Understanding and targeting PI3K downstream of oncogenic Met mutant

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Submitted in partial fulfilment of the requirements
of the Degree of Doctor of Philosophy

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DECLARATION OF AUTHORSHIP

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ABSTRACT

The Receptor Tyrosine Kinase (RTK) Met, overexpressed or mutated in cancer, plays a major role in cancer progression and represents an attractive target for cancer therapy.

This study aimed to investigate whether PI3K plays a role in Met oncogenicity.

Three cell models were used: (i) NIH3T3 cells expressing WT Met or the constitutively active mutant M1268T Met; (ii) U87MG glioblastoma cells, with endogenous WT Met constitutively activated due to an autocrine loop; (iii) A549 lung cancer cells expressing endogenous WT Met, activated upon binding exogenous HGF.

Met dependent Rac1 translocation to the plasma membrane, actin cytoskeleton organisation, cell migration, anchorage independent growth in soft agar and tumour growth were studied in the presence of inhibitors of pan-PI3K / mTOR, various PI3K Class I isoforms, mTOR or Akt, or following siRNA knock-down of PI3K isoforms.

We report that PI3K class I (but not class III) regulates Met dependent cell migration. The PI3K class I isoforms required varies among the cell models. Interestingly, the combined inhibition of all p110 Class I isoforms lead to the strongest reduction of Met dependent cell migration. Met dependent phosphorylation of Akt, an effector of PI3K class I, is reduced upon endocytosis inhibition, suggesting that Met signals to PI3K Class I on endosomes.

Our results indicate that mTOR is responsible for Met dependent anchorage independent growth and tumour growth *in vivo*. Surprisingly, PI3K class I (and class III) are not required. Moreover, Rac1 is required for Met dependent mTOR activation, (phosphorylation of mTORC1's effector, p70 S6K) subcellular translocation of mTOR

and anchorage independent growth. Finally, our results suggest that this Met-Rac1-mTOR pathway occurs on endosomes.

Thus while PI3K class I regulates Met dependent cell migration, mTOR regulates Met driven anchorage independent growth and *in vivo* tumorigenesis. Thus PI3K Class I / mTOR may be targeted in Met driven cancers.

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LIST OF ABBREVIATIONS

4E-BP1: eukaryotic translation Initiation Factor 4E (eIF4E)-Binding Protein 1

ABD: Adaptor Binding Domain

AGC: protein kinase A/protein kinase G/protein kinase C

AMPK: 5' Adenosine Monophosphate-activated Protein Kinase

AP180: Adaptor Protein 180

APPL: Adaptor Protein containing PH domain, PTB domain, and Leucine zipper motif

Arf : ADP ribosylation factor

ATG13: Autophagy-related Gene 13

BAD: Bcl-2 Antagonist of cell Death

BAR: Bin-Amphiphysin-Rvsp

Bcl: Beclin

BH: BCR homology

BIF1: Bax-Interacting Factor 1

Ca^{2+}: Calcium ion

Cbl: Casitas B-lineage lymphoma

CCV: Clathrin-Coated Vesicles

CHC: Clathrin Heavy Chain

CIN85: Cbl-interaction protein of 85 kDa

CLASP: Clathrin-Associated Sorting Protein

Crk: CT10 regulator of kinase

DAG: Diacylglycerol

DEP: Dishevelled, EGL-10 and pleckstrin domain

DEPTOR: DEP domain-containing mTOR-interacting protein

DYN-1: Dynamin-1

EEA1: Early Endosome Antigen 1

eEF: eukaryotic Elongation Factor

EGF: Epithelial Growth Factor

EGFR: Epithelial Growth Factor Receptor

eIF: eukaryotic Initiation Factor

Eps15: Epidermal Growth Factor Receptor substrate 15

ER: endoplasmic reticulum

ERC: Endocytic Recycling Compartments

ERK: Extracellular signal-Regulated Kinase

FAK: Focal Adhesion Kinase

FAT: FRAP, ATM and TRRAP domain

FATC: FAT domain at the *C* terminus

FGF: Fibroblast Growth Factor

FIP200: Family-Interacting Protein of 200 kDa

FLIP: FLICE-Like Inhibitory Protein

FoxO1/3a: Forkhead box O1/3a

FRAP: FKBP12-rapamcyin-associated protein

FRB: FKBP12-Rapamycin Binding domain

FYVE: Fab1, YOTB, Vac1, EEA1

Gab1: Grb2-associated binding protein 1

GAP: GTPase Activating Proteins

GDI: Guanine nucleotide Dissociation Inhibitors

GDP: Guanosine diphosphate

GEF: Guanine nucleotide Exchange Factor

GGA3: Golgi-localized Gamma ear-containing Arf-binding protein 3

GM-CSF: Granulocyte Macrophage-Colony Stimulating Factor

GPCR: G Protein Coupled Receptor

Grb2: Growth factor receptor bound protein 2

GSK3 beta: Glycogen Synthase Kinase 3 beta

GTP: Guanosine triphosphate

GTPase: Guanosine triphosphatase

HEAT: huntingtin, elongation factor 3, a subunit of protein phosphatase 2A and TOR1 motif

HEK: Human Embryonic Kidney cells

HGF: Hepatocyte Growth Factor

HIF-alpha: Hypoxia Inducible Factor 1 alpha

HOS: Human Osteogenic Sarcoma cell line

Hrs: HGF-regulated tyrosine kinase substrate

IGF: Insulin-like Growth Factor

IGFR: Insulin-like Growth Factor Receptor

IKKbeta: IkappaB kinase beta

INPP4B: Inositol Polyphosphate 4-phosphatase II

IP3: Inositol-1, 4, 5-triphosphate

IPT: Immunoglobulin-Plexin-Transcription

IRS: Insulin Receptor Substrate

iSH2: inter-Src Homology 2

KO: knock-out

LCD: Leucine charged domain

LPA: Lysophosphatidic Acid

Lys: Lysine

MAPK: Mitogen Activated Protein Kinase

MBS: Met Binding Site

MEF: Mouse Embryonic Fibroblast

MCP1: Monocyte Chemotactic Protein 1

MDCK: Madin Darby Canine Kidney

MDM2: Mouse double minute 2 homolog

MEF: Murine embryonic fibroblast

mIMCD-3: mouse Inner Medullary Collecting Duct-3

mLST8: mammalian lethal with SEC13 protein 8

MNNG: N-Methyl-N-nitro-N-nitroguanidine

MRS: MET-Related Sequence

mSIN1: mammalian stress-activated map kinase-interacting protein 1

MT: polyoma middle T antigen

mTOR: mechanistic Target of Rapamycin

mTORC1/2: mTOR complex 1/2

MVB: MultiVesicular Body

NLS: Nuclear Localization Signal

p120-ras-GAP: p120 ras-GTPase Activating Protein

p70S6K1: p70 ribosomal S6 kinase 1

PAE: Porcine Aortic Endothelial cells

PDCD4: programed cell death protein 4

PDGF: Platelet-Derived Growth Factor

PDK1: Phosphoinositide-dependent kinase-1

PDZ: postsynaptic density of 95 kDa, Discs large and zonula occludens domain

PGC-1alpha: PPARgamma coactivator-1alpha

PH: Pleckstrin Homology domain

PI3K: Phosphatidylinositol 3-Kinase

PI3P: Phosphatidylinositol-3-Phosphate

PIKK: PI3K-related protein kinases

PIP2: Phosphatidylinositol-4,5-bisPhosphate

PIP3: Phosphatidylinositol-3,4,5-triPhosphate

PKB: Protein Kinase B

PKC: Protein Kinase C

PLC: Phospholipase C

PP2A: Protein Phosphatase 2A

PPARgamma: peroxisome proliferator-activated receptor-gamma

PRAS40: Proline Rich Akt Substrate of 40 kDa

PROTOR: protein observed with RICTOR

PSI: plexin-semaphorin-integrin

PTB: PhosphoTyrosine Binding

PtdIns: Phosphatidylinositol

PtdIns3P: Phosphatidylinositol-3-Phosphate

PtdIns4P: Phosphatidylinositol-4-Phosphate

PtdIns(4,5)P_2: Phosphatidylinositol-4,5-bisPhosphate

PtdIns(3,4,5)P_3: Phosphatidylinositol-3,4,5-triPhosphate

PTEN: Phosphatase and Tensin homologue deleted on chromosome 10

PX: Phox homology

RAFT1: rapamycin and FKBP12 target

RAPT 1: rapamycin target 1

RAPTOR: Regulatory associated protein of mTOR

RBD: Ras binding domain

REDD1: regulated in development and DNA damage response 1

Rheb: Ras homolog enriched in brain

RICTOR: rapamycin-insensitive companion of mTOR

RD domain: repressor domain

RNC: RAPTOR *N*-terminal conserved

rpS6: 40S ribosomal S6 protein

RTK: Receptor Tyrosine Kinase

S1P: Sphingosine-1-Phosphate

S6K: ribosomal protein S6 kinase

SCF: Stem Cell Factor

SCLC: Small Cell Lung Cancer

SEP: sirolimus effector protein

Ser: Serine

SF: Scatter Factor

SGK1: serum- and glucocorticoid-induced protein kinase 1

SH2: Src Homology 2

SH3: Src Homology 3

Shc: Src homology-2-containing

SHIP: Src Homology domain-containing 5' Inositol Phosphatase

SPH: serine protease-homology

Src: Sarcoma

SREBP1/2: Sterol Regulatory Element Binding Protein 1/2

STAT3: Signal Transducer and Activator of Transcription 3

TCR: T-Cell Receptor

Tel2: telomerase maintenance 2

TFEB: Transcription Factor EB

TIF-IA: Transcription Initiation Factor-IA

TNFalpha: Tumour Necrosis Factor alpha

TOP: terminal oligopolypyrimidine

TOR: Target of Rapamycin

TOS: TOR signalling motif

TPR: Translocated Promoter Region

TRRAP: Transformation/transcription domain-associated protein

TSC1: Tuberous Sclerosis 1

TSC2: Tuberous Sclerosis 2 / Tuberin

Tti1: Tel2 interacting protein 1 homolog

Tyr: Tyrosine

ULK1: Unc-51-Like Kinase 1

UTR: 5'Untranslated Region

UVRAG : Ultraviolet radiation Resistance Associated Gene

v-ATPase: vacuolar H+adenoside triphosphate-ATPase

Vps34: Vacuolar protein sorting 34

WT: Wild Type

YY1: Ying-Yang 1

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INTRODUCTION

I. Met receptor/Hepatocyte growth factor

I. a) Met receptor

Met is the Receptor Tyrosine Kinase (RTK) for Hepatocyte Growth Factor (HGF) also called Scatter Factor (SF) [1]. HGF and Met are ubiquitously expressed in human tissues. Met is expressed at the surface of epithelial and endothelial cells, and HGF is secreted by mesenchymal cells.

I. b) Role of Met/HGF

In vivo, HGF-dependent Met signalling controls a complex biological program known as *invasive growth program* [2, 3]. This program is required for many biological processes as tissue morphogenesis and homeostasis during embryogenesis, or wound healing and tissue regeneration during adulthood. Once Met is activated by HGF binding, Met induces cell migration, cell proliferation, and protects cells from apoptosis [4, 5]. Also, Met promotes angiogenesis [6, 7]. This is achieved through the activation of several pathways **(see I. i) Met adaptors and signalling molecules, IV. Crosstalk between Met, PI3K, and mTOR signalling pathways)**.

I. c) Discovery of Met / Hepatocyte growth factor receptor

In 1984, Cooper *et al*. showed that DNA from a Human Osteogenic Sarcoma cell line (HOS) treated with the carcinogenic chemical N-Methyl-N-nitro-N-nitroguanidine

(MNNG), transformed NIH3T3 mouse fibroblasts in a DNA transfection experiment [8, 9]. They isolated the specific proto-oncogenic gene called *Met* [9], which was fused with a portion of Translocated Promoter Region (TPR) instead of the regions of the Met gene encoding for Met transmembrane and extracellular domains [10, 11]. It turned out that TPR/Met was constitutively activated [10]. In 1990, Met was identified as the Hepatocyte Growth Factor receptor [1].

I. d) c-Met gene

The proto-oncogene c-Met, located on chromosome 7q21-31, has a length of 120 kb and is composed of 21 exons separated by 20 introns [12]. At least 6 Met transcripts have been found in human cell lines and tissues. The most abundant Met isoform in tissues and cell lines lacks 18 amino acids in the extracellular region compared to the longest form [13].

I. e) Met structure

The Met protein is synthesised as a single chain precursor of 170 kDa, which is glycosylated and gets cleaved into two chains alpha and beta. The mature form of Met is a heterodimer consisting of an extracellular alpha chain and a beta chain which spans the membrane. They are linked together by disulphide bridges. The first 212 amino acid of the alpha chain and the beta chain (N-terminus), which are homologous, constitute the HGF binding domain. In addition, the beta chain is composed of the Met extracellular domain, the transmembrane helix and the intracellular portion [3, 14] **(Fig. 1)**.

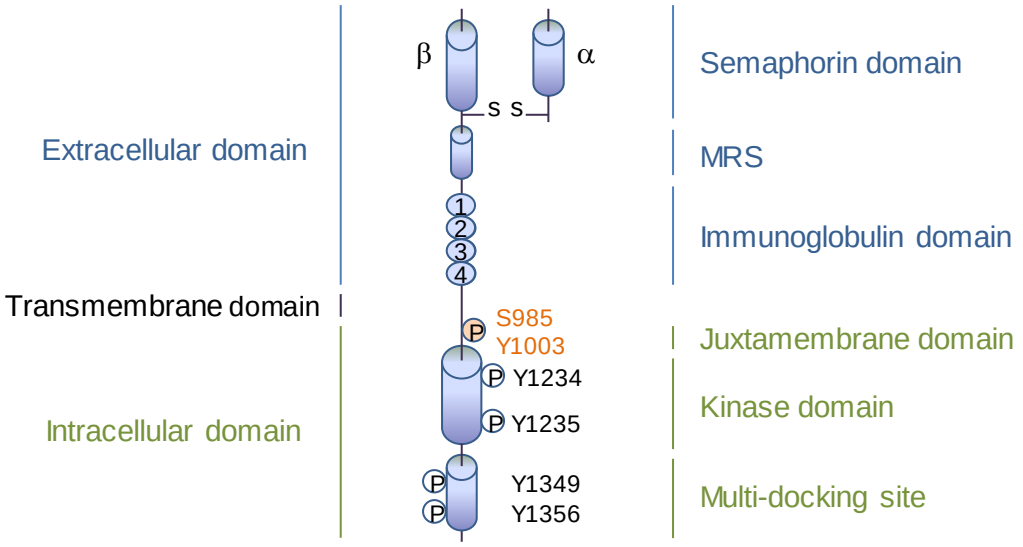


Figure 1: Met receptor structure

The mature form of Met is a heterodimer consisting of an extracellular alpha chain and a beta chain which spans the membrane. The extracellular domain, involved in HGF binding, contains a region of homology to semaphorins (Semaphorin domain), a cysteine-rich MET-Related Sequence (MRS domain), and a four immunoglobulin-like domain called Immunoglobulin-Plexin-Transcription (IPT) domains. The intracellular domain contains three regions: (1) a juxtamembrane segment with: (a) a serine residue (Ser 985) and (b) a tyrosine (Tyr 1003) negatively regulating the receptor; (2) the tyrosine kinase domain including Tyr 1234 and Tyr 1235; (3) the C-terminal region, containing Tyr 1349 and Tyr 1356, a multi-docking site involved in signal transduction. S, serine; Y, tyrosine.

The extracellular domain involved in HGF binding contains a region of homology to semaphorins (Semaphorin domain), a cysteine-rich MET-Related Sequence (MRS domain) also referred as plexin-semaphorin-integrin (PSI), and a four immunoglobulin-like domain called Immunoglobulin-Plexin-Transcription (IPT) domains [3, 14]. The intracellular domain contains three regions: (1) a juxtamembrane segment with: (a) a serine residue (Ser 985) [15] and (b) a tyrosine (Tyr 1003) [16] negatively regulating the receptor; (2) the tyrosine kinase domain including Tyr 1234 and Tyr 1235; (3) the C-terminal region, containing Tyr 1349 and Tyr 1356, a multi-docking site involved in signal transduction [3, 14].

I. f) HGF

HGF was discovered for the first time in 1984 as a powerful mitogen for mature hepatocytes in primary culture [17]. In 1987, it was purified and its structure described[18]. In parallel, in 1987, a new protein was found to be responsible for epithelial cell migration and called Scatter Factor (SF) [19]. In 1991, HGF cloning and SF cloning revealed that HGF and SF were the same molecule, the ligand for the Met receptor [20].

I. g) HGF gene and structure

The HGF gene is located on chromosome 7q21.1, has a length of 71 kb and consists of 18 exons separated by 16 introns [21]. It encodes for the full-length HGF of 728 amino acids or the truncated isoforms. All of the isoforms are able to bind [1, 22, 23] and activate Met, however the truncated isoforms are less potent than the full-length HGF isoform [24, 25].

HGF is synthesised as a pre-pro-peptide and pro-HGF is secreted as a single chain precursor into the extracellular environment. HGF activators perform the proteolytic cleavage and are extracellular serine proteases such as the urokinase-type and tissue-type plasminogen activators and the coagulation factors XII [26, 27]. To become biologically active, pro-HGF is cleaved between the Arginine 494 and the Valine 495 into two chains linked by a disulfide-bond: (1) the alpha (or heavy) chain (69 kDa) with an amino-terminal heparin binding domain (N), and 4 kringle domains; and (2) the beta (or light) chain (34 kDa) with a serine protease-homology (SPH) domain [28] **(Fig 2)**.

I. h) Met activation

HGF binds to the extracellular domain of Met on two sites: The SEMA domain of Met binds to the SPH domain of HGF, and the ITP3 and ITP4 domains of Met bind to the N-terminal domain of HGF. HGF forms homodimers by the kringle 1 domain of one monomer binding to the N-terminus of another. Binding of HGF to Met induces Met dimerisation and stabilisation, conferring to Met an active conformation [29]. The tyrosines of Met in the kinase domain are autophosphorylated, which is followed by the transphosphorylation of its tyrosines in the multi-docking site.

I. i) Met adaptors and signalling molecules

Once activated, Met recruits most of its downstream signals to the multi-docking site either directly or indirectly, thanks to scaffolding molecules or adaptors **(Fig. 3)** [30, 31]. Such a multi-docking site is unique to the Met receptor and has not been observed in other RTKs. Most RTKs have, in their cytoplasmic tail, several tyrosines that bind to

their respective signaling molecules, while only two tyrosines (Tyr 1349 and Tyr 1356) constitute the multi-docking site on the *C*-terminal of Met.

The main Met adaptors include Growth factor receptor bound protein 2 (Grb2) [32], Sarcoma (Src) homology-2-containing (Shc) [33] and Grb2-associated binding protein 1 (Gab1) which can bind Met directly or indirectly through Grb2 [34].

These adaptors can supply binding sites for signalling molecules such as for p85 regulatory subunit of Phosphatidylinositol 3-Kinase (PI3K), Src Homology domain-containing 5' Inositol Phosphatase (SHIP) [35], Phospholipase C (PLC) gamma, and p120-ras-GTPase Activating Protein (p120-ras-GAP) [34, 36-41]. The tyrosine kinase Src [31], p85 regulatory subunit of PI3K [42], PLC gamma, and the transcription factor Signal Transducer and Activator of Transcription (STAT3) [43-45] can interact directly with Met **(Fig. 3)**.

One pathway can induce various cell functions, while several pathways can be interconnected to induce one cell function **(Fig. 3)**. For example, following Met activation, the small guanosine triphosphatase (GTPase) Ras triggers the activation of the Mitogen Activated Protein Kinase (MAPK) cascade, leading to Extracellular signal-Regulated Kinase (ERK) 1 and ERK2 activation. ERK1/2 activated by Met induces cell proliferation, migration and survival [46-48]. After Met activation, STAT3 binds to Met, becomes phosphorylated, dimerises and goes to the nucleus to act as a transcription factor. STAT3 functions have been found to be responsible for Met induced tubulogenesis, anchorage independent growth, tumour growth, and cell invasion [43-45]. Furthermore, PI3K/Akt signaling pathway is activated directly by Met or indirectly through Ras or Gab1 [31, 49, 50]. Akt is well known for its role in inhibiting apoptosis.

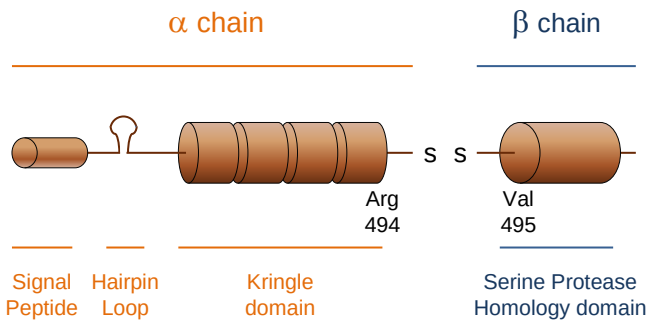


Figure 2: Hepatocyte Growth Factor structure

HGF is synthesised as a pre-pro-peptide and pro-HGF is secreted as a single chain precursor into the extracellular environment. To become biologically active, pro-HGF is cleaved between the Arginine (Arg) 494 and the Valine (Val) 495 into two chains linked by a disulfide-bond: (1) the alpha (or heavy) chain (69 kDa) with an amino-terminal heparin binding domain (N), and 4 kringle domains; and (2) the beta (or light) chain (34 kDa) with a serine protease-homology (SPH) domain

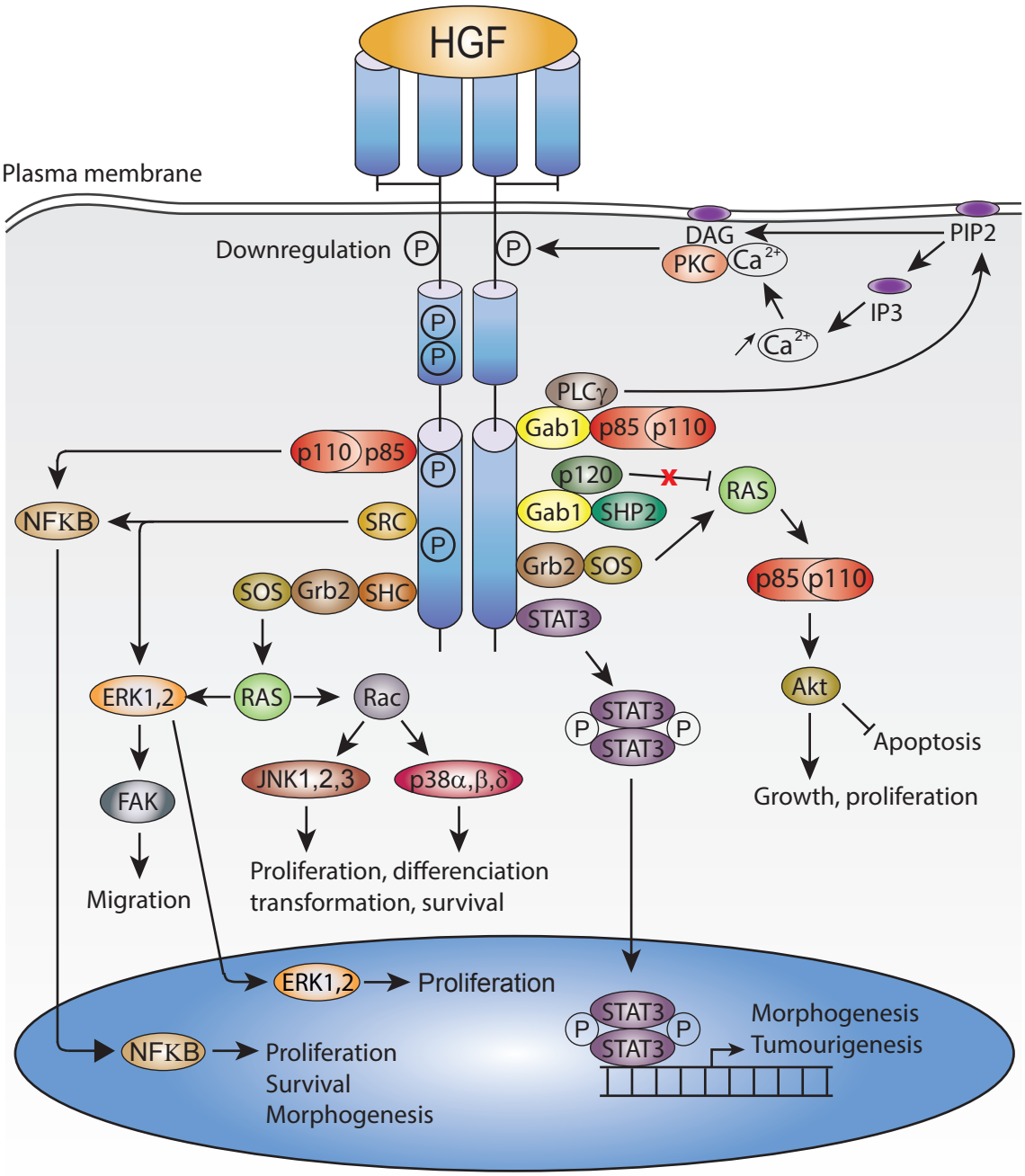


Figure 3: Met signalling network

Once activated, Met recruits most of its downstream signals directly or indirectly to the multi-docking site thanks to scaffolding molecules or adaptors. One pathway can induce various cell functions, while several pathways can be interconnected to induce one cell function.

Hepatocyte Growth Factor (HGF); p110-p85 (PI3K); Growth factor receptor bound protein 2 (Grb2); Sarcoma (SRC) homology-2-containing (Shc); Grb2-associated binding protein 1 (Gab1); Src Homology domain-containing 5' Inositol Phosphatase 2 (SHP2); Phospholipase C (PLC); p120-ras-GTPase Activating Protein (p120); Son Of Sevenless (SOS); Signal Transducer and Activator of Transcription (STAT3); Extracellular signal-Regulated Kinase (ERK); Diacylglycerol (DAG); Inositol-1, 4, 5-triphosphate (IP3); Calcium ion (Ca^{2+}); Protein Kinase C (PKC); Phosphatidylinositol-4,5-bisPhosphate (PIP2).

Other cell functions induced through this pathway under Met activation will be described further in more details **(see IV. Crosstalk between Met, PI3K, and mTOR signalling pathways)**.

I. j) Met downregulation

As an alternative to Met signalling and executing cell functions, Met activation induces its own downregulation through different ways: (1) by Met degradation **(see I. k) 3. Degradation)**; or (2) by phosphorylation of the Ser 985. PLC gamma recruitment to activated Met **(see I. i) Met adaptors and signalling molecules)** induces the production of Diacylglycerol (DAG) and Inositol-1, 4, 5-triphosphate (IP3). IP3 activates Calcium ion (Ca^{2+}) channels, which release intracellular Ca^{2+} from the endoplasmic reticulum (ER). This results in the activation of Protein Kinase C (PKC), Ca^{2+} dependent kinase, which phosphorylates the Ser 985 of Met, prompting the inhibition of Met activation [15, 51] **(Fig. 3)**. It has been suggested that the PKC isoforms involved are PKC delta and PKC epsilon [46], and that the phosphorylation of the Ser 985 of Met is antagonised by the Protein Phosphatase 2A (PP2A) [46].

I. k) Met trafficking

Met was first described as a receptor that signals from the plasma membrane and receptor internalisation was thought to be solely a desensitisation mechanism. However, recent studies have shown that it is also a mechanism that modulates the receptor signalling.

1. Endocytosis

Four mechanisms of endocytosis are known: 1) macropinocytosis, 2) the caveolin-mediated pathway, 3) the clathrin-mediated pathway, and 4) the clathrin and caveolin independent pathway. The most well characterised is clathrin mediated endocytosis and appears to be the main pathway for RTK endocytosis, including for Met [52]. In T47D cells expressing Wild Type (WT) Met [53], HeLa cells [54, 55], and NIH3T3 cells expressing a mutant of Met that is constitutively activated [56], it has been shown that Met internalisation requires dynamin, as inhibitors of dynamin or expression of a dominant negative of dynamin strongly reduces Met internalisation. Similarly, Clathrin Heavy Chain (CHC) knock-down inhibits Met endocytosis in Hela cells [55, 57] and NIH3T3 cells expressing a constitutively active Met mutant [56].

When HGF binds to Met, the complex moves along the membrane until it reaches a specific area called clathrin coated pits **(Fig 4)**. Clathrin is recruited to this area by adaptors which also bind Phosphatidylinositol-4,5-bisPhosphate (PIP2) located at the plasma membrane and sorting signals on the receptor, which include Met phosphorylation and/or ubiquitination. Clathrin, helped by the endocytic adaptors, curves the membrane and forms a mechanical scaffold for budding vesicles. Met endocytic adaptors include Epidermal Growth Factor Receptor (EGFR) substrate 15 (Eps15) [53, 58, 59] and Adaptor Protein 180 (AP180) [60], part of the Clathrin-Associated Sorting Protein (CLASP) class of monomeric adaptor proteins. At this stage, the ubiquitin ligase Casitas B-lineage lymphoma (Cbl) is required for two reasons: 1) as an endocytic adaptor, allowing the formation of Cbl - Cbl-interaction protein of 85 kDa (CIN85) - endophilin complex to help the invagination of the plasma membrane [61, 62]; 2) for its ubiquitin ligase activity, interacting indirectly with Met through Grb2, it is required to induce Met endocytosis [62, 63].

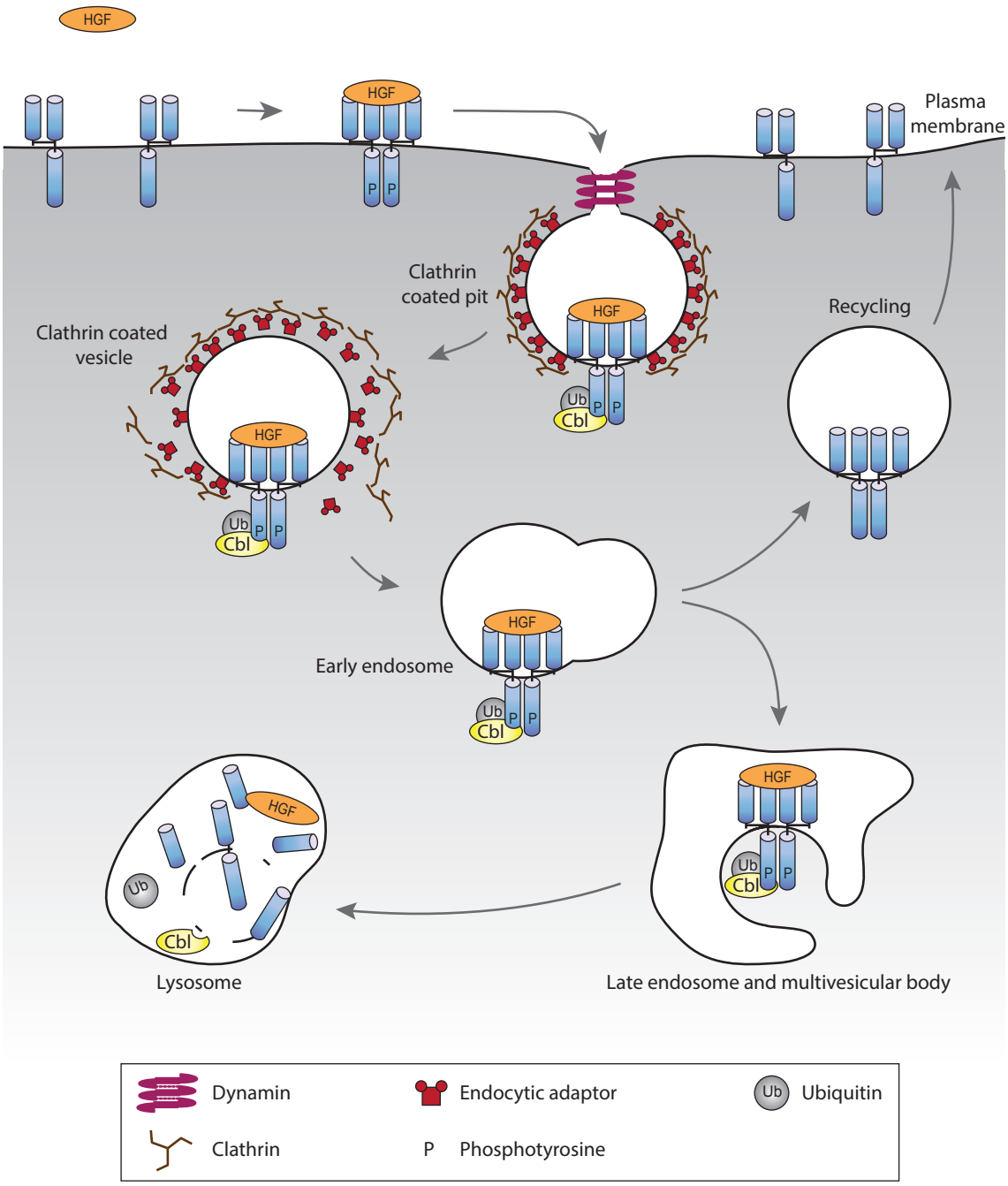


Figure 4: RTK internalisation via the clathrin-mediated pathway

When HGF binds to Met, Met is internalised through the clathrin-mediated pathway. Internalised vesicles fuse with early endosomes, from where several destinations are possible. Once the receptor reaches the MVB, the cytoplasmic tail of the receptor is not available for signalling anymore, ending the signalling, and the receptor gets degraded.

Hepatocyte Growth Factor (HGF); Casitas B-lineage lymphoma (Cbl).

Ubiquitin is a small protein that is highly conserved and ubiquitously expressed. Its molecular weight is 8.5 kDa and consists of 76 amino acids which bind to Lysine (Lys) residues of the target protein. The knock-down of Cbl or of Grb2 inhibits Met endocytosis in NIH3T3 expressing a constitutively active Met mutant [56].

The GTPase dynamin is recruited to the neck of nascent vesicles forming a spiral and is anchored at the membrane via its Bin-Amphiphysin-Rvsp (BAR) domain. Dynamin permits the pinching off of the nascent vesicles from the plasma membrane [64] **(Fig. 4)**.

2. Trafficking

Once internalised, Clathrin-Coated Vesicles (CCV) loose their clathrin coats and fuse with early endosomes, from where cargo is sorted **(Fig 4)**.

Generally, several destinations are subsequently possible for receptors from the sorting endosomes: 1) recycle back to the plasma membrane (it is classically thought that the receptor is available to be stimulated once again by its ligand), 2) be directed to the trans-Golgi network, or 3) go to late endosomes from where cargo can traffic to Endocytic recycling Compartments (ERC) or the MultiVesicular Body (MVB). It is usually considered that the cytoplasmic tail of the receptor is not available for signalling anymore in the MVB, ending the signalling and leading to receptor degradation **(Fig 4)**.

Immunofluorescence and confocal microscopy studies in HeLa cells demonstrated that at 15 minutes of HGF stimulation, Met has internalised and is located in Early Endosome Antigen 1 (EEA1) positive endosomes, mostly peripheral endosomes [60].

Then, Met progressively traffics towards a perinuclear region. At 120 minutes, Met accumulates in a perinuclear compartment and its localisation in early endosomes is strongly reduced [54]. Met trafficking to the perinuclear location is controlled by PKC

alpha activity and occurs along the microtubule network. Specific inhibition of PKC alpha or inhibition of microtubule polymerisation by pharmacologic inhibitors, reduces the perinuclear accumulation of Met without preventing its internalisation **(Fig. 5)** [57, 60].

Although most studies have shown that internalised Met gets progressively degraded, a recent study has reported that upon HGF stimulation in HeLa cells, a proportion of Met recycles requiring Golgi-localized Gamma ear-containing Arf-binding protein 3 (GGA3). Depletion of GGA3 is consequently followed by an increase in Met degradation. Activated Met recruits GGA3 through the CT10 regulator of kinase (Crk) adaptor protein and ADP ribosylation factor (Arf) 6-Guanosine triphosphate (GTP) within early endosomes in order to recycle back to the plasma membrane [65].

It is interesting to note that PI3K does not seem to be involved in Met trafficking, as demonstrated using a high concentration of a PI3K inhibitor in T47D expressing WT Met [53].

3. Degradation

Met degradation occurs progressively such that after 2 hours of HGF stimulation, 50% of Met is degraded in HeLa cells [60]. Studies have reported that proteasomal or lysosomal inhibition both impair Met degradation [49, 62]. Interestingly, inhibition of post-endocytic Met traffic in HeLa cells stimulated for 2 hours with HGF does not modify Met degradation, showing that Met does not need to traffic to the perinuclear area to be degraded [60].

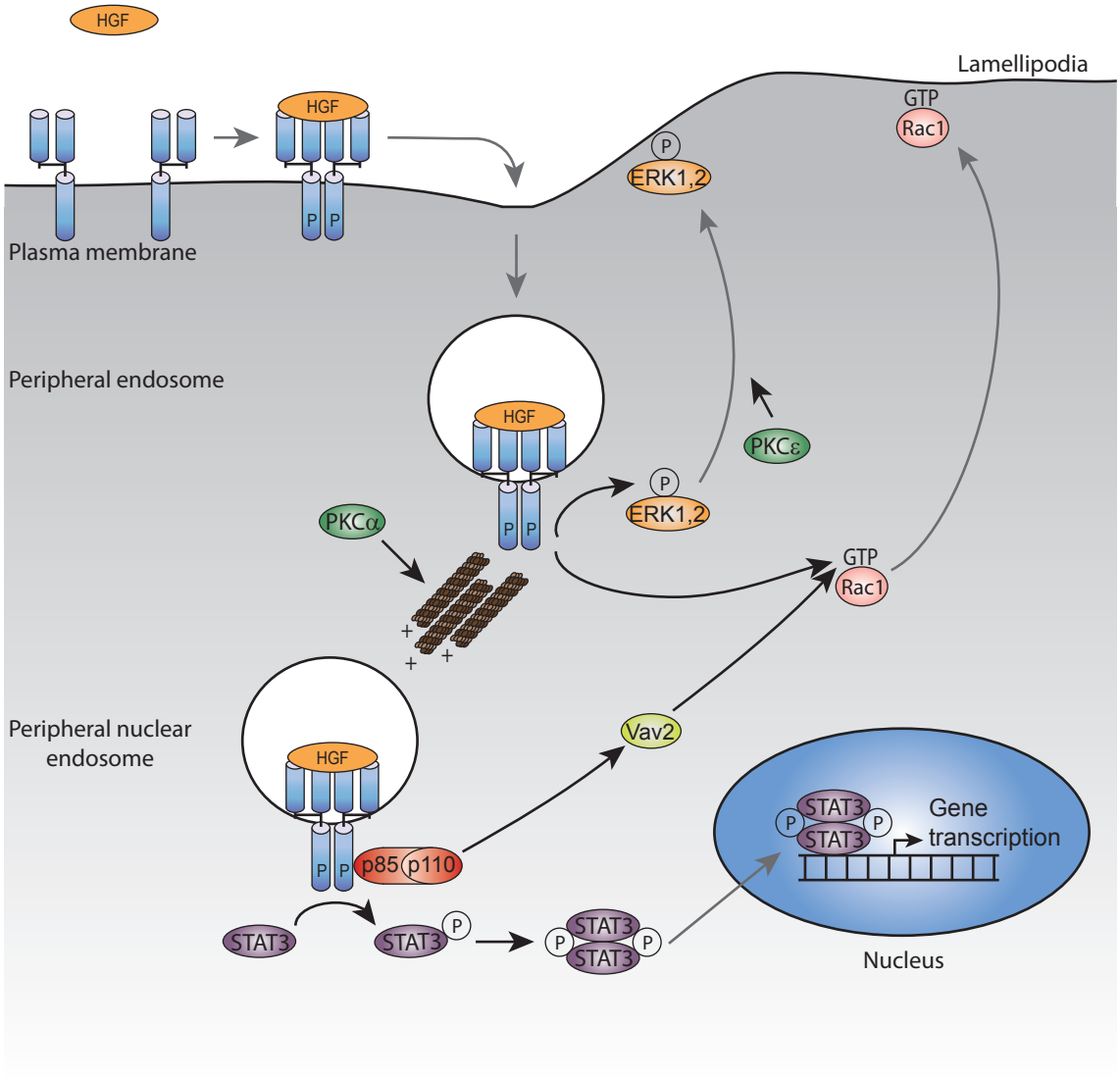


Figure 5: Compartmentalisation of Met signalling

Once Met is recruited to peripheral endosomes, it activates of ERK1/2 and Rac1, which are relocalised to the plasma membrane to induce cell migration. ERK relocalisation depends on PKC epsilon. Met continues to traffic to perinuclear endosomes along microtubules (dependent on PKC activity), from where it activates STAT3 and Rac1.

Hepatocyte Growth Factor (HGF); Protein Kinase C (PKC); Extracellular signal-Regulated Kinase (ERK); p110-p85 (PI3K); Signal Transducer and Activator of Transcription (STAT3).

To be degraded, phosphorylated Met gets ubiquitinated. It has been reported that phosphororylated Met gets polyubiquitinated [54, 66] but as well multi-mono-ubiquitinated [67]. The ubiquitination occurs by the E3 ubiquitin-ligase Cbl [67, 68], which is also involved in Met endocytosis, as previously described **(see I. k) 1. Endocytosis)**. Cbl needs to bind directly to Met on its phosphorylated Tyr 1003 for Met to get degraded, as loss of this binding in the Y1003F Met mutant results in greatly reduced Met degradation. However it can still internalise [16, 68] **(Fig 3)**. Ubiquitinated Met is recognised by HGF-regulated tyrosine kinase substrate (Hrs) in endosomes and Met phosphorylates it. The Y1003F Met mutant fails to phosphorylate Hrs [68]. Hrs sends ubiquitinated receptors to be degraded, preventing them from being recycled back to the plasma membrane [68, 69].

Interestingly, PI3K inhibition by specific inhibitors reduces Met degradation induced by HGF stimulation in T47D cells expressing Met [53].

I. l) Met spatio-temporal signalling

Downstream of Met, many signaling pathways are activated to induce one cell function, and one signaling pathway can induce many cell functions **(Fig. 3)**. Moreover, there are only two tyrosines in the Met multi-docking site, which, once phosphorylated, bind to most of the signalling molecules. Thus, how Met generates specific signals to trigger specific cellular responses has been unclear. One possible explanation is spatial signalling through trafficking: recent studies reported that receptors do not signal only from the plasma membrane but also post-internalisation from endosomes [70, 71]. This has been described for EGFR [72-74], and more recently for Met **(Fig. 5)** [55, 57].

Kermorgant *et al.* showed in HeLa cells that Met remains activated in endosomes and that Met dependent ERK1/2 phosphorylation requires endocytosis [57]. Under the control

of PKC epsilon, ERKs are relocated to focal complexes to stimulate cell migration [57, 75]. They further showed that Met needs to be localised to perinuclear endosomes, under the control of PKC alpha [57, 60], to induce STAT3 activation. This perinuclear localisation protects STAT3 against phosphatase activity, which allows the nuclear accumulation of STAT3 [55, 76, 77].

Ménard *et al.* showed in 2014 in our laboratory that the Rho GTPase Rac1 is regulated distinctly by Met depending on their location [78]. In MDA-MB-468 breast cancer cells, activated Met promotes Rac1 activation from peripheral and perinuclear endosomes. The pharmalogical inhibition of PI3K reduced Met dependent GTP loading of Rac1 (and therefore its activation) from perinuclear endosomes but not peripheral endosomes, and furthermore did not interfere with the accumulation of Rac1-GTP from peripheral endosomes. This result indicated a distinct mechanism of regulation of Rac1 downstream of Met, depending on the peripheral or perinuclear endosome. Moreover, upon Met activation, the Guanine nucleotide Exchange Factor (GEF) Vav2 activated Rac1 only from the perinuclear endosome, and a Vav2 mutant which is unable to bind the PI3K's product Phosphatidylinositol-3,4,5-triPhosphate (PIP3) **(see II. c) PI3Ks substrates and products)** did not activate Rac1 anymore. This result suggests that Met dependent Rac1 activation from perinuclear endosomes requires PI3K and Vav2 **(Fig. 5)**. Once activated by Met in endosomes, Rac1 relocalises to the plasma membrane and induces cell migration [56, 79]. However, only Rac1 activated in the perinuclear endosome upon Met activation is capable of inducing cell migration; inhibition of Rac1 relocalisation at the plasma membrane when activated from peripheral endosome has no effect on Met dependent cell migration [78]. Altogether these results demonstrate that Met regulates distinct signalling pathways and distinct cell functions depending on its intracellular location.

In addition, Joffre *et al.* in our laboratory discovered in 2011 that two Met mutants, initially identified in human papillary renal carcinomas, are oncogenic not only because they are highly activated but also because they signal from endosomes [56] **(Fig. 6 and see I. o) Understanding the mechanisms involved in Met mutants oncogenicity)**.

I. m) Met oncogenicity

HGF and Met control cell functions that promote cancer progression such as growth, proliferation, migration, angiogenesis, and the prevention of apoptosis.

How does Met become oncogenic? Through overexpression of HGF and/or Met, Met has been shown to drive many different types of cancer including lung, liver, stomach, breast, and brain. Hundreds of Met mutations have also been discovered in all types of cancer such as breast, gastric, head and neck, liver and lung cancer [14, 80] **(Fig. 6, see I. n) Oncogenic Met mutants, and COSMIC: the Catalogue of Somatic Mutations in Cancer, http://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=MET#dist)**.

Met upregulation is associated with many mechanisms such as the deregulation of the oncogenes: Ras, Ets-1 and Pax-5 and possibly of the tumour suppressor, p53 [29]. Moreover Met and HGF overexpression have been associated with drug resistance in cancer therapy targeting EGFR [81, 82] and Raf [83] respectively.

Patients with overexpression of HGF and/or Met, or mutated Met have been shown to have a poor prognosis [14]. [84].

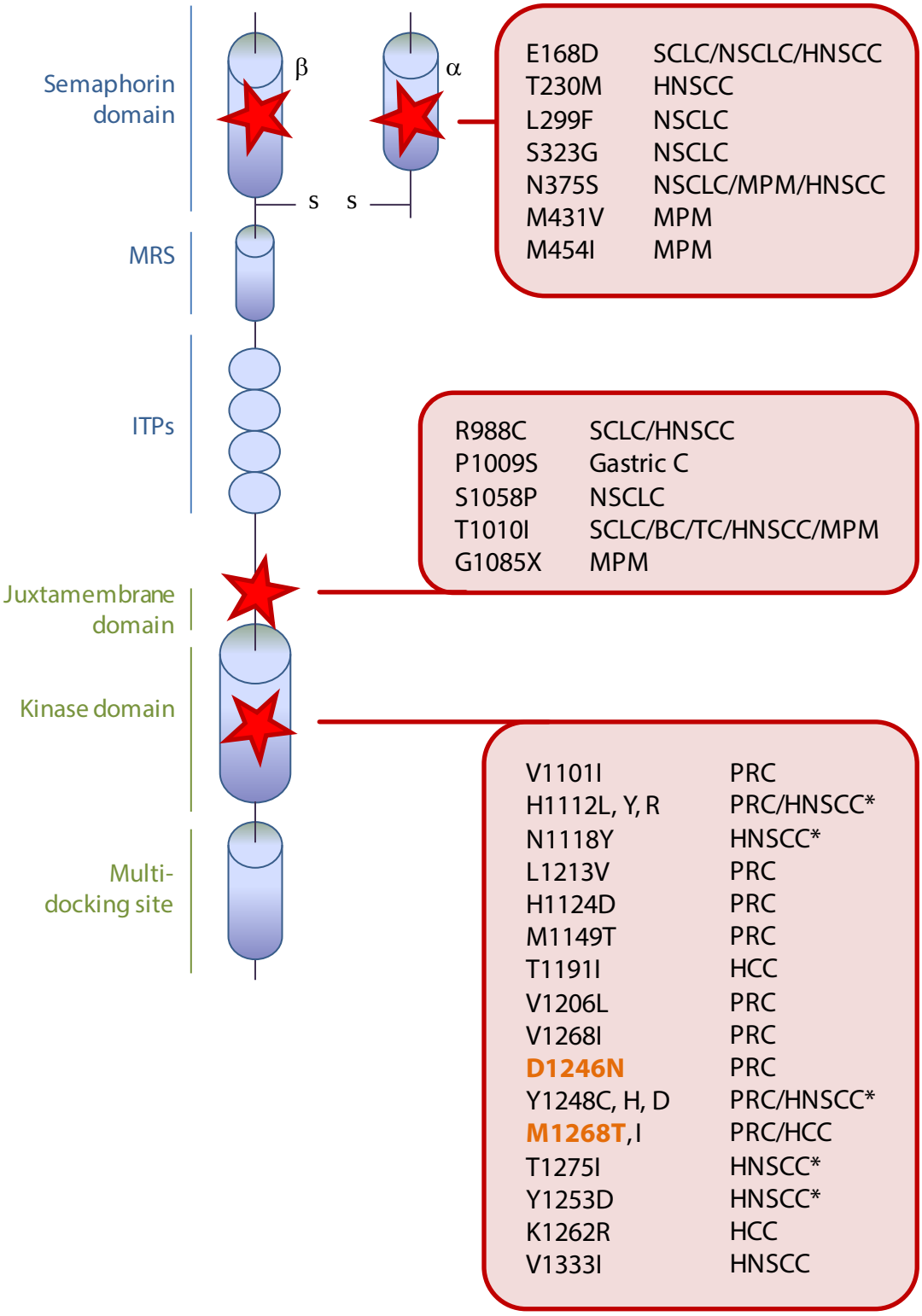


Figure 6: Met mutations found in human cancers

Summary of Met missence mutations found in cancer and the domains of Met where they are found.

MET-Related Sequence (MRS domain); immunoglobulin-like domain called Immunoglobulin-Plexin-Transcription (IPT) domains; Breast Cancer (BC), Small Cell Lung Cancer (SCLC), Non Small Cell Lung Cancer (NSCLC), Malignant Pleural Mesothelioma (MPM), Papillary Renal Carcinoma (PRC), Head and Neck Squamous Cell Carcinoma (HNSCC), HepatoCellular Carcinomas (HCC), Thyroid Carcinamo (TC). *: mutation found in metastasis.

Thanks to a better understanding of the ligand, the receptor, and activators, many HGF and Met inhibitors have been developed (inhibitors for HGF activators, HGF inhibitors, Met antagonists, and mainly Met kinase inhibitors) and some are being tested in clinical trials for cancer therapy **(Table 1)** [85]. Among these compounds, 4 of them have reached the phase III: Rilotumumab, Onartuzumab, Cabozantinib, and Tivantinib.

Currently, there are almost 240 clinical trials targeting HGF or Met, in monotherepies or combined with other treatments.

I. n) Oncogenic Met mutants

Met mutations found in cancer are located in the Met tyrosine kinase domain [86-91], the juxtamembrane region [91-97], and the semaphorin domain [91, 92, 97, 98] **(Fig. 6)**. So far, most studies have focused on Met tyrosine kinase domain mutations including the M1268T mutation initially found in papillary renal carcinomas and studied in our laboratory, as these mutations lead to the constitutive activation of Met. Met M1268T has also been found in childhood hepatocellular carcinoma and the lymph-node metastasis of head and neck squamous cell-carcinomas [99]. It is important to note that Met mutations in cancer are rare as compared to Met overexpression.

Amongst all the mutations discovered it seems that the mutation M1268T is the most strongly activated [100]. Molecular modelling studies suggest that Met mutation M1268T is located in the pocket substrate (P+1 loop), which leads to the destabilisation of the auto-inhibitory region of Met, and induces the constitutive activation [80, 88, 101]. The M1268T Met mutant induces cell transformation *in vitro* as well as tumour growth and metastasis *in vivo* [56, 100, 102].

Compound	Targets	Status
Monoclonal antibodies		
Rilotumumab (AMG-102)	HGF	Phase I - III
Ficlatuzumab (AV-299)	HGF	Phase I - II
TAK701	HGF	Phase I
Onartuzumab (MetMab)	MET	Phase III
ATP competitors		
AMG-337	MET	Phase I
EMD1204831	MET	Phase I
INC280 (INCB28060)	MET	Phase I - II
JNJ38877605	MET	Phase I
PF-04217903	MET	Phase I
SGX-523	MET	Phase I
Amuvatinib (MP-470)	MET, KIT, PDGFRα , FLT3	Phase II
BMS-777607	MET, AXL, RON, TYRO3	Phase I
Crizotinib (PF-2341066)	MET, ALK, ROS1, RON	Phase I
Cabozantinib (XL184)	VEGFR2, MET, RET, KIT, FLTs, TIE2, AXL	Phase I - III
Foretinib (XL-880) (GSK-1363089)	MET, VEGFR2, RON, FLTs, KIT, PDGFRs, TIE2	Phase I - II
golvatinib (E7050)	MET, VEGFR2	Phase I - II
MGCD-265	MET, VEGFRs, RON, TIE2	Phase I - II
MK-2461	MET, RON, FLT1, FGFRs	Phase I - II
Non-ATP competitor		
Tivantinib (ARQ-197)	MET and microtubing target(s)	Phase I – III

Table 1: Current strategies for Met inhibition in cancer therapy

Summary of Met inhibitors existing in clinical trials.

Modified from Cui., J.J., Targeting Receptor Tyrosine Kinase MET in Cancer: Small Molecule Inhibitors and Clinical Progress, Journal of Medicinal Chemistry, 2014.

Hepatocyte Growth Factor (HGF); stem cell growth factor receptor (KIT); Vascular Endothelial Growth Factor Receptor (VEGFR); Platelet-Derived Growth Factor Receptor (PDGFR); Fibroblast Growth Factor Receptor (FGFR); Fms-like tyrosine kinase 3 (FLT-3); Activin receptor-like kinase-1 (ALK1); Tyrosine-protein kinase receptor UFO (AXL), RON, FLT3, ROS1, RET, TIE2.

I. o) Understanding the mechanisms involved in Met mutants oncogenicity

In our group, Joffre *et al.* have studied the Met mutations M1268T and D1246N, both located in the kinase domain and leading to the constitutive activation of Met that is stronger than WT Met activated with HGF in NIH3T3 cells [56]. These mutations, expressed in NIH3T3 cells, cause *in vitro* and *in vivo* transformation [103].

Joffre *et al.* observed that, in basal conditions, M1268T and D1246N Met mutants are expressed at the plasma membrane but also on endosomes, while WT Met is present at the plasma membrane in basal conditions and localises to endosomes only upon HGF stimulation. Further investigation revealed that M1268T and D1246N Met were localised in early and recycling endosomes [56].

Biotinylation degradation and recycling assays brought to light the fact that Met mutants have a defect in degradation, and the fact that the M1268T and D1246N Met mutants are constitutively internalised and recycled back to the plasma membrane, travelling constantly from one compartment to the other [56] **(Fig. 7)**. Even more surprisingly, the inhibition of Met activity by a specific Met inhibitor PHA665752 inhibits M1268T Met (not D1246N Met) phosphorylation, but not its internalisation, demonstrating that the Met mutant constitutively internalises despite its dephosphorylation [56]. By performing knock-down, using inhibitors or using a mutated M1268T Met to abrogate its binding to Grb2 in the multi-docking site **(see I. k) 1. Endocytosis)**, they showed that Met internalisation requires clathrin, dynamin, Grb2 and Cbl [56]. The inhibition of M1268T Met and D1246N Met endocytosis by these techniques inhibits the cell transformation induced by the Met mutants such as (1) *in vitro*: Rac1 activation, loss of stress fibres, cell migration and anchorage independence growth, and (2) *in vivo*: metastasis and tumour growth [56] **(Fig. 7)**.

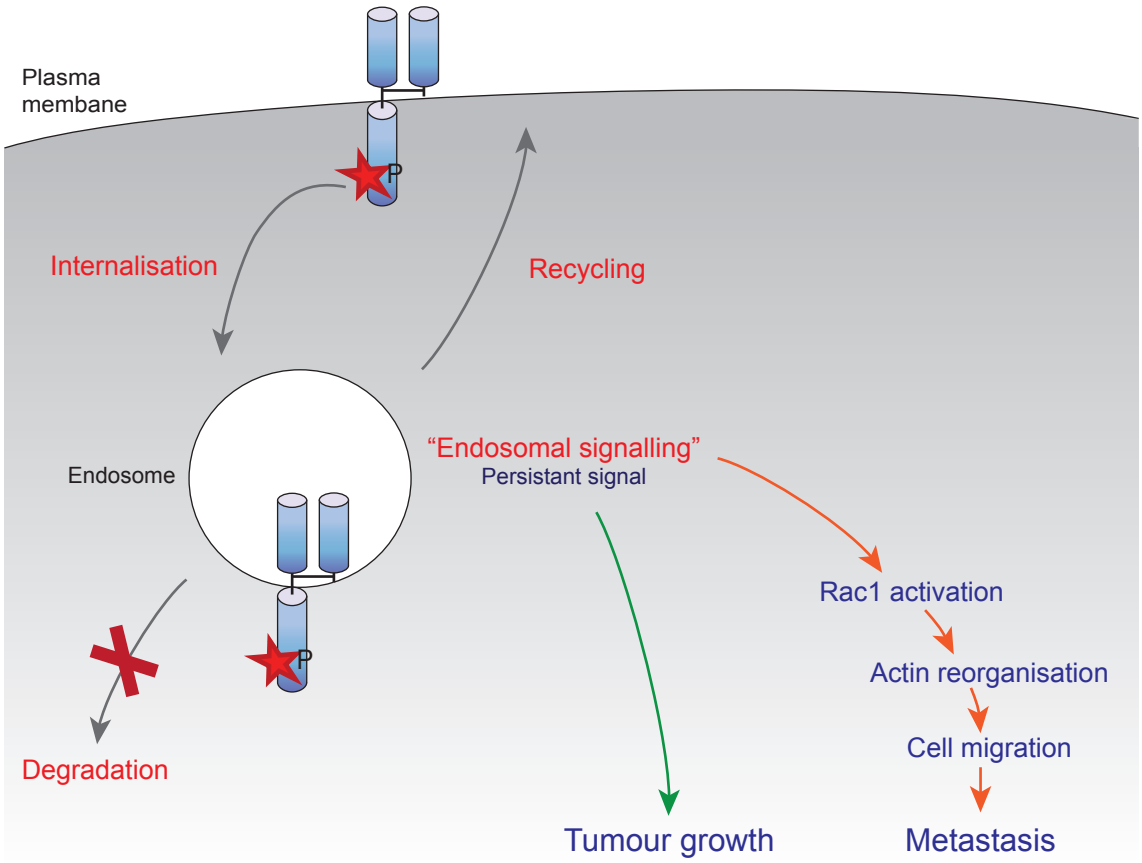


Figure 7: Met endosomal signalling

Constitutively phosphorylated (P) mutant Met shuttles between the plasma membrane and endosomes, escaping degradation. From endosomes, mutant Met signals from endosomes ("endosomal signalling"), which induces tumour growth and metastasis.

