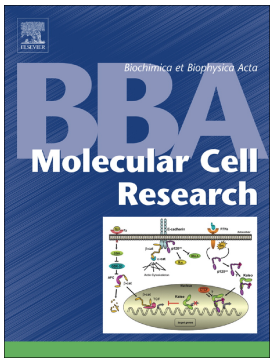

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Advances in understanding the mechanisms of evasive and innate resistance to mTOR inhibition in cancer cells

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Highlights:

- Aberrant activation of mTOR signaling is a common event in many human tumors, making mTOR an attractive target for cancer therapy.
- mTOR inhibitors have met with a very limited success as anticancer therapeutics.
- Understanding the reasons underlying the lack of efficacy of mTOR inhibition in cancer patients is of utmost importance for the designing of better therapies.
- mTOR inhibitors unleash activation of several compensatory signaling pathways that dampen their efficacy.
- Over the last few years, other mechanisms of resistance have emerged, including epigenetic alterations, compensatory metabolism rewiring and the occurrence of mTOR mutations.

ABSTRACT

The development of drug-resistance by neoplastic cells is recognized as a major cause of targeted therapy failure and disease progression. The mechanistic (previously mammalian) target of rapamycin (mTOR) is a highly conserved Ser/Thr kinase that acts as the catalytic subunit of two structurally and functionally distinct large multiprotein complexes, referred to as mTOR complex 1 (mTORC1) and mTORC2. Both mTORC1 and mTORC2 play key roles in a variety of healthy cell types/tissues by regulating physiological anabolic and catabolic processes in response to external cues. However, a body of evidence identified aberrant activation of mTOR signaling as a common event in many human tumors. Therefore, mTOR is an attractive target for therapeutic targeting in cancer and this fact has driven the development of numerous mTOR inhibitors, several of which have progressed to clinical trials. Nevertheless, mTOR inhibitors have met with a very limited success as anticancer therapeutics. Among other reasons, this failure was initially ascribed to the activation of several compensatory signaling pathways that dampen the efficacy of mTOR inhibitors. The discovery of these regulatory feedback mechanisms greatly contributed to a better understanding of cancer cell resistance to mTOR targeting agents. However, over the last few years, other mechanisms of resistance have emerged, including epigenetic alterations, compensatory metabolism rewiring and the occurrence of mTOR mutations. In this article, we provide the reader with an updated overview of the mechanisms that could explain resistance of cancer cells to the various classes of mTOR inhibitors.

Keywords

Target therapies; Drug-resistance; Cell Signaling Pathways; Epigenetics; Metabolism; Mutations

1. Introduction

Over the last 20 years many small molecules have been developed for selective targeting of oncogenic pathways. However, with a few notable exceptions, such as imatinib and its derivatives that revolutionized the treatment of chronic myelogenous leukemia and changed the natural history of the disease [1, 2], targeted drugs have not led to a cure, either when used alone or in combination with other therapeutics. The very limited success of targeted therapy is due to several reasons, including drug-resistance of tumor cells. Two modes of cancer drug-resistance exist, innate (or intrinsic) and evasive (or acquired) [3]. While innate resistance implies non-responsiveness to a therapy from the beginning, evasive resistance is defined as an initial responsiveness (that could be robust in some cases) followed by tumor relapse. Innate resistance could be due to cancer cells diffusely containing a preexisting mutation that confers resistance in a cell-autonomous manner. In contrast, evasive resistance can be similarly inherent to the neoplastic cell, but with the change arising upon treatment, despite an initial clinical response. Importantly, evasive resistance can also be non-inherent (i.e. non-cancer cell-autonomous), whereby it relies on interactions with the tumor microenvironment cells [3]. Understanding the mechanisms that confer innate or evasive resistance is essential for patient stratification and the rational design of more efficacious targeted therapies, hence for personalized and precision medicine approaches to cancer cure [4].

In this article, we will review the mechanisms underlying both evasive and innate inherent resistance of cancer cells to the various classes of drugs trageting mechanistic (previously mammalian) target of rapamycin (mTOR).

2. mTOR

mTOR is a highly conserved Ser/Thr kinase that integrates a variety of stimuli including growth factors, hormones, cellular energy status, oxygen availability and stress to mainly regulate proliferation (increase in cell number), growth (increase in cell volume/mass) and survival [5]. mTOR is the core component of two structurally and functionally different multi-protein complexes: mTOR

complex 1 (mTORC1) and 2 (mTORC2) [6]. mTORC1 comprises mTOR, Tel2-interacting protein 1/telomere interacting protein 2 (Tti1/Tel2), regulatory-associated protein of TOR (Raptor), mammalian lethal with SEC13 protein 8 (mLST8), proline-rich Akt substrate 1 40-kDa (PRAS40) and disheveled/Egl-10/pleckstrin (DEP) domain-containing mTOR-interacting protein (Deptor) [7-9]. While mLST8, Tti1/Tel2 and Deptor are found in both mTORC1 and mTORC2, rapamycin-insensitive companion of TOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1) and protein observed with Rictor (Protor) are specific components of mTORC2 [10-13]. mTORC1/mTORC2 components and their roles are summarized in **Table 1 and Figure 1**.

Regarding mTORC1 activation, hormones and growth factors bind to receptor tyrosine kinases (RTKs) to activate phosphatidylinositol 3-kinase (PI3K). PI3K phosphorylates the inositol ring of the membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP2), to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3) [14]. PIP3 recruits phosphoinositide-dependent kinase 1 (PDK1) and Akt to the plasma membrane, whereby PDK1 phosphorylates Thr^{308} in the activation loop of Akt [11]. Akt then phosphorylates Tuberous sclerosis 2 (TSC2), thus inducing lysosomal release and inhibition of the TSC complex that comprises TSC2 itself, the TSC1 scaffolding protein and Tre2-Bub2-Cdc16-1 domain family member 7 (TBC1D7) [9]. The TSC complex is a GTPase-activating protein (GAP) for the lysosomal GTP-binding protein Ras homolog enriched in brain (Rheb) [15]. GTP-loaded Rheb interacts with the mTOR catalytic domain and activates mTORC1 [12]. However, mTORC1 can be activated by amino acids, high energy/oxygen [16, 17] and metabolic intermediates such as D-2-hydroxyglutarate [18], whereas a reduction in low energy [19], DNA damage and hypoxia inhibit mTORC1 [20]. Furthermore, mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling, by impinging on the TSC complex via p90 ribosomal S6 kinase (p90RSK), is another key positive regulator of mTORC1 activity [21] (**Figure 2**).

At variance with mTORC1, the mechanisms by which mTORC2 activity is controlled are not as well defined. mTORC2 activity was found physically associated with a subpopulation of ribosomes and

mitochondria [22, 23], thereby implicating cell endomembranes as potential sites of mTORC2 activity. A recent study by Liu and coworkers [24] identified a link between PI3K/PIP_3 and mTORC2 activity at the plasma membrane. Indeed, they found that the pleckstrin homology (PH) domain of mSIN1 interacts with the kinase domain of mTOR resulting in suppression of mTORC2 activity. PIP3, generated at the plasma membrane upon growth factor stimulation, interacts with the PH domain of mSIN1 to repress its inhibition on mTOR, whereby leading to mTORC2 activation (**Figure 2**). However, a different group [25] has subsequently reported that both the localization and activity of mTORC2 at the plasma membrane via the mSIN1 PH domain were independent of PIP3 synthesis, upon stimulation with insulin. In contrast, a subpopulation of endosomal vesicles displayed a PI3K-dependent mTORC2 activity, suggesting the existence of different mTORC2 subpopulations with distinct sensitivity to PIP3 [25] .

In addition to differences in their protein composition and activity regulation, mTORC1 and mTORC2 control distinct cellular processes through the phosphorylation of largely non-overlapping substrates. Notable downstream targets of mTORC1 are 70-kDa ribosomal protein S6 kinase 1/2 (p70S6K 1/2), eukaryotic translation initiation factor 4E binding protein (4E-BP1), La ribonucleoprotein domain family member 1 (LARP1), lipin 1 [26] and Unc-51-like kinase 1 (ULK1) [27-29]. In general, mTORC1 promotes anabolic-related pathways by regulating mRNA translation. Specifically, 4E-BP1 phosphorylation relieves its binding to eukaryotic translation initiation factor 4E (eIF4E). This in turn allows eIF4E to initiate translation by allowing eIF4F complex (eIF4E, eIF4A and eIF4G) formation and binding to the cap of mRNA, thus initiating cap-dependent translation [30]. Moreover, mTORC1 upregulates glycolysis, lipid metabolism, nucleotide synthesis and ribosome biogenesis, whereas it represses autophagy [31, 32] (**Figure 2**).

mTORC2 plays an important role in the regulation of cell survival through the phosphorylation and activation of several AGC family kinases, including Akt, serum and glucocorticoid-induced kinase 1 (SGK1) [33-35]. In particular, mTORC2 phosphorylates the hydrophobic motif of Akt at Ser^{473}, which increases Akt activity toward a well-defined subset of substrates, including the forkhead box

O (FoxO) transcription factor family and glycogen synthase kinase (GSK) 3α/β [36]. As to SGK1, it phosphorylates N-myc downstream regulated gene 1 (NDRG1) that is involved in angiogenesis, cancer growth and metastasis in a variety of tumors [37] (**Figure 2**).

In addition, mTORC2 is involved in actin cytoskeletal organization and cell motily via protein kinase (PKC) [38], lipid biosynthesis [39], as well as mitochondrial function, following its growth factor-stimulated recruitment to the mitochondrial-associated endoplasmic reticulum membrane [23]. Furthermore, emerging evidence indicates that also mTORC2 is somehow involved in glucose, amino acid and nucleotide metabolism [40].

It should be emphasized, however, that recent findings seem to indicate that mTOR exists in at least two other complexes different from either mTORC1 or mTORC2. One of these complexes has been identified in astrocytes and lacks either Raptor or Rictor [41]. The second novel complex, named mTORC3, is present in many cancer cells and, as we shall see in this article, contributes to innate resistance to the mTOR inhibitor, rapamycin [42].

