Adaptation to mTOR kinase inhibitors by amplification of eIF4E to maintain cap-dependent translation

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INTRODUCTION

The mechanistic target of rapamycin (mTOR, formerly known as mammalian target of rapamycin) senses growth factors, nutrients and energy status to coordinate cellular catabolic and anabolic processes (Zoncu et al., 2011; Laplante and Sabatini, 2012). Growth factor receptors activate mTOR by driving RAS, phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB)-dependent signals that converge to inhibit the tuberous sclerosis 1 and 2 heterodimer (TSC1 and TSC2) leading to activation of the small GTPase RHEB. Cells possess two discrete multi-protein mTOR complexes, mTORC1 and mTORC2. mTORC1, the molecular target of the immune-suppressant rapamycin, phosphorylates unc-51-like kinase 1 (ULK1) to repress autophagy and promote protein synthesis by phosphorylating the p70 (RPS6KB1) subunit of S6 kinase (S6K) and the eukaryotic initiation factor 4E-binding proteins (4EBPs) (Laplante and Sabatini, 2012). Phosphorylation of 4EBPs releases the eukaryotic translation initiation factor eIF4E, which then forms part of the eIF4F complex that initiates cap-dependent mRNA translation (Pause et al., 1994; Brunn et al., 1997; Gingras et al., 1998). mTORC2 phosphorylates PKB on Ser473 to increase its enzymatic activity and activates other protein kinases including SGK (Sarbassov et al., 2005; Laplante and Sabatini, 2012). mTOR activity is tightly regulated by negative feedback loops; for example, S6K phosphorylates insulin receptor substrate 1 (IRS1), promoting its degradation and decreasing PI3K–PKB signalling loops; for example, S6K phosphorylates insulin receptor substrate 1 (IRS1), promoting its degradation and decreasing PI3K–PKB signalling (Shi et al., 2004); mTOR also inhibits IRS1 by direct phosphorylation (Tzatsos and Kandror, 2006) and can phosphorylate Grb10 to inhibit RTK signalling (Hsu et al., 2011; Yu et al., 2011).

mTOR is hyper-activated in many cancers or familial over-growth syndromes owing to mutations in RAS, PI3K, PTEN, TSC1 and TSC2, LKB1 (Laplante and Sabatini, 2012) and mTOR itself (Gerlinger et al., 2012), and mTOR signalling is an essential component of tumour development and progression in the majority of cancers (Menon and Manning, 2008). Treatments using rapamycin, an allostERIC inhibitor of the mTORC1 complex, and related ‘rapalogues’ have shown some success in specific tumour types but have not exhibited broad anti-cancer activity (Benjamin et al., 2011). This might be because selective mTORC1 inhibition leaves PKB survival signalling intact: indeed, suppression of the mTORC1- and S6K-dependent feedback loops increases PI3K–PKB signalling (Shi et al., 2005; Tamburini et al., 2008). In addition, rapalogues cause only a partial de-phosphorylation of the 4EBPs (Choo et al., 2008) allowing cap-dependent translation, a driver of cell proliferation (Dowling et al., 2010), to persist in the presence of the drug. These results and others have prompted the development of new ATP-competitive mTOR kinase inhibitors that directly target the mTOR catalytic site, inhibit mTORC1 and mTORC2, and elicit potent and prolonged inhibition of mTOR targets; they include INK128, TORKi CC223, OSI027, AZD2014 levels and energy status to coordinate cellular catabolic and anabolic processes (Zoncu et al., 2011; Laplante and Sabatini, 2012). Growth factor receptors activate mTOR by driving RAS, phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB)-dependent signals that converge to inhibit the tuberous sclerosis 1 and 2 heterodimer (TSC1 and TSC2) leading to activation of the small GTPase RHEB. Cells possess two discrete multi-protein mTOR complexes, mTORC1 and mTORC2. mTORC1, the molecular target of the immune-suppressant rapamycin, phosphorylates unc-51-like kinase 1 (ULK1) to repress autophagy and promote protein synthesis by phosphorylating the p70 (RPS6KB1) subunit of S6 kinase (S6K) and the eukaryotic initiation factor 4E-binding proteins (4EBPs) (Laplante and Sabatini, 2012). Phosphorylation of 4EBPs releases the eukaryotic translation initiation factor eIF4E, which then forms part of the eIF4F complex that initiates cap-dependent mRNA translation (Pause et al., 1994; Brunn et al., 1997; Gingras et al., 1998). mTORC2 phosphorylates PKB on Ser473 to increase its enzymatic activity and activates other protein kinases including SGK (Sarbassov et al., 2005; Laplante and Sabatini, 2012). mTOR activity is tightly regulated by negative feedback loops; for example, S6K phosphorylates insulin receptor substrate 1 (IRS1), promoting its degradation and decreasing PI3K–PKB signalling loops; for example, S6K phosphorylates insulin receptor substrate 1 (IRS1), promoting its degradation and decreasing PI3K–PKB signalling (Shi et al., 2004); mTOR also inhibits IRS1 by direct phosphorylation (Tzatsos and Kandror, 2006) and can phosphorylate Grb10 to inhibit RTK signalling (Hsu et al., 2011; Yu et al., 2011).

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and AZD8055 (Thoreen et al., 2009; Feldman et al., 2009; Benjamin et al., 2011). AZD8055 potently (IC₅₀<1 nM) and selectively inhibits mTOR, resulting in strong sustained de-phosphorylation of both mTORC1 (4EBP1 and S6K) and mTORC2 (PKB) substrates, inhibition of cell proliferation, induction of autophagy and, in some cases, cell death (Chresta et al., 2010). In addition, it combines well with the MEK1/2 inhibitor selumetinib (AZD6244) in pre-clinical studies (Holt et al., 2012).