Importantly, the Met inhibitor PHA665752 also inhibits the cell transformation induced by the M1268T Met mutant *in vitro* and *in vivo* [56].

These results demonstrated that oncogenic Met mutants, M1268T and D1246N, are not oncogenic only because of their constitutive activation, but also because of their location [56]. Upon endocytosis inhibition, the mutated receptors are still activated but not oncogenic because they are not at the correct location [56].

II. Phosphatidylinositol 3-Kinase family

II. a) Phosphatidylinositol 3-Kinase

The Phosphatidylinositide 3-Kinase (PI3K) family of enzymes have dual activity, lipid kinase and protein kinase activity [104], which play a role in many key cell functions such as cell proliferation, migration, differentiation, survival and trafficking. This signalling pathway is one of the most deregulated pathways in cancer and is implicated in various types of cancer.

At the beginning of the 1980s, extended research focused on Phosphatidylinositol-4,5-bisPhosphate (PtdIns(4,5)P2) conversion into Diacylglycerol (DAG) and Inositol-1, 4, 5-triphosphate (IP3) by Phospholipase C (PLC) and its role in Protein Kinase C (PKC) activation and calcium release [105].

In 1982, Cantley *et al.* discovered a phosphoinositide kinase able to phosphorylate phosphoinositides on the 3' position of the inositol ring and have PtdIns(4,5)P2 as a substrate. This phosphoinositide kinase was called type I phosphatidylinositol kinase and nowadays called PI3K class I [106-108]. Since then, PI3K isoforms have been identified and classified: the PI3K family contains eight isoforms divided into three distinct classes (I, II, and III) **(Table 2)** [109], which could be responsible for the specificity of cellular responses [110]. The largest and the most understood is the class I while the roles of classes II and III still remain unclear.

II. b) PI3Ks structure

Class I PI3Ks are comprised of a catalytic and a regulatory subunit while class II and III consist of only catalytic subunits **(Fig. 8)**.

		Catalytic subunit	Regulatory subunit	Lipid Substrate *in vivo*	Main expression
Class I	A	p110 α p110 β	p85	PtdIns(4,5)P_2	Ubiquitous
		p110 δ			Haematopoietic system
	B	p110 γ	p101 p87		
Class II		PI3K-C2 α PI3K-C2 β		PtdIns PtdIns(4)P	Ubiquitous
		PI3K-C2 γ			Liver
Class III		Vps34	Vps15	PtdIns	Ubiquitous

Table 2: PI3K isoforms and classes

8 isoforms of PI3K divided into three classes depending on their lipid substrate, their catalytic subunit and their regulatory subunit. Phosphatidylinositol-4,5-bisPhosphate (PtdIns(4,5)P_2); Phosphatidylinositol (PtdIns); Phosphatidylinositol-4-Phosphate (PtdIns4P).

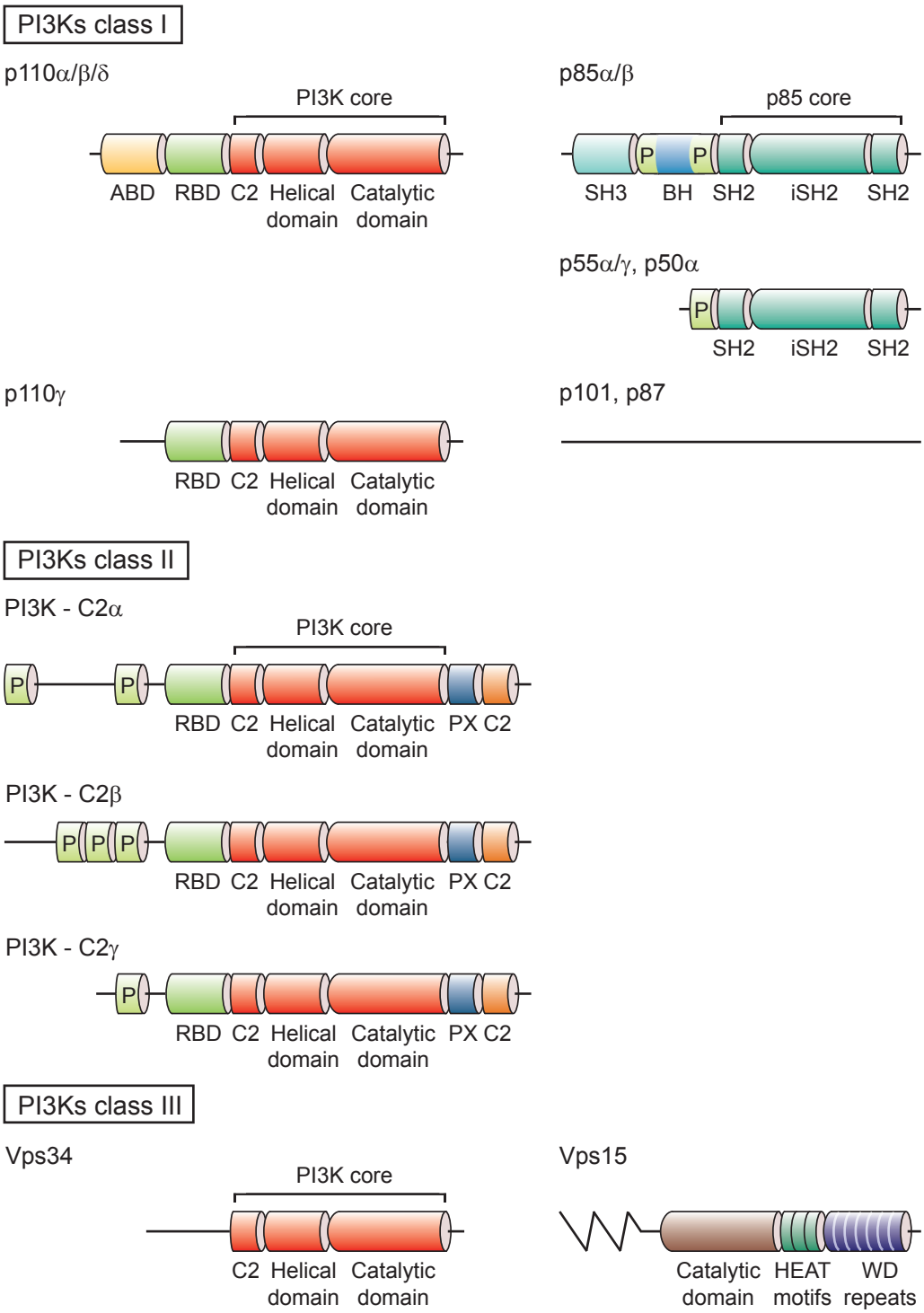


Figure 8: PI3Ks structures

PI3K class I is composed of a catalytic subunit (p110) and a regulatory subunit (p85/87/101); PI3K class II is composed of one catalytic subunit, and PI3K class III of two catalytic subunits.

Adaptor binding domain (ABD); Ras binding domain (RBD); inter-Sarcome (Src) Homology 2 (iSH2); Src Homology 2/3 (SH2/3); BCR homology domain (BH), Phox homology domain (PX); Proline rich region (P); Huntingtin, elongation factor 3, protein phosphatase 2A and TOR1 (HEAT) motif; tryptophan-aspartic acid (WD) dipeptide; Vacuolar protein sorting 34 (Vps34).

All PI3K catalytic subunits contain a PI3K core which contains a C2 domain binding phospholipids in a Ca^{2+} dependent manner, a helical domain, and a catalytic domain.

The catalytic subunit of class I PI3Ks includes the p110 alpha, beta, and delta isoforms (class IA) or the p110 gamma isoform (class IB). In addition to a PI3K core, all p110 isoforms have a Ras-binding domain and a p85-binding domain called Adaptor Binding Domain (ABD). The catalytic subunit binds the p85 regulatory subunit of which there are various isoforms: p85 alpha, beta, p55 beta, gamma and p50 alpha (for class IA) or p101 and p87 (for class IB). Class IA regulatory subunits have a p110-binding domain called inter-Sarcoma (Src) Homology 2 (iSH2) domain and two Src Homology 2 (SH2) domains. Additionally, p85 subunits have one Src Homology 3 (SH3) domain and a BCR homology (BH) domain flanked by proline rich regions. The BH domain is responsible for p85 interaction with the Rho GTPases Cdc42 and Rac1. For a long time, the role of p85 on p110 was unclear until the discovery that monomeric p110 alpha is heat labile [111]. The regulatory subunit p85 stabilises the catalytic subunit [112], and the binding between p85 and p110 appears to be very tight [113]. A monomer of p110 does not seem to exist under cellular condition, instead p85 and p110 exist as a heterodimer [114]. The contact between the N-terminal SH2 domain of the catalytic subunit p85 and the helical domain of the regulatory subunit p110 inhibits the catalytic subunit [112, 115]. The release of the inhibition presumably occurs when a phosphoprotein binds the N-terminal SH2 domain of p85 [115]. No homology to other protein has been found for the p101 and p87 regulatory subunits **(Fig 8)**.

Class II is composed of the catalytic subunit PI3K-C2 alpha, beta, or gamma. In addition to the PI3K core, they all have a Ras-binding domain and contain a carboxy-terminal extension with a Phox homology domain (PX) and a C2 domain. The amino-terminal extension (N-terminal) of each isoform is enriched with proline regions, known

to mediate clathrin-binding [116, 117] **(Fig 8)**. The N-terminal domain has been demonstrated to play a role in regulating the activity of these isoforms [117].

Class III is composed of the catalytic subunit Vacuolar protein sorting 34 (Vps34), also called PIK3C3 in mammals. Vps34 is present in unicellular and eukaryotic organisms. It constitutively binds the regulatory subunit Vps15, called PIK3R4 in mammals. Vps15 is myristoylated, allowing anchorage of the heterodimer to the plasma membrane. Vps15 also has a catalytic domain, a "Huntingtin, elongation factor 3, protein phosphatase 2A and TOR1" (HEAT) domain (which mediate protein-protein interactions), and WD repeats, which are repeated units often ending with a tryptophan-aspartic acid (W-D) dipeptide, and it is necessary for Rab5- Guanosine triphosphate (GTP) binding [118] **(Fig 8)**.

II. c) <u>PI3Ks substrates and products</u>

PI3Ks phosphorylate the 3-hydroxyl group of the inositol ring of their own lipid substrates **(Fig. 9)** [110].

The PI3K class I lipid substrate is the PtdIns(4,5)P_2, also known as PIP2 [119]. PtdIns(4,5)P_2 is the major Phosphatidylinositol (PtdIns) at the plasma membrane [120, 121]. When PtdIns(4,5)P_2 is phosphorylated by PI3K class I, it becomes Phosphatidylinositol-3,4,5-triPhosphate (PtdIns(3,4,5)P_3), also known as PIP3. The "Phosphatase and Tensin homologue deleted on chromosome 10" (PTEN) antagonises PI3K class I as it dephosphorylates PtdIns(3,4,5)P_3 to PtdIns(4,5)P_2, and Src Homology domain-containing 5' Inositol Phosphatase (SHIP) dephosphorylates PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2.

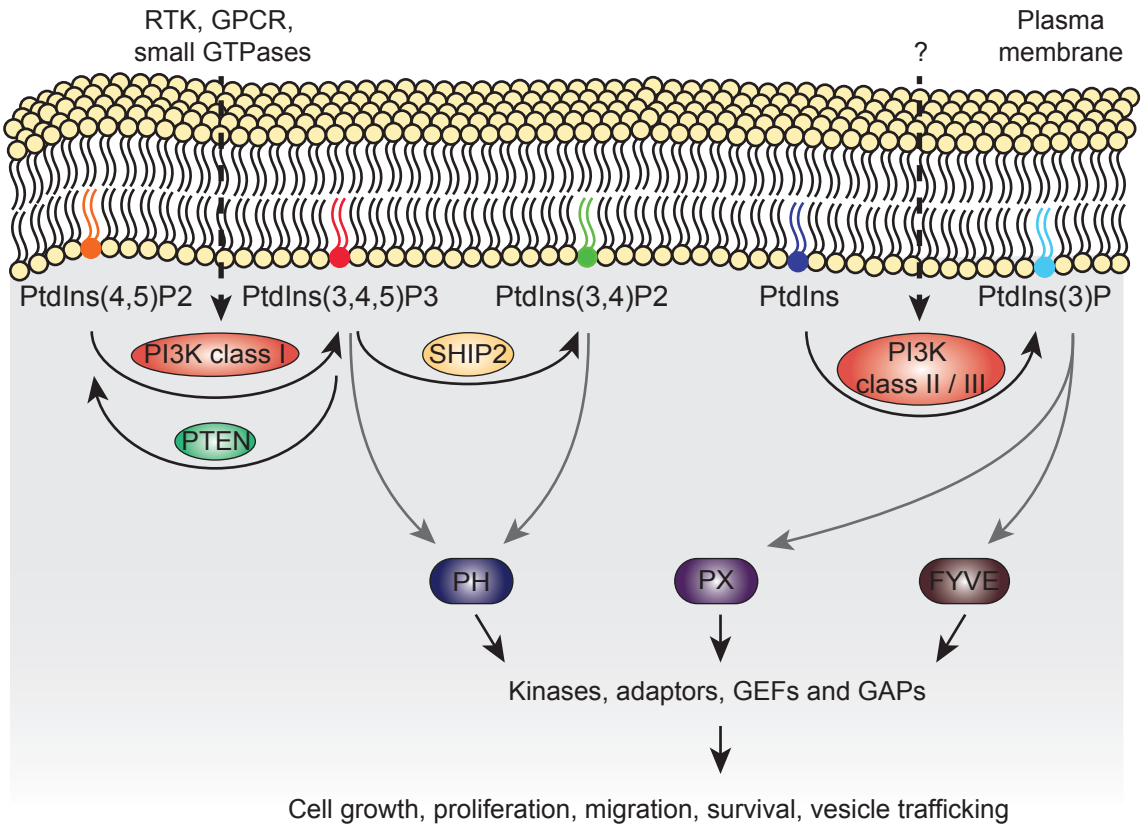


Figure 9: The 3-phosphoinositide and PI3K classes signalling network

Upon activation, PI3K class I phosphorylates PtdIns(4,5)P_2 to PtdIns(3.4,5)P_3, which is dephosphorylated by PTEN. SHIP2 dephosphorylates PtdIns(3.4,5)P_3 to PtdIns(3,4)P_2. PI3K class II/III phosphorylates PtdIns to PtdIns3P. The binding domains of the 3-phosphoinositide mediates the downstream signalling to induce cell functions.

Receptor Tyrosine Kinase (RTK); G-Protein Coupled Receptor (GPCR); Guanosine triphosphatase (GTPase); Phosphatidylinositol-4,5-bisPhosphate (PtdIns(4,5)P_2); Phosphatidylinositol-3,4-bisPhosphate (PtdIns(3,4)P_2); Phosphatidylinositol-3,4,5-triPhosphate (PtdIns(3.4,5)P_3); Phosphatidylinositol (PtdIns); Phosphatidylinositol-3-Phosphate (PtdIns3P); Phosphatase and Tensin homologue deleted on chromosome 10 (PTEN); Src Homology domain-containing 5' Inositol Phosphatase 2 (SHIP2); Pleckstrin Homology domain (PH); Phox homology (PX); zinc finger domain (FYVE); Guanine nucleotide Exchange Factor (GEF); GTPase Activating Proteins (GAP).

Class II has the PtdIns and Phosphatidylinositol-4-Phosphate (PtdIns4P) as lipid substrates, which are respectively phosphorylated to Phosphatidylinositol-3-Phosphate (PtdIns3P) and PtdIns(3,4)P_2. However, PtdIns3P, also known as PI3P, remains the main product of class II PI3K as demonstrated *in vitro* and *in vivo* [122]. PtdIns3P is mainly localised to microdomains within early endosomes and on internal vesicles of multivesicular endosomes [123, 124].

Class III PI3K shares the lipid substrate PtdIns with the class II [125]. In the same way as class II, class III PI3K phosphorylates PtdIns to PtdIns3P [126, 127], which implies in principle that they could share the same effectors.

Then, the produced 3-phosphoinositides can bind their effector proteins thanks to lipid binding domains such as Pleckstrin Homology (PH) domain (for PIP3), PX domain or "Fab1, YOTB, Vac1, EEA1" (FYVE) zinc finger domain (for PtdIns3P), and depending on their intracellular localisation. For example, PIP3 can recruit, through its PH domain, serine/threonine protein kinase like Protein Kinase B (PKB), also known as Akt [128-130], a major downstream effector of PI3K. Also, PtdIns3P binds the Rab5 effector Early Endosome Antigen 1 (EEA1) through its FYVE domain [131, 132].

- *Akt, the indirect and well known effector of PI3K*

Akt, an indirect substrate of PI3K, belongs to the serine/threonine kinases "protein kinase A/protein kinase G/protein kinase C" (AGC) family. Akt has 3 isoforms: Akt1, Akt2, and Akt3. These isoforms are not-redundant as illustrated by the fact that knock-out mice have different phenotypes. Akt1 knock-out mice present growth retardation and perinatal death; Akt2 knock-out mice develop insulin-resistant diabetes; and Akt3 knock-out mice have a reduced brain size [133].

The production of PIP3 by PI3K class I leads to: (1) the recruitment and activation of Phosphoinositide-dependent kinase-1 (PDK1) at the plasma membrane; (2) the recruitment of Akt at the plasma membrane; (3) the phosphorylation of the threonine 308 of Akt by activated PDK1; (4) 2 the phosphorylation of the serine 473 of Akt by mTORC2 [134] **(see III. The mTOR complexes)**. These two phosphorylations are necessary for the full activation of Akt. Due to PIP3 localisation, it is traditionally thought that Akt signals, when fully activated, from the plasma membrane; however some recent studies suggest otherwise **(see II. e) PI3Ks spatial signalling)**.

II. d) PI3Ks expression, activation and functions

Over the last two decades, there is an increasing interest to define the role of each PI3K isoforms [135]. Due to their similarities, can the isoforms compensate for each other? Or despite these similarities, does each isoform accomplish distinct functions? The roles of the individual PI3K isoforms have been investigated *in vitro* and *in vivo*. Firstly, mice knocked - out for the PI3K isoforms were generated, this resulted in the alteration of the expression of non-targeted proteins (compensatory effect) sometimes leading to no phenotype. Therefore, knock - in mice (PI3K kinase dead) were generated, to preserve the expression and thus preserve the signalling complex stoichiometry. In the case of the absence of a notable phenotype, it could indicate either that the studied isoform does not play an essential role in the cell, or it is compensated by another protein.

1. Class I PI3Ks

In the class I PI3Ks, p110 alpha and beta are ubiquitously expressed in mammal's tissue, whereas p110 delta and p110 gamma are highly enriched in immune cells [136]. So far, the roles of each of the class I regulatory subunits are unknown. However it is known that the isoforms of the regulatory subunit p85 can bind to each isoform of PI3K class IA when overexpressed [137].

- *Isoforms ubiquitously expressed: p110 alpha and p110 beta*

Homozygous null mice for p110 alpha (knock-out mice) [99, 138], or mice expressing a kinase dead p110 alpha (knock-in mice) [139] are embryonically lethal **(Table 3)**.

Heterozygous mice are viable but present vascular defects [141], and severe deregulation of signalling through Insulin Receptor Substrate (IRS) [139] involved in Insulin-like Growth Factor (IGF)-1, insulin, and leptin signalling. p110 alpha has been shown to play an important role in vascular development by regulating endothelial cell migration [99, 140].

p110 alpha is involved in several cell functions such as the regulation of the endothelial barrier by activating Rac1 through the recruitment of its Guanine nucleotide Exchange Factor (GEF) Tiam1 [141] and insulin signalling (through Akt phosphorylation) in several cell lines [142]. It is also implicated in the actin reorganisation induced by Platelet-Derived Growth Factor (PDGF) [143] and in DNA synthesis induced by PDGF and Epithelial Growth Factor Receptor (EGFR) [144].

p110 alpha is believed to be mainly activated by Receptor Tyrosine Kinases (RTKs) and by the small guanosine triphosphatase (GTPase) Ras [145-148].

PI3K isoform	Homozygous Knock - Out	Heterozygous Knock - Out	Homozygous Knock - In	Heterozygous Knock - In
p110 alpha	• Lethal (E9.5) • Proliferation [1] • Vascular defects [2]	• Viable	• Lethal (E10.5) [3] • Defective vascular development [4]	• Viable • Severe deregulation of signalling through IRS [3] • Vascular defects [4]
p110 beta	• Lethal [5]	• Viable	• Partially Lethal • Growth retardation and Insulin resistance [6] • *Kinase independent role in EGFR internalisation* [6]	• Viable
p110 delta	• Viable • Immunological defects [7, 8, 9]	• Viable	• Viable • Immunological defects [10,11]	• Viable
p110 gamma	• Viable • Immunological defects [12, 13] • Cardiac problems [14]	• Viable	• Viable • Immunological defects [14] • *Kinase independent role in negatively modulation of cardiac contractility* [14]	• Viable

Table 3: PI3K class I isoforms roles in transgenic mice

Summary of the phenotypes of transgenic mice that are knock-out or knock-in for various PI3K class I isoforms.

1. Bi, *et al.*, *J Biol Chem* 1999. **2.** Lelievre, *et al.*, *Blood* 2005. **3.** Foukas, *et al.*, *Nature* 2006. **4.** Graupera, *et al.*, *Nature* 2008. **5.** Bi, *et al.*, *Mamm Genome* 2002. **6.** Ciraolo, *et al.*, *Sci Signal* 2008. **7.** Clayton, *et al.*, *J Exp Med* 2002. **8.** Jou, *et al.*, *Mol Cell Biol* 2002. **9.** Puri, *et al.*, *Blood* 2004. **10.** Ali, *et al.*, *Nature* 2004. **11.** Okkenhaug, *et al.*, *Science* 2002. **12.** Hirsch, *et al.*, *Science* 2000. **13.** Rodriguez-Borlado, *et al.*, *J Immunol* 2003. **14.** Patrucco, *et al.*, *Cell* 2004.

The homozygous knock–out and knock–in of the p110 beta isoform in null mice induces a high proportion of lethally at an early embryonic stage **(Table 3)**. The mice which survive to adulthood present growth retardation and insulin resistance [149, 150].

The p110 beta isoform can be activated by both RTKs [143] and by G Protein Coupled Receptors (GPCRs) [147, 151-154]. Interestingly, there is some evidence that p110 beta can be activated by the small GTPase Rab5 [155-157], the Rho GTPase Cdc42 and Rac1 [148], and is present in Clathrin-Coated Vesicles (CCV) [155, 156].

The isoform p110 beta has been shown to be involved in a kinase-independent manner in cell proliferation, in insulin metabolism and trafficking [150, 158, 159] and in the internalisation of EGFR [150] and Insulin-like Growth Factor Receptor (IGFR) [160].

However, its kinase activity is required to trigger Lysophosphatidic Acid (LPA) and Sphingosine-1-Phosphate (S1P) signalling through GPCRs [150, 158, 161]. Following insulin stimulation, p110 beta has been reported to be involved in Akt phosphorylation [142] and in the cytoskeleton reorganisation in several cell lines [143].

Interestingly, the two isoforms p110 alpha and p110 beta have different kinetic properties (Vmax, Km) in both their lipid-kinase activity and protein-kinase activity. The authors Beeton *et al*. suggest that the differences obtained could indicate different functional roles *in vivo*: p110 alpha would be expected to be more efficient in membrane lipid rafts (high substrate density) and p110 beta in areas with low substrate density [162].

Recently, a novel model suggests a competition between the two isoforms p110 alpha and p110 beta for their binding to the receptor binding site of RTKs. The authors

demonstrate that in polyoma middle T antigen (MT) and HER2/Neu transgenic models of breast cancer, the tumourigenesis is inhibited when p110 alpha is ablated, while p110 beta ablation had the opposite effect and thus increased the tumourigenesis. Pharmalogical inhibition of p110 beta did not induce an increase of the tumourigenesis as it did not interfere with p110 beta binding to the activated receptor. Based on the fact that p110 alpha and p110 beta have distinct kinetic properties, and that p110 alpha is the most active, the authors suggest that binding competition could be the mechanism that regulates the level of PI3K activity downstream of RTKs [147, 163].

- *Isoforms expressed in the immune system: p110 delta and p110 gamma*

The isoform p110 delta is mainly activated by RTKs, while the p110 gamma can be activated by GPCRs [151, 153, 164] and RTKs [165]. Both isoforms p110 delta and p110 gamma can be activated by the small GTPase Ras [166, 167].

Homozygous null mice for p110 delta and p110 gamma, or kinase dead (inactive kinase) p110 delta [168-172] and p110 gamma [164, 173-175] are viable, however they present immunological defects. In addition, p110 gamma seems to play an important role in cardiac cells [176, 177].

Interestingly, it appears that the PI3K class IA is redundant with the class IB **(Table 2)**. A study describing the Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) growth factor survival response in human neutrophils shows that the inhibition of all of the PI3K isoforms together strongly induce apoptosis of the neutrophils, while the inhibition of any of the PI3K isoforms had only a small effect, indicating a redundant role between the PI3K isoforms [178]. Similarly, in NIH3T3 and Mouse Embryonic

Fibroblast (MEF), the expression of p110 gamma reduced the involvement of p110 beta in response to GPCR ligand stimulation [151].

2. Class II PI3Ks

Class II PI3Ks is the least understood class of the three PI3K classes, but this class has recently arisen more and more interest. Mammals express three isoforms [179]: C2 alpha, beta and gamma. *D. melanogaster* and *C. elegans* express only one isoform [180], and yeast do not express class II PI3Ks.

The isoform C2-alpha is ubiquitously expressed, with a higher expression in some tissues such as the heart, placenta, and ovaries [179]. C2-beta is widely expressed, but it is particularly highly expressed in the placenta and thymus. The expression of the isoform C2 gamma is more restricted to the liver, prostate, breast and salivary glands [181]. Class II PI3Ks are localised to intracellular membranes and the nucleus [182-185]. Once activated, they translocate to the plasma membrane [186-188].

The isoform C2 alpha can be activated by various stimuli including insulin, Monocyte Chemotactic Protein 1 (MCP1), leptin and Tumour Necrosis Factor alpha (TNFalpha) [186, 189-191]. The isoform C2 beta can be stimulated by growth factors such as Epithelial Growth Factor (EGF), PDGF, Stem Cell Factor (SCF) and LPA [187, 188, 192, 193]. So far, how C2 gamma is activated has not been demonstrated. Interestingly, it has been shown that clathrin and the clathrin-binding site regulate C2 alpha activity [116]. Moreover C2 beta, which does not have a clathrin-binding site, seems however to be able to bind to Clathrin Heavy Chain (CHC) [117]. Calcium can also activate the class II isoforms [187, 194, 195].

It is important to note that C2 alpha and beta, in particular alpha, are resistant to high concentrations of wortmannin and LY294002 [196], which are widely used to inhibit all the PI3K isoforms.

In vivo, the roles of PI3K class II have not been widely reported. Only C2 beta null mice have been studied and no specific phenotype has been noticed [197]. *In vitro*, more and more is known. C2 alpha has been shown to be involved in glucose metabolism [186], exocytosis [198, 199], endocytosis [116, 200], smooth muscle cell contraction [201], cell growth and survival in some cancer cells [202, 203]. In NIH3T3, C2 beta has been shown to be involved in cell migration via Rac [193, 204, 205]. When activated by T-Cell Receptor (TCR), C2 beta has been demonstrated to activate potassium channels [206]. C2 beta is also involved in cell growth, cell-cycle progression and survival [205, 207, 208]. Less is known about C2 gamma, which has been shown to be necessary for homing leukemic cells [209].

3. Class III PI3K

Vps34 and its regulatory subunit Vps15 are ubiquitously expressed in mammals. Vps34 is localised to membranes via its binding to Vps15 [210]. Vps34 activity can be regulated by nutrients and GPCR [211-213], and, in yeast, requires the kinase domain of Vps15 [210].

Vps34 is found with various complexes involved in different trafficking pathways leading invariably to the lysosome. Downstream of Bax-Interacting Factor 1 (BIF1) stimulation, Vps34 associates with Beclin1, ATGL14L, and Ultraviolet radiation Resistance Associated Gene protein (UVRAG), and is involved in autophagy [125, 214-217]. Rab5-GTP and Rab7-GTP, in early and late endosomes respectively, bind the Vps15/Vps34 complex; which has been shown to be involved in endocytosis and protein sorting [118, 126, 127, 218-220]. It also has been shown that Vps34 directly recruits

Dynamin-1 (DYN-1) to nascent phagosomes, which then recruits Rab5-Guanosine diphosphate (GDP). This work suggests that the conversion of Rab5-GDP to Rab5-GTP is responsible for the activation of the kinase activity of Vps34 [221]. However, although Vps34 is not necessary for the formation of the nascent phagosome, it is indispensable for phagosome maturation [222, 223].

II. e) PI3Ks spatial signalling

One explanation for the specific roles of these isoforms could be that their signalling depends on their spatial localisation. In 2008, Schenck and Zerial studied the role of the "Adaptor Protein containing PH domain, PhosphoTyrosine Binding (PTB) domain, and Leucine zipper motif 1" (APPL1), a Rab5 effector, in Akt signalling in zebrafish and HeLa cells [224]. They found that APPL1, which interacts with transmembrane receptors, is localised to vesicles underneath the plasma membrane corresponding to "APPL endosomes" [225]. Knock-down of APPL1 provoked cell apoptosis in zebrafish embryos, indicating that APPL1 is necessary for cell survival in zebrafish development. Moreover, knock-down of APPL1 was associated with dephosphorylation of Akt in zebrafish embryos. Interestingly, among the two downstream effectors of Akt, Glycogen Synthase Kinase 3 beta (GSK3 beta) and Tuberous Sclerosis 2 (TSC2), which are involved in cell survival and cell growth respectively, only GSK3 beta had its activity reduced. They observed that after stimulation with IGF-1, Akt colocalised transiently with APPL1 on endosomes in HeLa cells. Before stimulation, GSK3 colocalised with APPL1 endosomes, while after stimulation GSK3 had dissociated from APPL1 endosomes. TSC2 was not found to colocalise with APPL1 endosomes. Finally, they compared Akt activity in the following models of zebrafish embryos: the zebrafish embryos either (1) expressed WT APPL1

(presence of APPL1 endosomes); (2) were knocked down for APPL1 with morpholinos; (3) were knocked down for APPL1 and WT APPL1 was rescued with *in vitro* transcribed mRNA; (4) expressed Nuclear Localization Signal (NLS)-APPL1 (an APPL1 mutant which localised in the nucleus); (5) expressed the triple mutant (triM)-APPL1, bearing R147A, K153A, K155A mutations (this APPL1 mutant fails to associate with endosomes, and is therefore soluble). The results showed that only APPL1 on endosomes allows and is sufficient for Akt activation. This work is consistent with the hypothesis that spatial localisation is required and sufficient to induce specific PI3K signalling pathways.

II. f) PI3K in cancer

In cancer or aging, the PI3K signalling pathway is frequently deregulated [226, 227] and PI3K is more active due to mutations or amplification. In solid tumours these deregulations occur mainly on p110 alpha amongst all of the PI3K isoforms [228-230]. However, overexpression of the other PI3K class I isoforms can also transform cells *in vitro* [231]. Additionally, p110 beta is highly expressed in tissues and cancer cells [232, 233].

The increase of PI3K activity in cancer is often associated with the altered function of the tumour suppressor PTEN, due to either loss of heterozygosity or mutations [230, 234]. As mentioned earlier, the function of the PtdIns(3,4,5)P_3 phosphatase PTEN is to antagonise PI3K class I signalling **(Figure 9)**.

In cancer, mutations/deletion in the PIK3R1 gene, which encodes for 3 species of the p85 regulatory subunit (p85 alpha, p55 alpha and p50 alpha) have also been found [235-237].

For those reasons, cancer treatments so far have focused on targeting PI3K class I. **(Table 4)** [238].

However, deregulations can also occur downstream of PI3K. Indeed mutations of PDK1, PTEN or Akt have been discovered in cancer [227, 232], which affect Akt or "mechanistic Target of Rapamycin" (mTOR) signalling. mTOR is well known as a PI3K effector involved in mitogenesis [239] **(see III. The mTOR complexes)**.

Amongst pharmalogical inhibitors targeting PI3K/Akt/mTOR in clinical trials, 27% are PI3K class I inhibitors, 27% are Akt inhibitors **(Table 4)**, and 19% target both PI3K and mTOR [238, 240] **(Table 5, and see III. f) mTOR in cancer)**. These dual PI3K-mTOR inhibitors provide the benefit of targeting the signalling pathway at two levels and through inhibiting mTORC1 and its effector p70S6K, prevent Akt upregulation.

II. g) <u>RTKs & PI3K in cancer</u>

During this last decade it has been widely described that PI3K class IA activation in cancer is often due to RTK activation [227, 241-246] . Activated RTKs, or their adaptors, bind the SH2 domain of the p85 regulatory subunit, followed by the activation of the PI3K class IA signalling pathway [247, 248]. In cancer therapy, RTK inhibitors can lead to drug resistance [81, 249, 250], explaining the necessity to develop therapies targeting downstream signalling or to target multiple signalling pathways simultaneously.

Compound	Tumor type	Status
PI3K class I inhibitors		
BKM120	NSCLC, endometrial, thyroid, CRPC, breast, colorectal, head and neck, glioblastoma, renal cell, B cell lymphoma, GIST, melanoma, ovarian, prostate, pancreatic, leukaemia, oesophageal, cervical, non-hodgkin lymphoma, squamous NSCLC, advanced solid tumours	Phase I - III
GDC0941	Breast, NSCLC, non-hodgkin lymphoma, advanced solid tumours	Phase I - II
BAY80-6946	Non-Hodgkin lymphoma, advanced solid tumours	Phase I - II
ZSTK474	Advanced solid tumours	Phase I - II
PX866	Colorectal, SCCHN, melanoma, NSCLC, prostate, glioblastoma, advanced solid tumours	Phase I - II
XL147	Breast, endometrial, ovarian, lymphoma, glioblastoma, NSCLC, advanced solid tumours	Phase I - II
CH5132799	Advanced solid tumours	Phase I
Akt inhibitors		
MK-2206	Advanced/metastatic solid tumour, breast, NSCLC, pancreatic, ovarian, colorectal, leukemia, prostate, kidney, gastric, lymphoma, liver, NET, endometrial	Phase I - II
GDC-0068	Advanced/metastatic solid tumour, gastric, prostate, breast	Phase I - II
Uprosertib (GSK2141795) (GSK795)	Breast, cervival, myeloma, melanoma, ovarian, leukemia, endometrial	Phase I - III

Table 4: PI3K class I and Akt inhibitors in clinical trial

Summary of PI3K class I / Akt inhibitors existing in clinical trials.

Modified from Thorpe, L.M. et al., PI3K in cancer: divergent roles of isoforms, modes of activation and therapeutic targeting , Nature review, 2015.

Castration-resistant prostate cancer (CRPC); gastrointestinal stromal tumour (GIST); neuroendocrine tumors (NET); non-small-cell lung carcinoma (NSCLC); squamous cell carcinoma of the head and neck (SCCHN).

III. The mTOR complexes

III. a) The mechanistic target of rapamycin

The mechanistic (or originally “mammalian”) Target of Rapamycin (mTOR) is a serine/threonine protein kinase belonging to the PI3K-related protein kinases (PIKKs) [251, 252].

Highly conserved, mTOR is expressed in every eukaryote organism studied (yeasts, algae, plants, worms, flies and mammals). Initially, the Target of Rapamycin (TOR) was discovered in the budding yeast *Saccharomyces cerevisiae* treated with the macrolide fungicide rapamycin produced by *Streptomyces Hygroscopicus*. Rapamycin, also called sirolimus, induced the yeast cell cycle arrest in phase G1. Genetic analysis of yeasts resistant to rapamycin revealed mutations in TOR genes [253, 254]. Soon, its mammalian counterpart mTOR was described by biochemical approaches as the physical target of rapamycin [253, 255, 256].

Rapamycin forms a complex with the protein FKBP12, belonging to the FK506 binding protein family, and this complex binds mTOR on its FKBP12-rapamycin binding domain (FRB), thus inhibiting mTOR function by a possible allosteric effect. Therefore, mTOR is also named FKBP12-rapamcyin-associated protein (FRAP), rapamycin and FKBP12 target (RAFT1), rapamycin target 1 (RAPT 1), or sirolimus effector protein (SEP) [257, 258].

III. b) <u>The structure of mTOR</u>

mTOR is a protein of 289 *kDa*, and is the catalytic subunit of two multiprotein complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2), which are structurally and functionally distinct [259, 260] **(Fig. 10)**.

mTOR contains on its *N*-terminus two repeated "Huntingtin, elongation factor 3, protein phosphatase 2A and TOR1" (HEAT) motifs mediating protein-protein interaction. This is followed by a FRAP, ATM and "Transformation/transcription domain-associated protein" (TRRAP) (FAT) domain (common to PIKK family members), a FKBP12-rapamycin-binding (FRB) domain, which mediates the inhibitory action of rapamycin, a kinase domain sharing a strong homology with PI3K's catalytic domain, an auto-inhibitory repressor domain (RD domain), and a short FAT domain at the *C* terminus (FATC), interacting with other domains of mTOR and other proteins of mTORC1/2 to activate its kinase function [261] **(Fig. 11)**. Recently, in 2009, the existence of a splice variant of mTOR was demonstrated in most human and rat tissues (including brain, lung, heart, intestine, colon, kidney, pancreas). In contrast to the full length (described above), now called mTORalpha (289 kDa), the lately discovered mTORbeta is truncated (~80 kDa) and contains only the FRB domain, kinase domain, RD domain and FATC [262] **(Fig. 11)**.

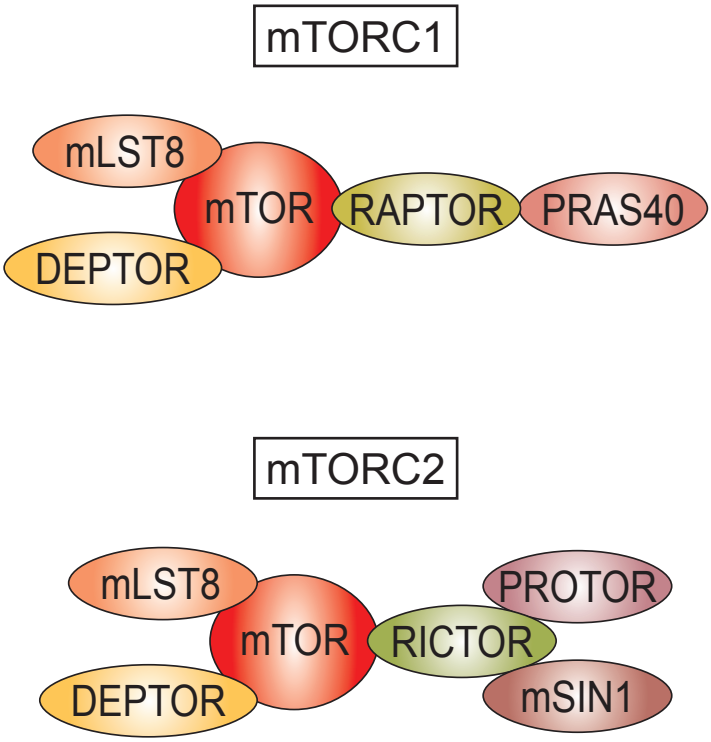


Figure 10: The structure of mTOR complexes 1 and 2

The complexes mTORC1 and mTORC2 have two regulatory subunits in common: 1) DEP domain-containing mTOR-interacting protein (DEPTOR); and 2) mammalian Lethal with SEC13 protein 8 (mLST8). In addition, mTORC1 contains Regulatory Associated Protein Of mTOR (RAPTOR), and Proline Rich Akt Substrate of 40 kDa (PRAS40); while mTORC2 contains Rapamycin-Insensitive Companion of mTOR (RICTOR), Protein Observed with RICTOR (PROTOR), and mammalian Stress-activated map kinase-Interacting protein 1 (mSIN1).

mechanistic Target of Rapamycin (mTOR).

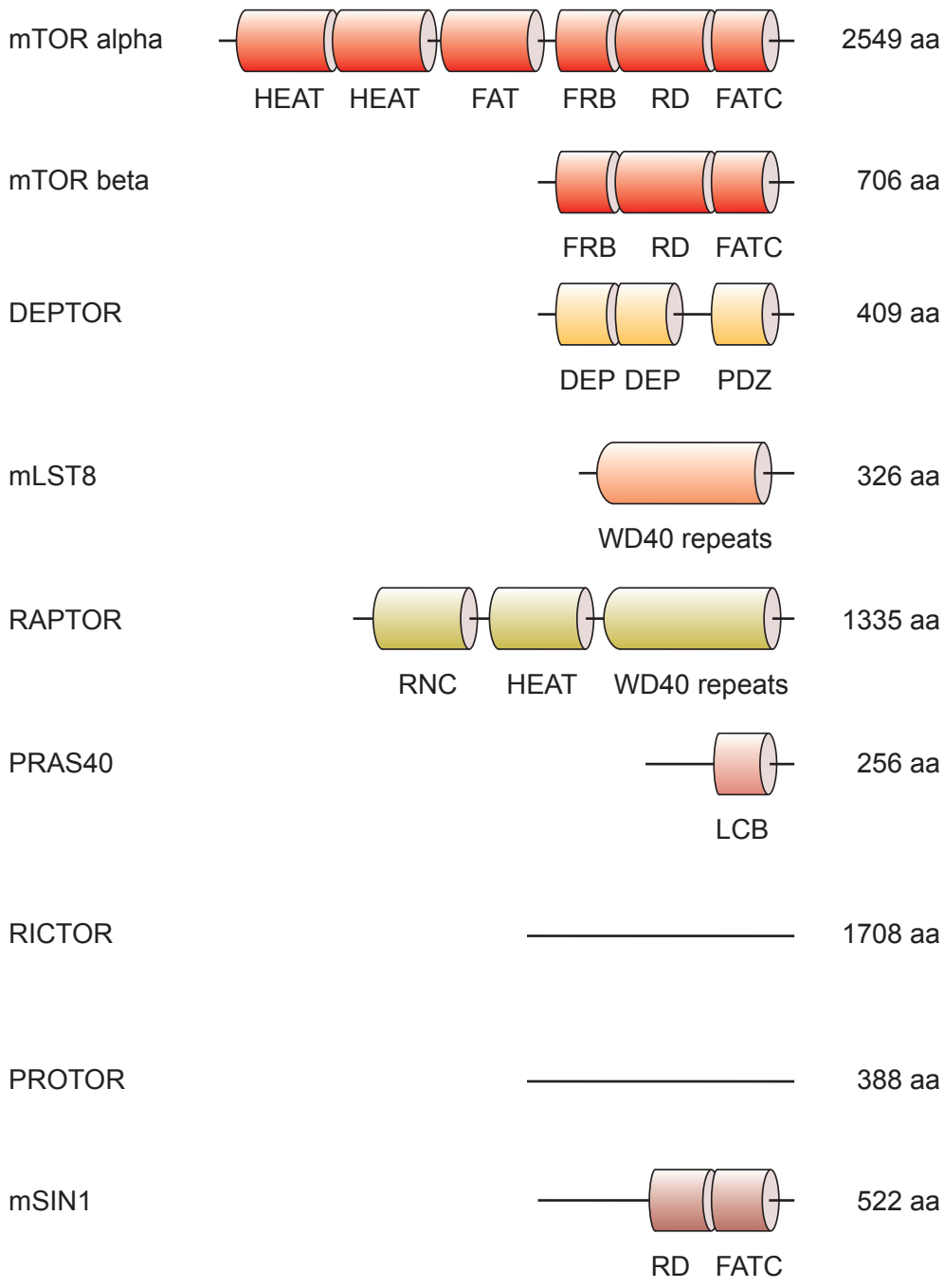


Figure 11: Protein domains of mTOR and mTORC1/2

The subunits that exist in mTORC1 and mTORC2 complexes and the protein domains that they contain.

"Huntingtin, elongation factor 3, protein phosphatase 2A and TOR1" (HEAT) motifs; FRAP, ATM and TRRAP (FAT) domain; FKBP12-rapamycin-binding (FRB) domain; repressor domain (RD domain); FAT domain at the *C* terminus (FATC); Dishevelled, EGL-10 and pleckstrin (DEP); postsynaptic density of 95 kDa, Discs large and zonula occludens" (PDZ) domain; tryptophan-aspartic acid (WD) dipeptide.

III. c) <u>The structure of mTOR complexes</u>

1. mTORC1/2 common regulatory subunits

The complexes mTORC1 and mTORC2 have two regulatory subunits in common **(Fig. 10)**.

- DEP domain-containing mTOR-interacting protein (DEPTOR).

The protein DEPTOR contains two "Dishevelled, EGL-10 and pleckstrin" (DEP) domains, followed by a "postsynaptic density of 95 kDa, Discs large and zonula occludens" (PDZ) domain. DEPTOR regulates mTOR activity by a complex feedback loop mechanism involving Akt [263] **(Fig. 11)**.

- Mammalian lethal with SEC13 protein 8 (mLST8).

mLST8, (also known as GβL), is highly conserved and contains seven WD40 domains forming a β-propeller to mediate protein–protein interactions **(Fig. 11)**.

mLST8 function is unclear, its ablation in mice does not affect mTORC1 signalling toward known substrates, but mLST8 is required to maintain mTOR-RICTOR interaction and for mTORC2 activity [264]. Another study suggests that mLST8 is required to maintain a nutrient and rapamycin-sensitive interaction between mTOR-RAPTOR (Regulatory Associated Protein Of mTOR) [265].

2. mTORC1 associated proteins

- RAPTOR

RAPTOR contains RAPTOR *N*-terminal conserved (RNC) motif, several HEAT repeats, and seven WD40 domains, possibly forming the β-propeller **(Fig. 11)**. RAPTOR has the role of a scaffold protein: it regulates the assembly, localisation and substrate binding of mTORC1 [266-269].

- PRAS40

PRAS40 (Proline Rich Akt Substrate of 40 kDa), also known as AKT1S1, contains a conserved Leucine charged domain (LCD) and is phosphorylated by Akt **(Fig. 11)**.

It associates with mTORC1 through RAPTOR and regulates mTORC1 negatively [270].

- Tel2 and Tti1

In 2010, it was suggested that telomerase maintenance 2 (Tel2) and Tel2 interacting protein 1 homolog (Tti1) bind mTOR complexes and stabilise them [271]

3. mTORC2 associated proteins

- mSIN1

mSIN1 (Mammalian stress-activated map kinase-interacting protein 1), also known as MAPKAP1, contains a Ras binding domain (RBD) and a Pleckstrin Homology (PH) domain, which possibly interact with phospholipids **(Fig. 11)**.

mSIN1 is required for mTORC2 activity [272, 273].

- RICTOR

RICTOR (rapamycin-insensitive companion of mTOR) has some regions conserved, but no functional domains have been identified.

Like RAPTOR for mTORC1, RICTOR plays the role of a scaffolding protein for mTORC2, regulating its assembly, localisation and substrates binding [260].

- PROTOR

PROTOR, which has no identified domains, binds RICTOR but it is not required for mTORC2 assembly [274]. PROTOR is specifically involved in the phosphorylation of the mTORC2's substrate serum- and glucocorticoid-induced protein kinase 1 (SGK1) [275].

- HSP70

HSP70 was reported to be required for mTORC2 formation, by binding and stabilising RICTOR [276].

4. Mechanism of action of rapamycin

Rapamycin inhibits specifically the assembly and the activity of mTORC1 by disrupting RAPTOR binding, with no effect upon acute treatment on mTORC2 [267, 277-280]. However, prolonged rapamycin treatment inhibits mTORC2 assembly, possibly by depleting mTOR availability to constitute the complex [281].

III. d) mTORC1/2 regulation

Although mTOR complexes have some common regulators, their mechanisms of action are divergent and have been far better characterised for mTORC1 than for mTORC2 [282, 283]. Common positive regulators of mTORC1/2 activation include growth factors and nutrients. Importantly, the regulation of both mTORC1 and mTORC2 depends on the location of the complexes, which is discussed in **III. f) mTOR spatial signalling**.

1. mTORC1 upregulation

mTORC1 is a key target of various signalling pathways. Nutrients activate directly mTORC1, while growth factors, inflammation and Wnt signalling pathway inhibit mTORC1's inhibitor Tuberous Sclerosis 1 (TSC1) / Tuberin (TSC2) heterodimer, thus resulting in mTORC1 activation [284-289]. TSC1/2 complex plays the role of a GTPase Activating Proteins (GAP) for the Ras homolog enriched in brain (Rheb) GTPase, localised in lysosomes. Rheb-GTP interacts with mTOR in mTORC1, and is responsible for the activation of mTOR kinase activity [290-294] **(Fig. 12)**.

- *Nutrients*

mTORC1 is considered to be a sensor for nutrients. The amino acids leucine and arginine are particularly required to allow mTORC1 activation [295, 296]. Amino acids, by a mechanism poorly understood, regulate the GEF activity of a complex called Ragulator through the vacuolar H+adenoside triphosphate-ATPase (v-ATPase) [297], both the Ragulator complex and v-ATPase being localised at the lysosomal surface [298] **(Fig.**

12). In turn, Ragulator loads the Rag GTPases RagA or RagB with GTP. There are four Rag GTPases, RagA, RagB, RagC, and Rag D. The RagA or RagB forms a heterodimer with RagC or RagD. Within the heterodimer, Rag proteins have opposite GTP/GDP states. The GTP loading on RagA/B involves the GDP loading on RagC/D. In the state RagA/B-GTP and RagC/D-GDP, Rag proteins interact with RAPTOR [299], relocalise mTORC1 into a Ragulator-Rag complex at the lysosomal surface and allow mTORC1 activation [300]. The mechanism of mTORC1 regulation controlled by nutrients is independent of others mechanisms of regulation induced by other cellular inputs discovered so far [301].

Interestingly, Vps34 knock down (PI3K class III) prevents mTOR activation stimulated by amino acids [211], and overexpression of Vps34 increases the activation of some mTORC1 effectors S6K1 and 4E-BP1 **(see III. d) mTORC1/2 functions and downstream pathways involved)** [212]. Amino acids increase intracellular calcium, which stabilise the binding of calcium/calmodulin to Vps34 and is required for the full activation of mTORC1 in a Vps34 dependent manner [302] **(Fig. 12)**.

- *Growth factors*

Growth factors, like insulin, stimulate the PI3K/Akt and MAPK/RSK signalling pathway, which inhibit the TSC1/2 complex, leading as mentioned above, to Rheb activation and subsequent mTORC1 activation [284-289] **(Fig. 12)**.

In parallel, and independently of TSC1/2 regulation, Akt can directly inhibit the mTORC1 inhibitor PRAS40 [270, 303-305] **(Fig. 12)**.

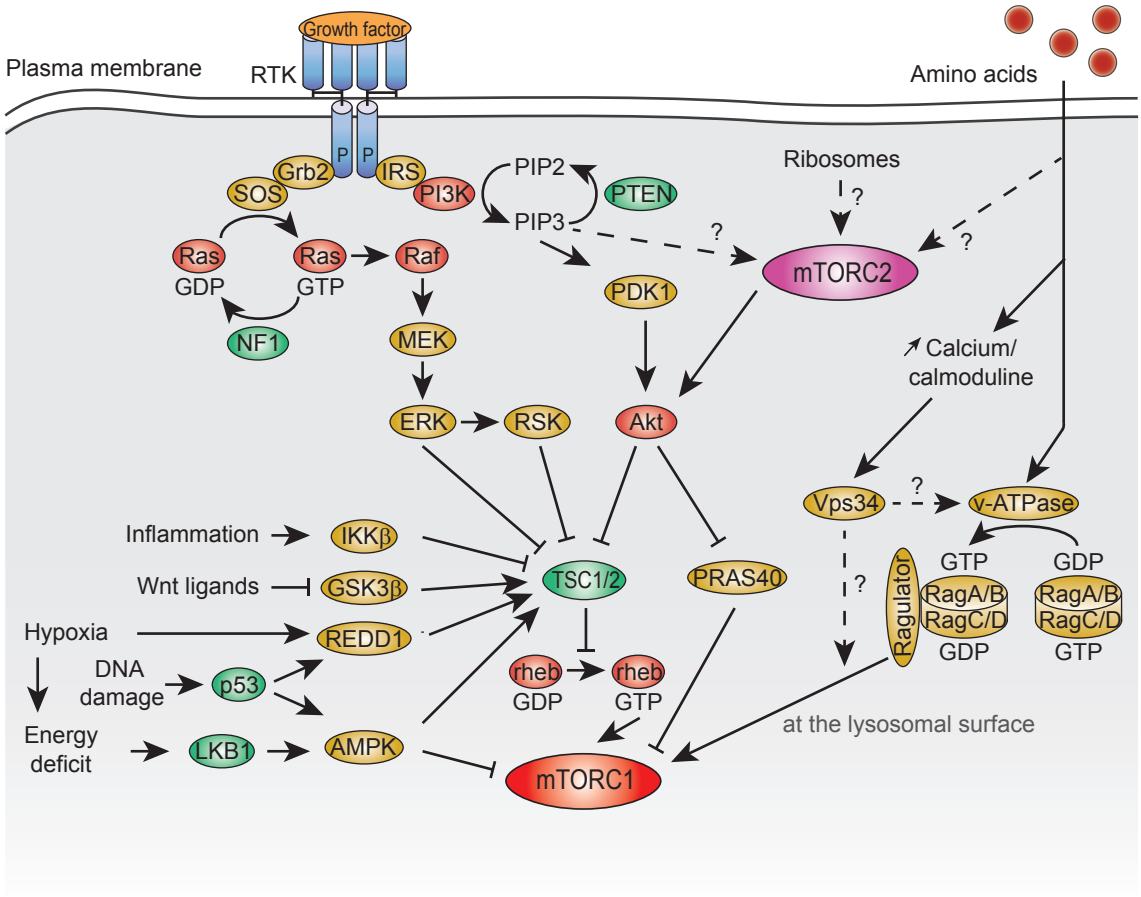


Figure 12: mTORC1/2 upstream signalling

mTOR complexes are regulated by multiple signalling pathways, downstream of growth factors, amino acids, stress and inflammation.

Receptor Tyrosine Kinase (RTK); Phosphatidylinositol 3-Kinase (PI3K); Insulin Receptor Substrate (IRS); Growth factor receptor bound protein 2 (Grb2); Son Of Sevenless (SOS); Phosphatidylinositol-4,5-bisPhosphate (PIP2); Phosphatidylinositol-3,4,5-triPhosphate (PIP3); Phosphatase and Tensin homologue deleted on chromosome 10 (PTEN); Phosphoinositide-dependent kinase-1 (PDK1); Guanosine triphosphate (GTP); Guanosine diphosphate (GDP); Extracellular signal-Regulated Kinase (ERK); Ribosomal S6 Kinase (RSK); mechanistic Target of Rapamycin complex 1/2 (mTORC1/2); Tuberous Sclerosis 1/2 (TSC1/2); vacuolar H+adenoside triphosphate-ATPase (v-ATPase); Vacuolar protein sorting 34 (Vps34); Proline Rich Akt Substrate of 40 kDa (PRAS40); Ras homolog enriched in brain (Rheb); : Glycogen Synthase Kinase 3 (GSK3) beta; IkappaB kinase beta (IKKbeta); 5' Adenosine Monophosphate-activated Protein Kinase (AMPK).

- *Inflammation*

IkappaB kinase beta (IKKbeta), effector of the proinflammatory cytokine Tumour Necrosis Factor alpha (TNFalpha), interacts with TSC1, consequently inhibiting TSC1/2, leading to mTORC1 activation [306] **(Fig. 12)**.

- *Wnt signalling pathway*

Another signalling pathway that regulates mTORC1 activity by inhibiting TSC1/2 is the Wnt signalling pathway. Wnt (important for cell growth, proliferation and development) inhibits Glycogen Synthase Kinase 3 beta (GSK3 beta), which when active, stimulates TSC2 activation [307].

2. mTORC1 downregulation

Various cellular stresses such as hypoxia, energy deficit and DNA damage induce mTORC1 inhibition **(Fig. 12)**.

- *Hypoxia*

Hypoxia leads to the expression of "regulated in development and DNA damage response 1" (REDD1), which promotes TSC2 activation and mTORC1 inhibition [308-310].

- Energy deficit

Energy deficit, also induced by hypoxia, stimulates the human tumour suppressor LKB1 responsible for 5' Adenosine Monophosphate-activated Protein Kinase (AMPK)

activation [311]. AMPK is considered to be a key metabolic sensor [311] and its activation results in mTORC1 inhibition, either indirectly by increasing TSC1/2 GAP activity for Rheb [312], or directly by phosphorylating RAPTOR which is then bound by 14-3-3 protein and induces allosteric inhibition of mTORC1 [268].

- DNA damage

In the case of DNA damage, p53 leads to mTORC1 down-regulation through both REDD1 and AMPK signalling, similarly as describe above [313-315] **(Fig. 12)**.