3. mTOR inhibitors

mTORC1 and mTORC2 activities are deregulated in a wide array of tumors. Neoplastic cells exploit mTOR oncogenic signaling for driving their proliferation, survival, metabolic transformation and metastatic potential [3]. Therefore, mTOR lends itself very well as a therapeutic target for innovative cancer treatments. mTOR was originally discovered as the target of rapamycin (sirolimus), a macrolide antibiotic purified from a bacterium (*Streptomyces hygroscopicus*) growing in the soil collected on Easter Island (Rapa Nui in the local language) [43]. Rapamycin and its derivatives (everolimus and temsirolimus, also referred to as rapalogs) were the first class of mTOR inhibitors that displayed anticancer properties *in vitro* and in xenografted tumors *in vivo* [31]. mTORC1 and mTORC2 display a different sensitivity to rapamycin/rapalogs, that are considered to be allosteric inhibitors mainly of mTORC1 activity. Together with the immunophilin FK506-binding protein of 12 kDa (FKBP12), rapamycin/rapalogs associate with the FKBP12-rapamycin-binding (FRB)

domain of mTOR [44]. This association results in decreased interactions between mTOR and Raptor with a consequent downregulation of mTORC1 activity [45]. The rapamycin-FKBP12 complex prevents binding of mTORC1 to its substrates by steric hindrance through reduction in the size of the active-site cleft of mTOR [44]. The steric hindrance model explains the differential sensitivity of mTORC1 substrates to rapamycin/rapalogs. For instance, rapamycin/rapalogs usually potently suppress p70S6K 1/2 phosphorylation whereas they have only marginal effects on 4E-BP1 phosphorylation levels [46, 47]. Differently from mTORC1, mTORC2 is much less sensitive to acute inhibition with rapamycin/rapalogs, i.e. under conditions wherein the drugs have been applied for less than 12 h in cell culture [48]. Indeed, in mTORC2 Rictor/mSIN1 mask the FRB domain of mTOR [49]. However, there are many reports showing that rapamycin is capable of inhibiting mTORC2 upon longer exposure, most likely by negatively affecting the assembly of new mTORC2 complexes [50].

To date, rapalogs are the only class of mTOR inhibitors approved for the treatment of various human advanced cancers, including renal clear cell carcinoma (RCC), pancreatic/lung/gastrointestinal neuroendocrine tumours, postmenopausal hormone receptor-positive breast cancer in combination with exemestane and refractory mantle cell lymphoma [31].

Rapamycin/rapalogs only partially inhibit mTORC1-dependent outputs (see above), cause feedback activation of oncogenic pathways, including PI3K/Akt (see further on) and display a weak pro-apoptotic activity in cancer cells [31]. These observations, coupled to the structural similarities between the catalytic domains of PI3K and mTOR [51], provided the rationale for the development of ATP-competitive dual PI3K/mTOR inhibitors, a class of drugs that target PI3K and both mTOR complexes [52]. Dual PI3K/mTOR inhibitors were followed by mTOR kinase inhibitors (TORKIs), a class of molecules that block only the mTOR catalytic domain. TORKIs were designed to reduce toxicity due to the use of dual PI3K/mTOR inhibitors [53]. The newest class of mTOR inhibitors consists of RapaLink-1, a drug that simultaneously acts as an allosteric inhibitor while targeting the

active site of mTOR. RapaLink-1 exploits the juxtaposition of the corresponding two drug-bindings pockets, i.e. the FRB domain and the kinase domain of mTOR [54].

4. Evasive mTOR inhibitor resistance due to overactivation of compensatory signaling pathways

Several lines of evidence indicate that both mTORC1 and mTORC2 mediate potent negative feedback loops that restrain upstream signaling networks through insulin receptor (IR), insulin-like growth factor 1 (IGF1) receptor (IGF1R) and other RTKs in both healthy and neoplastic cells. Therefore, pharmacological inhibition of either mTORC1 or mTORC2 unleashes a series of compensatory phenomena that explain some types of evasive resistance to mTOR-targeting drugs.

4.1. Feedback loops leading to PI3K/PDK1/Akt overactivation

A commonly observed effect of rapamycin/rapalog treatment in cultured tumor cells, preclinical cancer models and clinical trials is a striking increase in Akt phosphorylation at Thr^{308} by PI3K/PDK1 and at Ser^{473} by mTORC2 [55-63].

Regarding Thr^{308} Akt phosphorylation, it should be considered that both mTORC1 and its substrate, p70S6K1, provide a negative feedback to insulin and IGF1 signaling networks via inhibitory serine phosphorylation of insulin receptor substrate (IRS) 1 and 2 [64]. The IRS proteins are a family of docking proteins that integrate and coordinate the transmission of signals from the extracellular to the intracellular environment through transmembrane receptors. IRS proteins are the major molecules that mediate cell responses to either insulin or IGF1 stimulation [65]. Specifically, IRS 1/2, by interacting with the p85 regulatory subunit of PI3K, stimulate PIP3 synthesis [66].

mTORC1-dependent phosphorylation sites of IRS 1/2 include $Ser^{422/636/639}$, while p70S6K1 targets $Ser^{270/307/636/1001}$ [67, 68] (**Figure 3**). Once phosphorylated at these residues, IRS1/2 are targeted for proteasomal degradation via Skp/Cullin/F-box containing complex/β-transducin repeats-containing

protein (SCF β-TRCP) E3 ubiquitin ligase [68-70]. Hence, insulin/IGF1-dependent, IRS-induced signals are switched-off and PI3K/Akt signaling is downregulated [71].

An additional mTORC1 substrate that negatively impinges on PI3K/PDK1 signaling is growth factor receptor-bound protein 10 (Grb10) [72]. Grb10 is an adaptor protein that inhibits signals elicited by either insulin or IGF1 [73]. Once phosphorylated by mTORC1, Grb10 is stabilized and this leads to feedback inhibition of the Akt phosphorylation [74, 75]. Several Grb10 residues phosphorylated by mTORC1 were identified by two independent groups *in vitro* and in cells. These include $Ser^{501/503}$ [76], as as well as $Ser^{104/150/155/428/476}$ [77]. Rapamycin-sensitive sites include $Ser^{476/501/503}$ residues, whereas $Ser^{104/150/155/428/476}$ (and presumably also $Ser^{503/505}$) were dephosphorylated only by the TORKI, Torin-1 [76, 77]. Therefore, Grb10 is similar to 4E-BP1, in that it displays both rapamycin-sensitive and -insensitive residues [78] (**Figure 3**). In addition to inhibiting IR/IGF1R tyrosine kinase activity by direct binding, Grb10 mediates degradation of the receptors through ubiquitination [79]. Importantly, mTORC1-mediated phosphorylation of Grb10 increases its stability, while chronic mTOR inhibition decreases Grb10 protein abundance without significantly affecting mRNA levels [77]. As a consequence, acute mTORC1 inhibition leads to dephosphorylation of Grb10, while chronic mTORC1 inhibition leads to changes in the expression levels of Grb10 proteins which are likely to be the most important effects of rapamycin/rapalogs to consider in their clinical use. When the feedback negative loops based on IRS 1/2 and Grb10 are interrupted by exposure to rapamycin/rapalogs, a hyperphosphorylation of Akt at Thr^{308} is usually observed.

Regarding mTORC2-mediated Ser^{473} Akt phosphorylation, it should be considered that mSIN1 is targeted by p70S6K1 at both Thr^{86} and Thr^{389} residues located at the N-terminus and PH domain, respectively [80]. Phosphorylation at Thr^{86} interferes with SIN1-N-terminus binding to Rictor, while phosphorylation at Thr^{398} impairs SIN1-PH domain interactions with the kinase domain of mTOR. Both phosphorylation events are required for mSIN1 dissociation from the mTORC2 and inhibition of mTORC2 activity [80]. Therefore, p70S6K1-dependent phosphorylation of mSIN1 provides yet another negative feedback mechanism downstream of mTORC1 in response to several growth factors

important for tumor cell growth, that include not only insulin and IGF1, but also platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) [81, 82]. Also Rictor is direct target of p70S6K1 that phosphorylates it on Thr1135 in a rapamycin-sensitive manner and mediates 14–3-3 protein binding to Rictor, whereby inducing a conformational change that prevents mTORC2 from phosphorylating Akt at Ser^{473} [83] (**Figure 3**). As a consequence, when the inhibitory loops based on mSIN1 and Rictor are switched off by treatment with rapamycin/rapalogs, a hyperphosphorylation of Akt at Ser^{473} is observed that is independent from either IRS 1/2 or Grb10.

The existence of all the aforementioned feedback loops unleashed by rapamycin/rapalogs, has been one of the reasons that provided impetus for the synthesis of dual PI3K/mTOR inhibitors (e.g. PI-103, NVP-BEZ235), as well as of TORKIs [84], that indeed do not cause p-Akt upregulation in some settings [63, 85-87]. However, as we shall see later in this article, both dual PI3k/mTOR inhibitors and TORKIs could hyperactivate Akt through other mechanisms.

4.2. Feedback loops leading to MEK/ERK overactivation

Constitutive activation of the MEK/ERK network is a commonly occurring event in cancer, where it frequently coexists with PI3K/Akt upregulation [88]. Aberrant MEK/ERK signaling has been implicated in the initiation, progression and metastasis of both solid and hematologic malignancies [89, 90]. Over the years, extensive cross-talk have demonstrated to occur between these two signaling cascades [91, 92]. A phonomenon that is frequently observed in response to cancer cell treatment with mTOR inhibitors is a hyperphosphorylation at the Thr^{202} and Tyr^{204} residues of the ERK activation loop. Carracedo and coworkers [93] were the first to report a marked increase in Thr^{202}/Tyr^{204} p-ERK levels in biopsies of breast cancer patients treated with everolimus. Experiments based on the use of a PI3K inhibitor and a dominant-negative form of Ras, led to the conclusion that ERK overactivation partly depended on the existence of a mTORC1/p70S6K1/IRS/PI3K/Ras/Raf/MEK/ERK negative feedback loop control mechanism which is interrupted by rapamycin/rapalogs, similarly to the previously described loop involving

mTORC1/p70S6K1/IRS/PI3K/PDK1/Akt. Comparable results have been subsequently reported in colon cancer cells that became became resistant to everolimus [94].

Furthermore, given that phosphorylation of Grb10 by mTORC1 potentiates its inhibitory activity on IR/IGF1R signaling, acute suppression of Grb10 phosphorylation by mTOR inhibitors elicits not only PI3K/Akt, but also MEK/ERK overactivation [76].

As highlighted previously, dual PI3K/mTOR inhibitors usually do not induce Akt activation, however it has been reported that they upregulated $Ser^{217/221}$ MEK and Thr^{202}/Tyr^{204} ERK phosphorylation levels in several ductal pancreatic adenocarcinoma cell lines [95]. ERK phosphorylation was abrogated by the MEK inhibitors, UO126 or PD0325901. The molecular mechanisms leading to such an activation are not completely understood, but they are PI3K-independent, as the dual PI3K/mTOR inhibitor NVP-BEZ235 prevented PIP3 generation at the plasma membrane, but increased ERK phosphorylation. MEK/ERK upregulation was also independent of EGF receptor (EGFR), human epidermal growth factor receptor 2 (HER2), IR and IGF1R activities (see Subsection 4.3 of this article). However, the increased phosphorylation was mTORC2-dependent, as Rictor knockdown via siRNA attenuated the enhancing effects of NVP-BEZ235 on ERK phosphorylation [95].