Acquired drug resistance has limited the impact of even the most successful targeted agents, such as BCR-ABL and BRAF inhibitors (Rosenzweig, 2012; Little et al., 2013) and, in all likelihood, tumour cells will adapt and acquire resistance to mTOR kinase inhibitors. Anticipating this, we have studied the effects of AZD8055 in human colorectal cancer cells. We show that cells respond to acute treatment with AZD8055 by switching from cap-dependent to internal ribosome entry site (IRES)-dependent translation. In contrast, acquired resistance to chronic AZD8055 exposure arises through selective upregulation of just one arm of the mTORC1 signalling pathway, the eukaryotic translation initiation factor eIF4E. Notably, the mTOR and MEK1/2–ERK1/2 pathways converge on eIF4E, and we find that cells that are resistant to AZD8055 through increased eIF4E are also cross-resistant to the MEK1/2 inhibitor selumetinib. These results provide important new insights into how tumour cells respond and adapt to chronic mTOR inhibition.

RESULTS

Identification of AZD8055-sensitive colorectal cancer cells

We screened seven human colorectal cancer cell lines for their sensitivity to AZD8055 in cell proliferation assays. Most were very sensitive, such as SW620 with an IC₅₀ of 30–50 nM, but a few were relatively resistant, such as CO115 (IC₅₀>10 μM) (Fig. 1A). These differences in sensitivity did not reflect differences in target inhibition given that AZD8055 inhibited mTORC1 (P-S6K, P-S6) and mTORC2 (P-Ser473 PKB) signalling at 100 nM in both sensitive (COLO205) and resistant (CO115) cells (supplementary material Fig. S1A). Rather they suggest that CO115 cells exhibit little ‘mTOR addiction’ and/or mechanisms of intrinsic or innate resistance to mTOR kinase inhibition operate in some cell lines.

When SW620 cells were subjected to a time course of drug treatment, AZD8055 inhibited mTORC1 and mTORC2 signalling within 1 hour, causing loss of P-S6K, P-Thr37/Thr46 4EBP1 and P-Ser473 PKB, this effect persisted for at least 48 hours (Fig. 1B). Total levels of 4EBP1 also increased, commensurate with the rapid de-phosphorylation of 4EBP1; because dephosphorylation protects 4EBP1 from proteasome-dependent degradation (Elia et al., 2008) these results suggest that AZD8055 may stabilize 4EBP1, although we cannot rule out the possibility that the non-phospho-4EBP1 antibody detects the de-phosphorylated 4EBP1 more avidly. In comparison, rapamycin failed to inhibit P-Ser473 PKB (consistent with it being an mTORC1 inhibitor) but did inhibit P-S6K and P-S6 (supplementary material Fig. S1B). Notably, rapamycin failed to inhibit 4EBP1 phosphorylation at Thr37/Thr46 (supplementary material Fig. S1B), consistent with previous reports that rapalogues cause little inhibition of 4EBP1 phosphorylation (Choo et al., 2008; Thoreen et al., 2009; Feldman et al., 2009; Chresta et al., 2010). AZD8055 treatment of SW620 cells caused a loss of cyclin D1 (CCND1) expression (Fig. 1B) and a G1 cell cycle arrest, with no evidence of dead cells (sub-G1 DNA content) even after 72 hours (Fig. 1C). A similar G1 arrest was observed in other AZD8055-sensitive cell lines including COLO205 and HT29 (C.L.C., unpublished data).

eIF4E-driven, cap-dependent mRNA translation is thought to be one of the key effector pathways controlling cell proliferation downstream of mTORC1 (Dowling et al., 2010). To monitor changes in cap-dependent mRNA translation we employed a bicistronic dual Renilla–Firefly luciferase reporter construct (pRL-IRES-FL, Fig. 1D) (Li et al., 2002). This assay was validated by showing that expression of eIF4E increased cap-dependent translation, whereas a dominant 4EBP1 mutant (4EBP1ΔA, which sequesters eIF4E) strongly inhibited it; in both cases we observed reciprocal regulation of IRES-dependent translation (supplementary material Fig. S2A–C). In addition, treatment of transfected SW620 cells with AZD8055 did not impair the integrity of the dual pRL-IRES-FL reporter in these assays as assessed by qRT-PCR for Renilla and firefly (supplementary material Fig. S2D). When SW620 cells were treated with AZD8055, we observed inhibition of cap-dependent translation and a compensatory increase in IRES-dependent translation (Fig. 1D). Finally, AZD8055 caused an increase in processing of LC3 and a reduction in the expression p62 consistent with the induction of autophagy (C.L.C., unpublished data). Thus AZD8055 effectively inhibited both mTORC1 and mTORC2, inhibited cap-dependent translation and exerted a strong anti-proliferative effect in SW620 cells, causing a G1 cell cycle arrest.