3. mTORC2 regulation

As mentioned earlier, mTORC1 and mTORC2 seem to share some regulators that act differently according to the complex considered **(Fig. 12)**.

- Growth factors

Growth factors like insulin stimulate mTORC2 activation possibly through Phosphatidylinositol-3,4,5-triphosphate (PIP3) [316], which induces the activation of mTORC2 substrates [134, 272, 317, 318]. The mechanism by which PIP3 contributes to mTORC2 activation is unknown. The TSC1/2 complex, which down-regulates mTORC1, has an opposite effect on mTORC2. TSC1/2 interacts directly with RICTOR and activates mTORC2 independently of the TSC1/2 GAP activity towards Rheb and mTORC1 [319, 320].

One characteristic of mTORC2 activation is its requirement for mature ribosomes. Upon insulin stimulation, ribosomes bind and activate mTORC2. The knock-down of

the ribosome maturation factor mNIP7 reduces mTORC2 activity and signalling [321]. How ribosomes activate mTORC2 through their association remains to be determined **(Fig. 12)**.

- Amino acids

Stimulation of starved cells with amino acids leads to mTORC2 substrates' phosphorylation [322], and leucine stimulation increases mTORC2 activity [323], indicating a possible regulation of mTORC2 by nutrients **(Fig. 12)**.

III. e) mTORC1/2 functions and downstream pathways involved

mTOR has an essential role in development and metabolism. Double knock-out of mTOR in mice is embryonically lethal at E5.5-6.5 stage. The cell proliferation and the gastrulation were impaired [324, 325]. mTOR conditional knock-out model mice presented severe myopathy and premature death due to metabolism impairment [326], [327].

Each complex, mTORC1 and mTORC2, possesses specific effectors involved in distinct cell functions. As for the upstream signals, the downstream signals are better characterised for mTORC1 than mTORC2. Interestingly, the splicing isoform mTORbeta seems to have a distinct role as compared to mTORalpha.

1. mTORC1

Due to the abundance of mTORC1 upstream signals/regulators, and its role as a nutrient sensor, mTORC1 controls many essential cell functions.

- Protein synthesis

mTORC1 is the central regulator of protein synthesis in cells, and this has been widely described. When inhibited with selective inhibitors such as Torin-1, protein synthesis is repressed, resulting in the cell cycle arrest in G1, cell proliferation inhibition, and apoptosis activation **(see below Cell survival)**. The main proteins described to be involved downstream of mTORC1 to regulate protein synthesis are (1) the ribosomal protein S6 kinase (S6K) and (2) the eukaryotic translation Initiation Factor 4E (eIF4E)-Binding Protein 1 (4E-BP1) [328, 329].

- S6K

S6K belongs to the protein kinase A/protein kinase G/protein kinase C (AGC) group serine/threonine protein kinases (as well as Akt), and includes 2 members, S6K1 and S6K2, each comprising several isoforms [330, 331]. The isoform the most studied is the p70 ribosomal S6 kinase 1 (p70S6K1), which has been described to regulate initiation and elongation factors **(Fig. 13A, Appendix 2 *p.243* for the description of the steps of protein synthesis regulated by S6K)**.

S6K is well inhibited by rapamycin, however S6K is involved in a negative feedback loop downregulating IRS, therefore S6K inhibition or silencing relieves this negative feedback loop and results in PI3K pathway upregulation. For this reason S6K is becoming a target of interest for cancer therapy **(see III. g) Targeting mTOR pathway in cancer)**.

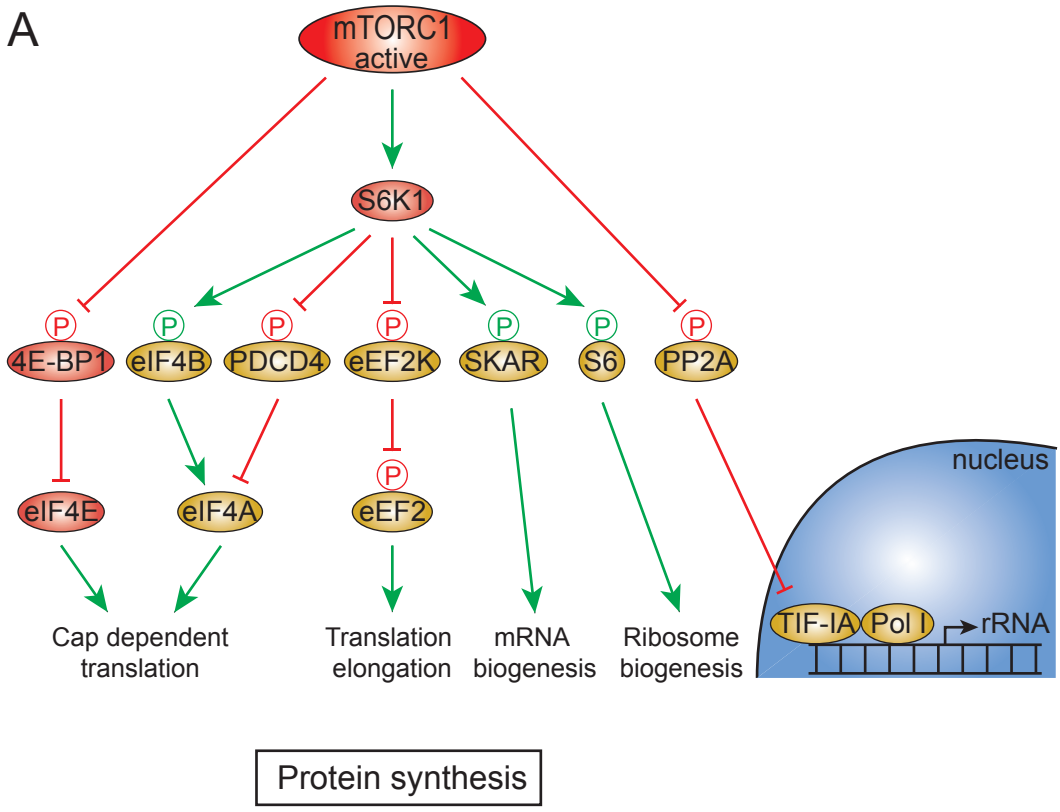


Figure 13: Processes regulated by mTORC1

A) Downstream effectors of mTORC1 involved in protein synthesis.

Mechanistic Target of Rapamycin complex 1 (mTORC1); ribosomal protein S6 kinase (S6K)1; eukaryotic translation Initiation Factor (eIF)4E -Binding Protein 1 (4E-BP1); programed cell death protein 4 (PDCD4); eEF: eukaryotic Elongation Factor (eEF); 40S ribosomal S6 protein (S6); Protein Phosphatase 2A (PP2A); Transcription Initiation Factor-IA (TIF-IA); polymerase (Pol)1.

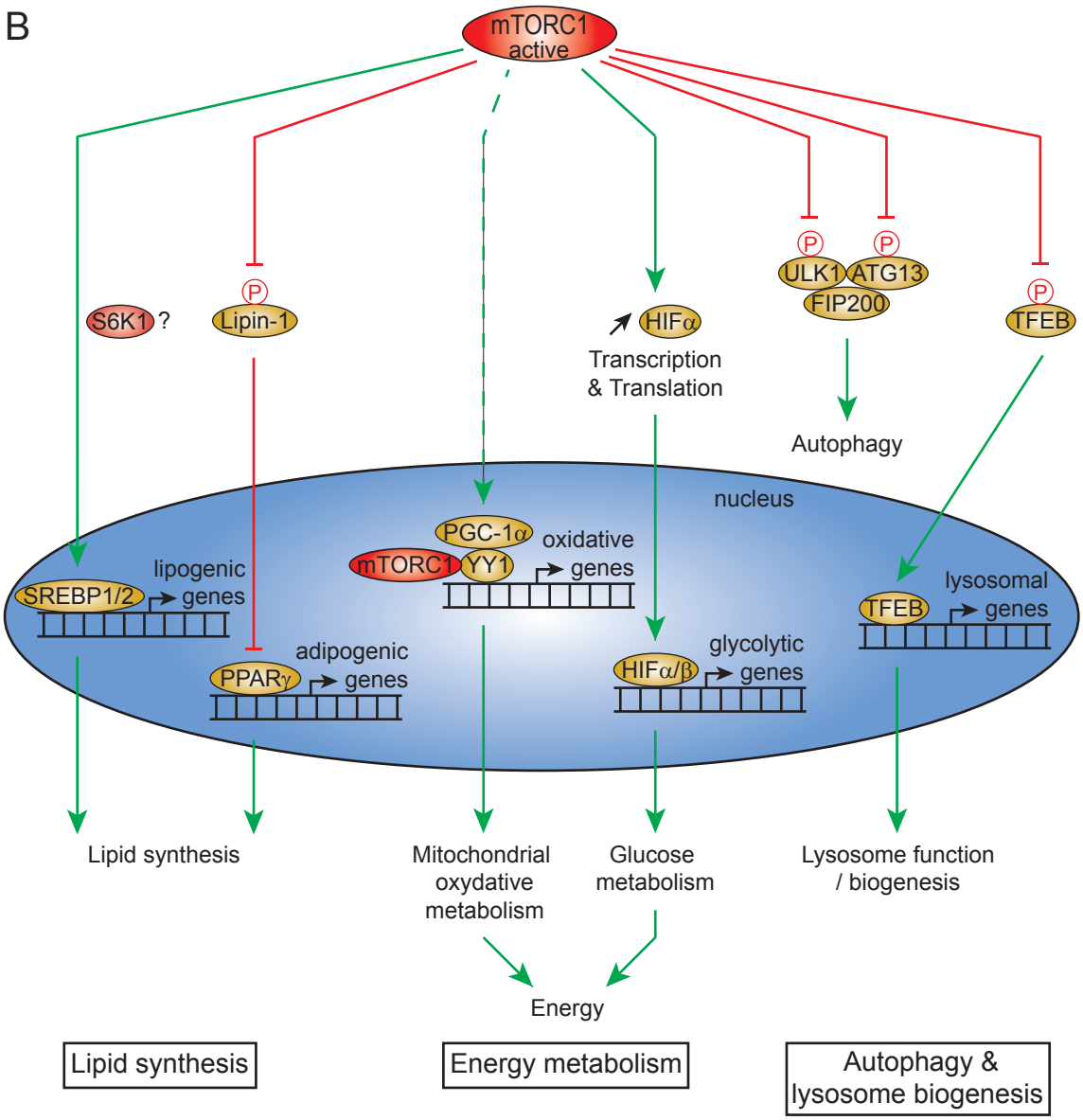


Figure 13: Processes regulated by mTORC1

B) Downstream effectors of mTORC1 involved in lipid synthesis, energy metabolism, autophagy and lysosome biogenesis.

Mechanistic Target of Rapamycin complex 1 (mTORC1); ribosomal protein S6 kinase (S6K)1; Sterol Regulatory Element Binding Protein 1/2 (SREBP1/2); peroxisome proliferator-activated receptor (PPAR) gamma; PPARgamma coactivator-1alpha (PGC-1alpha); Ying-Yang 1 (YY1); Hypoxia Inducible Factor 1 (HIF) alpha/beta; Unc-51-Like Kinase 1 (ULK1); Autophagy-related Gene 13 (ATG13); : Family-Interacting Protein of 200 kDa (FIP200); Transcription Factor EB (TFEB).

- 4E-BP1

mTORC1 substrate 4E-BP1 is a translational regulator [332] **(Fig. 13A)**. Once mTORC1 phosphorylates and inhibits 4E-BP1, the eukaryotic translation Initiation Factor 4E (eIF4E) can then induce initiation of translation [333, 334]. Interestingly, it has been demonstrated recently that rapamycin treatment has only a partial effect on dephosphorylating 4E-BP1 [335], which possibly justify the failure of clinical trials **(see III. g) Targeting mTOR pathway in cancer).**

- Lipid synthesis

Lipid synthesis regulation is required for cell growth and proliferation to generate membranes. mTORC1 positively regulates the expression of genes encoding for proteins involved in lipid and cholesterol synthesis such as the transcription factors Sterol Regulatory Element Binding Protein 1/2 (SREBP1/2). Upon insulin treatment or sterol starvation, the active forms of SREBP1/2 travel to the nucleus to activate transcription. In some cell lines, this has been shown to be regulated by S6K [336-338]. Another transcription factor regulated by activated mTORC1 is the peroxisome proliferator-activated receptor-gamma (PPARgamma), which is important for adipogenesis [339, 340] **(Fig. 13B)**.

- Energy metabolism

mTORC1 regulates the production of energy by upregulating the mitochondrial metabolism [341-343]. Additionally, in hypoxic conditions, mTORC1 promotes ATP production by activating the Hypoxia Inducible Factor 1 alpha (HIF-alpha) [336, 344-346],

which promotes, with HIF-beta, the metabolism of glucose (as well as angiogenesis and erythropoiesis) **(Fig. 13B)**.

- Autophagy and lysosomes biogenesis

Autophagy is a mechanism by which cells degrade cellular components (including damaged or unnecessary organelles), and compounds are recycled. In the case of cellular starvation, autophagy allows cells to maintain anabolic processes like protein synthesis and energy production. Firstly, cellular components are sequestrated in autophagosomes, and secondly the autophagosomes fuse with lysosomes and their content get degraded. mTORC1 activation reduces autophagy, and conversely, mTORC1 inhibition increases autophagy [347-350]. Moreover, mTORC1 also regulates lysosome biogenesis [351-353] **(Fig. 13B)**. Thanks to these mechanisms, cell starvation inhibits mTORC1 activity and therefore mTORC1 negative regulation is prevented.

- Cell survival

In addition to their role in protein synthesis, S6K isoforms promote cell survival and this through different pathways. While upon Fibroblast Growth Factor-2 (FGF-2) stimulation, S6K2 was shown to protect cell from apoptosis [354] through forming a complex with PKCepsilon and B-raf, leading to the up-regulation of the anti-apoptotic proteins Bcl-XL and XIAP [355-358], upon IGF-1 stimulation, S6K1 phosphorylates the pro-apoptotic Bcl-2 Antagonist of cell Death (BAD) which is then unable to promote cell death [359].

Also, S6K1 contributes to cell survival by phosphorylating Mouse double minute 2 homolog (MDM2), the p53 ubiqutin ligase [360].

2. mTORC2

- Cell survival and metabolism

mTORC2 phosphorylates two substrates belonging to the AGC family serine/threonine protein kinases to induce cell survival: (1) Akt [134], (2) serum- and glucocorticoid-induced protein kinase 1 (SGK1) [361] **(Fig. 14)**.

- Akt

The best described mTORC2 substrate is Akt, which is known to be involved in numerous cell functions such as survival, metabolism, but also proliferation [362]. mTORC2 phosphorylates Akt on the serine 473 [134]. To be fully active, Akt also requires phosphorylation on threonine 308, mediated by PDK1, post activation by PI3K class I **(see II. c) PI3Ks substrates and products)**.

mTORC2 depletion affects the regulation of some Akt substrates, such as the transcription factors Forkhead box O1/3a (FoxO1/3a), without altering other substrates such as TSC2 and GSK3 beta [264, 350] **(Fig. 14)**.

- SGK1

mTORC2 seems fully responsible for SGK1 activation as mTORC2 depletion totally reduces SGK1 activity. SGK1 is involved in ion transport, cell proliferation and apoptosis [318]. Like Akt, SGK1 phosphorylates FoxO1 and Fox3a. Active FoxO1/3a upregulates the expression of genes involved in stress resistance, metabolism, cell-cycle arrest and apoptosis [363] **(Fig. 14)**.

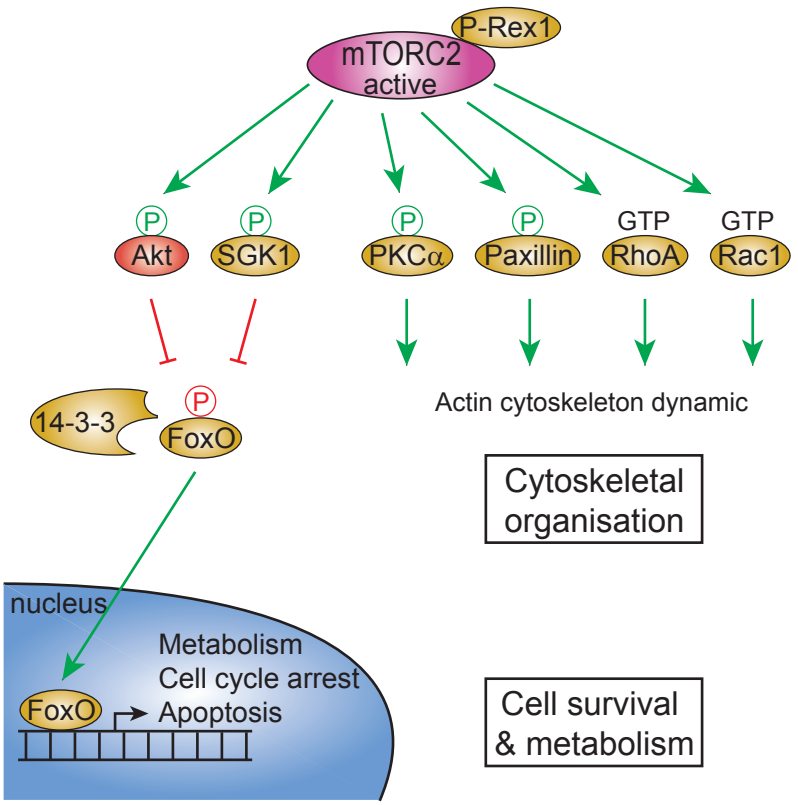


Figure 14: mTORC2 downstream signalling

Downstream effectors of mTORC2 involved in cytoskeletal organisation, cell survival and metabolism.

Mechanistic Target of Rapamycin complex 2 (mTORC2); serum- and glucocorticoid-induced protein kinase 1 (SGK1); Forkhead box O (FoxO); Protein Kinase C (PKC); Guanosine triphosphate (GTP).

- Cytoskeletal organisation

Finally, the third mTORC2 substrate belonging to the AGC family is the Protein Kinase C alpha (PKC alpha). In Hela cells PKC alpha is a mediator of actin reorganisation regulated by mTOR and RICTOR [260]. mTORC2 also regulates paxillin phosphorylation and localisation, and the GTP loading of the Rho GTPases RhoA and Rac1, leading to the regulation of the actin cytoskeleton [260, 350].

Rac1 activation relies on the binding of the GEF P-Rex1 to mTOR in mTORC2 [323] **(Fig. 14)**. How mTORC2 regulates the other proteins has not been identified yet.

3. mTORbeta vs mTORalpha

The specific role of the splice variant mTORbeta has been poorly studied so far. It has been shown that mTORbeta is able to phosphorylate similar downstream signalling molecules than the full length mTORalpha, including S6K1, 4E-BP1 and Akt [262]. Human Embryonic Kidney (HEK) cells overexpressing mTORbeta have increased cell proliferation as compared to HEK cells overexpressing mTORbeta kinase dead. Also in HEK cells, mTORbeta but not mTORalpha overexpression increases anchorage independent growth [262]. In nude mice, tumourigenesis is increased when mTORbeta is overexpressed in NIH3T3 cells as compared to parental NIH3T3 cells [262]. To do so, it was shown in HEK cells that mTORbeta shortens the cell cycle by reducing the time for G1 phase, with no effect on S and G2 phases [262].

III. f) <u>mTOR spatial signalling</u>

mTORC1/2 regulation depends on multiple cellular cues as mentioned earlier, but not only. mTORC1/2 signalling seem strongly dependent on their cellular location;

however, where do mTOR complexes form, how do they translocate, and their role in the distinct cellular localisation is poorly understood. mTORC1 needs to localise at the lysosomal surface to get activated and signal [299, 300], and mTORC2 at the mitochondria-associated endoplasmic reticulum [364]. In addition, mTORC1 has been observed in the nucleus, Golgi, endoplasmic reticulum (ER), mitochondria, stress granules, cytoplasm, and at the plasma membrane [364]. Interestingly, rapamycin treatment does not affect mTORC1 localisation [299], and kinase-dead mTORC1 is still localised at the lysosomal surface [365]. mTORC2 has also been localised to the ER, the mitochondrial associated ER membrane, in the nucleus and at the plasma membrane [364].

mTOR anchoring to the endoplasmic reticulum seems mediated by its HEAT motifs **(Fig. 11)**, as their deletion leads to mTOR mislocalisation in the cytoplasm from where it is unable to phosphorylate S6K [366].

mTORC1/2 localisation at the plasma membrane might be regulated by the Rho GTPase Rac1. Thus Rac1, independently of its GTP-loading, binds directly to mTOR. Mutation in the Rac1 binding domain to mTOR mislocalises mTOR complexes and reduces the phosphorylation of their substrates [367].

These studies highlight the importance of the localisation of mTOR complexes for mTORC1/2 signalling.

III. g) Targeting mTOR pathway in cancer

As mTOR role is essential for cell functions such as proliferation and cell growth, its deregulation leads to various pathologies, including cancer (tumour growth, angiogenesis and metastasis) [334, 368].

Many component of the mTOR signalling pathway can be deregulated or mutated leading to cancer. Proto-oncogenes upstream of mTOR include RTK, Raf, Ras, PI3K **(see II. f) PI3K in cancer)**, Akt, and Rheb **(Fig. 12)** and downstream of mTOR include 4E-BP1, and eIF4E **(Fig. 13A)**; tumour suppressors upstream of mTOR include PTEN, NF1, p53, LKB1 and TSC1/2 [368, 369] **(Fig. 12)**. The deregulation of one or multiple of these components results in mTOR induced cell proliferation, observed in many cancer types such as breast, colon, ovarian, renal, head and neck cancers [369].

There are two types of inhibitor targeting mTOR: allosteric and kinase inhibitors.

1. mTORC1 allosteric inhibitors

Rapamycin was the first mTORC1 allosteric inhibitor discovered **(see III. a) The mechanistic target of rapamycin)**. However, the poor solubility and pharmacokinetics properties of rapamycin led to clinical trial failures in the treatment of cancer patients. Therefore, rapamycin analogues called rapalogues were generated with better solubility and improved pharmacokinetics properties. The rapalogues temsirolimus and everolimus are both currently in clinical trials as single agent or combined with other drugs [see ClinicalTrials.gov (https://clinicaltrials.gov/)]. So far, rapalogue monotherapies has not met expectations. Several reasons could justify this failure: (1) rapamycin and rapalogues have no pro-apoptotic effect; (2) some mTORC1 effectors are poorly downregulated upon rapamycin treatment such as 4E-BP1 phosphorylation [335]; (3) S6K1 downregulation relieves S6K negative feedback loop on IRS which can result in PI3K signalling pathway upregulation **(see III. e) 1. mTORC1)** and increased Akt activity in patient tumours [370, 371]; (4) mTORC1 inhibition increases cytoprotective

autophagy which allows cells to escape cell death [372]. Thus, the combination of rapalogues with additional treatment seems more efficient. For example, the combined treatment of the rapalogue temsirolimus and the autophagy inhibitor hydroxychloroquine is more effective and stabilised cancer progression [373].

2. mTOR kinase inhibitors

PI3K and mTOR belong to the PIKK family. To avoid S6K feedback loop effect, inhibitors that target both PI3K and mTOR catalytic domains are being used. PI-103 was the first dual PI3K / mTOR inhibitor discovered. Dual PI3K and mTOR inhibitors are ATP-competitors, therefore the mechanism of inhibition is distinct from rapamycin and rapalogues. Numerous dual PI3K and mTOR inhibitors are currently in clinical trials **(see Table 5)**.

In an effort to design drugs less toxic than the dual PI3K and mTOR inhibitors, recent inhibitors target only mTOR kinase domain and inhibit both mTORC1 and mTORC2. The mTOR ATP-competitive inhibitors seem more efficient than rapamycin or rapalogues, and have potent pro-apoptotic and anti-proliferative effects **(see Table 6)**. However, cancer therapy with these drugs results in drug resistance. For example, RTK signalling, such as the Human Epidermal Growth Factor Receptor 3 (HER3), is increased, resulting in Akt re-activation [374]. In addition, a key role played by 4E-BP1 in the resistance against the ATP-competitive mTORC1/2 inhibitor PP242 has been reported in several studies **(Fig. 13A)**: (1) in colon carcinoma cell line, 4E-BP1 has been found poorly dephosphorylated after treatment; (2) in HegG2 and SK-HEP-1, both liver cancer cell lines, have 4E-BP1/eIF4E level of expression deregulated; with 4E-BP1 poorly expressed and/or eIF4E overexpressed [375].

Compound	Tumor type
BEZ-235	Pancreatic cancer Breast cancer Renal cancer Leukemias
BGT226	Advanced solid tumors Breast cancer
GSK2126458	Solid tumors, non-Hodgkin lymphoma Advanced solid tumors
GSK1059615	Breast cancer, non-Hodgkin lymphomas
PF05212384	Breast cancer, ovarian cancer Breast cancer, endometrial cancer, colorectal cancer
PF04691502	Endometrial cancer Breast cancer Advanced solid tumors
VS-5584	Solid tumors, non-Hodgkin lymphomas
XL765 (SAR245409)	Solid tumors, non-Hodgkin lymphomas Glioblastoma Non-Hodgkin lymphomas, leukemias
DS-7423	Colorectal cancer, endometrial cancer
GDC-0980	Renal cancer Endometrial cancer Advanced solid tumors, non-Hodgkin lymphomas

Table 5: Dual PI3K/mTOR inhibitors as monotherapies in clinical trials Summary of dual PI3K/mTOR inhibitors existing in clinical trials that are used as a monotherapy.

Modified from Chiarini, F. et al., Current treatment strategies for inhibiting mTOR in cancer, Trends in pharmacological sciences, 2014.

Compound	Tumor type
AZD-2014	Advanced solid tumors Prostatic cancer Metastatic clear cell renal cancer
CC-223	Glioblastoma, hepatocellular carcinoma, multiple myeloma
MLN0128 (formerly INK128)	Glioblastoma, gliosarcoma Multiple myeloma
OSI-027	Advanced solid tumors, non-Hodgkin lymphomas
CC-115	Glioblastoma, prostate cancer, Ewing's osteosarcoma, chronic lymphocytic leukemia
GDC-0349	Advanced/metastatic solid tumors, non-Hodgkin lymphomas

Table 6: mTOR ATP-competitive inhibitors as monotherapies in clinical trials Summary of mTOR ATP-competitive inhibitors existing in clinical trials that are used as a monotherapy.

Modified from Chiarini, F. et al., Current treatment strategies for inhibiting mTOR in cancer, *Trends in pharmacological sciences*, *2014.*

So far, mTOR inhibition as a monotherapy in cancer has not been successful but the numerous clinical trials and on-going scientific investigations hopefully will provide some answers in the coming years on how to improve mTOR inhibition for cancer therapies.

III. h) Targeting S6K in cancer

Due to the central role of S6K downstream of mTOR, there is a growing interest in targeting S6K in cancer and specific inhibitors are currently being developed. However, the feedback regulation occurring when S6K is inhibited by mTOR inhibitor might not be prevented with specific S6K inhibitors. Alternatively, the use of dual inhibitors of S6K and Akt (as they belong to the same family) might help to prevent the feedback regulation.

Interestingly, the S6K2 isoform, but not S6K1, might greatly contribute to tumourigenesis. S6K2 in complex with B-raf and PKCepsilon, mediated chemoresistance against Fibroblast Growth Factor 2 (FGF-2) inhibition in small lung cancer cells [355] and S6K2 seems to contribute to cell proliferation by shortening the cell cycle and allowing the cell entrance from G1 to the S-phase [376]. In normal tissues S6K2 expression is very low; however, S6K2 expression is often increased in tumour samples as compared to normal tissues [355 377-379], moreover constantly in a greater extent than S6K1. [380]. Strinkingly, S6K2 is found in the cytoplasm in normal breast cells while it is relocalised in the nucleus in breast cancer cells [377]; in endometrial cancer, the increase of S6K2 in the nucleus correlates with the tumour grade [378]. The compilation of these data, and the fact that knock-out mice for S6K2 have a normal phenotype, suggest a role for S6K2 only in transformed cells but not in normal cells. S6K2 would be therefore an

ideal target for cancer therapy. So far no specific inhibitors for S6K isoforms are available.

IV. Crosstalk between Met, PI3K, and mTOR signalling pathways

IV. a) Interaction of Met and PI3K class I: mechanisms of activation

Studies in 1990s aimed to identify Met effectors, their direct binding sites, and their molecular adaptors allowing indirect binding. Among the Met effectors discovered, there is PI3K [381]. In parallel, in 1990s, PI3K structure and lipid substrates/products were being more understood. Today three PI3K classes have been described.

The existing literature suggest that PI3K class I can act downstream of Met. However, so far, no studies have investigated which PI3K class I isoform(s) is / are downstream of Met and whether PI3K class II and III also are involved in Met signaling.

- *Direct interaction*

Met is able to bind directly to PI3K class I isoforms through its SH2 domains [31, 382], while a direct interaction between Met and PI3K class II or class III is unknown.

The regulatory subunit p85 co-immunoprecipitate with phosphorylated Met [381], and can bind synthetic Met phosphopeptide Y1307, Y1313, Y1349, and Y1356 *in vitro*. In cells, p85 presents a high affinity for the double binding site Y1349-Y1356 [382] **(Fig. 15)**.

- *Indirect interaction through Gab1*

Alternatively, upon Met activation, PI3K class I can bind Met indirectly through binding the Met adaptor Grb2-associated-binding protein 1 (Gab1) [31, 36] **(Fig. 15)**.

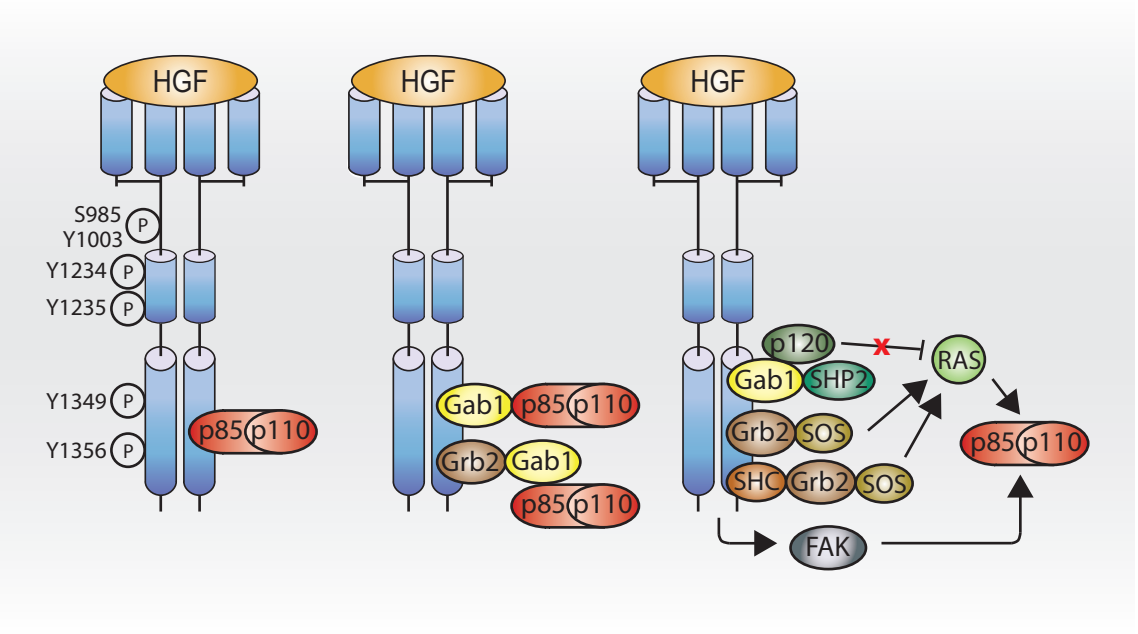


Figure 15: PI3K class I activation by Met

Met, directly or indirectly, through adaptors, scaffolding molecules or other downstream signalling molecules, regulates PI3K class I activation.

Hepatocyte Growth Factor (HGF); p110-p85 (PI3K); Growth factor receptor bound protein 2 (Grb2); Sarcoma (SRC) homology-2-containing (Shc); Grb2-associated binding protein 1 (Gab1); Src Homology domain-containing 5' Inositol Phosphatase 2 (SHP2); p120-ras-GTPase Activating Protein (p120); Son Of Sevenless (SOS); Focal Adhesion Kinase (FAK).

Gab1 contains a specific 13 amino acid binding site to Met called Met Binding Site (MBS) that can interact with Met docking tyrosines Y1349 and Y1356 in its multifunctional docking site [383, 384]. The binding of PI3K class I to Gab1 upon Met activation might be dependent on the integrin alpha3beta1. Upon HGF stimulation, Gab1 and PI3K co-immunoprecipitate with Met in immortalised epithelial cells generated from mice expressing WT integrin alpha3beta1, however this was not the case in epithelial cells from mice knocked out for alpha3beta1 [385].

- *Indirect interaction through Ras*

In addition, activated Met could signal to PI3K class I through the GTPase Ras [47, 386] **(Fig. 15)**, however Met signalling through Ras is better described to activate ERK pathway. Met activates Ras through several mechanisms:

1) the adaptor Grb2 (growth factor receptor-bound protein 2) binds to Met multifunctional docking site and associates with Son Of Sevenless (SOS), a Guanine nucleotide Exchange Factor (GEF) for Ras [31]

2) Grb2 associates indirectly to Met via SH2 domain-containing transforming protein (SHC) [33].

3) Grb2, bound directly to Met, can recruit the adaptor Gab1, which promotes Met dependent Ras activation by binding the tyrosine phosphatase SHP2 which, presumably like for EGFR, dephosphorylates the Gab1 binding site for p120-Ras-GAP leading to the recruitment and inhibition of p120-Ras-GAP, which, when active, inhibits Ras activation [37, 41].

The catalytic subunits of PI3K class I, p110 subunits, present a Ras binding domain (RBD) and it is well documented that Ras plays an important role in p110 subunits

activation [110], however Met dependent p110 subunits activation through Ras has not been shown yet.

- *Indirect interaction through FAK*

PI3K may be activated through Focal Adhesion Kinase (FAK) in mouse Inner Medullary Collecting Duct-3 (mIMCD-3) epithelial cells following HGF stimulation [387] **(Fig. 15)**.

IV. b) Met and PI3K effectors and their cell functions

PI3K, Akt, and mTOR are involved in various cell functions induced by Met. Cell survival and cell migration are the best described.

1. Cell survival

In primary embryonic hepatocytes, HGF treatment leads to the activation of Akt and mTOR, and the subsequent inhibition of apoptosis by activating the E3 ubiquitin-protein ligase MDM2, inhibitor of p53 **(Fig. 16)**. MDM2 activation is inhibited by LY294002, the Akt inhibitor A-443654, and rapamycin [388]. Moreover, GSK3beta (downstream of Akt) is phosphorylated, which results in its inhibition, leading to p53 inhibition as GSK3beta activation is involved in the activation of p53.

In addition, Akt was shown to inhibit Beclin (Bcl)-2 Antagonist of cell Death (BAD) upon HGF treatment, leading to inhibition of apoptosis, which is inhibited by wortmannin at low concentration [389].

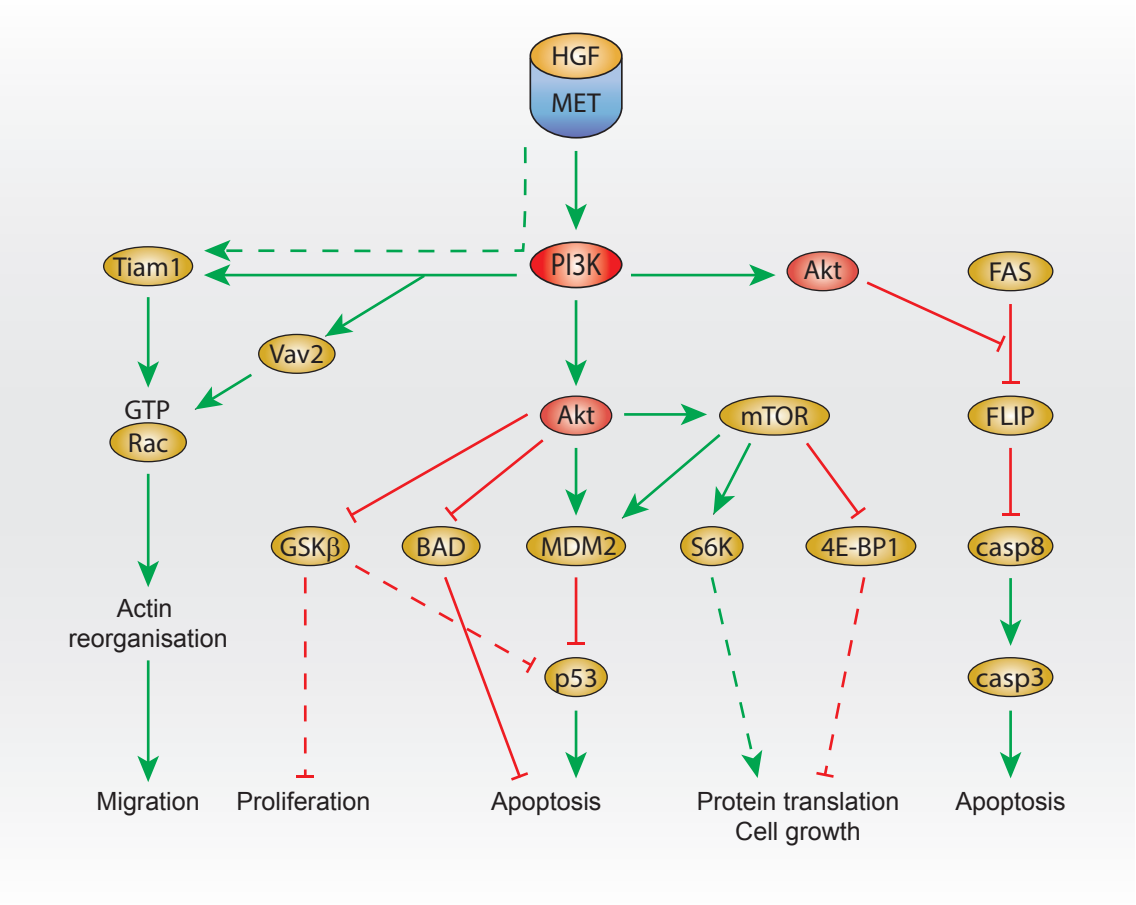


Figure 16: PI3K signalling activated by Met

Summary of Met/PI3K class I downstream signalling, that regulates migration, proliferation, apoptosis, protein translation and cell growth.

Hepatocyte Growth Factor (HGF); Phosphatidylinositol 3-Kinase (PI3K); Guanosine triphosphate (GTP); Glycogen Synthase Kinase 3 beta (GSK3 beta); Bcl-2 Antagonist of cell Death (BAD); Mouse double minute 2 homolog (MDM2); Mechanistic Target of Rapamycin (mTOR); eukaryotic translation Initiation Factor (eIF)4E -Binding Protein 1 (4E-BP1); FLICE-Like Inhibitory Protein (FLIP); caspase (casp) 3/8.

Another pathway that seems to be regulated by Met and PI3K and protects cells from apoptosis is the signalling pathway of FLICE-Like Inhibitory Protein (FLIP). FLIP inhibits the caspase-8 which is involved in apoptosis. Upstream of FLIP, Fas promotes FLIP degradation. In embryonic hepatocyte cells, HGF treatment prevents FLIP degradation, and this is inhibited by treatment with LY294002 or Akt inhibitor A-443654 [390].

The signalling pathway NFkappaB, which is involved in numerous cell functions (e.g. survival, proliferation, morphogenesis) is also regulated by Met through PI3K and Akt. In DU-145 prostate cancer cells and Madin–Darby canine kidney (MDCK) epithelial cells, HGF induces the activation of the transcription factor NFkappaB, and this is inhibited by the expression of a dominant negative form of Akt, or overexpression of PTEN. This was mediated by the serine/threonine p21-activated kinase 1 (Pak1), downstream of Akt, and led to the expression of the antiapoptotic factor TRAF-2 and cIAP [391].

Interestingly a study suggests distinct roles for PI3K depending on how it is activated by Met [50]. When Met activates PI3K through Met Grb2 binding domain, cell survival and Akt phosphorylation are induced. When a mutant of Gab1 that is unable to bind PI3K is overexpressed, there is no effect and Met is still able to induce a similar level of cell survival and Akt phosphorylation. However, when wild type Gab1 is overexpressed, cell survival and Akt are reduced. The authors concluded then that PI3K recruitment through Gab1 downstream of Met is responsible for the redirection of PI3K signalling for other function than cell survival.

2. Cell migration through Rac1

Studies using the specific panPI3K / mTOR inhibitor LY294002 reported that PI3K is required for Met dependent Rac activation [387, 392].

Rac is a member of the small GTPase Rho family (with Cdc42 and Rho) and has 3 isoforms: Rac1 (ubiquitously expressed), Rac2 (mainly expressed in hematopoietic cells) and Rac3 (highly expressed in the brain, and found in many other tissues).

Amongst Rac isoforms, only Rac1 has been described downstream of Met to mediate cell scattering through the promotion of actin reorganisation [393]. Rac binds GTP when activated, and GDP when inactivated. Switching between these two conformations is positively regulated by Guanine nucleotide Exchange Factors (GEFs) and negatively regulated by GTPase Activating Proteins (GAPs) and Guanine nucleotide Dissociation Inhibitors (GDIs). GEFs promote GDP dissociation and GTP association. Their activation can occur directly and indirectly by RTKs [394]. PI3K class I stimulates the production of PIP3, leading to binding and activation of a GEF, through its PH domain, resulting ultimately to an increase of Rac activity [395].

Interestingly, the GEF Tiam1 is activated by HGF treatment [396] and is required for HGF dependent Rac1 activation in HeLa cells [79]. Recently, Ménard *et al.* in our laboratory have shown that, in breast cancer cells, Met dependent Rac1 activation on the late endosome and subsequent cell migration require the GEF Vav2 and can be inhibited by LY294002. Moreover, downstream of Met, the binding of Vav2 to PIP3 is required for Rac1 activation **(Fig. 16)**.

3. Chromosome instability

PI3K and Akt might be involved in Met dependent chromosome instability. HeLa cells overexpressing M1268T Met have supernumerary centrosomes and aneuploidy, which is inhibited by LY294002, knock-down of Akt, or overexpression of PTEN [397].

IV. c) Spatial signalling of Met and PI3K pathway

Increasing amounts of studies demonstrate the importance of spatial signalling to regulate cell functions **(see I. l) Met spatio-temporal signalling, II. e) PI3Ks spatial signalling, III. f) mTOR spatial signalling)**, and some recent evidence seem to indicate that Met might activate the PI3K pathway from endosomes.

In 2010, Tan *et al.* have generated mice knock-out (KO) for Appl1 [398]. Appl1 binds to Rab5 on peripheral early endosomes. It has been shown that Appl1 is required in endosomes to induce cell survival in zebrafish. Rapidly, while endosome maturation progresses, Appl1 is replaced by Early Endosome Antigen 1 (EEA1). The conversion from Appl1 to EEA1 is promoted by Phosphatidylinositol-3,4,5-triPhosphate (PI3P) [399]. Murine embryonic fibroblast (MEF) cells were stimulated with several growth factors (insulin, EGF, and HGF) [398]. WT Appl1 and KO Appl1 MEF cells increased in the same proportion Akt phosphorylation when treated with insulin and EGF. However, Akt phosphorylation Met dependent was strikingly reduced in MEF cells KO Appl1 as compared to WT Appl1. This reduction was observed in both Akt phospho-sites: serine 473 and threonine 308. The remaining phosphorylation of Akt induced by Met in MEF cells KO Appl1 was further reduced by the knock down of Appl2. These results indicate that Met requires Appl proteins to be able to phosphorylate Akt. Moreover, due to

Appl1/2 location, and the fact that Appl proteins are effective only when located in endosomes, these results suggest that Met could activate Akt from Appl1 endosomes.

Another piece of evidence, suggesting that PI3K is activated by Met on endosomes, was provided by Ménard *et al.* in 2014. In MDA-MB-468 breast cancer cells, confocal live imaging detected the colocalisation of fluorescently labelled HGF with p85alpha-GFP (PI3K class I regulatory subunit) in perinuclear endosomes [78]. He also demonstrated that the above-described requirement of GEF Vav2 and subsequent Rac1 activation occurred in the perinuclear Rab7 positive endosome.

Together these studies suggest that Met could induce distinct cell functions through PI3K/Akt depending on the location of the signalling. Met may activate PI3K signalling pathway from Appl1 endosomes to induce cell survival, or from perinuclear endosomes and induce cell migration.

IV. d) Met and PI3K/Akt/mTOR pathway in cancer

As mentioned earlier **(see I. m) Met oncogenicity, II. f) PI3K in cancer, and III. g) Targeting mTOR pathway in cancer)**, extensive research is ongoing to test the possible benefit of targeting Met or PI3K/Akt/mTOR for cancer therapy. Interestingly, an increasing number of investigations report the deregulation of Met and PI3K/AKT/mTOR pathway simultaneously in various cancers, including in therapy resistance [400-404]. Due to the failure of monotherapies, mostly due to drug resistance due to cellular compensations, the combined targeting of Met and PI3K/Akt/mTOR is currently tested in clinical trials. However, ongoing research investigation is required to design / generate / improve these therapies and define / stratify the patients that will respond to the therapy.

AIM OF THE PROJECT

The receptor tyrosine kinase Met, overexpressed or mutated in cancer, plays a major role in cancer progression, it is associated with poor prognosis and represents an attractive target for cancer therapy.

PI3K, a lipid kinase involved in many cell functions and a major pathway downstream of receptor tyrosine kinases, plays an important role in promoting cancer cell growth and survival.

The aim of my PhD was to investigate whether and how the PI3K signalling pathway plays a role in cell transformation and tumourigenesis triggered by the M1268T Met mutant found in human cancer and to determine which isoform(s) are involved.

This Met mutant is constitutively activated, transforms cells *in vitro* and promotes tumourigenesis *in vivo*. Moreover, it is oncogenic not only because it is highly activated but also because it signals on endosomes.

In the first chapter, I validated the cell model and the published results in my hands. The cell model used consisted of NIH3T3 cells stably transfected to express mutant Met or Wild type Met. I then determined whether the cell transformation triggered by the M1268T Met mutant is dependent on PI3K activity using a pan-PI3K / mTOR inhibitor.

In the second and third chapter, I aimed to determine whether the PI3K isoforms, Akt, and /or mTOR are responsible for mutant Met dependent cell transformation (migration and anchorage independent growth / *in vivo* tumour growth), using specific inhibitors and siRNA knock-down experiments. I also aimed to determine where and how the PI3K/Akt/mTOR pathway promotes Met dependent cell transformation.

Some of the results obtained were compared to endogenous Met signalling in human cancer cell lines: U87MG glioblastoma cells, with Wild type Met that is constitutively activated due to an autocrine loop, and A549 lung cancer cells expressing Wild type Met that is acutely activated with exogenous HGF.

MATERIALS AND METHODS

I. Reagents

I. a) Antibodies

The following antibodies were used for western blotting:

Antibodies targeting phosphorylated Met (Y1234-Y1235) (rabbit polyclonal; 1:1000), phosphorylated Akt (Ser473) (rabbit polyclonal; 1:1000), Akt (rabbit polyclonal; 1:1000), phosphorylated p70 S6K (Thr389) (rabbit polyclonal; 1:1000), p70 S6K (rabbit polyclonal; 1:1000), p110 alpha (rabbit monoclonal; 1:1000) and Vps34 (rabbit polyclonal; 1:1000) were obtained from Cell Signalling. Antibodies against murine Met (B2) (mouse monoclonal; 1:200), p110 beta (rabbit polyclonal; 1:500), p110 delta (rabbit polyclonal; 1:1000) and HSC70 (mouse monoclonal; 1:5000) were bought from Santa Cruz Technologies. Anti-C2 alpha (mouse monoclonal; 1:1000) and anti-C2 beta (mouse monoclonal; 1:1000) antibodies were purchased from BD biosciences. The antibody anti-human Met (CVD13) (rabbit polyclonal; 1:1000), anti-tubulin (mouse monoclonal; 1:5000) were bought respectively from Invitrogen Life Technologies and Sigma Aldrich. Horseradish Peroxydase (HRP) linked secondary antibodies, sheep anti-mouse and donkey anti-rabbit (1:2000) were purchased from GE Healthcare Life Sciences (VWR International).

The following antibodies were used for immunofluorescence:

The antibody anti-Rac1 (mouse monoclonal; 1:100) was purchased from Merck Millipore, human and mouse anti-Met (goat polyclonal; 1;100) from R&D, anti-EEA1 (rabbit polyclonal; 1:100) from Santa Cruz Technologies, and anti-mTOR (7C10)

(rabbit monoclonal; 1:50) from Cell Signalling. F-actin was stained using Alexa Fluor®-546 phalloidin (Molecular probes, Life Technologies). Alexa Fluor®-488 coupled anti-goat IgG, (Molecular Probes, Life Technologies), Cy3-coupled donkey anti-rabbit or anti-mouse IgG and Cy5-coupled donkey anti-rabbit or anti-mouse (Jackson Immunoresearch) were used as secondary antibodies (1:500).

I. b) Drugs and Growth factor

Purified human recombinant HGF (R&D Systems) was used at 50 ng/mL unless otherwise stated. Met inhibitors PHA-665752 (100 nM) and PF-2341066 (100 nM) were kindly provided by Dr. James Christensen (Pfizer, La Jolla). Dynasore (80 µM) and cycloheximide (50 µg / mL) were bought from Sigma Aldrich. LY294002 (10 µM) was purchased from Calbiochem. The pharmaceutical inhibitors wortmannin (100 nM), A66 (500 nM), TGX221 (40 – 100 nM), IC87114 (1 µM), GDC0941 (100 nM – 10 µM), Akt (1 µM), rapamycin (2 nM), and NSC23766 (100 µM were purchased from Selleckchem. Stock dilutions of each were prepared in DMSO.

I. c) siRNAs

Listed below are the sequences of siRNAs used, provided from Dharmacon. The negative control used is AllStars Neg. Control siRNA (20 nmol) from Qiagen (no sequence provided).

Target	Sequence number	Sequence
PI3K - p110 alpha	8	GGAGAACCCUUAUGUGACA
PI3K - p110 beta	7	GAACGAAAUGGACCUUAUU
Vps34	8	CUAUGUACGUUCUUGAUAU
Appl1	4	UGAAUUGAGUGCAGCAACA
Rac1	1	GGACGAAGCTTGATCTTAG
	2	AGACGGAGCTGTTGGTAAA
	3	GATCGGTGCTGTCAAATAC
	4	GCAAAGTGGTATCCTGAAG
Met	1	GGACUUUGCUGGACAAUGA
	2	GAACAGCAGCUAAAUAUA
	3	GGGAAGAAGUGUUUAAUAU
	4	CCAGAGACAUGUACGAUAA

II. Cell culture

The mouse fibroblast NIH3T3 cell line expressing either murine wild-type (WT), M1268T, or D1246N Met [56], were a gift from Prof. G. Vande Woude [100]. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L of glucose and L-Glutamine, supplemented with 10% Donor Calf Serum (DCS, from Gibco, Life Technologies).

Cells were starved in DMEM supplemented with 1% DCS for 24 hrs prior to migration assays and serum starved with 0% DCS in DMEM for 1 hour when required for western blot analysis.

The A549 lung cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L of glucose and L-Glutamine, supplemented with 10% Foetal Bovine Serum (FBS, from Gibco, Life Technologies).

The U87MG brain cancer cells were cultured in MEM alpha containing 1 g/L of D-glucose and 292 mg/L L-Glutamine (from Gibco, Life Technologies), supplemented with 10% FBS.

A549 and U87MG cells were serum starved for 24 hrs in their medium with no serum for migration assays, western blots and immunofluorescence.

All cell lines were maintained in humidified incubators at 37°C and 8% CO_2 and were passaged every three days. During passaging or harvesting, cells were detached with a trypsin/EDTA solution (0.5%/0.2%). For long-term preservation, pelleted cells were resuspended in 60% complete medium, 30% serum, and 10% DMSO and frozen step-wise, first at -80°C overnight then placed at -196°C (liquid nitrogen). Cells were not used beyond passage 10 to ensure experimental consistency.

III. Cell transfection

NIH3T3 and U87MG cells were transfected with Amaxa® Nucleofector® technology following the manufacturer's instructions (Lonza). The kit "R" and the program of nucleofection "U-030" were used for NIH3T3 cells, and kit "T" and program "X-001" for U87MG. Transfected cells were harvested after 48 hrs.

IV. Cell morphology assay

50,000 cells were seeded per well of a 6-well plate in complete medium and grown for 24 hrs in order to attach. Then, cells were treated with DMSO or 100 nM of PHA-

665752. After 24 hrs of incubation, changes in morphology were observed by microscopy and pictures were taken.

V. Western Blotting

V. a) Cell treatment and preparation of total cellular proteins

Using 6-well plates, 100,000 cells/well were seeded and allowed to attach for 24 hrs in complete medium. Cells were treated for 1 hour unless otherwise stated. NIH3T3 cells were treated with Dynasore for 30 min. In A549 cells, a pre-treatment with Dynasore for 40 min was performed before HGF treatment at 50 ng/mL. Cells were washed with cold PBS (4°C), lysed and harvested in RIPA buffer containing 0.2 nM tyrosine phosphatase inhibitor NaOV, 200 µM of serine/threonine phosphatase inhibitor NaF and protease inhibitor cocktail (Calbiochem) at 1/100 dilution. Lysates were rotated/mixed for 30 min at 4°C, then centrifuged at 18,000*g* for 10 min at 4°C to pellet cellular membrane fragments. Alternatively, cells were lysed and harvested in 2X Sample buffer (Invitrogen, Lifer Technologies) containing 200 mM DTT.

V. b) Quantification of protein concentration

Protein concentrations were determined using the Bio-Rad Protein Assay Kit. Absorbances were measured at 650 nm using plate reader. Concentrations were calculated against BSA standards ranging in concentration from 0.1 to 2 mg/mL prepared in the RIPA/inhibitors and detergent as per manufacturer's instructions.

V. c) SDS PAGE

Equal quantities of total cellular proteins from each sample were mixed with 4X loading/sample buffer (Invitrogen, Life Technologies, N70007) which included 400mM of the reducing agent dithiothreitol (DTT), and then denatured at 95°C for 5 min. Samples were loaded either onto 4-12% gradient polyacrylamide gels or 10% acrylamide gels containing sodium dodecyl sulphate (SDS-PAGE), and resolved at 135V for 120 min at room temperature in either NuPAGE MES SDS Running Buffer (Invitrogen, Life Technologies) for gradient gels or Tris-Glycine-SDS buffer (Fisher Scientific) for 10% acrylamide gels. Protein sizes were determined by comparison to a set of standards, Molecular Weight Marker Full Range Rainbow™ (RPN800E Recombinant Protein, from GE Healthcare Life Sciences) and Novex Sharp Pre-stained Protein Standard (Invitrogen, Life Technologies).

V. d) Protein immunoblot

Proteins were transferred to a nitrocellulose membrane (Schleider & Schuell) at 30V for 3 hrs while at 4°C in a 1.5 M glycine, 200 mM Tris, 10% methanol transfer buffer. Ponceau S red solution was used to stain the membrane-bound proteins to check for equal protein loading and transfer efficiency. Non-specific binding sites were blocked with a 3% BSA, 0.1% Tween-20 Tris Buffer (TBS-T) for 1hr. Primary antibodies, were diluted in 3% BSA/TBS-T, and incubated overnight at 4°C. Excess antibody was removed by washing with TBS-T. Appropriate HRP-coupled secondary antibodies, diluted to 1/1000 in 3% BSA/TBS-T, were incubated for 1 hour at room temperature. Antibody binding was visualized using ECL reagent (GE Healthcare Life Sciences). Membranes were incubated for 1 min in ECL reagents A and B (1:1 ratio) then labelled

proteins were revealed by chemiluminescence exposure of photographic film for variable durations. Protein expression were analysed and compared by densitometry.

V. e) Densitometry

Density measurements of non-saturated bands were determined using ImageJ software to evaluate and compare the intensities of protein expression. Relative protein phosphorylation levels were obtained by normalising the level of protein phosphorylation to total protein level, while the expression of total proteins were normalised to tubulin levels from the same membrane.

VI. Transwell chemotactic migration assay

VI. a) Migration assay

NIH3T3 cells were seeded at 175,000 cells, A549 and U87MG cells at 300,000 cells in 10 cm plates and allowed to attach for 24 hrs at which point they were serum deprived for a further 24 hrs prior to the experiment.

Transwell filters (Corning, 8 µm pore size) were coated with fibronectin (Sigma Aldrich) for 30 min, which was the removed and they were allowed to dry for 45 min. Harvested cells were suspended in 0.1% BSA/serum free media at 45,000 cells/mL for NIH3T3 and U87MG cells, and 100,000 cells/mL for A549 cells, with the appropriate drug. 200 µL of the cell suspension (0.9 or $2x10^4$ cells) was added to the upper chamber of each transwell insert, while complete medium (with or without HGF at 50 ng/mL for the A549 cells) was added to the lower chamber to create a chemoattractant gradient.

Cells were allowed to migrate at 37°C for 90 min for NIH3T3 and U87MG cells, and for 3 hrs for A549 cells. After this time, cells on the top of the filters, which had not migrated, were removed with a wet cotton bud. The remaining migrated cells (on lower side of the membrane) were fixed with 4% paraformaldehyde (PFA) for 10 min and washed with PBS. Cell nuclei were stained with haematoxylin (Sigma Aldrich) for 20 min. Filters were washed 3 times in PBS, then removed from their insert and mounted onto a glass slide with Aqueous medium (Aquatex, VWR).

VI. b) Quantification

Migrated cells were counted by microscopy at x20 with a Zeiss Axiophot microscope 10 fields were counted per membrane and each condition was performed in triplicate in each experiment.

