT appear transformed, which may be a result of increased microtubule instability and diminished actin stress fibres (da Costa, Wang et al. 2000). Cytoskeleton re-organisation is an important process for cell adhesion and migration. It is not clear whether MT directly or indirectly influences cytoskeletal structures. Some reports suggest that MT may associate with actin or actin binding proteins directly. Early antibodies raised against the MT sequence surrounding the PI3K p85binding site have been found to immune-label microfilaments in untransformed cells similar to those labelled with anti-actin antibodies (Sugawara, Fujinaga et al. 1983). In a later study, phosphorylated MT molecules were immunoprecipitated by anti-vinculin serum (Ballmer-Hofer, Ziegler et al. 1988). Vinculin is an actin-binding protein, which mostly localises to focal contacts. These are sites of focal adhesion which anchors the cell basal membrane to the extracellular matrix. Focal adhesion is mediated by integrin binding to fibronectin in the extracellular matrix (ECM) or other integrin molecules on the surface of adjacent cells, which then activates distinct integrin-associated signalling molecules, such as integrin-linked kinase (ILK), focal adhesion kinase (FAK) and c-Src. Integrin also connects to the actin cytoskeleton via a wide range of actin-binding proteins including vinculin and affects cell movement by re-modelling the actin cytoskeleton. It has been suggested that MT/pp60c-src complexes may be bound to vinculin
at focal contacts, as was found with v-Src, which may contribute to the disorganisation of actin filament network required for transformation morphology (Ballmer-Hofer, Ziegler et al. 1988). Normal migration in polarised cells involves regulated actin cytoskeleton rearrangement with coordinated focal adhesion assembly and disassembly. Actin polymerisation into actin bundles or stress fibres forms protrusions at the leading edge of the cell. Focal adhesion complex assembly is important for the cell to lay down new processes in the direction of migration. Disassembly of focal adhesion complexes and actin stress fibres at the rear of the cell are required to release the bulk of the cell for it to move forward (reviewed by Wehrle-Haller 2012).

1.14.1 Focal Adhesion Kinase

In cancer cells, many of the components involved in cell migration and adhesion are deregulated, which may lead to enhanced migration potential and metastasis (Wehrle-Haller 2012, Wehrle-Haller 2012). One of these components is FAK, a protein tyrosine kinase localised to focal adhesion sites. Upon integrin complexes engagement on the ECM, FAK is recruited to the integrin cluster and is phosphorylated. Its activation plays an important step in initiating cell migration. FAK is highly phosphorylated in v-src transformed chicken fibroblasts and rat fibroblast transformed by MT (Kanner, Reynolds et al. 1990, Guan and Shalloway 1992, Bachelot, Rameh et al. 1996). Similar to Src family tyrosine kinases, FAK activity is stimulated by the removal of an auto-regulatory domain from the central kinase region and the phosphorylation of a tyrosine residue on the activation loop. Unlike Src, FAK lacks SH2 and SH3 domains and does not possess a myristoylation site. The focal adhesion targeting (FAT) domain at the C-terminus is responsible for the localisation of FAK to focal adhesion plaques probably through interaction with actin-linker proteins such as talin and paxillin (reviewed by Zhao and Guan 2009). The N-terminal region of FAK contains a
FERM domain that binds to the cytoplasmic domain of integrin β or other activators. Upon engagement with integrin, FAK is phosphorylated on Y576 within the activation loop of its kinase domain. Once activated, FAK auto-phosphorylates on Y397, which serves as a binding site for the SH2 domain of activated pp60c-src and pp59c-fyn non-receptor tyrosine kinases (Cobb, Schaller et al. 1994, Schlaepfer and Hunter 1996), which can then phosphorylate FAK on multiple sites on the FAK C-terminus to create additional binding sites for signalling molecules such as Grb2 and the PI3K p85 subunit (Schlaepfer, Hanks et al. 1994, Bachelot, Rameh et al. 1996). Src is also activated by binding to FAK phospho-Y397 as a result of displacing the auto-inhibitory Y527 of Src from its kinase domain. Therefore, FAK and Src tyrosine kinases can be mutually activated to propagate a wide range of signalling events. In addition to integrin, FAK has been found to interact with activated PDGF, EGF and HGF receptors via its N-terminal FERM domain. FAK association with these growth factor receptors is important for ligand stimulated cell migration (Sieg, Hauck et al. 2000). Therefore FAK mediates cross talk between integrin and growth factor receptor signalling.

Given the similarity between MT mitogenic signalling and growth factor activation, there are likely to be many ways by which FAK can be recruited to the MT signalling complex. However, direct interaction between MT and FAK has not been reported. Also, the role of FAK in normal and oncogenic signalling is unclear. Over-expression, deletion or deregulation of FAK catalytic activity did not affect normal cell growth or morphology (Hildebrand, Schaller et al. 1993). However, deletion of FAK inhibits tumorigenesis in MMTV- MT, -Ras and Neu mouse model by induces apoptosis in tumour cells (Pylayeva, Gillen et al. 2009).
1.14.2 Vinculin

Vinculin is a membrane associated cytoskeletal protein found in focal adhesion plaques, which are large focal adhesion complexes at the cell periphery. It plays an important role in linking integrin adhesion molecules to the actin cytoskeleton. The association of receptors with the actin cytoskeleton is often mediated by cytoskeletal proteins, such as talin, vinculin, paxillin and α-actinin. Using a GFP fusion with the PI3K subunit p85, Gullick and co-workers showed that EGF-ligand stimulation redistributed PI3K to the peripheral cell membrane, where it colocalises with vinculin (Gillham, Golding et al. 1999). Vinculin plays an important role in regulating migration, transmitting mechanical signals and establishing cell polarity (Carisey, Tsang et al. 2013). Vinculin consists of head and tail domains separated by a flexible Proline-rich neck region. In the inactive form, the head and tail domain bind to each other by intramolecular interaction, forming a globular structure, which prevents vinculin from binding to the cytoskeleton and to receptors (Johnson and Craig 1994). Stable integrin complex formation at the sites of focal adhesion is often accompanied by a high concentration of talin and vinculin at the plasma membrane (Burridge, Molony et al. 1987). Talin contains both a FERM domain for interaction with the integrin β cytoplasmic tail and a vinculin binding site. Upon recruitment to the integrin-talin complex at focal adhesions, vinculin adopts an open, rod-like conformation that allows access to its binding partners. Other examples of vinculin binding proteins include α-actinin that binds to the head domain; Arp2/3, an actin nucleation factor, that binds to the neck region; filamentous or F-actin, paxillin and PIP2 that bind to the tail domain (Humphries, Wang et al. 2007, Carisey, Tsang et al. 2013).
1.14.3 Integrin

Adhesion molecules such as integrin have been heavily implicated in tumour initiation and metastasis. Integrins are integral transmembrane proteins complexes consisting of heterodimeric α and β subunits. The long extracellular domain of integrin binds to components of extracellular matrix (ECM). The short cytoplasmic tails of integrin serves as a platform for the recruitment of protein tyrosine kinases including FAK and Src, and actin binding proteins. In mammalian cells there are 8 isoforms of each subunit. Antibody – induced integrin clustering and adhesion to fibronectin coincides with FAK activation and its recruitment to focal adhesion plaques (Kornberg, Earp et al. 1992). Integrin β1 - FAK signalling has been shown to activate cyclin-dependent kinases and is implicated in the proliferation of mammary epithelial cells (Li, Zhang et al. 2005). Integrin β1 has been shown to be required for mammary gland tumorigenesis in MMTV-MT mice but not the MMTV-Neu strain, although β1 integrin is required for metastasis at a later stage of MMTV-Neu induced mammary gland carcinoma (White, Kurpios et al. 2004, Huck, Pontier et al. 2010). Ablation of β1 integrin limits the proliferative capacity of cells derived from established tumours driven by MT (White, Kurpios et al. 2004).

1.15 Lipid Rafts

Cellular membranes can be divided into regions or domains with distinctive features, according to their lipid composition or resident proteins, which differentiate them from the contiguous membrane (Anderson and Jacobson 2002). Membrane regions enriched with bulky lipids such as cholesterol, which increase the rigidity of phospholipid bilayer, serve to attract and accommodate integral membrane proteins and proteins with lipid modifications. Lipid rafts is a term that is generally used to describe these micro-domains that are rich in
glycosphingolipids, sphingomyelin, or glycoproteins. They are detergent insoluble and their functions are dependent on cholesterol (Carter and Hakomori 1981, Chang, Rothberg et al. 1992).

Some membrane proteins are targeted to cholesterol-rich lipid rafts. Several mechanisms have been proposed, namely lipid modification especially acylation, palmitoylation, and the interaction between TMD sequence and membrane lipids (Scheiffele, Roth et al. 1997, Melkonian, Ostermeyer et al. 1999); (Levental, Lingwood et al. 2010). Interestingly, a lipid modification sequence, CAAX box at the C-terminus of H-Ras can functionally replace the C-terminus of MT (Elliott, Jones et al. 1998), which seems to suggest a common specificity to MT and H-Ras membrane binding. Ras GTPases have been widely used as a model to study cluster formation on the inner leaflet of the plasma membrane. H-Ras and K-Ras have been shown to form distinct separate nano-clusters, mediate by their C-terminal lipid modifications. Wild type H-Ras normally resides in both lipid rafts and cholesterol-independent microdomains in an equilibrium (Prior, Muncke et al. 2003). But nano-clusters of activated H-Ras, when it is GTP-bound, are cholesterol-independent therefore it was thought that the activated H-Ras has lower affinity for lipid rafts (Yang, Nickerson et al. 2012). H-Ras is targeted to the membrane by the C-terminal CAAX box. The Cysteine in the CAAX box is farnesylated. Farnesylpyrophosphate is a lipid intermediate in the cholesterol biosynthesis pathway. The final three residues, –AAX, is removed by proteolytic cleavage and the C-terminal Cysteine can then be methylated (Hancock, Magee et al. 1989). There are two additional Cysteine residues immediately upstream of the CAAX box, which can be palmitoylated and this targets H-Ras to cholesterol–dependent lipid rafts (Hancock, Magee et al. 1989). K-Ras, which lacks palmitoylation sites, is localised at cholesterol-independent non-raft micro-domains. Palmitoylation can be reverted by
thioesterases, and the state of palmitoylation of H-Ras influences its location to cholesterol-dependent or cholesterol-independent microdomains (Yang, Nickerson et al. 2012).

Other examples of well defined raft-like domains in the plasma membrane include clathrin or caveolin-1 coated membrane micro-domains involved in endocytosis. Caveolae is a well-studied raft structure. These are flask-shaped membrane structures lined with caveolin-1 on the inner leaflet of the plasma membrane. They sequester both small or macro-complexes of cell surface receptors and internalise them upon ligand binding, a process that differs from clathrin-dependent endocytosis and is sometimes described as potocytosis (Mineo and Anderson 2001). It has been proposed that caveolae rafts measuring 50 to 200 nm in size can aggregate to form even larger lipid domains that function to segregate membrane proteins during cell signalling and endocytosis (Anderson and Jacobson 2002).

The bulky hydrophobic residues of the TMD of membrane proteins that are located near the outer leaflet of the lipid bilayer could shape the TMD structure in a way to accommodate cholesterol in the microdomain (Scheiffele, Roth et al. 1997). The hydrophobic sequence near the C-terminus of MT is enriched with residues with bulky side chains (ICLMLFILI) but it is not known whether MT activated partition into cholesterol-rich microdomains (see Figure 1.3 below for TMD sequence and supplementary figure S2.1 for complete MT protein sequence).

1.16 Membrane Localisation Region

Membrane localisation of MT was first reported by Ito et al (Ito, Brocklehurst et al. 1977). Association with the cell membrane is absolutely required for MT to induce cellular transformation (Novak and Griffin 1981, Carmichael, Schaffhausen et al. 1982). MT with a truncated C-terminal region was unable to transform (Carmichael, Schaffhausen et al. 1982).
Membrane binding requires a 22 residue stretch of hydrophobic amino acids located near to
the MT C-terminus (Soeda, Arrand et al. 1979) (see underlined sequence in Figure 1.3 or
supplementary figure S2.1 for complete MT protein sequence). The C-terminal membrane
anchoring sequence is flanked on each side by positively charged amino acids, see Figure 1.3.
The positively charged Arginine residues are important for MT transformation (Dahl,
Thathamangalam et al. 1992). Glutamate substitution of the Arginine immediate N-terminal
to the hydrophobic sequence abolished MT transformation potential (Dahl, Thathamangalam
et al. 1992). However, it is not clear why the Arginine-rich sequence is important, although
interaction with the cytoskeleton has been suggested. The basic residues C-terminal to the
hydrophobic sequence can be deleted without affecting transformation (Dahl,
Thathamangalam et al. 1992). However, some past mutants generated in our lab with
mutations made to the hexapeptide have suggested otherwise (unpublished observation).

AHSM QRHLRRLGRTLLLVTFLAALLGICLMFLIKRSRHF C-terminus

Figure 1.3 Transmembrane domain sequence of MT
Above sequence represents residue 381 to 421 on the C-terminus of MT. The hydrophobic sequence is
underlined and is followed by a hexapeptide. Basic amino acids surrounding the hydrophobic sequence
are in bold.

Despite a number of attempts, MT was undetectable on the outside of cells, so it was
proposed that it is probably situated on the cytoplasmic face of the membrane since it was not
degraded by trypsin in intact cells and was only phosphorylated when cells were made
permeable before in vitro $\gamma^{32}$P ATP labelling (Schaffhausen, Dorai et al. 1982). It is not
clear how the MT C-terminus is inserted into the membrane. Some proteins spontaneously
insert into the membrane, for example, cytochrome $b_5$. Using isolated microsomes, Kim et
al., have demonstrated that MT TMD does not insert into the membrane spontaneously.
Therefore, MT probably requires an active insertion mechanism. Those TMDs that passively/
spontaneously insert into the membrane tend to form hair-pin structures in the membrane (Kim, Janiak-Spens et al. 1997). Therefore, the C-terminus of MT probably spans the lipid bilayer, however, this is not proven for MT. Efforts to generate monoclonal antibodies targeting the MT C-terminus have not succeeded so far (unpublished data).

MT with a truncated C-terminus is found mostly in the soluble fraction (Carmichael, Schaffhausen et al. 1982). Although this mutant can still bind to PP2A core dimers, it fails to associate with any of the c-Src tyrosine kinases. However, it was later found that MT molecules that fail to associate with membranes were relocated to the nucleus, probably through interaction with PP2A, as a double mutant lacking both PP2A binding region and the C-terminus showed a cytoplasmic distribution (Glover, Brewster et al. 1999). Therefore, although MT does not seem to be targeted to a particular intracellular compartment, the ability to bind PP2A seems to influence MT localisation. Further work is needed to investigate the requirement for membrane targeting mechanism and the role of the MT hydrophobic region.

MT is located in most cell membranes (Dilworth, Hansson et al. 1986), so the hydrophobic region presumably does not seem to target one specific membrane compartment site. However, this region must do more than specify general membrane association, as hydrophobic domain mutants have been isolated that still bind to membranes but fail to transform (Markland, Oostra et al. 1986). Similarly, replacement with the transmembrane region from vesicular stomatitis virus glycoprotein G (Templeton, Voronova et al. 1984)or cytochrome b$_5$ (Kim, Janiak-Spens et al. 1997), resulted in transformation defective mutants that still associate with the membrane. So far, only the lipid modified CAAX sequence at the C-terminus of H-Ras can functionally replace the C-terminus of MT (Elliott, Jones et al. 1998). CAAX box is responsible for targeting H-Ras to cholesterol-dependent lipid rafts (see
1.14 above). Consequently, it is likely that the hydrophobic domain in MT does more than target generalised membrane location, but what this extra activity consists of is unknown.

The similarity between MT and activated tyrosine-kinase-associated growth factor receptors are striking. Both are membrane bound. When phosphorylated on multiple tyrosine residues both GFR and MT act as docking sites for the recruitment and activation of cellular signalling proteins such as PI3K and PLC-γ1 as well as bringing adaptor protein SHC to the complex to form a scaffolding for signal propagation. However, many GFRs such as the EGFR family and PDGFR family growth factor receptors dimerise upon ligand binding to the extracellular domains. It is not yet established whether MT TMD sequence mediates dimerisation in the way of RTK activation and whether dimerisation is required for MT transformation. However, it has been shown that dimer formation between MT molecules requires association with 14-3-3, but mutants lacking 14-3-3 binding site transformed cell just as well if not better (Senften, Dilworth et al. 1997). Some RTKs such as the EGFR family receptor kinases are well known for their ability to homo- or hetero-dimerise, through the interaction of their transmembrane α-helices. It is not known whether MT TMD can interact with other transmembrane proteins, although it has been recently reported that MT immunoprecipitates with insulin receptor and IGF-I receptors in tumours derived from MMTV-MT mouse models and this association seems to be required for the formation of new tumours (Novosyadlyy, Vijayakumar et al. 2009).
1.17 Aims:

The aim of this project is to study the role of the TMD sequence that is specific for MT function. In particular:

i) to clarify the topology of MT C-terminus and how the MT hydrophobic sequence is inserted into the membrane;

ii) to investigate in which compartment of the cell that transformation signals are generated;

iii) with the aid of site directed mutagenesis, to identify any additional property encoded by the TMD that is important for MT transformation;

iv) to relate our results to growth factor receptor signalling.
CHAPTER 2 - MATERIAL AND METHODS

2.1 Cell Culture

Rat2 rat fibroblast cell line was maintained in Dulbecco’s Modified Eagles Medium (DMEM), high glucose (Invitrogen 41965, + D-glucose, + L-glutamine, - pyruvate) supplemented with 0.29 mg/ml L-glutamine, 100 units of penicillin, 100 µg streptomycin (from Gibco® 100x Penicillin-Streptomycin-Glutamine stock and 10% foetal bovine serum (FBS), and incubated at 37°C and 10% CO₂.

To freeze cells for long term storage, trypsinised cells were collected to the bottom of 15ml Falcon tube by centrifugation then re-suspended in DMEM with 20% FBS, + 10% dimethylsulfoxide (DMSO) (Sigma).

Inhibitors used in this project can be found in supplementary Table ST 2.4.

2.2 Plasmids

All mutations were made to the pUC MT plasmid, consisting of vector pUC19 containing the BamHI–EcoRI fragment of polyomavirus lacking the MT intron (Nicholson, Empereur et al. 2001).

_In vitro_ transcription requires T7 promoter. Wild type MT or MTKDEL cDNA were cloned into pcDNA3.1 from BlpI to EcoRI site after excision from pUC MT plasmids.

pYX containing mouse ASNA1 cDNA cloned into NotI to EcoRI restriction sites (pYX-Asc) was obtained through Open Biosystems (clone ID:5717763). Plasmid contains both T3 and T7 promoters.

N-terminal 2XMYC-tagged human Ras family small GTP binding protein H-Ras G12V (constitutively active) cloned into pcDNA3.1+ at KpnI (5’) and XhoI (3’) was obtained from UMR cDNA Resource Centre, University of Missouri-Rolla. The constitutively activated mutant H-Ras (G12V) with Myc–tag is expressed under CMV promoter.
2.3 Mutant Generation

To construct wild-type MT with a FLAG tag, a forward primer (HA for), which is an exact match to the MT cDNA sequence upstream of the hydrophobic domain-encoding region, was used together with a reverse primer that contained an EcoRI site and the sequence encoding the FLAG tag (DYKDDDDK), and a priming sequence that matches MT C-terminus coding sequence. PCR reaction was performed with 4ng pUC MT and 20ng appropriate forward and reverse oligos using Phusion polymerase (NEB) according to manufacturer’s conditions.

PCR conditions: step 1: 94°C for 180 sec; step 2: 94°C for 45 sec; step 3: 57°C for 60 sec; step 4: 72°C for 120 sec; step 5: 25 cycles from step 2. The reaction was finished off by incubation at 72°C for 600 sec.

The PCR products and pUC19MT plasmids were digested with restriction enzymes BplI and EcoRI (NEB) in Buffer 4. Digested fragments were purified by 1% Agarose gel electrophoresis followed by Qiagen Gel Extraction kit. The purified fragments of PCR products and large fragment of pUC19 MT double-digest products were combined and ligation was performed at room temperate (RT) with Quick Ligase (NEB). The resulting plasmids were transformed into 50 µl chemically competent DH5α E.coli in LB broth by incubation on ice for 40 min before heat-shock at 42°C for 60 sec, followed by 5 min incubation on ice. 250 µl LB media without ampicillin and incubated at 37°C with shaking for 2 hrs. 100 µl of the transformed bacteria were spread and grown over night on LB-agar containing ampicillin. Plasmid DNA was purified using QIAprep Spin Miniprep Kit for clonal selection and Qiagen HiSpeed Mini prep for subsequent large scale purification. ∆416-421 MT and 416G6 MTHA+ were generated using a similar method (see supplementary table ST2.1–2.3 for primer sequences).

All mutants were verified as correct by DNA sequencing before use. Sequencing data were obtained using Imperial College MRC CSC Genomics Core Laboratory service. DNA sequences were analysed by EditSeq DNASTAR software. The complete protein sequence of MT can be found in supplementary figure S2.1.

2.4 Agarose Gel Electrophoresis

50 ml of Agarose was prepared by dissolving 0.5 g agarose powder in 50 ml 1x Tris Borate EDTA (TBE). 5 µl of nucleic acid stain (SafeView) was added to the 0.9 % agarose solution
highlight the DNA. The agarose solution was poured into a gel tank with a gel comb and the gel was left to set. 1x TBE buffer was poured to cover the gel after setting. 0.5 - 2 µg of DNA sample was added to 6x DNA loading buffer (NEB, B7021S). The gel was run at 50V and 50mA for 30 min or until the bromophenol blue dye run to the middle of the gel. The gel was removed and place on top of a UV light box to visualise the DNA. The band containing PCR products was excised into a clean, pre-weighed 1.5 ml eppendorf tube using a clean scalpel. DNA was extracted by Qiagen Gel Extraction kit according to manufacturer’s protocol.

2.5 Ethanol Precipitation of DNA

After confirming plasmid sequence for cloned MT sequence, scale-up productions of appropriate plasmids was carried out using HiSpeed Plasmid Midi kit (Qiagen). The purified plasmids were then concentrated to 1 µg/µl. Concentrations of plasmid was measured by NanoDrop ND-1000 Spectrophotometer, UV absorbance wavelength = 260 nm. 1 volume of purified plasmids were mixed with 1/10 volume of 3 M sodium acetate pH 6.5, followed by 2 x volumes of 100% ethanol, then stored at – 20°C overnight. The precipitated DNA was centrifuged at 13,000x g for 10 min. Supernatant was discarded and 0.5 ml of 70% ethanol was added to remove residual salts. After 5 min incubation at RT, the mixture was centrifuged at 13,000x g for 5 min. Supernatant was removed and the pellet of DNA was allowed to air-dry at RT. The dried DNA was re-suspended in sterile 10 mM Tris-HCl pH7.4 to a final concentration of 1 µg/µl. Concentrations of plasmids were re-assessed by NanoDrop.

2.6 Foci Formation Assay and Stable Cell Line Isolation

Sub-confluent Rat2 fibroblasts grown in 10 cm diameter (Φ) tissue culture dish were incubated in 5% FBS culture media for 6 hours prior to transfection. Cells were transfected with 10 µg plasmids containing MT or mutant DNA overnight using calcium-phosphate method. Plasmids were mixed in 125 mM CaCl2, 0.7 mM Na2HPO4, 0.7 mM NaH2PO4, 1x (w/v) Heps buffer pH7.1, with aeration then incubated at RT for 15 min, which were then added to the cells. The following day, media was changed to 10% FBS tissue culture media. Cells were cultured in the same dish and fed every 3 - 4 days for 14 days. Foci were stained
by incubation with Leishmann’s staining solution (VWR-BDH Chemicals, Leichmann’s Eosin Methylene Blue Solution in methanol) for 5 minutes at RT, followed by extensive washing with distilled water.

To generate stable cell lines expression MT or its mutant, sub-confluent Rat2 fibroblasts in 60 mm (Φ) dishes were transfected with 5µg plasmid DNA plus 0.5µg pSV2neo plasmid using above method. pSV2neo contains the neomycin resistance gene under the control of the SV40 early promoter. MTFLAG expression Rat-2 fibroblast cell line 2.11 was transfected with MTHA expression plasmid plus puromycin-resistance plasmids. After transfection, cells were selected by addition of 750 µg/ml G418 (Calbiochem) for 14 days. Individual colonies were picked and subcloned. Clones were evaluated for MT or mutant content by WB and IF.

2.7 Attractene Transfection and Transient Expression

In order to obtain higher copy number/ higher expression level, cells were transfected with Attractene Transfection Reagent (Qiagen). The day before transfection, cells were seeded on to 24-well plate to achieve 80% confluency on the day of transfection. 0.4 µg DNA was diluted with DMEM without serum, antibiotics or additional L-glutamate to a total volume of 60 µl. 1.5 µl Attractene Transfection Reagent was added to the diluted DNA and incubated at RT for 15 min to allow complex formation. During DNA-lipid complex formation, cells were fluid changed to 500 µl fresh DMEM supplemented with 10% FCS, antibiotics and glutamate. The transfection complexes were added drop-wise onto the cells, followed by incubation at 37°C and 10% CO₂ for 6 – 8 hrs. Media containing transfection complexes were removed and cells were allowed to grow in normal 10% FCS-DMEM media. The day after transfection, or 24 hrs post-transfection, cells were trypsinised and seeded on to glass coverslips for IF staining on the next day, 48 hrs post-transfection.

2.8 Cell Lysis, SDS-PAGE and Western Blotting

Confluent cells grown in a 10 cm petri dish were washed twice with cold PBS the lysed in 1 ml ice cold lysis buffer: 100mM TrisCl, pH8.3, 100mM NaCl, 0.5% Nonidet P40 (Roche), containing complete EDTA-free protease inhibitor cocktail tablet (Roche). After incubation for 20 min on ice, the supernatant was removed and centrifuged at 15,000xg before the final
supernatant was taken and frozen at -80°C. To analyse for MT content, lysate was mixed at 2:3 dilution with Laemmli SDS-sample buffer (Laemmli. Nature, 1970) then was heated to 90°C for 5 min. When analysing for GFR expression, cells were lysed in above lysis buffer containing 1% Triton-X100 and proteins samples were prepared without boiling.

Polypeptides were separated by electrophoresis on a 10% polyacrylamide gel containing SDS, then transferred onto 0.2µm pore size nitrocellulose (GE Healthcare) by Western blotting. The membrane was blocked by addition of 5% powdered skimmed milk in PBS and incubation for 30 min at room temperature. Membranes were sometimes stained with Ponceau S to visualise protein bands (Sigma Adrich P3504, dissolve 0.2 g in 5% acetic acid) before blocking in milk. After washing with 1xTBS-T the membrane was incubated for 60 min at room temperature with the appropriate dilution of primary antibody in 5% milk in TBS-T. After washing again for 30 minutes with frequent buffer changes, antibody binding was detected by incubation with a 1:5000 dilution of either anti-mouse, or anti-rabbit IgG coupled to HRP (DAKO) for 60 min at room temperature. After a further 30 minutes of washes, the proteins were revealed by incubation with Immobilon Western Chemiluminescent substrate (Millipore) and exposure to X-ray film.

When necessary, protein concentrations were analysed using Pierce BCA Protein Assay (Thermo Scientific #23227) according to manufacturer’s protocol. Serial dilutions of decreasing concentrations of BSA were made from 2 mg/ml stock solution provided with the kit. Protein samples were prepared in 96-well plate at 10 µl per well. Reagents added to each well containing protein samples or BSA standards changed colour after 30 min incubation at 37°C. Absorbance at 562 nm (λ) was measured using OPTImax microplate reader.

2.9 Co-immunoprecipitation (IP) and in vitro Kinase Assay

Confluent plates of cells grown in 100-mm petri dishes were lysed in 1 ml per plate lysis buffer containing 0.5 % Nonidet P-40 as described above. 50 µl samples of lysates were analysed for MT content as described before while the rest of the lysates were kept at -80°C. A volume of lysate corrected for equal amounts of MT was thawed and incubated with 3 µg PAb762 for 60 min on ice. 30µl of (50% w/w) Protein A-Sepharose CL-4B (GE Healthcare) in TBS-N buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8.3, 0.05% Nonidet P-40) was added and incubated on ice for 30 min with constant mixing. The Sepharose beads were collected to the bottom of the 1.5 ml eppendorf by very brief centrifugation at 4,800 x g and then washed
4 times by re-suspension in TBS-N buffer. Following removal of the last wash supernatant, proteins were eluted from the Protein A-Sepharose by incubation with 30 µl of SDS sample buffer at RT for 10 min, and then centrifuged at 13,000 x g for 5 min. The protein samples were removed from the Sepharose and then analysed as described above. The same membrane was often used for the detection of multiple MT-binding proteins in the order of pp62c-yes, pp60c-src, ShcA, PI3K p85, PLC-γ1, PP2A A and C subunit. In between different antibody detection, the membrane was washed extensively for at least three times 0.5 hr in TBS-T.

For In vitro protein kinase assays, MT was immunoprecipitated as described above until the last wash of the Protein A-Sepharose, which was collected to the bottom of the eppendorf tube. The Sepharose beads with immunoprecipitated MT and Src kinase protein complex were incubated with 50 µl of 25 mM Tris-Cl, pH 7.4, 5 mM manganese acetate, and 2µ Ci of [γ-32P] ATP for 30 min at 30˚C. The Sepharose was then washed twice with TBS-N. Proteins were eluded and separated by SDS-FAGE as described. After separation, the gel was fixed with 40% methanol and 10% acetic acid for 60min, then dried and autoradiographed using X-ray film.

2.10 Antibodies

2.10.1 In-house Antibodies:

PAb762, a mouse monoclonal directed against the N-terminus of MT; biotinylated anti-MT monoclonal PAb762 . F2.5G4, anti-PP2A PR35 (subunit C); G3 8F11, anti-PP2A PR65 (subunit A); rabbit anti-ASNA1 antibody was a kind gift from Dr Matthias Seedorf from Heidelberg University.

2.10.2 Commercial Antibodies:

Rabbit monoclonal IgG anti-Src (32G6); (Cell Signaling Technology, #2123); rabbit polyclonal anti-Shc (BD Signal Transduction Lab, S14630); rabbit PI3K p85 (Upstate Biotechnology, 06-497); mouse monoclonal anti-PLC-γ1, clone 10 IgG1 (BD Biosciences, 610027); rabbit monoclonal anti-Yes1 [EPR3173] (Abcam, ab109265); rabbit anti-phospho-FAK (Y397), clone D20B1 (Cell Signaling Technology #8556); mouse monoclonal [P1F6]
anti-integrin alpha V+beta 5 (Abcam, ab24694); rabbit anti-phosphorylated tyrosine residues (Y317 and Y239) of ShcA (Upstate Biotechnology #07-206 for pY317 and #07-209 for pY239); mouse monoclonal IgG1 anti-HA epitope HA.11 (clone 16B12) (Covance MMS-101P); rabbit anti-FLAG polyclonal antibody (Cell Signaling Technology #2368).

2.11 In Vitro Translation

Vector pcDNA3.1 containing wild type or mutant MT cDNA were used for in vitro translation (with or without pYX-Asc plasmid) using TNT® T7 Quick Coupled Transcription/Translation System (Promega, #L1170). 2 µl plasmid DNA were added to 40 µl TNT T7 Quick Master Mix (formulated rabbit reticulocyte lysate), together with 1 µl of 1 mM methionine, and 7 µl of nuclease-free water to give a final volume of 50 µl. For MT and ASNA1 co-translation, 2µl of each plasmid were used with adjusted water volume. The mixture was then incubated at 30˚C for 90 min. 1 µl of the newly translated MT was added to SDS sample buffer for Western Blot analysis of MT content. PAb762 was added to the rest of the reticulocyte lysate immunoprecipitate MT as described in before. The immunoprecipitates were analysed for ASNA1 by Western Blotting.