Also TORKIs have been reported to activate ERK, for example in PANC1 and MiaPaCa2 pancreatic cell lines stimulated with either insulin or neurotensin, a G-protein coupled receptor agonist [96], and in multiple myeloma (MM) cells [97]. In MM cells, ERK overactivation was a clear mechanism of resistance to the PP242 TORKI, and was not dependent on PI3K activity but rather on a mTORC1/4E-BP1/eIF-4E signal cascade that led to Raf/MEK/ERK upregulation [97]. Raf overactivation was not downstream of Ras in MM cells, as demonstrated by the absence of any increase in Ras bound to GST-Raf in an *in vitro* assay as well as by the lack of effects of PP242 on the levels of the Ser^{338} residue of Raf, a Ras-inducible activating phosphorylation site that is critical for its activation. In contrast, Rictor genetic ablation via shRNA or overexpression of eIF-4E led to an increase in Raf activity in an *in vitro* kinase assay. The molecular mechanisms leading to such an upregulation are

still unclear, however they could be partly explained by PP242 dephosphorylation of 4E-BP1 and sequestering of eIF-4E [97].

As a consequence of all the aforementioned findings, combined treatments with drugs targeting PI3K/Akt/mTOR and MEK/ERK display improved efficacy compared with inhibition of either cascade alone in a wide variety of preclinical settings of hematological and solid cancers [91, 98]. However, initial clinical studies where mTOR and MEK /ERK inhibitors were combined together, have yielded so far much less promising results than expected [99, 100]. For example, two recently published Phase Ib studies where a MEK inhibitor was combined with a dual PI3K/mTOR inhibitor, showed poor long-term tolerability and limited antineoplastic activity in patients with advanced solid tumors [101, 102].

4.3. Overexpression of RTKs and adaptor proteins leading to PI3K/PDK1/Akt and MEK/ERK overactivation

The FoxO transcription factors, which includes FoxO1/3/4/6 in mammalian cells, are major downstream targets of Akt. FoxO phosphorylation by Akt creates docking sites for 14–3–3 proteins. Once bound to FoxO factors, 14–3–3 proteins promote FoxO translocation from the nucleus to the cytoplasm. Reciprocally, Akt inhibition releases a feedback loop that promotes nuclear localization of FoxOs [103, 104]. It should be emphasized here that mTORC2-dependent phosphorylation of Akt at Ser^{473} is essential for Akt activity on FoxO proteins [36]. Once in the nucleus, FoxO factors stimulate transcription of EGFR, IR, IGF1R, HER2 and HER3, as well as that of IRS1, in a wide spectrum of cancer cells [105-107]. Moreover, FoxOs upregulate Rictor expression, thereby enhancing mTORC2 activity and Akt phosphorylation at Ser^{473} and creating an amplification loop [108, 109].

Accordingly, it has been shown that long-term exposure to rapamycin, dual PI3K/mTOR inhibitors (e.g. NVP-BEZ235) or TORKIs (e.g. AZD8055), initiate transcriptional responses that lead to RTK (EGFR, IGFR, HER2, HER3) or adaptor protein (IRS) overexpression [110], or RTK

phosphorylation [111]. Knockdown of FoxOs by siRNA can block not only RTK/IRS overexpression, but also RTK phosphorylation induced by the mTOR inhibitors [111]. However, it remains to be elucidated how activation of FoxOs lead to phosphorylation of growth factor receptors, although increased c-Src activity has been implicated in case of EGRF phosphorylation induced by rapamycin [112].

In some cases, FoxO activation positively impacted on MEK/ERK activity, as observed in HER2-overexpressing breast cancer cells, where NVP-BEZ235 treatment resulted in abolished Akt activation that, however, was paralleled by a compensatory activation of MEK/ERK signaling [110]. The enhanced MEK/ERK signaling occurred as a result of activation of HER family receptors, as demonstrated by induction of HER receptors dimerization and phosphorylation, increased expression of HER2/HER3 and binding of adaptor/regulatory molecules (Grb2, p85 PI3K) to HER2/HER3. MEK/ERK activation was prevented with either a MEK inhibitor (AZD6244) or anti HER2 monoclonal antibody (trastuzumab) and tyrosine kinase inhibitor (lapatinib). Combined administration of PI3K inhibitors with either HER2 or MEK inhibitors resulted in decreased proliferation, enhanced cell death and superior antitumor activity compared with NVP-BEZ235 alone [110].

In pancreatic cancer models, it was found that AZD8055 induced a transient Akt inhibition that, however, was followed by the expression/activation of EGFR via FoxO1/3a and feedback reactivation of Akt. *In vitro* and *in vivo* experiments further indicated that a combination consisting of AZD8055 and erlotinib (an EGFR inhibitor) synergistically inhibited mTORC1/mTORC2 signaling, EGFR/Akt feedback activation, and cell growth, as well as suppressed the progression of pancreatic cancer in a xenograft model [113]. Reactivation of Akt through FoxO1 and RTKs has been also reported in acute myelogenous leukemia (AML) cells treated with the TORKI, Torin-1 [114].

In ovarian cancer cells, NVP-BEZ235 induced a much more complex program that involved both FoxO-regulated transcription and cap-independent translation, leading to expression of RTKs and survival proteins, including EGFR, HER2, IGF1R, Bcl-2, Bcl-xL, XIAP1. However, this response

did not result in MEK/ERK signaling overactivation [106]. Interestingly, NVP-BEZ235 treatment of ovarian cancer-spheroids led to death of inner matrix-deprived cells whereas matrix-attached cells were resistant. Resistance to NVP-BEZ235 could be abrogated by a Bcl-2/Bcl-xL inhibitor (ABT-737), EGFR inhibitors (PD16839, gefitinib) or downregulation of IGF1R with shRNA strategy, whereas a MEK inhibitor (PD98059) had no effects.

In conclusion, cancer cell treatment with inhibitors targeted to the mTOR pathway induces concerted transcriptional responses mediated, at least in part, by FoxO family members. Depending on the setting, FoxOs could oppose the anticancer effects of mTOR inhibitors by upregulating PI3K/Akt and/or MEK/ERK activity through RTKs.

5. Activation of WNT/β-catenin signaling

Dysregulated WNT/β-catenin signaling is important for cancer cell proliferation as well as for progression, metastases and relapse in several types of tumor [115-117]. A key component of the WNT/β-catenin axis is GSK3β that is part of a degradative multiprotein complex including adenomatous polyposis coli (APC), casein kinase 1 (CKI), axis inhibition protein 2 (AXIN2) and β-catenin [118]. This complex acts as a negative regulator of WNT/β-catenin signaling, as GSK3β phosphorylates β-catenin, marking it for proteasomal degradation [119, 120]. GSK exists as two isofrms (α and β) and is mostly known as a tumor suppressor. However it also functions in promoting the proliferation of many types of cancer cells. In particular, GSK3β is thought to play both positive and negative roles in the context of WNT/β-catenin signaling, but the precise mechanisms have not yet been established [121, 122].

Moreover, GSK3α/β is a central hub that orchestrates signals from several signaling cascades, including PI3K/Akt and MEK/ERK, to elicit regulatory influences on cancer initiation, epithelial-mesenchymal transition and resistance to therapy [122-124].

A type of neoplasia characterized by high levels of WNT/β-catenin signals is colorectal cancer (CRC). In CRC, WNT/β-catenin upregulation is mostly, although not exclusively, due to mutations in APC tumor suppressor [125, 126]. Furthermore, CRC patients frequently display increased mTOR signaling due to upregulation of both PI3K/Akt and Ras/Raf/MEK/ERK networks [127, 128].

Very recent findings have documented how WNT/β-catenin signaling activation is involved in resistance to mTOR inhibition in CRC cells. In this setting, it has been demonstrated that all cell lines that displayed innate resistance to PF05212384 (gedatolisib, a dual PI3K/mTOR inhibitor) expressed high levels of active GSK3β and harbored the same frameshift mutation (c.465_466insC; H155fs*) in T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) 7 (TCF7) [129]. TCF7 is a transcription factor that mediates and positively regulates the WNT/β-catenin signaling pathway by inducing expression of several target genes, including *CCND1, AXIN2, TCF7, LEF1, MET* and *WNT3*A [130, 131]. It was found that the gedatolisib-resistant cell lines expressed much lower levels of inactive p-GSK3β Ser^{9} (an Akt target) and higher levels of active p-GSK3β Tyr^{216} (that is targeted by an as yet unidentified kinase) compared to the sensitive cell lines, suggesting that GSK3β was more active in the resistant cells. This difference could be related to the TCF7 frameshift mutation, as in resistant cells siRNA-mediated knockdown of TCF7 reduced p-GSK3β Tyr^{216} whereas it increased the levels of p-GSK3β^{Ser9}. However, it is unclear how the mutation could positively impact on GSK3β phosphorylation and activity. In any case, active GSK3β resulted in increased association of mTOR to Raptor and upregulated mTORC1 activity that was resistant to gedatolisib [129].

Downregulation of GSK3β expression in PF05212384-resistant cells via siRNA-mediated knockdown or treatment with a GSK3β inhibitors (CHIR99021, SB216763, LiCl) reduced mTORC1 activity, while also decreasing signaling through the WNT/β-catenin pathway. Importantly, GSK3β inhibition rendered the resistant cell lines sensitive to the cytotoxic effects of PF05212384, both *in vitro* and in a murine xenograft model [129]. Therefore, a combined treatment with GSK3β inhibitors

may be a strategy to overcome innate resistance to PI3K/mTOR-targeted therapies in CRC characterized by high levels of active GSK3β.