VII. Immunofluorescence and confocal microscopy

VII. a) Immunocytochemistry/Immunofluorescence

13 mm coverslips were placed into the wells of 24-well plates. 20,000 NIH3T3 cells were seeded on poly-L-Lysine pre-coated coverslips (BD BioCoat™, BD Biosciences) and allowed to attach for 24 hrs. 50,000 A549 cells were seeded on 13mm coverslips, allowed to attach for 24 hrs and starved for 24 hrs. NIH3T3 cells were then treated with appropriate drugs for 1hr, and A549 cells were pre-treated with dynasore for 40 min prior to HGF treatment at 50 ng/mL. Cells were fixed with 4% paraformaldehyde (PFA) in PBS. After a PBS wash, excess PFA was quenched by incubation with 50 mM of ammonium chloride (NH_4Cl). PBS containing 3% BSA, 0.1% Triton X-100 (Sigma Aldrich) was used for membrane permeabilisation and blocking non-specific sites.

Primary antibodies were diluted at 1/100 in 3% BSA/PBS and, incubated for 30 min, followed by washing with PBS. To visualise the actin cytoskeleton, cells were incubated with Alexa Fluor®-546 phalloidin (Molecular probes, Life Technologies) at 1/500 for 20 min during secondary incubation. Secondary antibodies, diluted at 1/500 in 3% BSA/PBS were incubated for 20 min, followed by sequential washing in PBS, then distilled water prior to mounting with the Prolong® Gold Antifade Mountant with DAPI (Life Technologies).

VII. b) Confocal microscopy

Immunofluorescence was detected and captured using a Zeiss LSM710 confocal microscope, fitted with a Plan-Apochromat oil immersion objective lens (63x/1.4). Alexa Fluor® 488 fluorochrome was excited at 488 nm wavelengh using an argon laser and emitted at 519 nm (green), while Cy3 and Alexa Fluor® 546 were excited at 543 nm by HeNe laser and emitted at 568 nm (red), and Cy5 was excited at 633 nm line by HeNe laser to emit at 672 nm (far red).

VII. c) Image analysis

Each image represents a single section of 0.7 μM thickness of the basal section of cells for phalloidin staining or the middle section of cells for Rac1, EEA1, Met and mTOR staining. Scale bars are 10 μM. The counting of cells with no visible stress fibres or with Rac1 or mTOR present at the plasma membrane were performed on at least 100 cells in each experiment.

VIII. Met internalisation biotinylation assay

400,000 cells were seeded in 10 cm plates for 24 hrs to allow attachment and cycloheximide was added to the medium for 5 hrs to inhibit protein synthesis prior the assay. Cells were then pre-treated at 37°C for 15 min with the appropriate drugs. Cells were then placed on ice, washed twice with PBS at 4°C, and incubated with NHS-SS-biotin (0.2 mg/mL) at 4°C for 45 min to label the cell surface. Cells were washed with PBS at 4°C and divided into 3 conditions:

- Total surface: Biotin at the cell surface is not cleaved to allow the analysis of the total amount of Met at the cell surface
- 0 min: biotin is cleaved to confirm the efficiency of the cleavage
- 15 min: cells are incubated at 37°C for 15 min with the appropriate drug to allow the internalisation of biotinylated cell surface proteins. Biotin is then cleaved from the remaining biotinylated proteins at the cell surface.

"TS" and "0 min" cells were kept at 4°C, while "15 min" were incubated at 37°C. After incubation of the cells, cells were returned to ice and washed twice with PBS at 4°C. Remaining biotin at the cell surface of "0 min" and "15 min" conditions were cleaved with 8 mL per plate of 180 mM MesNa at 4°C for 20 min while gently rocking. The Mesna (from Sigma Aldrich) solution also contained 50 mM Tris and 100 mM NaCl, and the pH was adjusted to 8.6. Residual MesNa was quenched by adding 1.6 mL 180 mM Iodoacetamide (Sigma Aldrich) per plate and left at 4°C while gently rocking for another 10 min. Cells were washed twice with PBS at 4°C, then lysed in RIPA buffer and scraped. Lysates were passed 3 times through a 27-gauge needle and centrifuged for 10 min at 13,000*g*. The protein concentration of the supernatant was determined using the Bio-Rad Protein Assay Kit **(see V. b) Quantifcation of protein concentration)**. Equal amounts of protein per condition was added to 100 µL of streptavidin-coated

agarose beads (Merck Millipore) in lysis buffer, which was then incubated on a rotating wheel at 4°C for 2 hrs. Samples were then centrifuged for 1 min, at 7000*g*, while at 4°C. The beads were washed 3 times in lysis buffer at 4°C after which sample buffer was then added to the beads and the proteins bound to the beads were dissociated by heating at 95°C. Proteins were then analysed by western blotting for Met.

After densitometry **(see V. e) Densitometry)**, the percentage of internalised Met was calculated as follow:

$$\text{Internalised Met} = \frac{\text{Met level at time 15} - \text{Met level at time 0}}{\text{Met level at the total surface}} \times 100$$

IX. Soft agar assay

500 NIH3T3, 1000 A549 and 10,000 U87MG cells in single cell suspensions were mixed with 5 ml 0.3% agarose (type IX-A, Sigma Aldrich)/medium per condition and placed on ice to solidify. After 20 min, 1 mL of complete medium was added to the top of the agar and cells were cultured for 5 days (NIH3T3 and U87MG) or 14 days (A549 cells). Then, treatment was started and the medium was changed daily. After 4 days (NIH3T3 and U87MG) or 6 days (A549 cells), pictures were taken of the whole wells using a Zeiss, Stemi SV11 microscope. The total area of the colonies was measured with ImageJ software.

Colonies were collected with a needle under the Stemi SV11 microscope and were transferred to an Eppendorf containing PBS at 4°C. Colonies were centrifuged gently (*3000g* for 5 min) and washed twice in PBS at 4°C. Colonies were then collected and lysed in RIPA and the proteins were quantified and analysed by western blotting.

X. Tumour growth assay

Female athymic nude mice (CD1 Nu/Nu, Charles River UK) at 4 - 6 weeks old were used in accordance with the United Kingdom Coordination Committee on Cancer Research guidelines and Home Office regulations.

A cell suspension of 5 x 10^6 cells/mL was prepared is PBS and 100 µL was injected subcutaneously into the right flank of each mouse. Tumours volumes were measured daily using callipers and the volumes were calculated as follow:

$$\text{Tumour volume} = \text{lengh} \times \text{width} \times \frac{\pi}{6}$$

Once the tumours reached the volume of 50 mm^3, they were treated with 100 µL of the appropriate treatment. DMSO was used as negative control. Reagents were applied topically on the skin over the tumour and surrounding area.

Once tumours reached 500 mm^3, mice were sacrificed humanely and the tumours were dissected and half of each tumour was preserved in formalin while the other half was snap frozen in liquid nitrogen.

XI. Statistical analysis

All experiments were performed at least three times and a Student's t-test was used to test the significance. A Two-tailed Student's t test was performed, unpaired between the different cells, paired between different conditions for a given cell line. Quantitative data of the indicated number of independent experiments ("n=" in figure legends) are expressed as mean ± SEM.

RESULTS - CHAPTER I

M1268T Met induces cell transformation which is inhibited by the pan-PI3K / mTOR pharmacological inhibitor, LY294002

In this Chapter I, I had two main objectives: (1) validate in my hands the published cell model in which cell transformation is triggered by the M1268T Met mutant; (2) determine whether the cell transformation triggered by the M1268T Met mutant is dependent on PI3K/Akt/mTOR signalling pathway using a pan-PI3K/mTOR inhibitor.

I. a) M1268T Met is constitutively activated

NIH3T3 cells, with a low level of endogenous Met, have been stably transfected to express murine Wild type (WT) Met or Met that is mutated on M1268T **(Fig. 1A)** [100].

In the presence of DMSO (negative control), while WT Met is poorly phosphorylated on the tyrosines 1234/5 in the kinase domain, M1268T Met is highly and significantly more phosphorylated (by 3.1 times, $p<0.05$) **(Fig. 1A, B)**. This occurs in basal conditions (no stimulation with HGF). Thus, M1268T Met is constitutively activated. The total quantity of Met (mature protein at 145 kDa and precursor at 170 kDa) is similar in both cell lines. PHA665752 treatment (Met kinase inhibitor) inhibits totally and significantly M1268T Met phosphorylation (by 91%, $p<0.05$). LY294002 treatment (PI3K class I/III, and mTOR inhibitor) has no effect on M1268T Met phosphorylation **(Fig. 1A, B)**. It was also noted that the levels of expression of WT and M1268T Met were similar and that PHA665752 or LY294002 had no effect on these **(Fig. 1C)**.

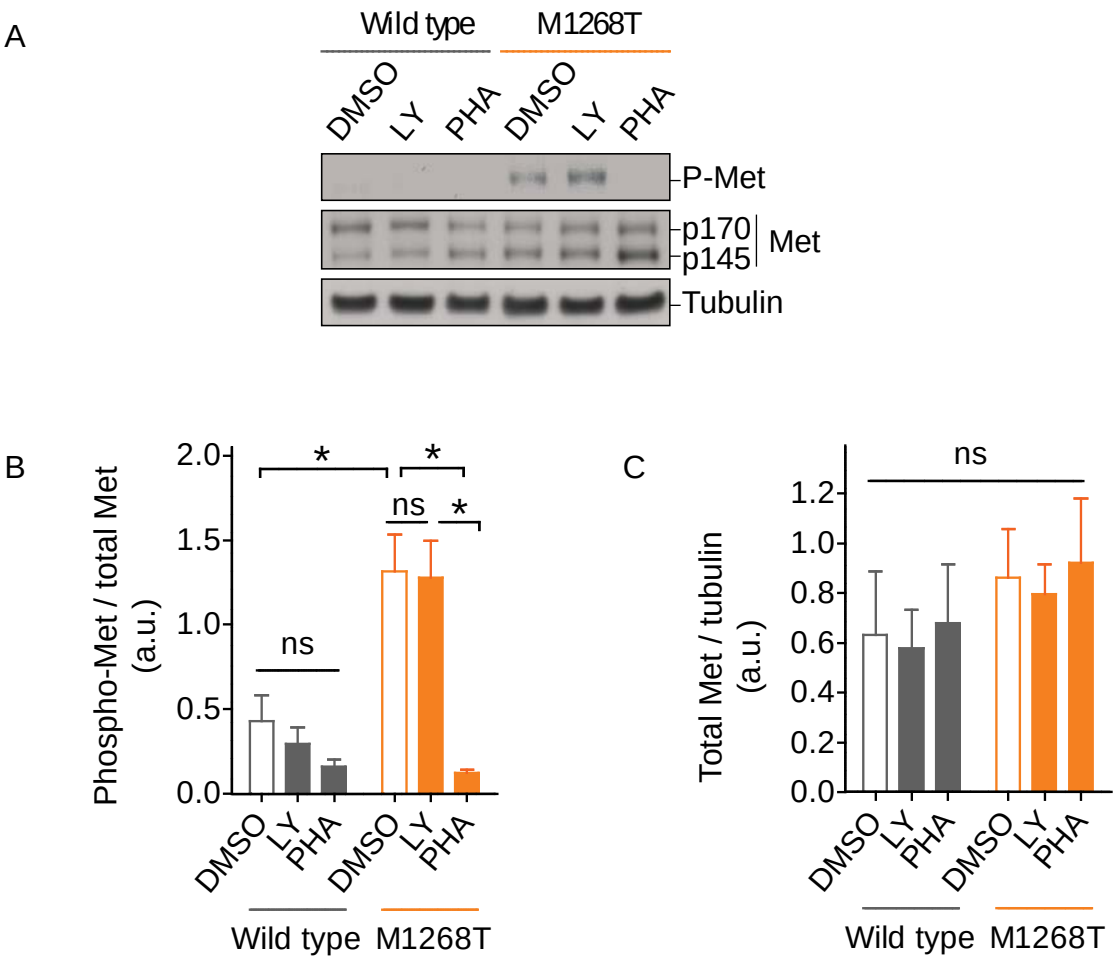


Figure 1: M1268T Met is highly phosphorylated

(A) Western blots for phosphorylated Met (Y1234/5) (P-Met), Met (p170, the precursor form and p145, the mature form of the beta chain) and tubulin were performed on NIH3T3 Wild type and M1268T Met expressing cells treated with DMSO, LY294002 (LY) (10 µM) or PHA665752 (PHA) (100 nM) for 1 hour. **(B,C)** Quantification of western blots, obtained by densitometry, of **(B)** phosphorylated Met (Y1234/5) (P-Met) normalised on total Met, or **(C)** the mature form of Met normalised on tubulin. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, **p<0.01.

I. b) M1268T Met promotes changes in cell morphology

It was observed that WT Met expressing cells are very well spread, corresponding to a better cell attachment to the plastic support, while M1268T Met expressing cells are rounded and less attached **(Fig. 2)**. This difference in attachment was observed during the day-to-day tissue culture: while WT Met expressing cells are very adherent to the support, M1268T Met expressing cells can be detached easily. Inhibiting Met phosphorylation, with the Met inhibitor PHA665752 for 24 hours, changes the morphology of the M1268T Met expressing cells to resemble the WT Met expressing cells, while having no effect on WT Met expressing cells. This indicates that the observed cell rounding is triggered by Met activity.

I. c) M1268T Met does not alter NIH3T3 cell growth as compared to WT Met

A growth curve was performed **(Fig. 3)**. 2,500 or 5,000 WT Met and M1268T Met expressing cells were plated in 24 well plates and the number of cells per well was counted every day for 5 days. The cell growth was proportional to the number of cells seeded for both the WT and M1268T Met expressing cells. As previously observed in the lab, no obvious difference between the growths of the two cell lines was observed in these conditions. However, the growth of these cell lines in soft agar was different **(see I. f LY294002 reduces M1268T Met dependent anchorage-independent growth)**.

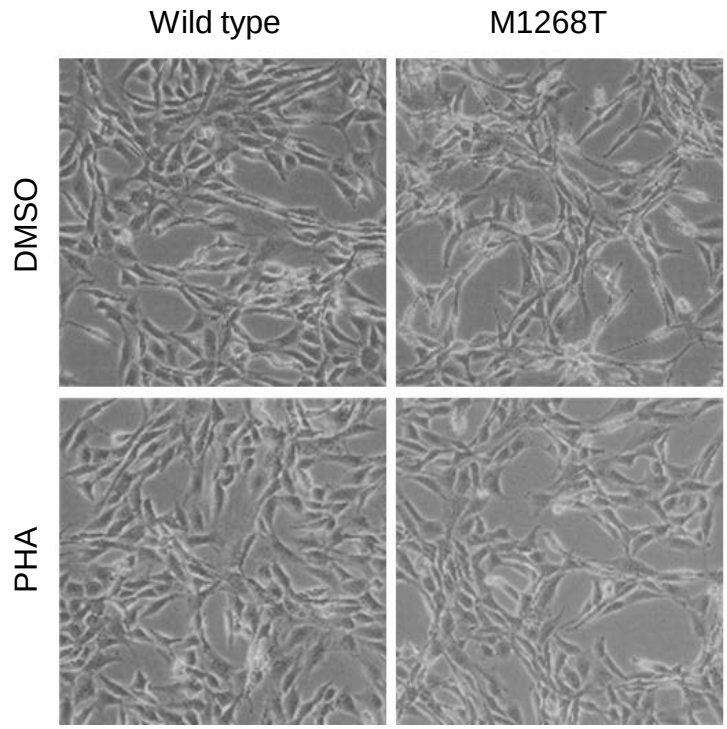


Figure 2: M1268T Met promotes changes in cell morphology

NIH3T3 Wild type and M1268T Met expressing cells were seeded on plastic, treated with DMSO or PHA-665752 (PHA) (100 nM) for 24 hours. Morphological changes were observed on an inverted microscope.

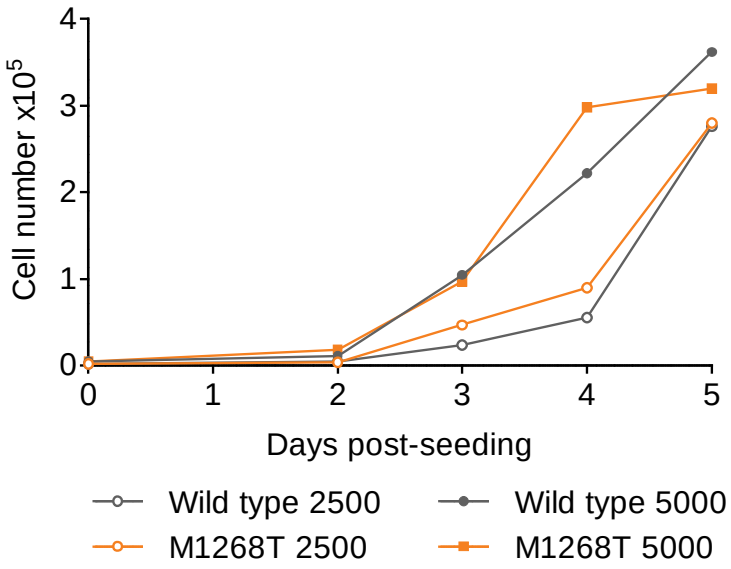


Figure 3: M1268T Met does not alter NIH3T3 cell growth as compared to Wild type Met

NIH3T3 Wild type and M1268T Met expressing cells were seeded at 2,500 or 5,000 cells per well in a 24 well plate and the number of cells per well was counted every day for 5 days (n=1).

I. d) LY294002 inhibits the migratory phenotype of M1268T Met cells

1. M1268T Met expressing cells exhibit an increased Rac1 localisation at the plasma membrane, which is decreased by LY294002

The Met receptor is well known to induce cell motility, and this has been shown to be dependent on the activity of the Rho GTPase, Rac1, in M1268T Met expressing cells [56]. I first verified this result **(Fig. 4)**. When Rac1 is bound to GTP, it is localised at the plasma membrane. Thus, the localisation of Rac1 at the plasma membrane was monitored as a read-out of Rac1 activation by immunofluorescence and confocal microscopy. I found that twice more cells have Rac1 at the plasma membrane in M1268T Met expressing cells than in WT Met expressing cells (27% for WT and 53% for M1268T Met expressing cells, $p<0.001$), **(Fig. 4B)**. Inhibition of Met activity, using PHA665752, has no effect on WT Met expressing cells, while it significantly decreases the percentage (%) of M1268T Met expressing cells that had Rac1 at the plasma membrane to the same level as was observed in WT Met expressing cells (27%, $p<0.001$) **(Fig. 4)**.

Consistent with previously published data from the laboratory, my results show that M1268T Met induces Rac1 relocalisation to the plasma membrane, which is a consequence of Met activation [56].

Interestingly, PI3K/mTOR inhibition using LY294002 also significantly decreases the percentage (%) of M1268T Met expressing cells that have Rac1 at the plasma membrane to 35% ($p<0.01$) while it has no effect on WT Met expressing cells **(Fig. 4)**, indicating that PI3K and/or mTOR activity is required by M1268T Met to induce Rac1 relocalisation.

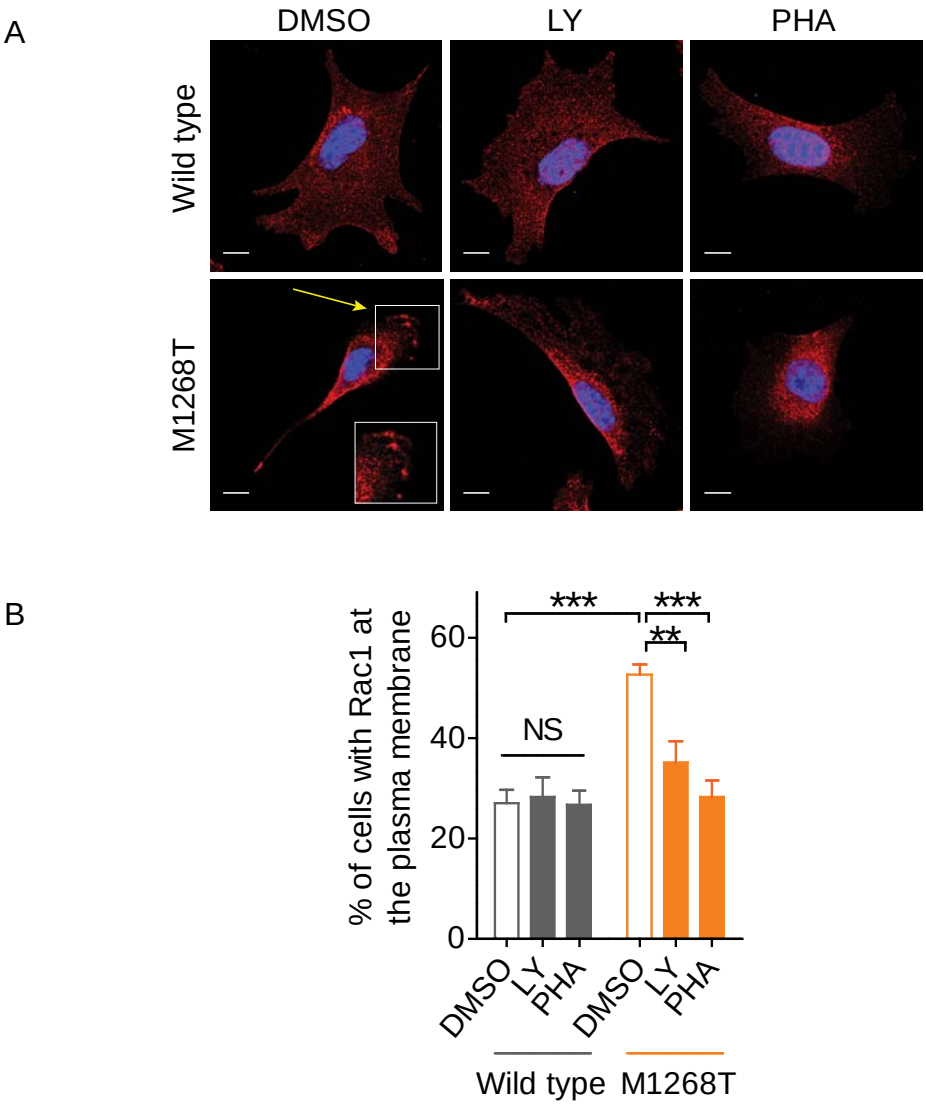


Figure 4: LY294002 reduces Rac1 localisation at the plasma membrane in M1268T Met expressing cells in a Met dependent manner

(A) Confocal sections of NIH3T3 Wild type and M1268T Met expressing cells treated with DMSO, LY294002 (LY) (10µM) or PHA665752 (PHA) (100 nM) for 1 hour. Cells were then stained with DAPI (blue) and immunostained with an antibody against Rac1 (red). Scale bar=10µm. **(B)** Percentage of cells with Rac1 at the plasma membrane. 100 cells were counted per condition per experiment. Mean values +/- SEM (DMSO: n=8, LY: n=7, and PHA: n=8). ns: non significant, **p<0.01, ***p<0.001.

2. M1268T Met expressing cells exhibit a loss of stress fibres, which are partially restored by LY294002

The motility of M1268T Met expressing cells is associated with actin reorganisation, especially the loss of stress fibres, which depends on Rac1 activity [56]. Thus in our model, WT Met expressing cells exhibit F-actin organised in stress fibres (visualised by phalloidin-Alexa-Fluor®-546 staining), suggesting a rather non-migratory cell behaviour. By contrast, in M1268T Met expressing cells, the organisation of F-actin is perturbed: stress fibres are lost (represented by the arrow) while cortical actin is increased [56] **(Fig. 5A)**.

Indeed, quantification indicated that the percentage of cells lacking stress fibres is higher in M1268T Met expressing cells (63%) compared to in WT expressing cells (23%, $p<0.001$) **(Fig. 5B)**. Inhibition of Met activity, using PHA665752, has no effect on WT Met expressing cells. In contrast it restores stress fibres in M1268T Met expressing cells **(Fig. 5A)**, and thus decreases the percentage of M1268T Met expressing cells without stress fibres to 44% ($p<0.001$) **(Fig. 5B)**. Interestingly, PI3K/mTOR inhibition by LY294002 restores the stress fibres in M1268T Met expressing cells **(Fig. 5A)**, and thus significantly decreases the percentage of M1268T Met expressing cells lacking stress fibres to a similar percentage as inhibition of Met activity (43%) **(Fig. 5B)**. No effect occurs on WT Met expressing cells.

These results indicate that M1268T Met induces the loss of stress fibres through PI3K and/or mTOR activity.

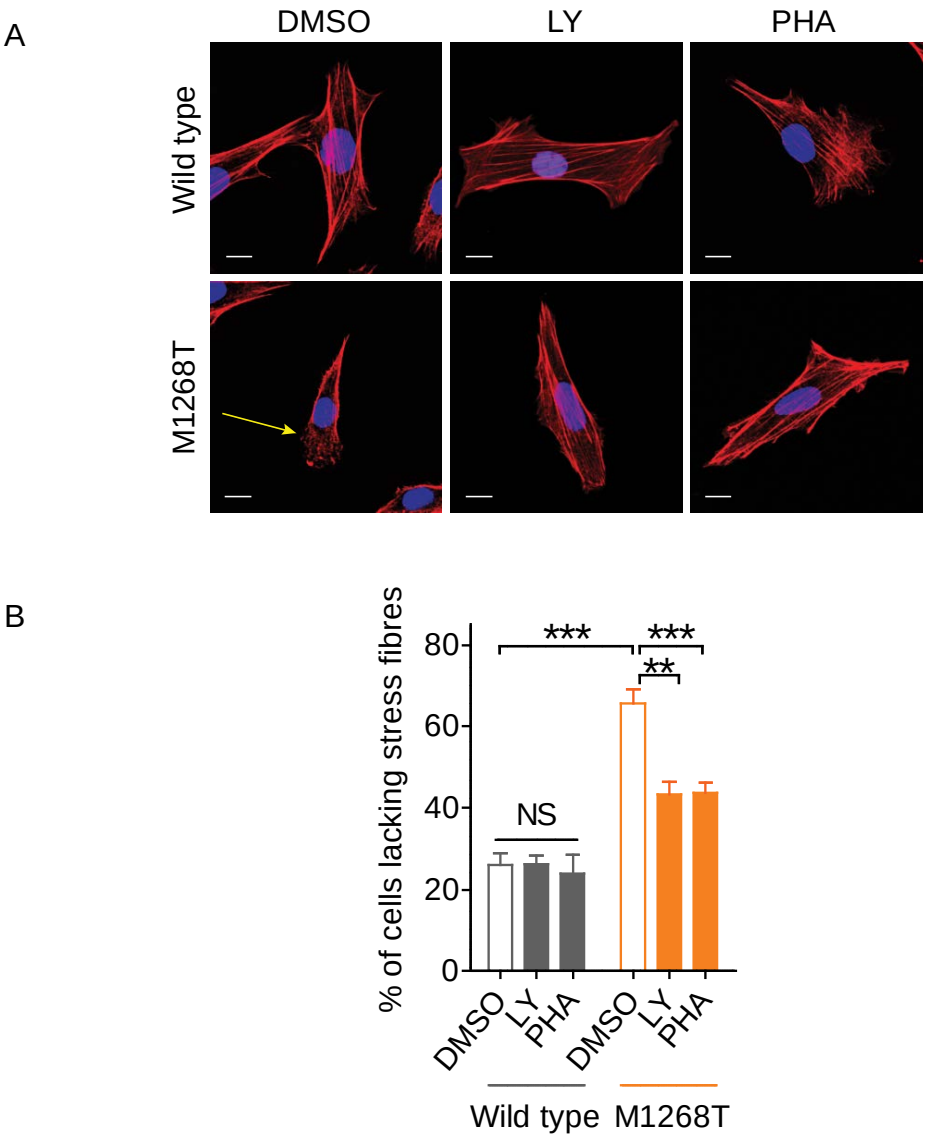


Figure 5: LY294002 restores stress fibres in M1268T Met expressing cells in a Met dependent manner

(A) Confocal sections of NIH3T3 Wild type and M1268T Met expressing cells treated with DMSO, LY294002 (LY) (10 µM) or PHA665752 (PHA) (100 nM) for 1 hour and stained with DAPI (blue) and rhodamin-phalloidin (red). Scale bar =10µm. **(B)** Percentage of cells lacking stress fibres. 100 cells were counted per condition per experiment. Mean values +/- SEM (n=6). ns: non significant, **p<0.01, ***p<0.001.

I. e) LY294002 reduces M1268T Met dependent cell migration

Migration assays were performed using Transwell chambers. In control cells, treated with DMSO**,** a 2.6 fold increase in migration was observed in M1268T Met expressing cells versus WT expressing cells ($p<0.001$) [56]. Met or PI3K/mTOR inhibition using PHA665752 or LY294002 respectively do not affect the migration of WT Met expressing cells. However, the migration of M1268T Met expressing cells is reduced to the same level as WT Met expressing cells ($p<0.05$) **(Fig. 6)**.

Altogether **(Fig. 4, 5, 6)**, these results suggest that, through the PI3K/mTOR pathway, M1268T Met induces Rac1 relocalisation, actin reorganisation and consequent cell migration.

I. f) LY294002 reduces M1268T Met dependent anchorage-independent growth

The ability of WT Met and M1268T Met expressing cells to undergo anchorage independent growth was tested by performing a soft agar assay. 500 single cells were grown in soft agar for 10 days. At day 5, they were treated daily with DMSO (negative control), the PI3K/mTOR inhibitor, LY294002, or the Met inhibitor, PHA665752. In the control conditions, the total area occupied by colonies formed by M1268T Met expressing cells is 3.8 times higher than the ones formed by WT Met expressing cells, ($p<0.001$) **(Fig. 7B)**, confirming the published results [56]. Treatment with PHA665752 reduces the area occupied by M1268T Met expressing colonies by 50% and does not have any have effect on WT Met expressing colonies, confirming that the anchorage independent growth of the cells is promoted by M1268T Met. Similarly and interestingly, LY294002 also leads to a 50% reduction in the area occupied by M1268T

Met expressing colonies and does not have any effect on WT Met expressing cells, suggesting that PI3K/mTOR is involved in M1268T Met expressing cells' transformation.

These results indicate that M1268T Met promotes cellular anchorage independent growth through the PI3K/mTOR pathway.

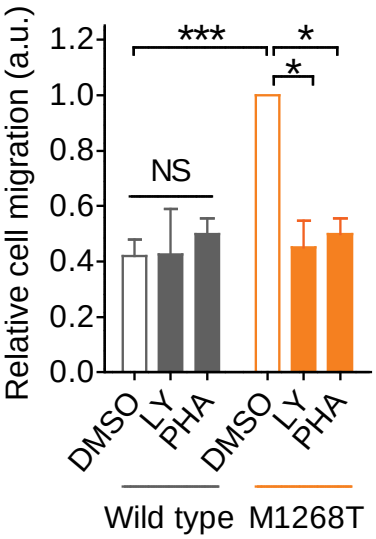


Figure 6: LY294002 reduces M1268T Met expressing cells migration to the same level as Wild type Met expressing cells

Transwell migration assay performed with 9,000 Wild type or M1268T Met expressing cells treated with DMSO, LY294002 (LY) (10 µM) or PHA665752 (PHA) (100 nM). Cells were incubated for 90 min. Mean values +/- SEM (DMSO: n=5, LY: n=4, and PHA: n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, ***p<0.001.

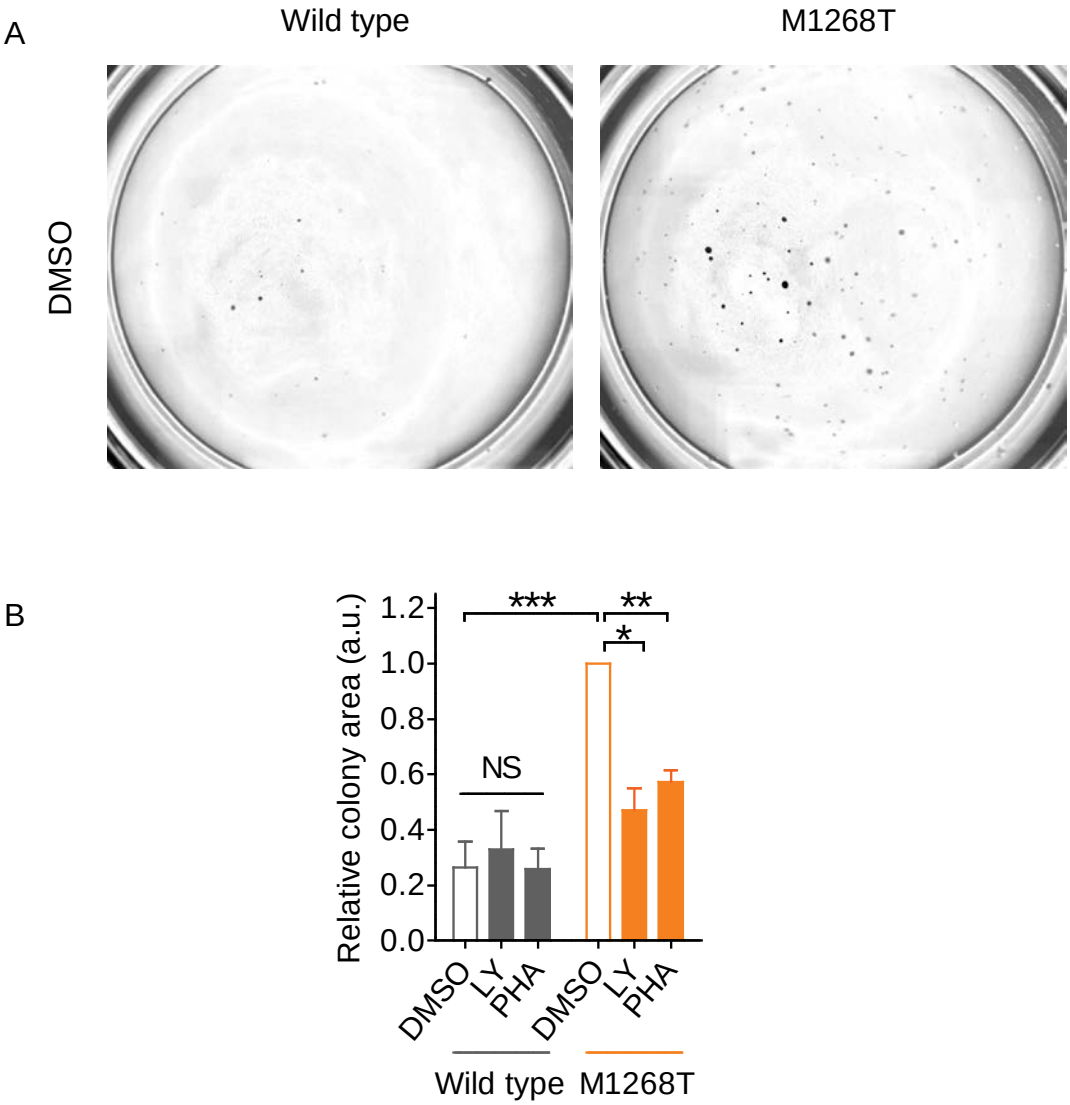


Figure 7: M1268T Met expressing cells induce anchorage independent growth, which is inhibited by LY294002

(A,B) 500 single cells from NIH3T3 Wild type or M1268T Met expressing cells were grown in soft agar for 10 days. At day 5, they were treated daily with DMSO, LY294002 (LY) (10 µM), or PHA665752 (PHA) (100 nM). **(A)** Colonies were photographed and **(B)** the area were calculated by ImageJ. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, **p<0.01, ***p<0.001.

DISCUSSION – CHAPTER I

M1268T Met induces cell transformation which is inhibited by the pan-PI3K / mTOR pharmacological inhibitor, LY294002

In this work, I have firstly confirmed that in my hands the M1268T Met mutant, found in papillary renal carcinomas, increases cell transformation of NIH3T3 cells *in vitro*, as described [56]. NIH3T3 cells expressing the M1268T Met mutant are therefore a good model to study the Met signalling pathway and its role in cancer.

Secondly, while the involvement of PI3K/mTOR downstream of WT Met signalling has been previously described [381, 388, 405], my original results, using pharmacological inhibition with LY294002, indicate that the PI3K/mTOR pathway is required for the induction of cell functions such as cell migration (through Rac1 activation), and anchorage independent growth by the M1268T Met mutant. The PI3K family includes 8 isoforms divided into 3 classes, and mTOR is a major downstream effector of PI3K class I. LY294002 targets PI3K class I, PI3K class III and mTOR, but PI3K class II is not inhibited. These results clearly indicate that PI3K and/or mTOR are involved in Met mutant signalling, however at this stage it is not known whether LY294002 effect was due to PI3K inhibition, mTOR inhibition or dual inhibitions.

I. a) M1268T Met induces cell transformation

As observed previously [100, 406], the M1268T mutation leads to a constitutively activated Met receptor **(Fig. 1, *p.117*)**. NIH3T3 cells expressing M1268T Met exhibit increased anchorage independent growth **(Fig. 7, *p.128*)**, an increased migratory phenotype (Rac1

at the plasma membrane, lack of stress fibres) **(Fig. 4, *p.122*; 5, *p.124*)** and increased cell migration **(Fig. 6, *p.127*)** as compared to NIH3T3 cells expressing WT Met.

Equal Met levels in WT and M1268T Met expressing cells allow the comparison of these two cell lines. WT Met is not activated in basal conditions and requires HGF to be activated **(Fig. 1, *p.117*)**. Importantly, Met inhibition or Met knock-down has no effect on the phenotype and functions of WT Met expressing cells, while they revert the phenotype of Met mutant expressing cells to the WT Met expressing cells phenotype [56]. Thus, these two cell lines represent a good model to study Met dependent signalling and cell functions.

I. b) The pharmacological inhibitor LY294002 inhibits the cell transformation induced by M1268T Met

The PI3K/Akt/mTOR signalling pathway is involved in numerous cell functions induced by various GPCRs and RTKs. Similarly, the involvement of the PI3K signalling pathway downstream of WT Met stimulated with HGF has been already demonstrated with LY294002 treatment in cell scattering of Madin Darby Canine Kidney (MDCK) [392], or cell migration of Small Cell Lung Cancer (SCLC) H69 cells [42]. But the role of the PI3K/Akt/mTOR signalling pathway downstream of oncogenic Met mutants has been poorly studied [397].

By inhibiting PI3K class I and III, and mTOR, using the pharmaceutical inhibitor LY294002, I reverted the cell transformation triggered by the M1268T Met mutant in the same way as inhibiting Met activity **(Fig. 4,5,6,7, *p.122, 124, 127, 128*)**, while no effect was observed on the basal functions of the cells expressing WT Met.

These results indicate that PI3K/mTOR appears to be involved in cell transformation driven by the constitutively active Met mutant in concordance with the literature reported on Met activated with HGF.

How Rac1 is activated by PI3K or mTOR remains to be established. One possibility is that PI3K/mTOR activity is required for the activation of specific GEFs operating in the Met mutant – Rac1 signalling pathway. In MDCK stimulated with HGF, it was shown that Rac1 activation occurs in a PI3K/mTOR dependent manner, but no mechanism was provided [392]. On the other hand, in HeLa cells, the GEF Tiam1 was reported to activate Rac1 upon HGF stimulation [79]. Recently, Ménard *et al*, in our group, have shown that upon HGF, Met signals through PI3K and the GEF Vav2 to activate Rac1 and induce cell migration [78]. Moreover, when Vav2 is mutated in its PH domain (binding domain for the lipid product of PI3K class I PIP3), it is unable to induce Met dependent cell migration. This suggests a key role for PI3K class I in Met dependent cell migration. Interestingly, mTOR, as part as the mTORC2 complex, has been reported to modify actin organization upon serum stimulation [258]. Could both PI3K and mTOR be regulated by M1268T Met to induce cell migration?

Moreover, LY294002 inhibits PI3K class III, which is composed of one isoform, Vps34. Vps34 is known to have a role in endosomal sorting and in mTORC1 activation upon amino acid stimulation [125]. It was shown in our group that M1268T signals from endosomes [56]to promote cell migration and anchorage independent growth, and that WT Met signaling, upon activation by HGF, varies depending on the type of endosome in which Met is located [55, 57, 78]. Therefore, is Vps34 involved in Met endosomal signalling? This has not been investigated so far.

In conclusion

I have observed that the M1268T Met mutant transforms cells, inducing a migratory cell phenotype, cell migration and anchorage independent growth, as previously shown. I have demonstrated that this cell transformation is dependent on PI3K/mTOR activity.

Cell transformation triggered by M1268T Met depends on its constitutive activation but also on its internalisation. Is PI3K/mTOR activation dependent on Met internalisation? Which PI3K isoform(s) play a role in M1268T Met dependent cell transformation? Where and how PI3K/mTOR pathway is activated will be studied in this thesis **(see Chapter II and III)**.

Importantly, LY294002 targets mainly PI3K class I/III and mTOR. PI3K class II, and in particular C2 alpha, are resistant to this drug at the concentration used [407]. This means that the role of the PI3K class II isoforms in Met mutant signalling remains to be established.

In the next chapters, I aim to determine the role and the mechanism of action of each protein inhibited by LY294002 downstream of M1268T Met. For this, I used specific pharmacological inhibitors, and knock-down by RNAi.

RESULTS – CHAPTER II

PI3K class I isoforms promote Met dependent cell migration and Akt activation

In this chapter, the aim was to understand better how the PI3K/Akt/mTOR pathway regulates the migratory phenotype and the migration of M1268T Met expressing cells through:

(1) Identifying which LY294002 targets are required: PI3K Class I and III isoforms, Akt and mTOR;

(2) Determining if the activation of the identified signalling molecules require M1268T Met endocytosis.

II. a) Met dependent cell migration is reduced by PI3K class I inhibition, but not by PI3K class III knock down

1. Wortmannin reduces M1268T Met dependent cell migration

To evaluate whether the effect of LY294002 observed previously in M1268T Met expressing cells is due to PI3K inhibition or mTOR inhibition, I used the pharmacological inhibitor wortmannin, an irreversible PI3K inhibitor. The IC50 of LY294002 in enzyme assay is 1 µM for PI3K class I, 23 µM for PI3K class II, 4 µM for PI3K class III and 4 µM for mTOR. The IC50 of wortmannin obtained in an enzyme-based *in vitro* assay is 10 nM for human PI3K class I, 450 nM for PI3K class II, 10 nM for PI3K class III and 200 nM for mTOR. Therefore, wortmannin is more potent at inhibiting PI3K class I and III than mTOR and PI3K class II.

To determine the optimum concentration in cells that would target PI3K class I and II but hopefully not mTOR, I performed a dose response assay. Cells expressing M1268T Met were exposed to DMSO as a negative control, LY294002 at 10 μM as a positive control for PI3K and mTOR inhibition, or increasing concentrations of wortmannin (from 25 nM to 500 nM). It is believed that PI3K class I is the main PI3K class leading to Akt phosphorylation. However, the PI3K class II isoform C2 alpha seems to also be involved in Akt phosphorylation [408]. Thus, Akt phosphorylation was used as read out in western-blot for PI3K activity **(Fig. 1A, B)**. It was observed that wortmannin partially reduces Akt phosphorylation at 25 and 50 nM. At 100 and 500 nM, wortmannin reduces the phosphorylation of Akt by 90%, which is greater than achieved with LY294002 (70%). It was decided that wortmannin would be used at 100 nM, which is the concentration that strongly inhibits PI3K class I and III but should not target mTOR. Western blots to evaluate the level of phosphorylation of mTOR effectors would have allowed to check whether mTOR is not targeted when the cells are treated with 100 nM wortmannin.

In the presence of wortmannin at 100 nM, the migration of M1268T Met expressing cells is significantly reduced to the same level that is achieved by WT Met expressing cells treated with DMSO ($p<0.01$). Wortmannin also has an effect on the migration of WT Met expressing cells, however this is not statistically significant **(Fig. 1C)**.

It was concluded that wortmannin inhibits M1268T Met dependent cell migration, indicating a role for PI3K class I and/or III in M1268T Met dependent cell migration. However, since an additional role for mTOR could not be excluded, I later investigated whether mTOR also plays a role in M1268T Met dependent cell migration **(Fig. 12)**.

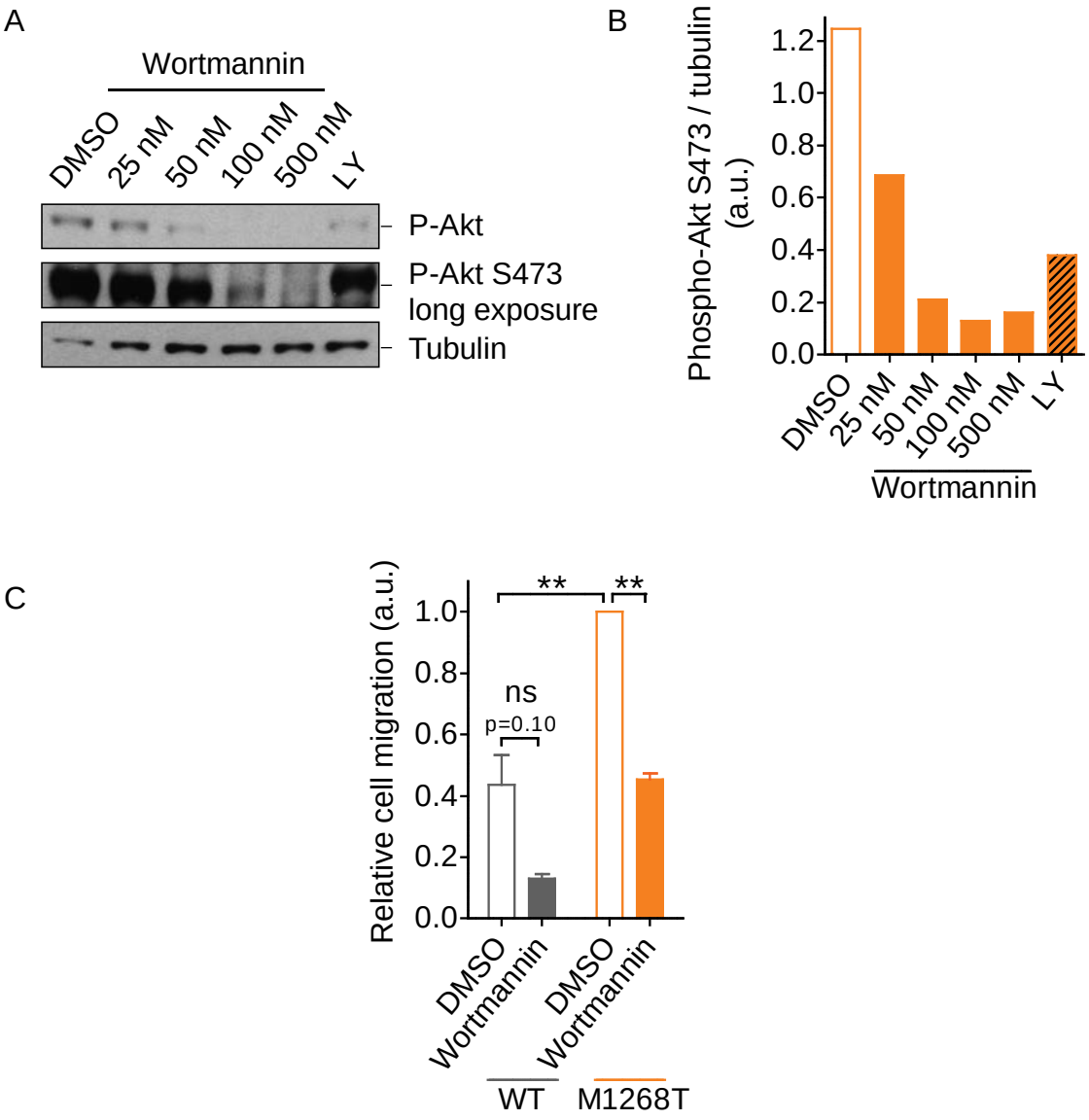


Figure 1: Wortmannin reduces M1268T Met dependent cell migration

(A) Western blots for phosphorylated Akt (S473) (P-Akt), and tubulin were performed on NIH3T3 cells expressing M1268T Met treated with increasing concentrations of Wortmannin or LY294002 (LY) (10 µM) for 1 hour. **(B)** Quantification of phosphorylated Akt (S473) normalised on tubulin, obtained by densitometry of western blots. **(C)** Transwell migration assay performed with 9,000 Wild type (WT) or M1268T Met expressing cells treated with DMSO or Wortmannin (100 nM). Cells were incubated for 90 min. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, **p<0.01.

2. PI3K isoforms expressed in NIH3T3 Met expressing cells

I then aimed to investigate which PI3K isoform is implicated in M1268T Met signalling. The PI3K family is comprised of 8 isoforms, divided into three classes, I, II, and III, which are classified depending on their structure and their lipid substrates. Western blots were first performed with specific antibodies to investigate which PI3K isoforms are expressed in NIH3T3 WT and M1268T Met expressing cells **(Fig. 2A)**. Amongst PI3K class I, the isoforms p110 alpha and beta are clearly detected. However, only a weak signal from the isoform p110 delta is detected. The isoform p110 gamma does not seem to be present, as previously observed [151]. Regarding PI3K class II, the isoforms C2 alpha and beta are present. Vps34, the only isoform of the PI3K class III, is clearly detected. Quantification of three independent experiments, using densitometry, shows that the levels of expression of each of these isoforms are similar in WT and M1268T Met expressing cells **(Fig. 2B)**, suggesting that M1268T Met does not modify the expression of PI3K isoforms.

3. PI3K class III Vps34 do not seem to be involved in M1268T Met dependent cell migration

M1268T Met needs to be internalised and sorted to the right endosomes to induce optimal cell signalling and cell migration [56, 57, 142], and Vps34 (the PI3K class III) is involved in vesicles trafficking (in endocytosis, phagocytosis and autophagy); therefore Vps34 could be involved in M1268T Met signalling.

As no drugs were available, I aimed to determine if Vps34 has a role in M1268T Met dependent cell migration by using siRNA to mediate transcriptional gene silencing.

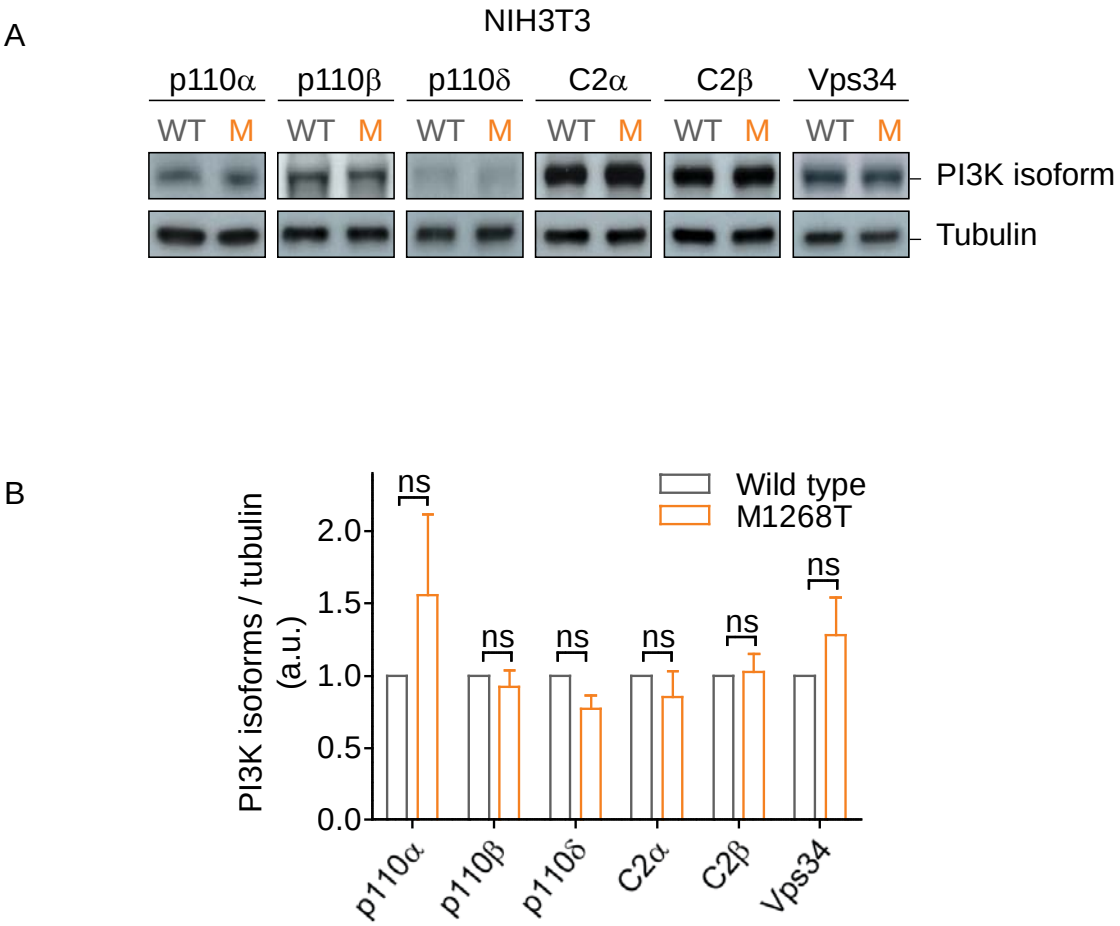


Figure 2: Expression of PI3K isoforms

(A) Western blots for PI3K isoforms (class I: p110 alpha, beta and delta; class II: C2 alpha and beta; class III: Vps34) and tubulin in NIH3T3 cells expressing Wild type (WT) or M1268T (M) Met. **(B)** Quantification of western blots for each PI3K isoform normalised on tubulin, obtained by densitometry. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant.

NIH3T3 cells expressing WT or M1268T Met were transfected with negative control or for Vps34 siRNA **(Fig. 3A)**. In negative control conditions, M1268T Met expressing cells migrate 2.4 fold more than WT Met expressing cells ($p<0.01$) **(Fig. 3B)**. Meanwhile, upon Vps34 knock-down, the migration of WT and M1268T Met expressing cells was not decreased and in fact it was slightly increased (by 59 and 47%) although not significantly.

This result suggests that Vps34 does not promote cell migration induced by M1268T Met.

4. PI3K class I is responsible for Met dependent cell migration

- *Optimisation of drug concentrations for inhibiting PI3K class I isoforms*

I then investigated the role of PI3K class I, focusing on the two isoforms, p110 alpha and beta, which are strongly expressed in my cell model **(Fig. 2)**. Additionally, inhibitors that specifically bind these PI3K isoforms in an ATP-competitive manner were available. I used several inhibitors:

- GDC0941, which targets all of the PI3K class I isoforms [407]. Its IC50 *in vitro* is 3 nM for p110 alpha and delta, 33 nM for p110 beta, and 75 nM for p110 gamma;
- A66, which is a potent inhibitor of the isoform p110 alpha [409]. Its IC50 *in vitro* is 32 nM for p110 alpha, and it is more than 100 times less active on the others isoforms at high concentration such as 10 µM.
- TGX221, which specifically targets the isoform p110 beta. Its IC50 *in vitro* is between 5 to 9 nM for p110 beta, 100 to 211 nM for p110 delta, from 1 to 5 µM for p110 alpha, and more than 10 µM for p110 gamma [142, 410].

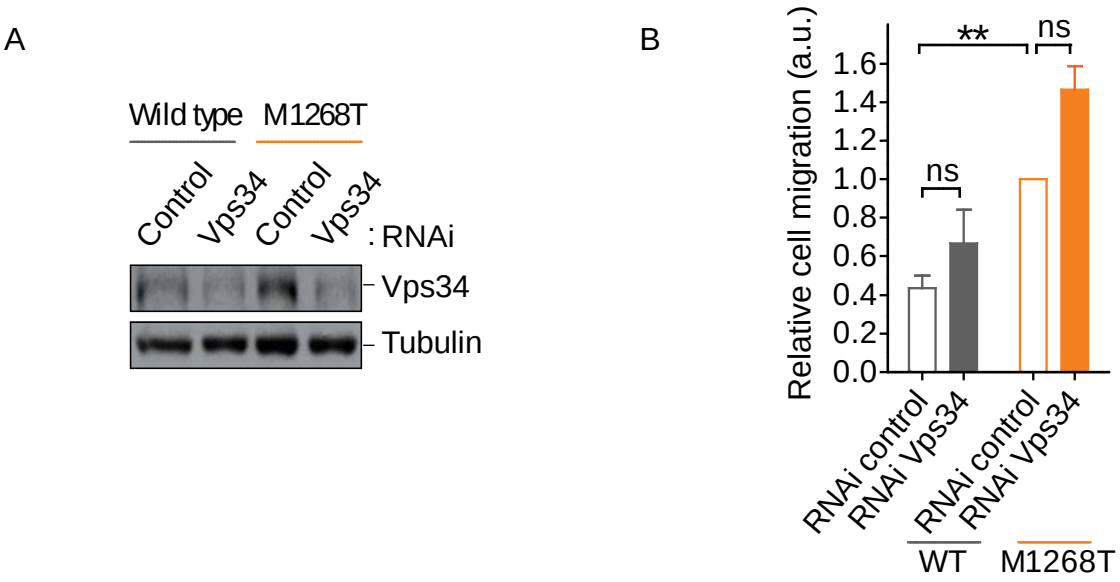


Figure 3: Knock-down of PI3K class III isoform Vps34 has no effect on the migration of M1268T Met expressing cells

(A,B) NIH3T3 Wild type and M1268T Met expressing cells were transfected with negative control or Vps34 siRNA. **(A)** Western blots for Vps34 and tubulin were performed. **(B)** Transwell migration assays were performed with 9,000 Wild type or M1268T Met expressing cells. Cells were incubated for 90 min. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, **p<0.01.

I first aimed to determine the optimal drug concentrations necessary to target the isoforms studied as specifically as possible in the M1268T Met expressing cells. Thus, dose responses for the inhibitors were performed and Akt phosphorylation was detected by western blot as a read-out for the activity of PI3K isoforms. It is worth noting that Akt has two phospho-sites: the threonine 308 (Thr308), and the serine 473 (S473). PI3K class I regulates phosphorylation of Thr308 through PDK1, and S473 through mTORC2.

Western blots were performed on proteins from the cell lysates of cells that have been treated with DMSO as a negative control, PI3K inhibitor LY294002 as a positive control, or increasing concentrations of PI3K class I inhibitors.