2.12 Detergent Resistance Membrane Fractionation

Confluent cells on 10 cm tissue culture dishes were trypsinised and centrifuged at 3000 g for 5 min at RT. The pellet of cells was then washed in cold PBS (4˚C) and centrifuged again at 4˚C. Cells were then re-suspended in 500 µl of 20 mM Tris-HCl pH7.0 with protease inhibitors (Roche). The suspension of cells was then passed through a 23G needle for at least 10 times on ice to break open the cells. A sample of 50 µl was taken as whole cell lysate (WCL). The broken cells were centrifuged at 45000 g at 4˚C for 1 hr with Beckman’s ultracentrifuge. The supernatant was kept as a sample for non-membrane fraction (NMF). The pellet was the re-suspended in 500 µl cold PBS with above protease inhibitors to wash the membranes. An aliquot of 50 µl re-suspended membrane was kept as membrane fraction sample (MF). The remaining membranes were centrifuged again at 45000 g at 4˚C for 0.5 hr. Supernatant was discarded. The pellet was re-suspended in 200 µl cold PBS, 1% Triton X-100 with above inhibitors and incubated on ice for 0.5 hr to solubilised cell membranes. The suspension was then centrifuged at 45000 g at 4˚C for 1 hr. Supernatant was stored as
detergent soluble fraction (DSF). The remaining pellet was solubilised in 100 µl 1% SDS in PBS plus inhibitors. To fully solubilise the detergent insoluble fraction (DIF), the pellet was passed through a 21G needle for at least 15 time then a 27G needle for at least 20 times.

For immunoprecipitation, the detergent insoluble pellet was re-suspended in 200 µl cold PBS with protease inhibitors. 30 µl Protein-A Sepharose (50% w/w) was added to DSF and DIF and incubated on ice for 20 min with agitation every 2 – 5 min. Sepharose beads were collected to the bottom of the eppendorf tubes by centrifugation at 4800 g for 20 sec. The pre-cleared supernatant was transferred to a new eppendorf tube. 100 µl of DSF or DIF was incubated with PAb762 anti-MT monoclonal mouse IgG on ice for 1 hr and immunoprecipitated as described before.

2.13 Immunofluorescent (IF) Staining

2.13.1 Total MT

Cells were grown on 13mm glass coverslips in a 24 well plate for 24 hr. For staining of total MT in fixed-cells, the cells were washed twice with complete Dulbecco’s phosphate-buffered saline (PBS supplemented with CaCl₂ and MgCl₂ or DPBS) at 37°C, and then fixed by incubation with 0.5 ml 4% formaldehyde solution (stock: 16 % formaldehyde solution w/w, Methanol-free, Thermo Scientific, # 28908) in DPBS for 10 min at RT. After washing with DPBS three times, the cells were permeabilised by incubation with 1% NP-40 (Roche, Nonidet P40 aqueous solution, 10 % w/v) diluted in DPBS, for 5 minutes at RT. Cells were washed three times 5 min in DPBS, and then treated with 30 µl per coverslips Image-iT FX Signal Enhancer solution (Invitrogen, # I36933) for 30 min at RT. After further three washes, the cells were incubated with a 1:200 dilution of primary purified monoclonal antibody against MT N-terminus (PAb762) for 60 min at RT. After the cells washing three times 5 min with DPBS, the antibody was detected by incubation with a 1:1000 dilution of Cy3- or Alexa Fluor 488-conjugated anti-mouse antibody (Jackson Immunoresearch or Invitrogen). The cells were washed a further three times 5 min in PBS, and then two times 5 min in purified water to reduce non-specific signals coming from salt precipitates in PBS before the coverslips were mounted in ProLong Gold anti-fade mounting reagent with DAPI nucleic acid stain (Invitrogen, P36935).
Immunofluorescent labelled cells were viewed on a Nikon inverted fluorescent light microscope and captured using a Princeton Instruments MicroMax Charge-Coupled Device (CCD) camera using Metamorph software.

For high resolution analysis, and multi-colour immunofluorescent labelling, images were viewed and captured with Leica Microsystems TCS SP5 laser scanning confocal microscope and Leica LAS AF software system. Images were taken at 20% overall laser power, with HCX PL APO lambda blue 63.0x1.40 oil UV lens. Area of 41 µm x 41 µm was selected for multi-channel laser scanning (zoom factor = 6). Only cells with intact nuclei were selected for imaging.

2.13.2 Surface MT

For labelling surface HA- or FLAG-tagged MT, cells on coverslips were fluid changed to 1 % FBS DMEM 15 min prior to immune-labelling in order to reduce non-specific binding to coverslips and cell debris. In the meantime, antibody solution was prepared in 1 % FBS, DEME, with anti-HA antibody used at 1:200 dilution and anti-FLAG antibody used at 1:100 dilution. 250 µl solution per well was required to cover the cells completely. Antibody solutions were centrifuged at 13,000 x g for 5 min before use. Surface tags were labelled by incubating the living cells on coverslips with the antibody solutions at 37°C for 20min. Cells were then washed twice in DPBS at 37°C then fixed by incubation with 4% formaldehyde solution in warm DPBS for 10 min at RT and carried forward with above protocol but without permeabilisation of the cell membranes.

For co-staining surface MTHA with phosphorylated ShcA on internal membranes, cell membranes were permeabilised after surface HA labelling and formaldehyde fixation as described above then incubated with rabbit anti-activated ShcA (pY317 and pY239), followed by Alexa Fluor 488-conjugated anti-mouse or Alexa Fluor 555-conjugated anti-rabbit secondary antibodies. For cold staining, cells were incubated in DMEM with 5% FBS, 20 mM Hepes buffer at RT for 15 min, before anti-epitope tag antibodies were added and incubated at 4°C for 30 min.
2.13.3 IF Labelling of Actin

LifeAct-MARS plasmids were kind gifts from Dr Nicola Brownlow at London Research Institute. The plasmid contains sequence that encodes a 17-amino-acid peptide, with F-actin binding property, fused to red fluorescent protein (Riedl, Crevenna et al. 2008). Plasmids were transfected into Rat-2 fibroblast cell lines expressing wild type epitope-tagged MT using Attractene Transfection Reagent. Green-fluorescent labelling for surface tags of MT was performed on transfected cells 48 hrs post-transfection as described before.

2.13.4 Vybrant® Lipid Raft Labelling

Cholesterol-rich lipid rafts on living cells were labelled with Vybrant® Alexa Fluor 555 Lipid Raft Labelling Kit (Invitrogen, V-34404). The Alexa Fluor-conjugated cholera toxin subunit B (CT-B) binds to the polysaccharide chain of monosialotetrahexosyl ganglioside (GM1), which is a membrane lipid that selectively partitions into lipid rafts.

Living cells were incubated in anti-HA or anti-FLAG antibody in the presence of Alexa Fluor 555-conjugated CT-B in 1% FCS tissue culture media for 20 min at 37°C. Lipid rafts labelling at 4°C were performed in some experiments.

2.13.5 Plasma Membrane Labelling

Plasma membranes on live cells were labelled with Image-iT™ LIVE Plasma Membrane Labelling Kit (Invitrogen, I34406) according to manufacturer’s recommendation.

2.14 Image Analysis with FIJI ImageJ Software

Image in channel 1 (red, total phospho-SheA) or channel 2 (green, surface HA) was processed using macro 1. The resulting images from the two channels were then merged to give rise to a combined image. Scale bar = 2 µm.

An area of 20.5 µm x 20.5 µm from the combined image was selected for colocalisation analysis using JACoP. M1 is the fraction of red pixels colocalising with green pixels while M2 is the green pixels colocalising with red pixels. Since green pixels were generated from labelling HA-tags on cell surface, the value of M2 represents the fraction of
surface MT colocalising with activated ShcA. Pearson’s coefficient is measured using Costes’ randomisation based colocalisation analysis with automatic threshold as part of JACoP (Bolte and Cordelieres 2006).

When measuring complex size, images of surface HA or FLAG tagged MT were processed with Macro 1. An area of 20.5 µm x 20.5 µm was selected. A mask was made from the selected area to exclude background signals by setting threshold to 15. Using the mask, go to ‘Analyse: set measurement’ and redirect analysis to the actual fluorescent image, measuring ‘Area’ on the parameter list. Then using the ‘Analyse particles’ function and setting ‘Area covered (µm²): 0-infinity’, the size of individual complex were measured.

Macro 1:
run("8-bit");
run("Median...", "radius=1");
run("Subtract Background...", "rolling=50");
run("Multiply...", "value=2");

JACoP setting:
Measure M1 & M2, using threshold 60 for channel 1, 30 for channel 2
Costes’ randomisation based colocalisation analysis with automatic threshold
Number of randomisation rounds: 200
Bin width: 0.001

2.15 Receptor Expression Level Analysis

2.15.1 RNA Extraction and Conversion into cDNA

Confluent cells grown in 6-well plates were lysed in 1 ml Trizol at RT. After vigorous scrapping, lysates were transferred to a 1.5 ml eppendorf tube. 200 µl chloroform were added to each 1 ml trizol sample and mixed well. Cellular RNAs were separated by centrifugation at 5,000 x g at 4°C for 15 min. The top aqueous phase containing RNA was removed carefully,
avoiding white precipitates of genomic DNA in the middle, and transferred into a sterile 1.5 ml eppendorf. Each sample of extracted RNA were mixed with 0.5 ml isopropanol and incubated at RT for 10 min. The precipitated RNA was centrifuged at 11,000 x g for 10 min at 4˚C. The isopropanol supernatant was discarded and the pellet was washed with 1 ml 75% ethanol. After centrifugation at 7,500 x g for 5 min at 4˚C, the ethanol was carefully removed without disturbing the pellet containing extracted RNA and the pellet was allowed to air-dry. The dried pellet of RNA was re-dissolved in 30 µl TE buffer (10 mM Tris-HCl, pH 7.4) and the concentration analysed by NanoDrop. 300 ng of RNA extract were used for generating cDNA using RNA Superscript (invetrogen) according to Table T2.1. Contents were mixed gentle and spun down to the bottom of the eppendorf, and then incubated at 42˚C for 30 min, then 50˚C for 30 min to increase specificity.

\[
\begin{array}{|c|c|}
\hline
\text{Reagent} & \text{Volume (µl)} \\
\hline
\text{Sample of RNA extract} & 300 \text{ ng (~}0.2\text{µl)} \\
\text{Random primers} & 0.5\text{µl} \\
\text{Water} & 27.3 \text{ µl} \\
\text{dNTP} & 1 \text{ µl} \\
\text{5x buffer} & 8\text{µl} \\
\text{0.1M DTT} & 1 \text{ µl} \\
\text{Superscript III} & 1 \text{ µl} \\
\text{RNase OUT} & 1 \text{ µl} \\
\text{Total volume} & 40 \text{ µl} \\
\hline
\end{array}
\]

65°C for 5min to initiate RNA

\text{2.15.2 Primer Design and Expression Assay for Growth Factor Receptors}

Genomic DNA sequences of rat EGFR1, PDGFRβ, ErbB2, ErbB3 and ErbB4 were obtained through UCSC genome browser(Kent, Sugnet et al. 2002). Forward and reverse primer-pairs were designed by inputting two exon regions of a receptor into Primer3 programme (Koressaar and Remm 2007, Untergasser, Cutcutache et al. 2012). Primer sequence were checked by UCSC BLAT of rat genome to make sure each primer only produce one complementary hit in the rat genome (Kent, Sugnet et al. 2002).
PCR reactions were performed with each cDNA sample generated and a pair of forward and reverse primer designed specifically for the receptor of interest according to Table T2.2. 20 µl PCR products were separated on a 5% 19:1 Bis-Acrylamide gel in 0.5% TBE buffer. After electrophesis, gels were incubated in 150 ml 0.5% TBS buffer with 1 µl ethidium bromide for 15 min at RT. DNA bands were visualised under UV.

**Table T2.2 PCR mix preparation for receptor expression analysis**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward +Reverse mixed primers at 1:5 dilution</td>
<td>2 µl</td>
</tr>
<tr>
<td>cDNA sample</td>
<td>1 µl</td>
</tr>
<tr>
<td>PCR master mix</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>9.5 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

Websites used for primer design:


[http://genome.ucsc.edu/](http://genome.ucsc.edu/)
3.1 INTRODUCTION:

MT association with the cell membrane association requires a 22-residue stretch of hydrophobic amino acids located near to the MT C-terminus (Soeda, Arrand et al. 1979). Deletion of this region abolishes transforming activity and prevents binding to all signalling molecules except PP2A (Novak and Griffin 1981, Carmichael, Schaffhausen et al. 1982). However, the role of the C-terminus in MT-induced transformation is not well studied. The protein cannot be labelled with protein-modifying reagents from the extracellular side of the plasma membrane (Schaffhausen, Dorai et al. 1982). However, the very hexapeptide following the hydrophobic regions might be too short for an interaction with modifying reagents (Hofer, Wehrle et al. 1995). The position of the MT hydrophobic sequence is very close to the C-terminus, which suggests MT may be a tail anchored (TA) protein (Borgese, Brambillasca et al. 2007). TA proteins are typically characterised by the location of hydrophobic sequence near the C-terminus. This is followed by a short translocated polar region orientated towards the extracellular surface of the cell, which is made up of no more than 30 amino acid residues. The N-terminal functional domain faces the cytosol (Borgese, Brambillasca et al. 2007).

To examine how MT is inserted into membranes we have isolated and studied a series of mutations of the C-terminal region. We report here that an epitope tag added to the C-terminus of MT can be detected on the surface of transformed cells, demonstrating that MT is a tail anchored (TA), integral membrane protein. Inserting the ER retention sequence KDEL at the C-terminal end of MT located it to the ER and cis-Golgi only, showing that MT is first
inserted into membranes at the ER. This ER localised MT species was able to bind to PP2A and Src but failed to bind ShcA, PI3K and PLC-γ1, and did not transform, indicating that MT has to migrate out of the ER to associate with further signalling proteins in order to generate a mitogenic signal. Replacing the MT TMD with one from an ER-resident membrane protein (Sec61β) did not entirely trap the MT in the ER, suggesting that other properties of MT in addition to the TMD allow MT to exit the ER. Mutations in the N-terminus of MT suggest this property is the binding of PP2A.
3.2 RESULTS:

3.2.1 MT is a tail-anchored transmembrane protein

The MT hydrophobic domain is positioned only six amino acids from the C-terminus. The location of the hydrophobic domain so close to the C-terminus suggests strongly that MT belongs to a class of tail-anchored (TA) transmembrane proteins (Borgese, Brambillasca et al. 2007). However, one important requirement for TA protein is that the hydrophobic sequence must span the lipid bilayer and this has not been proven for MT. In order to study MT C-terminus topology, a slightly extend hemagglutinin epitope (HA) tag, AYPYDVPDYASL, was added to the C-terminal end of MT sequence to see whether this epitope tag can be detected on the cell surface. The resulting protein (wtMTHA+) was transformational competent as shown by similar levels of foci formed by the transfection of wild-type or tagged MT cDNAs (Figure 3.1 B). The focus formation assay is routinely used to assess the ability of various oncogenes to induce cellular transformation (Morgan, Kaplan et al. 1988, Sparkowski, Anders et al. 1995). Cells transformed by oncogenes such as MT, lose contact inhibition and exhibit anchorage independent growth. A raised lesion or focus is formed by the piling-up of cells, which can be visualised by Leishmann’s Stain labelling cytoplasmic structure (stained by eosin) and the nuclei (stained by methylene blue). The HA tag was detected on the surface of the cells stably expressing wtMTHA+ using antibody specific for HA added to living cells prior to formaldehyde fixation (Figure 3.1 C (ii)). An in-house mouse monoclonal antibody directed against the N-terminal sequence of MT (PAb762 anti-MT) did not react with wild type or HA-tagged MT when added to living cells. This antibody reacted strongly to wild type MT and MTHA+ only after the cells were fixed and permeabilised (Figure 3.1 C (a)). Results shown in Figure 3.1 C suggested that at least a detectable amount of MT C-termini were translocated to the extracellular surface of plasma membrane while the bulk of the MT N-termini were facing the cell interior.
Figure 3.1 MT C-terminus is translocated across to the cell surface

A. Amino acid sequences of the MT C-terminus and the epitope tags added. Wild type MT sequence is shown at the top. Positions in the complete protein sequence are indicated with numbers at 10-residue interval above the corresponding amino acids. Designation of each MT species is listed on the left. Single-spacing separates the hydrophobic sequence from sequences on either side. MTHA+ plasmid was generated by Dr N. Ichaso.

B. Epitope tag did not affect transformation. Rat2 fibroblasts on 10 cm tissue culture dishes were transfected with 10 µg plasmids carrying wild-type MT (a) or tagged MT sequence (c). Mock transfection was performed without any plasmid (b). Cells were cultured for 14 days before staining in Leishmann’s solution.

C and D. Epitope tags can be detected on cell surface. Cell lines stably expressing wtMT (i) or epitope tagged MT (ii) were established and immunofluorescence (IF) staining was performed with primary antibody against MT N-terminus (anti-MT) or epitope tag, anti-HA (C), anti-FLAG (D) added before (Live) or after (Fixed) cell fixation. Cells were imaged at low magnification on Nikon inverted epifluorescence microscope. Experiment in C was performed by Professor Stephen Dilworth.
In order to verify our findings with MTHA+, a different epitope, FLAG tag (DYKDDDDK), was added to the C-terminus of MT. The resulting wtMTFLAG transformed Rat-2 fibroblasts to the same level as wild-type MT (Figure 3.1 B lower panels). The FLAG tag can be detected by incubating MTFLAG expressing cells live, in a primary antibody specific for the tag before fixation (Figure 3.1 D), hence further confirmed that MT C-terminus was translocated across to the cell exterior with the hydrophobic sequence spanning the lipid bilayer in a single-pass topology.

3.2.2 MT localised in the Endoplasmic Reticulum failed to transform

We demonstrated that a short amino acid sequence can be added to the MT C-terminus without affecting its ability to transform Rat-2 fibroblasts. A similar approach was used to examine the role of subcellular localisation in MT function. MT is found in most internal cell membranes but little is known about where the oncoprotein is first inserted into a membrane and where the transforming signal is generated (Dilworth, Hansson et al. 1986). To determine whether MT located solely in the endoplasmic reticulum (ER) can produce a transforming signal, a specific ER- retention sequence, KDEL, was added to MT C-terminus (MTKDEL). Proteins bearing the KDEL sequence in the ER lumen are recognised and bound by KDEL receptors in the cis Golgi membranes and are subsequently returned to the ER through retrograde transport (Lewis and Pelham 1992). The amino acid sequence, GGDV, was used to make a control (MTGGDV), which lacks the four critical amino acids recognised by the KDEL receptor thus should not be retained in the ER lumen. These are the same sequence additions that demonstrated that bovine papillomavirus E5 must locate to the distal Golgi compartments to transform fibroblasts (Sparkowski, Anders et al. 1995).
Figure 3.2 ER-retention sequence abolishes MT transformation of fibroblast monolayer

A. ER retention sequence KDEL or a non-functional control mutation GGDV was added to MT C-terminus. In addition, the MT hydrophobic sequence was replaced with that of an ER-resident TA protein, Sec61β. Designation of each MT species is listed on the left. Single-spacing separates the hydrophobic sequence from sequences on either side.

B. Foci formation assay for transformation ability. Sub-confluent Rat2 fibroblasts on 10 cm tissue culture dishes were transfected as described previously. The MT cDNA that were used are indicated above or below each panel.
The resulting MTKDEL failed to transform Rat-2 fibroblasts while MTGGDV transformed Rat2 fibroblasts at the same level as wild type (Figure 3.2 B). IF labelling of MT in cells expressing MTKDEL showed a distinctive distribution of the protein. While the wild-type MT can be found throughout the cell including the peripheral plasma membrane, the mutant located solely in reticular structures in the cytoplasm which resembled the ER (Figure 3.3 A). The distribution of MTGGDV was the same as wild-type MT (Figure 3.5 A). Co-staining MT with a known ER marker – calreticulin, confirmed that MTKDEL resided in the ER compartment (Figure 3.3 B). Wild-type MT was targeted to membrane sites beyond the ER, such as the Golgi and the plasma membrane, so did not colocalise strongly with this ER marker. MTKDEL was unable to reach the Golgi apparatus as the protein failed to colocalise with the Golgi marker giantin (Figure 3.4). Retention of MT in the ER by the KDEL sequence suggests strongly that the protein is initially inserted into membranes at the ER as integration at any other membrane organelle would bypass the KDEL receptor and generate a different distribution. ER-retention of MTKDEL also provided further evidence that the C-terminus of MT must have translocated across the lipid bilayer exposing the KDEL sequence to the ER/ cis Golgi lumen.
Figure 3.3 MTKDEL is localised to the ER membrane

A. MT distribution in cells stably expressing wtMT or MTKDEL. Cells were fixed and permeabilised before incubation with PAb762 anti-MT mouse monoclonal antibody alone.

B. Dual-fluorescent labelling of MT and an ER-marker calreticulin. Mouse anti-MT primary antibody PAb762 was added in conjunction with rabbit anti-calreticulin monoclonal antibody. Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 633-conjugated anti-rabbit secondary antibodies were added in order to label total MT in green or calreticulin in red respectively. Cells were imaged on Leica SP5 confocal microscope using 63.0x1.40 oil UV lens. Co-localisation between MT and calreticulin is seen as yellow in the combined channel.
We then investigated whether MTKDEL localised to the ER associated with known MT-binding proteins. All protein binding sites were unchanged in the MTKDEL polypeptide. To determine whether these sites were occupied, immunoprecipitation of MT was performed using PAb762 anti-MT antibody on cell lines stably expressing each mutant and then analysed by SDS-PAGE and Western blotting for known MT associating proteins required for oncogenic signalling. Figure 3.5 B shows that MTKDEL failed to bind ShcA, PI3K and PLC-γ1 even though it was able to bind to PP2A core dimer and pp60c-src at levels comparable to those of the wild type MT. The pp60c-src bound by MTKDEL was fully

Figure 3.4 MTKDEL is absent from the Golgi apparatus

Rat-2 fibroblasts stably expressing MTKDEL (i) or wtMT (ii) were fixed and permeabilised before incubation with PAb762 anti-MT mouse monoclonal antibody (a) and with Alexa Fluor 488-conjugated rabbit anti-giantin monoclonal antibody (b). Alexa Fluor 555 anti-mouse secondary antibody was subsequently added to fluorescently label MT. Total MT staining is shown in red. Giantin in Golgi is shown in green. DAPI nuclear staining is shown in blue in the combined channel (c). Cells were imaged on Leica SP5 confocal microscope using 63.0x1.40 oil UV lens with zoom factor 4.5. An area of 30 µm x 30 µm was selected for analysis. Scale bar = 5 µm.

We then investigated whether MTKDEL localised to the ER associated with known MT-binding proteins. All protein binding sites were unchanged in the MTKDEL polypeptide. To determine whether these sites were occupied, immunoprecipitation of MT was performed using PAb762 anti-MT antibody on cell lines stably expressing each mutant and then analysed by SDS-PAGE and Western blotting for known MT associating proteins required for oncogenic signalling. Figure 3.5 B shows that MTKDEL failed to bind ShcA, PI3K and PLC-γ1 even though it was able to bind to PP2A core dimer and pp60c-src at levels comparable to those of the wild type MT. The pp60c-src bound by MTKDEL was fully
active as this mutant in the MT immunoprecipitates was found to be phosphorylated by in vitro kinase assay (performed by Prof. Dilworth, Figure 3.6). Low level of PI3K activity was detected MTKDEL by in vitro kinase assay, probably due to high sensitivity of the assay (Figure 3.6). Two very weak bands that correlate to PI3K p85 and PLC-γ1 can sometimes be detected in MTKDEL immunoprecipitates, which is probably due to the leakage of a small amount of MTKDEL from the ER compartment. Alternatively, some protein binding might have occurred in the after cell lysis. Therefore the absence of ShcA, PI3K and PLC-γ1 in the MTKDEL precipitate was not due to the lack of phosphorylated binding sites on this mutant but was likely a consequence of these signalling molecules not having access to MT localised in the ER.
Figure 3.5 Analysis of MTKDEL location and association with known MT-binding proteins

A. Rat-2 fibroblasts were transfected with expression plasmids carrying MT mutants described in Figure 3.2 A, using Attractene transfection kit, see Material & Methods. IF staining for total MT was performed at 48 hr post transfection, using PAb762 antibody. Plasmids transfected are indicated above or below each panel. Cells were photographed using a Nikon inverted epifluorescence microscope at 100x magnification.

B. Protein binding properties of each of the mutants described in figure 3.2 A. MT was immunoprecipitated (IP) by PAb762 from equal volumes of lysates collected from stable cell lines, as indicated above each lane. The immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose membrane for Western blotting. Antibodies used to immune-bind (IB) known MT-associating proteins are shown above each panel. The migratory position of each detected polypeptide species is indicated by an open arrow to the right. Experiments were performed by Prof. S. Dilworth.
Since MT retained in the ER cannot transform, there must be signals on MT that target itself to additional membrane locations once inserted into the ER membrane. In other TA proteins, the TMD sequence alone is thought to determine where the polypeptide is finally located (Borgese, Brambillasca et al. 2007). To investigate whether MT hydrophobic sequence alone directs MT out of the ER to additional membrane sites, we replaced the sequence with that from an ER-resident TA protein, Sec61β. It is part of the ER co-translational membrane insertion complex and resides mainly on ER membranes (Hartmann, Sommer et al. 1994). Although Sec61β does not have retrieval peptide sequences like KDEL, this TA is found exclusively in the ER-Golgi intermediate compartment (ERGIC) but not the trans Golgi network, a property thought to be mediated by its TMD sequence only.

Figure 3.6 Kinase activity associated with MTKDEL

MT from stable expression cell lines was immunoprecipitated then incubated with [γ-32P] ATP. The radio-labelled immunoprecipitates were separated by SDS-PAGE and autoradiographed. The MT species is indicated above each lane and the migration positions of MT and its associated proteins are indicated with closed arrows on the right. Experiment was performed by Prof. S. Dilworth.
(Greenfield and High 1999, Butler, Lee et al. 2007, Butler, Watson et al. 2011). This new hydrophobic region should target the new mutant MTSec61β to the ER membrane only. Foci formation induced by MT was reduced but not abolished by the substitution of Sec61β hydrophobic sequence (Figure 3.5 B). Unlike MTKDEL, MTSec61β was not trapped in the ER (Figure 3.5 A). It was found not only in the ER but also in peripheral plasma membranes (Figure 3.5 A). Apart from the ability to bind PP2A core dimers and pp60c-src, MTSec61β was able to bind ShcA, PI3K and PLC-γ1 (Figure 3.5 B, in vitro kinase assay can be found in Figure 3.6). The lower level of ShcA and PLCγ1 may reflect a lower level of the mutant present in cell lysate. An HA tag added to the C-terminus of MTSec61β showed the tag was detect on cell surface although at reduced levels (data not shown). Therefore MTSec61β was targeted to membranes other than the ER. Together, these results suggest the TMD of MT plays a role in targeting the polypeptide to the plasma membranes otherwise MT transformation would have been unaffected by the substitution of the ER-targeting TMD sequence from Sec61β. However, component(s) outside the hydrophobic sequence may also contribute to membrane targeting as the MTSec61β inhibition of ER exit was incomplete.

3.2.3 Association with PP2A is required for MT to migrate out of the ER

The observation that the TMD may not be sufficient to specify MT migration out of the ER prompted us to examine whether another property of MT plays a role in the relocation of MT in the ER to additional membrane sites. Our lab had previously reported that a MT mutant lacking the ability to bind PP2A was retained in cytoplasmic membranes, probably the ER (Brewster, Glover et al. 1997). In a series of deletion mutants generated in the study, amino acid sequence 185 – 210 on MT was identified as the region required for pp60c-src binding. Mutant 200Δ10 is defective in pp60c-src binding while 180Δ10 has a shortened PP2A-binding region and therefore lacks both PP2A and Src binding (Brewster, Glover et al. 1997).
(see List of Mutants). Using PAb762 IF labelling of MT (Figure 3.7 A) showed that mutant 180Δ10 accumulated in the perinuclear region and was completely absent from the plasma membrane. This mutant had a distribution pattern most similar to MTKDEL while mutant 200Δ10 was distributed to the peripheral plasma membrane just like wild type MT. Treatment with okadaic acid (OA), an inhibitor of PP2A, resulted in accumulation of MT in cytoplasmic membranes after 48 hrs. However, the level of MT in the plasma membrane was not affected by the level of OA tested. Similar observations were made when the treatment period was shortened to 24 hrs. The experiment was repeated at 5 nM OA but required a treatment period of 48 hrs to achieve a similar level of MT staining at the ER. Treatment with dasatinib, a dual Bcr-Abl and Src tyrosine kinase inhibitor, did not change MT distribution in the cells (Figure 3.7).
Figure 3.7 MT mutant 180Δ10 lacking PP2A binding is localised to ER region

A. MT distribution in mutants defective in PP2A or pp60c-src binding. MT was labelled by PAb762, which targets the MT N-terminus, after cell fixation and permeabilisation. MT species in stably cell lines are described in the top panel. The ability of each MT mutant to bind PP2A and pp60c-src is indicated below each image.

B. Cells stably expressing wtMT were treated with 10 nM okadaic acid (OA) for 48 hrs before IF labelling of total MT using PAb762 anti-MT.

C. In a separate experiment, wtMT cells were treated with 50 nM dasatinib for 48 hrs before IF labelling MT as above.

Cells were imaged on a Leica SP5 confocal laser scanning microscope using 63.0x1.40 oil UV lens. Scale bar, 10 µm. Images are representative of at least 8 independent frames. Experiments with inhibitor treatment have been repeated for at least two times.
In order to determine whether other mutants defective in PP2A binding also fail to reach the plasma membranes, the location of the MT polypeptide in cell lines expressing mutants NS2, DC1, and DC2 (Dilworth, Brewster et al. 1994) were examined by IF using a number of anti-MT monoclonal antibodies (see Figure 3.8 for each mutant lesion position and the antibody recognition region). In all cases, MT mutants that are defective for PP2A binding were observed to be present only on cytoplasmic membranes and not the plasma membrane, irrespective of the antibodies that were used to detect MT (Figure 3.9, see Table T3.1 for a summary of IF results). With antibodies PAb762, PAb754 and PAb750, significant amounts of wild type MT was detected at the plasma membrane (Figure 3.9, panels a, b and c). It has been suggested that the binding of Hsc70 to the J domain in PP2A binding defective mutants of MT might account for their cytoplasmic retention (Whalen, de Jesus et al. 2005). To determine whether this was case, a mutant that contained both the deletion of the Hsc70 and PP2A binding sequence was generated by Professor S. Dilworth (mutant ∆J-DC1) (Markland and Smith 1987; Whalen, de Jesus et al. 2005, Zhou, Ichaso et al. 2011). HPDKGG sequence at position 42-47 in MT J domain is required for interaction with Hsc70 (Whalen, de Jesus et al. 2005) and the DC1 deletion disrupts PP2A binding (Markland and Smith 1987). In mutant ∆J-DC1 both sites and the sequence in between were deleted, see Figure 3.6. This mutant was then transfected into Rat2 cells and the MT was detected by immunofluorescence with antibody PAb762 after 48 hours post-transfection. It was found that ∆J-DC1 MT does not locate to the plasma membrane, and the location resembles that of DC1 (Figure 3.9, panel g). It seems likely, therefore, that Hsc70 binding is not responsible for maintaining PP2A binding defective MT mutants within cytoplasmic membranes (Zhou, Ichaso et al. 2011). Finally, to examine the role of PP2A binding in MT subcellular distribution in the absence of a mutation, we performed immunofluorescence analysis on cells that expressed wild type MT using monoclonal antibody PAb701. This antibody reacts
with the N-terminal region of MT required for PP2A binding and fails to bind to the MT-PP2A complex (Dilworth and Horner 1993). The MT detected by PAb701 therefore is not associated with PP2A and showed very little plasma membrane staining (Figure 3.9, panel h). Table T3.1 summarises the immunofluorescence labelling of MT in mutants examines in section 3.2.3. It represents clearly the correlation between the lack of PP2A association and MT localisation in cytoplasmic membranes. Taken together, our data suggests strongly that association with PP2A is required for MT to relocate from the ER to the plasma membrane.