β-catenin mRNA and protein levels are also upregulated in human glioblastoma (GBM) where they correlate with malignancy. Indeed, an increased nuclear localization of β-catenin and an elevated expression of β-catenin target genes, such as cyclin D1 and c-Myc, have been observed in high-grade GBM. These findings suggest that increased WNT/β-catenin activity is crucial for GBM progression [132, 133]. On the other hand, the PI3K/Akt/mTOR pathway is activated in over 50% of GBM patients [134]. mTOR inhibitors have proven their efficacy in preclinical models of GBM and have therefore been tested in combination with other therapeutics in clinical trials, although with disappointing results [60, 135-137]. It was recently demonstrated that exposure of GBM cells to the dual PI3K/mTOR inhibitors, NVP-BEZ235 and DS-7423, markedly induced the expression of mitogen- and stress-activated protein kinase 1 (MSK1) while downregulation of MSK1 by shRNA strategy attenuated acquired resistance to PI3K/mTOR inhibitors in glioma-initiating cells [138]. Furthermore, it was shown that MSK1 phosphorylates β-catenin at Ser^{552}, thus regulating its nuclear localization and transcriptional activity. Depletion of β-catenin potentiated PI3K/mTOR inhibitor-induced cytotoxicity and inhibition of MSK1 synergized with PI3K/mTOR inhibitors to improve survival in an intracranial animal model [138]. These findings suggest that MSK1/β-catenin signaling serves as an escape survival signal for GBM cells upon PI3K/mTOR inhibition and provides a rationale for the combined use of PI3K/mTOR and MSK1/β-catenin inhibitors in this setting.

6. GSK3-mediated resistance

It has also been documented that, in GBM cells chronically exposed to rapamycin, GSK3β phosphorylation was not regulated through the WNT/β-catenin pathway, but was rather dependent on MEK/ERK activity, as it was attenuated by the MEK inhibitor, AZD6244. [139]. To reach such a conclusion, the phosphoproteome of HK296 GBM cells chronically treated with rapamycin was

analyzed. Kinase enrichment analysis revealed that GSK3β was significantly associated with 52 out of 425 proteins hyperphosphorylated in drug-treated cells. Interestingly, combinatorial treatment of GBM cells with either rapamycin or NVP-BEZ235 in the presence of CHIR99021, a selective GSK3β inhibitor, conferred resistance to both mTOR inhibitors. Furthermore, depletion of GSK3β via shRNA resulted in a dramatic increase in resistance to either rapamycin or NVP-BEZ235. These findings indicate that GSK3β modulates resistance to mTOR pathway specific inhibition, even when mTORC2 and PI3K are additionally targeted by the dual inhibitor, NVP-BEZ235 [139]. Even more interesting, Rictor knockdown by shRNA prevented the development of resistance to mTOR inhibitors, suggesting that mTORC2 was involved. Therefore, resistance that develops in response to chronic exposure to mTOR inhibitors, including TORKIs such as AZD8055 [140], could be, at least in some cases, due to interruption of a mTORC2/MEK/ERK/GSK3β axis. We have previously highlighted in this article how mTORC2 inhibition could lead to MEK/ERK overactivation via FoxO transcription factors (see Subsection 4.3.). It is still controversial whether ERK could phosphorylate directly GSK3β, although the available evidence indicates that ERK associates with and phosphorylates GSK3β at Thr^{43}, which primes GSK3β for its subsequent phosphorylation at Ser^{9} by p90RSK (a downstream target of ERK), resulting in inactivation of GSK3β [141] (**Figure 4a**). Importantly, the MEK/ERK/p90RSK/GSK3β axis is a general signal, as it was observed in cells in which ERK-primed inactivation of GSK3β was regulated by IGF1R and HER2, and is further supported by immunohistochemical staining in a variety of human tumors, including liver, breast, kidney and stomach cancer [141].

Laks and coworkers [139] identified microtubule-associated protein (MAP)1B, as the downstream target of MEK/ERK/GSK3β signaling that was fundamental for the development of evasive resistance to either rapamycin or NVP-BEZ235 in human GBM cells. Both rapamycin treatment and depletion of GSK3β via shRNA strategy, decreased phosphorylation of MAP1B at Thr^{1270}.

MAP1B is a well-known GSK3β substrate, however phosphorylation by this kinase has been mapped to the Ser^{1260} and Thr^{1265} residues of MAP1B [142]. We could infer that when GSK3β activity is switched off, ^{Thr}1270 p-MAP1B levels somehow decrease while drug-resistance increases. This could be due either to upregulation of a protein kinase or downregulation of a protein phosphatase that are regulated through GSK3β (**Figure 4**).

Accordingly, it was documented that a combined treatment with a MEK inhibitor (AZD6244) decreased resistance of GBM cells to rapamycin or NVP-BEZ235 both *in vitro* and *in vivo* in a xenograft model of human GBM [139]. However, it is still unclear how MAP1B could determine resistance to mTOR inhibitors, although this protein has several signaling functions in addition to its canonical role in the regulation of the microtubule and actin microfilament polymerization [143]. In any case, these findings are interesting as they provide a bridge between mTORC2 inhibition, ERK activation and GSK3β-mediated mechanism of resistance to mTOR-targeted drugs.

They also further support the contention that GSK3β activity is a critical determinant for the therapeutic response to mTOR inhibitors [144]. This is due to the fact that GSK3β and mTORC1 are linked through complex and not well-defined cross-talks. For example, GSK3β phosphorylates both TSC2 and Raptor [145, 146], while p70S6K1 could target and inactivate GSK3β in some settings [147].

More specifically, it has been demonstrated that GSK3β interacts with and phosphorylates 4E-BP1 at Thr37/46 residues, thereby inactivating 4E-BP1 [148, 149]. This phoshorylation increased eIF4E-dependent protein synthesis in breast and renal cancer cell lines that were either resistant to rapamycin or that became insensitive after prolonged exposure [149, 150]. Rapamycin treatment did not block proliferation of resistant cancer cell lines, while a GSK3β inhibitor or GSK3β stable knockdown negatively affected both translation and cell proliferation [149]. As we shall see later on in this review, GSK3α/β is also involved in resistance to mTOR inhibition due to metabolic rewiring. Therefore, targeting both GSK3α/β and mTOR may be a rational strategy for inhibiting cancer cell growth and

proliferation in some tumor types. However, the clinical development of selective GSK3α/β inhibitors has been so far problematic [151, 152].

7. Resistance due to epigenetic disregulation

Epigenetics refers to processes that change chromatin structure and gene expression without altering primary DNA sequence. Over the past 15 years it has become evident that epigenetic modifications, such as DNA methylation/demethylation and histone acetylation/deacetylation, play important roles in cancer cell biology even in the absence of DNA mutations [153, 154]. It is now emerging that epigenetic mechanisms are involved in resistance to mTOR inhibitors.

The first report hinting at a possible epigenetic regulation of mTOR inhibitor-resistance dates back to 2010, when it was discovered that in > 90% of human colorectal cancer (CRC) cell lines and primary samples there is an epigenetic silencing of the protein phosphasphatase 2A (PP2A) regulatory B55β subunit, due to promoter DNA hypermethylation of *PPP2R2B*, i.e. the gene encoding for the B55β subunit. In contrast, normal colon mucosa samples did not display such hypermethylation [155]. Importantly, the PP2A B regulatory subunits confer substrate specificity for dephosphorylation events in a cell- and context-dependent manner [156]. It was found that *PPP2R2B* reexpression led to to downregulation of p-c-Myc Ser^{62} and sensitized CRC cells to rapamycin both *in vitro* and *in vivo*, while either rapamycin or Raptor knockdown, induced c-Myc Ser^{62} phosphorylation and protein accumulation in cells lacking the PP2A B55β subunit, although the authors were unable to detect physical interactions between c-Myc with B55β. Remarkably, the dual PI3K/Akt inhibitor PI-103 reduced p-Akt Ser^{473} levels, but enhanced p-c-Myc Ser^{62} phosphorylation, suggesting that rapamycin induces c-Myc phosphorylation through a distinct mechanism that does not depend on PI3K activity [155]. Surprisingly, either genetic knockdown or pharmacological inhibition of PDK1 abolished rapamycin-induced c-Myc phosphorylation. Lastly, it was documented that B55β binds to and inhibits PDK1 recruitment to cell membrane, whereby blunting PDK1 activation. However, since c-Myc accumulated mainly in the nucleus in response to rapamycin treatment, the effects of the cytoplasmic

B55β-PDK1 complex on c-Myc are most likely to be indirect and may route through as yet unidentified PDK1 downstream kinase substrate(s) [155]. Overall, these findings supported PDK1 as a therapeutic target in CRC, as inhibition of PDK1 reduces c-Myc signaling and alleviates rapamycin resistance. However, also the development of selective PDK1 inhbitors has proven so far to be quite a challange [157].

Similar results were subsequently reported by an independent group that exploited a setting consisting of nasopharyngeal carcinoma cells displaying evasive resistance to NVP-BEZ235. These cells showed upregulation of DNA methyltransferases that induced *PTEN* and *PPP2R2*B promoter DNA hypermethylation, resulting in downregulation of their expression. Reduced *PTEN* and *PPP2R2B* expression correlated with activated PI3KAkt/mTOR and PDK1/c-Myc pathways and conferred resistance to NVP-BEZ235 [158]. However, the authors took a different approach for overcoming mTOR inhibitor resistance as they targeted DNA methyltransferases with decitabine (a DNA-hypomethylating agent [159]) in combination with NVP-BEZ235. This combined therapy sensitized resistant cells to the NVP-BEZ235 both *in vitro* and *in vivo*, suggesting a potential clinical application of this strategy to overcome evasive resistance to dual PI3K/mTOR inhibitors [158].

The importance of histone deacetylation in driving resistance to mTOR inhibition has also started to emerge. The human RCC cell line, RCC4-EV, was used to generate a model of *in vitro* evasive resistance by continuous culture in the presence of NVP-BEZ235. NVP-BEZ235 blocked phosphorylation of mTORC1 downstream targets S6 ribosomal protein (S6RP) and 4E-BP1 in parental cells, however 4E-BP1 levels were unchanged in resistant cells, suggesting a NVP-BEZ235-refractory mTORC1 activity. NVP-BEZ235-resistant cells were cross-resistant to the TORKI, AZD2014 [160]. Sensitivity was regained after 4 months of drug withdrawal, and resistance was partially suppressed by the histone deacetylase (HDAC) inhibitor, pabinostat, whereby supporting the existence of an epigenetic mechanism. Interesingly, NVP-BEZ235-resistant cells upregulated and/or activated numerous signaling molecules including tyrosine kinases (c-Met, c-Abl, IR, IGF1R) and MEK/ERK. However, resistance was not reversed by inhibiting or depleting these pathways,

suggesting that many of the observed changes were passengers and not drivers of resistance. Consistent with this, resistant cells overexpressed the mTORC1 component Raptor at both mRNA and protein level. Furthermore, NVP-BEZ235-resistance was suppressed either by Raptor genetic depletion or by rapamycin. These findings demonstrate that Raptor upregulation, presumably due to epigenetic alterations, contributes to dual PI3K/mTOR inhibitor-resistance and suggest that Raptor expression might be included in the pharmacodynamic assessment of clinical effects of this class of mTOR inhibitors [160].