Inhibition of PI3K by LY294002 at 10 μM (the dose inhibiting M1268T Met induced cell migration **(as described in Chapter I)**) poorly reduces Akt phosphorylation on Thr308, but strongly reduces Akt phosphorylation on the S473 **(Fig. 4, 5, 6)**. The poor inhibition of the phospho-site Thr308 has been already described [411], and might be due to the poor inhibition of PDK1 by LY294002 [412]. It has been suggested that PKCbeta could be involved in the phosphorylation of Akt on this site [413].

Inhibition of PI3K class I by GDC0941 reduces Akt phosphorylation on Thr308 by 60% at low concentrations (30, 100 and 500 nM) **(Fig. 4A, B)** and by 75% at higher concentrations (1 and 10 μM). Akt phosphorylation on S473 is more sensitive to the inhibition by GDC0941 than Thr308 **(Fig. A, C)**. Consequently, GDC0941 strongly reduces Akt phosphorylation on S473 at low concentrations (30, 100 and 200 nM) **(Fig.**

4A, C, D, E) and abolishes Akt phosphorylation on S473 from 400 nM or higher concentrations.

It was decided that GDC0941 would be used at 100 nM, in order to strongly inhibit PI3K class I, but still maintain a level of Akt phosphorylation on S473 equivalent as when the cells are treated with LY294002, because at this level of inhibition LY294002 reverts Met dependent cell transformation **(see Chapter I)**.

Inhibition of p110 alpha by A66 decreases Akt phosphorylation (both Thr308 and S473) from 100 nM **(Fig. 5A, B, C)**, and strongly decreases it at 10 µM. The concentration of A66 chosen to use was 500 nM, to greatly reduce p110 alpha activity for the studies but also to avoid potential off-target effect observed with high concentration of drugs. To confirm p110 alpha inhibition by A66 at 500 nM, p110 alpha was stimulated indirectly with lysophosphatidic acid (LPA), a ligand for the GPCR P2Y5 [151] **(Fig. 5D, E)**. LPA treatment induces the increase of phospho-Akt S473 through PI3K class I following activation of the GPCR. Akt phosphorylation is decreased following inhibition of p110 alpha by A66 from 500 nM, confirming the suitability of concentration of A66 chosen at 500 nM.

Inhibition of p110 beta by TGX221 has a poor effect on phospho-Akt (Thr308 and S473) in basal conditions **(Fig. 6)**. This could indicate that the basal level of Akt phosphorylation in M1268T Met expressing cells is mainly due to p110 alpha activity. To ensure the effectiveness of TGX221 in inhibiting p110 beta, p110 beta activation was induced with LPA **(Fig. 7A, B),** or HGF **(Fig. 7C, D)**. The Akt phosphorylation induced by LPA or HGF is decreased by inhibition of p110 beta with TGX221 from 100

nM or higher. Akt phosphorylation induced by LPA is therefore reduced by both p110 alpha inhibition with A66 at 500 nM and p110 beta inhibition with TGX221 at 100 nM. This could indicate that either 1) Akt phosphorylation induced by LPA requires both p110 alpha and p110 beta activity; or 2) one of the two inhibitors, A66 or TGX221, is used at a concentration that has off-target effect. TGX221 was initially chosen to be used at 100 nM. Numerous assays were performed with TGX221 at 100 nM, including assays evaluating the M1268T Met dependent migratory phenotype **(see II. b) The migratory phenotype induced by M1268T Met requires the expression of the PI3K class I isoforms p110 alpha and p110 beta)** and anchorage independent growth **(see Chapter III)**. But later, it emerged that when TGX221 was used at 100 nM it had a strong and significant effect on the cell migration of NIH3T3 cells expressing mutant but also WT Met. Met is not active in these cells, therefore leading to the hypothesis that p110 beta might be involved in Met independent cell migration in NIH3T3 cells. Consequently, it was not possible to conclude about the effect of TGX221 at 100 nM on the cell migration driven by M1268T Met. For this reason, and taking into account the previously described potency of TGX221 toward p110 beta, it was decided that TGX221 would be used at 40 nM in Transwell migration assays, for a second time. Higher concentrations than this, such as 50 and 75 nM, seemed to also have an effect on the cell migration of WT Met expressing cells.

The treatment with each of these inhibitors, LY294002, GDC0941, A66, and TGX221, does not seem to modify total Akt levels **(Fig. 4, 5, 6, and 7)**.

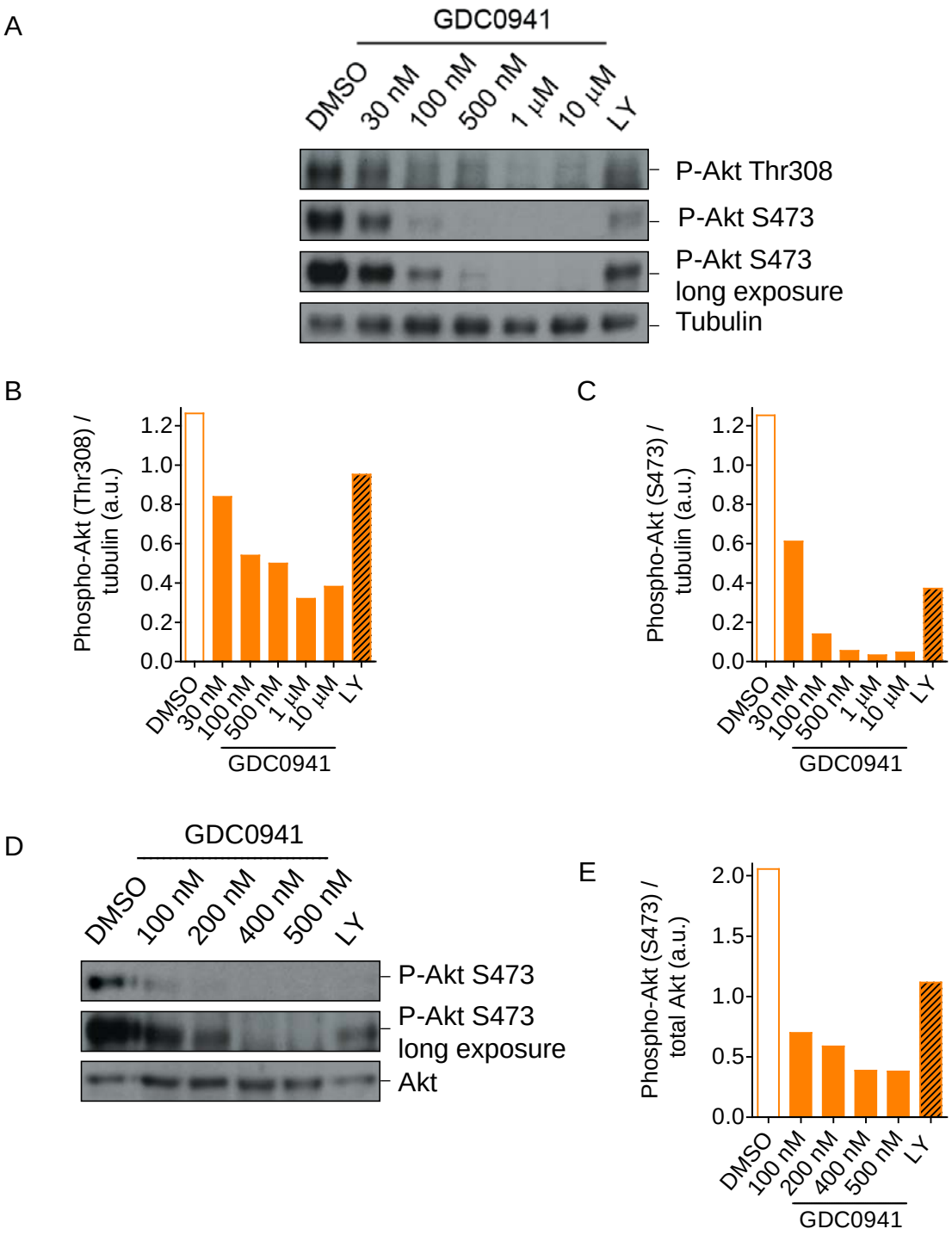


Figure 4: Treatment of M1268T Met expressing cells with GDC0941

(A-E) M1268T Met expressing cells were treated with DMSO, LY294002 (LY) (10µM) or GDC0941 **(A-C)** 30 nM - 10 µM and **(D,E)** 100 nM - 500 nM). **(A)** Western blots for phosphorylated Akt (Thr308 and S473) (P-Akt) and tubulin. **(B,C)** Quantification of **(B)** P-Akt Thr308 or **(C)** P-Akt S473 normalised on tubulin, obtained by densitometry of western blots **(D)** Western blots for phosphorylated Akt (S473) (P-Akt) and Akt. **(E)** Quantification of P-Akt (S473) normalised on Akt, obtained by densitometry of western blots. (a.u. = arbitrary units).

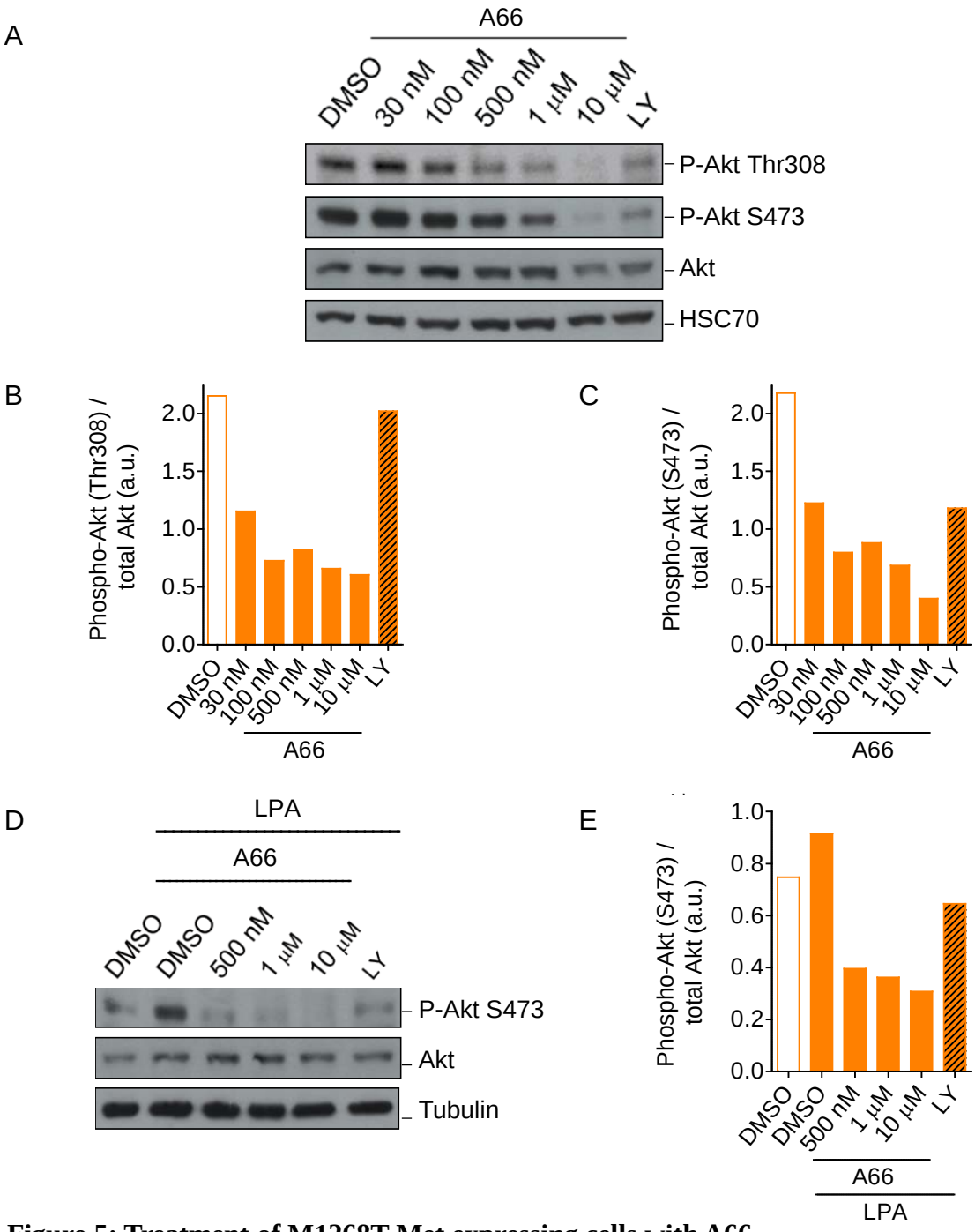


Figure 5: Treatment of M1268T Met expressing cells with A66

(A-E) M1268T Met expressing cells treated with DMSO, LY294002 (LY) (10 µM) or A66 (30 nM-10 µM). **(A)** Western blots for phosphorylated Akt (Thr308 and S473) (P-Akt), Akt, and HSC70. **(B,C)** Quantification of **(B)** P-Akt Thr308 or **(C)** P-Akt S473, normalised on AKT, obtained by densitometry of western blots. **(D)** Western blots for phosphorylated Akt (S473) (P-Akt), Akt and tubulin on cells stimulated or not with lysophosphatidic acid (LPA) (1 µM). **(E)** Quantification of P-Akt (Thr308 and S473) normalised on Akt, obtained by densitometry of western blots. (a.u. = arbitrary units).

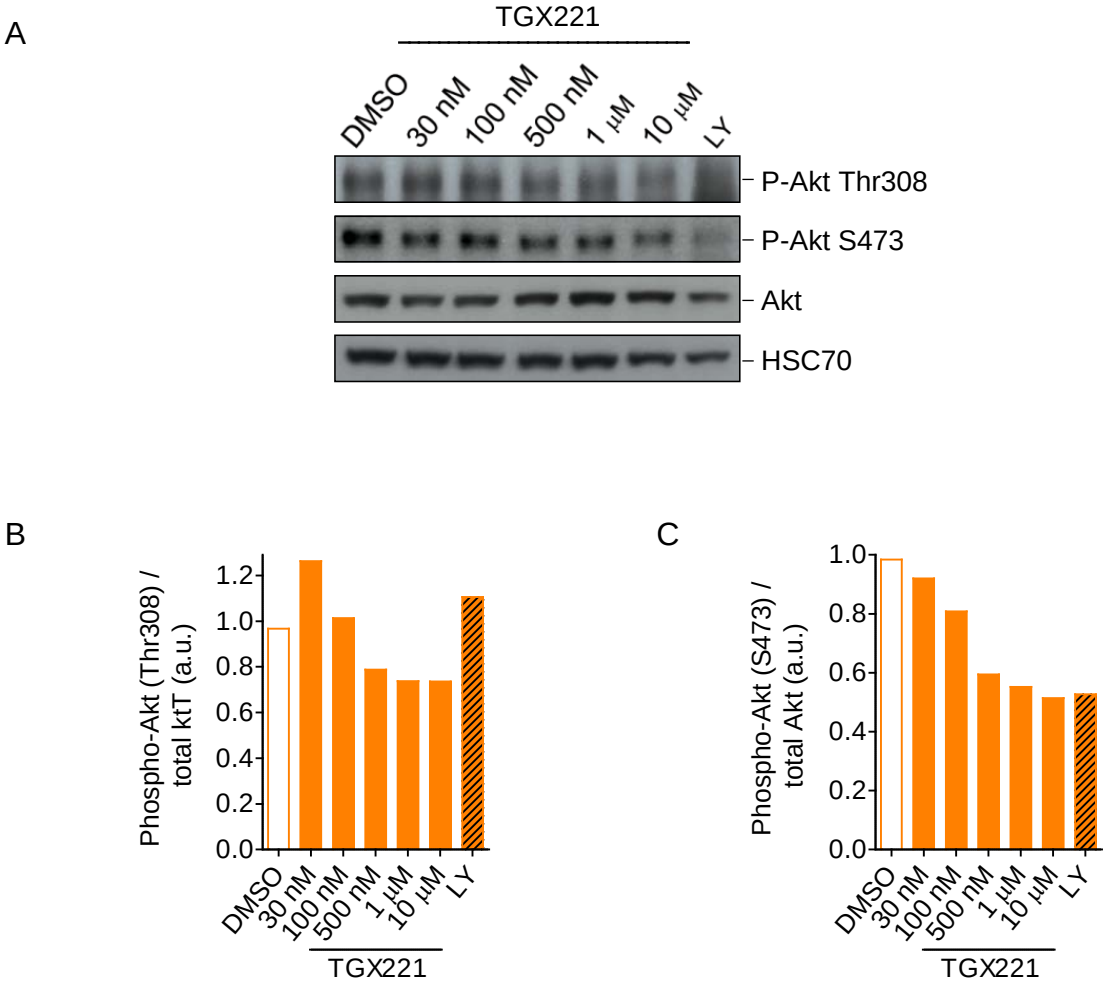


Figure 6: Treatment of M1268T Met expressing cells with TGX221

(A-C) M1268T Met expressing cells treated with DMSO, LY294002 (LY) (10 µM) or TGX221 (30 nM - 10 µM). **(A)** Western blots for phosphorylated Akt (Thr308 and S473) (P-Akt), Akt, and HSC70. **(B,C)** Quantification of **(B)** P-Akt Thr308 or **(C)** P-Akt S473 normalised on AKT, obtained by densitometry of western blots. (a.u. = arbitrary units).

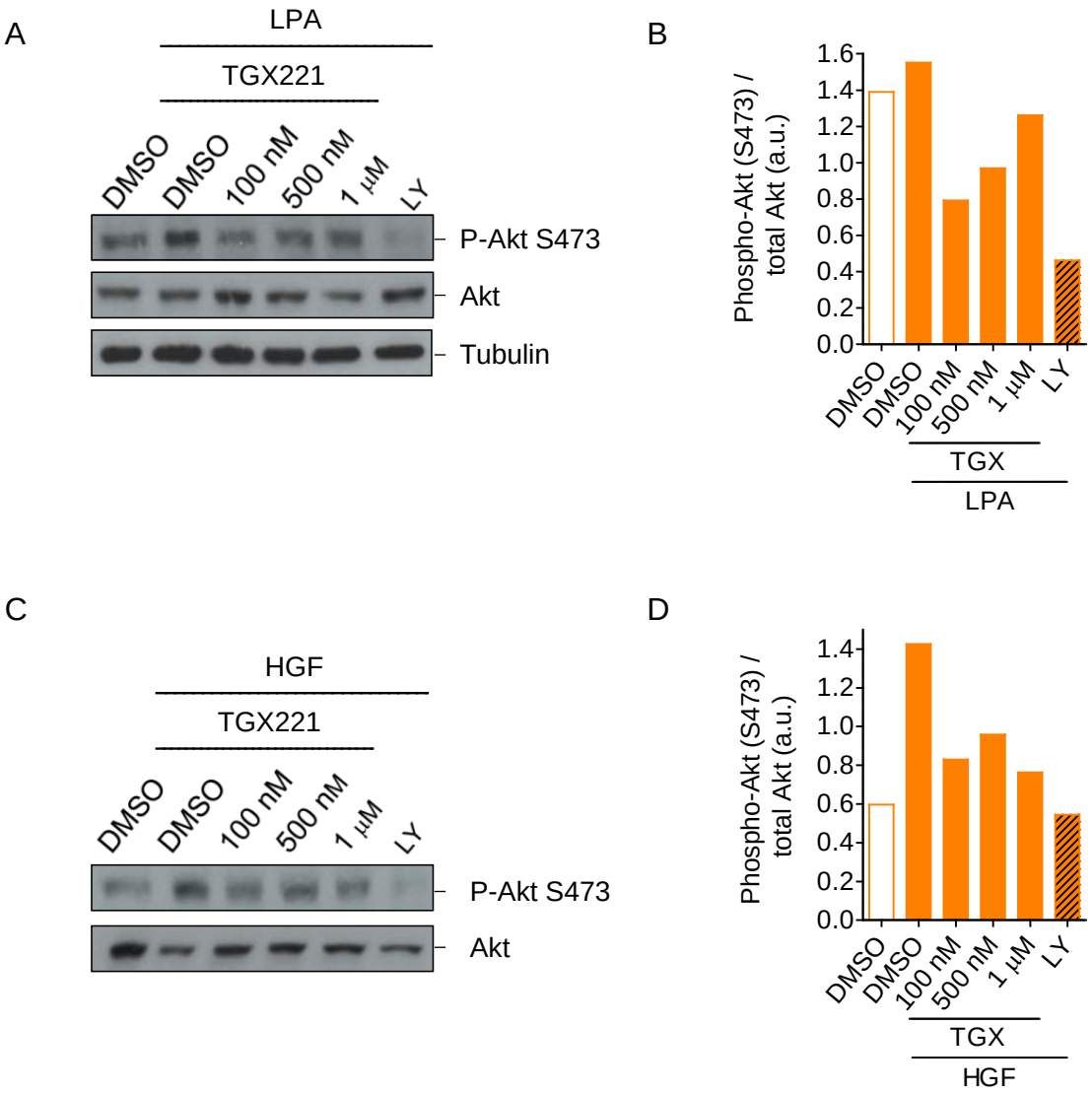


Figure 7: TGX221 treatment in M1268T Met expressing cells

(A-D) M1268T Met expressing cells treated with DMSO, LY294002 (LY) (10 µM) or TGX221 (100 nM – 1 µM). **(A,B)** Cells were stimulated with or without lysophosphatidic acid (LPA) (1 µM). **(A)** Western blots for phosphorylated Akt (S473) (P-Akt), Akt, and tubulin. **(B)** Quantification of P-Akt (S473) normalised on Akt, obtained by densitometry of western blots. **(C,D)** Cells were stimulated with or without hepatocyte growth factor (HGF) (50ng/ml) **(C)** Western blots for phosphorylated Akt (S473) (P-Akt) and Akt. **(D)** Quantification of P-Akt (S473) normalised on Akt, obtained by densitometry of western blots. (a.u. = arbitrary units).

- *Targeting PI3K class I reduces the migration of M1268T Met expressing cells*

Transwell migration assays were performed on the NIH3T3 cells expressing either WT or M1268T Met, treated with the PI3K class I inhibitors at the concentrations determined above, or knocked-down for the PI3K class I isoforms expressed in these cells (p110 alpha and p110 beta).

M1268T Met expressing cells migrate 2 fold more than WT Met expressing cells ($p<0.001$) in the presence of the negative control **(Fig. 8A)**.

PI3K class I inhibition by GDC0941 does not have any effect on WT Met expressing cells, while it significantly reduces the cell migration of M1268T Met expressing cells by 49%, thus back to the level of migration observed in the WT Met expressing cells ($p<0.05$) **(Fig. 8A)**.

Inhibition of the individual PI3K isoform p110 alpha with A66 decreases the cell migration of WT Met expressing cells by 45%, however this is statistically not significant. The inhibition of p110 beta with TGX221 had no effect on these cells. Similarly, the inhibition of both p110 alpha and p110 beta individually had no effect on M1268T Met dependent cell migration **(Fig. 8A)**.

However the combined inhibition of PI3K class I isoforms p110 alpha and p110 beta by combining A66 and TGX221 significantly reduces the migration of M1268T Met expressing cells by 43% ($p<0.05$) to the same level of migration as in WT Met expressing cells, while the inhibitors have no effect on these cells **(Fig. 8A)**.

PI3K p110 alpha or p110 beta knock-down do not influence the migration of WT and M1268T Met expressing cells **(Fig. 8A, B)**. Importantly, the knock-down of each isoform does not seem to modify the expression of the other isoform **(Fig. 8B)**.

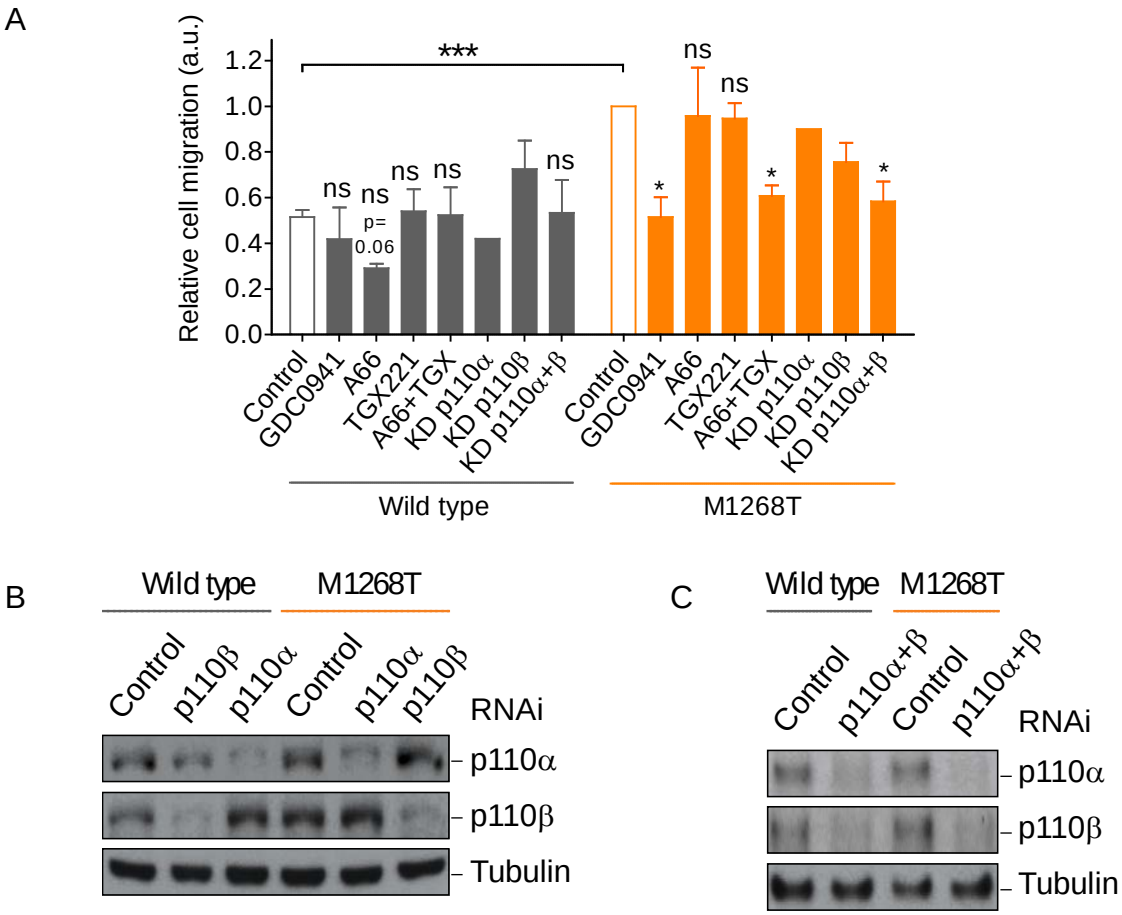


Figure 8: PI3K class I inhibition and knock-down reduces the migration of M1268T Met expressing cells to the same level as Wild type Met expressing cells

(A) Transwell migration assays performed with 9,000 Wild type or M1268T Met expressing cells. Cells were treated with DMSO (control), GDC0941 (GDC, n=4) (100 nM), A66 (n=3) (500 nM), TGX221 (TGX, n=3) (40 nM) or A66 and TGX221 combined (n=4); or transfected with negative control (control), p110 alpha (n=1), p110 beta (n=2), or both p110 alpha and beta combined (n=3) siRNA. Cells were incubated for 90 min. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, **p<0.01. **(B, C)** Western blots for p110 alpha, p110 beta and tubulin were performed on NIH3T3 Wild type and M1268T Met expressing cells transfected with **(B)** negative control (Control), p110 alpha, or p110 beta siRNA, or with **(C)** negative control (Control) and p110 alpha and beta combined siRNA.

The combined knock-down of PI3K p110 alpha and p110 beta has no significant effect on WT Met expressing cell migration, while it totally and significantly abrogates the cell migration induced by M1268T Met (-43%, $p<0.05$) to the level of migration observed in the WT Met expressing cells **(Fig. 8A, B)**.

Altogether these results indicate that M1268T Met dependent cell migration is inhibited only when both PI3K class I isoforms p110 alpha and p110 beta are inactivated.

- *Targeting PI3K class I in Met dependent cell migration of human cancer cell lines*

I aimed to establish if WT Met in human cancer cells signals through PI3K class I in the same manner as M1268T Met in NIH3T3 cells to induce Met dependent cell migration. For this, I used two different cell models:

• The non-small lung cancer (NSLC) cell line, A549, which expresses endogenous WT Met **(Fig. 9)**. At 15 minutes of HGF treatment, the phosphorylation of Met is increased. At 120 minutes of HGF treatment, Met phosphorylation is maintained although it appears reduced compared to 15 minutes due to a high proportion of Met that has been degraded, consistent with previously published data in other cell lines [56, 60, 142].

• The human glioblastoma-astrocytoma, epithelial-like cell line U87MG, which expresses endogenous WT Met but also secretes HGF, resulting in a Met-HGF autocrine loop. Therefore Met is phosphorylated in basal conditions in these cells **(Fig. 9)**.

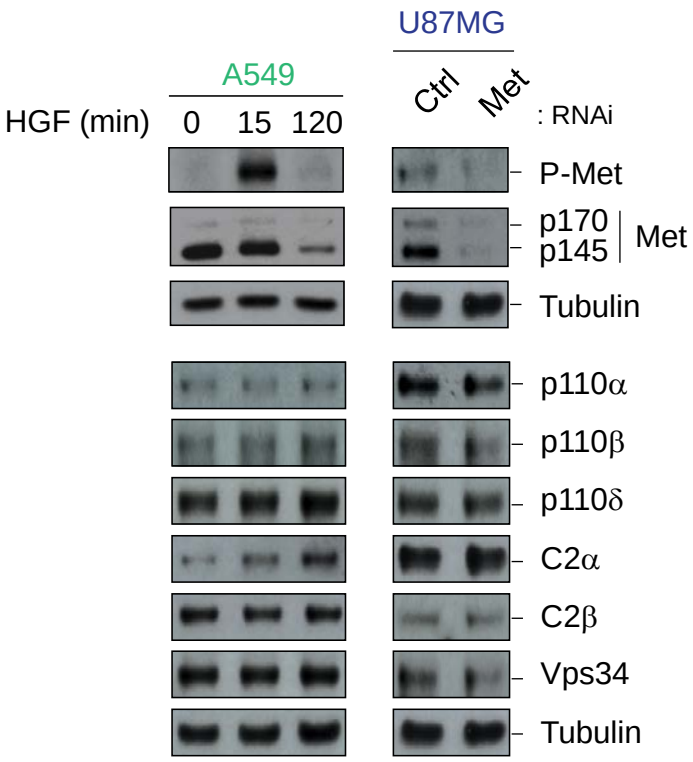


Figure 9: Expression of PI3K isoforms in A549 and U87MG human cancer cell lines

Western blots for phosphorylated Met (Y1234/5) (P-Met), Met (p170, the precursor form and p145, the mature form of the beta chain), PI3K isoforms (class I: p110α, p110β, p110δ; class II: C2α and C2β; class III: Vps34) and tubulin in A549 cells stimulated with HGF at 50 ng/mL for 0, 15 or 120 minutes, or U87MG cells transfected with negative control or Met siRNA.

Amongst the PI3K class I, as observed in the NIH3T3 cells, the two human cell lines express p110 alpha and beta but also delta (p110 delta was poorly detected in NIH3T3 cells), C2 alpha and beta amongst the PI3K class II as well as the PI3K class III Vps34 **(Fig. 9)**. Neither the activation of Met by HGF stimulation in A549 cells, nor the knock-down of Met in U87MG cells, seems to modify the level of expression of PI3K isoforms, except for PI3K isoform C2 alpha which seems to increase when A549 cells are treated with HGF **(Fig. 9)**.

To study the role of the PI3K class I isoforms in Met dependent cell migration in these two cell lines, the same inhibitors of PI3K class I isoforms were used at the same concentrations as determined earlier. In contrary to NIH3T3 cells, the human cancer cells A549 and U87MG express the PI3K class I isoform p110 delta. To specifically inhibit p110 delta, the pharmacological inhibitor IC87114 was used at 1 µM. The IC50 of IC87114 *in vitro* is 500 nM for p110 delta, more than 100 µM for p110 alpha, 75 µM for p110 beta and 29 µM for p110 gamma.

A549 cells were stimulated or not with HGF and were treated with DMSO as a negative control or with PI3K class I inhibitors, either alone or combined **(Fig 10A)**. In the control conditions, A549 cells stimulated with HGF migrate 2.9 times more than the non-stimulated cells ($p<0.001$). The individual and combined inhibition of PI3K class I isoforms has no effect on the basal migration of A549 cells. While the HGF dependent A549 cell migration is not reduced by the inhibition of p110 alpha with A66 alone, it is significantly reduced by 35% ($p<0.05$) and 29% ($p<0.01$) with the p110 beta and p110 delta individual inhibitors TGX221 and IC87114 respectively.

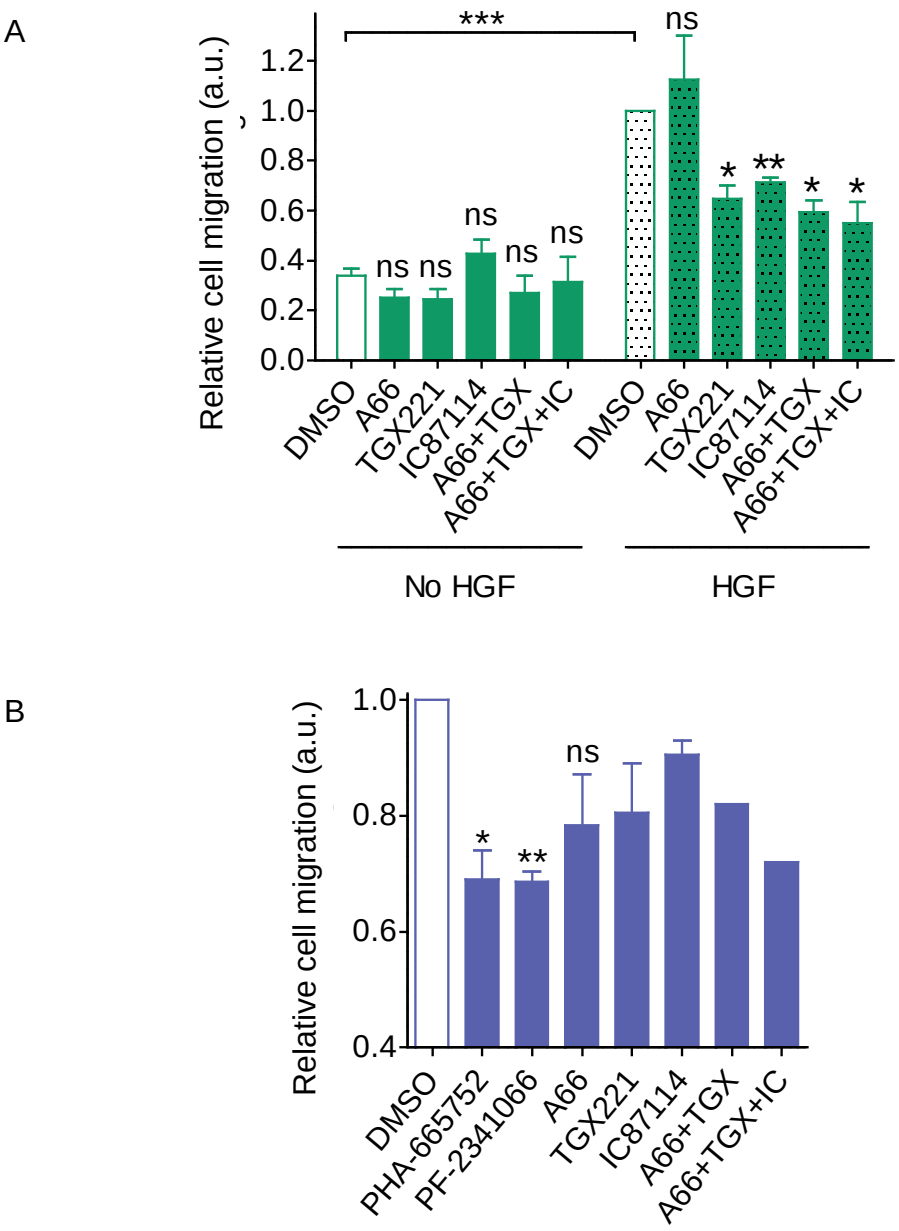


Figure 10: The role of Met and PI3K class I isoforms in the migration of human cancer cells

(A,B) Transwell migration assays. **(A)** 20,000 A549 cells stimulated or not with 50 ng/mL of HGF, treated with DMSO, A66 (500 nM), TGX221 (TGX) (40 nM), or IC87114 (IC) (1 μM) alone or combined and incubated for 180 min (n=3). **(B)** 9,000 U87MG cells incubated for 90 min, treated with DMSO, PHA-665752 (n=3) (100 nM), PF-2341066 (n=3) (100 nM), A66 (n=3) (500 nM), TGX221 (TGX, n=2) (40 nM), or IC87114 (IC, n=2) (1 μM) alone or combined (n=1) and incubated for 90 min. Mean values +/- SEM (a.u. = arbitrary units). ns: non significant, *p<0.05, **p<0.01, ***p<0.001.

In addition, the combined inhibition of p110 alpha (with A66) and p110 beta (with TGX221) or of p110 alpha (A66), p110 beta (TGX211) and p110 delta (IC87114) significantly reduces the HGF dependent migration of A549 cells by 40% ($p<0.05$) and 45% ($p<0.05$) respectively **(Fig 10A)**.

In U87MG cells, WT Met is constantly activated by the HGF that these cells produce. Therefore, Met dependent cell migration in these cells was verified by the use of two Met inhibitors: PHA-665752 and PF-2341066. U87MG cell migration is significantly reduced by 30% upon treatment with PHA-665752 or PF-2341066 ($p<0.05$ and $p<0.01$) **(Fig 10B)**. U87MG cell migration is reduced, although not significantly, by 22% upon p110 alpha inhibition (with A66, n=3), by 19% upon p110 beta inhibition (by TGX221, n=2), and by 10% upon p110 delta inhibition (by IC87114, n=2). The combined inhibition of p110 alpha and p110 beta did not appear to further reduce U87MG cell migration as compared to the individual inhibitions (-18%, n=1). However the combined inhibition of p110 alpha, beta and delta reduced U87MG cell migration by 29% (n=1), almost to the same level as obtained by Met inhibition **(Fig 10B)**. As the experiments with TGX221, or IC87114 were however repeated only twice, and the experiments with A66+TGX221, or A66+TGX221+IC87114 were performed only once, this work would need to be completed to draw a clear conclusion.

Altogether, these results indicate that p110 beta inhibition, and to a lesser extent, p110 delta inhibition, reduces the Met dependent migration of both cell lines, although this is so far more obvious in A549 cells. Moreover, p110 beta and delta inhibitors combined, possibly with alpha, appear to further reduce both cells' migration.

p110 alpha inhibition has no effect on A549 cell migration while it tends to reduce U87MG cells' migration although not significantly.

5. mTOR inhibition reduces M1268T Met dependent cell migration

I then investigated the potential role of mTOR in M1268T Met dependent cell migration.

The protein mTOR has been described as active only when included in the complexes mTORC1 or mTORC2. Each of these complexes is regulated distinctly, and has specific effectors. The mTOR inhibitor rapamycin prevents the formation of mTORC1. In some cell lines, it has been described that rapamycin can also inhibit mTORC2, in particular after a long period of treatment, however the mechanism by which rapamycin inhibits mTORC2 is not well understood. The IC50 of Rapamycin *in vitro* for mTOR is ~ 0.1 nM.

In order to determine the optimal concentration of rapamycin to inhibit mTORC1, and to establish if such a concentration has a role on mTORC2 in NIH3T3 cells, I performed a dose-response assay. M1268T Met expressing cells were treated for 24 hours with DMSO as a negative control, LY294002 as a positive control, and a range of increasing doses of rapamycin (from 2 to 20 nM). As read-out of mTORC1 and mTORC2 activity, the level of phosphorylation of their respective effectors was analysed by western blot: (1) p70 S6K for mTORC1, and (2) Akt phospho-site S473 for mTORC2 **(Fig. 11A)**.

The phosphorylation of p70-S6K by M1268T Met expressing cells is reduced by 74% when exposed to rapamycin (from 2 to 20 nM) as compared to DMSO. LY294002 reduces the p70-S6K phosphorylation by 67% **(Fig. 11B)**. The Akt phosphorylation on S473 is progressively reduced by 48% to 58% when cells were treated with 2 to 20 nM

of rapamycin and by 70% with LY294002 as compared to the DMSO treatment **(Fig. 11C)**.

I chose to use rapamycin at 2 nM, which efficiently inhibits mTORC1, and seems to only partially inhibit mTORC2 (allowing some Akt phosphorylation).

In transwell migration assays, rapamycin significantly reduces M1268T Met expressing cells' migration by 38% ($p<0.05$), while it had no significant effect on WT Met expressing cells **(Fig. 12)**.

Therefore, mTOR seems to be involved in M1268T Met dependent cell migration. As PI3K class I is upstream of mTOR, PI3K class I might be regulating mTOR to induce M1268T Met dependent cell migration. M1268T Met might therefore induce cell migration through the PI3K/mTOR pathway.

II. b) The migratory phenotype induced by M1268T Met requires the expression of the PI3K class I isoforms p110 alpha and p110 beta

In Chapter I, it was established that the M1268T Met dependent migratory phenotype (Rac1 relocalisation at the plasma membrane, as a read-out of its activation; and reorganisation of the actin, evaluated by the lack of stress fibres) is inhibited by the drug LY294002 in NIH3T3 WT and M1268T Met expressing cells. However, LY294002 inhibits multiple targets, so here I aimed to identify which targets of LY294002 are involved in the M1268T Met dependent migratory phenotype.

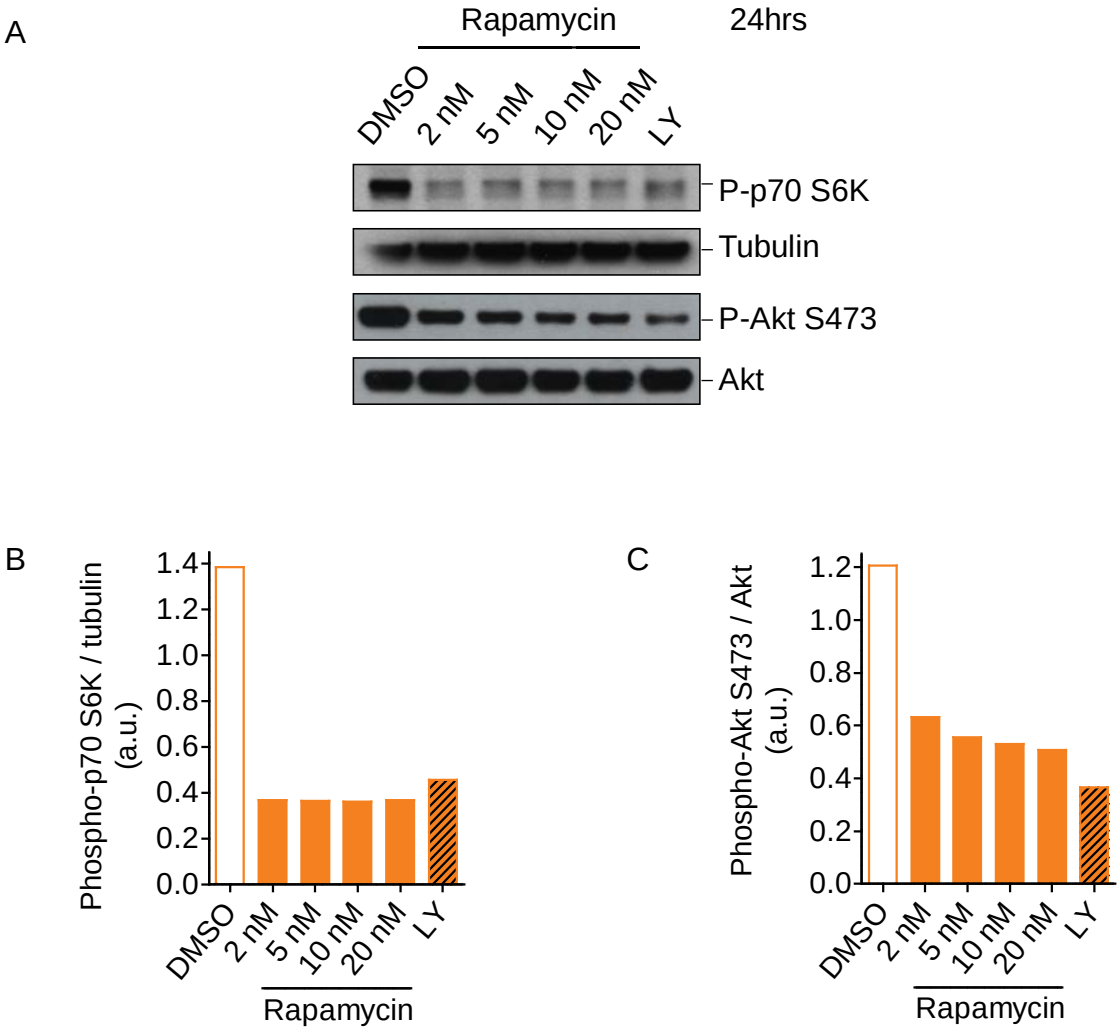


Figure 11: Treatment of M1268T Met expressing cells with rapamycin

(A) Western blots for phosphorylated p70-S6K (Thr389), phosphorylated Akt (S473) (P-Akt), Akt, and tubulin performed on M1268T Met expressing cells treated with DMSO, rapamycin (2 – 20 nM), or LY294002 (LY) (10 μM) for 24 hours. and densitometries obtained for **(B,C)** Quantification of **(B)** phospho-p70-S6K normalised on tubulin, or **(C)** P-Akt S473 normalised on Akt, obtained by densitometry of western blots (a.u. = arbitrary units).

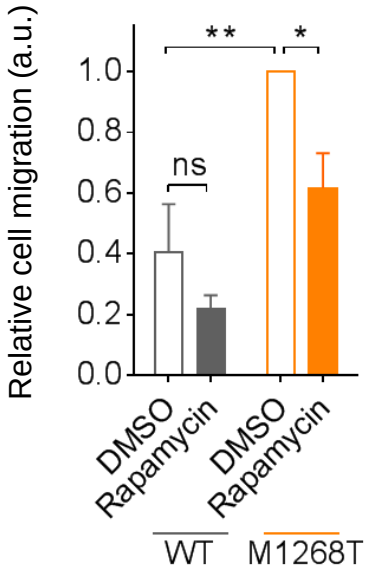


Figure 12: mTOR inhibition reduces migration of M1268T Met expressing cells

Transwell migration assays performed with 9,000 Wild type (WT) or M1268T Met expressing cells treated with DMSO or rapamycin (2 nM). Cells were incubated for 90 min. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, **p<0.01.

1. Inhibition of PI3K class I weakly reduces the migratory phenotype of M1268T Met expressing cells

PI3K class I inhibition with GDC0941 and PI3K p110 alpha and p110 beta inhibition with A66 and TGX221 combined, reduces the number of cells with Rac1 at the plasma membrane in cells expressing M1268T Met (reduction of 19%, $p<0.01$ and $p<0.05$). Although the reduction is weaker than with LY294002, it is statistically significant, and no reduction was observed in WT Met expressing cells **(Fig. 13)**. However GDC0941 and combined A66+TGX221 do not have a significant effect on the number of cells lacking stress fibres whether they express WT or M1268T Met **(Fig. 14)**.

2. The expression of the PI3K class I isoforms p110 alpha and p110 beta, but not Vps34, is required to promote the M1268T Met dependent migratory phenotype

The combined knock-down of p110 alpha and p110 beta efficiently and significantly reduces the M1268T Met dependent migratory phenotype to the WT level: Rac1 localisation at the plasma membrane is reduced by 40% ($p<0.05$), and the number of cells lacking stress fibres is reduced by 38% ($p<0.05$) **(Fig. 13, 14)**. The combined knock-down of p110 alpha and p110 beta is therefore as efficient as LY294002.

However it seems that, to be efficient, the expression of both p110 alpha and p110 beta must be reduced as their individual knock-down has no significant effect on the migratory phenotype of WT and M1268T Met expressing cells **(Fig. 13, 14)**.

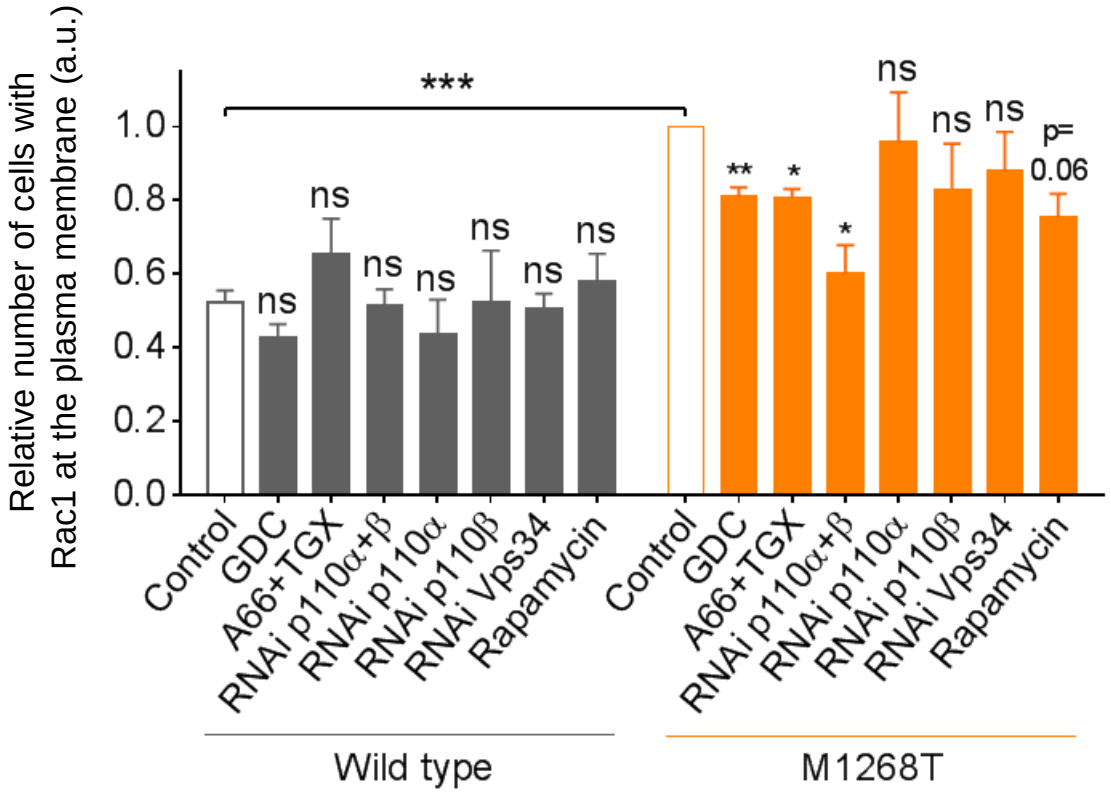


Figure 13: PI3K class I knock-down and inhibition reduces Rac1 localisation at the plasma membrane in M1268T Met expressing cells

NIH3T3 Wild type and M1268T Met expressing cells were treated with DMSO (control), GDC0941 (100 nM) , a combination of A66 (500 nM) and TGX221 (100 nM), or rapamycin (2 nM) for 1 hour; or cells were transfected with negative control (control), p110α, p110β, a combination of p110α and p110β, or Vps34 siRNA. Cells were immunostained with an antibody against Rac1 and stained with DAPI. The percentage of cells with Rac1 at the plasma membrane was counted. 100 cells were counted per condition per experiment. Mean values +/- SEM (GDC: n=4, others: n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, **p<0.01.

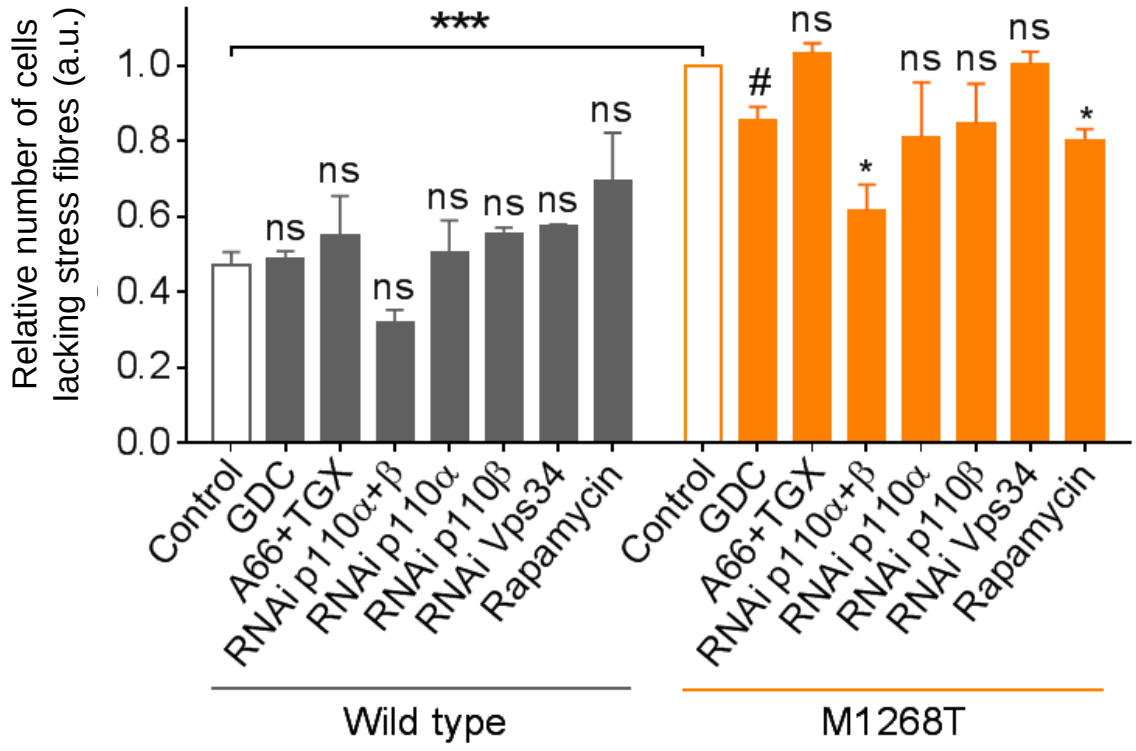


Figure 14: PI3K class I knock-down but not inhibition restores stress fibres in M1268T Met expressing cells

NIH3T3 Wild type and M1268T Met expressing cells were treated with DMSO (control), GDC0941 (100 nM) , a combination of A66 (500 nM) and TGX221 (100 nM), or rapamycin (2 nM) for 1 hour; or cells were transfected with negative control (control), p110α, p110β, a combination of p110α and p110β, or Vps34 siRNA. Cells were immunostained with rhodamin-phalloidin and stained with DAPI. Percentage of cells lacking stress fibres were counted. 100 cells were counted per condition per experiment. Mean values +/- SEM (GDC: n=4, others: n=3) (a.u. = arbitrary units). ns: non significant, #p= 0.05, *p<0.05.

Another target of LY294002 is PI3K class III Vps34. The knock-down of Vps34 did not affect the migratory phenotype of WT and M1268T Met expressing cells **(Fig. 13, 14)**, similarly to what I observed for the cell migration. These observations are consistent with the migration data and indicate further that Vps34 might does not play a role in M1268T Met dependent cell migration, at least in the conditions tested.

Altogether the results obtained with siRNA demonstrate that the expression of p110 alpha and beta together is required for M1268T Met dependent Rac1 localisation at the plasma membrane and the disorganisation of stress fibres. However, these results are not fully reproduced with the PI3K isoform-specific inhibitors and thus are not consistent with the results obtained with the LY294002 compound. One hypothesis is that, as the inhibition on Rac1 and actin obtained with GDC0941 or A66+TGX221 are rather weak, the remaining level of Rac1 activity is sufficient to trigger an optimal stress fibre disorganisation; while the knock-downs lead to stronger reduction of Rac1 activation, which are possibly below the threshold for an efficient actin disorganisation.

Another hypothesis is that in fact, the role of PI3K class I p110 alpha and beta on Met-Rac1 pathway, or moreover on actin remodelling, is partially independent from its kinase activity and may instead play the role of a scaffold. Furthermore, the efficient inhibitions obtained on Rac1 and actin with LY294002 may in fact be due to the inhibition of mTOR instead of class I PI3K.

3. mTOR inhibition with rapamycin reduces more the number of M1268T Met expressing cells lacking stress fibres than PI3K class I inhibition

I then investigated the potential role of mTOR in M1268T Met dependent Rac1 translocation at the plasma membrane and the reduction of actin stress fibres.

Rapamycin reduces the number of M1268T Met expressing cells with Rac1 at the plasma membrane by 25% (although not significantly, p=0.06) **(Fig.13),** and significantly the number of M1268T Met expressing cells lacking stress fibres by 20% ($p<0.05$) **(Fig.14)**, without modifying the migratory phenotype of WT Met expressing cells. Although the effect of rapamycin is not as strong as LY294002, the inhibition of mTOR significantly restores stress fibres in M1268T Met expressing cells, in contrast to the inhibition of PI3K class I, which has an effect on M1268T Met dependent relocalisation of Rac1 at the plasma membrane, but not on actin reorganisation **(Fig. 13, 14)**.

These results might suggest that LY294002 inhibits M1268T Met dependent migratory phenotype by targeting several of its targets. Rac1 relocalisation at the plasma membrane seems to require both PI3K class I and mTOR activity, while M1268T Met induced reorganisation of the actin seems to require mTOR activity, and the expression of p110 alpha and p110 beta.

Altogether however, the same proteins are involved in M1268T Met dependent migratory phenotype and cell migration **(see II. a) Met dependent cell migration is reduced by PI3K class I inhibition, but not by PI3K class III knock-down)**.