Figure 3.8 Schematic representations of the MT mutations and antibodies used in Chapter 3.2.3 studies

Lesions deleted are indicated with black bars on the MT sequence. DC1, DC2, NS2 and 180Δ10 carry deletion lesions in the PP2A binding region. ΔJ-DC1 is truncated from dnaJ-domain to DC1 region. 200Δ10 carries a deletion in the Src-binding region. The approximate binding regions for PAb762, PAb701, PAb754 and PAb750 are indicated by black lines. The gray bar represents the TMD. PP2A and pp60c-src binding regions are indicated by brackets below the MT sequence. Also see List of Mutants for description. Monoclonal antibodies and mutants were generated by Professor S. Dilworth and others.
Figure 3.9 Mutants lacking PP2A binding failed to reach the plasma membrane

Mutants DC1, DC2 and NS2 are defective in PP2A binding. ∆J-DC1 fails to bind Hsc70 and PP2A. Cells expressing each mutant were fixed and permeabilised before incubation with individual monoclonal antibody to detect MT. MT species and the antibody used are indicated above each image. Antibody PAb762 reacts with MT N-terminus, PAb701 reacts with region required for PP2A-binding, PAb754 and 750 reacts with regions near the C-terminus. See figure 3.6 for detailed lesion positions on MT sequence and the region targeted by each antibody. Cells were photographed using Nikon inverted epifluorescence microscope at 100x magnification.

Immunofluorescence analysis was performed on cells expressing various MT mutants by Prof. S. Dilworth.
Table T3.1

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<td>Wild Type / PAb762</td>
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Table T3.1 Summary of MT distribution in chapter 3.2.3 immunofluorescence staining analysis

The MT polypeptides expressed and the antibodies used are listed on the left. The ability of the MT and antibody combination to IP with PP2A is indicated in the second column. Strength of immunofluorescence signals in cytoplasmic or plasma membranes are presented by ‘+’ in the last two columns. IF staining of MT in above mutants was performed by Prof. S. Dilworth.
3.2.4 MTKDEL and Src Tyrosine Kinase Association

3.2.4.1 MTKDEL fails to bind pp62c-yes

Tyrosine kinase activity associated with MT is attributed to the Src-family tyrosine kinases bound to MT. This association with Src-family tyrosine kinases is essential for polyomavirus-induced cellular transformation and the ability to induce tumour formation in animals (Dunant, Senften et al. 1996). MPyV MT has been shown to preferentially associate with pp60c-src (Courtneidge and Smith 1983), pp62c-yes at lower affinity (Kornbluth, Sudol et al. 1987), and, to a lesser extent, pp59c-fyn (Cheng, Harvey et al. 1988, Kypta, Hemming et al. 1988). MT association with pp62c-yes had not been investigated previously in this lab due to the lack of a good antibody against this non-receptor tyrosine kinase. A number of commercially available antibodies targeting pp62c-yes were purchased and screened by MSc student Ewelina Pino Lopez for their recognition of pp62c-yes in Rat-2 fibroblasts lysates. It was found that only the rabbit anti-Yes1 monoclonal antibody (EPR3173) effectively labelled pp62c-yes by Western-Blotting and by indirect immunofluorescence (IF) labelling in permeabilised Rat-2 fibroblasts (Figure 3.10 A and data not shown).

Curiously, it was found that MTKDEL failed to bind pp62c-yes despite its ability to bind pp60c-src (Figure 3.10 A). This differential tyrosine kinase binding in MTKDEL was verified by immunofluorescence labelling of MT with each of the two Src-family tyrosine kinase members. Immunofluorescence staining of the pp62c-yes showed granular distribution throughout the cytoplasm to the plasma membrane, Figure 3.10 B. Unlike wild-type MT that shared similar subcellular distribution with pp62c-yes, ER-localised MTKDEL was devoid of pp62c-yes labelling (Figure 3.10 B). Co-immunofluorescence labelling of pp60c-src and MT produced colocalisation in both wild-type and MTKDEL expressing cells (Figure 3.10 C).

Therefore the lack of MTKDEL and pp62c-yes association is probably due to the mutant not having access to this tyrosine kinase while pp60c-src remains accessible to MTKDEL.
Figure 3.10 MTKDEL failed to associate with pp62c-yes

A. MTKDEL failed to bind pp62c-yes. MT polypeptides were immunoprecipitated as described in figure 3.4. The presence of Src-family tyrosine kinases were detected with antibody specific to pp60c-src (Cell Signaling Technology) or pp62c-yes (Abcam), as indicated above each lane. Non-transfected Rat-2 fibroblast lysate was used as a negative control. Experiment was performed by student Ewelina Pino Lopez.

B. MT trapped in the ER does not have access to pp62c-yes. Cell lines stably expressing wt (i) or KDEL MT (ii, iii) were fixed and permeabilised then incubated with both mouse PAb762 and rabbit monoclonal antibody against pp62c-yes. Alexa Fluor 488 or 555-conjugated secondary anti-mouse or rabbit IgGs were added to label MT (green) or pp62c-yes (red) respectively.

C. MTKDEL co-localises with pp60c-src in the ER. Dual labelling MT and pp60c-src was performed on cells expressing wtMT or MTKDEL as described in B.
3.2.4.2 MTKDEL redistributes pp60c-src but not pp62c-yes

We noticed that pp60c-src distribution was changed in cells expressing MTKDEL. Figure 3.11 and 3.12 represent the results of colocalisation studies between MT and pp60c-src in Rat-2 fibroblasts with wild type or ER-localised MTKDEL in low and high magnifications respectively. In normal non-transfected Rat-2 fibroblasts, this tyrosine kinase was asymmetrically distributed in the perinuclear region as well as the plasma membrane. Staining of pp60c-src was observed at the edge of cells as well as area of cell-cell contact (white arrows in Figure 3.11 panel i). In cells expressing wild-type MT, pp60c-src can be found in membrane ruffles and area where strong MT fluorescence signal was observed (Figure 3.12 panel ii). However, in cell expressing MTKDEL, pp60c-src distribution was limited to cytoplasmic area around the nuclei and was almost symmetrical located with very little fluorescence signal detected at the cell periphery (Figure 3.11 and Figure 3.12). The distribution of pp60c-src colocalised perfectly with MTKDEL in cells (Figure 3.10 – 12). Unlike pp60c-src, pp62c-yes subcellular distribution was not affected by this MT mutant (Figure 3.13). Therefore it is likely that the formation of MTKDEL-pp60c-src complexes redistributed this tyrosine kinase in cells.
Figure 3.11 MTKDEL redistributes pp60c-src

Cells were fixed and permeabilised before staining for total MT (ii, green) and pp60c-src (i, red) as described before. Open arrows indicates pp60c-src at the cell periphery. Cell type was indicated above each column of images. Non-transfected Rat-2 fibroblasts were used as control. Images were taken on TCS SP5 confocal microscope using 63.0x1.40 oil UV lens with zoom factor 1.
Figure 3.12 Single-cell images showing pp60c-src and MT distribution in cell lines

Single-cells from images shown in Figure 3.11 were selected for high resolution imaging. Antibodies used are indicated above the top panel. Cell types are indicated on the right. Images were taken on a TCS SP5 confocal microscope using 63.0x1.40 oil UV lens. Scale bar, 5 µm.
Several lines of evidence suggest MT is inserted by the newly discovered GET/TRC 40 system. The hydrophobic TMD is so close to the C-terminus that it must be released from the ribosome before it can interact with the membrane machinery for insertion (Borgese, Brambillasca et al. 2007). Membrane insertion of MT C-terminus does not happen spontaneously but is likely to require post-translational targeting machinery (Kim, Janiak-Spens et al. 1997). The TMD of MT has an estimated hydrophobicity value of 63.7, calculated as described by Kyte and Doolittle (Kyte and Doolittle 1982) as the sum of hydrophobicity values for the amino acids in MT hydrophobic sequence (see Table T3.2).
Table T3.2

<table>
<thead>
<tr>
<th>Side-chain</th>
<th>Single letter amino acid code</th>
<th>Hydrophobicity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoleucine</td>
<td>I</td>
<td>4.5</td>
</tr>
<tr>
<td>Valine</td>
<td>V</td>
<td>4.2</td>
</tr>
<tr>
<td>Leucine</td>
<td>L</td>
<td>3.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>F</td>
<td>2.8</td>
</tr>
<tr>
<td>Cysteine/cystine</td>
<td>C</td>
<td>2.5</td>
</tr>
<tr>
<td>Methionine</td>
<td>M</td>
<td>1.9</td>
</tr>
<tr>
<td>Alanine</td>
<td>A</td>
<td>1.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>G</td>
<td>-0.4</td>
</tr>
<tr>
<td>Threonine</td>
<td>T</td>
<td>-0.7</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>W</td>
<td>-0.9</td>
</tr>
<tr>
<td>Serine</td>
<td>S</td>
<td>-0.8</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Y</td>
<td>-1.3</td>
</tr>
<tr>
<td>Proline</td>
<td>P</td>
<td>-1.6</td>
</tr>
<tr>
<td>Histidine</td>
<td>H</td>
<td>-3.2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>E</td>
<td>-3.5</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Q</td>
<td>-3.5</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>D</td>
<td>-3.5</td>
</tr>
<tr>
<td>Asparagine</td>
<td>N</td>
<td>-3.5</td>
</tr>
<tr>
<td>Lysine</td>
<td>K</td>
<td>-3.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>R</td>
<td>-4.5</td>
</tr>
</tbody>
</table>

Table T3.2 Kyte and Doolittle's hydrophobicity scale
Amino acids with increasingly hydrophobic side chains are assigned with more positive values while hydrophilic side chains are assigned with more negative values.
Adapted from Kyte and Doolittle, 1982.

The same hydrophobicity scale was used by Rabu et al, as well as Kalbfleisch et al in a bioinformatics study to identify TA proteins in the human genome (Kalbfleisch, Cambon et al. 2007, Rabu, Wipf et al. 2008). Integration of TA proteins with such a high hydrophobicity is not obligatory clients for the Hsp40/Hsc70 chaperone (Rabu, Wipf et al. 2008), but more likely to utilise the high ATP-input route mediated by the recently discovered cytosolic TMD-recognition complex (TRC) (Stefanovic and Hegde 2007) or a combination of the two pathway. The 40 kDa subunit of TRC is an ATPase, identified as
TRC40/ASNA1. Here we investigate whether MT can physically associate with the TRC core ATPase, ASNA1 (a mammalian homologue to arsenite-stimulated ATPase in bacteria).

Commercially available antibody raised against ASNA1 (Abcam, ab81979) did not bind strongly to ASNA but cross-reacted with a high molecular weight polypeptide in rabbit reticulocytes lysate (results not shown). A rabbit anti-ASNA1 serum was kindly donated to us by Dr Matthias Seedorf from Heidelberg University. After careful optimisation of this antibody, immunoprecipitation of MT in cells expressing wild type or MTKDEL was analysed for ASNA1 association but did not yield any detectible band in the region of 40 kDa, Figure 3.14.

![Figure 3.14 Analysis of MT and ASNA1 association by immunoprecipitation](image)

Equal volumes of lysates from wtMT or MTKDEL stable cell lines were immunoprecipitated with PAb762. Non-transfected Rat-2 lysates was used as a control. The volume of lysates used was 5 times more than the volume used for MT-binding signalling molecule analysis. Wash buffer with 0.005% NP40 was used instead of the normal 0.05% NP40. 5 µl lysate samples were analysed for total ASNA1 content using a rabbit antibody against ASNA1 from Dr Matthias Seedorf (i). Immunoprecipitated samples were analysed for ASNA1 (ii) and MT (iii) using biotinylated mouse monoclonal PAb762. Migration positions of target molecules are indicated with open arrows on the right.
Using a TNT reticulocyte lysate in vitro translation system, we attempted to assess whether newly synthesised MT polypeptide can associate with ASNA1. In this system, RNA is synthesised in vitro from T7 RNA polymerase promoters in cell-free rabbit reticulocyte lysate. These mRNAs are subsequently translated into polypeptides, which are capable of binding to cellular proteins already present in the reticulocyte lysate as well as co-translated proteins. pcDNA3.1 plasmids carrying MT cDNA under the T7 promoter were previously constructed by Prof. Dilworth. Initial immunoprecipitates of the in vitro translated MT failed to show the presence of ASNA1 (results not shown). This may be due to the low level of native ASNA1 in the reticulocytes lysate. An expression plasmid of mouse ASNA1 with a T7 promoter (pYX-ASNA1) was subsequently obtained from Open Biosystems. Co-translating equal amounts of wild-type MT and ASNA1 expression plasmids in vitro, the newly synthesised MT molecules were immunoprecipitated with PAb762 and separated by SDS-PAGE. Rabbit polyclonal antibody against ASNA1 was used to detect the presence of the ATPase. Analysis of post-reaction samples showed there is a small increase of ASNA1 when pYX-ASNA1 plasmid was added (Figure 3.15 A (ii)). There is a very weak signal in MT immunoprecipitates that co-migrates with the band detected in the positive control when both plasmids were added (Figure 3.15 A (i)). When MT and ASNA1 were co-translated, there was also a strong lower molecular weight protein (~ 30 kDa) that was reactive to anti-ASNA1 antibody. However, this molecule was not seen again in subsequent in vitro co-translation experiments (Figure 3.15 B (i) and results not shown). Immunoblotting for PP2A subunit A on the same nitrocellulose membrane showed the MT binding partner was co-immunoprecipitated with the newly synthesised MT by PAb762, thus indicating that in vitro translated MT was capable of associating with signalling molecules proteins present in reticulocyte lysates.
MTKDEL which localises to the ER should in theory be more accessible to the membrane insertion machinery that includes TRC40/ASNA1 and therefore a larger population of MTKDEL may associate with the ATPase. In an attempt to trouble shoot the MT – ASNA1 binding assay, pcDNA3.1 plasmid carrying MTKDEL cDNA was constructed by substituting the pcDNA3.1 wtMT C-terminus sequence with that of pUC19 MTKDEL. However, pcDNA3.1MTKDEL plasmid did not translate well in vitro despite DNA sequencing data confirming the presence of the MTKDEL sequence (data not shown).
Figure 3.15 *In vitro* co-translated MT and ASNA1 failed to associate

A. Analysis of MT immunoprecipitates after in vitro translation. 1 µg of pCDNA3.1-MT plasmid was added with or without 1 µg pYX-ASNA1 to a TNT reticulocyte lysate *in vitro* translation system according to the manufacturer’s recommendation. PAb762 was added to reaction mixtures after in vitro translation to immunoprecipitate MT and subsequently analyse for ASNA1 association (i). 1 µl of reticulocyte lysate without DNA was loaded in lane d as a positive control. Mouse IgG band at 55 kDa was cut away to reduce non-specific signals. 1 µl post-translation lysate from each reaction mixtures was analysed for ASNA1 content (ii). cDNA added is indicated above each lane. Migration positions of target molecules are indicated with open arrows on the right.

B. Independent repeat of experiment described in A. Antibodies used are indicated above each blot. Rat monoclonal antibody against PP2A subunit A (6G3) (Santa Cruz) or rabbit antibody against ASNA1 were used to detect their presence in the MT immunoprecipitates (i). 1 µl post-translation lysate from each reaction mixtures was analysed for ASNA1 or MT content (ii).
3.2.6 The C-terminal hexapeptide has a functional role

The MT hydrophobic sequence is followed by a hexapeptide, KRSRHF, rich in positively charged amino acids and is exposed on the extracellular surface of the cell. Although Dahl and co-workers concluded that the hexapeptide C-terminal to the MT hydrophobic sequence is dispensable for transformation, some mutants generated in this lab suggest that positively charged amino acids on both sides of the hydrophobic sequence are equally important for transformation (data not shown). We next looked at the effect on MT transformation when the hexapeptide is partially or completely deleted. Deletion mutants of the C-terminus were generated from MTHA+ plasmid by Dr N. Ichaso. Δ419-421MTHA+ is missing the latter half of the hexapeptide (RHF) while Δ416-421MTHA+ has the whole hexapeptide (KRSRHF) sequence removed (see Figure 3.16 A for sequence comparison). Transformation abilities of the two mutants were assayed by foci formation in Rat-2 fibroblast monolayer. Removal of the final three amino acids from the MT C-terminus had no effect on transformation ability while deletion of the whole hexapeptide completely abolished foci formation (Figure 3.16 B). In stable cell lines, the amount of Δ416-421MTHA+ was similar to wtMTHA+ (Figure 3.16 C). In fact, the transforming mutant Δ419-421MTHA+ was expressed less well. Therefore the lack of foci formation was not due to difference in expression level. Δ416-421MTHA+ was able to associate with PP2A core dimer and pp60c-src, but associated weakly with ShcA and PI3K p85 subunit. The amount of pp62c-yes in 416-421MTHA+ was very low (Figure 3.17 A). The weak detection of signalling molecules bound to Δ419-421MTHA+, a transformation-competent mutant was probably due to a lower level of MT immunoprecipitated from cell lysate. Immunoprecipitation analysis had been repeated and the findings were consistent (data not shown). HA tags can be detected on the cell surface of Δ419-421MTHA+ but not Δ416-421MTHA+ (Figure 3.17 B upper panels). Analysis of MT distribution revealed that Δ416-421MTHA+ appeared to be trapped in
intracellular membranes (Figure 3.17 B lower panels). However, Western-blotting analysis of \( \Delta416-421MTHA^+ \) showed that the HA tag had been cleaved in this mutant, Figure 3.17 C.

\[
\begin{array}{cccc}
\text{wtMT} & \text{wtMTHA}^+ & \Delta419-421MTHA^+ & \Delta416-421MTHA^+ \\
\text{RRLGR TLLVTFALGLCMLFLIL KRSHF} & \text{RRLGR TLLVTFALGLCMLFLIL KRSHF AYPYDPYASL} & \text{RRLGR TLLVTFALGLCMLFLIL KRS AYPYDPYASL} & \text{RRLGR TLLVTFALGLCMLFLIL AYPYDPYASL}
\end{array}
\]

**Figure 3.16** Loss of hexapeptide is inhibitory in HA-tagged MT

A. Amino acid sequences of MTHA+ C-terminus with the deletion of three or six final residues. Designation of each MT species is listed on the left. MT mutant plasmids were generated by Dr N. Ichaso.

B. Transformation potential is assayed by foci formation. Sub-confluent Rat2 fibroblasts on 10 cm tissue culture dishes were transfected with 10 \( \mu \)g of plasmids carrying the above sequences. Images are of two independent foci assays.

C. Expression of MT in mutants shown in part A. Cell lines expressing each of the mutant MTs shown in panel A were isolated. Lysates were collected from confluent plates of cells. An equal volume of cell lysate from each line was separated by SDS-PAGE, Western-blotted, and then probed with anti-MT monoclonal antibody PAb762.
Figure 3.17 Immunofluorescence and immunoprecipitation analysis of Δ416-421MTHA+

A. Analysis of ability to bind signalling molecules in mutants shown in Figure 3.12. MT-immunoprecipitates were obtained as described before. Antibodies used to immune-bind (IB) known MT-associating proteins are shown above each panel. The migratory position of each detected polypeptide species is indicated by an open arrow to the right.

B. Hexapeptide mutant distribution was assessed by IF. Antibody against HA (HA11) or MT (PAb762) was added before or after fixation to label surface or total population of MT molecules, respectively, in cell lines indicated above the top panels. Cells were photographed using a Nikon inverted epifluorescence microscope at low magnification. Images of MT mutants are representative of an average of 11 independent frames.

C. Cleavage of HA tag in mutant Δ416-421MTHA+. Lysates from stable expression cell lines were separated by SDS-PAGE, Western-blotted, and then probed for HA. Membrane was then stripped of HA antibody before re-probing for MT using PAb762. Volume of mutant lysates were normalised against MT level in wtMTHA+. Two clones of Δ416-421MTHA+ were tested as indicated above the top panel.
In order to further investigate the sequence specificity of the C-terminus hexapeptide, a deletion mutant was made without the HA tag (Δ416-421MT) (Figure 3.18 A), based on the one designed by Dahl et al. (Dahl, Thathamangalam et al. 1992), along with a six-Glycine substitution of the region in HA-tagged MT (416G6MTHA+) (Figure 3.19 A). The number of foci formed by Δ416-421 MT was clearly lower than the wild-type MT-transfected cell monolayer (Figure 3.18 B). Uneven drying of the plates resulted in large circles of darker colours in the background. Similar to Δ416-421MT, 416G6MTHA+ show low frequency of foci formation when transfected into Rat-2 fibroblasts (Figure 3.19 B). Expression levels of both Δ416-421MT and 416G6MTHA+ were comparable to wild type (Figure 3.20). This suggests the sequence of the hexapeptide has a functional purpose for MT transformation of fibroblasts.

Cell lines expressing Δ416-421MT showed normal MT distribution, in perinuclear and peripheral membrane (Figure 3.18 C). The mutant 416G6MTHA+ can be detected at perinuclear region as well as peripheral membrane, much like wtMTHA+. However the HA tag was not detected on the cell surface of 416G6MTHA+ (Figure 3.19 C). Western blotting for total MT in both Δ416-421MT and 416G6MTHA+ revealed lower molecular weight fragments reactive to anti-MT antibody, and only the upper band of 416G6MTHA+ was reactive to the HA antibody (Figure 3.20). Δ416-421MT and 416G6MTHA+ were able to associate with all signalling molecules, including pp62c-yes, to the same level as corresponding wild type. Immunoprecipitation experiments had been repeated with additional clones for each mutant (data not shown).
Figure 3.18 Loss of C-terminal hexapeptide reduced the ability of MT to transform fibroblasts

A. Δ416-421 MT sequence compared to wild type. Designation of each MT species is listed on the left.

B. Transformation potential was assayed by foci formation. Sub-confluent Rat2 fibroblasts on 10 cm tissue culture dishes were transfected with 10 µg of plasmids carrying the above sequences. Images are representative of two independent foci assays.

C. Δ416-421 MT distribution at the plasma membrane. PAb762 was used to label MT in cells after fixation and permeabilisation. Cells were photographed using Nikon inverted epifluorescence microscope at low magnification. Images of Δ416-421 MT are representative of at least 11 independent frames from two independent experiments. Open arrows indicate the presence of MT at peripheral plasma membranes.

D. Analysis of the ability of Δ416-421 MT to bind signalling molecules. Lysates from Rat-2 fibroblasts stably expressing each mutant, as indicated above each lane, were analysed for MT content then normalised volumes of lysates were used for IP with PAb762. The immunoprecipitates were separated by SDS-PAGE for Western blotting. Cell line used for IP is indicated above each lane. Two different clones of Δ416-421 mutants were analysed (see numbers in brackets). Antibodies used to immunobind (IB) known MT-associating proteins are shown above each panel. The migratory position of each detected polypeptide species is indicated by an open arrow to the right.
Figure 3.19 Six-Glycine substitution of the C-terminal hexapeptide inhibited transformation

A. 416G6 MTHA+ sequence compared to wild type.

B. Transformation potential was assessed by foci formation, as described in Figure 3.18.

C. 416G6 MTHA+ distribution at the plasma membrane. Antibody against HA (HA11) or MT (PAb762) was added before or after fixation to label surface or total population of MT molecules, respectively, in cell lines indicated above the top panels. Open arrows indicate the presence of MT at peripheral plasma membranes. Images of cells were obtained with Leica TCS SP5 confocal microscope using 63.0x1.40 lens.

D. Analysis of 416G6 MTHA+ ability to bind signalling molecules. MT molecules were immunoprecipitated from lysates after normalising against of MT expression level as described in Figure 3.18.
Figure 3.20 Expression levels of hexapeptide region mutants

A. Δ416-421 mutant expression levels are comparable to wild type. MT cDNAs carrying deletion or glycine substitution of sequence Δ416-421 were expressed in Rat-2 fibroblasts. Lysates were collected from confluent stably clones grown on 10 cm tissue culture dishes. Varying volume of each lysate were analysed for MT contents compared to wild type by Western blotting using PAb762 anti-MT antibody. MT species expressed are indicated above the panels. Volumes (µl) of lysate loaded are indicated below each lane.

B. HA-tag analysis in 416G6 MTHA+. Equal volumes of lysates from cell expressing MT species indicated above each lane were separated by SDS-PAGE and transferred on to membrane by Western blotting. Membrane was first probed for HA tag then thoroughly washed in 1x TBS-T before MT detection. The upper half of the membrane was separated and probed for Hsp90 as a loading control. MT species expressed is indicated above each lane Antibodies used for immune-binding are indicated above each panel. Migratory positions of target molecules are indicated by open arrows.
3.3 DISCUSSION:

3.3.1 Summary

Murine PyV MT is a transmembrane protein that binds signalling molecules in discrete subcellular membrane sites (Figure 3.21). In this study, we have demonstrated that MT is a true tail-anchored integral transmembrane protein with a C-terminus translocated to the surface of the cell and a cytosolic N-terminal domain that binds signalling molecules. Newly translated MT can probably bind PP2A subunit A and C core dimer in the cytosol as MT lacking TMD has been shown to co-immunoprecipitate with PP2A (Messerschmitt, Disela et al. 1996). MT is first inserted in to ER membrane where it can bind pp60c-src but must migrate out of the ER in order to associate with additional signalling molecules such as pp62c-yes, ShcA, PI3K p85 and PLC-γ1 en route to the plasma membrane to fully transform fibroblasts (Figure 3.21). The ability of MTKDEL to selectively sequester pp60c-src in the ER provides the possibility of differentiating signal pathways controlled by pp60c-src and pp62c-yes. The TMD sequence plays a role in targeting MT to the membrane insertion machinery, which is mostly likely to involve the recently discovered TRC40/ASNA1 post-translational membrane insertion complex. In addition, association and probably the activation of PP2A are required for MT migration out of the ER to higher membrane sites.
Figure 3.21 A schematic representation of the sequence of events during the maturation of a transforming MT complex

Starting in the bottom right hand corner, where MT is synthesised in the cytoplasm on free polysomes, the stages in MT maturation through the ER, exocytic membranes to eventually reach the plasma membrane are shown. At each stage, the cellular proteins added to the MT complex are illustrated. A representative mutant that blocks each individual step is also indicated. Published on Zhou, et al. J Virol, 2011.
3.3.2 MT is an integral transmembrane protein that is first inserted in the ER

By the addition of two independent epitope tags, HA and FLAG, to the MT C-terminus, we can now confirm that MT is a transmembrane tail-anchored protein with a cytoplasmic N-terminus, a hydrophobic sequence spanning the lipid bilayer and that the C-terminus is translocated to the outer surface of cells. HA- and FLAG-tagged MT can be detected on the surface of transfected cells when primary antibodies specific for each tag sequence are added to living cells before fixation and without the need for permeabilisation of cell membranes (Figure 3.1). Both tags are widely used for studying the function and distribution of a wide range of proteins as well as purification and identification of the fusion proteins. Due to their small sizes, these epitope tags can be added to the C-terminus of MT without affecting its ability to transform cells, unlike a GFP insertion into MT sequence, which disrupted MT function (unpublished data).

In order to assess whether MT located in the endoplasmic reticulum (ER) can generate transforming signals, a specific ER-retention sequence, KDEL, was added to MT C-terminus. The complete localisation of MTKDEL in the ER suggests MT must be first inserted into membrane at the ER as insertion in any other localisation would bypass the KDEL receptor. Our results agree with previous observation that newly synthesised MT first accumulated in the perinuclear membranes soon after MPyV infection (Dilworth, Hansson et al. 1986).

We found that MT localised to the ER by KDEL cannot transform fibroblasts and that MTKDEL fails to associate with ShcA, p85 subunit of PI3K and PLC-γ1 despite binding and activating pp60c-src (Figure 3.2 - 3.5). Therefore the inability of MTKDEL to transform fibroblasts is probably due to the lack of access to ShcA, PI3K and PLC-γ1 rather than the lack of phospho-tyrosine sites on MT as MTKDEL was phosphorylated by the activated pp60c-src bound to this mutant (see Figure 3.6 for kinase assay results).
Our results correlate well with reports that BPV E5 localised in the ER inhibited transformation despite the mutant’s ability to induce PDGFRβ activation and auto-phosphorylation (Sparkowski, Anders et al. 1995) (see section 1.12 for introduction). Bovine papilloma virus (BPV) E5 is thought to activate the PDGFRβ in the Golgi by stabilising the active conformation of the receptor that is normally induced by the binding of ligands to the extracellular domains at the cell surface (Sparkowski, Anders et al. 1995, Lai, Henningson et al. 1998). We observed that mutant Δ402-406 is retained in the Golgi but can associate with all known signalling molecules of MT, indicating that they are all accessible to MT at this location in the cell (see Figure 4.14 in Chapter 4 or List of Mutants). However, in addition to the association with mitogenic signalling molecules, additional properties are required for MT to transform cells as mutant Δ402-406 failed to transform fibroblasts.

### 3.3.3 MTKDEL redistributes pp60c-src but not pp62c-yes

MTKDEL binds and redistributes pp60c-src but not pp62c-yes to the ER (Figure 3.10 - 3.12). This is most probably because MT has a higher affinity for pp60c-src over pp62c-yes (Kornbluth, Sudol et al. 1987). It is also possible that the known differences in trafficking between pp60c-src and pp62c-yes might be of relevance. Newly synthesised pp62c-yes initially accumulates in the Golgi region and is then trafficked to the plasma membrane through the exocytic pathway, a process mediated by the palmitoylation sites in the N-terminus (Sato, Obata et al. 2009). Unlike pp62c-yes, pp60c-src is not palmitoylated and does not traffic through the Golgi (McCabe and Berthiaume 1999, Resh 1999, Sato, Obata et al. 2009). pp62c-yes is of known importance to MT transformation because in mouse models of hemangioma (benign tumours of the blood vessels), pp62c-yes knock-out mice respond much more weakly to MT, forming fewer tumours and with longer latency that do pp60c-src or pp59c-fyn knock-outs (Thomas, Aguzzi et al. 1993, Kiefer, Anhauser et al. 1994).
contrary, pp60c-src is more important for mammary tumour formation in the MMTV-MT mouse model (Guy, Muthuswamy et al. 1994). Consequently it would be of interest to study the general role of pp62c-yes in signalling in isolation from the much better studied pp60c-src. MTKDEL may be a useful tool for this purpose.

3.3.4 PP2A plays a role in MT migration to higher membrane sites

Since ER-localised MT cannot transform fibroblasts, there must be some kind of signal on the MT sequence that targets the tumour antigen to the plasma membrane where it can associate with additional signalling molecules such as ShcA adaptor proteins, p85 subunit of PI3K and PLC-γ1. The generally accepted view is that the plasma membrane targeting signal is contained within and round the TMD of the TA protein (Borgese, Brambillasca et al. 2007). We investigated whether the hydrophobic sequence alone can dictate where in the cell MT is eventually located and we found two lines of evidence that the hydrophobic sequence in itself is not sufficient for targeting MT to the plasma membrane, association with the PP2A core dimer is also important. We first substituted the MT hydrophobic sequence with a transmembrane sequence from another TA transmembrane protein Sec61β, which resides mainly in the ER and its localisation is thought be mediated through its TMD sequence (Butler, Watson et al. 2011). Transformation efficiency assayed by foci formation was reduced in MT Sec61β but not inhibited (Figure 3.2). More of MTSec61β was located in the ER compared to normal MT but a substantial amount still reached the plasma membrane (Figure 3.5). This indicates that there is a dominant MT property outside of the transmembrane region which contributes to directing MT to the plasma membrane.