The role of histone deacetylaton in driving evasive resistance to temsirolimus in prostate cancer cells was recently demonstrated [161]. The authors generated temsirolimus-resistant PC3 cells and were able to document that FDA-approved valproic acid (VPA), a selective inhibitor of class I and IIa HDACs [162], reverted resistance to mTOR inhibition. Interestingly, temsirolimus-resistance was characterized by reduced binding of cells to endothelium, immobilized collagen and fibronectin as compared to parental cells, however displayed increased adhesion to laminin. The expression of several integrins was altered, with some (α2, α3, β1, and β4) subtypes being distinctly elevated, while α5 was nearly lost in resistant cells. VPA significantly counteracted temsirolimus-resistance by downregulating tumor cell–matrix interactions, chemotaxis and migration. Analysis of integrin expression in the presence of VPA revealed a significant downregulation of integrin α5 in resistant cells. Blocking studies demonstrated a close association between α5 expression on resistant cells and chemotaxis. It was therefore concluded that temsirolimus-resistance could drive prostate cancer cells to become highly motile through an epigenatic modulation of integrin expression, while HDAC inhibition reversed the potential metastatic activity [161].

Overall, the findings on epigenetic alterations and mTOR inhibitor-resistance are very important in light of the growing emphasis on using epigenetic therapies to reprogram neoplastic cells toward a normal phenotype [163]. Many agents targeting epigenetic regulation are under development and have entered clinical trials [164, 165]. Remarkably, the HDAC inhibitor, pabinostat, has been

approved in combination with bortezomib and dexamethasone for third-line treatment of relapsed/refractory MM patients by both the FDA and the EMA [166].

8. Metabolic remodeling

mTOR signaling controls cancer cell metabolism by altering expression and/or activity of a number of key metabolic enzymes [167, 168]. Both mTORC1 and mTORC2 are involved in regulating glucose, amino acid, lipid and nucleotide metabolism (see ref [169] for an updated review on mTOR and the regulation of metabolism in cancer cells). The increased conversion of glucose to lactate even in the presence of O_2 (aerobic glycolysis), discovered by Otto Warburg, was the first noted change in cancer metabolism [170]. However, mitochondria are intact in cancer cells, allowing tricarboxylic acid (TCA) cycle intermediates to feed biosynthetic pathways [171]. Therefore, neoplastic cells can become addicted to glutaminolysis, a limiting step in the TCA cycle. Hence, glutamine, the most common amino acid, may represent a major source of molecules, including ATP, that sustain metabolic pathways necessary for tumor growth and survival [172]. Moreover, cancer cells require lipids, including fatty acids, sphingolipids, glycerophospholipids and sterols for ATP production, as well as for the synthesis of membranes and signaling molecules [173]. It is now emerging that metabolic reprogramming is among the mechanisms of resistance to mTOR inhibition.

8.1. Aerobic glycolysis upregulation

Neoplastic cells display increased glucose uptake and glycolytic flux to sustain their growth and proliferation. In addition, aerobic glycolysis, one of the cancer cell hallmarks, provides a source of carbon moieties for anabolic processes including lipid, amino acid and nucleotide synthesis [174]. As a result of increased glycolysis, tumour cells often secrete excess lactate via monocarboxylate transporter 4 (MCT4). This is particularly true for cancer cells distant from blood vessels that, for surviving in a hypoxic microenviroment, become hyperglycolytic [175]. This in turn causes acidification of the cancer microenviroment [176]. Interestingly, it has been shown that the acidic

tumor microenvironment abrogates the efficacy of mTORC1 inhibitors as shown by a recent study, where human cancer cell lines were treated with rapamycin under either acidic (pH 6.4) or physiological (pH 7.4) conditions and cell proliferation was investigated. Exposing cancer cells to acidic pH *in vitro* significantly reduced the antiproliferative effects of rapamycin. This decreased efficacy was not due to rapamycin inactivation by low pH, as it was found that the inhibitor, previously exposed to acidic pH, still significantly decreased S6RP phosphorylation. At the molecular level, acidity decreased rapamycin-sensitive mTORC1 activity as evidenced by a decreased phosphorylation of p-4E-BP1 Ser^{65}, but not of p-4E-BP1 $Thr^{37/46}$. In contrast, the activation of either MEK/ERK or Akt were not affected by acidity, and both MEK and Akt selective inhibitors maintained their efficacy at low pH. In xenograft models, sodium bicarbonate increased mTORC1 activity in cancer cells and potentiated the efficacy of rapamycin. Indeed, combining sodium bicarbonate with rapamycin resulted in increased tumor necrosis and cancer cell apoptosis, as well as decreased cancer cell proliferation, when compared with single treatment. Taken together, these results highlighted the inefficacy of rapamycin under acidic conditions [177]. The molecular mechanisms leading to this phenomenon are still unclear, however a previous report demonstrated that the TSC1/TSC2 complex is required for mTORC1 inactivation by low pH [178]. The findings by Faes and coworkers [177] further susbstantiate the potential of combining sodium bicarbonate with rapamycin to improve its anticancer effects. In this context, it should be emphasized that the use of existing drugs such as proton pump and carbonic anhydrase inhibitors or even buffers (sodium bicarbonate, citrate) have been proposed as a strategy to improve cancer therapies [179-181].

In another recent report, a hyperglycolytic phenotype and mTOR inhibitor-resistance have been associated with a mitochondrial DNA variant in H1975 lung cancer cells, harboring an EGFR T790M mutation which confers resistance to EGFR inhibitors. The cells became resistant to NVP-BEZ235 (but not to MEK inhibitors), after prolonged (8 months) *in vitro* treatment with the drug [182] and displayed upregulated Akt and S6RP phosphorylation levels, as well as features consistent with elevated glycolysis (increased levels of glucose, lactate, glucose transporter expression, extracellular

acidification, and a decreased rate of oxygen consumption). A combined treatment consisting of NVP-BEZ235 and the glycolysis inhibitor 3-bromopyruvate, was synergistic in resistant clones, but only additive in parental cells. DNA sequencing revealed the presence of a mitochondrial DNA (mtDNA) encoded cytochrome c oxidase I (MT-C01) variant (ENST00000361624.2: c.1367T>A, G456E) in resistant but not parental cells [182]. MT-CO1 is a protein found within complex IV of mitochondrial redox carriers that catalyzes the reduction of oxygen to water [183]. Complex IV is a major regulator of oxidative phosphorylation and MT-CO1 mutations have previously been associated with weak oxidative phosphorylation in the settings of oxidative stress [184]. Interestingly, depletion of mitochondrial DNA in parental H1975 cells induced resistance to NVP-BEZ235 and other dual PI3K/mTOR inhibitors (PI103, KU-0063794), and was accompanied by increased glycolysis. The results of this study provided the first evidence that a metabolic switch associated with a mtDNA mutation can be an underlying mechanism for evasive resistance to mTOR inhibitors and highlighted the usefulness of glycolysis inhibitors in such a setting. However, it is not clear whether also resistance to dual PI3K/mTOR inhibitors is dependent on acidification due to increased glycolysis or could be related to other mechanisms.

8.2. Compensatory glutamine metabolism

Many studies have shown that several types of tumors are dependent on glutamine metabolism for energy production to meet the demand of accelerated growth and proliferation. Therefore, these cancer cells are sensitive to changes in exogenous glutamine levels. Moreover, evidence suggests that the catabolism of glutamine (glutaminolysis) is associated with known oncogenic drivers such as c-Myc [185]. Glutaminase (GLS) is the enzyme that catalyzes the first step in the glutaminolysis of glutamine to glutamate. Two GLS isoforms exist, GLS1 and GLS2, originally identified as kidney and liver GLS, respectively. GLS1 is more ubiquitously expressed than GLS2, and exists as two splice variants, the kidney-type glutaminase (KGA, longer variant) and the glutaminase C (GAC, shorter

variant), both of which are located in the mitochondria. Interestingly, GLS1 expression is associated with tumor growth [186].

Increased glutamine metabolism has been recently implicated in innate resistance to both rapamycin and PP242 (a TORKI), in a model of GBM overexpressing an activating EGFR mutation (U87/EGFRvIII). Rapamycin or PP242 exposure did not result in the death of U87/ EGFRvIII cells, although they significantly suppressed their glucose consumption, lactate production and proliferation. These events could be related to downregulation of mTORC1 activity [187]. However, it was noticed that glutamine metabolism was increased due to upregulated expression of the KGA mRNA, while expression of GAC mRNA decreased in response to mTOR inhibitors. To determine whether KGA expression could be detected *in vivo* in response to mTOR-targeted treatment, EGFRvIII-expressing tumor tissues from a xenograft model after 5 days of PP242 or CC214 (a different TORKI) treatment were analyzed. It was indeed found that KGA expression was significantly elevated relative to that of controls. Importantly, combined genetic and/or pharmacological inhibition of mTOR kinase activity (with PP242) and GLS1 activity (with compound 968) resulted in massive synergistic tumor cell death and growth inhibition in tumor-bearing mice. Moreover, this study showed that GBM cells were dependent on KGA to survive mTOR inhibition in an α-ketogluratate (αKG) -dependent manner, as αKG was required for TCA cycle as a source of succinic acid, fumaric acid and malic acid [187].

Similar results were subsequently reported by a different group that studied innate PP242 resistance in SKOV3 and C13K human ovarian cancer cells. Indeed, despite evidence of mTORC1/mTORC2 activity inhibition by the drug, these cell lines did not undergo apoptosis upon treatment with PP242. Also in this setting, either genetic or pharmacological downregulation of GLS1 activity rendered resistant cells sensitive to PP242. Furthermore, the anticancer activity of the GLS1 inhibitor CB-839 and PP242 was abrogated by the addition α-KG, indicating the critical function of glutaminolysis in ovarian cancer cell resistance to TORKIs [188].