SW620 cells with acquired resistance to AZD8055 exhibit loss of S6K signalling but increased expression of eIF4E

The G1 arrest observed in response to AZD8055 peaked at 24 hours before subsiding somewhat. To determine how tumour cells adapted to chronic mTORC1/mTORC2 inhibition, we grew SW620 cells in 2 μM AZD8055 until they acquired resistance and grew apparently normally: these were named SW620:8055R cells. These cells were >100-fold more resistant to AZD8055 (Fig. 2A) than SW620 cells and were cross resistant to other highly selective mTOR kinase inhibitors such as PP242 and WYE-125132 (supplementary material Fig. S3A,B) but retained normal sensitivity to cytotoxic chemotherapy drugs such as doxorubicin, paclitaxel and etoposide (supplementary material Fig. S3C–E). SW620:8055R cells were also partially cross-resistant to the dual mTOR and PI3K inhibitor PI-103 (supplementary material Fig. S4A). Parental SW620 cells were not very sensitive to ZSTK474 (a pan-PI3K inhibitor with little or no activity against mTOR) suggesting that although these cells are addicted to PI3K. Regardless, the SW620:8055R derivatives showed a similar poor sensitivity (supplementary material Fig. S4B). This suggested that the mechanism of resistance was related to the target, mTOR. Finally, in contrast to mTOR kinase inhibitors, SW620:8055R cells were not cross-resistant to the mTORC1-selective inhibitor rapamycin (supplementary material Fig. S4C), correlating with the inability of rapamycin to inhibit 4EBP1 phosphorylation (supplementary material Fig. S1B).

Prompted by these results we examined mTOR signalling. AZD8055 caused normal inhibition of mTORC2 in both SW620 and SW620:8055R cells, as judged by the rapid loss of P-Ser473 PKB (Fig. 2B) but we observed striking changes in signalling downstream of mTORC1. Although AZD8055 inhibited P-S6K and P-S6 normally in SW620 cells, basal P-S6K and P-S6 signals were completely lost in the SW620:8055R cells. This was not due
to a complete loss of mTORC1 activity because SW620:8055R cells exhibited normal basal 4EBP1 phosphorylation at Thr37/Thr46 (mTORC1 sites) and normal de-phosphorylation of this site in response to AZD8055 (Fig. 2B). However, the mobility of total 4EBP1 on SDS-PAGE gels indicated that additional phosphorylation sites were constitutively de-phosphorylated in SW620:8055R cells. SW620:8055R cells also exhibited a higher basal level of 4EBP1 than parental SW620 cells, again consistent with stabilization (Elia et al., 2008) or enhanced detection of de-phosphorylated 4EBP1 by the non-phospho-4EBP1 antibody.

In addition to these changes, we also observed a striking increase in the expression and phosphorylation of the eukaryotic translation initiation factor eIF4E: both total eIF4E and P-Ser209 eIF4E were greatly elevated in SW620:8055R cells compared to parental SW620 cells (Fig. 2B). Quantification of these changes from 11 experiments revealed that total eIF4E and P-Ser209 eIF4E were elevated to similar extents in the SW620:8055R cells suggesting that there was no change in the stoichiometry of phosphorylation of P-Ser209 (Fig. 2C). Indeed, we did not observe any difference in the phosphorylation of the MNK proteins (R.G., K.B. and S.J.C. unpublished data), the kinases responsible for Ser209 phosphorylation (Topisirovic et al., 2004; Furic et al., 2010; Ueda et al., 2010). We also performed an eIF4E immunoprecipitation from SW620 and SW620:8055R cells (Fig. 2D). SW620:8055R cells again exhibited higher levels of eIF4E and this was reflected in the greater amount of eIF4E precipitated from the drug-treated SW620:8055R cells (lane 8) compared to SW620 cells (lane 6). Despite this, the amount of co-precipitating 4EBP1 was the same indicating that there was an excess of eIF4E that was not bound to 4EBP1 in the SW620:8055R cells. Finally, we generated five additional AZD8055-resistant clones (SW620:8055C1, C2, etc) all of which exhibited the same features: loss of P-S6K, constitutive de-phosphorylation and increased expression of 4EBP1 and
increased eIF4E and P-eIF4E when compared to parental SW620 cells (Fig. 3A, B). Thus, a switch away from S6K and increased expression of eIF4E was a common response to chronic mTOR inhibition in SW620 cells.

AZD8055-resistant SW620 cells exhibit amplification of eIF4E and enhanced cap-dependent translation

To determine the cause of the increase in eIF4E we employed quantitative RT-PCR [(q)PCR]. This demonstrated that although acute treatment with AZD8055 did not increase eIF4E mRNA in parental SW620 cells, SW620:8055R cells exhibited a 9–10-fold increase in eIF4E mRNA levels; 4EBP1 mRNA levels were unaffected (Fig. 4A). Genomic (q)PCR revealed no difference in 4EBP1 copy number between SW620 and SW620:8055R cells (Fig. 4B). Parental SW620 cells actually exhibited a copy number of <1 for eIF4E when compared to normal healthy volunteers (Fig. 4B). In comparison to SW620 cells, eIF4E copy number was increased by a factor of five to six in SW620:8055R cells (Fig. 4B), consistent with the amplification of eIF4E.

To define the nature of the eIF4E amplification, we performed fluorescence in situ hybridization (FISH) with a bacterial artificial chromosome (BAC) clone from the eIF4E genomic locus. We examined metaphase nuclei, where chromosomes are visible, and interphase nuclei, where chromatin is less condensed, providing a greater chance of distinguishing individual copies of an amplified locus. In interphase, SW620 cells contained two copies of chromosome 4 but only one FISH signal from the eIF4E BAC; SW620:8055R cells also contained two copies of chromosome 4 but showed around six eIF4E signals (Fig. 4C). These results were consistent with the genomic (q)PCR data above and suggest that there is eIF4E amplification in SW620:8055R cells (Fig. 4C). Metaphase FISH analysis of SW620 cells confirmed the presence of two copies of the chromosome 4 centromere. Only one copy of eIF4E was detected, consistent with spectral karyotyping data for SW620, which suggests one normal copy of chromosome 4 and a
second copy with a large deletion (http://www.path.cam.ac.uk/~pawefish/). All SW620:8055R cells analysed by metaphase FISH exhibited one chromosome 4 with multiple copies of elf4E (typically six to eight) and one chromosome 4 with a deletion of elf4E (Fig. 4C). In addition, the majority of metaphase spreads analysed also exhibited the presence of the elf4E locus on a chromosome that lacked the chromosome 4 centromere; we speculate that this might have arisen through a translocation event with an unknown chromosome. Regardless, these results suggest that the increase in elf4E mRNA and protein in SW620:8055R cells reflects elf4E gene amplification.