II. c) The role of Akt in M1268T Met dependent cell migration and Met dependent phosphorylation of Akt through PI3K class I isoforms

The important role of PI3K class I in the migratory phenotype induced by M1268T Met, and in Met dependent cell migration of several cell lines, directed the investigation toward Akt. It is often assumed that the role of PI3K class I is mediated through Akt signalling.

1. The role of Akt in M1268T Met dependent cell migration

To verify if Akt is involved in M1268T Met dependent cell migration, I performed Transwell migration assays targeting Akt with the available allosteric inhibitor MK2206, with *in vitro* IC50s of 8, 12 and 65 nM for Akt isoforms 1, 2, and 3, respectively. I first determined the optimum drug concentration to inhibit Akt in M1268T Met expressing cells. In addition, I monitored if Akt inhibition affects the activation of p70-S6K, as Akt is upstream of mTORC1. A dose response assay was performed on M1268T Met expressing cells. Cells were treated for 1 hour with DMSO as a negative control, PI3K inhibitor LY294002 as a positive control, or increasing concentrations of MK2206. Western blots were performed on proteins from cell lysates to analyse the level of Akt and p70-S6K phosphorylation, as read-outs of their activation **(Fig. 15)**.

LY294002 reduces the level of Akt and p70-S6K phosphorylation by 62% and 51% respectively. MK2206 at 100 nM reduces Akt phosphorylation by 63%, and by ~83% at higher concentrations (250 nM, 500 nM, and 1 µM). Interestingly, MK2206 also reduces p70-S6K phosphorylation from 54 to 71% **(Fig. 15)**.

Thus, MK2206 seems to efficiently inhibit Akt, which in turn seems to inhibit p70-S6K.

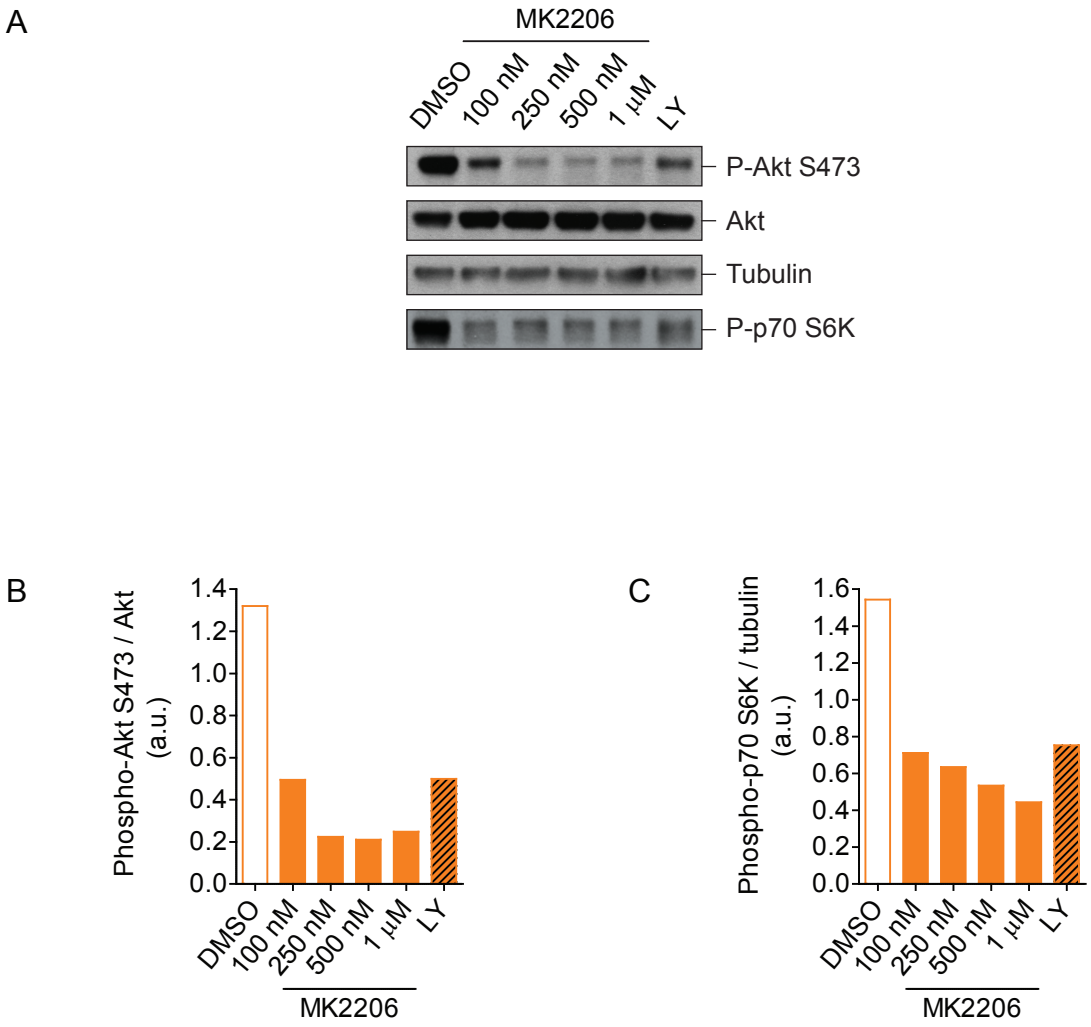


Figure 15: Treatment in M1268T Met expressing cells with MK2206

(A) Western blots for phosphorylated Akt (S473) (P-Akt), Akt, phosphorylated p70-S6K (Thr389), and tubulin performed on M1268T Met expressing cells treated with DMSO, MK2206 (100 nM-1 μM), or LY294002 (LY) (10 μM) for 1 hour. **(B,C)** Quantification of **(B)** P-Akt normalised on Akt and **(C)** phospho-p70-S6K normalised on tubulin, obtained by densitometry of western blots (a.u. = arbitrary units).

Transwell migration assays were performed with NIH3T3 cells expressing WT or M1268T Met, first with a low concentration of MK2206 (250 nM). Despite the strong inhibition of Akt and p70-S6K, the migration of WT and M1268T Met expressing cells was not modified as compared to the DMSO negative control **(Fig. 16A)**. However, at 1 µM MK2206 significantly reduces the migration of M1268T Met expressing cells by 37% as compared to the negative control, and has no effect on WT Met expressing cells ($p<0.05$) **(Fig. 16B)**. Nevertheless, as compared to the inhibition induced by LY294002 **(Chapter I Fig. 6)**, or PI3K class I inhibitors **(Fig. 8)**, MK2206 has a poor effect on M1268T Met dependent cell migration. Moreover, this high dose of MKK2206 probably has a reduced specificity, questioning whether the inhibition in cell migration observed does really result from Akt inhibition. Therefore, the role of Akt on M1268T Met dependent cell migration remains unclear and more investigation is required such as siRNA knock down experiments.

2. WT and M1268T Met dependent phosphorylation of Akt through PI3K class I isoforms

Akt activation downstream of WT Met stimulated by HGF has been widely described in the past, however it has not been shown downstream of M1268T Met. Firstly, I investigated whether or not M1268T Met regulates Akt activation and expression; and secondly, if so, the role of PI3K class I isoforms in this regulation.

In complete medium containing 10% serum, no difference in the level of Akt phosphorylation is detected by western blot between WT and M1268T Met expressing cells. Cells were therefore starved for 1 hour in medium containing no serum and treated with DMSO as a negative control, LY294002, or the Met inhibitor, PHA-665752

(Fig. 17). In these serum-deprived conditions, Akt phosphorylation is significantly higher in M1268T Met than in WT cells ($p<0.001$) and is significantly reduced with LY294002 ($p<0.05$) or the Met inhibitor ($p<0.05$) **(Fig. 17A, B)**. It was also noted that the expression levels of Akt is significantly higher in M1268T Met expressing cells compared to WT Met expressing cells ($p<0.05$) **(Fig. 17A, C)**.

It was concluded that M1268T Met triggers Akt phosphorylation in basal conditions.

Then, I investigated which PI3K class I isoform(s) is/are involved in M1268T Met dependent phosphorylation of Akt.

In the same conditions of starvation, cells were treated with DMSO as negative control, PHA-665752 as positive control, or PI3K class I inhibitors at the concentration determined previously (PI3K class I inhibitor GDC0941 at 100 nM, p110 alpha inhibitor A66 at 500 nM, TGX221 inhibitor p110 beta at 40 nM).

As expected, M1268T Met dependent phosphorylation of Akt is significantly reduced by 73% ($p<0.05$) with the Met inhibitor **(Fig. 18)**. The inhibition of PI3K class I with GDC0941, and the combined inhibition of p110 alpha and p110 beta with A66 and TGX221, have comparable effects to Met inhibition with significant reductions of M1268T Met dependent phosphorylation of Akt by 66% ($p<0.05$) and 76% ($p<0.05$). The effects of individual inhibition of p110 alpha and p110 beta are not statistically significant. However, p110 alpha inhibition (reduces M1268T Met dependent phosphorylation of Akt by 52%) is borderline significant ($p=0.05$) and is greater than p110 beta inhibition (inducing a reduction of 37%, $p=0.07$). These treatments do not seem to modify Akt expression.

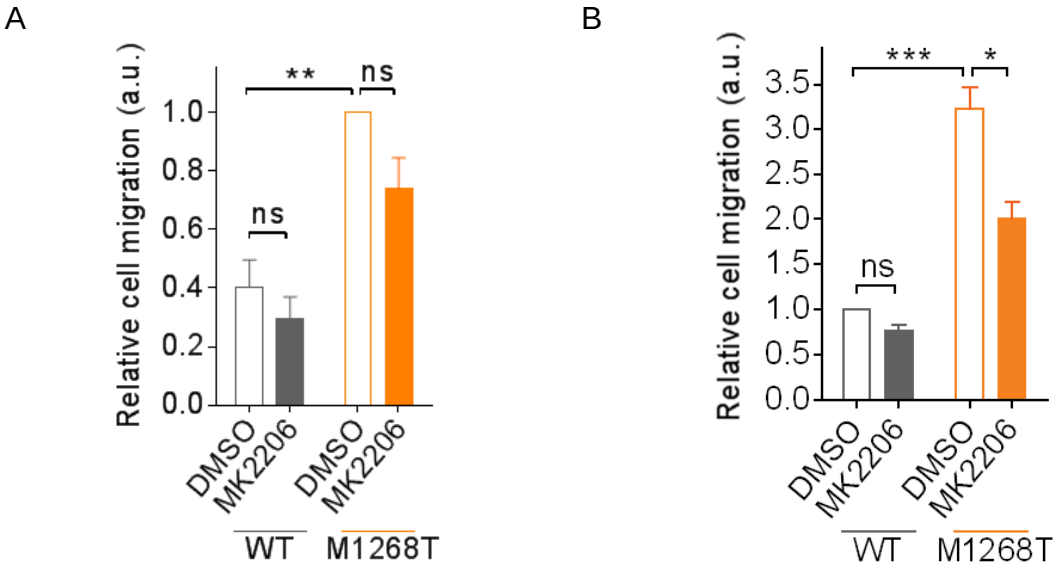


Figure 16: Akt inhibition reduces the migration of M1268T Met expressing cells

Transwell migration assays performed with 9,000 Wild type (WT) or M1268T Met expressing cells treated with DMSO, **(A)** MK2206 (250 nM) or **(B)** MK2206 (1 µM). Cells were incubated for 90 min. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, ***p<0.001.

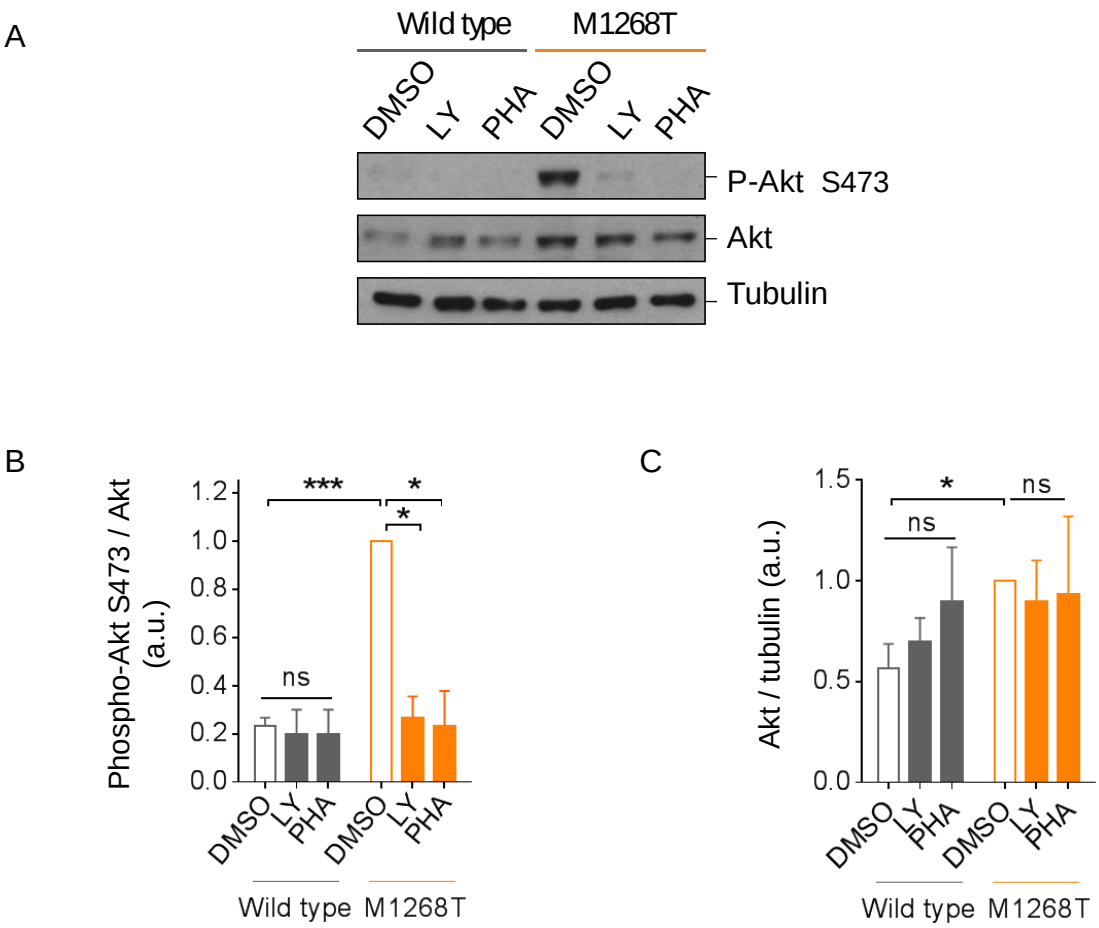


Figure 17: Akt is highly phosphorylated in M1268T Met expressing cells but not in Wild type Met expressing cells

(A) Western blots for phosphorylated Akt (S473) (P-Akt), Akt, and tubulin were performed on NIH3T3 Wild type and M1268T Met expressing cells starved in medium with no serum and treated with DMSO, LY294002 (LY) (10 µM) or PHA-665752 (PHA) (100 nM) for 1 hour. **(B,C)** Quantification of **(B)** Phosphorylated Akt (S473) (P-Akt) normalised on Akt and **(C)** Akt normalised on tubulin, obtained by densitometry of western blots. . Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, ***p<0.001.

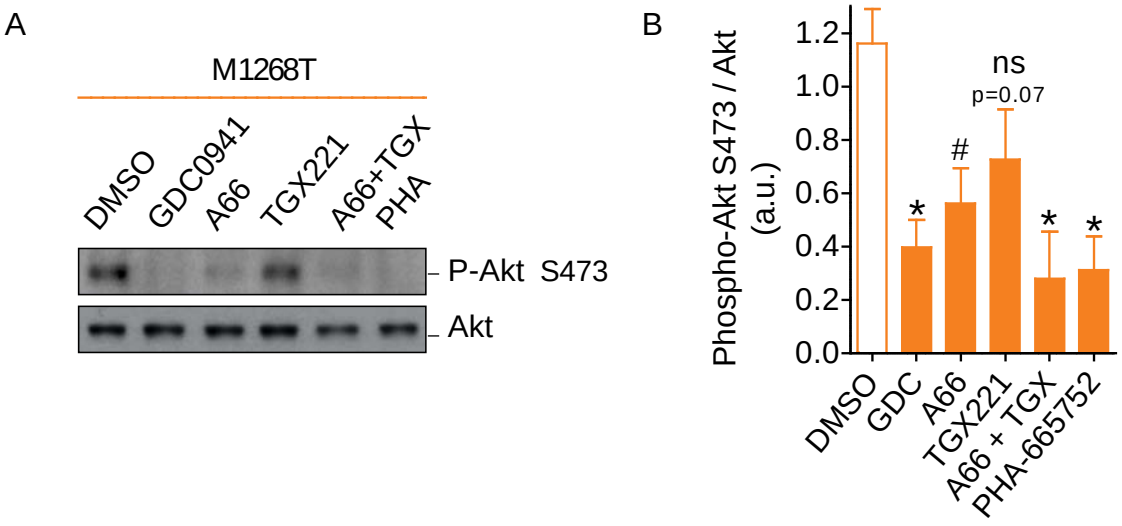


Figure 18: PI3K class I isoform p110 alpha regulates M1268T Met dependent phosphorylation of Akt

(A) Western blots for phosphorylated Akt (S473) (P-Akt), and Akt were performed on NIH3T3 M1268T Met expressing cells starved in medium with no serum and treated with DMSO (negative control), GDC0941 (GDC) (100 nM), A66 (500 nM) and TGX221 (TGX) (40 nM) alone or in combination, or PHA-665752 (PHA) (100 nM) for 1 hour. **(B)** Quantification of phosphorylated Akt (S473) (P-Akt) normalised on Akt, obtained by densitometry of western blots. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, # = 0.05, *p<0.05.

These results suggest that the PI3K class I isoforms, p110 alpha and p110 beta, are together involved in M1268T Met dependent phosphorylation of Akt. The individual inhibition of each isoform tends to reduce the total level of Akt phosphorylation (although not significantly), particularly p110 alpha inhibition. However, the individual inhibition of p110 alpha or beta had no effect on M1268T Met dependent cell migration **(Fig. 8)**.

I aimed to check in human cancer cells whether the PI3K class I isoforms involved in WT Met dependent Akt phosphorylation are the same as those involved in WT Met dependent cell migration **(Fig. 10)**. Therefore, A549 cells were starved for 24 hours, treated for one hour with DMSO as negative control or PI3K class I inhibitors, stimulated or not with HGF for 15 minutes.

A549 cells starved for 24 hours have a very low level of phospho-Akt on S473 as detected by western blot **(Fig. 19)**, which is intensely and significantly increased upon 15 minutes of HGF stimulation. After 120 minutes of HGF stimulation, Akt phosphorylation has decreased (although it is sustained compared to time 0), which is consistent with the observed Met degradation **(Fig. 9)**.

Inhibition of the PI3K class I with the combined drugs GDC0941 (Class I inhibitor), A66 (p110 alpha inhibitor), TGX221 (p110 beta inhibitor) and IC87114 (p110 delta inhibitor) reduces Met dependent phosphorylation of Akt by 95% ($p<0.001$) **(Fig. 20)**. An equivalent level of inhibition is achieved when p110 alpha inhibitor is combined either with p110 beta (-94%, $p<0.001$), or p110 delta (-92%, $p<0.01$) inhibitor, but not when p110 beta and delta are inhibited without inhibiting p110 alpha (-68%, $p<0.05$).

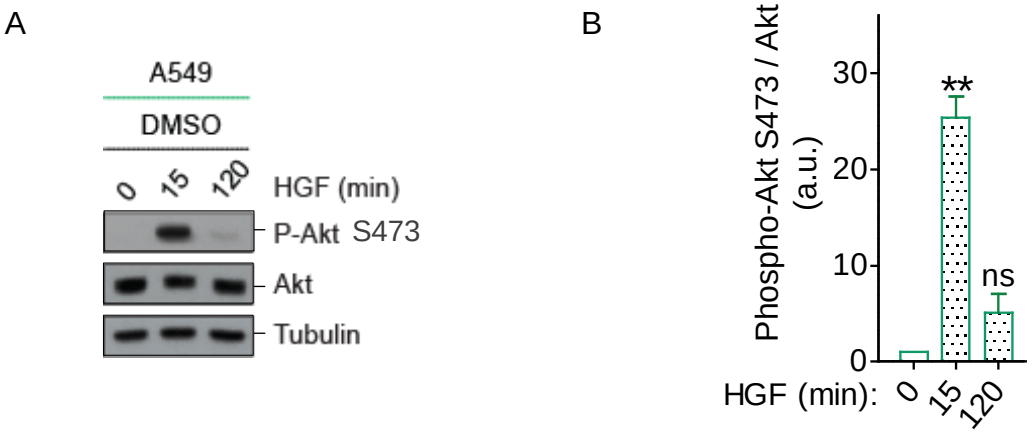


Figure 19: Met activation induces Akt phosphorylation in A549 cells

(A) Western blots for phosphorylated Akt (S473) (P-Akt), Akt and tubulin were performed on A549 starved for 24 hours in medium with no serum and stimulated with HGF (50 ng/mL) for 0, 15 or 120 minutes. **(B)** Quantification of phosphorylated Akt (S473) (P-Akt) normalised on Akt, obtained by densitometry of western blots. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, **p<0.01.

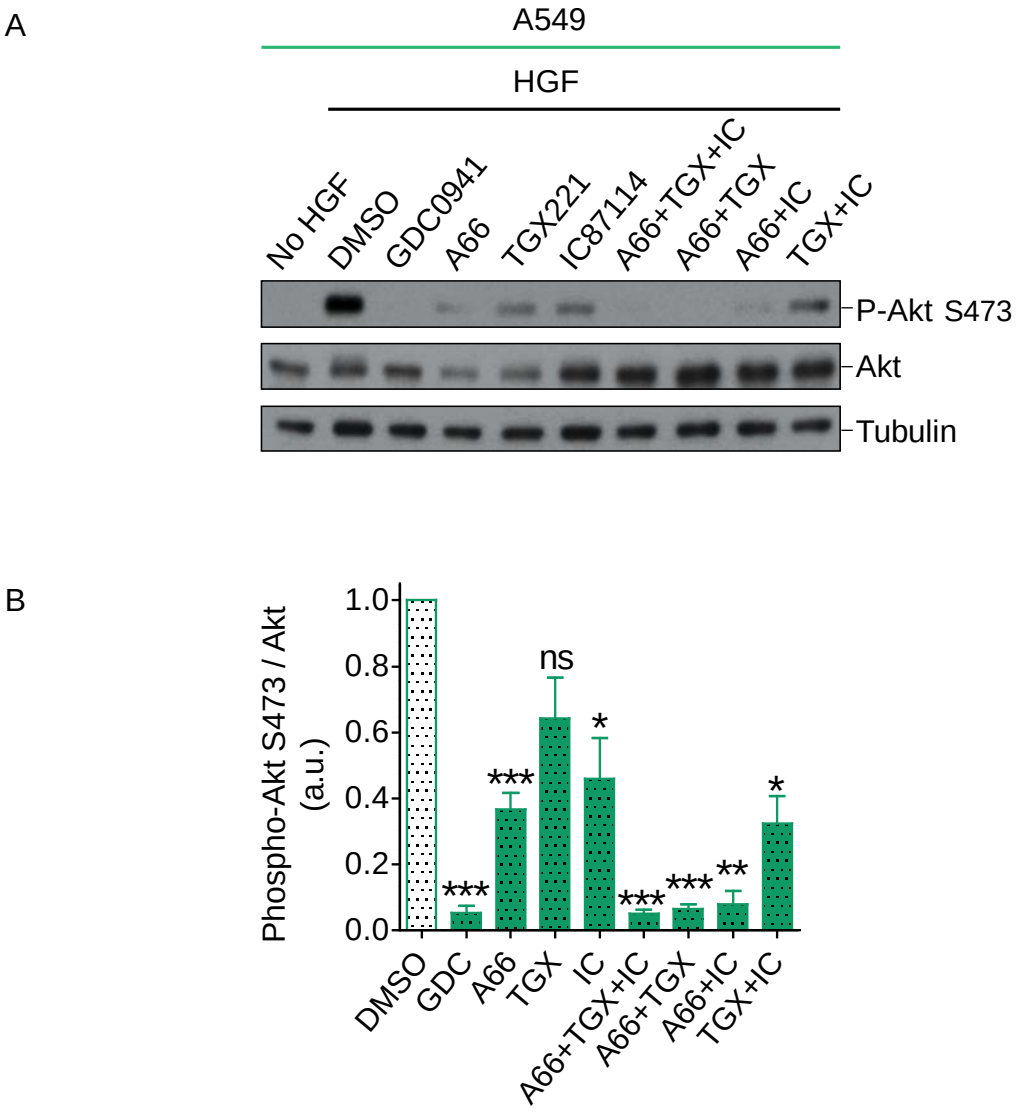


Figure 20: Met induces Akt phosphorylation in A549 cells mainly through p110 alpha

(A) Western blots for phosphorylated Akt (S473) (P-Akt), Akt and tubulin were performed on A549 starved for 24 hours in medium with no serum, treated for 1 hour with DMSO (negative control), GDC0941 (GDC) (100 nM), A66 (500 nM), TGX221 (TGX) (40 nM), or IC87114 (IC) (1 µM) alone or in combination, and stimulated with HGF (50 ng/mL) for 15 minutes. **(B)** Quantification of phosphorylated Akt (S473) (P-Akt) normalised on Akt, obtained by densitometry of western blots. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, **p<0.01, ***p<0.001.

Furthermore, Akt phosphorylation induced by Met is also reduced by 63% ($p<0.001$) when only p110 alpha is inhibited, by 36% (not statistically significant) when p110 beta is inhibited, and by 54% ($p<0.05$) when p110 delta is inhibited. The inhibition of multiple PI3K class I isoforms have a tendency to increase Akt expression levels, however this was not statistically significant. This might indicate a role for Akt to downregulate its own expression when active, thus Akt inhibition would result in the increase of Akt expression.

Thus, WT Met in A549 cells mediates Akt phosphorylation mainly through p110 alpha, which is the only PI3K class I isoform expressed that has no effect in Met dependent cell migration in these cells.

Overall, in the different cell lines studied, it seems that Met activates Akt mainly through p110 alpha and that combining inhibition of alpha with other p110 isoforms such as beta or delta enhances the inhibition.

II. d) Met localisation regulates Met dependent Akt phosphorylation

Met signalling is highly dependent on its endocytosis and trafficking within the cell and Met endosomal signalling regulates cellular outcome. I first tested if the effect observed previously with the pharmacological inhibitors could be due to alteration of Met endocytosis / trafficking.

1. LY294002 does not seem to interfere with M1268T Met internalisation

It has been established that M1268T Met is oncogenic not only because it is constitutively activated, but also because it is constitutively internalised [56]. Interestingly,

the inhibition of phospho-M1268T Met by PHA-665752 does not prevent its internalisation [56].

I performed a biotin internalisation assay to evaluate if LY294002, which inhibits M1268T Met dependent cell transformation **(see Chapter I)**, interferes with M1268T Met internalisation.

While 28% of M1268T Met internalises from the cell surface within 15 minutes when cells are treated with DMSO (negative control), 24% is internalised when cells are treated with LY294002 **(Fig. 21)**.

This poor reduction seems to indicate that LY294002 does not interfere with M1268T Met internalisation. However, could LY294002 interfere with the endosomal sorting of M1268T Met, resulting in a mislocalisation of M1268T Met which prevents its signalling?

2. M1268T Met localisation regulates Akt phosphorylation

I then investigated if Met dependent phosphorylation of Akt requires Met to be present on endosomes.

Once activated, Met internalises via a clathrin dependent pathway, which requires the GTPase dynamin to detach the nascent vesicle from the plasma membrane. The vesicle can then traffic to peripheral and perinuclear endosomes. Dynasore, a pharmacological inhibitor, inhibits the GTPase dynamin, inhibiting the detachment of nascent vesicles from the plasma membrane. Our team has already demonstrated the efficiency of dynasore in preventing M1268T Met internalisation and signalling from endosomes [56]. I used this tool to investigate if M1268T Met activates Akt from endosomes.

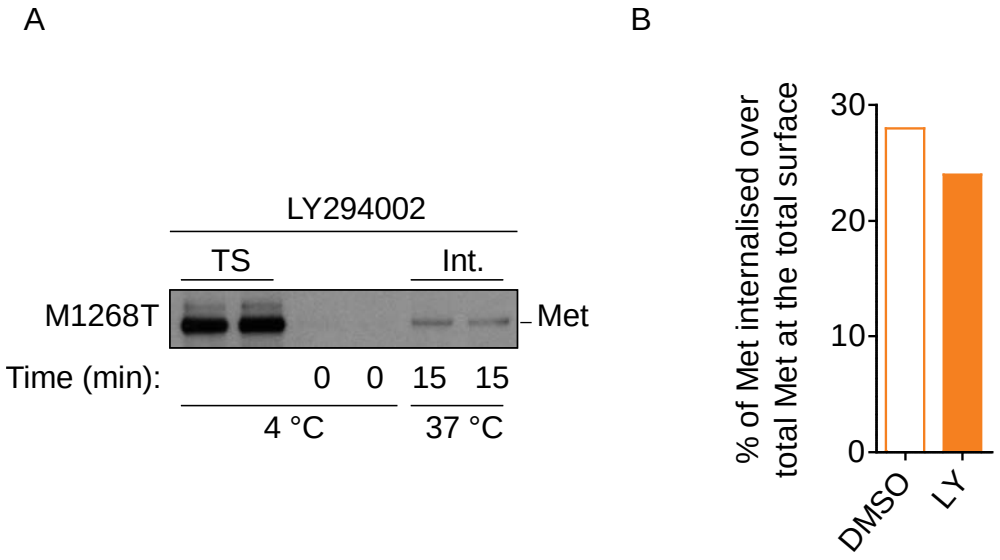


Figure 21: The constitutive internalisation of M1268T Met does not seem to be reduced by LY294002

(A) Biotin internalisation assay was performed on NIH3T3 cells expressing M1268T Met. Cells were pretreated with DMSO or LY294002 (10μM) for 15 minutes, surface biotinylated at 4 C and then either remained at 4 C or were incubated at 37 C for 15 minutes with treatment. Biotin on the cell surface was cleaved (except TS), and the remaining biotinylated proteins (internalised) were pulled down using Strepdavidin beads. **(A)** Western blot was performed for Met, and **(B)** the percentage of internalised Met was calculated (n=1). TS: total surface Met, Int: internalised Met.

NIH3T3 cells expressing M1268T Met were starved for 1 hour in medium with no serum and treated for 30 minutes with dynasore **(Fig. 22)**.

The treatment of M1268T cells with dynasore lead to a reduction of Akt S473 phosphorylation by 73% compared to DMSO (negative control) ($p<0.05$).

This result suggests that M1268T Met needs to be internalised to be able to phosphorylate Akt efficiently.

3. WT Met localisation regulates Akt phosphorylation

I then verified if this is also the case for WT Met in A549 cells. I first confirmed by immunofluorescence that dynasore inhibits WT Met internalisation upon HGF stimulation in these cells. A549 cells, serum starved for 24 hours, were first pre-treated with DMSO (as negative control) or dynasore for 40 minutes and then treated for 0, 15 and 120 minutes with HGF. Cells were then stained with DAPI and an antibody against Met **(Fig. 23)**.

In control conditions, when not stimulated with HGF (0 min), Met is mainly localised at the plasma membrane. After 15 minutes of HGF stimulation, Met has disappeared from the plasma membrane and is present in peripheral vesicles. At 120 minutes, it is mainly located in perinuclear vesicles. In contrast, Met in cells treated with dynasore is constantly located at the plasma membrane. HGF stimulation does not induce Met internalisation anymore. So dynasore prevents HGF stimulated Met internalisation in these cells.

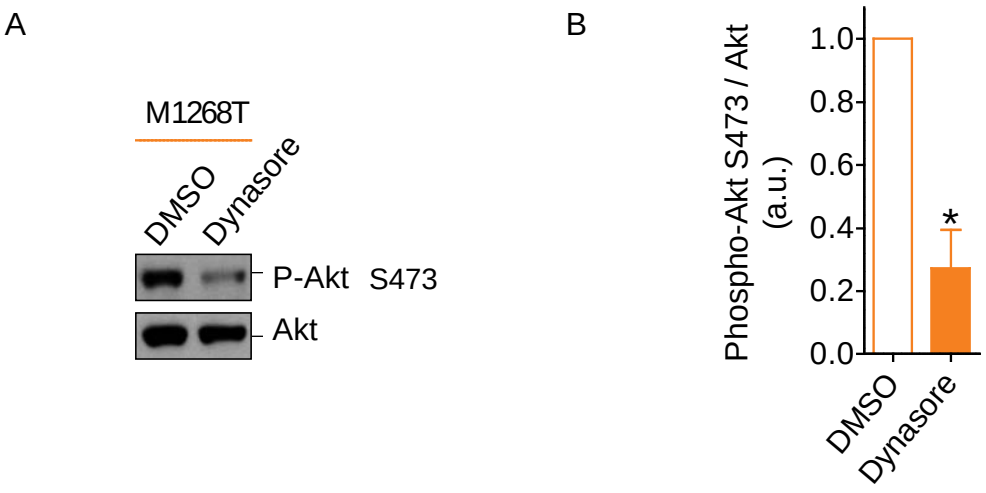


Figure 22: M1268T Met dependent phosphorylation of Akt requires Met internalisation

(A) Western blots for phosphorylated Akt (S473) (P-Akt), and Akt were performed on NIH3T3 M1268T Met expressing cells starved in medium with no serum for 1 hour and treated with DMSO (negative control) or dynasore (80 μM) for 30 minutes. **(B)** Quantification of phosphorylated Akt (S473) (P-Akt) normalised on Akt, obtained by densitometry of western blots. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05.

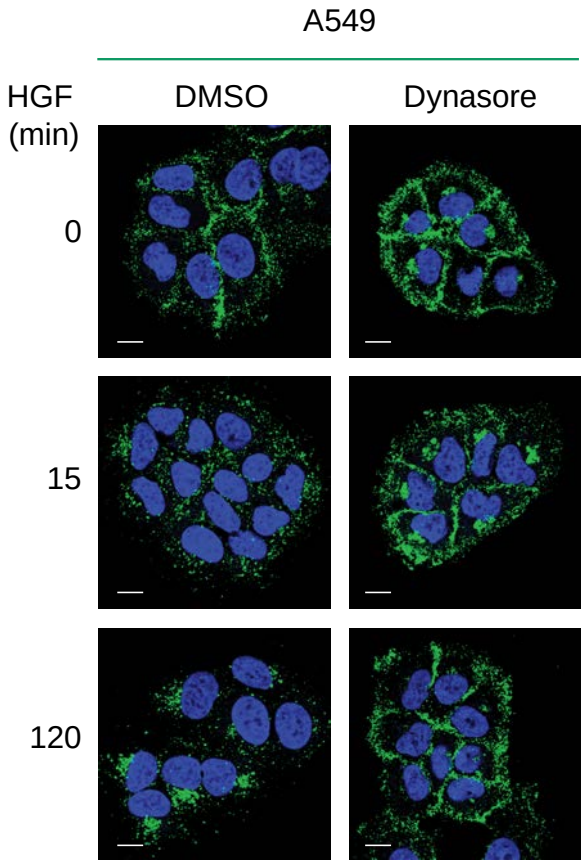


Figure 23: Dynasore inhibits the internalisation of activated Met

Confocal sections of A549 cells immunostained with an antibody against Met (green) and stained with DAPI (blue). Cells, starved for 24 hours in medium with no serum, were pre-treated with DMSO (negative control) or dynasore (80 μM) for 40 minutes and then stimulated with HGF (50 ng/mL) for 0, 15, and 120 minutes.

By western blot, we can observe that dynasore treatment does not reduce HGF stimulated Met phosphorylation **(Fig. 24A)**. With DMSO or dynasore, no phosphorylation of Akt on S473 is detected in absence of HGF stimulation **(Fig. 24A, B)**. After 15 minutes of HGF stimulation, phospho-Akt is strongly and significantly increased ($p<0.01$) in presence of DMSO but not in presence of dynasore ($p=0.06$).

In fact, at 15 minutes of HGF stimulation, dynasore reduces Akt phosphorylation by 48%. After 120 minutes of HGF stimulation, phospho-Akt is poorly detected with both DMSO and dynasore treatment.

These results suggest that in A549 cells, WT Met activated by HGF needs to internalise to phosphorylate Akt **(Fig. 24)**. Interestingly, at 15 minutes of HGF stimulation, WT Met is found in peripheral endosomes **(Fig. 23)**. Thus, in sum, these results suggest that WT Met activates Akt from peripheral endosomes.

II. e) M1268T Met does not need to localise in EEA1 and Appl1 endosomes to signal

Recent results suggest that the nature of the endosome on which Met signals influences Met signalling. I aimed to investigate which endosome(s)| M1268T Met signals from.

1. M1268T Met and EEA1 colocalisation requires Vps34

Once internalised M1268T Met colocalises with EEA1, marker of early endosomes [56] **(Fig. 25A)**. In order to evaluate if the PI3K pharmacological inhibitors alter Met endosomal localisation, which could possibly alter its signalling, I quantified Met and EEA1 colocalisation in WT and M1268T Met expressing cells treated with DMSO as a negative control, or LY294002 **(Fig. 25B)**.

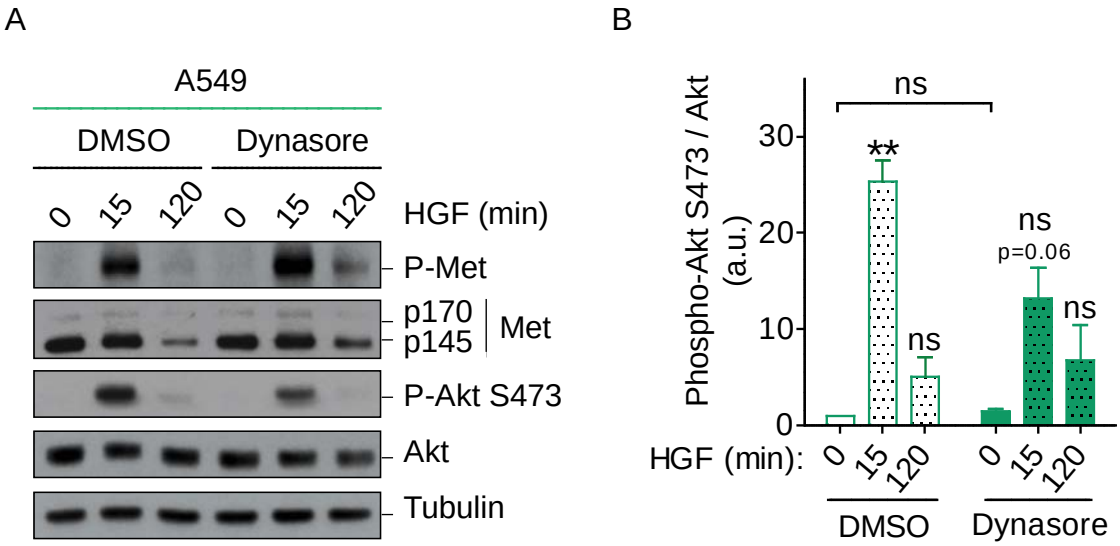


Figure 24: Wild type Met dependent phosphorylation of Akt requires Met internalisation

(A) Western blots for phosphorylated Met (Y1234/5) (P-Met), Met (p170, the precursor form and p145, the mature form of the beta chain), phosphorylated Akt (S473) (P-Akt), Akt, and tubulin were performed on A549 cells. Cells were starved in medium with no serum for 24 hours, then pretreated with DMSO (negative control) or dynasore (80 μM) for 40 minutes, followed by stimulation with HGF (50 ng/mL) for 0, 15, and 120 minutes. **(B)** Quantification of phosphorylated Akt (S473) (P-Akt) normalised on Akt, obtained by densitometry of western blots. Mean values +/- SEM (n=3) (a.u. = arbitrary units). Unless indicated, statistics are compared to 0 min of the same treatment. ns: non significant, *p<0.05, **p<0.01.

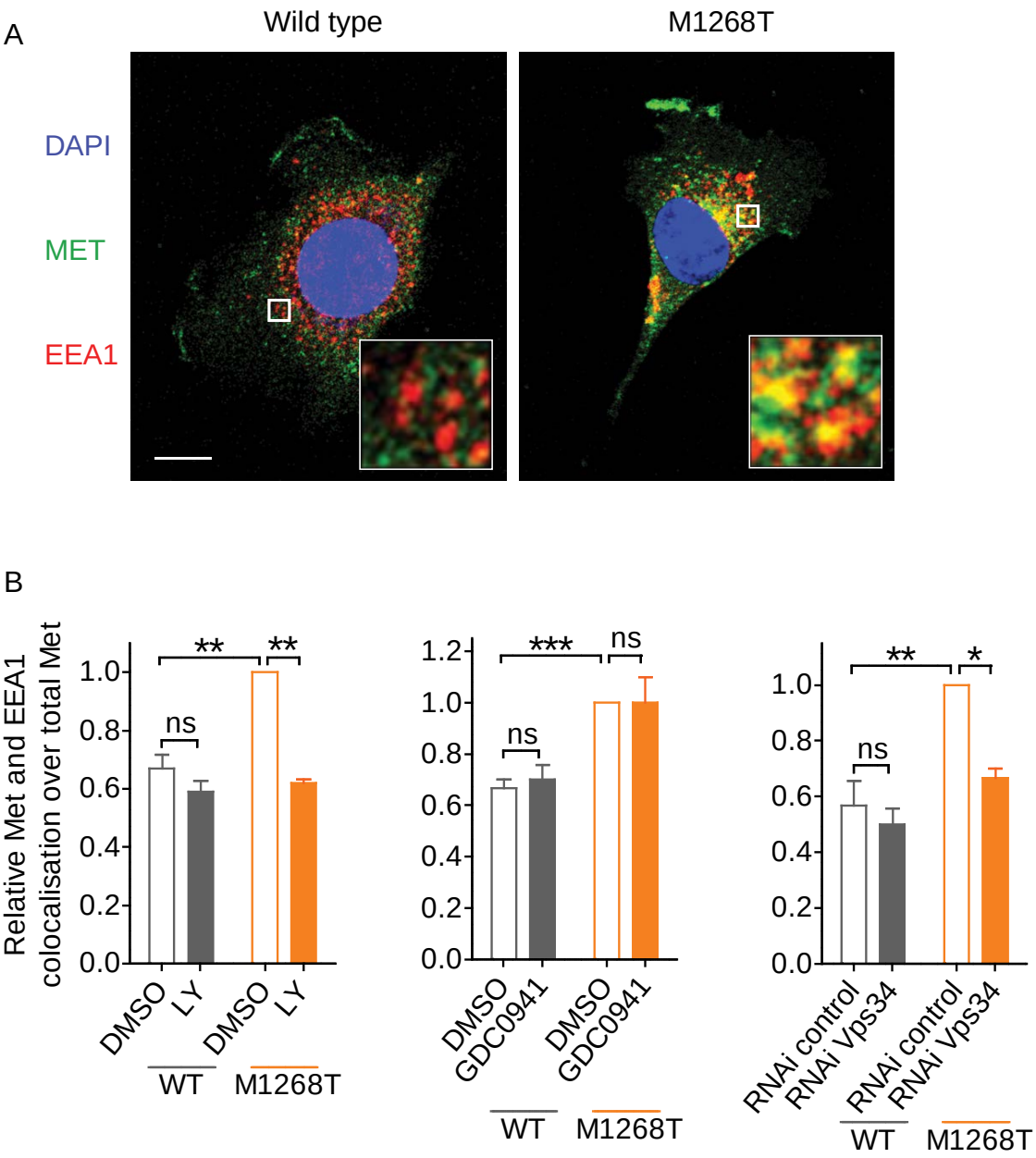


Figure 25: Inhibition of M1268T Met and EEA1 colocalisation by LY294002 is mediated by inhibition of Vps34

(A) Confocal sections of NIH3T3 Wild type and M1268T Met expressing cells immunostained with antibodies against Met (green), and EEA1 (red), and stained with DAPI (blue). **(B)** Quantification of Met and EEA1 colocalisation in cells treated with DMSO, LY294002 (LY) (10 µM), GDC0941 (100 nM) for 1 hour, or transfected with negative control or Vps34 siRNA. The percentage of colocalisation was calculated using Metamorph software. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, **p<0.01, ***p<0.001. Scale bar=10µm.

In control conditions, M1268T Met colocalises with EEA1 50% more than WT Met ($p<0.01$). This increase of Met-EEA1 colocalisation is lost upon LY294002 treatment ($p<0.01$), suggesting that one of the LY294002's targets is responsible for the recruitment of M1268T Met into EEA1 positive endosome.

Amongst LY294002's targets: PI3K class I p110 beta has been shown to be present in Clathrin-Coated Vesicles and regulate the internalisation of EGFR and IGFR; PI3K class III Vps34 is involved in endosomal sorting and maturation of Appl1 endosomes to EEA1 endosomes; The role of mTOR has not been investigated as, to our knowledge, no report suggests its potential implication in endosomal sorting.

Inhibiting PI3K class I with GDC0941 has no effect on WT / M1268T Met and EEA1 colocalisation; however the knock-down of Vps34 reduces the colocalisation of M1268T Met and EEA1 (-33%, $p<0.05$) to the same level as WT Met and EEA1 colocalisation, on which it does not have any effect **(Fig. 25B)**.

These results indicate that the effect of LY294002 on M1268T Met and EEA1 colocalisation is mainly mediated through the inhibition of Vps34, with no apparent role played by PI3K class I. However, Vps34 knock-down has no effect on M1268T Met dependent cell migration **(Fig. 3)**. These results suggest that M1268T does not need to localise in EEA1 positive endosomes to trigger cell migration.

2. M1268T Met induces Akt phosphorylation independently of Appl1 expression

Akt was shown to signal on endosomes, regulated by Appl1. Appl1 binds to Rab5 on the "Appl1 endosomes", corresponding to peripheral endosomes that mature to Early "Endosome Antigen 1 (EEA1) endosomes" where EEA1 binds to Rab5 instead of Appl1. Appl1 endosomes were proposed to act as platforms allowing the assembly of

complexes leading to MAPK and Akt activation [224, 414, 415]. The conversion from Appl1 to EEA1 is promoted by Phosphatidylinositol-3,4,5-triPhosphate (PI3P) [399], a product of Vps34 (PI3K class III). Interestingly, HGF dependent Akt phosphorylation is reduced in MEF cells generated from mice knock-out for Appl1, as compared to MEF cells from WT mice [339].

To verify whether M1268T Met phosphorylates Akt through Appl1, Appl1 was knocked-down in NIH3T3 cells expressing WT or M1268T Met **(Fig. 26A)**.

As expected, in control condition (control siRNA), Akt is strongly phosphorylated in M1268T Met but not in WT Met expressing cells **(Fig. 26A, B)**. The knock-down of Appl1 had no influence on Akt phosphorylation in M1268T Met cells. Moreover, it does not significantly modify M1268T Met dependent cell migration **(Fig. 26C)**.

These results indicate that M1268T Met does not signal to Akt in Appl1 endosomes, Consistent with this, I did not observe M1268T Met colocalisation with Appl1 (not shown).

Thus the question remains open: from which compartment does M1268T Met signal? Future studies will be required to determine this.

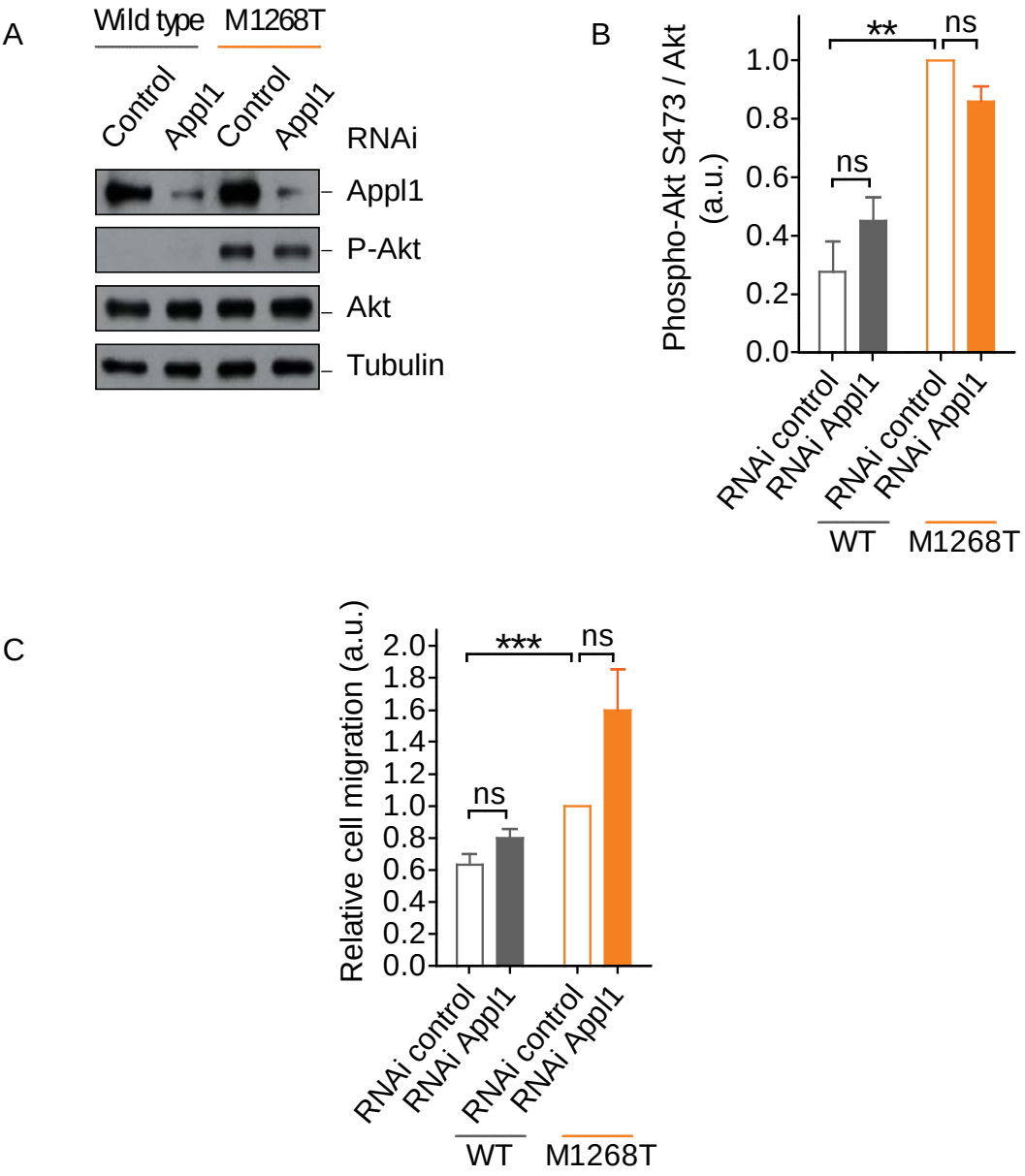


Figure 26: Appl1 knock-down does not reduce M1268T Met dependent Akt phosphorylation and cell migration

(A-C) NIH3T3 Wild type (WT) and M1268T Met expressing cells were transfected with negative control or Appl1 siRNA. **(A)** Western blots for Appl1, phosphorylated Akt (S473) (P-Akt), Akt, and tubulin were performed on cells starved in medium with no serum for 1 hour. **(B)** Quantification of phosphorylated Akt (S473) (P-Akt) normalised on Akt, obtained by densitometry of western blots. **(C)** Transwell migration assays performed with 9,000 cells and incubated for 90 min. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, **p<0.01, ***p<0.001.

DISCUSSION – CHAPTER II

PI3K class I isoforms promote Met dependent cell migration and Akt activation

The PI3K family includes 3 classes. It is now generally accepted that the PI3K class I triggers RTK signalling including downstream of EGFR [144] or IGFR [233]. However the precise role and contribution of each isoform is poorly understood and remains to be defined. Moreover, nothing was known regarding Met signalling. Therefore, my work aimed to determine which PI3K isoform(s) plays a role in the signalling of Met (M1268T mutant and endogenous WT).

The inhibition of cell transformation induced by M1268T Met with the pharmacological inhibitor LY294002, which inhibits PI3K class I/III and mTOR, led me to investigate which of LY294002 target(s) are implicated in Met signalling. I first confirmed the role of PI3K in Met dependent cell migration by using wortmannin, another PI3K inhibitor, and extended my research to understand which PI3K isoform(s) regulate Met signalling.

In this chapter, I provided a detailed investigation on M1268T Met signalling through PI3K class I to induce a migratory phenotype, cell migration and Akt activation, and I aimed to determine where this signalling occurs in the cells. I further investigated some of the results obtained in human cancer cells. Finally, I excluded any potential role of Vps34 (PI3K class III) in M1268T Met dependent cell migration and I defined the role of mTOR in the migratory phenotype and cell migration induced by M1268T Met.

II. a) PI3K isoforms that are expressed in the cell models used

The PI3K family is classified in 3 classes and divided into 8 isoforms. Most of these isoforms are ubiquitously expressed and some are tissue-dependent.

In this work, I used 3 different cell models: (1) NIH3T3 cells stably transfected to express either the constitutively active M1268T Met mutant, leading to cell transformation, or WT Met as a control **(see Chapter I)**; (2) A549, non-small lung cancer (NSLC) cells, expressing WT Met that is acutely activated by HGF; (3) U87MG, human glioblastoma-astrocytoma, epithelial-like cell line, presenting a Met-HGF autocrine loop. All of the cell models used express the ubiquitous PI3K isoforms p110 alpha and p110 beta (class I), C2 alpha and C2 beta (class II), and Vps34 (class III). Interestingly, A549 and U87MG cancer cells also express the isoform p110 delta (class I). Importantly, the expression of these isoforms is not regulated by Met activity **(Fig. 2 *p.137*, and 9 *p.150*)**. The expression of p110 delta in normal cells is assigned to immune cells, and over-activation of p110 delta has been detected in haematological malignancies. The overexpression of p110 delta has been observed in only a few types of cancer (glioblastoma, prostate carcinoma, neuroblastoma, and breast cancer) [416], but not in lung cancer. The role of p110 alpha in cancer has been well established but p110 delta is also emerging as a potential target for cancer therapy.

II. b) Tools to study PI3K/Akt/mTOR pathway

Many studies on PI3K signalling downstream of Met have involved the use of the pharmacological inhibitor LY294002. Although it is a pan PI3K inhibitor, it was somehow considered as a PI3K class I inhibitor but it is now clear that LY294002 inhibits efficiently PI3K class I and III and moreover mTOR. PI3K class II is not

inhibited (or poorly) by LY294002. Nowadays, studies involve different techniques such as genetic means and novel specific inhibitors to confirm the role of PI3K downstream of Met. Older studies in which only LY294002 was used to study the role of PI3K downstream of Met might actually be incomplete to allow clear conclusions considering that the effect mediated by LY294002 at high concentrations (10 to 50 µM) could be due to mTOR activity instead of PI3K, or off targets. Thus an alternative method is to use the inhibitor wortmannin which inhibits PI3K class I and III efficiently, with a poor effect on mTOR if used at 100 nM or less.

LY294002 and wortmannin high potential in therapy for cancer and autoimmune diseases encouraged pharmaceutical companies to develop more potent and selective inhibitors for PI3K classes and isoforms, in particular PI3K class I, which is the most understood class [340]. I took advantage of the availability of these inhibitors to study the role of PI3K class I isoforms downstream of Met. I used GDC0941 as a PI3K class I inhibitor, and A66, TGX221 and IC87114 to selectively inhibit the PI3K class I isoforms p110 alpha, p110 beta, and p110 delta, respectively. Importantly, I first investigated for each drug the optimal concentration to use, in order to strongly inhibit the main target but also to reduce as much as possible any off-target effects. This was particularly important as my aim was to identify the specific role of each isoform. As a read-out of P3K class I activity, I used the phosphorylation of Akt. The drugs were tested on NIH3T3 cells expressing M1268T Met in complete medium to evaluate their efficiency in full serum condition.

Wortmannin was chosen to be used at 100 nM, a concentration which strongly reduces phospho-Akt **(Fig. 1 *p.135*)**, consistent with the literature. Importantly, Wortmannin, in contrast to LY294002, does not inhibit mTOR at the concentration chosen. PI3K class

II, and in particular C2 alpha, is also not inhibited by wortmannin. To date, no pharmacological inhibitor is available for PI3K class II. GDC0941 concentration was chosen at 100 nM, to be slightly permissive and thus allow basal cell functions **(Fig. 4 *p.143*)**. A66 and TGX221 were chosen at concentrations that reduced acute stimulation induced by LPA or HGF **(Fig. 5 *p.144*, and 7 *p.146*)**, which were at 500 and 100 nM respectively. TGX221 at 100 nM had an effect on basal cell migration of NIH3T3 cell expressing WT Met. For this reason and after testing several concentrations, the final concentration chosen was 40 nM of p110 beta inhibitor to inhibit Met dependent activation.

In addition to PI3K class I inhibition, I tested the inhibition of the PI3K effectors Akt, with MK2206 **(Fig. 15 *p.164*)**, and mTORC1, with rapamycin **(Fig. 11 *p.156*)**.

Akt indirectly regulates the activation of the complex mTORC1. MK2206 reduces Akt and mTORC1's effector S6K activation at low concentrations such as 100 nM. It is often claimed that Akt is involved in Met dependent cell migration [417], however I found that MK2206 had no effect on M1268T Met dependent cell migration except partially at high concentration such as 1 µM **(Fig. 16 *p.167*)**. These results suggest two hypotheses: (1) MK2206 does not inhibit Akt function efficiently at low concentrations although this is unlikely as Akt phosphorylation appears to be strongly reduced; (2) Akt has no role in M1268T Met dependent signalling to induce cell migration, and at high concentrations, MK2206 has unspecific targets which are in part responsible for M1268T Met dependent cell migration **(see II. d) Re-evaluation of the role of Akt downstream of Met)**. Further work using siRNA to knock-down Akt isoforms is required to fully understand whether Akt, and which isoform(s), mediate Met dependent cell migration.

mTORC1 activates S6K which has its own effectors to induce protein synthesis. However S6K also triggers a feed-back loop which downregulates the PI3K/Akt pathway. Rapamycin efficiently inhibits the formation of mTORC1 which prevents the phosphorylation of S6K, however long exposure to rapamycin also inhibits mTORC2 and thereby phospho-Akt by a mechanism poorly understood. NIH3T3 cells expressing M1268T Met that were treated with rapamycin at 2 nM for 24 hours, had phospho-S6K strongly reduced and only partially inhibited mTORC2.