Very recently, using models of squamous cell lung carcinoma (SCC, a very aggressive subset of non-small cell lung cancer that displays high levels of glucose metabolism), Momcilovic and coworkers [189] identified GSK3α/β as a molecular switch that reprograms cancer metabolism from glycolysis to glutaminolysis in response to chronic mTOR inhibition with the TORKI, MLN128. It is worth highlighting here that MLN128 effectively inhibited mTORC1 activity (as shown by reduced 4E-BP1 phosphorylation levels) and suppressed glucose metabolism [as documented by ^{18}F-fluorodeoxyglucose (FDG) postive em-ssion tomography (PET) imaging] but failed to restrict tumor growth in a murine model of SCC that displayed a high influx of glutamine (as documented by elevated ^{11}C-labeled glutamine [189, 190]). Using the RH2 human SCC line, it was then demonstrated that both MLN128 and rapamycin suppressed glucose uptake while concomitantly inducing an increase in glutamine uptake *in vitro*. Similar results were observed when RH2 cells were xenografted in mouse. Since it was known that SCC tumors that escaped MLN128 treatment in mice had increased levels in Thr^{308} p-Akt and of the phosphorylated (inactive) form of the Akt substrate GSK3α/β, the pathways downstream of Ser$^{21/9}$ p-GSK3α/β were investigated for a better understanding of the metabolic adaptation in SCC tumors. It was found that upregulated Ser$^{21/9}$ p-GSK3α/β levels led to increased stability of c-Myc and c-Jun, that are both critical for regulating the levels of the KGA GLS1 splicing variant, as active (unphosphorylated) GSK3α/β facilitates c-Myc/c-Jun degradation by E3 ubiquitin ligases [191] [192]. Importantly, Momcilovic at al. [189] also demonstrated that Ser$^{21/9}$ p-GSK3α/β was a predictive marker of MLN128 response in human patient-derived xenografts (PDXs) of lung SCCs, and that the GLS inhibitor CB-839 overcame metabolic adaptation and resistance to MLN128 in human lung SCC cell lines and PDXs. Furthemore, Momcilovic and coworkers [189] discovered a conserved metabolic signature in lung SCC, head and neck squamous cell carcinoma and osteosarcoma suggesting that hypermetabolic, ^{18}F-FDG-avid tumors may be responsive to a combined treatment with MLN128 and CB-839. Such a metabolic signature is defined by positive staining for glucose transporter 1 (GLUT1), the glutamine transporter solute carrier family 1 member 5 (SLC1A5), p-4EBP1, p-GSK3α/β Ser$^{21/9}$ and nuclear p-cJUN Ser^{73} (**Figure 4b**).

Overall, these findings emphasize the relevance of compensatory glutamine metabolism in driving innate mTOR inhibitor resistance in cancer cells and suggest a rational combination therapy with GLS inhibitors having the potential to suppress resistance. They also indicate that GSK3α/β may serve as a key node that upregulates GLS1 expression and glutamine metabolism following treatment with mTOR inhibitors.

8.3. Activation of the purine salvage pathway

mTORC1 activation also enhances *de novo* purine synthesis through transcriptional effects on multiple enzymes feeding into the purine synthesis pathway, that include those of the pentose phosphate pathway, serine and glycine synthesis, and the mitochondrial tetrahydrofolate (mTHF) pathway [193, 194]. A key enzyme is methylene tetrahydrofolate dehydrogenase 2 (MTHFD2) as it provides cytosolic one-carbon formyl units required for purine synthesis. mTORC1 signaling upregulates MTHFD2 expression by increasing translation of the mRNA encoding activating transcription factor 4 (ATF4) transcription factor [193].

In a recent study performed in a small-cell lung carcinoma (SCLC) setting, it was found that cell lines resistant to the dual PI3K/mTOR inhibitor, gedatolisib, diplayed higher amounts of purine-related metabolites, including hypoxanthine, AMP and GMP [195]. Moreover, the levels of the mRNA encoding hypoxanthine phosphoribosyl transferase 1, a key component of the purine salvage pathway, were significantly lower in SCLC cell lines sensitive to gedatolisib if compared with resistant cells. Furthermore, complementation with purine metabolites could lower the efficacy to gedatolisib in SCLC cells normally sensitive to the inhibitor. Overall, these findings indicate that a resistance mechanism to dual PI3K/mTOR inhibition is mediated by the activation of the purine salvage pathway, that supplies purine resources to nucleotide biosynthesis independent of *de novo* purine biosynthesis. They also show that at least part of the anticancer effects of mTOR inhibitors are likely related to the blockage of nucleotide synthetic pathways.

It is remarkable that purine-related metabolites, such as hypoxanthine, were higher in human primary SCLC tumor tissues [195]. Therefore, high levels of purine-related metabolites seem to be characteristic of SCLC biology, and this feature might serve as novel therapeutic biomarker of dual PI3K/mTOR inhibitor efficacy.

9. mTOR mutations

MTOR mutations in tumors were first identified in 2010, when Sato and coworkers [196], by screening a human cancer genome database, described two different point mutations – S2215Y (from a CRC patient sample) and R2505P (from a kidney carcinoma sample) – that conferred constitutive activation of mTOR signaling even under nutrient-starvation conditions. More recently, next-generation sequencing (NGS) studies led to the discovery in multiple cancer types (including colon, lung, kidney and uterus) of additional mutations in *MTOR* that resulted in mTOR kinase activation [197, 198]. The activating mutations did not affect mTOR complex assembly, but a subset reduced mTOR binding to Deptor, that acts as an endogenous repressor of mTOR kinase activity [199]. Consequently, the mutations could activate either mTORC1 or mTORC2, whereby affecting the phosphorylation status of different downstream targets. Nevertheless, cancer cell lines with hyperactivating *MTOR* mutations displayed heightened sensitivity to rapamycin both *in vitro* and in *in vivo* xenografts, suggesting that such mutations conferred mTOR pathway dependency [31].

However, there is also *in vitr*o evidence that mTOR mutations could result in evasive resistance to rapamycin, as documented by a study where breast cancer BT474 cells were rendered resistant to rapamycin by prolonged culturing with increasing concentrations of the drug [200]. Rapamycin-resistant BT474 cells displayed a S2035F mutation in the FRB domain of mTOR. This mutation was previously known to interfere with mTOR–FKBP12 interactions and to confer rapamycin resistance [44, 201]. These findings may be highly relevant from a clinical point of view, as *MTOR* mutations may serve as biomarkers predicting tumor responses to mTOR allosteric inhibitors and explain evasive resistance to this class of drugs in patients. More recently, it was observed that resistant clones

emerged from the breast cancer cell line MCF-7 exposed for several weeks to either rapamycin or the TORKI, AZD8055. While AZD8055-resistant cells harbored an mTOR mutation located in the kinase domain at the M2327I position, two rapamycin-resistant clones displayed mutations located in the FRB domain, at positions A2034V and F2108L [54]. Interestingly, the F2108L mutation had been previously reported in a long-term (14-months) responder urothelial carcinoma patient who became resistant to everolimus treatment and relapsed [202]], while the M2371 mutation had been observed in five patients with different types of solid cancer [54]. In cells with FRB domain mutations, phosphorylation levels of the normally rapamycin sensitive residues on p70S6K1 (Thr^{389}) and S6RP ($Ser^{235/236}$) were unaffected even at high everolimus concentrations (100 nM). In contrast, the M237I mutation resulted in an increase in mTOR kinase activity and rendered cells resistant to a variety of TORKIs (PP242, WY354, KU-0063794) in addition to AZD8055, as documented by lower sensitivity of 4E-BP1 phosphorylation to this class of inhibitors. These observations led to the development of the novel bivalent mTOR inhibitor, RapaLink1, that could indeed reverse *in vitro* and *in vivo* resistance of breast cancer cells caused by either mTOR FRB or kinase domain mutations [54].

mTOR mutations have been mainly associated with long-term responders to rapalog treatment [31]. At present, there is no definitive evidence of their involvement in the development of evasive resistance to mTOR inhibitors in patients, as exemplified by a recent study on the possible existence of mTOR genetic alterations in a limited cohort of RCC patients who became resistant to everolimus after an initial response [203].

10. mTORC3

Very recently, Harwood et al. [42] described a novel rapamycin-resistant complex, named mTORC3, which assembles in the cytoplasm upon expression of E26 transformation specific (ETS) translocation variant 7 (ETV7) transcription factor, a protein interacting with mTOR independently from its transciptional activity. In humans, the ETS family of transcription factors consists of 27

members that are known to regulate a number of important biological processes in both healthy and cancer cells [204]. Of note, ETV7 overexpression is associated with carcinogenesis [205]. mTORC3 lacks crucial components of mTORC1/2 (Raptor, Rictor, mSIN1, mSLT8), displays mTORC1/2-specific kinase activity *in vitro* and has an estimated size comparable to that of mTORC2 (i.e. about 1.3 MDa [42, 206]). It is therefore likely that mTORC3 contains additional, as yet unidentified, components.

Interestingly, the mTORC3 *in vitro* kinase activity is resistant to rapamycin, whereas it is inhibited by TORKIs [42]. Moreover, upon loss of either Raptor or Rictor, exogenous ETV7 expression in EW8 cells (a Ewing's sarcoma cell lines that lacks ETV7) maintains mTORC3 *in vivo* kinase activity on p-p70S6K Thr^{389}, p-4E-BP1 $Thr^{37/46}$, p-Akt Ser^{473} and p-NDRG1 Thr^{346}. Harwood and coworkers [42] took advantage of the fact that mice lack *Etv*7, for generating a transgenic mouse carring a single copy of human *ETV7*. $ETV7^{TG+/-}$ mice displayed a normal phenotype and, upon maitenance up to two years, they did not show to be tumor-prone. However, when they were crossed onto the $Ptch1^{+/-}$ background (that predisposes to medulloblastoma and embryonal rhabdomyosarcoma [207]), transgenic *ETV7* expression accelerated tumor onset and promoted tumor penetrance. Furthermore, human cancer cell lines that lack ETV7 (EW8 cells) or where ETV7 was knocked down by shRNA (Karpas-299) were resistant to rapamycin. Based on the above -highlighted findings, Harwood and colleagues [42] came to the conclusion that mTORC3 signaling contributes to the poor efficacy of rapamycin/rapalogs observed in several preclinical cancer settings. *ETV7* is among the top 1-10% upregulated genes in many adult human cancers (e.g. B-cell acute lymphoblastic leukemia, ductal breast carcinoma, easophageal carcinoma, liposarcoma, gastric carcinoma, RCC, ovarian carcinoma, etc., see www.oncomine.org). Therefore, mTORC3 could have a relevant role in innate resistance to first generation mTOR inhibitors in patients and could be an attractive novel target for antitumor drug development.

11. Conclusions and future perspectives

mTOR signaling has had significant promise for the development of cancer therapeutics. Although we have at our disposal inhibitors that effectively target the two canonical mTOR complexes, they showed minimal benefit as anticancer drugs, except in few cases of exceptional responders [31]. As we have discussed in this review, over the last few years several reasons have emerged that could explain inherent mTOR inhibitor resistance in cancer cells. However, it should not be forgotten that evasive non-inherent resistance is of at least equal importance, as the tumor microenviroment induces, through a variety of signaling networks, changes in gene expression and protein activity that foster therapy-resistance in cancer cells Several of these signaling pathways converge on mTOR [208] and could explain the emergence of mTOR inhibitor-resistant tumor cell clones [209]. Furthermore, little is known regarding potential changes in the profile of tumor infiltrating immune cells in responsive versus nonresponsive (resistant) tumors [31]. Altered immune profiles may contribute to the development of resistance to mTOR inhibitors.