The increased levels of elf4E and P-elf4E should favour cap-dependent translation in SW620:8055R cells. Indeed, SW620:8055R cells exhibited a 3-fold increase in the ratio of cap-dependent:IRES-dependent translation compared to parental SW620 cells (Fig. 5A); this increase was reversed by expression of 4EBP1AA (Fig. 5A,B). The increase in cap:IRES ratio in SW620:8055R cells was observed across all AZD8055 doses, so that SW620:8055R cells maintained in their normal medium (2 μM AZD8055) exhibited up to a 4-fold increase in cap-dependent translation (Fig. 5C). We also examined the expression of CCND1 and MCL1, two proteins whose mRNAs are elf4E-sensitive and translated in a cap-dependent fashion (Tan et al., 2000; Wendel et al., 2007; Averous et al., 2008). Although CCND1 and MCL1 expression was inhibited by AZD8055 in parental SW620 cells, their expression was maintained and drug-refractory in SW620:8055R cells (Fig. 5D).

**Increased elf4E expression is necessary for AZD8055 resistance in SW620:8055R cells**

To determine whether increased elf4E expression was driving AZD8055 resistance we employed RNA interference. siRNA-mediated knockdown of elf4E was able to reverse the increase in cap-dependent translation in SW620:8055R cells, whereas a control non-targeting siRNA was ineffective (Fig. 6A,B). Similarly, elf4E siRNA caused a substantial re-sensitization of SW620:8055R cells to the growth inhibitory effects of AZD8055, so that the IC₅₀ for growth inhibition shifted from >10 μM to ~500 nM, much closer to that seen in parental cells (~100 nM) (Fig. 6C,D). Re-sensitization was not complete but this could be due to incomplete knockdown of elf4E (Fig. 6D). Nonetheless, these results indicate that the increase in elf4E expression in SW620:8055R cells increases cap-dependent translation and this is required to maintain the expression of key proliferation and survival proteins and to maintain resistance to AZD8055.

**Increased elf4E expression is sufficient for AZD8055 resistance**

To determine whether increased elf4E expression alone was sufficient to promote AZD8055 resistance we expressed elf4E in a separate, heterologous cell system. We used the ‘Tet-on’ (TO) expression system to generate stable HEK293 cell lines that exhibited inducible expression of either elf4E (TO-elf4E cells) or dominant-negative 4EBP1AA (TO-4EBP1AA cells); we also generated a control cell line that only expressed the Tet repressor protein (TR cell line) (supplementary material Fig. S5). Tet-inducible expression of elf4E was sufficient to reduce sensitivity to AZD8055 (Fig. 7A), whereas inducible expression of 4EBP1AA strongly inhibited cell proliferation in the absence or presence of AZD8055 (Fig. 7B); we did not observe any cell death in response to inducible expression of 4EBP1AA (R.G. and S.J.C., unpublished results). Tet treatment had no effect on basal proliferation or AZD8055 sensitivity in the control TR cell line (Fig. 7C). In parallel, we observed that inducible elf4E expression rescued the inhibition of CCND1 expression by AZD8055, whereas inducible expression of 4EBP1AA alone was sufficient to inhibit CCND1 expression (Fig. 7D) and inhibit cell proliferation as judged by loss of histone H3 phosphorylation (supplementary material Fig. S5). Indeed, AZD8055 treatment inhibited histone H3 phosphorylation and this was prevented by inducible expression of elf4E in TO-elf4E cells (supplementary material Fig. S5). Notably, inducible expression of 4EBP1AA reduced P-elf4E (consistent with elf4E sequestration) and then CCND1 expression and H3 phosphorylation prior to any loss of total elf4E expression. Thus loss of CCND1 expression was due to 4EBP1AA-induced sequestration of elf4E and not loss of elf4E, which was only observed after prolonged 4EBP1AA expression (supplementary material Fig. S5) and therefore might...
be a consequence of the cell cycle arrest. Taken together, these results demonstrate that increased expression of eIF4E alone is sufficient to reduce sensitivity to AZD8055, and that the mTOR-4EBP1-eIF4E pathway is a key determinant of CCND1 expression and cell proliferation in both SW620 and HEK293 cells.

SW620:8055R cells are cross-resistant to the MEK1/2 inhibitor selumetinib

Inhibition of the RAF–MEK1/2–ERK1/2 pathway is an attractive therapeutic approach in many cancers, exemplified by the success of vemurafenib in melanomas with BRAFV600E (Chapman et al., 2011). However, in tumour cells with KRAS mutations vemurafenib causes paradoxical ERK1/2 activation (Poulikakos et al., 2010) so that MEK1/2 inhibitors will be a better approach for ERK1/2 pathway inhibition. Given that KRAS activates multiple pathways in addition to ERK1/2, including the PI3K–PKB–mTOR pathway, it is likely that MEK1/2 inhibitors will be most effective in combination with other agents. Indeed, given that the ERK1/2 and mTOR pathways are both downstream targets of KRAS it is notable that selumetinib and AZD8055 combine well to inhibit the growth of KRAS mutant tumour cells (Holt et al., 2012). Prompted by this observation we examined whether combination with selumetinib could overcome AZD8055 resistance; however, combination with selumetinib did not really re-sensitise SW620:8055R cells to AZD8055 (Fig. 8A). Rather, we found that SW620:8055R cells were also resistant to selumetinib so that the IC₅₀ for growth inhibition by selumetinib shifted from 70 nM in SW620 cells to 5–10 μM in SW620:8055R cells (Fig. 8B). This cross-resistance was accompanied by a striking de-regulation of CCND1 and p27KIP1 (also known as CDKN1B) expression (Fig. 8C). In SW620 cells, selumetinib increased p27KIP1 and decreased CCND1, as expected, consistent with the strong anti-proliferative
effect in these cells; in contrast, both of these responses were greatly reduced in SW620:8055R cells (Fig. 8C). These results suggest that eIF4E, a point of convergence of ERK1/2 and mTOR signalling, is a key determinant of CCND1 and p27KIP1 expression and cell proliferation for both pathways.