Our lab and others have previously reported that MT mutants lacking the ability to bind PP2A are retained in cytoplasmic membranes, probably the ER (Brewster, Glover et al.
1997). Re-examining the subcellular distribution of these mutants confirmed the correlation between the lack of PP2A association and localisation of these mutants in cytoplasmic membranes resembling the ER (Figure 3.5 – 3.9 and Table T3.1). Using antibody PAb701 which specifically binds to the MT N-terminal region required for PP2A association (Figure 3.8), we visualised the sub-population of MT, which has not yet associated with PP2A or has lost this association in the cell, and found that this subpopulation of MT is localised to mostly cytoplasmic or perinuclear regions (Figure 3.9 and Table T3.1). Taken together, our data suggests strongly that association with PP2A is required for MT to relocate from the ER to the plasma membrane (Zhou, Ichaso et al. 2011).

Interestingly, treatment with OA, an inhibitor of PP2A Ser/Thr phosphatase activity, also resulted in accumulation of MT in the cytoplasmic membrane, while inhibition of Src kinase activity with dasatinib did not affect MT distribution (Figure 3.7). This observation could be due to OA inhibition of PP2A or to general disruption of protein export through the Golgi which has also been reported (Dinter and Berger 1998, Pryde, Farmaki et al. 1998). The nano molar concentrations of OA used in our study are likely to inhibit PP2A with high specificity. In fact, PP2A in complex with MT becomes extra sensitive to OA inhibition, probably due to changes in PP2A substrate specificity upon binding to MT (Cayla, Ballmer-Hofer et al. 1993, Favre, Turowski et al. 1997).

There is increasing evidence to support a role for PP2A in regulating exocytosis. PP2A has been shown to regulate cell surface expression of the T cell receptor (Lauritsen, Menne et al. 2001), mast cell degranulation (Sim, Ludowyke et al. 2006) and insulin secretion in rat pancreatic cell line INS-1 cells (Kowluru, Seavey et al. 1996). It has been suggested that phosphatases such as PP2A bound to multi-unit complexes such as the MT signalosome, which contains kinases, may act as a constitutive regulatory complex for rapid
response to positive or negative stimuli (Mandavia and Sowers 2012). Overall, we have found strong evidence that PP2A plays a regulatory role in trafficking.

3.3.5 Membrane Targeting and Insertion Mechanism for Tail-Anchored Proteins

While the C-terminal hydrophobic sequence of MT is responsible for association with cellular or artificial membranes, in vitro microsome association assays demonstrate that the wild-type protein does not spontaneously insert into membranes (Hofer, Wehrle et al. 1995, Kim, Janiak-Spens et al. 1997). Unlike many cell surface GFRs which are inserted by a co-translational insertion mechanism, MT lacks the ‘signal peptide’ N-terminal to the hydrophobic sequence (Templeton, Voronova et al. 1984). Because of the close proximity of the transmembrane hydrophobic region to the C-terminus of TA proteins, the translated peptide needs to be released from the ribosome first before membrane integration. There are two distinct pathways for assisted insertion of TA proteins, the Hsp40/Hsc70-mediated pathway and the newly discovered TRC40/ASNA1 mediated pathway, which is homologous to the post-translational Guided Entry of Tail-anchored (GET) protein pathway found in yeast (Stefanovic and Hegde 2007, Favaloro, Spasic et al. 2008). Several lines of evidence suggest MT is inserted by the Transmembrane Domain Recognition (TRC) pathway. The hydrophobicity of the TA region dictates whether a precursor is delivered to the ER via the molecular chaperones Hsp40/Hsc70 or the TRC40/ASNA1-dependent route (Stefanovic and Hegde 2007, Favaloro, Spasic et al. 2008, Rabu, Wipf et al. 2008). The hydrophobicity value of MT TMD is similar to those found in the hydrophobic region of many SNARE proteins which regulate the transport of vesicles inside the cell. It was found that SNARE proteins preferentially utilise the TRC40/ASNA1 pathway for their insertion into the ER membrane (Borgese, Brambillasca et al. 2007, Rabu, Wipf et al. 2008). However, many obstacles prevented the elucidation of possible MT and ASNA1 association. Among all the known
GET pathway components in yeast (Get1/Get2/Get3/Get4/Get5/Sgt2), only Get3 ATPase homology, TRC40/ASNA1 has been identified in the mammalian system so far (Jonikas, Collins et al. 2009, Stefer, Reitz et al. 2011, Kubota, Yamagata et al. 2012). Therefore, it is possible that MT does not have to directly bind to TRC40/ASNA1. In order to study MT membrane insertion mechanism, expression of MT mutants in the yeast system is probably required.

There are over 300 Tail-anchored (TA) proteins found in mammalian cells, examples include many SNARE proteins, subunits of the cytochrome complex (cytochrome \( b_5 \)) found on the outer membrane of mitochondria and Bcl-2 family proteins that regulate apoptosis (Borgese, Colombo et al. 2003, Rabu, Schmid et al, 2009). MT and its C-terminal mutants may be additional tools for the study of the process of TA transmembrane protein biogenesis in mammalian cells.

3.3.6 Residues KRS adjacent to the hydrophobic sequence are important for MT function

We have also found that deletion or mutation of the three residues immediately C-terminal to the hydrophobic sequence can also affect transformation (mutant \( \Delta 416-421 \) and 416G6 MT).

It has been suggested amino acids immediately surrounding the hydrophobic regions are important for the function of MT in transformation and may act as anchors at the membrane borders (Templeton, Voronova et al. 1984). Focus assay results in Figure 3.18 and 3.19 suggest sequence KRS (416-418), has a role in MT function. Removal or Glycine substitution of this sequence resulted in proteolytic cleavage from the C-terminal end as the lower fragment of 416G6MTHA+ was only reactive to anti-MT but not anti-HA antibody. It seems that the C-terminus hexapeptide enriched with positively charged amino acids may serve to
protect the MT C-terminus from degradation. Both mutant Δ419-421MT and 416G6MTTHA+ bind to all tested MT-associating signalling molecules, including pp62c-yes, at levels comparable to wild type MT (Figure 3.18 and 3.19). Therefore, additional properties other than binding to Src and signalling molecules are important for MT transformation. The HA-tag added to 416G6 was not detected on the cell surface (Figure 3.19). The lack of HA-tagged MT on cell surface suggests that the C-terminus of mutant 416G6MTTHA+ fails to translocate across the lipid bilayer, such that both the N- and C-termini are orientated towards the inside of the cell. Positively charged residues, often located near the boundaries of transmembrane segments, appear to be involved in specifying the topology of membrane proteins (Dalbey 1990) (Dahl, Thathamangalam et al. 1992). The juxtamembrane region near the cytosol-membrane or extracellular-membrane interface of a GFR often modulates TMD dimerisation, oligomerisation and kinase domain activation (Irusta and DiMaio 1998, Arkhipov, Shan et al. 2013). The role of MT TMD topology is explored in the next chapter.
CHAPTER 4 – ONCOGENIC SIGNALLING BY MPyV MT OCCURS IN DISCRETE MEMBRANE COMPLEXES

4.1 INTRODUCTION:

We have demonstrated in the previous chapter that once inserted into the ER, the MT binds to pp60c-src, and then a combination of the TMD sequence and the PP2A binding site is responsible for MT migrating into the membrane compartment beyond, where it interacts with PI3K, ShcA and PLC-γ1 (Figure 3.21). However, there must be an additional function for the TMD as hydrophobic domain mutants have been isolated that still bind to membranes but fail to transform (Markland, Cheng et al. 1986). In the previous chapter, we also demonstrated that deletion of the three amino acids immediately C-terminal to the hydrophobic sequence, KRS, also reduced foci formation, but still associated with membranes and all known MT-binding proteins (Figure 3.18 and 3.19). Hydrophobic sequence of VSV G protein and cytochrome b5 failed to functionally replace MT TMD sequence. Both hybrid MT species associated with membranes but failed to transform (Templeton, Voronova et al. 1984, Kim, Janiak-Spens et al. 1997). All of this evidence points to additional properties encoded in the MT TMD sequence, which is probably required for transformation.

To examine the membrane binding and location requirements for MT to transform, a series of mutants in the C-terminal region were generated and studied in detail by immunofluorescence (IF) and immuno-electron microscopy (IEM). We report here that MT can be detected in discrete signalling clusters in the outer cell membrane. New non-transforming mutants defective in their ability to form clusters, despite their association with all known signalling partners, have been isolated from this study, therefore suggesting that
assembly of a macromolecular complex is an essential requirement for MT oncogenic signalling. Activated growth factor receptors are also found in similar clusters suggesting complexes are also required for their activity. In addition, we show that the length and sequence of the TMD are important for targeting MT from the Golgi apparatus to the plasma membrane.
4.2 RESULTS:

4.2.1 MT forms large complexes on cell surface

Previously, we have shown that a short epitope tag, such as an extended version of the HA tag or the FLAG tag can be added to the C-terminus of MT without affecting its transforming potential. Both epitope tags can be detected on the surface of cells expressing these tagged MT species. Therefore at least a detectable population of MT is located on the cell surface. Close examination of surface MT with either an anti-HA or anti-FLAG antibody at high magnification revealed that surface MT molecules are not uniformly distributed but are present in discrete patches (Figure 4.1 A). When a single cell was imaged from top to bottom at high magnification, the punctate spots were observed throughout the whole external membrane, from the top of the nucleus (where the spots are observed in a single small area) to the bottom under the bottom surface of the cell (Figure 4.1 B). Some of these patches are over one micron across (Figure 4.2), which is too big to contain only one MT molecule.

These complexes are not likely to be caused by endocytosis induced by antibody cross linking because permeabilisation of the plasma membranes after live incubation with anti-HA antibodies did not increase the level of epitope tag staining (Figure 4.3) and surface MT clusters did not colocalise with markers for endocytosis. APPL-1 is a marker for peripheral recycling vesicles and EEA-1 and Rab-5 are early endosome markers (Figure 4.4). Upon binding to primary antibody, MT does not readily enter into the cells, unlike transferrin receptors. The process of endocytosis can be tracked by incubating cells expressing MT-FLAG in media containing Alexa Fluor 488-conjugated human transferrin and rabbit anti-FLAG antibodies for 30 min at 37°C and very little FLAG antibody was found inside the cells (results not shown).
Figure 4.1 Surface MT is found in discrete focal patches over the whole surface of the cell

A. IF analysis of surface labelled MT viewed under x 100 high magnification. Cells expressing epitope-tagged MT, as indicated below the panel, were incubated with anti-HA or anti-FLAG primary antibody at 37°C before fixing. Images were acquired using Nikon inverted epifluorescence microscope with 100x magnification lens. Surface labelled of HA was performed by S. Dilworth. Surface FLAG labelling was performed on a separate occasion.

B. Surface MT clusters were distributed on the whole of cell surface. IF labelling surface MT was performed, as described above. Z-stack images were taken at 0.13 μm intervals on Leica SP5 confocal microscope at high magnification. Cell types are indicated on the left. Antibodies added are indicated on the right. Images are shown from the bottom (a), middle (b) and top (c) of the stack. Scale bar, 5 μm. Images in A and B are representative of at least 4 cells for each mutants from at least 4 repeated experiments.
Cells expressing epitope-tagged MT, as indicated on the left were incubated with anti-HA (a, c) or anti-FLAG (b, d) primary antibody before fixing. Non-transfected Rat-2 fibroblasts were used as a control (c, d). High magnification images of cell under surface are acquired using confocal microscope. Antibody used is indicated above and below the panels. Examples of MT surface clusters with an equivalent diameter of 1 µm are circled. Scale bar, 2 µm.
Figure 4.3 Permeabilisation of plasma membrane does not enhance the fluorescence signals of externally labelled HA-tags

Cells expressing HA-tagged MT were incubated with anti-HA primary antibody before fixing with (b) or without (a) membrane permeabilisation step. Fluorescent-conjugated secondary antibody was added to label HA. Non-transfected Rat-2 fibroblasts were used as control (ii). X20 magnification images of cells were acquired using Nikon fluorescent light microscope. Cell types are indicated on the left.

Figure 4.4 Surface MT does not colocalise with endosome markers

MT on cell surface was labelled by incubating live cells in media containing anti-HA or anti-FLAG antibodies (green) for 30 min before fixing. Cells were subsequently permeabilised and antibodies targeting individual endosome markers were added, as indicated in red. Nuclei were stained by DAPI (blue). Scale bar = 5 µm.
Surface MT analysis was performed by adding antibody for 20–30 minutes to living cells as we found it impossible to exclude a small amount of antibody penetration into cells after fixation. However, even though the time was short, it is still feasible that the patches observed were a consequence of cross-linking the MT through the use of divalent antibodies. To exclude this possibility, we first varied the time of addition of the anti-HA primary antibody between 1 minute and 6 hours, and in various buffers. In Dulbecco’s PBS (DPBS), the number of complexes and their size did not vary with time, only the intensity of staining altered (data not shown). However, in DMEM the number and size of complexes remained similar up to 4 hours, but then smaller quantities of larger complexes were observed. It seems likely, therefore, that incubations of 30 minutes or less are unlikely to cross link the MT. To

Figure 4.5 MTFLAG clusters on the cell surface can be labelled at 4°C

Surface labelling of FLAG tag at 4°C and 37°C was performed with the same antibody dilution as described in material and methods. After fixing, fluorescent-conjugated secondary antibody was subsequently added at RT to label the FLAG tag. The fluorescent signals on the basement membrane of a cell were captured using Leica SP5 confocal microscope with 63.0x1.40 oil lens, zoom factor 6. Each image represent an area of 20.5 x 20.5 μm. Scale bar = 2 μm. 3 independent frames are shown for IF labelling FLAG at 4°C (top panels) and 37°C (bottom panels), respectively.

Surface MT analysis was performed by adding antibody for 20–30 minutes to living cells as we found it impossible to exclude a small amount of antibody penetration into cells after fixation. However, even though the time was short, it is still feasible that the patches observed were a consequence of cross-linking the MT through the use of divalent antibodies. To exclude this possibility, we first varied the time of addition of the anti-HA primary antibody between 1 minute and 6 hours, and in various buffers. In Dulbecco’s PBS (DPBS), the number of complexes and their size did not vary with time, only the intensity of staining altered (data not shown). However, in DMEM the number and size of complexes remained similar up to 4 hours, but then smaller quantities of larger complexes were observed. It seems likely, therefore, that incubations of 30 minutes or less are unlikely to cross link the MT. To
provide confirmation of this, we next performed live cell labelling at 4°C when the surface proteins are unlikely to move within the 30 minute time scale of the experiment. Previous experiments performed by Stainman et al had established that the process of endocytosis slows down at lower temperatures and become negligible below 10°C (Steinman, Silver et al. 1974, Silverstein, Steinman et al. 1977). The number and size of MT containing clusters labelled with anti-FLAG antibodies on the surface of cells expressing wild type MTFLAG did not change between incubation at 4°C or 37°C (Figure 4.5), only the intensity varied. Therefore it seems likely that these clusters are not caused by the addition of the antibody to living cells, but already exist on the cell surface. These patches are much too large (generally larger than 0.1 µm², an area equivalent to a circular complex with a diameter of 400 nm) to represent a single MT molecule, so it seems likely that MT is present in discrete complexes on the surface of cells. This is verified by a high degree of colocalisation between the FLAG-tags labelled from the cell surface and MT labelled internally (Figure 4.6). Therefore the MT complexes detected by IF are a genuine representation of MT distribution on the cell surface.
In order to quantify the level of colocalisation between surf ace MT complexes and MT on the cytoplasmic leaflet of the plasma membrane, pixels from the two different fluorescent channels were analysed by using the JACoP plug-in for Image J developed by Bolte and Cordelieres (Bolte and Cordelieres 2006). The degree of colocalisation between the red (MT N-terminus labelled with PAb762) and green (surface labelled MT) channel is measured by Mander’s overlap coefficient, M1 and M2, respectively. M1 represents the fraction of red pixels co-localising with the pixels in the green channel and M2 represents the fraction of green pixels co-localising with the red pixels at the same position. Since M2 is generated from the surface MT signals, it can be interpreted as the surface MT complexes co-localising with internally labelled molecules. Pearson’s co-efficient (Pr) is a measurement of the quality of colocalisation that is how much each pixel is deviating from the central axis of colocalisation between the two channels. A Pr value of +1 indicates perfect colocalisation; 0, reflects an independent relationship between the two channels; -1, represents mutually

Figure 4.6 MT clusters labelled from the cell exterior correlate with MT molecules labelled from the inside of cells

A. Dual IF labelling of surface FLAG and total MT. Rabbit anti-FLAG antibodies were added to living cell. After fixation and permeabilisation, cells were incubated in PAb762 mouse anti-MT N-terminus to label total MT from the inside. Species-specific Alexa Fluor 488- and 555-conjugated secondary antibodies were added to label FLAG (green) and MT (red) respectively. Non-transfected cells were labelled in the same conditions. An area of 20.5 µm x 20.5 µm on cell basement membrane was shown. M1 and M2 values for each image are indicated below the panel.

B. Surface FLAG tag colocalising with MT labelled from the inside of a cell. Zoom-in images from above highlighted area are shown (10.25 µm x 10.25 µm). The population of MT labelled in each channel is indicated above the panel, with surface labelled FLAG in green and total MT in red. Scale bar, 2 µm.

C. Quantitative co-localisation analysis for surface MTFLAG complexes and MT at the plasma membrane compared to control cells. Zoom-in images of 20.5 x 20.5µm were used for colocalisation analysis with ImageJ Plug-in (JACoP). 6 independent frames of MTFLAG and 4 of control were used for analysis. Results shown are representative from at least 3 independent experiments.

See image on previous page.
exclusive relationship (www.svi.nl/ColocalizationCoefficients). As Figure 4.6 C shows, colocalisation between surface MT complexes and MT on the inside of the cell occurs at about 80%. Although Pr and M2 values are low, these may be distorted by the much more numerous and intense pixels from the red channel.

4.2.2 Surface MT complexes contain signalling molecules

Incubation of living cells expressing epitope-tagged MT with tag-specific antibodies allows the detection of the MT located on the extracellular surface of the plasma membrane, therefore separating the population of MT on the cell surface from those located on the cytoplasmic leaflet of the plasma membranes. Figure 4.6 shows this is a valid method for analysing surface MT colocalisation with molecules located on the inner membrane leaflet. Next, we investigated whether these large surface complexes of MT contain active signalling molecules. Most of the MT present in cells is associated with PP2A, but less than 20% binds to a Src family tyrosine ((Bolen, DeSeau et al. 1987) and unpublished data). Association between MT and pp60c-src causes a rapid phosphorylation of ShcA on tyrosine 239, 240 and 317 (Nicholson, Empereur et al. 2001).
Figure 4.7 Colocalisation level between activated ShcA and surface MT complexes in cells expressing wild type or the non-transforming mutant 200Δ10 MTHA+

A. Dual IF labelling of surface HA complexes with activated ShcA. Surface MT was labelled with a mouse anti-HA antibody added to living cells before fixation. Total internal phosphorylated ShcA was labelled with rabbit antibody specific to phosphorylated Y317 and Y239 after membrane permeabilisation. Secondary anti-mouse IgG coupled to AlexaFluor488 and anti-rabbit IgG antibody coupled to AlexaFluor555 was added to label MT (green) and phospho-ShcA (red). Images were taken on Leica SP5 confocal microscope. An area of 20.5 x 20.5 µm was shown for each channel. Scale bar, 2 µm. The MT species expressed are indicated to the left of the panel, and the primary antibody used above each image.

B. Colocalisation analysis for surface MT and phospho-ShcA Pixels in an area of 20.5 x 20.5 µm were analysed using the ImageJ Plug-in (JACoP) using Costes’ randomisation method. At least 12 independent frames from each cell type were used for analysis.
In order to determine whether the clusters of HA-tagged MT on the cell surface membrane represent active signalling complexes or ‘uncomplexed’ inactive MT, we performed dual immunofluorescence labelling to detect the location of surface wtMTHA+ and phosphorylated ShcA (Y239/Y240/Y317) in the same cells. Figure 4.7 A shows there is a high degree of co-localising between surface MT complexes and phosphorylated ShcA in confocal micrographs with wild type MT expressing cells, but very little overlap in cells expressing a mutant 200Δ10 MTHHA+, which fails to bind c-SRC and subsequent signalling molecules including ShcA (Brewster, Glover et al. 1997). This mutant does not possess Src tyrosine kinase binding region near the N-terminal region while its PP2A binding sequence is preserved (Brewster, Glover et al. 1997). Despite not associating with SRC tyrosine kinase and other signalling molecules, 200Δ10 MTHA+ does still form surface clusters (Figure 4.7 A). To confirm this qualitative observation of co-localisation, a series of these images were analysed by the ImageJ JACoP plug-in (Bolte and Cordelieres 2006). Figure 4.7 B shows the results of this analysis and demonstrates a significant co-localisation between surface MT and phospho-ShcA in wild type MT expressing cells, but little with the non-ShcA binding mutant 200Δ10. Therefore, these surface MT clusters contain active ShcA molecules, so are likely to represent active signalling complexes, but probably do not require the binding of Src tyrosine kinase to form.

To investigate whether the activity of Src tyrosine kinase is required for MT cluster formation, we next treated cells that express wild type MTHA+ with dasatinib, a tyrosine kinase inhibitor with dual specificity for Src and Bcr-Abl, for 48 hrs before labelling surface HA and activated ShcA. Figure 4.8 A shows the signal of phospho-ShcA was reduced dramatically by dasatinib treatment without affecting the formation of MT complexes on cell surface. Colocalisation analysis showed that the amount of surface MT complexes containing activated ShcA dropped to the basal level observed in mutant 200Δ10. A similar graph was
produced when Pearson’s coefficient was analysed (data not shown). Therefore the colocalisation between surface MT complexes with activated ShcA is specific to MT transformation activity. The formation of large MT complexes on the cell surface membrane does not appear to require Src tyrosine kinase activity.

Figure 4.9 demonstrates the effectiveness of dasatinib concentration used in above assays. Tissue culture media containing 10 or 100 nM dasatinib inhibited foci formation normally induced by MT transfection when the inhibitor was added one day after the transfection with fresh drug added every 3 or 4 days. Transformed cells preferentially utilise the glycolytic pathway to generate energy to meet their high metabolic demands. The lactic acid produced as a by-product of glycolysis reduces the pH in the culture media and causes a colour change in the media from pink to yellow. Even at 1 nM, dasatinib slowed down the proliferation of cells expressing MT and prevented this colour change during the 48hr incubation period.
Figure 4.8 Dasatinib reduces colocalisation level between activated Shc and surface MT complexes

A. Surface MTHA+ complexes and total internal phospho-Shc A were labelled as described in Figure 4.5A. An area of 20.5 x 20.5 µm is shown. Scale bar, 2 µm. Treatment is indicated to the right of the panel, and the primary antibody used above each image. Wild type MTHA+ cells were treated with either 50 nM dasatinib or vehicle (DMSO) in 10% serum tissue culture media for 48hr before IF staining.

B. Fraction of surface MT complexes colocalising with phospho-Shc A. Analysis was performed as described in 4.5B. At least 12 independent frames from each cell type were used for analysis. M2 values, representing the fraction of surface HA colocalising with internal phospho-Shc A, were obtained from 5 frames of DMSO-treated MTHA+ cells, 9 frames of dasatinib-treated cells and 7 frames of 200Δ10HA+ cells.
Figure 4.9 Growth inhibition of MT-expressing cells by dasatinib

A. Colorimetric determination of effective dasatinib concentration. Subconfluent Rat-2 fibroblasts expressing wtMT or non-transfected cells were seeded on to 24-well plate. Next day, increasing concentrations of dasatinib were added to cells in 10% FCS tissue culture media. Equal volume of solvent, DMSO, was added to the well above or below the well with dasatinib. Cells were grown for 48hr before scanning. Effectiveness of dasatinib at various concentrations was inversely correlated to the colour change of the media.

B. Dasatinib inhibits MT-induced foci formation. Subconfluent Rat-2 fibroblasts grown on 10 cm tissue culture dishes were transfected with 10 µg wtMT plasmids overnight (b – e). Cells were grown for two weeks in tissue culture media with dasatinib at 10 nM (d), 100 nM (e) concentration, or, vehicle (DMSO) equivalent to 100 nM dasatinib (d). Tissue culture fluids were changed every 3 to 4 days with fresh inhibitor / DMSO added. Mock transfection without DNA or drug treatment was used as a control.
4.2.3 New non-transforming mutants of MT TMD isolated

The transmembrane region of MT must have properties important for transformation, in addition to its role in localising MT to the membrane since not all transmembrane domains can replace the MT hydrophobic sequence (Templeton, Voronova et al. 1984, Markland, Cheng et al. 1986) (Kim et al., 1997), suggesting that another property in addition to membrane binding is also required.

Upon ligand binding, many receptor tyrosine kinases dimerise via a central segment in the TMD (Sternberg and Gullick 1989). Introduction of a charged residue in the central region of the hydrophobic membrane spanning sequence of some receptors results in close packing of neighbouring α-helices by generating additional hydrogen bond links, hence constitutive dimer formation and receptor activation, which contributes to naturally occurring diseases (Sternberg and Gullick 1989, Gullick, Bottomley et al. 1992). Examples of constitutively activating mutant growth factor receptors include the oncogenic Neu/ErbB2 mutation V664E (Hynes and Stern 1994) and FGFR3 mutation G380R implicated in achondroplasia, a form of dwarfism (Shiang, Wasmuth, et al. Cell, 1994). There is some similarity between a five-amino-acid stretch of the TMD of MT and Neu (Figure 4.10), although it should be noted that the two membranes have opposite topology. Nevertheless, we were interested in testing the effect of equivalent neu mutation upon MT function. Consequently, the membrane spanning sequence on MT was replaced by those from wild type neu receptor and constitutively activated neu-mutant (V664E) by Dr N. Ichaso and Prof. S. Dilworth. In addition, MT L405E, which simulates the activating neu V664E, and MT G406L, which is equivalent to an inhibitory Neu mutation, were also generated (Gullick, Bottomley et al. 1992). Interestingly, the results were exactly the opposite of those expected from their effects on receptor kinase activation. The wild type neu hydrophobic sequence was
able to replace the MT TMD and induced foci formation, but the *neu* mutant sequence with a V664E mutation could not. G406L transformed almost as well as wild type MT, whereas mutant L405E completely inhibited foci formation (Figure 4.10). Deletion of the central five amino acids (Δ402-406, Figure 4.10) also abolished transformation but this may reflect the shortening of the hydrophobic sequence rather than any specific effect (see 4.2.5 below). Substituting short stretches of MT hydrophobic sequence with Alanine did not affect the transformation potential of MT (394A4 to 412A4, Figure 4.10) so there seems to be little sequence specificity in the hydrophobic region of MT. However, during this study, new mutants had been isolated which completely abolished foci formation and some of these were characterised here.

Cell lines stably expressing various MT mutants shown in Figure 4.10 were isolated. Analysis of immunoprecipitated L405E and the *neu*-mutant showed that the two non-transforming mutants are capable of binding to all of the signalling molecules that bind directly to the transformation competent MT (Figure 4.11 A). Although there seems to be less ShcA in the L405E immunoprecipitates, this is likely due to a variation in the amount of mutant MT pulled down from that particular experiment. Repeated immunoprecipitation experiments found comparable level of ShcA and PI3K in complex with L405E as with wild type (see Figure 4.16). A kinase assay, performed by Prof. S. Dilworth, on the immunoprecipitated MT mutant, showed L405E and the signalling molecules bound to it were phosphorylated, including Grb2 (data not shown). Therefore, the lack of transforming activity in mutant L405E and *neu*-mutant was not due to a deficiency in association, and subsequent activation, with known MT-interacting signalling proteins. These results also imply that the mutant MT species must have inserted into cellular membranes as expected, as mutants that fail to interact with membranes do not bind to pp60*c-src* and other signalling polypeptides (Carmichael, Schaffhausen et al. 1982, Markland, Cheng et al. 1986).
<table>
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Figure 4.10 Mutagenesis of MT hydrophobic sequence and their effects on MT transformation. Residues or sequence mutated are highlighted in gray. Bold letter indicates residues mutated in constitutive activation mutants of neu(neu-mutant) or FGFR3 receptor (FGFR3-mutant). Transformation efficiency was assayed by the ability to induce foci formation when transfected into Rat-2 fibroblast cell monolayer and results are summarised on the right with ‘-’ being non-transforming and ‘++++’ being equivalent to wild type levels of transformation. Mutants were constructed by Dr Ichaso and Prof. Dilworth. Foci assays were performed by Kunal Shah and others.
Figure 4.11 Characterisation of non-transforming TMD mutants L405E and neu-mutant

A. New hydrophobic domain mutants bind to signalling molecules. Cell lines expressing each MT species were generated and lysates were collected. Each MT expressed was immunoprecipitated from a volume of lysates normalised against MT level. WB analysis was performed to identify each MT-associated polypeptide, as indicated above each panel. Open arrows to the right indicate the migratory position of the target polypeptide labelled above each panel. IP and WB were performed by Prof. S. Dilworth.

B. Lack of surface staining in non-transforming neu-mutant. IF staining for surface tag was performed using an anti-HA antibody added to living cells followed by fixation and then fluorescent-conjugated secondary added. Total MT was labelled by PAb762 after fixation. Cells were imaged at low magnification using a Nikon inverted epifluorescence microscope. IF was performed by Prof. S. Dilworth.

C. HA tags on hydrophobic domain mutants remain intact. After normalising MT level, lysates from various hydrophobic domain mutants with added HA+ were analysed by Western-blotting for HA (i). The membrane was stripped and re-probed for total MT (ii). Lane f and g are from two clones of Δ416-421MTHA+ (b1 & d3), which were found to have lost their HA-tags.
4.2.4 Subcellular location and distribution of MT hydrophobic domain mutants

The lack of transformation by MT mutants L405E and neu-mutant, despite their interaction with all the known MT associated proteins, suggested yet again that an unidentified property of MT related to membrane binding is also required for its transforming ability. To determine whether precise location could be this additional activity, the subcellular distribution of these mutants in stable cell lines were examined by immunofluorescence (IF) with the mouse monoclonal PAb762 reactive to MT N-terminal sequence (Figure 4.12 B (i)). The results for mutants G406L and L405E are compared to wtMT. Very little difference between all three cell lines could be observed. Like wild-type MT, the non-transforming mutant L405E is localised to both intracellular membranous compartment as well as the plasma membrane. We had previously observed the formation large complexes of wild type MT on the outer cell membrane (Figure 4.1 and 4.2). To examine whether these complexes are present on the surface of cells expressing these novel TMD mutants, an HA+ tag sequence was added to the C-terminal tails of these MT mutants and cell lines expressing each species were established. Properties of these epitope-tagged mutants, including transformation, protein binding and total MT immunofluorescence patterns, were similar to those of the untagged mutant (data not shown). The amount of HA+ tag exposed on the cell surface of these lines was then detected with an anti-HA antibody added to living cells before fixing. As Figure 4.12 B (ii) shows, there is very little HA+ exposed on the surface of L405E MTHA+ expressing cells compared to the wild type and transforming mutant G406L. Again, the results were verified by adding an HA+ tag to the MT neu-wt and MT neu-mutant constructs and examining surface HA-tags (Figure 4.11 B). Western blot analysis showed that the HA-tag was not cleaved from the C-terminus of L405E (Figure 4.12 C) and that the amount of HA tag present in the lysates of L405E and G406L were comparable after normalising MT content.
Therefore, it is likely that the C-terminus of the hydrophobic domain mutant, L405E, failed to translocate across to the cell surface membrane. Similar findings were observed with \textit{neu}-mutant MTHA+ (Figure 4.11 B & C), so we concentrated on the MT L405E and MT G406L mutations.