We face several major challenges if we want to improve the clinical efficacy of mTOR targeting drugs. Cancers are very rarely dependent on mTOR signaling alone [31] and this, coupled with the modest efficacy data garnered for all classes of mTOR inhibitors, highlights the necessity for more work focusing on using these agents in combination therapy, as we have discussed in this article.

A daunting hurdle to the development of successful targeted anticancer strategies, is represented by the spatial and temporal intratumor/intertumor heterogeneity, that facilitates tumor branched evolution and the emergence of drug-resistance [210, 211]. This issue is even more critical in light of the findings showing that targeted agents themselves may be the driving force leading to the selection and emergence of evasive resistance to mTOR inhibition, not only in the bulk of the tumor cell population but also in cancer-initiating cells [212-215]. Nevertheless, tumor evolution and signaling rewiring could also provide opportunities for developing alternative therapeutic strategies, as demonstrated in a study highlighting that brain metastases displayed changes associated with

sensitivity to PI3K/Akt/mTOR and HER2 inhibitors not detected in the matched primary tumor samples [216].

A major advance for overcoming resistance could be represented by the use of multi-omic based molecular profiling of cancer patients. This approach should include NGS (whole genome and whole exome sequencing, RNA sequencing), epigenetics, metabolomics, proteomics/phosphoproteomics, high-throughput drug screening and kinase inhibition data coupled with bioinformatics and computational biology. All these emerging platforms have the potential to enable the design of more effective and durable personalized anticancer therapies [217]. Preliminary studies have already shown the efficacy of such an approach, for example in AML cells [218]. Furthermore, emerging single-cell and primary patient derived-tumor organoid technologies provide a new opportunity to profile individual cells within tumors and investigate what roles they play in drug-resistance [219, 220].

The increasing popularity of umbrella trials also represents an opportunity for a reliable identification of the most efficacious agents and the pathway alterations they can target, so that the most promising drugs could be studied in the next generation of clinical trials [221].

All these approaches are paving the way for a wider and more efficient use of personalized and precision medicine in the context of cancer therapy, and a better patient stratification based on tumor genotype/phenotype should improve the response rates of targeted therapeutics.

In conclusion, despite all the limitations and the formidable challenges associated with these therapeutic agents, mTOR signaling inhibitors remain an exciting frontier in cancer therapy and hold great potential in the optimization of patient outcome in the future.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure legends

Figure 1. mTOR domains and components of mTORC1/mTORC2. FAT, FKBP/ATM/TRRAP; FATC, FRAP/ATM/TRRAP/Carboxy terminal; FKBP-12, FK506-binding protein-12; FRB, FKBP rapamycin-binding; HEAT, Huntingtin/Elongation factor 3/A subunit of protein phosphatase-2A/ TOR1;RAPA, rapamycin/rapalog.

Figure 2. Regulations and funtions of mTORC1/mTORC2. For the details see the text. Arrows indicate stimulatory events, while perpendicular lines indicate inhibitory events.

Figure 3. Feedback loops controlling the activity of mTORC1 and mTORC2. Only the signaling circuits going that are downstream of mTORC1 and mTORC2 are shown for the sake of clarity. For the details see the text. Arrows indicate stimulatory events, while perpendicular lines indicate inhibitory events.

Figure 4. GSK and metabolic rewiring lead to mTOR inhibitor resistance. (**a**): In GBM cells, prolonged exposure to mTOR inhibitors (rapamycin, NVP-BEZ235) inhibits mTORC2 (**1)**; mTORC2 downregulation causes activation of FoxO/MEK/ERK/p90RSK axis (**2**); as a consequence, GSK3β activity is inhibited (**3**), Thr^{1270} p-MAP1B levels increase (**4**) and mTOR inhibitor resistance is induced (**5**). (**b**): Lung SCCs adapt to chronic mTOR inhibition and suppression of glycolysis through the GSK3α/β signaling pathway, which upregulates glutaminolysis mainly via cJUN and increased expression of the GLS1 gene.

Table 1

mTORC1 and mTORC2 components and their roles.

Component	Complex	Roles
mTOR	mTORC1, mTORC2	Serine/threonine kinase
Tti1/Tel2	mTORC1, mTORC2	Assembly and stabilization of both complexes [8]
Deptor	mTORC1, mTORC2	Inhibition of kinase activity in both complexes [199]
mLST8	mTORC1, mTORC2	Stabilization of mTOR active site; essential for functions of mTORC2, but not of mTORC1 [36, 47]
PRAS40	mTORC1	Blocking of substrate recruitment sites [222]
Raptor	mTORC1	Scaffolding protein [223]; binding and presentation of substrates to the mTOR active site via TOR signaling (TOS) motifs [224]
Rictor	mTORC2	Scaffolding protein; assembly, stabilization and activation; recognition and recruitment of downstream substrates [225]
mSIN1	mTORC2	Subcellular localization of the complex; assembly and activation [24, 25]
Protor	mTORC2	Interaction with Rictor [226]; regulation of some mTORC2 functions [227]

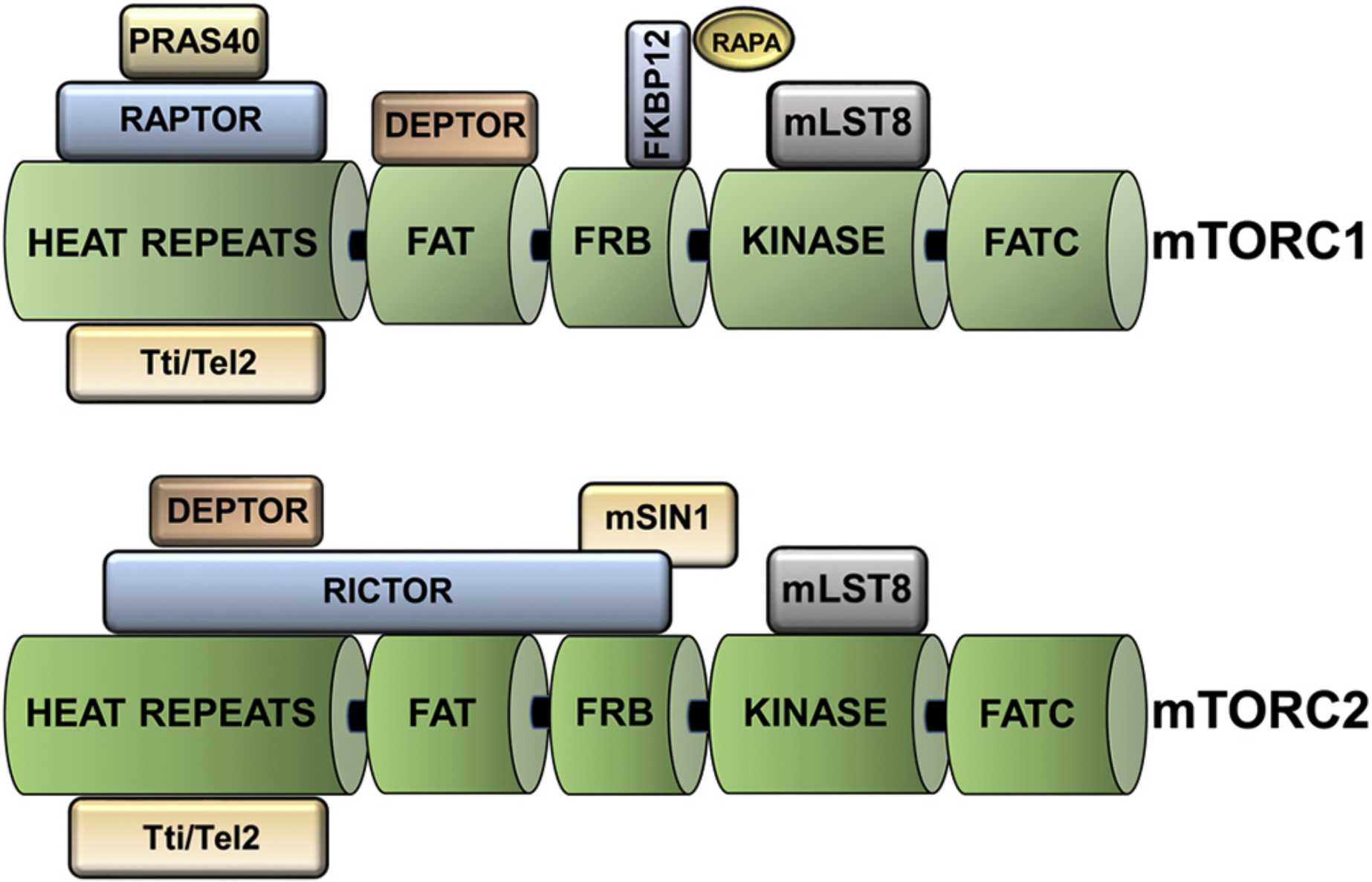
PRAS40
RAPTOR
DEPTOR
FKBP12
RAPA
mLST8
HEAT REPEATS
FAT
FRB
KINASE
FATC
mTORC1
Tti/Tel2
DEPTOR
mSIN1
RICTOR
mLST8
HEAT REPEATS
FAT
FRB
KINASE
FATC
mTORC2
Tti/Tel2