**DISCUSSION**

When faced with the selection pressure of chronic mTORC1 and mTORC2 inhibition by AZD8055, SW620 cells remodelled mTOR signalling to allow them to continue to proliferate. We anticipated that this adaptation might involve a switch from cap-dependent to IRES-dependent translation because: (1) compensatory IRES-dependent translation is seen upon inhibition of cap-dependent translation (supplementary material Fig. S2) (Svitkin et al., 2005); (2) this was observed upon acute AZD8055 treatment (Fig. 1D); and (3) some oncogenes are translated by IRES-dependent mechanisms (Stoneley and Willis, 2004). However, IRES-dependent translation was not upregulated in SW620:8055R cells. Rather the cells adapted to chronic mTORC1 and mTORC2 inhibition by amplifying eIF4E (Fig. 4) to increase eIF4E protein levels, thereby maintaining or even increasing cap-dependent translation (Fig. 5). RNAi to eIF4E revealed that SW620:8055R cells remained ‘addicted’ to the increased expression of eIF4E to maintain AZD8055 resistance (Fig. 6). Finally, conditional overexpression of eIF4E was sufficient to confer resistance to AZD8055 (Fig. 7).

Recent studies suggest that the signalling pathways controlling protein synthesis downstream of mTORC1 are not equally required in oncogenesis: deregulation of protein synthesis through the 4EBP–eIF4E signalling arm appears to be more important in tumour formation than S6K signalling. For example, loss of 4EBP1/2 and the resultant release of eIF4E to activate cap-dependent translation promotes cell proliferation in culture (Dowling et al., 2010), and active eIF4E is required for the transforming effects of PKB on mRNA translation, cell growth, tumour formation and maintenance (Wendel et al., 2007). In contrast, S6K makes a more modest contribution to the oncogenic action of ERK1/2 or PKB compared to the 4EBP–eIF4E pathway (Hsieh et al., 2010; She et al., 2010). Our results are entirely consistent with this notion. In addition to increased eIF4E expression, we observed a loss of P-S6K and P-S6, suggesting that even basal S6K signalling is dispensable for acquired resistance to mTOR kinase inhibitors. The precise cause of the decrease in P-S6K is unclear but might be related to the increase...
in basal levels of 4EBP1 that was seen in all SW620:8055R clones. Given that 4EBP1 mRNA levels were unaffected this might reflect stabilization of 4EBP1 protein following dephosphorylation (Elia et al., 2008), although we cannot rule out the possibility that the non-phospho-4EBP1 antibody detects de-phosphorylated 4EBP1 with greater avidity. It is known that S6K1 binds much more weakly to mTORC1 than 4EBP1, so that co-purifying S6K1 with mTORC1 is difficult (Choo and Blenis, 2009). Furthermore, mTORC1-dependent phosphorylation of S6K1 and 4EBP1/2 is determined by competition between these two substrates for interaction with Raptor, such that loss of 4EBP1/2 (which have a higher affinity for mTORC1) dramatically increases S6K1 activation (Dennis et al., 2013). Based on these results, an increase in basal 4EBP1 levels in SW620:8055R cells might out-compete S6K for access to Raptor, resulting in the loss of P-S6K in these cells. Given that 4EBP1 normally sequesters eIF4E an increase in 4EBP1 might be the actual ‘driver’ that selects for amplification and increased expression of eIF4E to maintain cap-dependent translation.

Although eIF4E can act as an oncoprotein (Lazaris-Karatzas et al., 1990; Ruggero et al., 2004; Wendel et al., 2004) and is overexpressed in some tumour types (Haydon et al., 2000; De Benedetti and Graff, 2004; Mamane et al., 2004) this is to our knowledge the first report of amplification of eIF4E in human cancer cells in response to selective mTOR inhibition. However, a recent study that employed engineered human mammary epithelial cells and the dual mTOR/P13K inhibitor BEZ235 did also observe eIF4E amplification (Ilic et al., 2011), consistent with the results herein. eIF4E promotes cell proliferation and tumorigenesis by promoting the translation of specific ‘eIF4E-sensitive mRNAs’ that code for proteins that promote cell proliferation, cell survival, energy metabolism and tumour metastasis (Mamane et al., 2004). Among the mRNAs targeted for cap-dependent translation by eIF4E are well-known oncoproteins that promote proliferation, such as CCND1, and pro-survival proteins, such as MCL1 (Tan et al., 2000; Mamane et al., 2004). Indeed, expression of CCND1 and MCL1 was refractory to AZD8055 in SW620:8055R cells overexpressing eIF4E (Fig. 5). Furthermore, the inducible expression of eIF4E alone was sufficient to prevent the loss of CCND1 protein expression and cell cycle arrest following AZD8055 treatment (Fig. 7). Taken as a whole, our data suggest that the refractoriness of CCND1 expression to AZD8055 in SW620:8055R cells is due to increased cap-dependent translation, although it is possible that eIF4E-dependent enhanced nuclear export of CCND1 mRNA might contribute (Culjkovic et al., 2005). Regardless, our results show that the de-regulated overexpression of eIF4E is not only a transforming event but also renders cells resistant to mTOR kinase inhibitors. Our results complement and extend those of a recent study, which found that resistance to mTOR kinase inhibitors was associated with downregulation of 4EBP1 or 4EBP2 to activate eIF4E and which concluded that the eIF4E:4EBP ratio predicted responses to mTOR kinase inhibitors (Alain et al., 2012). Thus tumour cells can arrive at a common phenotype, resistance to mTOR kinase inhibitors, by different mechanisms (decreased 4EBP1/2 or increased eIF4E) that share the common theme of maintaining cap-dependent translation in the presence of drug. Notably, SW620:8055R cells
were not cross resistant to the selective mTORC1 inhibitor rapamycin. Given that de-phosphorylation of 4EBP1 is a crucial aspect of the antiproliferative effects of mTOR kinase inhibitors (Dowling et al., 2010) these results are entirely consistent with the inability of rapamycin to elicit de-phosphorylation of 4EBP1 (supplementary material Fig. S1B), something that AZD8055 and other ATP-competitive mTOR kinase inhibitors do (Choo et al., 2008; Thoreen et al., 2009; Feldman et al., 2009; Chresta et al., 2010).