Figure 4.12 L405E C-terminus failed to translocate to the outer cell surface

A. Hydrophobic sequence of TMD mutants with HA+ tag. Cell lines stably expressing these mutants were generated by N. Ichaso and S. Dilworth.

B. Lack of surface HA staining in non-transforming L405E HA+. Total MT in cell lines expressing untagged MT mutants (a - c) was labelled by PAb762 after fixation (i). Surface MT in cell lines expressing MT with HA+ tag (d – f) was labelled by anti-HA antibody added to living cells prior to fixation (ii). Cells were photographed using a Nikon inverted epifluorescence microscope. Magnifications are indicated on the right; antibodies used are labelled on the left; cell lines are indicated above or below the panel.

C. HA tag is not cleaved in L405EHA+. After normalising MT level, lysates from hydrophobic domain mutants with HA+ tag were analysed by Western-blotting for HA (i). The membrane was stripped and re-probed for total MT (ii). Non-transfected Rat-2 lysate was used as control.
In order to verify our results with L405E HA+, FLAG-tagged versions of mutants L405E and G406L were constructed. The transformation potentials of the two mutants were assayed by their ability to induce foci formation when transfected into fibroblast cell monolayer (Figure 4.13 B). The mutant L405E MTFLAG was completely defective for its ability to transform, whereas G406L MTFLAG induced a moderate number of foci formation in Rat-2 fibroblasts (note that G406L also had a lower transformation potential compared to wild type, Figure 4.10). Stable cell lines were generated and L405E MTFLAG localisation to the plasma membrane was confirmed by labelling MT with PAb762 and the plasma membrane with Alexa Fluor 594-conjugated wheat germ agglutinin (WGA) (Figure 4.14 C). WGA binds to sialic acid residues, commonly found on glycosylated proteins and lipids on the cell surface membrane. The fluorescent conjugated WGA does not penetrate the cell membrane and therefore only labels the plasma membrane of cells. In stable cell lines, very little of the epitope tag was detected on the surface of stable cell lines expressing mutant L405E FLAG, while the transforming counterpart G406L FLAG showed translocation of the C-terminal tag to the cell exterior (Figure 4.13 D). The FLAG tag remains intact on the L405E C-terminus (Figure 4.13 E).
Figure 4.13 Hydrophobic domain mutants with FLAG-tag

A. Hydrophobic sequence of TMD mutants with FLAG tag. Mutations are indicated in bold with underline.

B. L405E mutation abolishes transformation. Foci formation assays were performed with 10 µg plasmids carrying wild-type or TMD mutants as described before. Mock transfection without plasmid was used as control.

C. L405E FLAG is present at the plasma membrane. Cell lines stably expressing mutants shown in A were generated. Plasma membranes in cells stably expressing wild-type or mutant MTFLAG were labelled with AlexaFluor594 WGA. After fixing and permeabilisation of membranes, total MT in cells was labelled with PAb762 anti-MT. Z-stack image of a single cell was obtained using a Leica SP5 confocal microscope 63.0x1.40 oil lens, step size = 0.13 µm. Only the cross-section near the bottom of the cell is shown. Open arrows indication colocalisation between MT (green) and WGA (red). Scale bar = 20 µm.

D. IF analysis of FLAG-tag on cell surface. IF labelling for the surface tag was performed using an anti-FLAG antibody added to living cells stabling expressing the mutants (indicated above the panel), prior to fixing. Cells were imaged using Leica SP5 confocal microscope at low magnification Scale bar = 50 µm.

E. FLAG tag is not cleaved in L405EFLAG mutant MT. After normalising MT level, lysates from wild-type and mutant MTFLAG were analysed for FLAG-tag (i) by Western-blotting. The membrane was then stripped and re-probed for MT using PAb762 (ii). Non-transfected Rat-2 lysates was used as a control.
The lack of surface fluorescence signal for the epitope tag in L405E mutant was not due to its lower expression level in stable cell lines. Cells transfected with plasmids carrying FLAG- or HA-tagged L405E using Attractene transfection reagent, which allows transient but higher expression level of plasmids, also did not show surface HA or FLAG tag fluorescent signal (results not shown). Mutant L405E is stably expressed. A commonly used protein synthesis inhibitor, cycloheximide (CHX), at concentrations known to impact on the stability of cellular proteins (Schneider-Poetsch, Ju et al. 2010), did not affect L405E protein level over a 48-hr period (Figure 4.14). Therefore the lack of L405E C-terminus presentation on the cell surface is a genuine feature of this mutant that correlates with its inability to transform fibroblasts. Interestingly, AKT but not MAPK activation is highly compromised in cells expressing L405E (Figure 4.15).
Figure 4.14 Mutant L405E MT is stable

A. Cycloheximide does not affect MT protein levels in the wild type MT and the L405E mutant. Cells stably expressing wtMT or L405E mutant were treated with 100 ng/ml cycloheximide (CHX) or the same amount of vehicle (ethanol). Lysates were collected at the indicated time points (hrs). Protein concentration for each sample was determined by BCA. After normalising lysate concentrations for each cell line, lysates were separated by SDS-PAGE and WB for MT using PAb762 (i) and α-tubulin (ii).

B. Ponceau S protein staining for the membrane shown in A. After polypeptides were transferred on to nitrocellulose membrane by WB, the membrane was incubated in Ponceau S protein stain for 15 min to evaluate protein levels prior to blocking in 5% milk.

Figure 4.15 Analysis of downstream signalling effectors in cells expressing mutant L405E

Lysates collected from cells expressing wild type or mutant L405E MT were normalised against protein concentration before loading on to a 10% bis-acrylamide gel. After SDS-PAGE protein separation and WB, the nitrocellulose membrane was probed for phosphorylated AKT, phospho-MAPK/ERK. Non-stimulated parental Rat-2 cells were used as a control for the activated signalling molecules. Amount of α-tubulin was analysed as a loading control.
Our results suggest that the defect of L405E may be the change of its C-terminus topology. In order to verify the topology of L405E hydrophobic domain, the KDEL sequence was added to L405E C-terminus (Figure 4.16 A). Since the KDEL receptor is in the lumen of the ER and cis- Golgi, the ER localisation sequence should have no effect on the distribution of L405E, in contrast to MTKDEL (Chapter 3, Figure 3.3). Cell lines expressing L405E KDEL was generated and the distribution of this mutant was assessed by immunofluorescent labelling. Instead of forming a distinctive tubular network pattern in the cytoplasm like MTKDEL, L405E KDEL was seen as much diffused granular staining throughout the cytoplasm that reaches the cell periphery (Figure 4.16 C). MTKDEL can bind to PP2A and pp60c-src but because it is trapped in the ER it cannot bind to pp62c-yes, ShcA, PLC-γ1 and PI3K p85. Analysis of immunoprecipitated L405E KDEL showed that the mutant binds to normal level of PP2A and pp60c-src but the level of associated pp62c-yes, ShcA and PI3K p85 was much reduced compared to wild type MT and L405E; he mutant, however, does bind to PLC-γ1 (Figure 4.16 D). The expression levels of L405E KDEL in all of the clonal lines generated were very low (data not shown). The double mutant L405E KDEL is therefore probably unstable, which may affect its association with some of the signalling molecules tested. Overall, L405E KDEL does not seem to be trapped in the ER compartment by the added ER-retention sequence. Therefore, the C-terminus of L405E is likely to orientate towards the inside of the cell, probably in a hair-pin conformation.
Figure 4.16 Characterisation of L405E KDEL

A. TMD sequence of L405E with KDEL added to the C-terminus. Positions in the complete sequence are indicated with numbers above corresponding amino acids at 10-residue intervals.

B. Foci assay for L405EKDEL. Cells were transfected with wild-type or L405EKDEL plasmid using method described previously. Plasmids used are indicated above the panel.

C. L405EKDEL can be found at the peripheral cell membrane. Total MT were labelled with PAb762 after cells were fixed and permeabilised. Cell lines used are indicated below the panel. Open arrow indicated MT localisation at the peripheral plasma membrane. Scale bar = 10 µm.

D. Protein binding property of L405EKDEL was analysed. PAb762 was used to IP MT from volumes of lysates normalised against MT level. Immunoprecipitates were separated and immunoblotted for known MT-binding partners. Mutants are indicated above each lane. Migration positions of polypeptides tested are indicated with arrows to the right.
Although the non-transforming mutants insert into the ER, and exit to other membrane compartments, they may still have a reduced level of surface expression that is difficult to observe (Figure 4.12 B). To investigate this further, immunofluorescent signals of the C-terminal HA-tag on cell surface were examined under high magnification using confocal scanning microscope, which is more sensitive than fluorescent light microscope and allows resolution of fluorescent beads of 170 nm apart (Bolte and Cordelieres 2006). The fluorescent signals generated from surface HA-tags were compared between wild type MTHA+, L405E HA+, dl1015 HA+ and non-transfected parental Rat-2 fibroblasts. Dl1015 HA+ is a non-transforming mutant with a deletion of the Proline-rich region of MT and is known to associate with all immediate MT-binding proteins but cannot transform (Magnusson and Berg 1979, Magnusson, Nilsson et al. 1981) (unpublished data). Expression of this non-transforming mutant is relatively low (data not shown).

Figure 4.17 Measuring surface HA fluorescence signals
A. Examples of high magnification images of surface labelled HA used for analysis. Cells expressing wtMTHA+ (a), L405EHA+ (b) or dl1015HA+ (d) were incubated with anti-HA antibody before fixation. The fluorescent signals on the cell basement membrane were captured using Leica SP5 confocal microscope with 63.0x1.40 oil lens, zoom factor 6. An area of 20.5 µm x 20.5 µm was selected for analysis. Scale bar = 2 µm.
B. Average number of complexes counted per frame in cell lines shown in A. Macro 1 was applied to each image. A binary mask of each image was made with threshold =15. Using ‘analyse particle’ the number of complexes were counted. 8 independent frames of each cell line were used for analysis.
C. Distribution of MTHA clusters across a range of cluster size. Using ‘analyse particle’ function in ImageJ, the area covered (µm²) by each complex was measured and converted to diameter (nm). MT species expressed by the cells are indicated above each graph.
D. Numbers of complexes larger than 300 nm across are compared between wild type and mutant MT. The total number of complexes with area greater than 0.071 µm² or a diameter of 300 nm was counted for each image.

See image on next page.
The C-terminal HA-tag can be detected on the basement membrane surface of cells expressing dl1015 HA+ despite at much lower intensity (Figure 4.17 A). The surface fluorescent signals detected on cells expressing L405E HA+ were similar to background level. The number of fluorescent signals on the cell basement membrane can be counted using ImageJ software (see Material & Method). The area covered by individual cluster of fluorescent signals was measured (µm²), which was then converted to equivalent size in diameter (nm) (see Figure 4.18 for examples). Although some HA complexes were detected on the surface of L405E mutant, the majority of these complexes are 300 nm or less in diameter, which can also be detected in the control (Figure 4.17 A & B). The number of these background clusters in L405E HA was higher than the control but extremely low compared to wild type and dl1015 MTHA (Figure 4.17 B). In cells expressing wild type and dl1015 MTHA, there are a lot of complexes with sizes larger than 300 nm in diameter, some of which are larger than 500 nm in diameter (Figure 4.17 C & D). The distribution of the L405EHA+ fluorescent signals counted follows the same pattern observed in control cells (Figure 4.17 C) with few complexes larger than 300 nm counted. These measurements seem to suggest that formation of large MT complexes on the outer cell surface may be required for transformation. However, confirmation would require a more sensitive method of microscopy.
Figure 4.18 Measuring surface fluorescent complexes

A. Steps of measuring surface complexes of varying sizes. (a) Confocal image of HA tags on the cell surface in an area of 20.5 µm x 20.5 µm. Surface HA-tag of wild type MTHA+ was labelled as described previously. Scale bar (black line) = 2 µm. (b) Using ImageJ, a mask is generated (threshold = 40). (c) Complexes with an area smaller than 0.05 µm² are excluded.

B. Defining complexes of increasing sizes or area covered by pixels. Left column: images of complexes matching the size limited are generated from (c). Middle column: limit on area covered by the pixels in each complex. Right column: equivalent numbers of complexes covered by the area defined on the left.
In order to resolve MT molecules with higher resolution, the location of MT in wild type MTHA+ and L405E MTHA+ was examined by immuno-electron microscopy with the help of our collaborators, Dr Forstova and Dr Zila. Here, PAb762 was used to label the total MT inside the cells and detected by binding with 10 nm gold particles (GPs) coated with anti-mouse IgG. Electron micrographs show that wtMTHA+ is present at the plasma membrane. The wild type MTHA+ labelled with gold particles were usually found in clusters of 4 or more gold particles, therefore confirming that MT exists in large multimeric complexes at the plasma membrane (Figure 4.19 A (a, c)). In cells expressing L405E MTHA+, gold particles were also observed at the plasma membrane (Figure 4.12 A (b, d)), but very few were found in clusters. To quantify this, a large number of electron micrograph fields were analysed and clusters were evaluated by counting the gold particles at the plasma membrane. A cluster was defined as four or more gold particles continuously occupying the same discrete area at the plasma membrane. Three or less gold particles in close proximity were counted as ‘non-clusters’. Figure 4.19 B (a) shows that over 20% of the gold particles labelled in cells expressing wild type MT are found in clusters of 4 or more particles, whereas less than 2% of the L405E MT was labelled in similar clusters. Counting clusters made of more than 4 GPs revealed a marked difference between WT MT and mutant L405E (Figure 4.19 B (b)).
Figure 4.19 IEM analysis of wild type and L405E MT at the plasma membrane

A. IEM images of MT at the plasma membrane. Cells expressing wild type (a, c) or L405E (b, d) were immune-labelled with a mouse anti-MT monoclonal primary antibody, PAb762, and then a goat anti-mouse secondary antibody conjugated to 10 nm gold particles. Scale bar, 50 nm. Experiments performed by Dr J. Forstova and Dr V. Zila.

B. Cluster analysis of gold particles at the plasma membrane. Many sections of each cell line were used for evaluation. A total number of 1165 gold particles (GP) were counted in wtMT, 261 gold particles were found in 54 clusters. For L405E cells, a total number of 1145 gold particles were counted and 24 of them were found in 6 clusters. Figures provided by Dr V. Zila.
Therefore, it seems likely that the defect in L405E MT is not its ability to bind to cellular membranes and to signalling proteins, but its incapacity to assemble efficiently into a multi-subunit complex once at the membrane, and this lack of complex formation may account for its inability to transform. Indeed, the ability to activate the downstream signalling effector AKT was highly compromised in mutant L405E when compared to cells transformed by wild type MT (Figure 4.15).

Together, these data indicate that L405E MTHA+ is inserted into the membrane but fail to translocate the C-terminus to the outer cell surface. As a consequence, L405E MTHA+ does not appear to form large multimeric complex, and does not transform. The ability to form large, active clusters in the plasma membrane is likely to be required for transformation of fibroblasts and is a new property discovered in the MT transmembrane domain.

4.2.5 Characterisation of Other Hydrophobic Domain Mutants

Although Alanine substitution in the hydrophobic domain did not affect MT-induced transformation, some different mutations in this region completely abolished foci formation. As it was demonstrated for neu-wt and neu-mutant MT, G406L and L405E MT, it is important to characterise the non-transforming mutants in order to deduce properties required for cellular transformation. Amongst the hydrophobic sequences of receptor tyrosine kinases tested, only those from wild type neu receptor and PDGFRβ can replace MT hydrophobic sequence without inhibiting transformation (Figure 4.10). Substitution of MT hydrophobic sequence with that of wild type FGFR3 (FGFR3-wt) or the activating mutation G380R (FGFR3-mutant), both failed to induce foci formation (Figure 4.10). Very little of the mutant FGFR3-wt or FGFR3-mutant MT was detected at the cell periphery (Figure 4.20).
Immunofluorescent labelling of the two MT mutants showed strong signals in the perinuclear region which colocalise with Golgi marker, giantin (results not shown).

Another non-transforming TMD mutant described earlier in Figure 4.10 is ∆402-406MT, which lacks the five amino acid sequence, AALLG, central to the hydrophobic sequence. An HA+ tag added to the C-terminus of this mutant was not detected on the surface membrane in cells transiently expressing this mutant (data not shown), therefore the lack of transformation was initially attributed to a change in TMD conformation. Here, a cell line expressing ∆402-406MTHA+ was generated and immunofluorescence analysis of MT distribution revealed an unusual localisation of this mutant. Figure ∆402-406MTHA+ was exclusively found in large distinctive patches next to the nucleus and colocalised perfectly with the Golgi marker, giantin (Figure 4.21 B). Cells expressing this mutant did not grow very well hence the low number of cells present in the image (Figure 4.21 A). Western blot analysis shows that some ∆402-406MTHA+ was degraded from the C-terminal end (Figure 4.21 D). Despite this, ∆402-406MTHA+ associated with all known MT-binding molecules at
levels comparable to wild type MT (Figure 4.21 C), including pp62c-yes, which was not observed in MTKDEL (Chapter 3, Figure 3.10). However, the phosphorylation states of the associated signalling molecules were not evaluated. Although it is possible that mutant Δ402-406 was relocated from the plasma membrane to the Golgi apparatus, fluorescence signal was not detected in endosome- or microsome-like structure near the cell periphery. It is more likely that the shortening of the hydrophobic sequence probably caused the retention of Δ402-406 MT in the Golgi membrane. The length of the MT hydrophobic sequence is, therefore, important for targeting MT to the plasma membrane.

Results with above transformation-defective mutants suggest that, once transported to the Golgi apparatus, MT probably will have access to all of its direct binding proteins such as pp62c-yes, ShcA, PI3K subunit p85 and PLC-γ1 in addition to the PP2A core dimer and pp60c-src bound in the cytoplasm and the ER, respectively.
Figure 4.21 MT localised to the Golgi apparatus associates with all signalling molecules

A. Δ402-406MT is localised in Golgi apparatus. Total MT were labelled with PAb762 after cells were fixed and permeabilised. Cell lines used are indicated above the panel. Scale bar = 50 µm.

B. Colocalisation between Δ402-406MT and Golgi marker. MT (red) was labelled as described above. Alexafluor-555-conjugated anti-mouse IgG was then added to cover-glass in conjunction with Alexafluor-488-conjugated rabbit anti-giantin antibody (green). Nuclei were labelled with DAPI (blue) imaged on Leica SP5 confocal microscope using 63.0x1.40 oil UV lens using zoom factor 4.5. Scale bar = 5 µm. Images are representative of at least 12 independent frames.

C. Protein binding properties of Δ402-406MT were analysed. PAb762 was used to IP MT. Immunoprecipitates were separated and immunoblotted for known MT-binding partners. Mutants are indicated above each lane. Migration positions of polypeptides tested are indicated with arrows to the right.

D. Analysis of MT expression level in Δ402-406MT mutant. Lysates proportional to the volume used for IP were analysed for C-terminal HA tag with HA11 (top) and then re-blotted for total MT with PAb762 (bottom).
4.2.6 Evaluating the amount of actively signalling MT

In previous sections, we have demonstrated that in order to transform fibroblasts, MT must migrate to the peripheral plasma membrane. In addition, MT molecules must assemble into large multimeric clusters in order to generate transforming signals and this requires the translocation of the C-terminus to the outer cell surface. Therefore MT detected at the cell exterior represents the most active signalosomes. We next tried to address a long standing question of how much of the MT polypeptides are actively signalling. Since we have demonstrated that surface MT can be immune-labelled by adding an antibody specific to the C-terminal epitope tag, we then applied the same technique to immunoprecipitate MT with a mouse monoclonal HA11 added to living cells before cell lysis. The remaining MT in cytoplasmic membranes were immunoprecipitated with a different mouse monoclonal PAb762 recognising the N-terminal region of MT. Figure 4.22 shows, only a small proportion of MT (approximately 1%) can be immunoprecipitated from the cell surface, which should represent the proportion of MT that is actively signalling. This is verified by the very small amount of PP2A catalytic subunit C in complex with surface MT. It has been previously established that MT associates with PP2A subunit A and C at a ratio of 1:1:1 (Glover, Brewster et al. 1999). However, while the bulk of the MT may be located on intracellular membranes, the levels of some of the MT-binding proteins immunoprecipitated from the cell surface are disproportionally high. An estimated 10% of the pp60c-src, 25% of PI3K p85 and 33% of PLC-γ1 was co-immunoprecipitated with MT from the cell surface. Inside the cells, MTKDEL is also immunoprecipitated with some PI3K p85, which probably indicates a small population of MTKDEL escaping into the Golgi.
4.2.7 Measuring the sizes of MT complexes on the cell surface

Since we have established that the formation of large complexes on the cell surface membrane is probably important for MT transformation, we then re-examined and compared the distribution and the size of these complexes on the surface of cells expressing wild type or the non-transforming mutant 200Δ10 MT in greater detail. Ligand stimulation seems to increase the number of larger EGFR clusters (Sako, Minoghchi et al. 2000). Therefore we wanted to investigate whether subtle changes in cluster size occur between active MT (wild type) and inactive MT 200Δ10, which lacks Src binding. First, we defined the minimum cut-
off size of a cluster. Images for cluster analysis were taken with a Leica SP5 confocal microscope with 63.0x1.40 NA (numerical aperture) UV oil immersion objective with a zoom factor of 6. Although a zoom factor of 8 or 12 had been used in some experiments and yielded images of slightly better resolution, they also caused rapid photo-bleaching of samples since laser power was focused in such a small area (data not shown). Since the smallest resolvable distance with this objective lens is 170 nm (Bolte and Cordelieres 2006), fluorescent signals covering an area smaller than 0.05 µm² were discarded. An area of 20.5 µm x 20.5 µm was selected for analysing particles to avoid counting out-of-focus complexes. The same threshold was used for analysing images taken from the same experiment, which is usually in the range of 30 - 40 %. Background level of fluorescence signals with a diameter (Φ) of 300 nm (area = 0.071 µm²) is relatively low (Figure 4.17). Therefore, fluorescent signal covering an area of 0.05-0.071 µm² was counted as one MT cluster on cell surface. Fluorescent signals covering any greater area were counted as complexes with more than one cluster of MT. Increments of 0.071 µm² (Φ 300 nm) was used to measure the number of equivalent clustered MT particles in a complex by using the ‘analyse particles’ function in FIJI ImageJ (Figure 4.18 for demonstration). For example, a fluorescence signal covering an area of 0.28 µm² would be assigned a value of 4 (see Figure 4.18).
Figure 4.23 Analysis of MT complexes on the cell surface

A. Surface MTHA complexes separated according to size. Surface HA labelling was performed on cells expressing wtMTHA+ (black) or 200Δ10HA+ (grey) as described before. The fluorescent signals on the basement membrane of a cell were captured using Leica SP5 confocal microscope with 63.0x1.40 oil lens, zoom factor 6. An area of 20.5 x 20.5 µm was selected for analysis. Macro 1 was applied to each image. A binary mask of each image was made with threshold =30. Using ‘analyse particle’ function in ImageJ, the size (in µm²) of each complex was measured. No complex was found in Rat-2 control with such setting. Numbers of complexes smaller or equal to 300 nm in diameter (area = 0.071 µm²) were counted and fractions of clustered MT complexes (> 300 nm) were presented.

B. Distribution of clusters across a range of complex size. Using complex size of 0.071 µm² as minimum, frequency of clusters within the defined size range was generated and presented as fractions to total complexes counted for each species of MT. 1145 wild-type MTHA complexes were counted from 7 independent frames. 714 complexes of 200Δ10HA were counted from 8 independent frames.
Out of the 1145 complexes counted for wtMTHA+, 65% of those in cells expressing wild-type MTHA+ were found to be bigger than 300 nm across and were considered to be clusters of MT molecules on cell surface (Figure 4.23 A). Similar figures were obtained with cells expressing 200Δ10HA+ in the same experiment. The pattern of distribution between wild-type and 200Δ10HA+ on the cell surface are very similar (Figure 4.23 B). 70% of these complexes are made up of two of those 300 nm clusters, therefore, are probably just over half a micron across in length. The remaining 30% of the clusters are one micron or bigger (Figure 4.23). Clearly 200Δ10 does form clusters but is unable to transform because it lacks the Src binding site. Therefore the size of surface MT complexes is not dependent upon active Src and does not necessarily reflect the transformation potential of the species expressed.

Similar complex measurements were made to cells treated with the Src kinase inhibitor, dasatinib (dasat.), or the PP2A phosphatase inhibitor, okadaic acid (OA). Figure 4.24 shows that 48 hrs treatment with 100 nM dasatinib causes dissociation of ShcA and PI3K from MT without affecting the formation of MT/PP2A/Src complex. A similar effect was observed using 50 nM dasatinib (data not shown). Dasatinib reverses the transformed morphology of cells expressing wild type MT without affecting the subcellular distribution of MT (wtMTHA+ in Figure 4.25 A). When surface MT complexes were measured using the methods described previously, there is no obvious difference between the size of complexes on the surface of cells treated with dasatinib or vehicle. Acute treatment with higher concentration of the inhibitor also did not affect surface MT complex size (data not shown).
Figure 4.24 Dasatinib inhibits MT association with signalling molecules

A. Effect of 48hr-treatment with dasatinib or okadaic acid on MT association with signalling molecules. Cells expressing MTHA+ were treated with 100 nM dasatinib (dasat.) or 20nM okadaic acid (OA) for 48hrs before lysis. MT after normalising MT content in each lysate, MT was immunoprecipitated using mouse monoclonal PAb762. The polypeptides were separated by SDS-PAGE and immunoblotted for known MT-binding partners as indicated above each panel. Non-transfected Rat-2 lysate was used as control. MTKDEL was used as a negative control for some signalling proteins. Migration positions of polypeptides tested are indicated with arrows to the right.
Figure 4.25 Analysis of MT distribution after dasatinib treatment

A. Dasatinib inhibits transformation but does not affect MT distribution. Cells expressing wtMTHA+ were cultured in media containing 100nM dasatinib or vehicle (DMSO) for 24 hrs before IF staining for surface HA (i) or total MT (ii). Scale bar = 50 μm.

B. Dasatinib does not affect the distribution pattern of surface MT clusters. Images generated from the above experiment were used for complex analysis. Fluorescence signals in the centre of the basement membrane after labelling surface HA were captured using Leica SP5 confocal microscope with 63.0x1.40 oil lens, zoom factor 12. Macro 1 was applied to each image. The area covered by individual surface complex was measured as described in Figure 4.16. Binary mask was made with threshold set at 30. Distribution of complex counts were generated for cells treated with dasatinib (grey) or DMSO (black).
We then investigate whether PP2A binding is required for MT macro-complex formation on the cell surface. However, mutants lacking PP2A binding, such as 180Δ10 and NS2, are trapped in the ER and do not get to the cell surface, therefore cannot be used (Zhou, Ichaso et al. 2011). It has been reported that 5 - 10 nM okadaic acid reduced the level of PP2A and Src tyrosine kinase binding to MT (Glover, Brewster et al. 1999). Here, we showed that treatment with the phosphatase inhibitor only slightly reduced the amount of PP2A subunit A and pp62c-yes bound to MT. However, association with ShcA and PI3K was not affected (Figure 4.24). Here OA was added to cells expression MT for 48 hrs before IF staining for surface MT. Figure 4.26 shows that similar surface MT complexes were observed in cells with and without treatment. Experiments were repeated with higher concentration of OA and again no difference was found between in the size of surface MT complexes compared to control. Altogether, results suggest the size of MT macro-complexes on the cell surface is not influenced by the binding of signalling molecules on the cytoplasmic bulk of the MT molecule.
Figure 4.26 Distribution of MT after treatment with okadaic acid

A. Dual IF labelling of surface FLAG and total MT with and without okadaic acid (OA). Cells were treated with 10 nM OA for 48 hrs before IF staining (ii). Rabbit anti-FLAG antibodies were added to living cell. After fixation and permeabilisation, cells were incubated in PAb762 mouse anti-MT N-terminus to label total MT from the inside. Species-specific AlexaFluor-488 and -555-conjugated secondary antibodies were added to label FLAG (green) and MT (red) respectively. Scale bar = 50 µm.

B. Images of a single cell from A. Scale bar = 10 µm.
4.3 DISCUSSION:

4.3.1 Summary

Here, we have observed large complexes of MT on the cell surface, some of which measure over one micron in diameter. These macro-complexes contain phosphorylated ShcA (Figure 4.7 and 4.8), so represent active signalling complexes. New mutations in the hydrophobic region (L405E and neu-mutant MT) prevented MT clustering, and also inhibited cell transformation without altering the association of MT with its known binding proteins, therefore suggesting that formation of a large macromolecular complex in the plasma membrane is required for MT signalling (see Figure 4.27) (see Mutant List for detailed mutation and phenotype). We have identified a mutant that is trapped in the Golgi (Δ402-406 MT). Despite the association with above signalling molecules, it cannot transform Rat-2 fibroblasts. Both the length and sequence of the TMD is important for targeting MT from the Golgi to the plasma membrane, where transformation signals are generated. A summary of the subcellular localisation of various MT mutants used in our study are represented in Table T4.1 together with their ability to associate with known MT-binding signalling proteins (see Mutant List).
Our results add support to the evidence that MT and certain oncogenic GFR must be correctly trafficked to the plasma membrane in order to transform fibroblasts (Nichols, Manger et al. 1985, Beerli, Wels et al. 1994, Morley, Uden et al. 1999). However, the requirement for the transformation of hematopoietic cells may be less stringent. This is particularly the case with a mutant form of receptor for human macrophage-colony stimulating factor (M-CSF or CSF-1), c-FMS, a member of the PDGFR family (Morley, Uden et al. 1999). The mutant c-fmsD802V is highly unstable and is rapidly degraded. As a result, very little of the receptor gets to the cell surface. It was found that this unstable mutant potently transforms FDC-P1

Figure 4.27 A schematic representation of the final step in the assembly of a transforming MT complex

MT at the plasma membrane (light blue disc) is represented linearly in red. The cellular proteins bound to MT are illustrated with phosphorylation tyrosine sites represented by yellow circles. Mutant (L405E MT) that blocks the assembly of a multimeric MT complex is represented under the arrow.

4.3.2 MT transformation signal is generated at the plasma membrane

Our results add support to the evidence that MT and certain oncogenic GFR must be correctly trafficked to the plasma membrane in order to transform fibroblasts (Nichols, Manger et al. 1985, Beerli, Wels et al. 1994, Morley, Uden et al. 1999). However, the requirement for the transformation of hematopoietic cells may be less stringent. This is particularly the case with a mutant form of receptor for human macrophage-colony stimulating factor (M-CSF or CSF-1), c-FMS, a member of the PDGFR family (Morley, Uden et al. 1999). The mutant c-fmsD802V is highly unstable and is rapidly degraded. As a result, very little of the receptor gets to the cell surface. It was found that this unstable mutant potently transforms FDC-P1
hematopoietic cells but not Rat-2 fibroblasts (Morley, Uden et al. 1999). Mutant MT species generated in this study can be useful tools to study how mitogenic signalling differs between fibroblasts and hematopoietic cells. For example, AKT is partially activated in fibroblasts expressing L405E despite the lack of a transformation phenotype (Figure 4.15 and data not shown). It is not known if this mutant is also unable to transform other cell types such as FDC-P1 cells.