The study of each inhibitor used was important to define the right balance between drug specificity and off-targets effects. However, it has to be noted that it is difficult to be sure that the chosen dose for a given inhibitor is really the most optimal / specific in our cell systems.

Alternatively, knock-downs were performed for PI3K class I and PI3K class III. Importantly, the knock-down of one PI3K class I isoform did not seem to induce the up-regulation of the other **(Fig. 8B *p.148*)**.

II. c) Targeting PI3K class I acting downstream of Met in cell migration

1. Both isoforms, p110 alpha and p110 beta, induce cell migration triggered by the M1268T Met mutant

The inhibition of pan-PI3K class I by wortmannin and GDC0941 fully inhibits the migration of M1268T Met mutant expressing cells **(Fig. 1 *p.135*, and 4 *p143*)**. Therefore, it was concluded that either one or both of the two PI3K class I isoforms

expressed in these cells, p110 alpha and p110 beta, trigger the cell migration induced by the M1268T Met mutant.

Neither the individual inhibition of p110 alpha and p110 beta nor their individual knock-down affected M1268T Met expressing cell migration **(Fig. 8 *p.148*)**. In contrast, when both isoforms are targeted at the same time, either by inhibition or by knock-down, M1268T Met dependent cell migration is fully reduced similar to GDC0941. When one isoform alone is downregulated, the second isoform may be activated by the M1268T Met mutant, which indicates a possible isoform redundancy between p110 alpha and p110 beta to induce M1268T Met mutant cell migration. Due to the fact that the combined downregulation of both p110 alpha and p110 beta fully decreases the cell migration that is induced by the M1268T Met mutant, this suggests that the p110 delta isoform, which is poorly expressed in the NIH3T3 cells, has no role in M1268T Met mutant cell migration in these cells. However, it may be interesting to verify this using p110 delta inhibition / siRNA knock down.

2. PI3K class I isoforms that are involved in endogenous WT Met dependent cell migration in human cancer cells

I determined that the M1268T Met mutant in NIH3T3 induces cell migration through all the PI3K class I isoforms expressed. Is this specific to M1268T Met mutant or does WT Met signal the same way? And, is this signalling dependent on the cell type? I used two different cancer cell lines with endogenous WT Met and, as NIH3T3, expressing p110 alpha and p110 beta, but also p110 delta.

In A549 cells, p110 alpha was not found to be involved in Met dependent cell migration **(Fig. 10A *p152*)**. The inhibition of either p110 beta or p110 delta similarly reduced Met

dependent cell migration, and the combination of p110 beta and delta inhibition did not have a synergic effect. Interestingly, the combined inhibition of all PI3K class I isoforms, while triggering the strongest inhibition, still did not fully reduce the Met dependent cell migration suggesting an additional role for another signalling pathway. The MAPK pathway has often been described downstream of Met and is involved in Met dependent cell migration [47]. Moreover, numerous studies demonstrate the cross-talk between the PI3K and MAPK pathways [418]. The hypothesis that the MAPK pathway is involved with PI3K class I to induce Met dependent cell migration in A549 cells could be checked by inhibiting the MAPK pathway with a pharmaceutical inhibitor. Another possibility is mTOR, independently of PI3K, as we found that mTOR plays a role in M1268T Met dependent NIH3T3 cell migration. This could be investigated using Rapamycin alone, or in combination with the three p110 isoform drugs.

In U87MG cells, although some experiments need to be repeated to obtain n=3, the results obtained so far indicate that the inhibitions of p110 alpha or p110 beta tend to induce a similar modest reduced cell migration (although not significant so far for p110 alpha) **(Fig. 10B *p.152*)**. Their combined inhibition did not have a synergic effect. However, the combined inhibition of p110 delta, p110 alpha and p110 beta give more promising results in the single experiment performed so far, where U87MG cell migration was reduced to the same level as with Met inhibitors. These results suggest that all PI3K class I isoforms are involved in U87MG cell migration or that they have a redundant role and thus all need to be inhibited. To confirm that the effects observed are downstream of Met, these assays should be reproduced with PI3K class I isoforms and Met inhibitors, combined. If the results observed are Met dependent **(Fig. 10B *p.152*)**, then when Met is inhibited, PI3K class I inhibition should not reduce the cell migration any further in U87MG cells.

Thus we can conclude that all p110 isoforms tested (alpha, beta and delta) play a role in Met (WT or mutant) dependent cell migration.

However, there are some differences: (1) while the combined inhibition of p110 alpha and beta are required to efficiently inhibit M1268T Met dependent NIH3T3 cell migration, inhibiting a single isoform can lead to significant reduction of WT Met dependent A549 cell migration (beta or delta) and trigger some reduction (although not significant) in U87MG cells (alpha or beta); (2) inhibiting a given single isoform can lead to very different effect in our three cell models. Hence, p110 alpha inhibition on its own has no effect at all in M1268T Met / WT Met dependent NIH3T3 / A549 cell migration cells while it tends to decrease WT Met dependent U87MG cell migration. Furthermore, p110 beta inhibition on its own has no effect in NIH3T3 cells but leads to a significant reduction of WT Met dependent cell migration in A549 cells.

Finally, our results so far suggest that in the three cell models, combined inhibition of all the p110 isoforms expressed triggers the best inhibition of Met dependent cell migration.

In this work I studied three different mechanisms for Met activation: a constitutive mutation, activation upon exogenous HGF, and an autocrine-loop. The limitation of this work is that the three types of Met signalling were studied in three different cell lines. Further work will confirm if PI3K class I isoforms that are involved in Met dependent cell migration depend on the cell type or really on the mode of Met activation (or both). For example, NIH3T3 cells expressing WT Met stimulated with exogenous HGF could be compared to M1268T Met expressing cells. Nevertheless, my results are important as they report unknown mechanisms of Met signalling to PI3K in human cancer cells.

Strikingly, p110 alpha activity, known to act downstream of RTKs, appears dispensable for M1268T / WT Met dependent NIH3T3 / A549 cell migration. In contrast to p110 alpha, p110 beta has mostly been described downstream of GPCRs, however it has been reported to also act downstream of RTKs [143]. Interestingly, p110 beta seems to be involved in Met dependent cell migration in all the cell models studied, similarly for p110 delta when expressed. The role of p110 delta is intriguing as this isoform is usually expressed in haematopoietic cells and associated with immune disease and some cancers; however the expression of p110 delta in other cancers is currently being uncovered [419].

Numerous pharmaceutical inhibitors are currently specifically targeting PI3K class I such as GDC0941. Such treatments could benefit cancer patients suffering from cancer induced by Met (WT and mutant).

3. Role of PI3K class I in the migratory phenotype induced by M1268T Met

Cell motility induced by the Met receptor depends on the Rho GTPase Rac1 activity and on the cell reorganisation induced by Rac1 activity [56, 142]. Cell reorganisation is observed by the loss of F-actin organised stress fibres such as in M1268T Met as compared to NIH3T3 WT Met expressing cells, which display F-actin organised in stress fibres [56]. I demonstrated that the migratory phenotype of the M1268T Met mutant expressing cells was inhibited by LY294002 **(see Chapter I)**.

My results suggest a role for p110 alpha and p110 beta together in the promotion of the cell migratory phenotype induced by M1268T Met mutant. Downstream of the pro-inflammatory cytokine TNF, only p110 alpha seems involved in Rac1 activation through the recruitment of its Guanine nucleotide Exchange Factor (GEF) Tiam1 [141].

Also, in Porcine Aortic Endothelial cells, PDGF or insulin were shown to trigger the cytoskeleton reorganisation through p110 alpha or p110 beta, respectively [143]. Interestingly, Rac was recently reported to instead act upstream of p110 beta. Thus Rac activates p110 beta downstream of GPCR [420], suggesting that, in fact, the pathway can operate in both directions. It would be interesting to investigate whether this mechanism also exists downstream of Met.

The pharmacological inhibition of PI3K class I reduced partially but significantly the relocalisation of Rac1 to the plasma membrane induced by the M1268T Met mutant **(Fig. 13 *p.159*)**. However, the stress fibres were not restored in these conditions **(Fig. 14 *p.160*)**. Nevertheless LY294002 strongly reduced the relocalisation of Rac1 to the plasma membrane and reduced partially but significantly the actin reorganisation **(Chapter I)**. The effect of LY294002 could have been mediated by another PI3K class than class I, but class II is not inhibited by LY294002 and the knock-down of Vps34 had no effect on M1268T Met mutant dependent cell migration **(Fig. 3 *p.139*)**. Therefore the inhibition of mTOR was tested and revealed a partial inhibition of Rac1 relocalisation at the plasma membrane, although not significant, and a partial but significant actin reorganisation downstream of M1268T Met mutant **(Fig. 13 *p159*, and 14 *p.160*)**. Rapamycin treatment lasted 1 hour, the length of time during which rapamycin is supposed to target only mTORC1 formation. Interestingly, mTORC2 has been suggested to meditate Rac1 activation and control the actin cytoskeleton, but not mTORC1 [258]. It would have been interesting to combine both PI3K class I and mTOR inhibition and compare to the level of inhibition obtained with LY294002.

Strikingly however, unlike the combined pharmacological inhibition, the combined knock-down of p110 alpha and p110 beta was able to reduce strongly and significantly

both M1268T Met mutant dependent Rac1 relocalisation at the plasma membrane and actin reorganisation.

From these combined observation, we conclude that p110 alpha and beta co-expression as well as mTOR activity are required in these events.

Two hypotheses can be issued to explain the discrepancy between pharmacological inhibition and siRNA knock-down results: (1) p110 alpha and p110 beta combined pharmacological inhibition (or class I inhibition with GDC0941) lead to a too weak Rac1 inhibition, thereby maintaining a level of Rac1 activation sufficient to trigger the loss of actin stress fibres; (2) PI3K class I alpha and beta activity are not required but only their expression is needed, suggesting that they act as scaffold proteins contributing to Rac1 activation and actin reorganisation. This is unlikely however, as class I inhibition with GDC0941 or combined A66 and TGX221, lead to a robust inhibition of cell migration triggered by M1268T Met **(Fig. 8 *p148*)**, unless class I alpha and beta play additional roles in the cell migration driven by M1268T Met. Further studies are required to clarify this.

4. PI3K class I isoforms involved in Akt phosphorylation that are triggered by Met

Which PI3K class I isoform(s) is/are required for Met dependent activation of Akt has never been studied so far.

To be able to observe Met dependent phosphorylation of Akt, cells were starved to fully reduce basal Akt activation in WT cells. In these conditions, M1268T Met cells still display a high Akt phosphorylation **(Fig. 17 *p.168*)**.

In NIH3T3 cells expressing M1268T Met, the phosphorylation of Akt was fully reduced when both PI3K class I p110 alpha and p110 beta were inhibited together **(Fig. 18 *p.169*)**. However, single inhibitions indicate that p110 alpha plays a bigger role than p110 beta.

In A549 cells stimulated with HGF, the phosphorylation of Akt was fully reduced only when all PI3K class I were inhibited, or when p110 alpha was inhibited in combination with either p110 beta or p110 delta **(Fig. 20 *p.172*)**. The combined inhibition of both p110 beta and delta only partially reduced Akt phosphorylation. Therefore, like in the NIH3T3 cells expressing M1268T Met mutant, p110 alpha seems to have a more important role than p110 beta and p110 delta in activating Akt downstream of Met.

So, interestingly, while in M1268T Met mutant expressing cells p110 beta seems to compensate for p110 alpha inhibition to activate Akt, in A549 cells stimulated with HGF both p110 beta and p110 delta are required to partially compensate p110 alpha inhibition.

Remarkably, similar observations have been made for the phosphorylation of Akt downstream of insulin [421]. The authors described some partial redundancy between the PI3K class I isoforms to phosphorylate Akt, and concluded that the requirement of each PI3K class I isoform to activate Akt was dependent on the level of expression of each isoforms. In NIH3T3 and A549 cells, the relative levels of PI3K isoforms activity could be compared to the isoforms responsible for Akt phosphorylation.

5. Activation of mTORC2 through PI3K class I

Monitoring the phosphorylation of Akt on serine 473 (S473) also provided information on mTORC2 activity as it is responsible for Akt S473 phosphorylation. WT Met

stimulated with HGF in A549 cells and M1268T Met in NIH3T3 cells both increase the phospho-Akt S473 in a PI3K class I dependent manner, in particular p110 alpha **(see II. c) 4. PI3K class I isoforms involved in Akt phosphorylation triggered by Met)**. Therefore, Met (WT and mutant) seems to activate mTORC2 mainly through the PI3K catalytic subunit p110 alpha, and partially through the other isoforms.

II. d) Re-evaluation of the role of Akt downstream of Met

Researchers have often targeted PI3K, frequently with LY294002, which also targets mTOR and at high doses, thus targeting potentially multiple other targets, and used Akt phosphorylation as a read-out [417]. Following such studies, which did not specifically target Akt, often a role for Akt downstream of Met has been assumed. Although, more recently, the role of Akt has been demonstrated in Met dependent cell survival [333, 363], thanks to the use of Akt pharmacological inhibitors, its role in Met dependent cell migration might now have to be reconsidered. My work is the first providing a detailed investigation on how Met signals to Akt, and reconsidering its role downstream of Met dependent cell migration.

Indeed, strikingly, in our three cell systems, p110 alpha is constantly involved in Met dependent Akt phosphorylation **(see II. c) 5. Activation of mTORC2 through PI3K class I)**, but not in Met dependent cell migration **(see II. c) 2. PI3K class I isoforms that are involved in endogenous WT Met dependent cell migration in human cancer cells, and 4. PI3K class I isoforms involved in Akt phosphorylation that are triggered by Met)**. Moreover, the Akt inhibitor MK2206 strongly inhibits Akt phosphorylation detected by western blots at 250 nM **(Fig. 14 *p.160*)**, but has no effect at this dose and only a partial effect at 1 µM on M1268T Met dependent cell migration

(Fig. 16 *p.167*). Altogether, these results suggest that Akt might not be involved in Met dependent cell migration, in contrast to what has been assumed so far. Consistent with this, recent literature on Akt isoform roles in invasion / migration *in vitro* and *in vivo* is controversial [133].

Further investigations are required, such as Akt siRNA knock-down, to establish whether Akt is involved or not in Met dependent cell migration.

II. e) Met endosomal signalling occurs through PI3K class I, but not class III

It is traditionally thought that RTKs signal from the plasma membrane, and get internalised to be degraded. However, the role of RTK internalisation is now being re-considered as an increasing amount of evidences highlights a link between RTK internalisation and signalling. Thus RTKs such as EGFR, insulin, PDGFR or Met, internalise and signal from endosomes to induce cell functions. Our laboratory focuses on Met endosomal signalling and on understanding how Met uses its sorting to different endosomes to activate specific signalling pathways. Joffre *et al.* have shown that the M1268T Met is constitutively internalised and colocalises with Early Endosome Antigen 1 (EEA1), a marker of early endosomes [56]. They demonstrated that M1268T Met needs to be internalised to induce cell transformation *in vitro* and tumourigenesis *in vivo*.

In parallel, the role of PI3K in spatial signalling is starting to be elucidated. The "Adaptor Protein containing PH domain, PhosphoTyrosine Binding (PTB) domain, and Leucine zipper motif 1" (Appl1), a Rab5 effector located in peripheral early endosomes, has been shown to be required for Akt activation. Importantly, Appl1 must be located at the membrane of endosomes to allow optimal Akt activation [224]. An Appl1 mutant,

which mislocalises from endosomal membranes, is unable to phosphorylate Akt and cell survival in zebrafish is consequently reduced.

Interestingly, a recent study reported that Akt phosphorylation upon Met activation with HGF is reduced in MEF cells generated from mice knocked-out for Appl1, as compared to WT MEFs [339].

I therefore investigated whether Akt is activated on endosomes by M1268T Met mutant in NIH3T3 cells or WT Met in A549 cells and if so, from which compartment.

1. Akt phosphorylation may be triggered by Met from endosomes

Joffre *et al.* demonstrated that the inhibition of dynamin, the GTPase responsible for the detachment of nascent vesicles from the plasma membrane, leads to a decreased internalisation of M1268T Met and a consequent inhibition of its signalling to Rac1. One of the tools they used to impair Met internalisation *in vitro* and *in vivo* is the dynamin pharmacological inhibitor dynasore [56]. I used this tool to investigate whether M1268T Met and WT Met in cancer cells need to be internalised to activate Akt. I first demonstrated that dynasore treatment leads to a strong reduction of HGF triggered WT Met internalisation in A549 cells **(Fig. 23 *p.178*)**.

Furthermore, I found that Met dependent phosphorylation of Akt is strongly reduced upon dynasore treatment in NIH3T3 cells expressing M1268T Met and in A549 cells **(Fig. 22 *p.176*, and 24 *p.179*)**, indicating that M1268T Met and WT Met in cancer cells activate Akt when internalised and not from the plasma membrane. However, as dynamin broadly controls endocytosis, these results should be confirmed with the use of more specific tools to inhibit Met endocytosis such as c-Cbl siRNA or a double Met mutant M1268T/N1358H, defective in endocytosis [56].

2. M1268T Met endosomal signalling seems to be independent of Appl1 and EEA1

I decided to first control if the effect mediated by LY294002 could in fact be due to the disruption of M1268T Met internalisation **(Fig. 21 *p.175*)** or endosomal sorting **(Fig. 25 *p.181*),** instead of operating in Met downstream signalling. Thus I performed a kinetic biotin internalisation assay and analysed Met–EEA1 colocalisations by confocal microscopy.

As compared to DMSO, LY294002 did not reduce the internalisation of M1268T Met **(Fig. 21 *p.175*)**. This result was also observed in T47D cells overexpressing WT Met; LY294002 did not inhibit WT Met internalisation upon HGF activation [53]. However, interestingly, the percentage of M1268T Met colocalising with EEA1 was strongly reduced **(Fig. 25 *p.181*)**, indicating a defect in sorting to EEA1 positive endosomes. The identification of LY294002's target involved in M1268T Met and EEA1 colocalisation revealed a role for Vps34, but not for PI3K class I. This was not surprising considering the role of Vps34 in endosomal sorting. However, the fact that Vps34 siRNA knock-down had no effect in M1268T Met dependent cell migration, suggesting that M1268T Met does not signal from EEA1 positive endosomes to stimulate cell migration was more surprising **(Fig. 3 *p.139*)**. Previous studies from the laboratory had assumed, but without clear demonstration, that Met signals from EEA1 positive endosomes to stimulate cell migration [57]. More work is however required to ascertain whether it is M1268T Met that is mislocalised or whether its is EEA1 that is not recruited anymore to the endosome (as Vps34 is responsible for the production of Phosphatidylinositol-3-Phosphate (PI3P) which is required for EEA1 to bind Rab5 [399]). Also, this result was obtained by knocking-down Vps34 as no specific drugs were available. Recently, a

pharmaceutical inhibitor has been made available [341], which could be used to validate these results using a different method.

Due to the role of Vps34 in promoting the conversion of Appl1 endosomes to EEA1 endosomes [399], and the literature suggesting that Met might signal to Akt from Appl1 endosomes [339], I investigated if the knock-down of Appl1 interferes with M1268T Met mutant endosomal signalling. However, the reduction of Appl1 did not modify M1268T Met dependent activation of Akt or cell migration **(Fig. 26 *p.184*)**. It would be interesting to verify by siRNA knock-down if alternatively Appl2 could have a role.

So M1268T does not appear to require Appl1 or EEA1 to fully induce Akt activation and cell migration. Considering that M1268T Met internalisation is not modified by LY294002 and that it was previously demonstrated in the laboratory that M1268T Met degradation is reduced compared to WT Met [56], one hypothesis could be that M1268T Met signals when it recycles from the early endosome to the plasma membrane. This could be tested firstly by evaluating the colocalisation of M1268T Met and Rab4 (marker of recycling from early endosomes), and secondly by inhibiting M1268T Met recycling (possibly by knock-down of GGA3, required for WT Met recycling from early endosomes to the plasma membrane) [78].

In conclusion

In this chapter, I provided for the first time a detailed study of the role of PI3K/Akt/mTOR pathway downstream of mutant M1268T Met signalling in NIH3T3 cells **(Figure 27)** and of WT Met in two human cancer cell lines **(Table 1)**. The table 1 summarises the results obtained for each PI3K class I isoforms in the three cell lines.

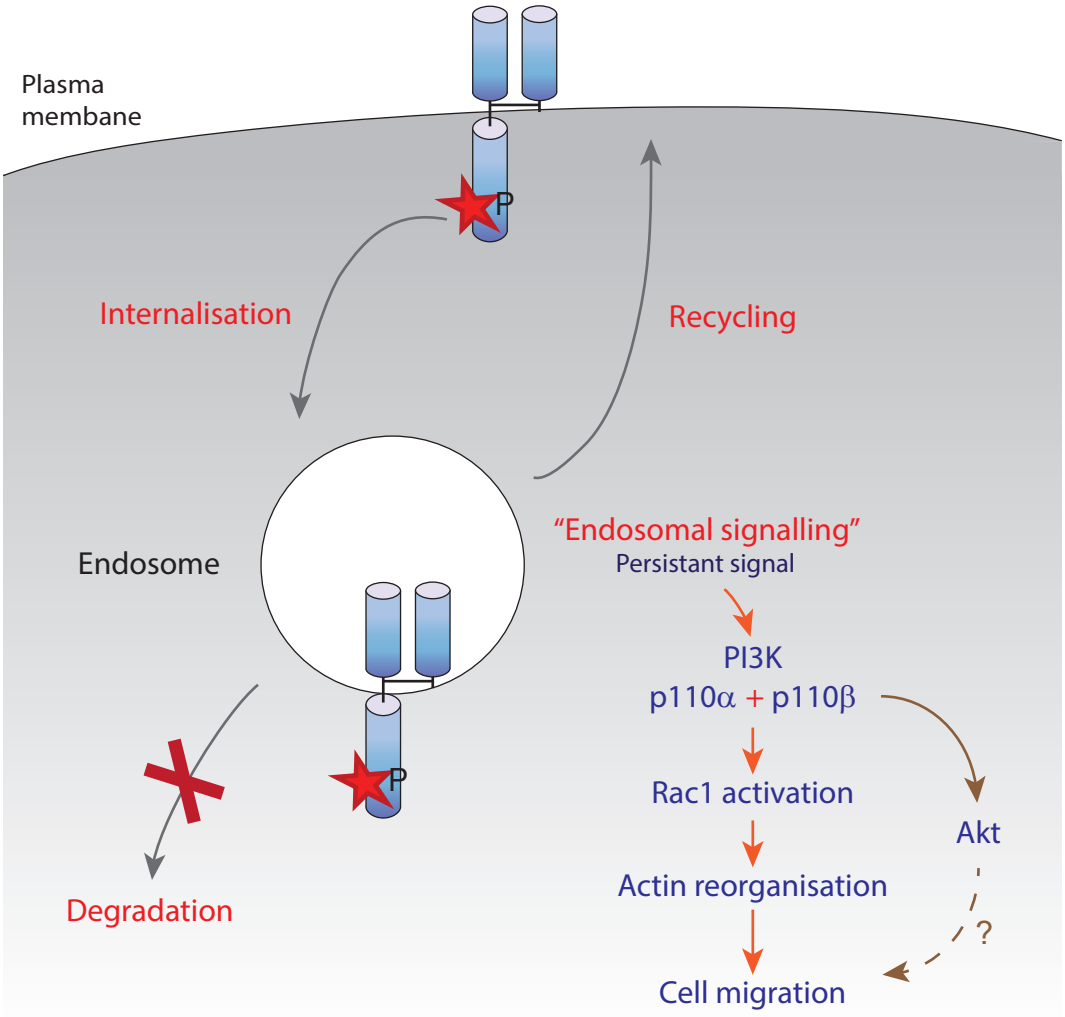


Figure 27: Model of M1268T Met signalling to induce cell migration

M1268T Met is constitutively activated and gets internalised and recycled back to the plasma membrane escaping degradation. From endosomes, M1268T Met induces PI3K class I activation, resulting in activation of Rac1, reorganisation of the cytoskeleton, cell migration and Akt activation.

Cell lines		NIH3T3	A549	U87MG
Met expressed Mode of activation		M1268T Met Constitutive activation	WT Met Upon exogenous HGF	WT Met Autocrine-loop
Cell functions	Cell migration	p110 alpha p110 beta	p110 beta p110 delta	p110 alpha p110 beta p 110 delta
	Migratory phenotype	p110 alpha p110 beta	-	-
	Akt activation	p110 alpha p110 beta	p110 alpha p110 beta p 110 delta	-

Table 1: PI3K class I isoforms involved downstream of Met

The PI3K class I isoforms that are downstream of Wild Type (WT) and M1268T Met, inducing cell migration, the migratory phenotype and Akt activation. The isoforms were investigated using specific inhibitors and siRNAs. Font size is proportional to the importance of the isoform. NIH3T3: mouse fibroblast cells; A549: non-small lung cancer cells; U87MG: human glioblastoma-astrocytoma, epithelial-like, cells.

Altogether my results indicate that PI3K class I and mTOR both mediate Met dependent cell migration. Depending on the Met forms and / or the cells used, different PI3K class I isoforms appear to play a role. Most of the time, the combined inhibition of several isoforms is required to efficiently block Met dependent cell migration. PI3K class I isoforms, p110 alpha and p110 beta, need to be knocked-down / inhibited together to significantly reduce M1268T Met dependent Rac1 pathway, actin stress fibres disorganisation, and cell migration. The reason for this could be that both isoforms are needed or that they compensate each other when one is removed / inhibited. In A549 cells, while inhibiting p110 alpha has no effect, the individual inhibition of p110 beta or p110 delta leads to a significant reduction of Met dependent cell migration. In U87MG cells, individual inhibitions of p110 alpha, p110 beta, and p110 delta have a poor effect on cell migration. Dual inhibition of p110 alpha and p110 beta did not improve it; however my preliminary data indicate that the inhibition of the 3 isoforms p110 alpha, p110 beta and p110 delta may present an advantage. The isoform p110 delta is normally expressed in haematopoietic cells, but it is interesting to note that p110 delta is expressed in these cancer cells, is functional and involved in Met dependent cell migration. Additional work is required to establish whether the differences observed in the requirement of different PI3K isoforms are dependent on the mode of Met signalling (constitutively active, stimulated by HGF or through an autocrine loop) or are in fact cell type dependent (or both).

Both WT and M1268T Met appear to require endocytosis to stimulate Akt. p110 alpha is the main and most common isoform promoting Met dependent Akt activation, although the other PI3K class I isoforms expressed are also involved to a smaller extent. Strikingly, the main isoform(s) involved in the cell migration of a given cell line do not correspond to the main isoform(s) responsible for Akt phosphorylation. Moreover the

inhibition of Akt phosphorylation with a low but specific and efficient dose of the Akt inhibitor had no effect on M1268T Met migration. Therefore the role of Akt in cell migration triggered by WT / M1268T Met remains to be clarified.

The endosomes from which Akt activation and cell migration occurs have not been determined, but a role for Appl1 and EEA1 has been excluded.

My work has uncovered the role of PI3K class I downstream of Met, however it appeared challenging to identify the precise role of each isoform: PI3K class I isoforms requirement appear to vary depending on the cell model studied and my results so far do not allow to distinguish between the requirement of several isoforms and their redundancies.

Numerous pharmacological inhibitors specific for one PI3K isoform are emerging, however it seems that, downstream of Met, the inhibition of the whole PI3K class I (alone or in combination with Met inhibition) might be more efficient.

In the following chapter, I aim to identify how PI3K/Akt/mTOR pathway is involved in the anchorage independent growth and tumour growth triggered by M1268T Met in NIH3T3 cells and by WT Met in human cancer cell lines.

RESULTS – CHAPTER III

M1268T Met driven anchorage independent growth and tumourigenesis requires Rac1 and mTOR independently of PI3K

In Chapter I, I observed that the anchorage independent growth induced by M1268T Met is inhibited by LY294002. In a similar way as for the cell migration induced by M1268T Met in Chapter II, I subsequently investigated which LY294002 targets, PI3K, Akt or mTOR, are involved in the anchorage independent growth that is induced by M1268T Met.

III. a) M1268T and D1246N Met induce anchorage independent growth through mTOR independently of PI3K

Cells were grown in soft agar and left to grow for 5 days to form colonies, then treated for 5 days with inhibitors targeting pan-PI3K, PI3K class I and mTOR at the same concentrations as determined in Chapter II, concentrations which efficiently inhibit their targets avoiding off-target effects as much as possible.

1. M1268T Met induces anchorage independent growth through mTOR independently of PI3K

First, wortmannin was used to confirm the role of PI3K in M1268T Met dependent anchorage independent growth **(Fig. 1A)**. Similarly to LY294002, wortmannin has no effect on colony areas formed by WT Met expressing cells. However, surprisingly, in contrast to LY294002, wortmannin has no significant effect on the colony areas formed

by M1268T Met expressing cells, which are significantly higher than the area formed by WT Met expressing cells by 8.5 times ($p<0.05$). As wortmannin inhibits PI3K class I and III, this result suggests that PI3K class I and III are not involved in M1268T Met anchorage independent growth; which would be surprising as it is commonly thought that PI3K Class I / Akt pathway promotes cell growth, including when induced by Met. To explore this further, PI3K class I inhibition by GDC0941 was tested first at a concentration of 100 nM, which is efficient in the cell migration assay **(Chapter II)** (not shown), and then at the higher concentration of 10 µM. The role of PI3K class III Vps34 was also investigated using siRNA. Similarly to wortmannin, none of these inhibition methods significantly affects the colony area formed by WT or M1268T Met expressing cells **(Fig. 1A, B)**. Furthermore, Akt inhibition by MK2206 at 100 nM, also has no effect on the anchorage independent growth of both cell lines **(Fig. 1A)**.

LY294002, in contrast to wortmannin, also inhibits mTOR. Could the effect of LY294002 be mediated by its effect on mTOR, which would explain why pan-PI3K, PI3K class I and Akt inhibition have no effect on M1268T Met dependent anchorage independent growth? The role of mTOR was therefore investigated using the inhibitor rapamycin. Rapamycin targets mTORC1 during short treatments, but also targets mTORC2 after long treatments. Interestingly, rapamycin at 2 nM reduces the colony areas of M1268T Met expressing cells by 61% ($p<0.05$) while it has no effect on WT Met expressing cells **(Fig. 1A)**.

These results suggest that M1268T Met might induce anchorage independent growth through mTOR, independently of PI3K and Akt.

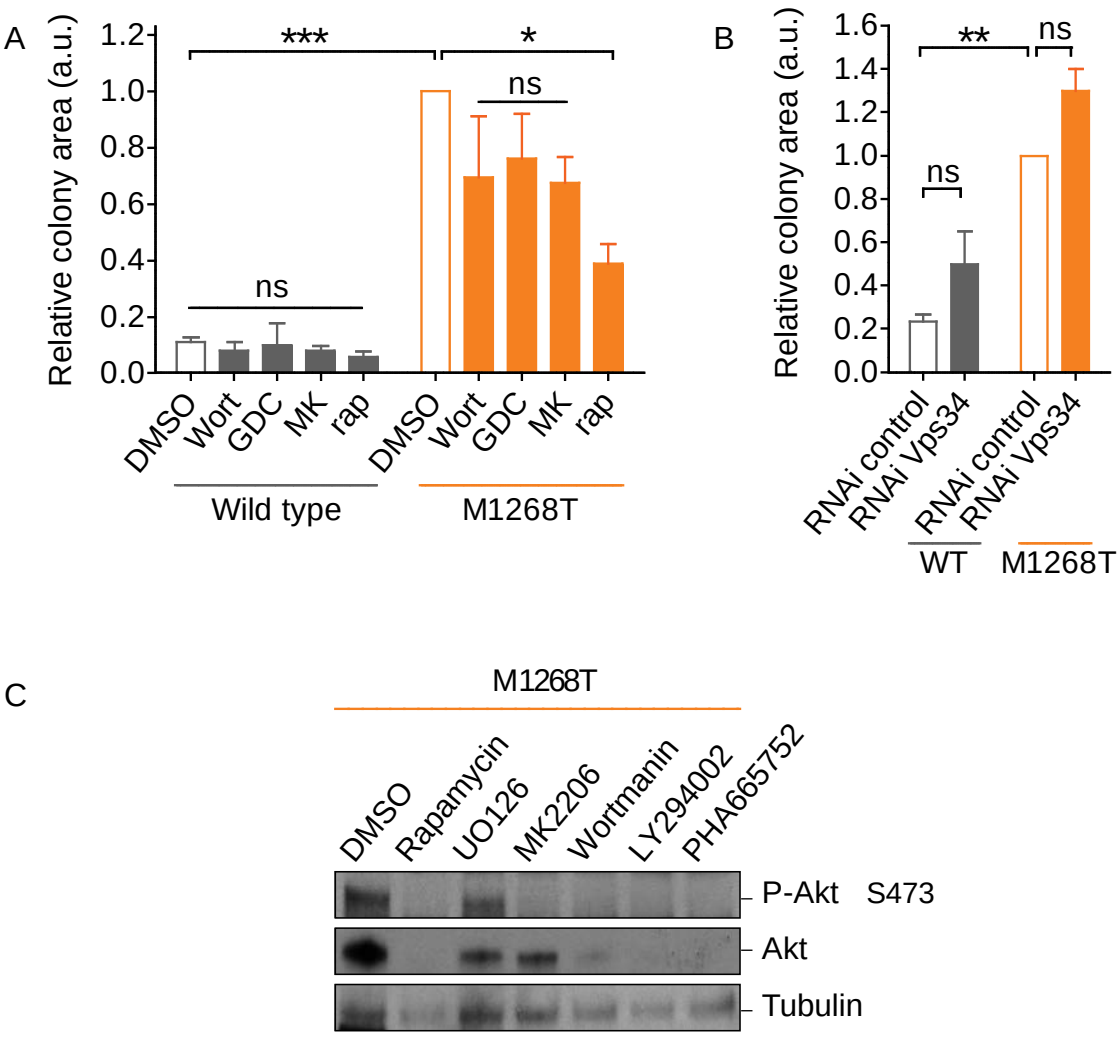


Figure 1: M1268T Met induces anchorage independent growth through mTOR, independently of PI3K

(A,B) Soft agar assays. Colonies were photographed and the areas were calculated by Image J. Mean values +/- SEM (n=3) (a.u. = arbitrary units). **(A)** 500 single cells from NIH3T3 Wild type (WT) or M1268T Met expressing cells were grown in soft agar for 10 days. At day 5, they were treated daily with DMSO, wortmannin (wort) (100 nM), GDC0941 (GDC) (10 µM), MK2206 (MK) (100 nM), or rapamycin (rap) (2 nM). **(B)** NIH3T3 Wild type or M1268T Met were transfected with negative control or Vps34 siRNA and 1000 cells were grown in soft agar for 10 days. **(C)** Western blots for phosphorylated Akt (S473) (P-Akt), Akt and tubulin were performed on colonies formed by M1268T Met expressing cells treated with DMSO, rapamycin (2nM), UO126 (10 µM), MK2206 (100 nM), wortmannin (100 nM), LY294002 (10 µM) and PHA-665752 (100 µM), which were extracted from the soft agar. ns: non significant, *p<0.05, **p<0.01, ***p<0.001.

Then, I aimed to confirm that M1268T Met anchorage independent growth can occur independently of Akt activation. I ensured that PI3K class I and Akt inhibition were effective within the soft agar. For this, M1268T Met colonies were collected at the end of the experiment, and a western blot was performed to evaluate the level of Akt phosphorylation **(Fig. 1C)**.

When treated with DMSO, as a negative control, the cells within in the colonies have a high phosphorylation of Akt, and pan-Akt is clearly detected. As expected, when the colonies were treated with MK2206, Akt phosphorylation is not detected anymore, while pan-Akt is still apparent. Surprisingly, following treatment with pan-PI3K inhibitors (LY294002 and wortmannin), or a Met inhibitor (PHA-665752), in addition to phospho-Akt, pan-Akt is also not detected **(Fig. 1C)**.

So, in this model (M1268T Met and pan-PI3K), the inhibition of upstream regulators of Akt appeared to lead to Akt degradation, possibly due to a feed-back loop switched on when Akt is not phosphorylated, since it is not really needed. Most of these inhibitors are ATP-competitive inhibitors, except the Akt inhibitor MK2206 which is an allosteric inhibitor. Interestingly, it was shown that because MK2206 protects the PH domain of Akt, Akt cannot be ubiquitinilated [342], which might therefore be protecting Akt from degradation.

Thus, although Akt activity is fully reduced in M1268T Met expressing cells cultured in soft agar with wortmannin and MK2206 **(Fig. 1C)**, M1268T Met dependent anchorage independent is not prevented **(Fig. 1A**). The result with wortmannin further suggests that downregulation of Akt does not interfere with the anchorage independent growth that is induced by M1268T Met.

Altogether, these results indicate that M1268T Met induces anchorage independent growth through mTOR, independently of PI3K class I/III and Akt.

Due to the fact that the treatment of colonies in soft agar lasts for 5 days, it is not possible at this stage to determine whether it is mTORC1 or mTORC2, or both, that are involved. Experiments using si/shRNA to target components of the complexes will need to be conducted in the future.

2. D1246N Met induces anchorage independent growth through mTOR independently of PI3K class I

Another Met mutation of interest is D1246N Met. The D1246N Met mutant has similar properties to the M1268T Met. D1246N Met was discovered in papillary renal carcinoma [406]. The mutation is located in the kinase domain and leads to Met being constitutively activated [56], and having a constitutive internalisation [56]. Similar to the M1268T mutant, it escapes degradation and recycles back to the plasma membrane [56]. When expressed in NIH3T3 cells, D1246N causes *in vitro* and *in vivo* transformation, which is significantly reduced with endocytosis inhibition using the dynamin inhibitor dynasore [56, 103]. The major difference between M1268T and D1246N is that D1246N is resistant to Met inhibitors [56].

I investigated whether D1246N Met expressed in NIH3T3 cells induces anchorage independent growth the same way as M1268T, through mTOR but independently of PI3K class I, and whether inhibition of mTOR reduces it.

Similarly, as shown by Joffre *et al*., I observed that the area of colonies formed by NIH3T3 cells expressing D1246N Met is 3.8 times higher than the colony area formed by WT Met expressing cells **(Fig. 2)**.

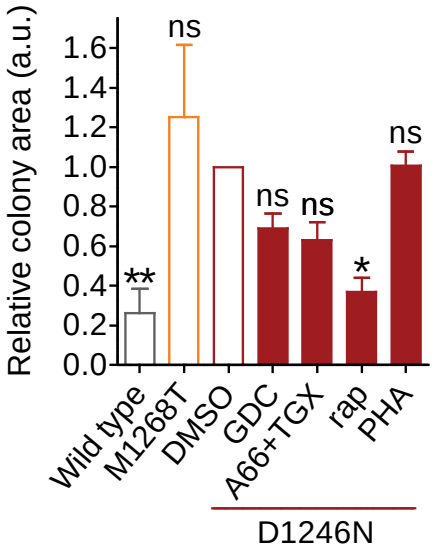


Figure 2: D1246N Met induces anchorage independent growth through mTOR, independently of PI3K

Soft agar assays. 500 single cells from NIH3T3 Wild type, M1268T or D1246N Met expressing cells were grown in soft agar for 10 days. At day 5, they were treated daily with DMSO, GDC0941 (GDC) (100 nM), A66 and TGX221 (500 and 100 nM), rapamycin (rap) (2 nM), or PHA-665752 (PHA) (100 nM). Colonies were photographed and the area were calculated by Image J. Mean values +/- SEM (n=3) (a.u. = arbitrary units). NS: non significant, *p<0.05, **p<0.01, ***p<0.001.

Importantly, the Met inhibitor PHA-665752 has no effect on D1246N Met induced anchorage independent growth [56]. The inhibition of PI3K class I with GDC0941 or the combined inhibitors A66 and TGX221 (p110 alpha and p110 beta inhibitors), does not modify the colony areas of D1246N Met expressing cells. However, the colony area formed by D1246N Met expressing cells is reduced by 63% upon rapamycin treatment ($p<0.05$).

Overall, these results indicate that like M1268T Met, D1246N Met mutant induces anchorage independent growth through the mTOR pathway and this is independent of PI3K class I. Therefore, mTOR might represent a good target for cancer therapy for patients with such mutations on Met, to overcome resistance to Met inhibitors.

III. b) WT Met in human cancer cells induces anchorage independent growth through mTOR independently of PI3K class I

I aimed to determine if the anchorage independent growth induced by endogenous WT Met in human cancer cells is also mediated by mTOR. Similarly to the Chapter II, I used non small lung cancer (NSLC) cell line A549, which express endogenous WT Met, and the human glioblastoma-astrocytoma, epithelial-like cell line U87MG, which express endogenous WT Met but also secrete HGF, resulting in a Met-HGF autocrine loop.

1. WT Met in A549 cells induces anchorage independent growth through mTOR independently of PI3K

A549 cells were grown for 2 weeks in soft agar and then were treated for one week with or without HGF, and with the various pharmacological inhibitors **(Fig. 3)**.

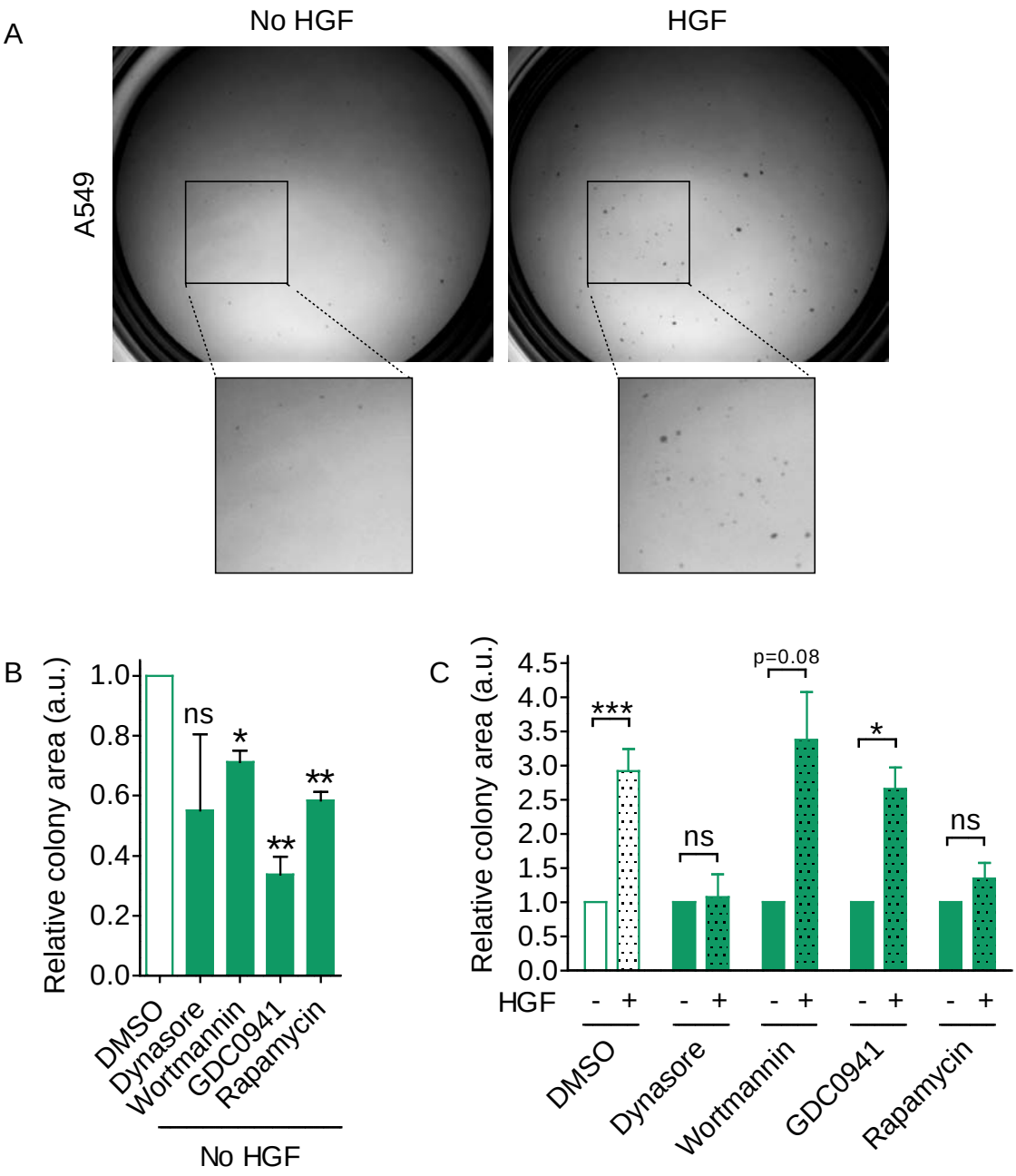


Figure 3: Met dependent anchorage independent growth in A549 cells is inhibited by mTOR inhibition

(A-C) Soft agar assays. 1000 single A549 cells were grown in soft agar for 14 days. At day 14, they were treated daily with or without HGF (15 ng/mL) and with DMSO, dynasore (80 µM), wortmannin (100 nM), GDC0941 (100 nM), or rapamycin (2 nM). Colonies were photographed and the area were calculated by Image J. **(A)** Pictures of colonies with and without HGF. **(B)** Relative colony area normalised to DMSO treated colonies. **(C)** Relative colony area with and without HGF normalised to each drug without HGF. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, **p<0.01, ***p<0.001.

DMSO was used as a negative control. Dynasore was used to investigate whether in this cell model, as in M1268T and D12246N expressing cells [56], Met induced anchorage independent growth requires intact endocytosis machinery. Wortmannin, GDC0941 and rapamycin were used to evaluate the role of PI3K and mTOR. The basal colony areas of A549 cells are reduced with all the inhibitors tested **(Fig. 3B)**. Thus, as compared to DMSO, the colony areas reduce with dynasore by 45% (not significant), with wortmannin by 29% ($p<0.05$), with GDC0941 by 66% ($p<0.01$), and with rapamycin by 42% ($p<0.01$) **(Fig. 3B)**. This indicates that the anchorage independent growth of A549 cells in basal conditions requires PI3K/mTOR signalling pathway.

Therefore, for a given drug, the effect of HGF was compared to the effect observed with the same drug in absence of HGF **(Fig. 3C)**. Thus, fold increases of relative colony area stimulated by HGF upon basal conditions were measured. In control conditions, HGF induces a significant increase of the colony area of 2.9 fold ($p<0.001$) **(Fig. 3A, C)**. When treated with HGF in presence of wortmannin or GDC0941, fold increases in colony areas are 3.4 and 2.7 fold respectively. However, dynasore and rapamycin fully inhibited the fold increase of colony areas induced by HGF; there are no significant differences between no HGF and HGF in the presence of these two inhibitors.

These results indicate that in A549 cells, Met induces anchorage independent growth once internalised, and through mTOR but independently of PI3K.

2. WT Met in U87MG cells induces anchorage independent growth through mTOR independently of PI3K class I

Rachel Barrow in our team has demonstrated that U87MG cells form tumours that are Met dependent. U87MG cells were injected subcutaneously into nude mice and the

tumours were measured daily. Once the tumours reached a volume of 30-50 mm^3, tumours were treated daily by topical application of the negative control DMSO, the Met inhibitor PF-2341066 or the dynamin inhibitor dynasore (unpublished, **Appendix 1 *p.242***). At day 28 post-graft, Met or dynamin inhibition significantly reduced the tumour volumes formed by U87MG cells by 41% ($p<0.05$) and 38% ($p<0.05$), respectively.

In soft agar assays, the anchorage independent growth of U87MG cells is reduced by 48%, when Met is inhibited with PF-2341066, and by 71% with dynasore ($p<0.05$) **(Fig. 4)**. Interestingly, the inhibition of PI3K class I with GDC0941 has no significant effect while rapamycin reduces the anchorage independent growth of U87MG cells by 51% ($p<0.01$).

Therefore, as in the other cell models tested, the anchorage independent growth of U87MG cells appears to require Met endocytosis and mTOR activity, but not PI3K class I activity.

III. c) <u>Met internalisation is required for mTORC1 activation</u>

As mentioned earlier, mTOR can be part of the complexes mTORC1 or mTORC2. mTORC1 is well known to be involved in cell growth and cell proliferation. In order to investigate if mTORC1 is activated downstream of WT and M1268T Met, western blots were performed to monitor the phosphorylation of the well-known effector of mTORC1, S6K1. S6K1 has several isoforms of different molecular weights, the most well studied being p70 S6K.

NIH3T3 cells expressing WT or M1268T Met were starved for 1 hour. In the presence of DMSO, p70 S6K phosphorylation on Thr389 is poorly detected in WT Met

expressing cells, while it is significantly increased by 2 fold ($p<0.01$) in M1268T Met cells **(Fig. 5A, B)**. M1268T Met dependent phosphorylation of p70 S6K is significantly reduced by 67% ($p<0.05$) with the Met inhibitor PHA-665752 and by 59% ($p<0.05$) with the pan-PI3K/mTOR inhibitor LY294002, while these drugs have no significant effect on the level of phospho-p70 S6K in WT Met expressing cells.

M1268T Met has been shown to require an intact endocytosis machinery to induce anchorage independent growth [56]. Therefore, using dynasore, I investigated whether M1268T Met requires endocytosis to induce p70 S6K phosphorylation. I found that the strong phosphorylation of p70 S6K in M1268T Met expressing cells treated with DMSO (negative control) is reduced by 40% ($p<0.05$) upon dynasore treatment **(Fig. 5C, D)**, suggesting that M1268T Met needs to be internalised to either induce or maintain the phosphorylation of p70 S6K.

Similarly, in A549 cells, 15 min of HGF stimulation increases the phosphorylation of p70 S6K by 3.3 fold ($p<0.01$) as compared to no HGF **(Fig. 6)**. At 120 min of HGF stimulation, phospho-p70 S6K is sustained and is still increased by 1.8 fold ($p<0.05$). The HGF mediated p70 S6K phosphorylation is lost upon dynasore **(Fig. 6)**, suggesting that, also in these cells, activated WT Met needs to be internalised to induce the phosphorylation of p70 S6K.

Altogether, these results suggest that both M1268T Met in NIH3T3 cells and endogenous WT Met activated with HGF in the lung cancer cell line A549 signal to mTORC1 from endosomes.

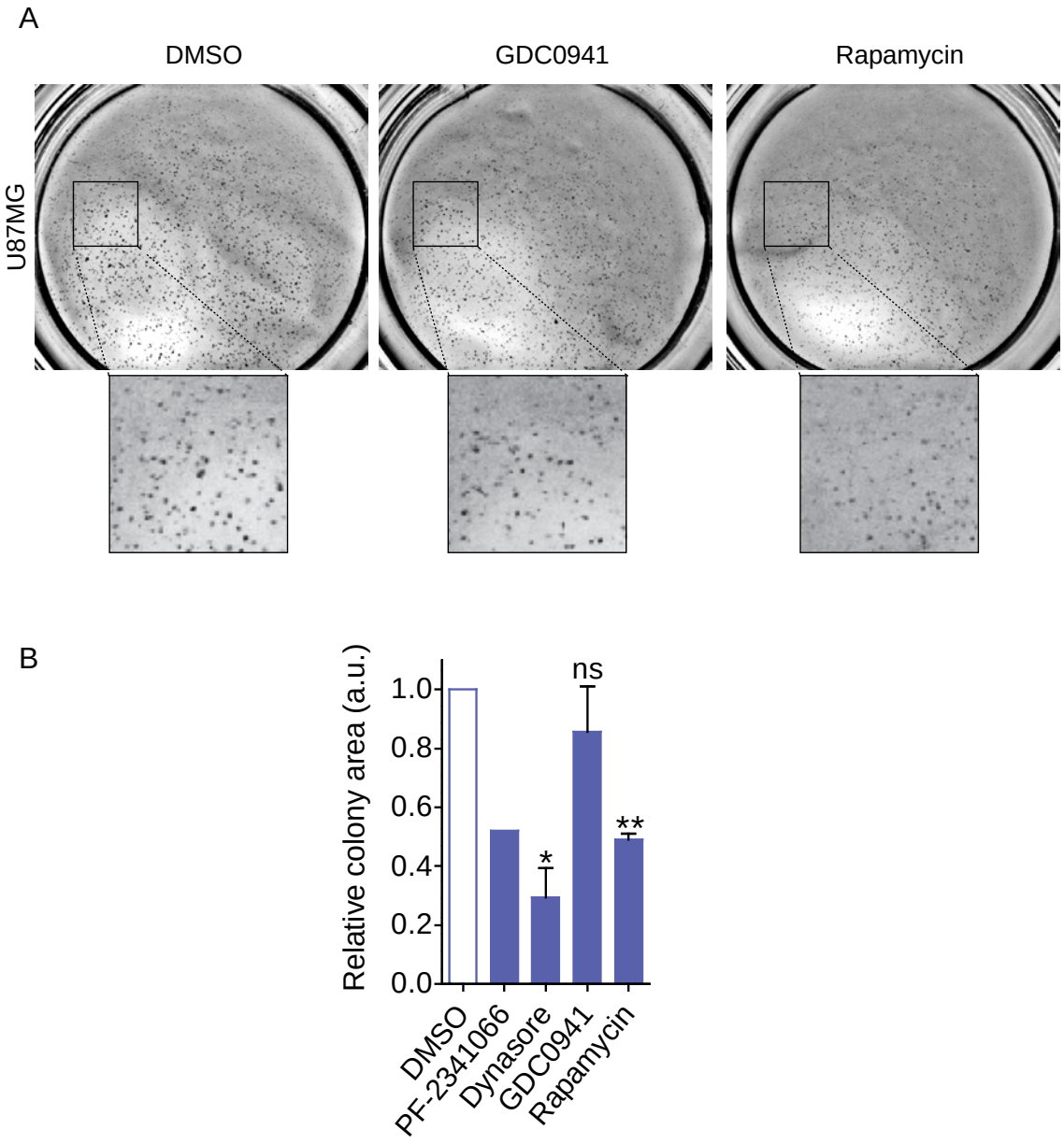


Figure 4: Met induces anchorage independent growth in U87MG cells, which requires mTOR but not PI3K class I activity

(A,B) Soft agar assays. 10,000 single U87MG cells were grown in soft agar for 5 days. At day 5, they were treated daily with DMSO, PF-2341066 (100 nM) (n=1), dynasore (80 µM), GDC0941 (100 nM), or rapamycin (2 nM). Colonies were photographed and the area were calculated by Image J. **(A)** Pictures of colonies treated with DMSO, GDC0941 or rapamycin. **(B)** Relative colony area normalised to DMSO treated colonies. Mean values +/- SEM (n=3, except PF-2341066 n=1) (a.u. = arbitrary units). ns: non significant, *p<0.05, **p<0.01.

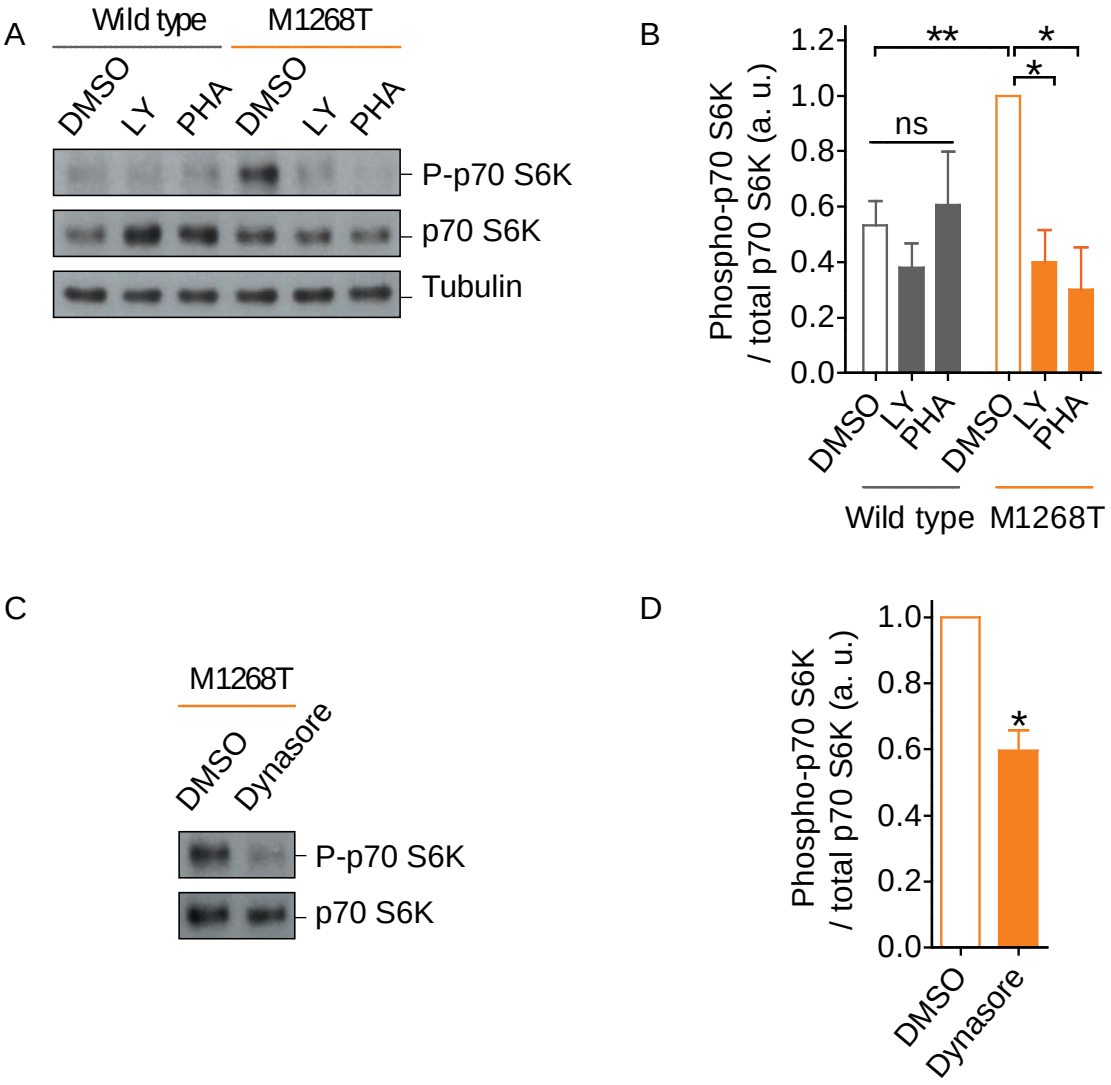


Figure 5: M1268T Met induces the phosphorylation of p70 S6K, which requires M1268T Met internalisation

(A-D) NIH3T3 cells expressing Wild type or M1268T Met starved in medium with no serum for 1 hour. **(A,B)** Cells were then treated with DMSO, LY294002 (LY) (10µM) or PHA-665752 (PHA) (100nM) for 1 hour. **(A)** Western blots for phosphorylated p70-S6K (Thr389) (P-p70 S6K), p70 S6K, and tubulin were performed. **(B)** Quantification of phosphorylated p70-S6K (Thr389) (P-p70 S6K) normalised on p70 S6K, obtained by densitometry of western blots. **(C,D)** Cells were treated with DMSO or dynasore (80 uM) for 30 minutes. **(C)** Western blots for phosphorylated p70-S6K (Thr389) (P-p70 S6K) and p70 S6K were performed. **(D)** Quantification of phosphorylated p70-S6K (Thr389) (P-p70 S6K) normalised on p70 S6K, obtained by densitometry of western blots. Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, **p<0.01.