These results suggest that, as the clinical evaluation of mTOR kinase inhibitors proceeds, increased eIF4E expression should be monitored and inhibition of eIF4E function should be considered as a potential adjunct to mTOR kinase inhibitors to increase efficacy and delay the onset of resistance. eIF4E itself might not be a readily druggable target; however, it forms part of the multi-protein eIF4F complex that includes the eIF4A RNA helicase. In addition, the transforming effects of eIF4E are enhanced by phosphorylation of Ser209, which is catalyzed by the MNK1 and MNK2 protein kinases (Topisirovic et al., 2004; Furic et al., 2010; Ueda et al., 2010). MNK1 and MNK2 are in turn activated by phosphorylation catalyzed by either ERK1/2 or the p38 stress kinases (Waskiewicz et al., 1997; Hou et al., 2012). Given that SW620 cells harbour a KRAS mutation and are strongly dependent on RAF–MEK1/2–ERK1/2 signalling for proliferation we anticipated that a MEK1/2 inhibitor might overcome eIF4E-driven acquired resistance to AZD8055. In fact we observed the opposite: SW620:8055R cells were cross-resistant to the MEK1/2 inhibitor selumetinib and this correlated with CCND1 expression being less sensitive to selumetinib (Fig. 8). We also observed that selumetinib failed to increase p27KIP1 (another important arbiter of the G1 to S phase transition) in SW620:8055R cells. Given that p27KIP1 expression can be promoted by a cap-independent mechanism (Jiang et al., 2007) through an IRES element in its 5’ UTR (Coleman and Miskimins, 2009) this might be due to preferential use of cap-dependent translation in SW620:8055R cells overexpressing eIF4E.

In summary, we have shown that although mTOR regulates numerous downstream signalling pathways, cells adapt to chronic mTORC1 and mTORC2 inhibition by amplifying just one of these, the eIF4E signalling arm downstream of mTORC1, to maintain cap-dependent translation; indeed, inducible expression of eIF4E is sufficient to render cells resistant to mTOR inhibition. This highlights the importance of eIF4E and its targets as key regulators of cell proliferation downstream of mTOR. Furthermore, increased expression of eIF4E can drive resistance to MEK1/2 inhibitors as well as mTOR kinase inhibitors, consistent with the convergence of both signalling pathways at the level of eIF4E (Hou et al., 2012). This suggests that although mTOR inhibitors and MEK inhibitors can combine well as a primary treatment (Holt et al., 2012), combination with MEK...
inhibitors might not be fruitful in seeking to overcome acquired resistance to mTOR inhibitors driven by eIF4E amplification.

**MATERIALS AND METHODS**

**Materials**

AZD8055 and selumetinib (AZD6244/ARRY-142886) were provided by AstraZeneca (Alderley Park, Macclesfield, UK). AZD8055 is a highly selective ATP-competitive inhibitor of mTOR that inhibits both the mTORC1 and mTORC2 complexes (Chresta et al., 2010). Selumetinib is a highly selective allosteric inhibitor of MEK1 and MEK2 (Davies et al., 2007). WYE-125132, ZSTK474 and PI-103 were purchased from Selleck Chemicals. All oligonucleotides are available upon request.

**Plasmids**

Plasmids encoding the following constructs were generous gifts from colleagues: HA–eIF4E was provided by Nahum Sonenberg, McGill University, USA; pRL-IRES-FL was provided by Peter Bitterman, University of Minnesota Medical School, USA and 4EBP1AA (4EBP1 R13A/F113A-Myc/His) from Chris Proud, University of Southampton, UK. The 4EBP1AA mutant harbour mutations in both the RAIP (R13A) and TOS (F113A) motifs of 4EBP1, fails to bind to mTOR and undergo mTOR-dependent phosphorylation and so acts in a dominant fashion to sequester eIF4E. TO-4EBP1AA and TO-eIF4E were generated by subcloning of fragments from 4EBP1AA–Myc/His and HA–eIF4E respectively into pcDNA 4/TO (Invitrogen). All inserts were verified by ABI automated sequencing. The details of cloning and sequences of all oligonucleotides are available upon request.