There have been various reports to suggest mitogenic signals can be initiated from endosomes, (Lipson, Pang et al. 1998, Wang, Pennock et al. 2002, Wang, Pennock et al. 2004). Unlike other cell surface receptors such as EGFR1 and integrin, which are endocytosed upon ligand or antibody binding to their extracellular domains (see Chapter 1, 1.13 for introduction), MT on the cell surface is not readily endocytosed (Figure 4.3 and 4.4). However, there is a substantial intracellular population of MT, which is associated with signalling molecules (Figure 4.22) and so is possibly actively signalling. However, this intracellular signalling is unlikely to be sufficient for fibroblast transformation, given the requirement for cell surface expression (Table T4.1), but it may contribute.
Table T4.1 Subcellular location of MT mutants and their ability to transform as well as their ability to associate with MT-binding partners

Final location of wild type MT and mutants in stable Rat-2 cell lines generated in Chapter 3 and 4 as analysed by IF staining for total MT or surface epitope tags (cell surface). Transformation efficiency was assayed by the ability to induce foci formation when transfected into a Rat-2 fibroblast cell monolayer and results are summarised with ‘-’ being non-transforming and ‘++++’ being equivalent to wild type levels of transformation. Mutants’ abilities to associate with known MT-binding partners were analysed by PAb762 anti-MT immunoprecipitation as indicated above the columns. The presence of each polypeptide is indicated with ‘+’. 180Δ10 lacks both PP2A and Src binding. 200Δ10 lacks Src binding only. See List of Mutants for detailed description of the other mutants.

<table>
<thead>
<tr>
<th></th>
<th>Final Location in Cells</th>
<th>Foci Formation</th>
<th>PP2A</th>
<th>pp60c-src</th>
<th>pp62c-yes</th>
<th>ShcA/PI3K p85/PLC-γ1</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtMT</td>
<td>Cell surface</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>200Δ10</td>
<td>Cell surface</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>180Δ10</td>
<td>ER</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MTKDEL</td>
<td>ER</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Δ402-406</td>
<td>Golgi</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δ416-419</td>
<td>Plasma membrane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>416G6</td>
<td>Plasma membrane</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Δ419-421</td>
<td>Cell surface</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L405E</td>
<td>Plasma membrane</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G406L</td>
<td>Cell surface</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>neu-mutant MT</td>
<td>Plasma membrane</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>neu-wt MT</td>
<td>Cell surface</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

4.3.3 Macromolecular complexes of MT on the cell surface is required for transformation

We introduced a single negatively charged residue into the middle of the MT hydrophobic sequence, which is known to stabilise dimerisation between two adjacent helices of the ErbB2/neu receptor by forming additional hydrogen and to cause cell transformation
(Sternberg and Gullick 1989, Gullick, Bottomley et al. 1992). However, the resulting MT mutant L405E failed to transform Rat-2 fibroblasts and to translocate its C-terminus outside the cell despite its localisation to the plasma membrane (Figure 4.12 and 4.13). The C-terminus of this mutant presumably adopts a hairpin structure instead of spanning the lipid bilayer. Hence the inability to transform cells is due to defect in membrane insertion and topology of the C-terminus. The finding with L405E MT is supported by the neu-mutant MT (Figure 4.11) and mutant 416G6 presented in the previous chapter (Figure 3.15). Charged residues are tolerated in GFR transmembrane domains probably because they utilise a different membrane insertion mechanism compared to MT (as discussed in Chapter 3, 3.3.5).

Rat-2 fibroblasts expressing wild type MT frequently showed a number of gold particles forming clusters in the plasma membrane when examined by IEM (Figure 4.19 A). This confirms the result seen with immunofluorescent surface labelling of MT (Figure 4.1-4.5), and this complex was shown to contain active phospho-ShcA, so probably represents an active signalling complex (Figure 4.7 and 4.8). This is the first demonstration that MT exists in the plasma membrane as discrete, active, clusters containing a number of MT molecules. The gold particles that bound to L405E MT, however, were rarely seen in groups (Figure 4.19 A and B). Therefore, perhaps as a consequence of the failure to translocate the C-terminal tail across the membrane, L405E MT in the plasma membrane fails to assemble into macromolecular complexes and perhaps for this reason does not transform. Table T4.1 summarises a range of mutant MT and how their final cell location and ability to bind to signalling molecules correlates with their ability to transform Rat-2 fibroblasts. Some of the mutants bind all of the known signalling proteins (Figure 4.16, 4.11 and Figure 3.19) but fail to show cell surface expression, to form clusters and to transform fibroblasts. This strengthens the argument that these clusters are an essential part of oncogenic signalling by
MT and are a final step in the establishment of a transforming MT complex (Figure 4.27 and 6.1).

Similarly to MT, many receptors have also been shown to cluster in the plasma membrane (Gillham, Golding et al. 1999). Using a GFP-85 PI3K fusion construct, Gullick and co-worker showed that cytoplasmic PI3K relocates to discrete patches on the PM upon EGF stimulation (Gillham, Golding et al. 1999). This occurs within as little as 1 min, prior to receptor internalisation and degradation. There is evidence that this clustering is associated with EGFR activation (Yang, Raymond-Stintz et al. 2007, Clayton, Orchard et al. 2008, Hofman, Bader et al. 2010). Dynamic clustering has been implicated in EGFR activation as a means to amplify activating signals locally. Ichinose and co-workers observed that only a small fraction of fluorescent-conjugated EGF ligand colocalises with activated EGFR at one time after a 30 min stimulation period. Instead the signal was found propagated through clustering of non-ligand associated but phosphorylated EGFR by their transient interaction with ligand-activated EGFR (Ichinose, Murata et al. 2004). Therefore, receptor clustering was proposed by many as an effect secondary to receptor dimerisation and activation as the initial response to ligand stimulation (Gillham, Golding et al. 1999, Nagy, Jenei et al. 1999, Ichinose, Murata et al. 2004).

Our data for the first time suggests that kinase activation alone may not be sufficient and that in addition, the assembly of a large macromolecular complex is important for signalling. The demonstration that MT clusters can form in the absence of Src tyrosine kinase activity (Figure 4.7, 4.8 and 4.25) may suggest these two processes can be separated, and explain why receptor clusters are seen in the absence of growth factor stimulation (Yang, Raymond-Stintz et al. 2007). It has been suggested that clustering of macromolecular complexes is a mechanism to provide efficient and rapid spatial confinement in order to avoid...
long-distance diffusion in the crowded environment of a cell (Cebecauer, Spitaler et al. 2010).

IEM analysis of the EGFR family members located to the cytoplasmic surface of the plasma membrane showed EGFR1, ErbB2 and ErbB3 receptors are pre-clustered in the absence of stimuli (Yang, Raymond-Stintz et al. 2007). In the SKBR3 breast cancer cell line, which is driven by ErbB2 over-activation, the sizes of ErbB2 pre-formed clusters do not change significantly following ligand stimulation or upon treatment with an ErbB2-specific kinase inhibitor but the level of receptor phosphorylation changes (Yang, Raymond-Stintz et al. 2007). These preformed clusters also contain signalling molecules such as ShcA, PI3K and STAT5. It is the distribution of these signalling molecules co-clustered with receptors which is changed in response to ligand stimulation. ShcA pre-associates with EGFR1 and the co-clusters move into distinctive ‘coated-pit’ structures in the membrane following stimulation, which is an indication of receptor internalisation (Yang, Raymond-Stintz et al. 2007). PI3K p85 is only recruited to ErbB3 after stimulation by heregulin and produces a distinctive membrane domain consists of ErbB3 cluster surrounded by a ring of p85, forming a ‘bull’s eye’ structure, which resembles a macro-complex made of signalling molecules and T-cell receptors often observed in immunological synapses (Bromley, Burack et al. 2001). STAT5 associates inherently with all EGFR family members. While ligand stimulation leads to rapid tyrosine phosphorylation of STAT5, the signalling molecule is re-distribution to separate membrane microdomains away from the receptor clusters (Yang, Raymond-Stintz et al. 2007).

Similar to our observation of surface MT clusters with sizes in the range between 500 - 1000 nm on confocal microscope, Nagy and co-workers estimated that Her2/neu clusters of 500 nm in diameter probably contain thousands of receptors using scanning near-field optical
microscope which has a much higher resolution (Nagy, Jenei et al. 1999). It is likely that the clusters observed in our system are also made up of tens of thousands of MT molecules.

Our data suggest that disruption of receptor cluster formation has the potential to inhibit receptor oncogenic signalling independently of kinase activity, so providing a wholly separate goal for drug development. Indeed, it has been demonstrated that cells expressing certain variation of neu transmembrane polypeptides, lacking ligand binding or intracellular kinase domains, in cells transformed by the activating neu V664E mutation reduced oncogenic potentials in mouse xenograft models by preventing neu receptor dimerisation (Lofts, Hurst et al. 1993). It is feasible that the MT transmembrane domain in isolation could also insert into membranes and act as a competitive inhibitor to prevent signalling by related complexes. In preliminary experiments (Figure 4.28 – 4.31), mutant 200Δ10 when co-expressed with wild type MT reduced the transformation potential of the wild type, probably by diffusing the local concentration of transforming signals via the formation of a heterogeneous macro-complex with wild type MT (Figure 4.28). However, foci formation was not completely inhibited. Despite the lack of Src binding site, all the tyrosine residues targeted by Src are completely intact on mutant 200Δ10 so it is possible that these tyrosine residues on the mutant can phosphorylated *in trans* by wild type MT in the same cluster. A better dominant negative mutant may be constructed by removing all major phospho-tyrosine sites from 200Δ10 MT.

Colocalisation between FLAG and HA-tagged MT complexes was also observed on the cell surface when a cell line expressing wild type MTFLAG was transiently expressing transfected with wtMTHA+ or 200Δ10 MTHA+ plasmids (Figure 4.29 – 4.31). However MTFLAG complexes do not always colocalise perfectly with MTHA complexes, probably as a result of different binding affinities or because of steric hindrance between anti-HA and anti-FLAG antibodies. Similarly, preliminary co-immunoprecipitation experiments have not
successfully proven the interaction between MT molecules with the two different epitope tags, possibly due to the large difference in expression level between the two MT species in co-expressing cells (data not shown).

Figure 4.28 Mutant 200Δ10 reduces the foci formation induced by wild type MT plasmid
A. Co-transfection of wild type MT and 200Δ10 mutant at 1:12 wild type to mutant ratio. Subconfluent Rat-2 fibroblasts on 10cm tissue culture plates were transfected with 1.4 µg pUC19 MT (wild type) plasmid with those carrying 200Δ10MT (16.8 µg) or empty plasmids at 1:12 ratio using calcium-phosphate method. Transfections without wtMT plasmids were also performed as controls (column on the left). Cells were cultured for 2 weeks before the foci were stained.

B. Co-transfection of wild type MT and 200Δ10 mutant at 1:8 (wild type: mutant) ratio. Subconfluent Rat-2 fibroblasts on 6cm tissue culture plates were transfected with 0.7 µg pUC19 MT (wild type) plasmid with those carrying 200Δ10MT (5.6 µg) or empty plasmids at 1:8 ratio using above methods.
Figure 4.29 Colocalisation between MTFLAG and 200Δ10 MTHA on the cell surface

A. Rat-2 fibroblast cell line 2.11 expressing wtMTFLAG was transfected with plasmids carrying 200Δ10MTHA+ sequence using Attractene transfection kit for 6 hrs at 37°C. 24 hrs post-transfection, cells were seeded on to glass slide and stained for surface FLAG- and HA-tags at the same time 48hrs post transfection. A cell with strong surface HA signals is shown in (a). An independent frame with weaker HA fluorescent signal is shown in (b). Each image represents an area of 30.60 x 30.60 µm. Scale bar = 5 µm.

B. Zoom-in images of area highlighted in A. Smaller area of 15.26 x 15.26 µm was selected for further analyses. Open arrows indicated colocalisation between surface MTFLAG and 200Δ10 MTHA. Scale bar = 2 µm.
A. Rat-2 fibroblast cell line 2.11 expressing wtMTFLAG was transfected with plasmids carrying wtMTHA+ sequence using Attractene transfection kit for 6 hr at 37°C. After 24 hrs, cells were seeded on to glass slide and stained for surface FLAG- and HA- tags at the same time 48 hrs post transfection. A cell with strong surface HA signals is shown in (a). An independent frame with weak HA fluorescent signal is shown in (b). Each image represents an area of 30.60 x 30.60 µm. Scale bar = 5 µm

B. Zoom-in images of area highlighted in A. Smaller area of 15.26 x 15.26 µm was selected for further analyses. Open arrows indicated colocalisation between surface MTFLAG and MTHA. Scale bar = 2 µm.

Figure 4.30 Colocalisation between MTFLAG and MTHA complexes on the cell surface
The indication that a MT cluster must form to initiate oncogenic signalling by MT raises the question why? Clustering of molecules within cell membranes has been reported recently in a number of systems, notably proteins involved in signal transduction (Cebeauer, Spitaler et al. 2010). Ras Family of GTPases in particular have been shown to exist in nano-clusters on the inner surface of the plasma membrane mediated by their C-terminal lipid modified region (Prior, Muncke et al. 2003). Accumulation of Ras in these nanoclusters is essential for signalling activity (Plowman, Muncke et al. 2005, Tian, Harding et al. 2007) and requires the actin cytoskeleton (Plowman, Muncke et al. 2005). It is interesting to note that one of the few membrane targeting domains that can functionally replace the MT hydrophobic sequence are the C-terminal regions of both H-Ras (Elliott, Jones et al. 1998) and K-Ras (unpublished observations), and MT has been shown to associate with cytoskeletal

![Fraction of Surface MTFLAG Colocalising with HA](image)

Figure 4.31 Colocalisation analysis between MTFLAG and MTHA complexes on the cell surface

Images of surface FLAG- and HA-tags co-staining in MTFLAG cell line transfected with 200Δ10 MTHA+ or wtMTHA+ were analysed for colocalisation. The average fraction of surface FLAG complexes colocalising with HA signals was calculated from 5 independent frames. Error bar = standard deviation.
structures (Schaffhausen, Dorai et al. 1982, Andrews, Gupta et al. 1993, Elliott, Jones et al. 1998, da Costa, Wang et al. 2000). Given these functional similarities between Ras and MT, it is tempting to speculate that the MT and Ras clusters are related and that close proximity is essential for transforming activity.

How MT is organised into large clusters in the plasma membrane is not clear. Cholesterol containing, ordered lipid structures, or lipid rafts, in external cell membranes (Cebecauer, Owen et al. 2009) may possibly contribute to the clustering of MT molecules seen in the plasma membrane. There has been intensive speculation concerning the role of lipid rafts in signalling, with conflicting views about their importance. The mechanism of MT macro-complex formation will be explored in Chapter 5.

A recent report has shown that there is a physical association between MT and the insulin receptor as well as the related IGF-I receptor and this association is required for transformation (Novosyadlyy, Vijayakumar et al. 2009). It will be interesting to determine whether this interaction occurs in the MT containing plasma membrane complexes, and if other receptors are also present. These results also provide an exciting potential new approach to drug development. It is interesting to note that the interaction of receptors within a cluster is probably dynamic in order to allow for rapid reversal when the initiating signal is removed (Cebecauer, Spitaler et al. 2010). MT has evolved to be permanently and strongly activating, and consequently, a competitive inhibitor based on the MT sequence may be considerably more potent than one designed on a receptor sequence. It is possible that hydrophobic compounds could also be devised that could prevent receptor oligomerisation. TMD-mediated signalling in GFR and the idea of artificial transmembrane polypeptide as competitive inhibitor for GFR signalling will be explored further in Chapter 6.
4.3.4 Estimating the proportion of active MT molecules

Since we have determined that MT localisation at the plasma membrane is important for transformation, it is intriguing to see how much of MT expressed is located at the plasma membrane. It has been a long standing question as to what proportion of MT produces mitogenic signals (Schaffhausen 1982). Overall, the amount of MT that can be immunoprecipitated from the cell surface in comparison to the bulk of the viral protein expressed inside the cell is very small (probably 1%). However, the amount of certain signalling molecules that associate with this surface population of MT is relatively high. These include a tenth of pp60c-src, a quarter of PI3K p85 and a third of PLC-γ1 immunoprecipitated from total MT (Figure 4.22). Therefore, the MT complexes immunoprecipitated from the cell surface represent a potentially more active population of MT. By IF, we found that only 15-20% of MT near the cell basement membrane is at the cell surface (Figure 4.6 C). The small proportion of active MT signalosome raises the question about the role of intracellular MT since they account for the bulk of the viral protein. After all, MT mutants localised to these cellular membrane sites have access to most of the signalling molecules associated with MT transformation (Table T4.1) but it is not clear whether MT in Golgi and microsomes are generating mitogenic signals.

About 30-50 % of surface MT colocalised with ShcA (Figure 4.7 B and 4.8 B). This raises the question as to whether MT bound to ShcA is also bound to all signalling molecules. The binding sites for PI3K p85 (pY215) and PLC-γ1 (pY322) are very close to each other on MT but it is not known whether this precludes binding of all of these signalling molecules to one MT protein. Cluster formation may overcome this possible limitation by increasing the number of available binding sites for different signalling molecules in the local region.
4.3.5 TMD targets MT from the Golgi to the plasma membrane

MT seems to be ubiquitously expressed throughout the cell membranes, with the bulk of the molecules found in the perinuclear region and the peripheral plasma membrane (Dilworth, Hansson et al. 1986). However, in the previous chapter, we have demonstrated, with mutant MT KDEL that MT located in the ER cannot transform and lacks access to most of the signalling molecules required for transformation. MT migration out of the ER to other membrane sites requires both the MT hydrophobic sequence as well as its association with the PP2A core dimer. Here, with the new TMD mutant ∆402-406 MT, which was found to localise solely in the Golgi apparatus (Figure 4.21), we demonstrated that once in the Golgi membrane, MT has access to all of the direct binding proteins required for generating mitogenic signals (Table T4.1). These include ShcA, PI3K and PLC-γ1 and pp62c-yes. Despite binding to tyrosine kinases and MT-associated signalling molecules, ∆402-406 MT failed to transform Rat-2 fibroblasts. Therefore, transformation signals must be generated from the locations beyond the Golgi apparatus, most likely at the plasma membrane. Characterisation of ∆402-406 MT demonstrates once again that subcellular localisation plays an important role in MT transformation.

Here we show that the length of MT hydrophobic sequence is important for the exocytic transport of this tail anchored protein out of the Golgi to the plasma membrane. Mutant ∆402-406 MT contains a hydrophobic sequence with deletions made to the central five residues. Shortening of the TMD sequence probably resulted in the retention of ∆402-406 MT in the Golgi apparatus. Using an ER-resident TA protein, Cytb₅, Pedrazzini and co-workers showed that extension of the C-terminal hydrophobic sequence from 17 to 22-residues relocated Cytb₅ to the plasma membrane (Pedrazzini, Villa et al. 1996). The anchor length in itself is a factor determining the subcellular localisation of many tail-anchored proteins. Other examples include Protein Tyrosine Phosphatase 1B (PTP1B), which has very
short anchor sequence and is targeted to microsomes and Sed5, a cis-Golgi located protein involved in vesicular transport (Banfield, Lewis et al. 1994). It was suggested that the plasma membrane is thicker in comparison to intracellular membranes as it has higher levels of cholesterol and sphingolipids (Pedrazzini, Villa et al. 1996). The ER-/ Golgi-resident enzymes with their shortened tail anchors are excluded from transport vesicles to the cell surface (Pedrazzini, Villa et al. 1996)(Masibay, Balaji et al. 1993, Pelham and Munro 1993), which supports the conclusion that the length of the hydrophobic sequence of MT is important for targeting MT to the plasma membrane.

Short stretches of alanine substitutions of the MT hydrophobic sequence had no impact on its ability to induce foci (394A4 to 412A4, Figure 4.10), which seems to suggest there is little sequence specificity in the hydrophobic domain of MT. However, for some transmembrane proteins, the specific sequence of the hydrophobic membrane spanning can sometimes play a role in the final localisation of the protein. In a collection of new TMD mutants generated in the lab, only sequences from wild type neu and PDGFRβ but not FGFR3 can functionally replace MT hydrophobic sequence. Replacement with the FGFR3 hydrophobic sequence, which is similar in length to the hydrophobic sequence of MT, resulted in mutants trapped in the intracellular membranes. We observed strong immunofluorescent-labelled signals of FGFR3 MT in the perinuclear region and cytoplasmic vesicles (Figure 4.20). Similar subcellular localisation pattern of FGFR3 was also observed in other fibroblast cell lines (Johnston, Cox et al. 1995). The TMD of FGFR3, when expressed alone, failed to reach the plasma membrane, thus confirming that the hydrophobic sequence of FGFR3 targets compartments other than the plasma membrane (He, Shobnam et al. 2011). Therefore, apart from the length of the hydrophobic sequence, there must be other properties in the sequence that target the MT to the plasma membrane, that are common to neu receptor and PDGFRβ TMDs, which will require more experiments to decipher.
CHAPTER 5 - MECHANISM OF MPyV MT MACRO-COMPLEX FORMATION

5.1 INTRODUCTION:

In the previous chapter, we have demonstrated that the ability to form large macro-molecular complexes on the cell surface membrane is an important requirement for MT-induced transformation and is mediated by the membrane anchoring transmembrane domain of MT. How MT molecules are organised into large multimeric complexes is unknown. Although the hydrophobic region does not seem to target MT to one particular membrane site, this region must have some specificity, as replacement with membrane targeting domains of cytochrome b$_5$ and pp60c-src myristylation sequence abolish transformation without disrupting membrane binding whereas H-Ras CAAX box restored oncogenic function (Elliott, Jones et al. 1998).

Ras proteins have been shown to exist in nano-clusters on the inner surface of the plasma membrane mediated by their C-terminal lipid modified regions (Prior, Muncke et al. 2003) (see Chapter 1, 1.14 for introduction). H-Ras can be found in cholesterol-dependent microdomains and its affinity for raft and non-raft microdomains is modulated by GTP-loading or palmitoylation (Roy, Plowman et al. 2005). Since the lipid modified CAAX sequences at the C-terminus of H-Ras can functionally replace the C-terminus of MT (Elliott, Jones et al. 1998), it is possible that MT and H-Ras share similar specificity for membrane domains.
Accumulation of H-Ras in nano-clusters requires the actin cytoskeleton (Plowman, Muncke et al. 2005, Tian, Harding et al. 2007) (Hancock and Parton 2005, Plowman, Muncke et al. 2005). MT has been shown to be associated with cytoskeletal structures (Schaffhausen, Dorai et al. 1982, Andrews, Gupta et al. 1993, Elliott, Jones et al. 1998, da Costa, Wang et al. 2000), but it is not clear how this association is mediated. It has been reported, amongst the early studies on MT, that phosphorylated MT can be immunoprecipitated by anti-serum raised against vinculin, an actin-binding protein that localises to focal contacts (Ballmer-Hofer, Ziegler et al. 1988). In MT transformed fibroblasts, PI3K p85 has been shown to associate with focal adhesion kinase (Bachelot, Rameh et al. 1996), but direct MT and FAK interaction has not been reported. It has been suggested that cholesterol-rich, ordered lipid structures, or lipid rafts, in the cell membranes can influence cluster formation of many cell surface molecules and may play a role in regulating signalling events (Cebeauer, Spitaler et al. 2010).

Protein-protein interaction may also be involved in cluster formation in the lipid bilayer. Some RTK such as the ErbB family receptor kinases are thought to hetero-dimerise, through part of their TMD (Gerber, Sal-Man et al. 2004). Cell surface receptors involved in adhesion, such as integrins have been shown to form complexes with growth factor receptors such as ErbB2, which enhances ligand-dependent phosphorylation of the GFR (Falcioni, Antonini et al. 1997). Integrin β1 was found to be required for mammary gland tumorigenesis in MMTV-MT mice (White, Kurpios et al. 2004). MT association with insulin receptor β (IRβ) and the insulin-like Growth Factor I (IGF-I) receptor have been detected (Novosyadlyy, Vijayakumar et al. 2009). Here we investigate how MT macro-complexes are formed, whether MT macro-complexes in the plasma membrane are related to lipid rafts or may also contain other membrane proteins.
5.2 RESULTS:

5.2.1 Membrane Micro-domains

5.2.1.1 Lipid Rafts

In order to investigate whether surface MT complexes are associated with or avoid lipid rafts, we used the lipid raft marker, Vybrant Alexa Fluor-conjugated cholera toxin subunit-B (CT-B) to label the cholesterol-rich lipid rafts in the membrane. CT-B binds specifically to monosialotetrahexosylganglioside (GM1), which is found in high concentration in cholesterol-rich lipid rafts in the membrane.

As Figure 5.1 shows, the level of colocalisation between the CT-B-labelled lipid rafts and surface MT complexes is relatively low. There is also no apparent difference between cells expressing wild type or non-transforming mutant 200Δ10 MTHA+ and lipid raft location (Figure 5.1). In fact, cells with more MT complexes on the cell surface showed much lower levels of lipid raft labelling (Figure 5.2 and 5.3). Although the fluorescent-conjugated CT-B does not permeate the lipid bilayer, it may be internalised with the plasma membrane during the incubation period at 37°C. Therefore the experiment was repeated at 4°C in cells expressing wtMTFLAG. Anti-FLAG antibody and fluorescent-conjugated CT-B were incubated together for 30 min to maximise lipid raft labelling. Some colocalisation between surface MT complexes and the lipid raft marker can be observed (arrows in Figure 5.4) but the majority of MT complexes do not colocalise with GM1-positive lipid rafts (Figure 5.4).
Figure 5.1 Little colocalisation between surface MTHA and raft marker at 37°C in cells expressing wild type or non-transforming mutant

A. Fluorescent labelling of surface MT and lipid rafts. Rat-2 fibroblasts expressing wtMTHA+ or 200Δ10 HA+ were incubated in mouse anti-HA antibody for 20 min at 37°C. 10 min before fixing, Vybrant AlexaFluor555-conjugated cholera toxin subunit B (CT-B) was added to cells, to label GM1 positive lipid rafts (red). After fixing, cells were incubated in AlexaFluor488-conjugated anti-mouse IgG to label surface HA-tags (green). Fluorescent signals on the basement membrane of a cell were captured using Leica SP5 confocal microscope with 63.0x1.40 oil lens, zoom factor 6. An area of 20.5 µm x 20.5 µm was shown. Scale bar = 2 µm.

B. Zoom-in image of highlighted area in A. Image for each cell line was representative of at least 6 independent frames. Scale bar = 2 µm.
Figure 5.2 Level of surface MT fluorescent signals inversely correlates with lipid raft staining.

Fluorescent labelling of surface MT and lipid rafts was performed as described in Figure 5.1. Images of a group of cells were captured with low zoom factors. Scale bar = 2 µm.
Figure 5.3 Cells with stronger surface MTHA signals have lower levels of the raft marker

A. Fluorescent labelling of surface MT and lipid rafts was performed as described in Figure 5.1. An area of 20.5 µm x 20.5 µm is shown.

B. Zoom-in image of highlighted areas in A. Scale bar = 2 µm for A and B.
Figure 5.4 Little colocalisation between surface MTFLAG and raft marker at 4°C

A. Fluorescent labelling of surface FLAG tag and lipid rafts. Rat-2 fibroblasts expressing wtMTFLAG were rested at RT for 15 min before the addition of rabbit anti-FLAG antibody and Vybrant AlexaFluor555-conjugated CT-B in 1% FCS media supplemented with Hepes buffer pH 7.4. Cells were incubated at 4°C for 30 min before fixing in cold formaldehyde. AlexaFluor488-conjugated anti-rabbit IgG was then added to label the FLAG-tag (green). Fluorescent signals on the basement membrane of a cell were captured using a Leica SP5 confocal microscope with 63.0x1.40 oil lens, zoom factor 6. An area of 30.04 µm x 30.04 µm is shown.

B. Zoom-in image of highlighted area in A. Image for each cell line is representative of at least 6 independent frames. Area shown = 20.5 µm x 20.5 µm. Scale bar = 2 µm for part A and B.
5.2.1.2 H-Ras G12V Non-raft Domains

It has been demonstrated that activated H-Ras nanoclusters are localised to cholesterol-independent microdomains in the membrane, therefore may be used as a non-raft marker in our study (Roy, Plowman et al. 2005). We then investigated whether MT at the plasma membrane colocalises with activated H-Ras microdomains. To do this,_FLAG-tagged MT was co-expressed with a constitutively activated mutant (G12V) of human H-Ras in Rat-2 fibroblasts, which has two consecutive Myc-tags added to the N-terminus (H-Ras-2XMyc G12V). Very little colocalisation were observed between MT labelled from the cell surface and internally labelled activated H-Ras using an antibody (mouse monoclonal 9E10) against Myc-tag (Figure 5.5). Most of the activated H-Ras mutants were located to large intracellular vesicles, with very little found at the basement membrane (Figure 5.5 A). Co-immunoprecipitation analysis between MT and H-Ras-2XMyc G12V also failed to show direct interaction between the two proteins (Figure 5.6). MTFLAG expression was relatively low compared to the cell line expressing MTFLAG alone. Any colocalisation between the two proteins is likely to be obscured by the strong H-Ras-2XMyc G12V staining from intracellular membranes (Figure 5.5 B).
Figure 5.5 Very little colocalisation between surface MT complexes and intracellular H-RasG12V-Myc

A. Cell lines co-expressing MTFLAG and H-Ras-2XMyc G12V were established. Surface MT was labelled as described previously using rabbit anti-FLAG antibody. After fixing and permeabilisation of cell membranes, mouse monoclonal 9E10 was added to bind Myc-tags of H-Ras-2XMyc G12V mutant. Then fluorescent-conjugated secondary anti-rabbit or anti-mouse IgG was added to label MT on the cell surface (green) and H-Ras (red). The ratios of MTFLAG to H-Ras-2XMyc G12V plasmids transfected are indicated above the panel. Images are representative of at least 12 independent frames.

B. Zoom-in images of highlighted area in A. Scale bar = 2 µm in both A and B.
5.2.1.3 Cholesterol Depletion by Methyl-β-cyclodextrin

Since we have observed inversely correlated staining of surface MT complexes and lipid rafts (Figure 5.1, 5.2 and 5.3), we next investigated whether cholesterol-rich lipid rafts may be a barrier for the formation of MT macro-complex on the cell surface. Methyl-β-cyclodextrin (MβCD) is a hydrophobic organic polymer commonly used to remove cholesterol. 60 min treatment in 1% MβCD has been shown to induce ligand-independent activation of the EGFR1 by depleting cholesterol from the plasma membrane (Chen and Resh 2002). Figure 5.7 shows the time-course of cell surface lipid raft staining after treatment in 1% MβCD. Incubation for 90 min or longer showed CT-B-labelled lipid rafts appear more diffused on the cell surface membrane.
Treatment of cells expressing wtMTFLAG with 1% MβCD 3 hrs before surface tag labelling, induced stronger overall fluorescent signals on the cell surface (Figure 5.8 A and B). After treatment in MβCD, MT complexes labelled from the external cell membrane appeared more numerous with stronger intensity for individual punctate. MβCD treatment did not increase background fluorescent level when non-transfected Rat-2 fibroblasts were used (results not shown). However, overall MT staining was also increased (Figure 5.8 C). The enhanced detection of MT was surprising and prevents us from concluding whether removal of cholesterol from the plasma membrane promotes macro-complex formation of MT. It is possible that treatment with MβCD causes exocytosis of intracellular MT to the cell surface (Takahashi, Hatakeyama et al. 2004), which complicates the interpretation of results.

Figure 5.7 Time course for MβCD treatment

Rat-2 fibroblasts expressing wtMT were incubated in 1% MβCD prepared in serum-free DMEM. At 10 min before each of the indicated time points, Vybrant fluorescent-conjugated CT-B was added to the media at 1:500 dilutions. After 10 min incubation with the lipid raft marker at 37°C, cells were subsequently fixed and mounted. Images of labelled lipid rafts on a single cell were captured with a confocal microscope. Scale bar = 10 μm.
Figure 5.8 Methyl-β-cyclodextrin increases surface and overall MT staining

A. Surface MT labelled after treatment in Methyl-β-cyclodextrin (MβCD). Cells expressing wtMTFLAG were incubated in 1% MβCD prepared in serum-free DMEM for 3 hrs. Media were removed and cells were incubated in rabbit polyclonal anti-FLAG prepared in 1% MβCD for 20 min before fixing and were subsequently incubated in AlexaFluor488-conjugated anti-rabbit IgG. Images of cells were captured with confocal microscope. Scale bar = 50 µm.