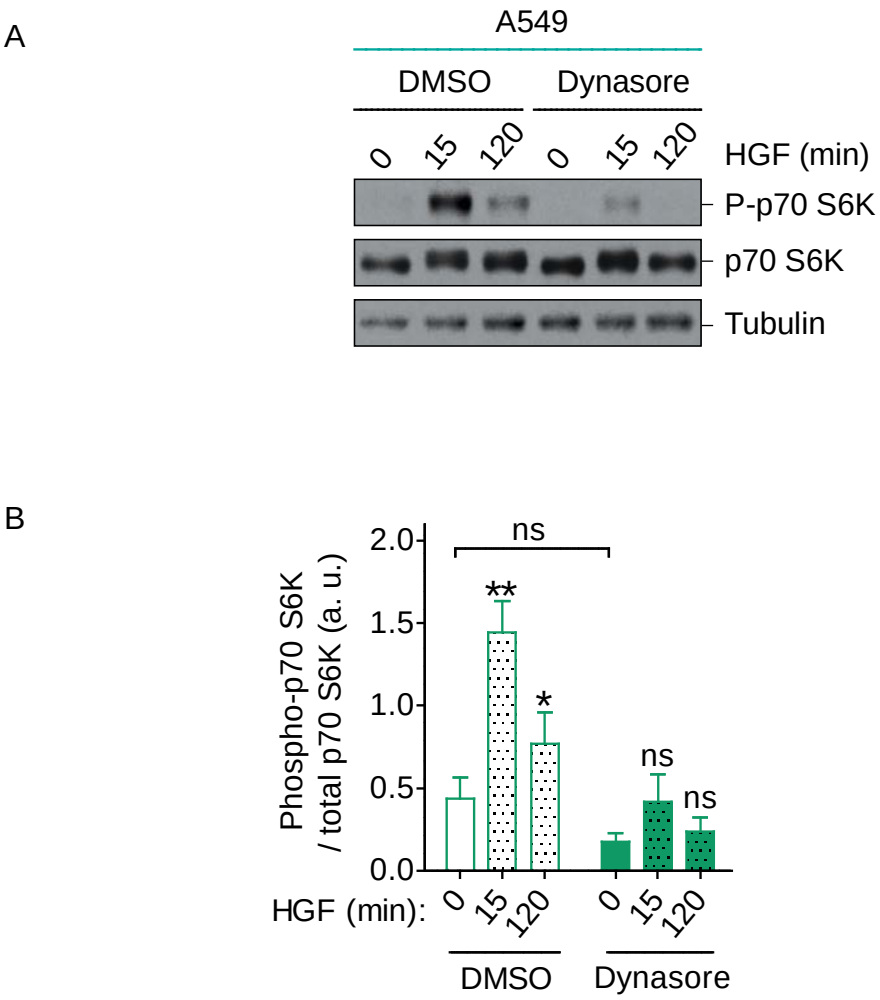


Figure 6: Met dependent phosphorylation of p70 S6K in A549 cells requires Met internalisation

(A) Western blots for phosphorylated p70 S6K (Thr389) (P-p70 S6K), p70 S6K, and tubulin were performed on A549 cells starved in medium with no serum for 24 hours, pre-treated with DMSO or dynasore (80 μM) for 40 minutes, followed by a stimulation with HGF (50 ng/mL) for 0, 15, and 120 minutes. **(B)** Quantification of P-p70 S6K normalised on total p70 S6K, obtained by densitometry of western blots. Mean values +/- SEM (n=3) (a.u. = arbitrary units). Unless indicated, statistics are compared to 0 min HGF of the same treatment. ns: non significant, *p<0.05, **p<0.01.

III. d) M1268T Met activates mTOR through Rac1

The previous results suggested that M1268T and WT Met in cancer cells activate mTORC1 independently of the PI3K/Akt pathway. Therefore, how does Met signal to mTORC1? The activation of mTORC1 is regulated by multiple factors. Growth factors can mediate mTOR activation through the PI3K/Akt pathway, but also through the MAPK pathway. Preliminary results obtained with M1268T Met expressing cells treated with the MEK inhibitor UO126 did not demonstrate any reduction in phospho-p70 S6K (data not shown). Therefore this pathway was excluded.

As described in Chapter I and published by Joffre *et al.*, M1268T Met activates Rac1 from endosomes, followed by Rac1 relocalisation to the plasma membrane [79]. Interestingly, in 2011, Saci *et al.* demonstrated that mTOR needs to be relocalised to the plasma membrane to induce cell growth, and this is dependent on Rac1, regardless of its activation status [422].

I therefore investigated the mechanism by which M1268T Met activates mTOR, studying the potential role of Rac1.

1. M1268T Met dependent relocalisation of mTOR to the plasma membrane requires Rac1

The localisation of mTOR and Rac1 was analysed by immunofluorescence in NIH3T3 cells expressing either WT or M1268T Met **(Fig. 7A)**. In WT Met expressing cells, mTOR and Rac1 are mainly intracellular, and colocalise poorly. In M1268T Met expressing cells, mTOR and Rac1 are both intracellular / perinuclear and at the plasma membrane, and they strongly colocalise in both locations.

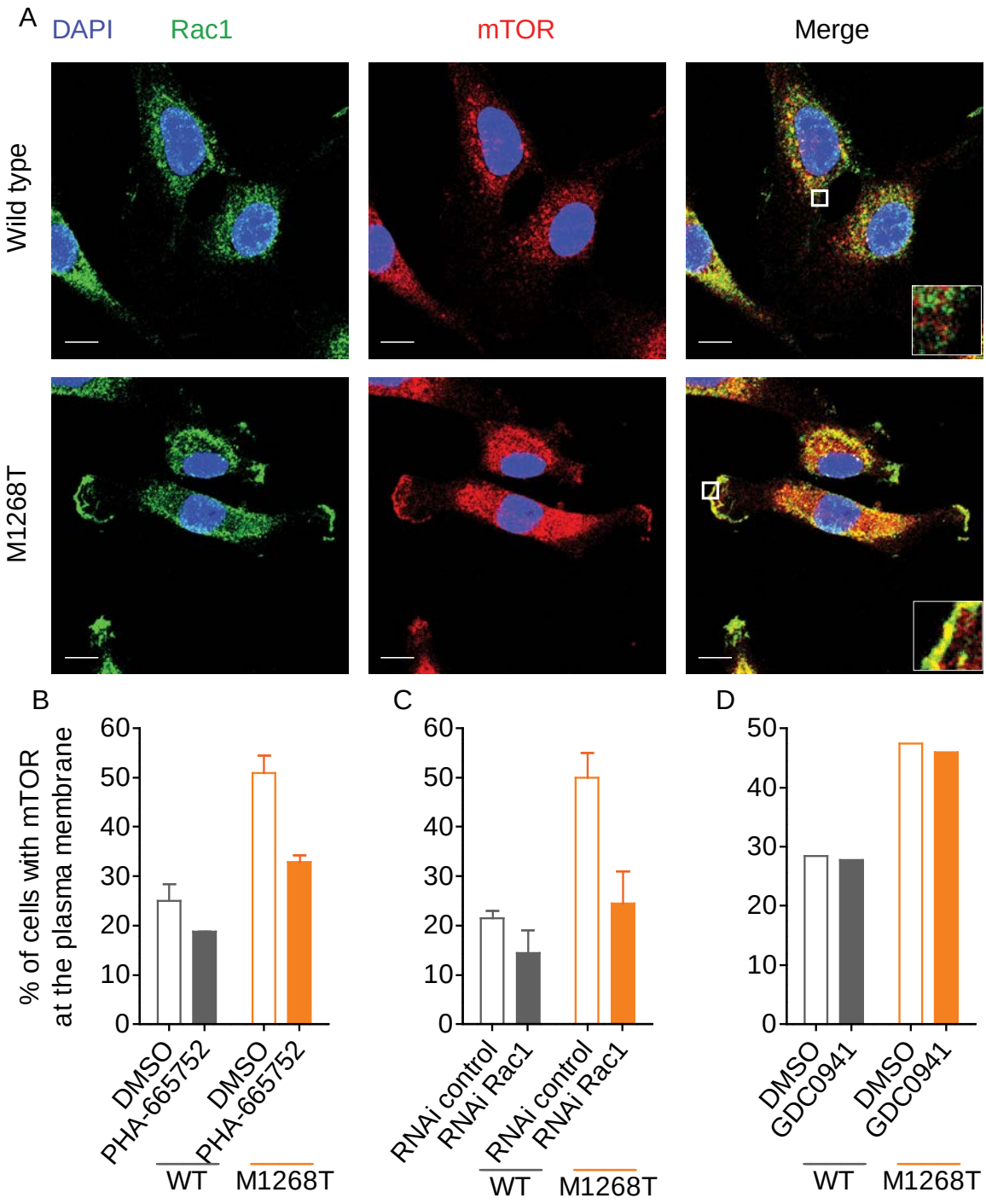


Figure 7: M1268T Met induces the relocalisation of mTOR to the plasma membrane through Rac1

(A) Confocal sections of NIH3T3 Wild type and M1268T Met expressing cells immunostained with antibodies against Rac1 (green), and mTOR (red) and stained with DAPI (blue). **(B-D)** Quantification of the percentage of cells with mTOR at the plasma membrane when **(B)** treated with DMSO or PHA-665752 (100nM) (n=2) for 1 hour, or **(C)** transfected with negative control or Rac1 siRNA (n=2), or **(D)** treated with DMSO or or GDC0941 (100 nM) (n=1) for 1 hour. 100 cells were counted per condition per experiment. Mean values +/- SEM.

I therefore evaluated whether the localisation of mTOR at the plasma membrane in M1268T Met expressing cells is due to M1268T Met activity. Cells were treated with DMSO as a negative control or with the Met inhibitor PHA-665752 and the number of cells with mTOR at the plasma membrane was counted **(Fig. 7B)**. Interestingly, I found that twice more M1268T Met expressing cells have mTOR at the plasma membrane than WT Met expressing cells when treated with the negative control DMSO (25% for WT and 51% for M1268T Met expressing cells, n=2). Met inhibition using PHA-665752 has no effect on WT Met expressing cells, while it decreases the percentage of M1268T Met expressing cells with mTOR at the plasma membrane by 35% (n=2).

Although, these experiments were performed twice and need to be repeated for statistical analyses, the results so far suggest that M1268T Met induces mTOR relocalisation to the plasma membrane.

Cells were transfected with siRNA against a negative control or for Rac1, to test the hypothesis that Rac1 mediates the relocalisation of mTOR induced by M1268T Met **(Fig. 7C)**. In the negative control, WT and M1268T Met expressing cells have equivalent percentage of cells with mTOR at the plasma membrane as when the cells were treated with DMSO (21% for WT and 50% for M1268T Met expressing cells, n=2) **(see above)**. Knock-down of Rac1 in M1268T Met expressing cells reduces the percentage of cells with mTOR at the plasma membrane to 24%, which is the same level as observed in WT Met expressing cells knocked-down for the negative control **(**n=2, **Fig. 7C)**. Rac1 knock-down has no effect on WT Met expressing cells.

Interestingly, this result indicates that Rac1 is required for the relocalisation of mTOR to the plasma membrane that is induced by M1268T Met.

Finally, I tested whether PI3K class I (that I have shown to promote M1268T Met dependent Rac1 relocalisation at the plasma membrane, **see Chapter II**) is involved in the relocalisation of mTOR to the plasma membrane induced by M1268T Met **(Fig. 7D)**. The inhibition of PI3K class I by GDC0941 has no effect on the percentage of cells with mTOR at the plasma membrane in both WT and M1268T Met expressing cells.

Therefore PI3K class I does not seem to be involved in the relocalisation of mTOR to the plasma membrane that is induced by M1268T Met, further suggesting that Rac1 translocation is not required for mTOR translocation to the plasma membrane but instead that Rac1 localisation on endosomes with mTOR is important.

2. M1268T Met induces mTORC1 activation through Rac1

Saci *et al.* observed that silencing Rac1 reduced mTOR at the plasma membrane but also downregulated the phosphorylation of its effectors [422]. I therefore tested whether M1268T Met is still able to phosphorylate p70 S6K when Rac1 is knocked-down **(Fig. 8)**.

Interestingly, in M1268T Met expressing cells knocked-down for Rac1, the phosphorylation of p70 S6K is reduced by 52% as compared to the negative control ($p<0.05$). This result indicates that Rac1 is involved downstream of M1268T Met in activating mTORC1 signalling.

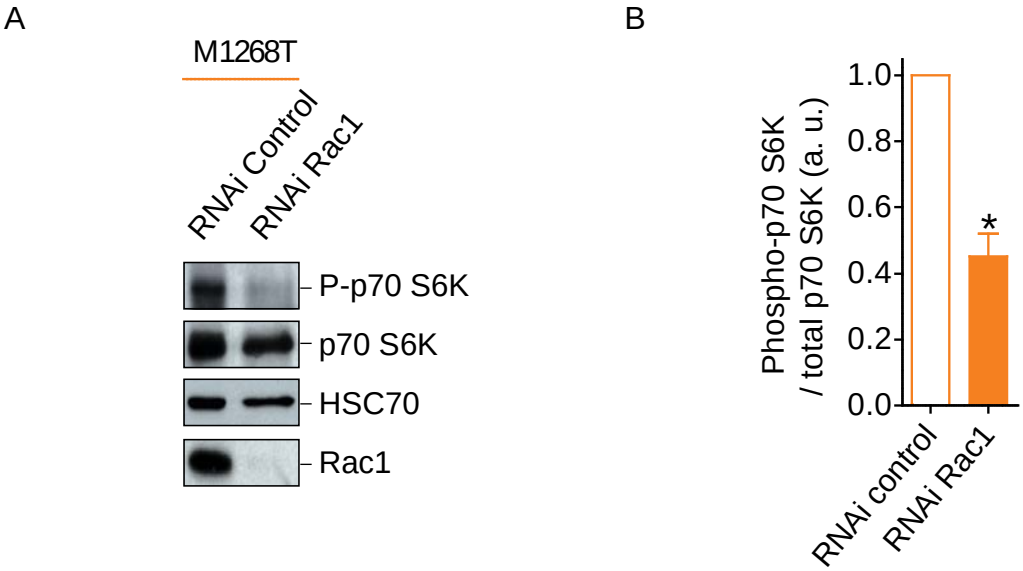


Figure 8: M1268T Met dependent phosphorylation of p70 S6K requires Rac1 expression

(A) Western blots for phosphorylated p70-S6K (Thr389) (P-p70 S6K), p70 S6K, HSC70 and Rac1 were performed on NIH3T3 cells expressing M1268T Met transfected with negative control or Rac1 siRNA and starved for 1 hour. **(B)** Quantification of phosphorylated p70-S6K (Thr389) (P-p70 S6K) normalised on p70 S6K, obtained by densitometry of western blots. Mean values +/- SEM (n=3) (a.u. = arbitrary units). *$p<0.05$.

3. Rac1 knock down reduces M1268T Met induced anchorage independent growth

So far, I observed that Rac1 seems to mediate the activation of mTOR downstream of M1268T Met, which induces mTOR relocalisation to the plasma membrane. mTOR is responsible for the anchorage independent growth induced by Met. Is Rac1 involved in this Met dependent anchorage independent growth?

Soft agar assays were performed with WT and M1268T Met expressing cells knocked-down for a negative control or Rac1 **(Fig. 9A)**.

Interestingly, while Rac1 knock-down has no effect on WT Met expressing cells, it significantly reduces M1268T Met expressing cells anchorage independent growth by 65% ($p<0.05$).

Saci *et al.* demonstrated that mTOR activation requires a direct binding to Rac1, regardless of the level of activation of Rac1. Therefore, is Rac1 activity is required in our system? There are currently no inhibitors available to directly inhibit Rac1 activity, however pharmacological inhibitors targeting Rac1-GEF interaction prevent Rac1 activation. Joffre *et al.* demonstrated that the Tiam 1 GEF inhibitor NSC23766 reduces M1268T Met dependent cell migration. Therefore I tested whether NSC23766 has an effect on M1268T Met dependent anchorage independent growth **(Fig. 9B)**. NSC23766 has no effect on the anchorage independent growth induced by WT or M1268T Met expressing cells, suggesting that Rac1 does not need to be activated downstream of M1268T Met to induce anchorage independent growth. Alternatively, it may be the case that NSC23766 does not inhibit the GEF(s) responsible for the Rac1 activation necessary for M1268T Met dependent anchorage independent growth.

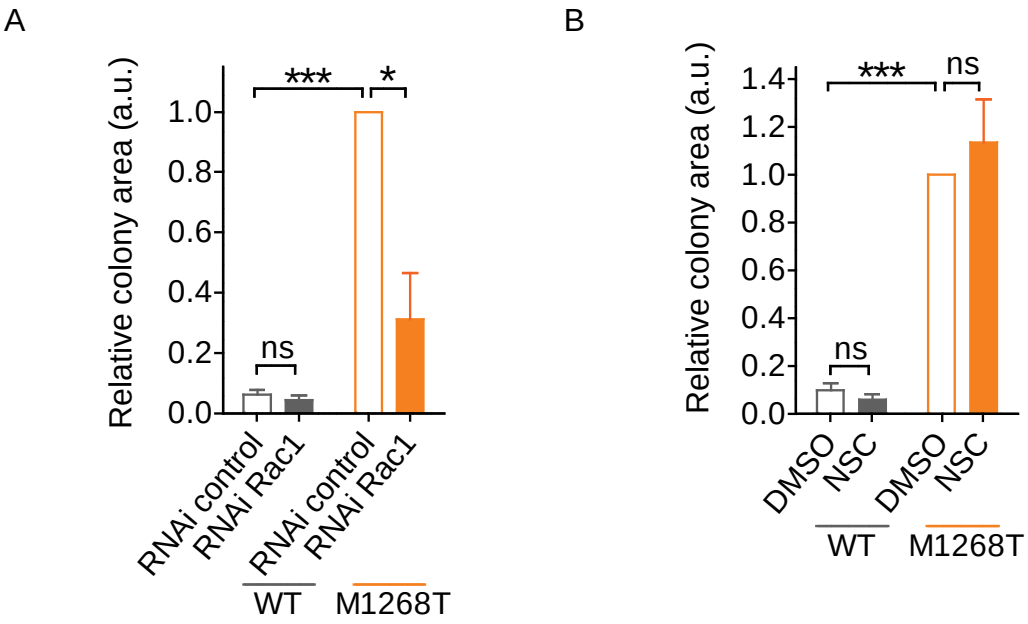


Figure 9: M1268T Met induces anchorage independent growth through mTOR independently of PI3K

(A,B) Soft agar assays. Colonies were photographed and the area were calculated by Image J. **(A)** 1000 cells from NIH3T3 Wild type (WT) or M1268T Met were transfected with negative control or Rac1 siRNA, were grown in soft agar for 10 days. **(B)** 500 single cells from NIH3T3 Wild type (WT) or M1268T Met expressing cells were grown in soft agar for 10 days. At day 5, they were treated daily with DMSO, or NSC23766 (NSC) (100 μM). Mean values +/- SEM (n=3) (a.u. = arbitrary units). ns: non significant, *p<0.05, ***p<0.001.

Altogether these results indicate a crucial role for Rac1 to activate mTOR downstream of M1268T Met and its anchorage independent growth, but the requirement for Rac1 activation, has not yet been clearly determined.

III. e) Inhibition of mTOR in vivo reduces M1268T Met dependent tumour growth

Finally, I aimed to assess the role of mTOR downstream of M1268T Met in tumour growth *in vivo*. NIH3T3 cells expressing either WT or M1268T Met were injected subcutaneously into nude mice and the tumours were measured daily. Once they reached a volume of 30-50 mm^3, tumours were treated daily by topical application of the negative control DMSO, or rapamycin **(Fig. 10)**.

Palpable tumours were formed by M1268T Met expressing cells on average at day 3 post-graft, and by WT Met expressing cells on average at day 8 post-graft **(Fig. 10A)**. Animals were sacrificed when tumours reached 500 mm^3, thus for M1268T Met tumours this was on average at day 9 post-graft, and for WT Met tumours it was on average at day 27. Thus, the tumour growth induced by M1268T Met expressing cells was faster than with cells expressing WT Met, as previously published [56]. Previous work in the laboratory has established that the WT Met tumours are insensitive to Met inhibition, consistent with the fact that WT Met is not activated [56].

At day 4, upon DMSO treatment, M1268T Met tumours were significantly bigger than WT Met tumours by 2 fold ($p<0.01$) **(Fig. 10B)**; Interestingly, Rapamycin treatment completely reduces the volume of M1268T Met tumours to the same level as the volume of WT Met tumours (-41, $p<0.05$), while it does not have any effect on WT Met tumours.

This result indicates that M1268T Met driven tumourigenesis *in vivo* is mediated through mTOR.

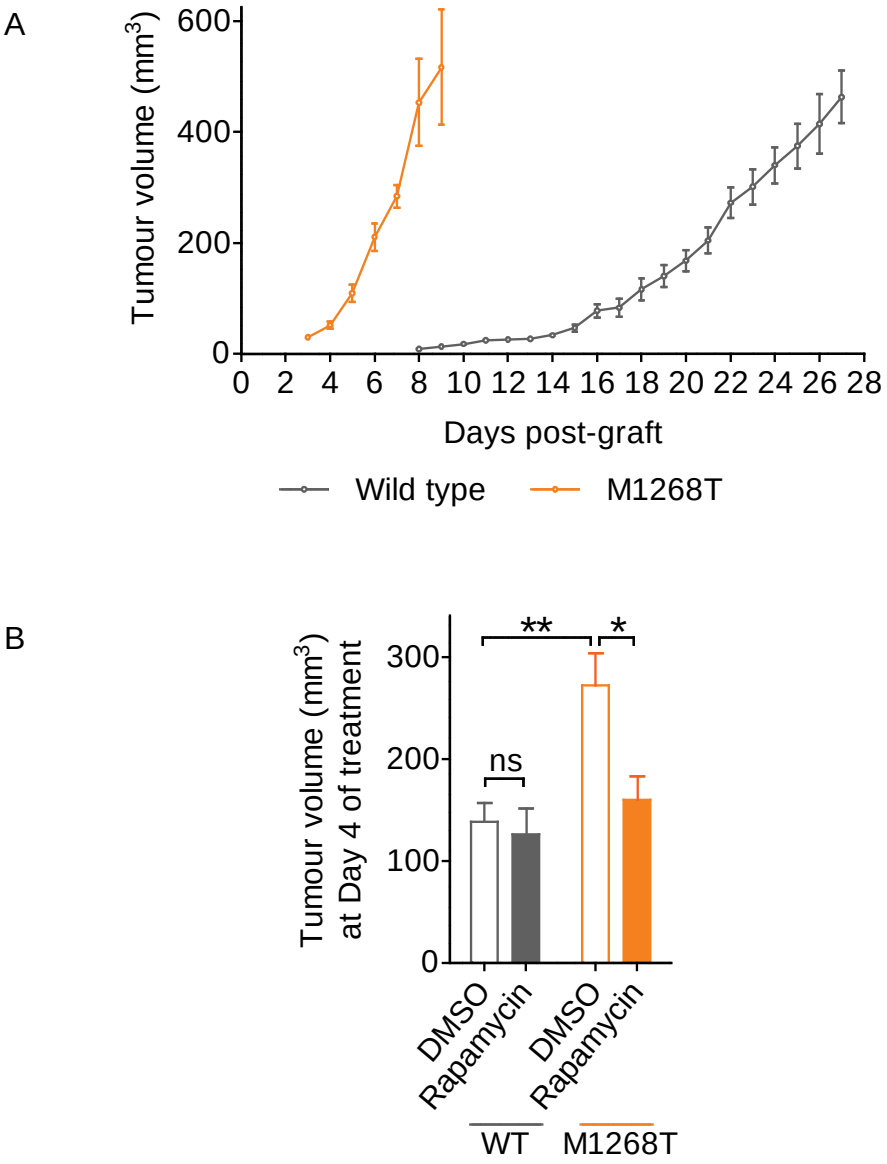


Figure 10: M1268T Met induces tumour growth through mTOR

$5x10^5$ NIH3T3 cells expressing either Wild type (WT) or M1268T Met were injected subcutaneously into nude mice. Tumours were measured daily and once they reached a volume of 30-50 mm^3, tumours were treated by topical application of either DMSO or rapamycin (2 nM) over the surface of the tumour. **(A)** Tumour growth curves over time of each cell lines. **(B)** Tumour volume at day 4 of treatment. Mean values +/- SEM. 5 mice per group. ns: non significant, *p<0.05, **p<0.01.

DISCUSSION – CHAPTER III

M1268T Met driven anchorage independent growth and tumourigenesis requires Rac1 and mTOR independently of PI3K

The lack of a specific investigation to understand whether and how PI3K, Akt and mTOR operate downstream of Met has led to the general acceptance that PI3K (regardless of the isoform) and AKT are regulating most of the cell functions that are induced by Met [32, 392, 405].

In Chapter III, I describe for the first time that Met induces anchorage independent growth through mTOR but not through PI3K. Moreover, I report a novel role for Rac1, in promoting mTOR dependent growth, which is distinct from the classical role of Rac1 in promoting cell motility.

I have shown that PI3K and Akt inhibition do not prevent Met anchorage independent growth, and moreover it seems that Akt is even degraded in these conditions. I determined that Met induces the anchorage independent growth through mTOR, which is activated when Met is internalised. Moreover, M1268T Met induces the relocalisation of mTOR to the plasma membrane. Importantly, I am describing for the first time a new role for Rac1. Downstream of Met, Rac1 seems to be responsible for mTOR activation, possibly on endosomes, the relocalisation of mTOR to the plasma membrane and anchorage independent growth. The role of Rac1 has been widely studied in cell migration, but not in anchorage independent growth.

III. a) The discrepancy in the results obtained with the pharmacological inhibitor LY294002 and wortmannin

LY294002 is a pharmacological inhibitor that is mostly used as a pan-PI3K inhibitor, without considering its other targets. LY294002, at the minimal concentration of 10 µM that is required for it to act as a pan-PI3K inhibitor (except the resistant PI3K class II), has numerous unrelated targets (such as calcium and potassium channels, phosphodiesterases, the estrogen receptor [340]). One of them is mTOR, which is fully inhibited by LY294002 at this concentration [407]. mTOR is a key regulator of numerous cell functions. Therefore, numerous studies concluded on the role of PI3K in these cell functions based on the use of LY294002 at high doses (from 10 to 50 µM); but they could be attributed to its other targets or off-target effects. This was well illustrated throughout Chapter I and III. In Chapter I, M1268T Met dependent anchorage independent growth was strongly inhibited by LY294002, but interestingly in Chapter III wortmannin, another pan-PI3K inhibitor, had no effect. Wortmannin was discovered at the same time as LY294002; it is a much more potent and specific inhibitor than LY294002. At the concentration used to inhibit PI3K (100 nM), wortmannin has no effect (or poorly) on mTOR activity.

This highlights the importance of carefully considering the potency of pharmacological inhibitors, and the need to confirm results whenever possible using different inhibitors and methods such as siRNA knock-down.

III. b) Anchorage independent growth induced by Met does not require PI3K/Akt

I have investigated the role of PI3K and Akt in Met dependent anchorage independent growth using several methods. Firstly, the use of pan-PI3K inhibitors LY294002 and

wortmannin in soft agar assays with NIH3T3 cells expressing either WT or M1268T Met led to distinct results **(Fig. 1 *p.208*, Chapter I, and see above)**. The use of PI3K class I, Akt inhibitors **(Fig 1 *p.208*, and 2 *p.211*)**, and PI3K class III knock-down **(Fig. 1 *p.208*)**, all resulted in having no effect on M1268T and D1246N Met dependent anchorage independent growth. These results were confirmed in two human cancer cell lines expressing endogenous WT Met activated by exogenous HGF stimulation or an autocrine loop **(Fig 3 *p.213*, and 4 *p.217*)**.

When controlling for the efficiency of the pharmaceutical inhibitors within the soft agar cultures, I noticed that the level of Akt expression was decreased except when the Akt inhibitor MK2206 was used **(Fig. 1 *p.208*)**, suggesting Akt degradation. Such degradation was observed downstream of VEGFR. VEGFR does not regulate Akt expression but it protects it from been degraded [343]. Interestingly, the protective effect of MK22066 in preventing Akt degradation has been already described [344]. The authors demonstrated that when MK2206 binds to Akt, Akt is not ubiquitinated and therefore not degraded.

Altogether these results indicated that Met, WT and mutants, do not induce anchorage independent growth through PI3K/Akt. However, we cannot exclude a role for PI3K class II which is resistant to the PI3K inhibitors. This requires further investigation using siRNA knock down experiments.

III. c) Met activates mTOR to induce anchorage independent growth

Because of the inhibitory effect of LY294002 on M1268T Met anchorage independent growth, mTOR was suspected to be involved. The use of rapamycin (mTOR inhibitor) at a low concentration confirmed that WT and mutant Met induce anchorage

independent growth through mTOR **(Fig 1 *p.208*, 2 *p.211*, 3 *p.213*, and 4 *p.217*)**. Rapamycin prevents the formation of mTORC1, but also mTORC2 depending on the cell type and if the time of treatment is long. Due to the fact that the treatment in soft agar assay is for 5 days, it is so far unclear which one of the mTOR complexes is/are involved. However, if mTORC2 is involved, my results suggest that it is not through its effector Akt **(see above)**, but possibly through another one of its effectors. siRNA experiments could be performed to understand the role of mTOR1 and mTOR2 in Met dependent anchorage independent growth. Finally, it would be interesting to confirm the role of mTOR in Met dependent anchorage independent growth using an mTOR ATP-competitive inhibitor such as AZD-2014.

III. d) Met endosomal signalling to mTOR

The phosphorylation of p70 S6K has already been observed downstream of WT Met [333], however not downstream of M1268T Met **(Fig. 5 *p.218*)**. Interestingly, I have shown for the first time that p70 S6K activation downstream of endogenous WT and M1268T Met is dependent on the endocytosis machinery and likely on Met internalisation **(Fig. 5 *p.218* and 6 *p.219*)**. This result is in accordance with the previously described M1268T Met endosomal signalling to induce anchorage independent growth [56]. Consistent with this result, the activation of mTORC1 has been shown to occur at the surface of lysosomes [423]. These mechanisms of activation and signalling highlight the importance of endosomal signalling. Ménard *et al.* has shown recently that, in breast cancer cells, Met colocalises and activates Rac1 on the late endosome [78]. It would be interesting to investigate whether Met activates mTOR on the late endosome through performing immunofluorescence studies.

As anchorage independent growth can be triggered by proliferation and / or survival, future proliferation and survival assays could establish more precisely the role played by mTOR in this process.

III. e) M1268T Met relocalises mTOR to the plasma membrane, independently of PI3K class I

Saci *et al.* observed in numerous cell lines such as MEFs, Hela, A549, and HEK293 cells the colocalisation of mTOR and Rac1 at the perinuclear region, and upon serum stimulation at the plasma membrane [422]. They demonstrated that Rac1 is involved in cell growth (function regulated by mTORC1 pathway), triggers the relocalisation of mTOR to the plasma membrane, and regulates mTOR signalling independently of PI3K.

Interestingly, I observed in NIH3T3 cells expressing M268T Met the colocalisation of Rac1 and mTOR at the perinuclear region and at the plasma membrane **(Fig. 7 *p.221*)**. mTOR plasma membrane localisation was inhibited by treatment with a Met inhibitor, and was not occurring in WT Met expressing cells. Furthermore, mTOR relocalisation to the plasma membrane induced by M1268T Met was inhibited by Rac1 knock-down but the inhibition of PI3K class I seemed to have no effect **(Fig. 7 *p.221*)**.

These results put Rac1 forward as a good candidate that is responsible for mTOR activation downstream of M1268T Met.

Only a few studies describe mTOR at the plasma membrane. In 2012, a study demonstrated the binding of mTORC1 to the plasma membrane through RAPTOR binding the PtdIns(3,5)P_2 at the plasma membrane after insulin or amino acid treatment

[424]. Concerning mTORC2, a study in 2010 demonstrated in MDA-MB-231 cells that RICTOR and PKC epsilon are relocalised and colocalise together at the plasma membrane on the leading edge upon EGF treatment, and PKC epsilon relocalisation is inhibited when RICTOR is knocked-down [425]. However, another study in 2011 did not find that IGF-1 treatment increased mTORC2 at the plasma membrane [302].

Therefore mTOR relocalisation to the plasma membrane might depend on the complex mTORC1/2 involved and might be specific to extracellular stimuli. No functional role has been demonstrated yet for mTOR at the plasma membrane. Thus further studies are required to determine the role of mTOR at the plasma membrane in promoting anchorage independent growth.

III. f) Novel role for Rac1 in anchorage independent growth downstream of M1268T Met

The role of the Rho GTPase Rac1 has been extensively studied in cell migration, but it is only recently that the role of Rac1 is being investigated in different cell functions such as cell survival, anchorage independent growth and tumourigenesis [426-428]. However, this has still not been broadly demonstrated and has not been studied downstream of Met.

In this chapter, I reported a role for Rac1 in activating mTORC1 downstream of M1268T Met **(Fig. 8 *p.224*)**, and its role in M1268T Met mediated anchorage independent growth **(Fig. 9 *p.226*)**. However, the requirement for Rac1 GTPase activity has to be identified. Rac1 can be activated by multiple GEFs and it has been suggested that the GEF Tiam1 can activate Rac1 downstream of Met [79]. The pharmaceutical inhibitor NSC23766 targets the GEFs Trio and Tiam1 and inhibits M1268T Met

dependent cell migration [56], however it did not have any effect on M1268T Met anchorage independent growth **(Fig. 9 *p.226*)**. More recently Ménard *et al.*, in our laboratory, demonstrated that Met dependent cell migration in breast cancer cells was dependent on the GEF Vav2, but not Tiam1, to activate Rac1 on the late endosome [142]. In their study, Saci *et al.* demonstrated that Rac1 did not need to be activated to induce mTOR activation and relocalisation [422]. However, mTORC2 activation requires the GEF P-Rex1 [323]. Therefore, the potential role of GEFs in the signalling pathway Met-Rac1-mTOR to induce anchorage independent growth remains to be elucidated. Also, do other RTKs signal in a similar way?

The anchorage independent growth assay is a preliminary test *in vitro* to assess the capacity of cells to form tumours *in vivo*.

Due to the observed role of Rac1 and mTOR downstream M1268T Met which is independent of PI3K, this pathway might represent a promising target for cancer therapy as an alternative when resistance to RTK inhibitors have developed.

In conclusion

In Chapter III, I revealed that mTOR, independently of PI3K, is a new signalling pathway downstream of WT and M1268T Met that induces anchorage independent growth **(Fig. 11)**. I confirmed the role of mTOR in *in vivo* M1268T Met dependent tumourigenesis.

Strikingly, PI3K or Akt appear to have no role in Met dependent anchorage independent growth.

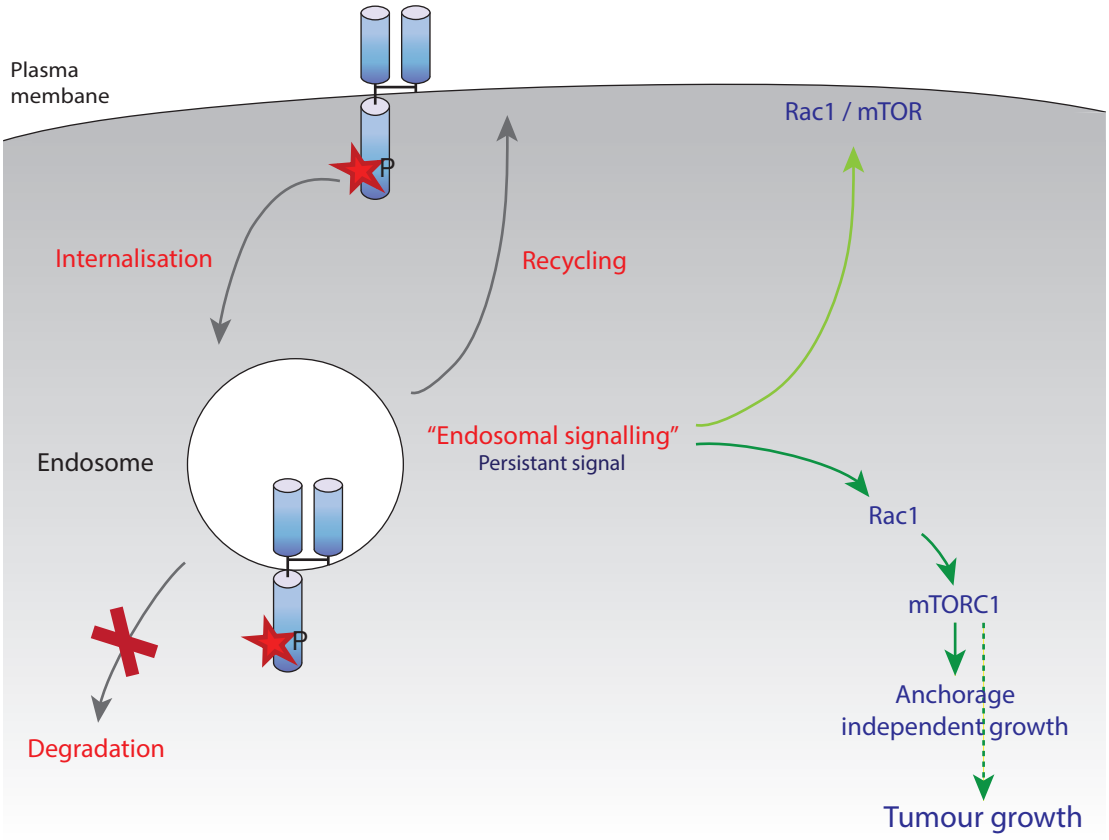


Figure 11: Model of M1268T Met signalling to induce anchorage independent growth and tumour growth

M1268T Met is constitutively activated and gets internalised and recycled back to the plasma membrane, escaping degradation. From endosomes, M1268T Met activates Rac1 and mTOR which induces anchorage independent growth and tumour growth. Downstream of M1268T, Rac1 and mTOR are relocalised to the plasma membrane.

My results also suggest that M1268T Met induces mTOR activation on endosomes, which is consistent with the previously published results that M1268T endocytosis is increased versus WT Met, that M1268T signals on endosomes and that M1268T Met dependent anchorage independent growth is impaired upon endocytosis inhibition [56].

Interestingly, Rac1 appears to be the protein that mediates Met signalling to mTOR. Met induces the relocalisation of Rac1 and mTOR to the plasma membrane, and mTOR membrane localisation is prevented upon silencing of Rac1. Moreover, Rac1 is required for mTOR signalling downstream of M1268T Met, and has been shown to get activated by M1268T Met from endosomes [56]. Finally, Rac1 expression is required for M1268T Met dependent anchorage independent growth; however, Rac1 activity may not be required, although this remained to be fully clarified.

Altogether these results suggest a new signalling pathway whereby M1268T Met associates with / activates Rac1 from endosomes, leading to mTOR activation and mTOR trafficking to the plasma membrane to induce anchorage independent growth. Such a mechanism has never been described downstream of any RTK.

Finally, the role of mTOR in M1268T Met driven tumourigenesis suggests a potential benefit in targeting mTOR to complement Met inhibition in cancer therapy. Preclinical studies targeting Met and mTOR combined are already ongoing for Epithelioid Sarcoma [346].

FINAL CONCLUSION

Receptor Tyrosine Kinases (RTK), including Met, regulate normal cell functions but their functional deregulation through overexpression or mutation leads to enhanced signalling playing a critical role in cancer development, progression, and metastasis. Met emerged as a major target for cancer therapy, therefore there is an increasing need to better understand Met signalling to target it efficiently.

When I started my PhD, it was well established that one of the major signalling pathway downstream of RTK was the PI3K/Akt/mTOR signalling pathway. Downstream of Met, this was mostly established through the use of broad PI3K inhibitors such as LY294002 and wortmannin. My team discovered that M1268T Met is oncogenic not only because it is constitutively activated, but also because it is constitutively internalised and signals from endosomes. Interestingly, it was demonstrated that Appl1, a Rab5 effector, is required for Akt signalling in zebrafish and HeLa cells [224] and for Met dependent phosphorylation of Akt in MEFs cells [398]. The existing literature suggests that PI3K class I is acting downstream of Met. However, so far, no studies have investigated which PI3K class I isoform(s) is / are downstream of Met and involved in Met signalling.

During my PhD, I investigated in detail the involvement of the PI3K/Akt/mTOR signalling pathway downstream of Met to regulate cell migration and independent anchorage growth. I had three main objectives corresponding to the three result chapters in this thesis. My first objective was to study the effect of the pan PI3K / mTOR inhibitor LY294002 on mutant Met dependent increase of cell transformation in NIH3T3 cells (Chapter I). The efficacy of this drug in reducing cell transformation led me to investigate which specific PI3K isoform(s) is involved in mutant Met dependent

cell migration (Chapter II), and mutant Met induced anchorage independent growth (Chapter III), in the hope of identifying a clear pathway for each cell function. However, my work led to numerous unexpected results **(Fig. 1)**:

- PI3K class I p110 alpha, p110 beta and p110 delta isoforms are required for Met dependent cell migration, but the degree of requirement for each isoform varies depending on the cell model / mode of Met signalling studied;
- Akt activated by Met might not be involved in Met dependent cell migration despite the role of PI3K class I;
- PI3K (at least class I and III) is actually not involved in Met induced anchorage independent growth;
- Mutant Met activates mTORC1 through Rac1;
- Rac1 has a role with mTORC1 in Met induced anchorage independent growth, while Rac1 is generally described as being responsible for cell migration.

To date, although most results have been obtained using the pan PI3K / mTOR inhibitor LY 294002 together with the detection of phosphorylated Akt (mostly on S473), it was assumed that class I PI3K-Akt was the major pathway activated by c-Met within PI3K – mTOR family of signalling molecules, leading to cell proliferation, survival and migration. Strikingly, no report so far have suggested that PI3K (at least class I and III) may not be involved in Met anchorage independent growth and none have questioned Akt role in Met dependent cell migration with the new generation of tools available (Akt pharmaceutical inhibitors and siRNAs targeting Akt). Moreover, the activation of mTORC1 by binding Rac1 has been described upon serum stimulation [367] but there is no upstream signalling pathway that has been previously defined as activating Rac1/mTOR independently of PI3K class I and having a role in anchorage independent growth.

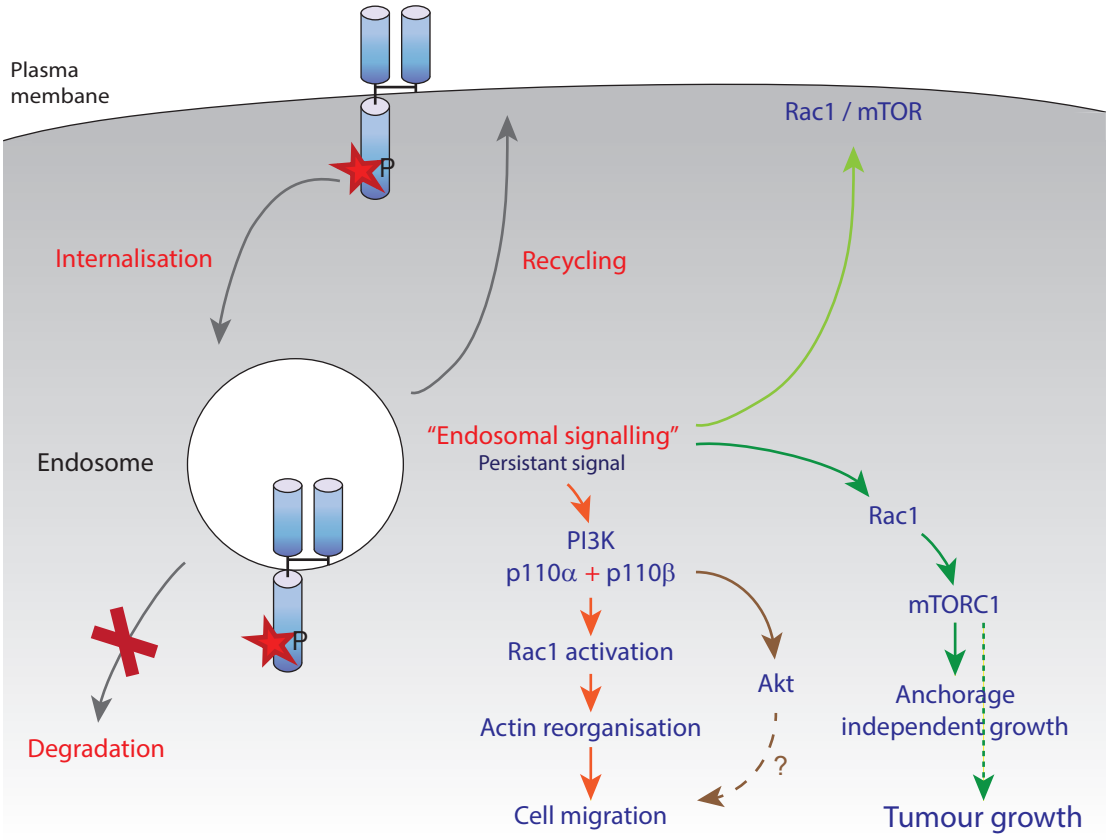


Figure 1: Model of M1268T Met signalling to induce cell migration, anchorage independent growth and tumour growth

M1268T Met is constitutively activated and gets internalised and recycled back to the plasma membrane, escaping degradation. From endosomes, M1268T Met induces PI3K class I activation, resulting in activation of Rac1, reorganisation of the cytoskeleton, cell migration and Akt activation; and activates Rac1 and mTOR which induces anchorage independent growth and tumour growth. Downstream of M1268T, Rac1 and mTOR are relocalised to the plasma membrane.

Thus my thesis provides a number of original and important results although several of my findings need to be completed to have a comprehensive overview of PI3K/Akt signalling pathway role in Met induced cell migration and of Rac1/mTOR role in Met dependent anchorage independent growth.

The results obtained were mostly analysed by Student t-test. It would have been interesting to apply other statistical test such as the analysis of variance (ANOVA) to confirm the results.

In experiments involving siRNAs, it would need to be repeated with different siRNAs.

The role of the PI3K class I isoforms p110 alpha, p110 beta and p110 delta have been determined in Met dependent cell migration, however the expression and role of p110 gamma remain to be investigated in A549 and U87MG cells. This could be done through the use of the pharmaceutical inhibitor AS-604850 or p110 gamma knock-down. NIH3T3 cells do not express p110 gamma [151].

The role of Akt in Met dependent cell migration could be investigated in NIH3T3, A549 and U87MG cells using the new generation of pharmaceutical inhibitors targeting Akt such as GDC0068 or performing Akt isoforms' knock-down.

In addition, it would be interesting to investigate if Rac1 has a role in activating mTORC2 downstream of mutant Met. This could be investigated by using as a read-out Akt phosphorylation on Serine 473. Also, does WT Met, upon HGF stimulation, activates mTORC1 (and potentially mTORC2) through Rac1 similarly to the mutant Met M1268T?

Finally, studying the role of Rac1 in Met dependent tumourigenesis in *in vivo* assay could provide useful information for Rac1 role in tumourigenesis downstream of Met.

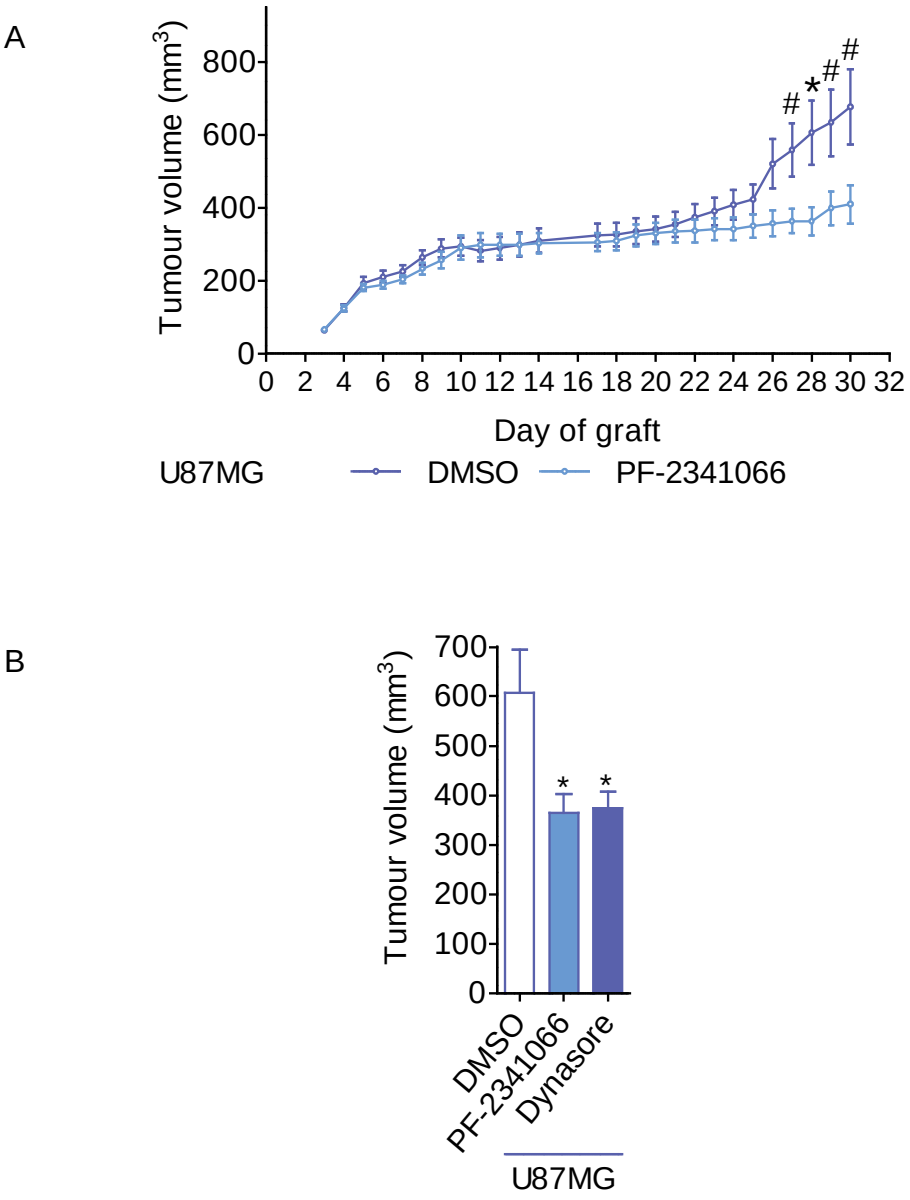
Overall, this work highlighted the complexity of the PI3K/Akt/mTOR pathway and the importance of considering the role of each of these proteins individually.

Accordingly, over the last decade, pharmaceutical companies have developed specific inhibitors for each PI3K isoform and for Akt, as well as mTOR ATP-competitive inhibitors or rapalogues (rapamycin analogues) [187, 383]. Due to the important role of the PI3K/Akt/mTOR pathway in cancer, many of these novel inhibitors have been / are being tested in clinical trials. However, monotherapies have not reached expectations as they showed limited efficacy. Therefore the new strategy recently adopted is to target both PI3K class I and mTOR with dual inhibitors.

Met has been reported to mediate resistance to various therapy in breast, lung, pancreatic and colorectal cancer [82, 257, 429-432]. Therefore, there is an increasing effort to design and test Met pathway inhibitors, many of which are in early clinical trials. However, chances are high that resistance will occur as experienced with other RTK inhibitors (such as the EGFR inhibitor gefitinib or the HER2 antibody Trastuzumab for example). Therefore, alternative or combined therapies may be required, such as Met inhibitor with PI3K/ mTOR inhibitors.

My work would benefit from further investigations, for example to identify in more detail the role of each PI3K/Akt isoform (including PI3K Class II) and of mTORC1/2 downstream of Met. However, I have demonstrated that Met activation is associated with PI3K/mTOR signalling in inducing several cell functions. Therefore it seems that PI3K class I/mTOR inhibition, alone or in combination with Met inhibition, could be beneficial for patients with Met driven cancers. More specifically, my results suggest that targeting PI3K class I may be used for advanced cancer with the intention of reducing the metastatic process while mTOR inhibition may be used to reduce tumour growth.

Interestingly, the discovery of Rac1 acting downstream of the Met mutant to activate mTOR and to induce anchorage independent growth, suggests that the role of Rac1 could be crucial for Met oncogenicity. Therefore, the role of Rac1 in Met driven tumourigenesis needs to be investigated, as well as its role downstream of other growth factors. Rac1-mTOR could represent a promising new pathway to be targeted in order to reduce the growth of cancers, driven by c-Met and possibly other oncogenic receptors.



Appendix 1: U87MG tumour growth is reduced by Met and dynamin inhibition

(A, B) 5x10^6 U87MG cells were injected subcutaneously into nude mice. Tumours were measured daily and once they reached a volume of 30-50 mm^3, the tumours were treated by topical application of either DMSO, PF-2341066 (200 nM) or dynasore (80 µM) daily over the surface of the tumour. **(A)** Tumour growth curves over time. **(B)** Tumour volume at day 28 post-graft. Mean values +/- SEM (9 mice per group). ns: non significant, #p=0.05, *p<0.05.

Steps of protein synthesis regulated by S6K

Protein synthesis is the result of 4 successive steps: transcription, translation initiation, translation elongation, and termination. When activated, S6K regulates **(A) translation initiation** and **(B) translation elongation**.

(A) Translation initiation can be cap dependent or cap independent.

Most mRNAs have cap dependent translation. A complex of proteins binds and holds together: (1) the 5'end of mRNAs (called 5'cap); (2) the 5'Untranslated Region (UTR) directly upstream of the mRNA's initiation codon; (3) the small ribosomal subunit 40S. This complex of proteins is formed by initiation factors including eukaryotic Initiation Factor (eIF) 3, and eIF4F complex (constituted of eIF4A, eIF4E, and eIF4G). eIF4G bounds eIF4F complex to eIF3. eIF4E is the cap binding protein. eIF4A is an RNA helicase, contributing to unwind mRNA structures.

In cap independent translation, the ribosome does not required to start at the 5'end, and can start directly at the start codon. This mechanism is particularly important for cells to respond to cellular stress or apoptosis.

(B) Translation elongation occurs following the step of initiation. Elongation factors as eukaryotic Elongation Factor (eEF) 2 promotes the GTP dependent translocation of ribosomes to the next codon so it can be translated and the produced peptide elongates to become the newly synthetized protein.

Appendix 2: Steps of protein synthesis regulated by S6K

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ACKNOWLEGEMENTS

My PhD at Barts Cancer Institute has been a truly memorable experience, and I would like to thank everyone who contributed to my self-fulfilment as a scientist.

First of all, I would like to thank my supervisor Dr Stéphanie Kermorgant. Thank you for giving me this life changing opportunity. You provided me with a constant source of motivation and encouragement. I learnt from you to demand the best out of myself. You have been a great boss, thank you for everything!

I would also like to thank Prof Bart Vanhaesebroeck for his valuable advice and for helping me to develop a critical point of view on my project.

I would particularly like to thank every member of spatial signalling group. It has been a pleasure to work with you all. You have been, for me, real team players, highly motivated, hard workers, always available to help or share your point of view; all of this, always smiling.

Among them, Dr Rachel Barrow McGee, who regularly helped me and shared her experience with me. Dear Rachel, thank you so much for everything. You have been a wonderful colleague and friend. I am going to miss working with you and being with you. Ludovic, Kishi, and Bakhouche, thank you so much for your constant good mood. Your jokes made me smile all of the time, or attempts of joke from Ludo. Waleed and Sara, I wish you much enjoyment from your experience in Met team and Tumour Biology department.

I would like to acknowledge all of my relatives and friends for their constant encouragement. Your support has always helped me to keep my feet on the ground.

A special thank you to my family,

You are my best support. I probably would not have gone this far in my studies and personal achievement without your constant blessing in the decisions I made. Merci!

And finally, I would like to thank you Mark.

You provided me with a home where my worries and stress were disappearing. You made this PhD a nice journey.