**Cell lines and cell culture**

All reagents used for routine tissue culture were obtained from Life Technologies or PAA. Human colorectal carcinoma cell lines were obtained from the American Type Culture Collection (Colo205, HT-29, SW620) or were provided by Richard Hammelin, INSERM U938, Paris (CO115), Kevin Ryan, the Beatson Institute (LoVo), Bert Vogelstein, Johns Hopkins University (HCT116) or Senji Shirasawa, School of Medicine Fukuoka University (DLD-1). Cells were maintained in DMEM (HCT116, DLD-1, LoVo, CO115), RPMI1640 (Colo205), or McCoy’s (HT29, SW620) or were provided by Richard Hammelin, INSERM U938, Paris (CO115), Kevin Ryan, the Beatson Institute (LoVo), Bert Vogelstein, Johns Hopkins University (HCT116) or Senji Shirasawa, School of Medicine Fukuoka University (DLD-1). Cells were maintained in DMEM (HCT116, DLD-1, LoVo, CO115), RPMI1640 (Colo205), or McCoy’s (HT29) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 mg/ml) and glucose (2 mM). SW620 cells were routinely cultured in Leibovitz’s L15 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 mg/ml) and glucose (2 mM). Exponentially growing SW620 cells were exposed to 2 μM AZD8055 until they could maintain a growth rate similar to that of the parental cells in the presence of inhibitor. This non-clonal resistant cell line was designated SW620:8055R. In addition, individual AZD8055 resistant clones of SW620 cells were obtained by limiting dilution, selection in 2 μM AZD8055 and ring cloning. All SW620:8055R cells were then routinely cultured in 2 μM AZD8055.

**Antibodies**

Plasmids encoding the following constructs were generous gifts from colleagues: HA–eIF4E was provided by Nahum Sonenberg, McGill University, USA; pRL-IRES-FL was provided by Peter Bitterman, University of Minnesota Medical School, USA and 4EBP1AA (4EBP1 R13A/F113A-Myc/His) from Chris Proud, University of Southampton, UK. The 4EBP1AA mutant harbour mutations in both the RAIP (R13A) and TOS (F113A) motifs of 4EBP1, fails to bind to mTOR and undergo mTOR-dependent phosphorylation and so acts in a dominant fashion to sequester eIF4E. TO-4EBP1AA and TO-eIF4E were generated by subcloning of fragments from 4EBP1AA–Myc/His and HA–eIF4E respectively into pcDNA 4/TO (Invitrogen). All inserts were verified by ABI automated sequencing. The details of cloning and sequences of all oligonucleotides are available upon request.

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**Fig. 8.** SW620:8055 cells are cross-resistant to the MEK1/2 inhibitor selumetinib. (A) SW620:8055R cells were exposed to increasing concentrations of AZD8055 with or without 100 nM selumetinib for 24 hours and cell proliferation was assayed by [3H]thymidine incorporation. Results are the mean±coefficient of variation (CoV) for three biological replicates from a single experiment; identical results were obtained in n=3 experiments. (B) SW620 and SW620:8055R cells were exposed to increasing concentrations of selumetinib for 24 hours in their normal growth medium (+2 μM AZD8055 for SW620:8055R cells). Cell proliferation was assayed by [3H]thymidine incorporation. Results are the mean±CoV for three biological replicates from a single experiment; identical results were obtained in n=3 experiments. (C) SW620 and SW620:8055R cells were treated with the indicated concentrations of selumetinib for 24 hours. Whole cell lysates were fractionated by SDS-PAGE and immunoblotted for the indicated proteins. In each case, results are taken from a single experiment; identical results were obtained in n=3 additional experiments.
Transfections

SW620 cells were transfected by Lipofectamine 2000 (Life Technologies) into penicillin-streptomycin-free medium at a ratio of Lipofectamine 2000: DNA of 4:1 according to the manufacturer’s instructions.

Preparation of cell extracts and western blotting

Cells were lysed in ice-cold TG lysis buffer (Todd et al., 2004), assayed for protein content, and fractionated by SDS-PAGE and then transferred onto PVDF membranes. The membranes were then blocked in 5% milk/ TBST before overnight incubation with the indicated antibodies. After washing and incubation with HRP-conjugated secondary antibodies, detection was with ECL.

Assay of cap-dependent protein translation

Cells were plated at 2x10^5 per well in 12-well plates, 24 hours later they were transfected with 500 ng of pRL-IRE-FL per well using Lipofectamine 2000 according to the manufacturer’s instructions. In co-expression experiments 0.5 µg of pcANHA, pHA-eIF4E, pcDNA 3.1 Myc His, pcDNA3.1 4EBP1 R13A/F113A-Myc/His or 50 µM of non-targeting (NT) or eIF4E siRNA were also included. Preparation of cell extracts and processing for Firefly and Renilla luciferase activity using the Promega Dual Luciferase Reporter Assay were performed according to the manufacturer’s instructions and the light emitted was then measured in 96-well format in a MicroLumat Plus LB96V.

Fluorescence in situ hybridization

Metaphase chromosome preparation and FISH were performed as previously described (Alsop et al., 2006; Pole et al., 2006; Little et al., 2011). BAC clone RP11-428B4 (BACPAC resources) corresponding to chromosome 4: 99727567–99839879 (GRCh37/HG19) was selected for localization to eIF4E and indirectly labelled using a nick translation kit (Abbott Molecular) and digoxigenin-dUTP (Roche Diagnostics) following the manufacturer’s instructions. A chromosome 4 centromere probe kindly provided by Suet-Feung Chin and Carlos Caldas for the chromosome 4 centromere probe. We are grateful to members of the Cook lab for their support and encouragement; Paul Edwards (Hutchison-MRC Research Centre) for providing facilities for, and advice with, DNA FISH; and to Teresa Klonowska (AstraZeneca) for her encouragement and helpful comments on the study and manuscript.