B. MT complexes on the basement membrane surface of a cell. Two independent frames are shown. Scale bar = 2 µm.

C. Total MT distribution after MβCD treatment. Cells were treated with MβCD under the same conditions described in A before fixing and permeabilisation of cell membrane. MT was labelled with PAb762 mouse monoclonal anti-MT (green). Nuclei were stained by DAPI (blue). Scale bar = 50 µm.
5.2.1.4 Detergent Resistance Membrane Fractionation

We then investigated whether MT signalling complexes are located in detergent soluble or insoluble membrane factions by fractionation. A similar protocol was used to show that many GPI-anchored proteins, such as caveolin-1, reside in the Triton-insoluble fraction while certain transmembrane proteins, such as EGFR1, preferentially reside in the detergent soluble fraction (Funatsu, Miyata et al. 1999, McKie, Vaughan et al. 2012). Cells were lysed by osmotic pressure and the cell membranes were collected by ultracentrifugation. The membrane faction was solubilised in 1% Triton-X 100. Any detergent insoluble membranes were then collected at the bottom of the eppendorf tube by ultracentrifugation. Caveolin-1 is a known lipid raft marker found exclusively in detergent-insoluble fraction of the membranes (Figure 5.9 A). Interestingly, non-stimulated PDGFRβ was fractionated mostly in the detergent soluble fraction (Figure 5.9 B and C), therefore can be used as an indicator for non-raft proteins. Figure 5.9 A shows similar amounts of wild type MT are found in both detergent soluble and insoluble fractions. Ponceau S protein stain was used as an indicator of the amount of proteins loaded in each lane.

Since mutant L405E seems to lack the ability to form large clusters in the membrane, fractionation experiment was also performed on cells expressing this mutant MT to see whether this non-transforming mutant partitions differently in the membrane. Figure 5.9 A shows L405E was also distributed almost equally between detergent-soluble and detergent-insoluble fractions, although a small amount of the mutant MT was detected in the non-membrane fraction, which indicates a weaker membrane association, probably as a result of the lack of C-terminus translocation across the lipid bilayer. However, the majority of L405E was still membrane bound. The non-transforming mutants 180Δ10 (lacks PP2A and Src binding sites) and 200Δ10 (lacks Src binding site only, see Mutant List) did not fractionate differently compared to wild type (Figure 5.9 C and data not shown). More importantly,
wtMT isolated from both detergent-soluble and detergent-insoluble fractions associates with signalling molecules such as PP2A core dimer, PI3K p85 subunit and p52 of Shc (Figure 5.9 D). Immuno-labelled surface MT complexes (wild type) showed very little colocalisation with caveolin-1 (results not shown). Together with previous IF data, it is likely that MT does not have any preference between raft and non-raft microdomains in the membrane.
Figure 5.9 Detergent resistance fractionation of cell membrane in cell lines expressing wild type or non-transforming MT mutant

A. Fractionation of wild type and L405E mutant MT. Cell lines stably expressing wtMT or L405E MT were fractionated as described in material & methods. Samples of whole cell lysates (WCL), non-membrane fraction (NMF), membrane fraction (MF), detergent-soluble faction (DSF) and detergent-insoluble faction (DIF) were normalised against protein concentration as determined by BCA assay, then analysed for MT content (i) or caveolin-1 (ii). Ponceau S protein stain in the migratory region of MT is show in (iii).

B. Fractionation of PDGFRβ in normal non-transfected Rat-2 fibroblasts. Ponceau S protein stain of PDGFRβ migratory region was shown in (ii).

C. Fractionation of non-transforming mutant 180∆10 MT. Fractionation samples were generated as described in A and analysed for the PDGFRβ.

D. Analysis of MT-associated signalling molecules in different membrane fractions. Cell lines expressing non-transforming mutant 180∆10 or wtMT were fractionated in to detergent soluble or insoluble fractions. After pre-clearing with Protein-A Sepharose beads, MT in each membrane fractions were immunoprecipitated with PAb762, and then analysed for the presence of known MT-binding molecules, as indicated above each panel. 5 µl of wtMT whole cell lysate was loaded in lane e as a reference (ref) for the migratory position of each polypeptide.
5.2.2 MT Association with the Cytoskeleton

Various sucrose gradient fractionation studies showed that MT co-fractionates with actin, vimentin and tubulin (Andrews, Gupta et al. 1993, da Costa, Wang et al. 2000). However, direct association between MT and cytoskeletal components has not been demonstrated so far. Here we investigate whether MT colocalises with actin cytoskeleton on the cell surface and if so, whether the formation of these MT macro-complexes in the plasma membrane is driven by actin polymerisation. Cells expressing epitope-tagged MT were transfected with the LifeAct-RFP plasmid, which encodes a short peptide with an actin binding property fused to red fluorescent protein (Riedl, Crevenna et al. 2008). Figure 5.10 (i) shows the detection of an extensive actin mesh near the membrane ruffle of a cell and its colocalisation with MT clusters labelled from the cell surface. Colocalisation analysis of the image suggests that about 50% of surface wild type MT clusters colocalise with actin.

Clusters of mutant 200Δ10, which lacks Src binding, also colocalise with actin. Instead of actin meshwork, 200Δ10 is located to actin cables or stress fibres running along the under surface of the cells (Figure 5.10 ii). These actin structures are also evident in non-transformed control Rat-2 fibroblasts. This suggests that Src tyrosine kinase binding is not requirement for MT association with actin cytoskeleton. Transformation of Rat-2 fibroblasts by MT is accompanied by a remodelling of the actin cytoskeleton (compare panels i and ii in 5.10 A). An additional picture of wild type MT complexes colocalising with actin cytoskeleton together with negative control are shown in Figure 5.11.
Figure 5.10 Surface MT complexes colocalise with actin cytoskeleton

A. Dual IF labelling of surface HA tag and actin cytoskeleton. Cell lines expressing wild type or 200Δ10 MTHA+ were transfected with LifeAct-Tag RFP plasmids using the Attractene transfection kit. 48 hrs post-transfection, cells were incubated with mouse monoclonal anti-HA antibody at 37°C for 20 min before fixing. AlexaFluor-488 conjugated anti-mouse secondary antibody was subsequently added to label surface MT (green). Images of the cell basement membrane were captured using a Leica SP5 confocal scanning microscope. An area of 20.5 µm x 20.5 µm is shown for each cell line indicated above the panel.

B. Zoom-in images of highlighted area in A. Individual and combined channels in an area of 10.25 µm x 10.25 µm are shown. Scale bar = 2 µm for both part A and B. Images shown are representative of at least 6 independent frames for each cell line.
Figure 5.11 Additional support images for surface MT complexes co-stained with actin-cytoskeleton

A. Dual IF labelling of surface HA tag and actin cytoskeleton. WtMTHA+ cells and parental Rat-2 fibroblasts were transfected with LifeAct-Tag RFP plasmids using the Attractene transfection kit. Staining conditions for surface HA is described in Figure 5.1. An area of 20.5 x 20.5µm is shown for each cell line indicated above the panel.

B. Zoom-in images of highlighted area in A. Individual and combined channels in an area of 10.25 x 10.25µm taken from A (white squares) are shown. Scale bar, 2 µm.
We then investigated whether actin disassembly affects surface MT cluster formation by treating cells with an inhibitor of actin polymerisation, cytochalasin D (CCD) before labelling surface MT. Cells were flattened and showed impaired cell division after 48hr-treatment in 500 nM CCD (Figure 5.12 A & B), which indicates that the actin cytoskeleton was effectively disrupted by the treatment conditions although actin staining was not performed. Figure 5.12 C shows MT complexes are observed on the cell surface with little difference in size and numbers compared to untreated cells. Therefore disruption of actin polymerisation probably does not affect the formation of MT macro-complexes in the plasma membrane.

Using similar methods, colocalisation between surface MT complexes and the intracellular micro-tubule network is demonstrated in Figure 5.13 A when α-tubulin (monomeric unit of microtubules) was labelled following surface FLAG-tag binding. Disruption of micro-tubules with nocodazole or colchicine, which bind to tubulin and inhibit the formation of microtubule, did not inhibit the formation of MT complexes on the cell surface despite inducing a lot of cell death (Figure 5.13 B and data not shown).
Figure 5.12 Cytochalasin D does not disrupt surface MT complex formation

A. Surface MT labelled after treatment with cytochalasin D (CCD). Cells expressing wtMTFLAG were incubated in culture media containing 500 nM CCD for 48 hrs before surface MT was immuno-labelled using rabbit polyclonal anti-FLAG. After fixing and permeabilisation of the cell membrane, mouse monoclonal PAb762 was added to label overall MT. AlexaFluor488/633-conjugated anti-rabbit/-mouse IgG were then added to label surface and total MT, respectively. Images of cells were captured with confocal microscope. Scale bar = 50 µm.

B. Total MT distribution of the images shown in A.

C. Surface MT complexes do not change after cytochalasin D treatment. High magnification images of surface MT complexes shown in A. Two independent frames are shown for each treatment and are representative of at least 20 images generated in 3 independent experiments. An area of 20.5 µm x 20.5 µm on the basement membrane is shown in each frame. Scale bar = 2 µm.
Figure 5.13 Surface MT complexes colocalise with micro-tubules inside cells

A. Surface MT complexes colocalise with tubulin. Cells expressing wtMTFLAG were incubated with rabbit anti-FLAG antibody for 30 min before fixing and permeabilisation of the cell membrane. Mouse polyclonal anti-α-tubulin was added to cells, followed by AlexaFluor 488 anti-rabbit IgG and AlexaFluor 633 anti-mouse IgG to label surface MT (green) and microtubule cytoskeleton (red). Images were captured on a confocal microscope. Areas of colocalisation are indicated with open arrows. Primary antibodies are indicated above the top panel.

B. Tubulin destabilising agents did not affect surface MT. Cells were treated with 25 ng/ml nocodazole or 25 µg/ml colchicine for 4 hr before IF labelling of surface FLAG tags as described above. Two independent frames were shown for each treatment with the upper panel showing cells with weaker fluorescence and the lower panel showing stronger fluorescent signals. Scale bar = 2 µm for A and B.
5.2.3 Focal Adhesion Associated Proteins

5.2.3.1 Vinculin

Although we have observed colocalisation between surface MT complexes and the actin cytoskeleton, co-immunoprecipitation experiments did not show direct interaction between MT and β-actin (result not shown). It is possible that MT may interact with the cytoskeleton indirectly via actin binding protein such as vinculin or molecules involved in actin remodelling, such as FAK and integrins. It has been reported that MT can be immunoprecipitated using anti-vinculin serum and that MT/pp60c-src associates with focal adhesion sites (Ballmer-Hofer, Ziegler et al. 1988) (see Chapter 1, 1.14 for introduction). We therefore investigated whether the MT macro-complexes associated with cytoskeleton on the cell basement membrane represent sites of focal contacts. Vinculin, an actin binding protein which connects integrin molecules on the cell surface with intracellular actin filaments, was used as a marker for focal contacts. Co-staining of surface MT complexes and vinculin produced very little colocalisation (Figure 5.14). The lack of colocalisation between MT complexes and vinculin may be attributed to the transformed phenotype of the cells. Therefore, in order to see whether more surface MT complexes colocalise with vinculin in non-transformed cells, cells expressing wild type MT were treated with 50 nM dasatinib for 48hrs before IF staining. Figure 5.14 shows that although dasatinib treatment increases the number of MT complexes at the edge of the cells, the majority of the surface MT complexes still do not colocalise with vinculin. Additional frames can be found in Figure 5.15.
Figure 5.14 Dual labelling surface MT complexes and intracellular vinculin

A. Co-staining surface MT with internal vinculin. Cells expressing wtMTHA were incubated in culture media containing 50 nM dasatinib for 48 hrs before surface MTs were immuno-labelled using mouse monoclonal anti-HA added to living cells. After fixing and permeabilisation of the cell membrane, rabbit monoclonal anti-vinculin was added. Cells were subsequently incubated in AlexaFluor488/555-conjugated anti-mouse/rabbit IgG to detect surface MT or intracellular vinculin, respectively. Images of cells were captured with a confocal microscope. For each treatment, the image shown is representative of at least 6 independent frames. Scale bar = 2 µm.

B. Zoom-in images of areas highlighted in A. The antibody detected in each channel is indicated above the top panel. Scale bar = 2 µm.
Figure 5.15 Little colocalisation between surface MT complexes and vinculin at the basement membrane

A. Co-staining surface MT with internal vinculin. Cells expressing wtMTHA with or without 50 nM dasatinib treatment were immuno-labelled for surface MT and total intracellular vinculin as described in Figure 5.14. Scale bar = 2 µm.

B. Zoom-in images of area highlighted in A. Antibody detected in each channel is indicated above the top panel. Scale bar = 2 µm.
5.2.3.2 Focal adhesion kinase

Focal adhesion kinase (FAK) is a protein tyrosine kinase often associated with focal adhesion plaques (see Chapter 1, 1.14.1 for introduction). It is activated by integrin clustering or by growth factor receptor activation and autophosphorylation on Y397, which recruits other signalling molecules to the integrin/FAK complex. PI3K p85and pp60c-src bind to phospho-Y397 on FAK in fibroblasts transformed by MT but the association between MT and FAK was not established (Bachelot, Rameh et al. 1996). It has been reported that dasatinib treatment abolishes the phosphorylation on all tyrosine residues of FAK except Y397, and autophosphorylation site (Caccia, Micciche et al. 2010). Therefore, we investigate whether the MT complexes observed on the basement membrane of transformed cells contain activated FAK (phospho-Y397) which regulate the formation of focal adhesion sites. Figure 5.16 shows that similar to vinculin, most of the phospho-FAK signals are located at the cell periphery with little colocalisation with surface MT complexes. After treatment with dasatinib, the number of MT complexes colocalising with phospho-Y397 increased at the cell periphery. However, complexes of surface MTHA and activated FAK do not always match perfectly in their shape and position (open circles, Figure 5.16). There are still a lot of surface MT complexes that do not colocalise with activated FAK. An additional frame can be found in Figure 5.17.
Figure 5.16 Surface MT complexes and activated focal adhesion kinase

A. Co-staining surface MT with activated FAK. Cells expressing wtMTHA were incubated in culture media with/without 50 nM dasatinib for 48 hrs before surface MT were immuno-labelled using mouse monoclonal anti-HA. After fixing and permeabilisation of the cell membrane, rabbit monoclonal antibody specific for phospho-FAK Y397 was added. Cells were subsequently incubated in AlexaFluor488/555-conjugated anti-mouse/rabbit IgG to detect surface MT or intracellular phospho-FAK, respectively. Images of cells were captured with a confocal microscope. For each treatment, the image shown is representative of at least 6 independent frames. Scale bar = 5 µm.

B. Zoom-in images of areas highlighted in A. Antibodies used are indicated above the top panel. Scale bar = 2 µm. Open circles highlight examples of colocalisation between surface MT and phospho-FAK at cell periphery.
5.2.3.3 Integrin αVβ5 Macro-complexes on the Cell Surface

Phosphorylation of FAK at focal adhesion complexes coincides with the clustering of integrins. These are well characterised integral transmembrane hetero-dimeric protein complexes found on the cell surface (see Chapter 1, 1.14.3 for introduction). Since MT formed macro-complexes on the cell surface membrane which colocalised with actin network, we asked whether MT complexes co-exist in domains in the plasma membrane occupied by integrins. Amongst the commercially available antibodies, only the mouse

Figure 5.17 Additional frames for surface MT complexes co-staining with activated FAK
A. Cells expressing wtMTHA+ were immuno-labelled for HA and phospho-FAK Y397 as described in Figure 5.16. Scale bar = 2 µm.
B. Zoom-in images of areas highlighted in A. Antibodies used are indicated above the top panel. Scale bar = 2 µm. Open circles highlight areas of strong phospho-FAK.
monoclonal antibody (P1F6), which binds to the extracellular domains of αVβ5 integrin, has been shown to produce strong immunofluorescent staining when added to living cells. Here, this mouse monoclonal antibody was added in conjunction with rabbit anti-FLAG antibody to produce co-staining of surface MTFLAG and integrin αVβ5 complexes simultaneously (Figure 5.18 and 5.19). Both MT and integrin complexes are distributed throughout the under surface of the cell. However, at 37°C, antibody cross-linking of integrin αVβ5 resulted in rapid internalisation of the cell surface receptor, whereas MT on the cell surface was not readily endocytosed (Figure 5.20). Therefore co-staining experiment was repeated at 4°C. At both temperatures, some colocalisation between MT and integrin αVβ5 complexes was observed (open arrows, Figure 5.18 and 5.19). Dasatinib treatment did not affect the level of colocalisation between the two surface molecules (Figure 5.18 (ii) and 5.19 (ii)). On average, 21% of surface MTFLAG and 16% of surface integrin αVβ5 were found to colocalise with one another (Figure 5.18 and 5.19). However, this level of colocalisation probably occurred by chance, as the integrin clusters were numerous. Despite various attempts, integrin has not been found in MT immunoprecipitates (data not shown). It is likely that the MT macro-complexes observed on the cell surface only passively associate with adhesion molecules.
Figure 5.18 Partial colocalisation between surface MT and integrin αVβ5

A. Surface labelling MT and integrin αVβ5 at 37°C and 4°C. Rat-2 fibroblasts expressing wtMT FLAG were incubated live in rabbit polyclonal anti-FLAG antibody and mouse monoclonal [P1F6] to integrin αVβ5 extracellular domains for 30 min at 37°C or 4°C. After fixing, cells were incubated with AlexaFluor488-conjugated anti-rabbit IgG and AlexaFluor555-conjugated anti-mouse IgG to label surface FLAG (green) and integrin αVβ5 (red) at the same time. The fluorescent signals on the basement membrane of a cell were captured using a Leica SP5 confocal microscope with 63.0x1.40 oil lens, zoom factor 6. Each image represents an area of 20.5 x 20.5 µm. Scale bar = 2 µm. Examples of colocalisation are indicated with open triangles.

B. Surface labelling MT and integrin αVβ5 at 4°C. The experiment in A was repeated at 4°C. Images for each staining condition are representative of at least 6 independent frames.
Figure 5.19 Zoom-in images of Figure 5.18
Examples of colocalisation are indicated with open triangles. Scale bar = 2 µm.
It has been reported that MT can bind to and activate the insulin receptor (IR) and the insulin-like growth factor I receptor (IGF-IR) (Novosyadlyy, Vijayakumar et al. 2009). We found that the MT TMD can be functionally replaced by the hydrophobic membrane spanning sequence from wild type neu and PDGFRβ (see Chapter 4, Figure 4.10). EGFR1 and PDGFRβ are well expressed in Rat-2 fibroblasts. Therefore, initially we intended to investigate whether MT macro-complexes in the plasma membrane may contain these two abundant receptor tyrosine kinases. However, initial experiments produced negative results (data not shown). In cells expressing wild type MT, very little EGFR1 was internalised following EGF stimulation (Figure 5.21).
Above result was followed up by assessing the protein level of EGFR1 and PDGFRβ in cell lines expressing wild type and various mutants of MT. Figure 5.22 shows that only cell lines transformed by MT, but not by the constitutively activating mutant H-Ras G12V, showed reduction in EGFR1 and mature PDGFRβ protein levels. Reduction of both PDGFRβ and EGFR1 was also observed with the non-transforming mutant L405E MT. The small amount of PDGFRβ in cells expressing wild type MT is not phosphorylated in the absence of ligand and the level of phosphorylated receptor is much lower compared to normal Rat-2 fibroblasts in response to stimulation by ligand human PDGF-BB (hPDGF-BB) (Figure 5.23).
B). Therefore in the absence of ligand, residual PDGFRβ is not activated in cells expressing wtMT.

Figure 5.22 Analysis of EGFR1 and PDGFRβ protein levels in Rat-2 fibroblast cell lines

Antibody used is indicated above each panel. Cell lines expressing wild type MT, non-transforming mutants L405E, MTKDEL, 180Δ10 and 200Δ10, activated H-Ras-Myc only or co-expressed with wtMTFLAG are compared to parental control Rat-2 fibroblasts. Cells were lysed in 1% Triton-X100 lysis buffer with scraping. Protein concentrations of lysates were normalised by a BCA test and analysed by 8% bis-acrylamide SDS-PAGE gel and Western Blotted for EGFR1 and PDGFRβ. The membrane of lower molecular weight region was blotted for α-tubulin as a loading control for each receptor blot.
Figure 5.23 Changes in expression levels of various growth factor receptors in Rat-2 fibroblasts transformed by MT

A. Residual PDGFRβ in MT transformed cells can be activated by ligands. Confluent control cells or cells expressing wtMT were cultured on 6-well plates. Ligand human PDGF-BB (PDGF-BB) was added to stimulate PDGFRβ activation. After normalising sample volume against protein concentration, lysates were analysed for PDGFRβ phospho-Y751. Ponceau S staining of the membrane after Western Blotting was used as a loading control (lower panel). Time-point of ligand stimulation (min) is indicated above each lane.

B. Analysis of various receptor levels in response to dasatinib. Cells expressing wild type MT or non-transforming mutant 200Δ10 and control cells were treated with 50 nM dasatinib (+) or vehicle for 48hrs then lysed in 1% Triton-X 100 lysis buffer. Lysates were analysed for the receptor indicated above each panel. Sample volume was normalised against lysate concentration determined by BCA assay. Western blot for α-tubulin was used as a loading control. X indicates a void sample loading.

C. PDGFRβ levels in transformed fibroblasts after removal of dasatinib. Cells expressing wtMT were treated with 50 nM dasatinib for 48 hrs to induce PDGFRβ expression. The inhibitor was then removed and cells were lysed at various time points (c-e).
Figure 5.24 Level of mRNA for EGFR family receptors and PDGFRβ in cell lines expressing MT

A. Analysis of mRNA levels of ErbB receptors and PDGFRβ in cell lines expressing wild type or mutant MT. Equal number of cells were seed on 6-well plates 24hrs before experiment. Cells were lysed in 1ml Trizol. Total RNA were purified and used as templates for cDNA conversion using Invitrogen Superscript cDNA conversion kit. Equal volume of each cDNA sample was analysed by PCR method for receptor tyrosine kinase DNA sequence using a pair of primers that bind to sequence specific for each receptor cDNA. PCR products were separated by PAGE and stained by ethidium bromide to visualise the band corresponding to the predicted PCR product size. A mistake was made in A (ii) Rat-2 PCR reaction mix, which resulted in an empty lane.

B. The same cDNA preparation from above experiment was re-analysed for EGFR1 and PDGFRβ with loading control ATP5B. Control primer from was a kind gift from Dr Jocelyn Mora.
In order to assess whether the expression level of receptors are affected in MT-transformed cells, RNA from cell lines expressing wild type or mutant MT were extracted and analysed for their mRNA levels of various EGFR family receptors and PDGFRβ by PCR. Figure 5.24 shows, that the mRNA of PDGFRβ is absent in cells expressing wild type MT but not in those expressing non-transforming mutants such as MTKDEL, a mutant trapped in the ER, and 200Δ10, a mutant lacking Src tyrosine kinase binding sequence. The reduction in EGFR1 mRNA level is less dramatic in cells transformed by MT. The mRNA levels of other EGFR family members are more variable between the cell lines expressing MT and mutant. Therefore, we focused on PDGFRβ expression in subsequent experiments.

We next tested whether inhibition of Src tyrosine kinase activity, which causes the transformation in cells expressing MT, could rescue the expression of PDGFRβ. Figure 5.25 A shows that 5 nM or higher concentrations of dasatinib can up-regulate PDGFRβ levels in cells expressing wtMT after 48hrs. Treatment with 50 nM dasatinib induced the maximum level of PDGFRβ expression without affecting the viability of the cells. The up-regulation of PDGFRβ by dasatinib was observed only in cells expressing wtMT but not 200Δ10 and control (Figure 5.25 B). Similar results were obtained for both confluent and growing cells that were transformed by MT (Figure 5.25 C). Dasatinib treatment did not affect PDGFRβ protein level in normal Rat-2 fibroblasts control (Figure 25 C) or those transformed by H-Ras G12V (data not shown). Attempts to analyse the mRNA level of the PDGFRβ after dasatinib treatment failed due to poor quality of the RNA extracted. After dasatinib was removed, cells become transformed again but the protein level of PDGFRβ was maintained for at least 4 hrs (Figure 5.23 C). Longer time points will be required to assess the stability of the receptor expression in transformed cells after the removal of dasatinib. The up-regulated PDGFRβ after dasatinib treatment is a fully mature polypeptide responsive to ligand stimulation (data not shown).
Dasatinib is also a potent inhibitor of many members of the PDGF receptor family, including c-KIT, PDGFRβ (Lombardo, Lee et al. 2004) and the FMS receptor (Brownlow, Mol et al. 2009). In order to confirm that the down-regulation of PDGFRβ was not due to constitutive activation of the receptor in MT-transformed cells, imatinib, a tyrosine kinase inhibitor that is specific for PDGFRβ but does not inhibit Src, was then used. The effects of dasatinib and imatinib on cells transformed by wild type MT are compared to their effects on non-transforming mutant L405E, which binds to all MT-associated protein and 180Δ10, which lacks both PP2A and Src binding sites. Figure 5.25 D shows imatinib treatment failed to up-regulate PDGFRβ expression in all three cell lines. Dasatinib (at 10 or 50 nM) induced re-expression of PDGFRβ in cells expressing wild type MT and up-regulated the level of PDGFRβ in cells expressing mutant L405E, which has reduced PDGFRβ expression in Figure 5.22 (iii). PDGFRβ expression was not affected by either drug in cells expressing...
mutant 180Δ10, which lacks both PP2A and Src binding sites (lane i – l in Figure 5.25 D).

The down-regulation of PDGFRβ, and possibly EGFR1, is therefore not a result of MT-induced constitutive receptor activation. After dasatinib up-regulation of EGFR1 and PDGFRβ, immunofluorescence-labelled surface MT complexes did not show colocalisation with either receptor (data not shown).

The effect of dasatinib treatment on other receptors has also been tested (Figure 5.23 B). In cells expressing wild type MT, ErbB2 expression was up-regulated after inhibition of Src, while ErbB4 and IRβ was reduced, but the differences are less dramatic compared to the changes in PDGFRβ protein level (Figure 5.23 B). Results also confirm dasatinib does not affect MT expression levels.
5.3 DISCUSSION:

5.3.1 Summary

Immuno-fluorescent microscopy experiments showed that wild type MT molecules are able to accumulate into clusters at the plasma membrane (see Chapter 4). We found little correlation between these MT-containing clusters with lipid raft markers (Figure 5.1 – 5.9), indicating that cholesterol-dependent membrane sub-domains are probably not involved in MT transformation. For the first time, we have demonstrated that MT macro-complexes on the cell surface are associated with cytoskeletal structures (Figure 5.7 and 5.9). However, we did not find direct interaction between MT and actin by immunoprecipitation methods. Therefore MT surface complex association with intracellular actin cytoskeleton is likely to be indirect. MT complexes on the cell surface are likely to utilise actin cytoskeletons as a means to limit free-diffusion of active MT signalosomes (see 5.3.3.1). The association between MT surface clusters and actin cytoskeleton does not depend on MT’s ability to bind Src tyrosine kinase though active MT complexes may play a role in re-modelling cytoskeleton to influence cell morphology (Figure 5.10). Disrupting cytoskeleton polymerisation with various inhibitors did not affect MT complex formation (Figure 5.12 and 5.13). Surface MT macro-complexes are probably not related to focal adhesion complexes (Figure 5.14 and 5.16), although partial colocalisation with cell surface integrin complexes have been observed (Figure 5.18 and 5.19). Despite report on MT association with the insulin receptor and insulin-like growth factor receptor (Novosyadlyy, Vijayakumar et al. 2009), we found very little colocalisation between surface MT complexes and several growth factor receptors tested in this study (data not shown). However we observed repression of PDGFRβ protein expression in Rat-2 fibroblasts transformed by MT, which complicated the study of MT association with cell surface receptors (Figure 5.22 – 5.25).
5.3.2 Lipid rafts do not play a role in MT cluster formation

Clustering of molecules within cell membranes has been reported recently in a number of systems, and may play a role in signal transduction (Cebecauer, Spitaler et al. 2010). Using a commercial lipid raft marker, we observed very little colocalisation between surface MT clusters with cholesterol-rich ganglioside GM1-positive lipid rafts (labelled by cholera toxin subunit-B in Figure 5.1 and 5.4). Chen and Resh have demonstrated that cholesterol depletion with Methyl-β-cyclodextrin (MβCD) caused ligand-independent activation of EGFR1 and suggested that cholesterol-rich rafts may act as a spacer that minimise EGFR dimerisation in the absence of ligands (Chen and Resh 2002). Cholesterol depletion by Methyl-β-cyclodextrin (MβCD) increased the level of surface MT clusters (Figure 5.8). However, due to the reported side effect that the compound has on exocytosis, we cannot conclude whether MT complexes reside in non-raft regions of the cell membrane. MβCD was found to promote exocytosis and the fusion of vesicles with the plasma membrane (Takahashi, Hatakeyama et al. 2004, Chen, Li et al. 2010, Hissa, Duarte et al. 2012). We have observed that cells with strong lipid raft marker staining were often accompanied by lower amount of MT clusters on the cell surface, therefore suggesting that MT may reside in non-cholesterol-enriched domains (Figure 5.2). We tried to use activating H-Ras mutant G12V as a marker for cholesterol-independent microdomains, but found that H-Ras was not a good indicator for non-raft domains in our system as it can only be labelled internally and the fluorescent signals masked the signals of MT clusters labelled from the cell surface (Figure 5.5). We then used detergent fractionation method to show that MT does not preferentially resided in raft or non-raft regions of the membrane, using caveolin-1 as an indicator for cholesterol-dependent detergent insoluble fraction (Figure 5.9).
Whether a membrane protein fractionates with caveolin-positive lipid raft is highly influenced by the method used. Some reports suggest that the PDGFRβ resides exclusively in detergent insoluble, caveolin-positive lipid rafts (Liu, Ying et al. 1996, Wu and Gonias 2005), while others reported half of the PDGFRβ resides in detergent insoluble fraction (Coats, Pledger et al. 1996). In non-transformed Rat-2 fibroblasts, we found that PDGFRβ fractionates predominantly in the detergent soluble membrane fraction (Figure 5.9), similar to the observation made with EGFR1 using the same fractionation protocol (McKie, Vaughan et al. 2012).

5.3.3 MT and Cytoskeleton

5.3.3.1 MT macro-complexes on the cell surface associate with actin cytoskeleton

Accumulation of Ras in nano-clusters is essential for signalling activity (Plowman, Muncke et al. 2005, Tian, Harding et al. 2007) and requires the actin cytoskeleton (Plowman, Muncke et al. 2005). We have also observed localisation of MT complexes to the intricate actin mesh near the membrane ruffles of transformed cells (Figure 5.7 i), and along the actin cables in non-transformed fibroblasts expressing mutant 200Δ10, which lacks Src-binding site (Figure 5.7 ii). In non-transformed cell type, cortical actin bundles are very prominent and surface 200Δ10 clusters seem to follow the track of these actin cables (Figure 5.6 ii). MT associated kinase activity has been shown to co-fractionate with cytoskeletal elements (Schaffhausen, Dorai et al. 1982). Cytoskeletons such as actin, tubulin and vimentin have all been detected in MT-enriched fractions using sucrose gradient fractionation methods (Andrews, Gupta et al. 1993, Krauzewicz, Elliott et al. 1994, da Costa, Wang et al. 2000). Here, for the first time, we have demonstrated MT complexes on the cell surface membrane are associated with networks of cytoskeleton (Figure 5.7 and 5.9).
Single-particle tracking have shown that a number of transmembrane proteins and lipids are restricted in their movement to ‘confinement zones’ that vary in size from 30 – 700 nm (Sako and Kusumi 1994, Simson, Sheets et al. 1995, Lillemeier, Pfeiffer et al. 2006). This favours our model that cell surface receptor clusters are pre-formed and are independent of extracellular stimuli-induced kinase domain activation. In the ‘picket-fence’ model proposed by Kusumi and co-workers, ‘Membrane proteins, like pickets, are anchored to and line up along a fence of cytoskeletal proteins surrounding the confinement zones’ – Kusumi, et al. Semin. Immunol. 2005, (Kusumi, Ike et al. 2005). This is then developed further into a recently described ‘active’ model of receptor clustering that is driven by the cortical actin meshwork just under the lipid bilayer of cell surface membrane (Sbalzarini 2012). This is supported by recent report that GPI-anchored folate receptors on the outer leaflet of the plasma membrane form clusters which are sensitive to inhibitors of myosin function, which are cellular motors that interacts with actin to form actomyosin and regulates contractility, but are not sensitive to inhibitors of actin polymerisation (Plowman and Hancock 2005, Gowrishankar, Ghosh et al. 2012, Sbalzarini 2012).