Figure 1

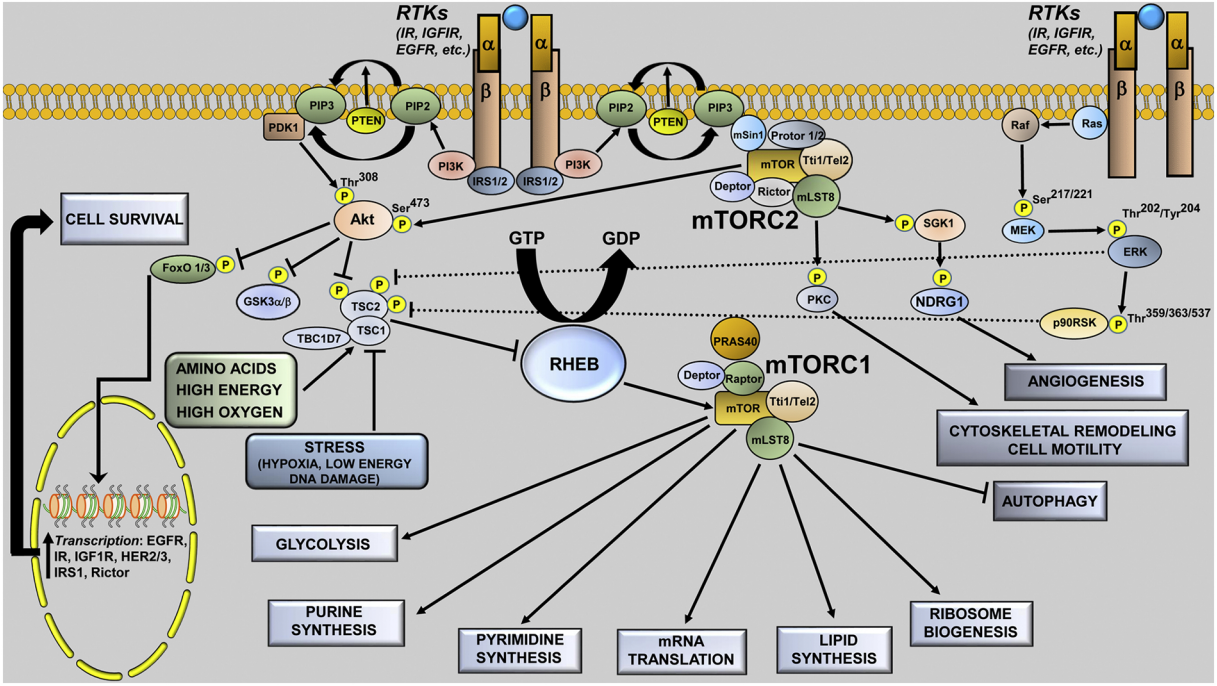
RTKs
(IR, IGFIR, EGFR, etc.)
RTKs
(IR, IGFIR, EGFR, etc.)
α
β
PIP3
PIP2
PTEN
PDK1
PI3K
IRS1/2
mSin1
Protor 1/2
mTOR
Tti1/Tel2
Deptor
Rictor
mLST8
mTORC2
Raf
Ras
MEK
ERK
Ser217/221
Thr202/Tyr204
Thr308
Ser473
Akt
SGK1
NDRG1
PKC
p90RSK
Thr359/363/537
CELL SURVIVAL
FoxO 1/3
GSK3α/β
TSC2
TSC1
TBC1D7
GTP
GDP
RHEB
PRAS40
Raptor
mTORC1
AMINO ACIDS
HIGH ENERGY
HIGH OXYGEN
STRESS
(HYPOXIA, LOW ENERGY
DNA DAMAGE)
ANGIOGENESIS
CYTOSKELETAL REMODELING
CELL MOTILITY
AUTOPHAGY
Transcription: EGFR, IR, IGF1R, HER2/3, IRS1, Rictor
GLYCOLYSIS
PURINE SYNTHESIS
PYRIMIDINE SYNTHESIS
mRNA TRANSLATION
LIPID SYNTHESIS
RIBOSOME BIOGENESIS

Figure 2

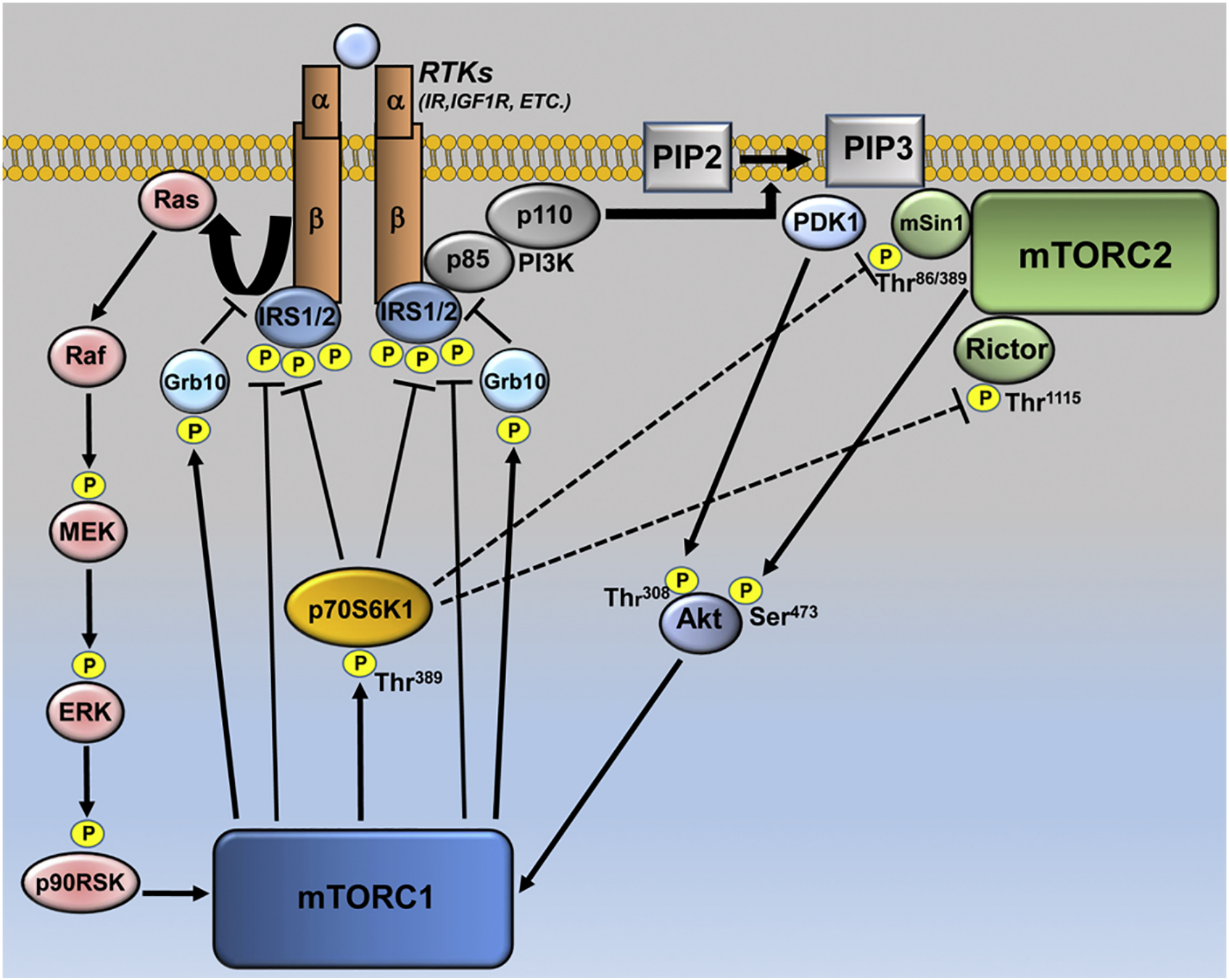
RTKs
(IR,IGF1R, ETC.)
α
α
β
β
PIP2
PIP3
Ras
p110
p85
PI3K
PDK1
mSin1
mTORC2
P
Thr86/389
IRS1/2
IRS1/2
Raf
Grb10
Grb10
Rictor
Thr1115
MEK
p70S6K1
Thr308
Akt
Ser473
Thr389
ERK
p90RSK
mTORC1

Figure 3

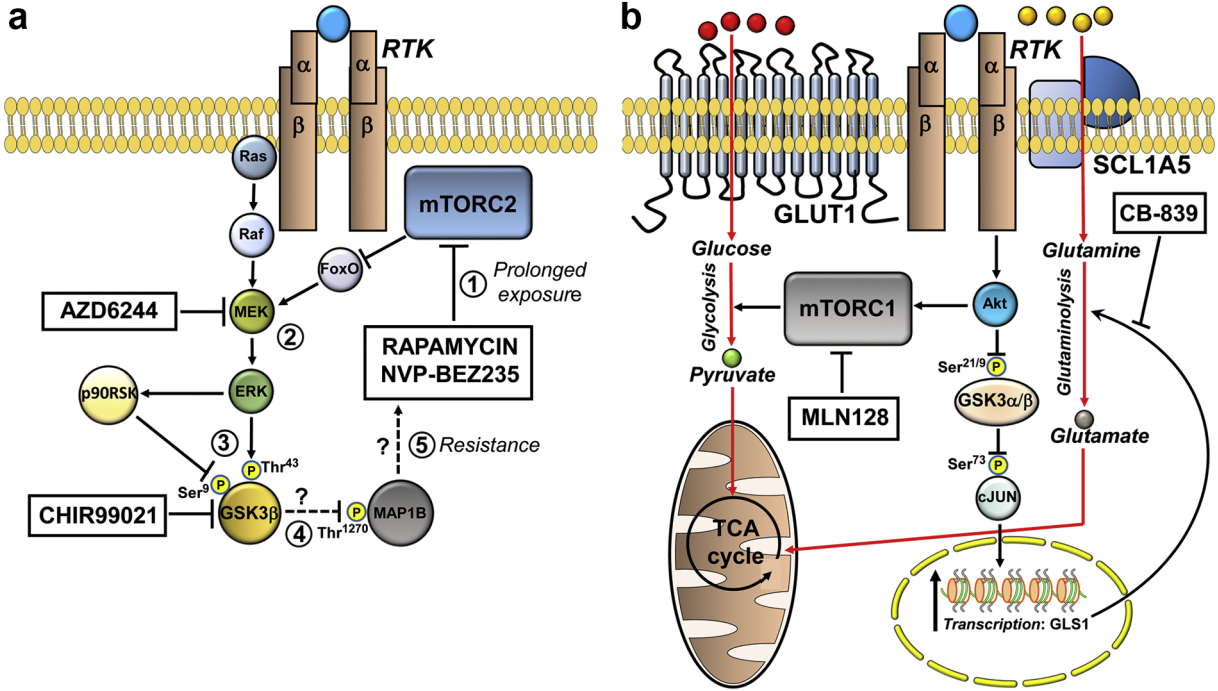
a
RTK
α
β
Ras
Raf
mTORC2
FoxO
AZD6244
MEK
① Prolonged exposure
RAPAMYCIN
NVP-BEZ235
②
p90RSK
ERK
③
? ⑤ Resistance
P Thr43
Ser9 P
CHIR99021
GSK3β
?
P
MAP1B
④ Thr1270
b
RTK
GLUT1
SCL1A5
CB-839
Glucose
Glycolysis
Pyruvate
mTORC1
Akt
MLN128
Ser21/9 P
GSK3α/β
Ser73 P
cJUN
Glutamine
Glutaminolysis
Glutamate
TCA cycle
Transcription: GLS1

Figure 4