The new model suggests that the actin snippets held together by myosin, form actin-rich domains under the inner membrane leaflet of cells. The movement of these actin-rich domains is influenced by myosin motors, which are actively regulated by receptors and signalling molecules in the local area. Membrane proteins localised to these actin-rich domains or rafts are dragged along by actomyosin movement. Therefore it is possible that the clustering of MT as reported in this study and GFRs reported by others are not formed by equilibrium, but instead, are driven by actin mesh on the cytosolic surface. The movement of such complexes in the plasma membrane is dependent on myosin motor activity instead of actin polymerisation. This model may also explain why cytochalasin D (CCD) did not affect complex formation on the cell surface in our system and others such as the nicotinic
acetylcholine receptor (Wenz, Borroni et al. 2010), since clustering is largely driven by
myosin activity instead of actin polymer stability (Gowrishankar, Ghosh et al. 2012,
Sbalzarini 2012).

5.3.3.2 MT macro-complexes may be involved in cytoskeleton re-modelling

It has been suggested that MT C-terminal region plays a role in targeting MT to the
cytoskeleton. Elliott and co-workers demonstrated that the Arginine clusters or R-rich region
(385-393: QRHLRRLGR) which precede the MT hydrophobic sequence, when fused to GFP,
can target the normally cytosolic protein to cytoskeletal fractionation of the cell lysate
(Elliott, Jones et al. 1998). Therefore, it is possible that the C-terminus of mutant L405E and
neu-mutant MT which fail to translocate across the membrane completely, may interfere with
the Arginine-rich, cytoskeleton-targeting region. This explanation was proposed for the lack
of transformation in MT mutant, NT-1d, which has a mutation of positively charged Arginine
to a negatively changed Glutamate at the cytoplasm-membrane interface (Dahl,
Thathamangalam et al. 1992). This non-transforming mutant is capable of binding and
activating pp60c-src and PI3K but showed weaker membrane association, a property similar
to L405E (Figure 5.9 A) (Dahl, Thathamangalam et al. 1992). Many transmembrane proteins,
such as glycophorin A, a heavily glycosylated protein on the red blood cell surface
membrane, have been shown to rely on the basic amino acids in the cytoplasmic
juxtaplasmic region to mediate interaction with cytoskeleton (Dahl, Thathamangalam et al.

MT complexes in the plasma membrane may also play a role in remodelling of the
cytoskeleton. In MT-transformed fibroblasts, actin cytoskeleton and microtubules appear
disorganised (Figure 5.10 and 5.13 A). Many of the proteins involved in regulating actin
dynamics are known effectors of Src tyrosine kinases including vinculin (El Sayegh, Arora et al. 2004) (reviewed by Reynolds and Rocznik-Ferguson 2004). Although some reports suggest that MT associates with actin-binding protein, vinculin, and MT/pp60c-src complexes localise at the focal adhesion sites (Ballmer-Hofer, Ziegler et al. 1988, Bachelot, Rameh et al. 1996), we found little colocalisation between surface MT clusters and vinculin or FAK (Figure 5.14 and 5.16). Due to the complexity of actin associated proteins, we have not been able to identify the mechanism by which MT is anchored to the cytoskeleton.

The microtubule associated protein tau, which plays a role in stabilising microtubules, was found to be a target of PP2A therefore PP2A regulates tubulin dynamics (Sontag, Nunbhakdi-Craig et al. 1996, Gong, Lidsky et al. 2000). In fact, there is an increased association between microtubules and PP2A in cells transformed by MT (da Costa, Wang et al. 2000). In addition, MT activates Rac-1 and the Cde42 Rho family GTPase by both PI3K and Shc – Ras pathways (Urlich, Senften et al. 1997). These GTPases play important roles in regulating filamentous actin organisation. Activated Rac induces rapid actin polymerisation in membrane ruffles (Ridley, Paterson et al. 1992)(Machesky and Hall 1997). Indeed, the disorganised nature of actin at the membrane ruffles of cells transformed by MT resembles that of PDGF-stimulated fibroblasts (Figure 5.10 and Machesky and Hall 1997). Therefore, a higher local concentration of Src tyrosine kinases and other signalling molecules in MT macro-complexes may influence cytoskeletal structures to favour cellular transformation (Krauzewicz, Elliott et al. 1994).

5.3.3.3 MT and Integrin

Hetero-dimeric integrin molecules have been known to form large clusters on the cell surface membrane, which mediates adhesion and migration by interacting with actin-binding
proteins. We have observed partial colocalisation between MT and integrin complexes on the cell (Figure 5.18 and 5.19). The antibody P1F6 targets only the αVβ5 integrin. We cannot rule out that whether MT colocalisation with other integrin subunit combinations may also occur. Although only about 20% of surface MT complexes colocalise with integrin clusters (Figure 5.18 and 5.19), this is not so low considering colocalisation between surface MTFLAG clusters and MTHA is only 30-40% on average (Figure 4.31). We did not find direct association between MT and integrin αVβ5 by immunoprecipitation (results not shown). This could be due to the small fraction of MT in complex with integrin or because the complex interaction was sensitive to detergent. Overall, we cannot conclude whether MT and integrin can co-exist in the same macro-clusters.

Integrin may be implicated in both tumorigenesis and metastasis in vivo. Integrin αVβ5 is expressed in most primary breast cancers, renal cancer, melanoma and its expression is particularly high in brain tumours metastasised from lung cancer and melanoma (Vogetseder, Thies et al. 2013). In the MMTV-MT mouse model for mammary gland carcinoma, β1 integrin-mediated phosphorylation of FAK, especially on Y576, is required for MT-induced tumorigenesis (White, Kurpios et al. 2004). However, Rac-induced actin reorganisation at membrane ruffles and lamellipodia was found to be independent of integrin and focal complex assembly (Machesky and Hall 1997). Therefore, integrin or FAK may not be absolutely required to mediate cell transformation.

5.3.4 Lack of PDGFRβ and reduced EGFR expression in MT-transformed cells

It has been reported that MT interacts with the insulin receptor and the closely related IGF-I receptor in a cell line derived from MMTV-MT mammary gland tumours. This association is required for the cells to initiate new tumours in animals (Novosyadlyy, Vijayakumar et al.
2009). Association between MT and IRβ was not observed in our system (data not shown). This discrepancy of results may be attributed to the difference in MT antibody and the cell type used in our study. Since replacement of the hydrophobic region of MT with sequence from wild type PDGFRβ did not affect MT transformation potential (Chapter 4, Figure 4.10), we then investigated the possible interaction between MT and this growth factor receptor by immunofluorescent labelling. However, instead of finding definitive answers, we observed significantly reduced PDGFRβ expression in MT-transformed Rat-2 fibroblasts at both protein and mRNA levels (Figure 5.22 and 5.24). The PDGFRβ expression is inversely correlated with the transformation potential of the MT expressed (Figure 5.22). PDGFRβ is re-expressed after inhibiting Src with dasatinib (Figure 5.25). Therefore the suppression of PDGFRβ is a specific consequence of MT activated mitogenic signalling. Reduced expression of PDGFRβ is also observed in cells expressing the mutant L405E, which was found to partially activate AKT signalling pathway (Figure 4.15). EGFR1 and ErbB2 protein levels are also reduced to a lesser extent in MT-transformed cells and the effect of MT signalling on their transcriptional levels are not very clear (Figure 5.22 - 5.24). Although dasatinib up-regulates EGFR1 and PDGFRβ expression in MT-expressing fibroblasts, we did not observe association between MT and GFRs by both immunofluorescent labelling and co-immunoprecipitation methods (data not shown).

Figure 5.24 indicates that MT transformation signals cause inhibition of the transcription of the PDGFRβ gene. The fact that dasatinib but not imatinib could suppress the down-regulation of the PDGFR shows that inhibition is caused by the activation of signalling pathways by MT and not by the PDGFR in response to MT (Figure 5.25 C). MT and normal mitogenic PDGF signalling share a lot of common effectors, so it is possible that the enhanced activation of a shared signalling pathway(s) may contribute to the down-regulation of PDGFRβ transcription. Activation of PI3K/AKT/mTORC signalling pathway has been
shown to down-regulate PDGFRβ expression by a negative feedback loop in mesenchymal stem cells stimulated with PDGF-BB ligands (Gharibi, Ghuman et al. 2012). Many of the PDGF early response genes such as *fos*, *jun*, and *myc* constitutively activated in cells expressing MT (Rameh and Armelin 1991, Armelin and Oliveira 1996). The PDGFRβ promoter contains a number of transcription factors binding sites for GATA-1, AP-1, AP-2 and NF-1 (Ballagi, Ishizaki et al. 1995). Some of these, such as AP-1, are known to be activated by MT oncogenic signalling. The implication of PDGFRβ down-regulation in MT-transformed cells will be discussed further in the next chapter.
6.1 Overall Summary

In summary our data illustrates that MT associates with signalling proteins at different sites in its maturation pathway. MT binds to PP2A core dimer in the cytoplasm, to pp60c-src at the ER, and to pp62c-yes, ShcA, PI3K, and PLC-γ1 at subsequent locations en route to the plasma membrane (Chapter 3). However, MT association with activated Src and other signalling molecules is not enough to transform fibroblasts and there appear to be at least two other requirements. One, MT needs to reach the cell surface in order to transform fibroblasts, as apparently do other cell surface signalling molecules (Chapter 3). Two, MT may also need to form a large macromolecular complex in order to transform fibroblasts (Chapter 4). These complexes are probably not associated with lipid rafts but there is interaction with the actin cytoskeleton, which may contribute to transformation (Chapter 5). Figure 6.1 summarises our current understanding of the requirement for fibroblast transformation by MT. We did not observe MT colocalisation with cell surface receptors such as PDGFRβ and EGFR1. However, we have observed that MT-induced transformation caused a dramatic down-regulation of PDGFRβ protein expression (Chapter 5).
Figure 6.1 A schematic representation of the maturation of a transforming MT complex.

Stages of the MT signalosome assembly are shown. Starting in the bottom right hand corner: MT is synthesised in the cytoplasm on free poly-ribosomes; MT maturation through the ER, exocytic membranes; the MT signalling complex eventually reaches the plasma membrane. At each stage, the cellular proteins added to the MT complex are illustrated. A representative mutant that blocks each individual step is also indicated. Adapted and extended from (Zhou, Ichaso et al. 2011).
6.2 Macro-complex Formation in GFR Activation

6.2.1 Receptor Clusters

Activation of growth factor receptor kinase activity by dimerisation of the receptor (reviewed by Lemmon and Schlessinger 2010), is an essential first regulatory step in signalling. However, recent evidence suggests receptor dimerisation probably takes place in the context of a higher order macromolecular complex structure (Yang, Raymond-Stintz et al. 2007, Arkhipov, Shan et al. 2013, Kozer, Barua et al. 2013). Cluster formation has been observed in many systems of Growth Factor Receptor (GFR) activation (Gillham, Golding et al. 1999, Ichinose, Murata et al. 2004, Yang, Raymond-Stintz et al. 2007, Kaufmann, Muller et al. 2011) (see Chapter 4 discussion). Our data supports the view that large signalling complexes can form on the cell surface without kinase involvement as observed with a MT mutant 200Δ10, which is unable to activate Src, and also in cells expressing wild type MT after treatment with the Src tyrosine kinase inhibitor, dasatinib (Figure 4.7, 4.8 and 4.25). In addition, mutants that failed to cluster efficiently cannot transform despite binding to Src tyrosine kinase and other signalling molecules (Figure 4.12 - 4.16). Together our results suggest that the assembly of a large macromolecular complex is important for transformation and that signalling by GFRs is dependent upon both kinase activation and upon preformed clusters. Large cluster formation may be necessary to achieve a sufficient threshold of signalling. This is supported by preliminary observation that AKT activation is much weaker in mutant L405E, which cannot cluster (Figure 4.15).

The significance of large complex formation is better established for a different class of GFR. EphA receptors are receptor tyrosine kinases that transduce signals mediated by cell-cell contact via their binding to ephrins on the surface of neighbouring cells. EphA receptor binding to corresponding ephrin ligands leads to activation of the kinase activity on the intracellular domain of the receptor, which results in the activation of signalling pathways
involved in growth, differentiation and pattern formation during development (Bocharov, Mayzel et al. 2008). It has been established that cluster formation is essential for EphA3 signalling. Cluster formation of EphA3 does not require a functional cytoplasmic signalling domain, therefore is independent of kinase activity (Wimmer-Kleikamp, Janes et al. 2004). Eph are highly promiscuous receptors that are known to form clusters (Surawska, Ma et al. 2004). Depending on the ephrin ligand densities and therefore the size of the clusters, Eph receptors can mediate kinase-dependent or kinase-independent signalling with different outcomes, with higher-order Eph receptor/ephrin clusters over-riding kinase-dependent signalling mechanism (Vearing and Lackmann 2005). Kinase-dead EphA8, when clustered with high density ephrin coated beads, is capable of binding to PI3K and promoting cell adhesion (Gu and Park 2001). Therefore, oligomerisation and dimerisation in the sense of ligand-induce conformational change in the cytoplasmic domain are likely to be two separate requirements for signal initiation, which are subjected to independent regulations. Since MT is strongly activating, it probably has a higher tendency to form large clusters and therefore, can be a useful model for studying the mechanism regulating macro-complex formation, which is likely to be required for normal GFR activation.

6.2.2 Inhibiting GFR oligomerisation

It has been demonstrated previously that expression of the transmembrane domain of neu-mutant V664E can inhibit transformation by prevention of receptor dimerisation (Lofts, Hurst et al. 1993). It is feasible that the MT transmembrane domain in isolation could also insert into membranes and act as a competitive inhibitor to prevent signalling by related complexes. Indeed, mutant 200Δ10 MT had an inhibitory effect on foci formation induced by wild type MT in preliminary experiments (Figures 4.28 - 4.30).
A similar approach has been used in the studies of transmembrane domain interaction between bovine papilloma virus (BPV) membrane oncoprotein E5 and PDGFRβ (see Chapter 1.13). It is thought that the transmembrane protein E5 dimer forms hydrogen bonds with PDGFRβ membrane spanning helix in the lipid bilayer in such way that it promotes the conformational change required for PDGFRβ cytoplasmic kinase domain activation independent of ligand binding (Lai, Henningson et al. 1998, DiMaio and Mattoon 2001, Talbert-Slagle and DiMaio 2009). Recently, screening of an artificial transmembrane peptide library derived from E5 sequence has identified a polypeptide (pTM36-4 T21N) that binds to PDGFRβ but does not transform cells. The artificial protein successfully blocked PDGFRβ activation and PDGF-BB induced mitogenic signalling (Petti, Talbert-Slagle et al. 2013). Similar hydrophobic peptide screens can be translated to identify hydrophobic sequence that inhibits GFR activation. It is worth noting that the most effective artificial activator and inhibitor of PDGFRβ signalling derived from the screen by Petti et al., has little resemblance of the original E5 amino acid sequence (Talbert-Slagle and DiMaio 2009, Petti, Talbert-Slagle et al. 2013). It is evident that our understanding of the mechanism behind TMD interaction in relation to GFR activation is still rather limited.

6.2.3 Mixed clusters: do they exist?

It is not clear whether MT has any ability to associate with other GFRs in the cell surface membrane and promote their activation in the same way as BPV E5. It has been recently reported that MT physically associates with the insulin and IGF-I receptors and that such an interaction is required for transformation in cell lines derived from MMTV-MT mouse model (Novosyadlyy, Vijayakumar et al. 2009). We did not find association between MT and GFRs such as EGFR1, PDGFRβ and insulin receptor by both immunofluorescent labelling and co-immunoprecipitation methods. Although some partial colocalisation between surface MT and
integrin αVβ5 have been observed (Figure 5.18 and 5.19), we cannot exclude the possibility that such a colocalisation occurred by chance. It has been reported that activated PDGFRβ co-immunoprecipitates with integrin αVβ3 (Schneller 2001). However, it only accounts for a very small fraction of the total integrin (Schneller 2001). It is likely that most of the MT surface complexes are not associated with other GFRs.

ErbB2/HER2/neu and ErbB3 have been widely studied for their propensity to heterodimerise. Since ErbB2 does not have a putative ligand and ErbB3 lack an active kinase function, it was thought that their signalling would have to involve hetero-oligomerisation with other members of the ErbB receptor family (Kashles, Yarden et al. 1991) (reviewed by Cymer and Schneider 2010). EGFR family receptor dimerisation is thought to be mediated by two GXXXG motifs, except ErbB3, which has only one of such motif. It has been proposed that the GXXXG motif near the membrane-cytosol interface is required for homodimer formation while the motif near the membrane-extracellular interface plays a role in heterodimerisation (Gerber and Shai 2001, Cymer and Schneider 2010). However, recent visualisation of ErbB2/neu and ErbB3 distribution in the plasma membrane failed to show conclusive evidence of mixed clusters between EGFR family members (Yang, Raymond-Stintz et al. 2007). Using a highly sensitive fluorescent microscopy method, Kaufmann and co-workers reconstructed Her2/neu and Her3 clusters in 3D and showed that most of the two types of receptors are found mostly in separate clusters (Kaufmann, Muller et al. 2011). Such segregation of ErbB family members provides a potential limitation to heterodimerisation to levels much lower than previously predicted, probably to allow specific signal propagation (Yang, Raymond-Stintz et al. 2007). This is favourable for rational drug design because short peptides or compounds that inhibit the oligomerisation of a receptor are more likely to be specific. Indeed, high throughput selection of a peptide library based on the TMD domain of ErbB2/neu found only a very few of the ~ 4000 peptides can dimerise with ErbB2/neu
receptor with a higher affinity than native sequence (He, Hoffmann et al. 2011). This seems to suggest that oligomerisation is mediated by highly specific sequence of the membrane spanning region of the transmembrane receptor, which also argues against mixed cluster formation.

6.2.4 Mechanism of Macro-complex Formation

Ichinose et al., have summarised in their study of EGFR1 clustering, the various mechanisms that have been proposed for cluster formation of GFRs in the plasma membrane (Ichinose, Murata et al. 2004). These including interaction between neighbouring transmembrane α-helices (Moriki, Maruyama et al. 2001), interaction between regions in the extracellular and cytoplasmic domains (Yu, Sharma et al. 2002), binding to the cytoskeleton (van Bergen en Henegouwen, den Hartigh et al. 1992), and accumulation into membrane domains (Mineo, Gill et al. 1999, Ichinose, Murata et al. 2004). We have found colocalisation between surface MT complexes and the cytoskeleton, especially actin network on the inner leaflet of the plasma membrane near the membrane ruffles of transformed cells (Figure 5.10). However, association between surface complexes and actin probably does not require binding to Src tyrosine kinase as mutant 200Δ10, which lacks Src binding, also localises to actin cables typical in non-transformed cells.

6.2.5 Additional components in MT clusters require further investigation

The exact mechanism behind macro-complex formation in MT is still not clear. Lipid rafts does not seem to play a role in the formation of these large clusters in the plasma membrane (Figure 5.4 - 5.8). Some reports suggest complex formation is supported by protein-protein interaction. Knock-down of galectin-1 reduces the clustering of H-Ras G12V and it has been
proposed that galectin-1 stabilises the interaction of activated H-Ras in non-raft microdomains (Paz, Haklai et al. 2001, Prior, Muncke et al. 2003, Rotblat, Belanis et al. 2010). It is worth noting that clusters of gold-particle labelled MT clusters are only found at the plasma membrane whereas the gold particles in microsomes or vesicles in the cytoplasm are mostly found in singlet (communication with Dr Jitka Forstova). It is possible that there are factors required for macro-complex formation of MT, which are only available at the cell surface membrane. It may be possible that there are not yet identified MT binding partners in plasma membrane. However, interaction in the plasma membrane can be easily disrupted by detergent-containing lysis buffers. Identification of potential MT binding partners on the cell surface will likely to require chemical cross-linking (see below in Future projects).

6.3 Changes in PDGFRβ in cells transformed by MT

During the investigation of MT and GFR complex formation, we observed diminished PDGFRβ expression in MT-transformed cell lines, which could be up-regulated by the Src/Bcr-Abl tyrosine kinase inhibitor, dasatinib (Figure 5.22 – 5.25). PDGFRβ down-regulation has been reported in other fibroblast cell lines transformed by SV40 or v-Src (Cook, Wang et al. 1993, Zhang and Baldwin 1994, Zhang, Walker et al. 1995). It was reported recently that ‘de-repression’ of PDGFRβ transcription confers resistance to targeted tyrosine kinase inhibitor therapy in human brain tumour driven by activated EGFR (Akhavan, Pourzia et al. 2013). It is not clear whether PDGFRβ re-expression confers any pro-survival signals in MT-transformed Rat-2 fibroblasts treated with dasatinib, which will be one area of future investigation.

PDGF-BB ligands stimulate mesenchymal stem cell (MSC) differentiation. However, persistent PDGFRβ activation can down-regulate expression of the receptor via a PI3K/AKT
negative feedback mechanism and this down-regulation of PDGFRβ promotes cell proliferation in the long term (Gharibi, Ghuman et al. 2012). It has been shown that activation of mTORC suppresses the transcription of PDGFRβ in mouse embryonic fibroblast (MEF) and also in some human brain tumours (Zhang, Bajraszewski et al. 2007). Fibroblasts expressing wild type MT are highly proliferative, with reduced cell-cell contact and other features associated with mesenchymal stem cells. Many of the PDGF early response genes such as fos, jun, myc are constitutively activated in cells expressing MT (Rameh and Armelin 1991, Armelin and Oliveira 1996). It is possible that the expression of genes including PDGFRβ may be switched off as cells return to a more stem-cell-like state. The observation of PDGFRβ down-regulation in MT-transformed fibroblasts raises interesting questions about the gene expression controlled by MT on a global scale in order to transform cells.

The changes in gene expression required to overcome contact inhibition in tumour initiation is poorly understood. We have generated mutants (L405E or neu-mutant MT) that bind to all major MT-binding partners known to induce transformation (Chapter 4, Figure 4.11 – 4.15) but still failed to transform Rat-2 fibroblasts. These new mutants of MT are likely to help elucidating the set of genes responsible for producing transformed cell morphology. Our system of MT and its mutants that block defined steps in oncogenic signalling initiation provides a simplified model for deciphering the signalling pathways and the associated transcription changes required for cell transformation.

6.4 A Story Unfinished

It is still unknown to us why mutant dl1015 does not transform. This mutant carries a deletion of a Proline-rich region immediately downstream of PI3K binding site (see List of Mutants). It seems to bind to known signalling proteins and forms clusters on the cell surface membrane. Mass-spectrometry analysis of proteins bound to dl1015 did not find significant
differences compared to those bound to wild type (unpublished data). Unlike L405E, dl1015 does not activate AKT or MAPK pathway (data not shown) therefore the defect in dl1015 appears more severe. Further characterisation of this mutant will likely to yield new knowledge on the requirement for tumorigenesis.

6.5 Future Projects:

i) Detailed investigation into the downstream signalling molecules including AKT, MAPKs and STATs in fibroblasts expressing various MT species in order to see how location to different subcellular compartments alters cell signalling. Similar analysis in other cell types, particularly haematopoietic cell lines where the signalling requirements for transformation may be different (Morley, Uden et al. 1999).

ii) Construct a potential dominant negative mutant of MT (Δ185-381) lacking intrinsic binding sites for Src, ShcA, p85 PI3K and PLC-γ1. Co-transfection of this mutant with wild type MT plasmid should inhibit foci formation (Figure 4.28). The mutant should retain the PP2A binding site in the N-terminal region, which is required for migration out of the ER to higher membrane sites; the hydrophobic sequence with juxtamembrane region on both sides, which contain positively charged residues; HA+ or FLAG-tag at the C-terminus, which allow dual immunofluorescent labelling of surface complexes with wild type MT, similar to those shown in Figure 4.29 - 4.30.

iii) Chemically cross-link MT and associated membrane proteins on the cell surface. N-hydroxysulfoosuccinimide (NHS) ester forms covalent bonds between the amine groups of Lysine residues. Since the extracellular region of MT C-terminus is very short and contains only one Lysine residue right at the membrane interface, cross-linking experiment should be
performed on cell line expressing MTFLAG, which contain two additional Lysines. Cross-linking experiment should be performed on live cells before lysis in detergent. However, cross-linker derivatives that cross the plasma membrane may be considered when comparing proteins associated with L405E mutant as the C-terminus is facing the cytosol. Cross-linking experiment like this may also help to identify key components affected in the non-transforming mutant dl1015. The MT-immunoprecipitates will be treated with β-mercaptoethanol or dithiothreitol, releasing proteins from MT complexes, which can be separated by 2D-gel SDS-PAGE and analysed by MALDI-TOF mass spectrometry after in-gel tryptic digest.

iv) Construction of an expression vector of PDGFRβ under a strong promoter, in order to investigate whether strong PDGFRβ expression has any inhibitory effect upon cell growth in fibroblasts transformed by MT. Use inhibitors targeting AKT or MAPK pathway to identify the driver behind PDGFRβ down-regulation in fibroblasts transformed by wild type MT. These can include mTORC-specific inhibitor rapamycin and MEK inhibitor PD325901. Past mutants of MT lacking the binding of individual signalling proteins are also useful tools to decipher the pathways responsible for PDGFRβ. It is still not clear whether re-expression of PDGFRβ after Src-inhibition by dasatinib confers any advantages for survival. Further experiments with combined treatment of dasatinib and imatinib in cells expressing MT will be required to investigate possible caspase-3 activation at various drug concentrations, combinations and time-points.

v) Analysis the difference in gene expression before and after dasatinib treatment in fibroblasts transformed by MT using microarrays. This will allow identification of genes regulating transformation.
Amino Acid Sequence of MPyV MT

1 MDRVLSRADK ERLLELLKLP RQLWGDFGRM QQAYKQQLL LHPDKGSHA LMQELNSLWG
61 TFKTEVYNLR MNLGGTGFQV RRLHADGWNL STKDTFGDRY YQRFCRMPLT CLVNVKYS
c
121 SCILCLLRKQ HRELKDKCDA RCLVLGECFC LECYMQWFGT PTRDVNLNYA DFIASMPI
181 LDLDVHSVYN PKRRSEELRR AATVHYTIMT GHSAMEASTS QGNGMISSES GTPATSRRL
241 LPSLLSNPTY SVMRSHSYPP TRVLQQIHPH ILEEDEILV LLSPMTAYFR TPPELLYP
301 DQDQLEPLEE EEEEYMPMED LYLDLIPGEQ VPQLIPPPIL PRAGLSPWEG LILRDLQRA
361 FDPILDASQR MRATHRAALR AHSMRQHLRR LGRTLMLVTFL AALLGICLM LFILIKSRH
421

Supplementary Figure S2.1 Complete amino acid sequence of MPyV MT
TMD region is highlighted in grey. NCBI Reference Sequence: NP_041265.1
**Supplementary Table ST2.1 Primers for MT Mutagenesis**

<table>
<thead>
<tr>
<th>Primers for MT Mutagenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oligonucleotide Sequence 5’ – 3’</strong></td>
</tr>
<tr>
<td>MT forward</td>
</tr>
<tr>
<td>Δ41-421 MTHA+ reverse</td>
</tr>
<tr>
<td>CTATTGAGATCAAAGATGCAAATACCAG</td>
</tr>
<tr>
<td>∆419-421MTHA+ reverse</td>
</tr>
<tr>
<td>CGGAACTTTTTATTTGAATAAATAGCATGAGAC</td>
</tr>
<tr>
<td>Δ416-421 MT reverse</td>
</tr>
<tr>
<td>416G6 MTHA+ reverse</td>
</tr>
<tr>
<td>MT FLAG reverse</td>
</tr>
<tr>
<td>MTGGDV reverse</td>
</tr>
<tr>
<td>MTKDEL reverse</td>
</tr>
<tr>
<td>MTSec61β reverse</td>
</tr>
<tr>
<td>wtMTHA+ reverse</td>
</tr>
</tbody>
</table>

**Supplementary Table ST2.2 Primers for Receptor Transcription Detection**

<table>
<thead>
<tr>
<th>Primers for Receptor Transcription Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oligonucleotide Sequence 5’ – 3’</strong></td>
</tr>
<tr>
<td>EGFR1 forward</td>
</tr>
<tr>
<td>EGFR1 reverse</td>
</tr>
<tr>
<td>PDGFRβ forward</td>
</tr>
<tr>
<td>PDGFRβ reverse</td>
</tr>
<tr>
<td>ErbB2 forward</td>
</tr>
<tr>
<td>ErbB2 reverse</td>
</tr>
<tr>
<td>ErbB3 forward</td>
</tr>
<tr>
<td>ErbB3 reverse</td>
</tr>
</tbody>
</table>

**Supplementary Table ST2.3 Oligonucleotides for Sequencing MT Plasmids**

<table>
<thead>
<tr>
<th>Primers for Sequencing MT Plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oligonucleotide Sequence 5’ – 3’</strong></td>
</tr>
<tr>
<td>MT-Apal forward</td>
</tr>
<tr>
<td>pUC reverse</td>
</tr>
<tr>
<td>pEMBL reverse</td>
</tr>
</tbody>
</table>
## Supplementary Table ST2.4 Inhibitor and Compound List

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Stock concentration</th>
<th>Working concentration</th>
<th>Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nocodazole</td>
<td>100 µg/ml</td>
<td>25 ng/ml</td>
<td>DMSO</td>
</tr>
<tr>
<td>Colchicine</td>
<td>100 mg/ml</td>
<td>25 µg/ml</td>
<td>100% Ethanol</td>
</tr>
<tr>
<td>Cytochalasin D (potassium salt)</td>
<td>2 mM</td>
<td>500 nM</td>
<td>100% Ethanol</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>20 µM</td>
<td>5 – 100 nM</td>
<td>100% Ethanol</td>
</tr>
<tr>
<td>dasatinib</td>
<td>200 µM</td>
<td>1 – 100 nM</td>
<td>DMSO</td>
</tr>
<tr>
<td>imatinib</td>
<td>20 mM</td>
<td>5 – 10 µM</td>
<td>DMSO</td>
</tr>
<tr>
<td>cyclohexamide</td>
<td>50 mg/ml</td>
<td>100 ng/ml</td>
<td>100% Ethanol</td>
</tr>
<tr>
<td>Methyl-β-cyclodextrin</td>
<td>2% w/w</td>
<td>1% w/w</td>
<td>DMEM</td>
</tr>
</tbody>
</table>
REFERENCES


Huerfano, S., V. Zila, E. Boura, H. Spanielova, J. Stokrova and J. Forstova (2010). "Minor capsid proteins of mouse polyomavirus are inducers of apoptosis when produced individually but are only moderate contributors to cell death during the late phase of viral infection." FEBS1277(5): 1270-1283.