Cell proliferation and cell cycle distribution assays

The assay of [3H]thymidine incorporation and determination of cell cycle distribution by propidium iodide staining and flow cytometry were as described previously (Todd et al., 2004).

RNA interference
eIF4E siGENOME SMARTpool and non-targeting (NT) siRNA oligonucleotides were purchased from Dharmacon. SW620:8055R cells were transfected by Lipofectamine 2000 according to the manufacturer's instructions. For eIF4E/4E-BP, each experiment was performed in triplicate under standard conditions and absolute quantity calculated by relating the CT value to a reference gene.

Quantitative RT-qPCR

RT-qPCR assays for eIF4E (Hs01689624_cn), 4EBP1 (Hs01094626_cn) and RNaseP (catalogue number 4316831) were purchased from Applied Biosystems. All assays were performed with the Mx3000P Real-Time PCR system (Agilent Technologies/Stratagene Products, catalogue number 401512). Each assay was performed on the cell lines and on genomic DNA samples from control healthy volunteers (HVP). Assays were run in triplicate under standard conditions and absolute quantity calculated by relating the CT value to a reference gene.

Acknowledgements

We thank Nahum Sonenberg for HA-eIF4E; Peter Bitterman for pRL-IRE-FL; Chris Proud for provision of 4EBP1 and useful discussions and Suet-Feung Chin and Carlos Caldas for the chromosome 4 centromere probe. We are grateful to members of the Cook lab for their support and encouragement; Paul Edwards (Hutchinson-MRC Research Centre) for providing facilities for, and advice with, DNA FISH; and to Teresa Klonowska (AstraZeneca) for her encouragement and helpful comments on the study and manuscript.

Competing interests

M.H., P.D.S. and S.M.G. are paid employees of AstraZeneca.

Author contributions

C.L.C., S.J.C., P.D.S. and S.G.M. conceived the project. C.L.C., R.G., K.B., M.S., and S.J.C. conceived and designed the experiments. C.L.C., R.G., K.B., M.S., H.D.K., and M.H. performed the experiments and analysed the data. All authors contributed to data interpretation. C.L.C., K.B., R.G. and S.J.C. wrote the manuscript with input from all other authors.

Funding

This work was supported by a Biotechnology and Biological Science Research Council (BBSRC) CASE PhD studentship awarded to AstraZeneca and awarded to the Babraham Institute (to C.L.C. and S.J.C.); a BBSRC studentship (to M.J.S. and S.J.C.); a grant from Cancer Research UK [grant number C1023/A14545 to K.D.H.]; and by The Babraham Institute (to K.B., R.G. and S.J.C.), which receives strategic funding from the BBSRC. Deposited in PMC for immediate release.

Supplementary material

Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.137588/-/DC1

References


Fig. S1. Changes in signalling in response to AZD8055 and Rapamycin.
(A) AZD8055 fully inhibits mTOR signalling in resistant (CO115) and sensitive (COLO205) cell lines. CO115 and COLO205 cells were treated with increasing doses of AZD8055 for 24 hours. Whole cell lysates were fractionated by SDS-PAGE and immunoblotted with the indicated antibodies.
(B) AZD8055 and Rapamycin causes strong dephosphorylation of 4EBP1 in SW620 cells. SW620 cells were treated for 1, 2 or 4 hours with 1μM AZD8055 or 1μM Rapamycin. Whole cell lysates were fractionated by SDS-PAGE and immunoblotted with the indicated antibodies.
In each case results are taken from a single experiment; identical results were obtained in n=3 experiments.

Fig. S2. Validation of pRL-IRES-FL as a reporter for cap-dependent translation.
(A-C) SW620 cells were transfected with a dual renilla/firefly luciferase construct, pRL-IRES-FL, together with either an empty vector control (EV), HA-eIF4E (eIF4E) or 4EBP1 R13A/F113A-Myc/His (4EBP1^AA). Lysates were prepared 24hr later and either used in a dual luciferase assay (A & B) or subjected to SDS-PAGE and immunoblotted with the indicated antibodies (C). In (A) both the separate cap-dependent Renilla and IRES-dependent Firefly values are shown whilst in (B) the cap/IRES ratio is shown.
(D) Parental SW620 or SW620:8055R cells were left untreated or treated with 2μM AZD8055 for 24 hours. Samples were prepared and the abundance of the firefly and renilla mRNAs was assessed by (q)PCR and normalized to YWHA, a stable mRNA control (left panels). In parallel, cells treated identically were assayed for firefly and renilla using the duaa luciferase assay. Results are taken from a single experiment; identical results were obtained in n=3 experiments.

Fig. S3. AZD8055-resistant SW620 cells are cross-resistant to other mTOR kinase inhibitors but not to cytotoxic chemotherapy drugs.
SW620 and SW620:8055R cells were treated with increasing concentrations of (A) PP242, (B) WYE-125132, (C) Doxorubicin, (D) Paclitaxel or (E) Etoposide for 24 hours and cell proliferation was assayed by [3H]thymidine incorporation. Results are the mean±CoV for three biological replicates from a single experiment; identical results were obtained in n=3 experiments.
Fig. S4. Response of AZD8055-resistant SW620 cells to a mixed PI3K/mTOR inhibitor (PI-103), a selective PI3K inhibitor (ZSTK474) or Rapamycin.
SW620 and SW620:8055R cells were treated with increasing concentrations of (A) PI103, (B) ZSTK474 or (C) Rapamycin for 24 hours and cell proliferation was assayed by [³H]thymidine incorporation. Results are the mean±CoV for three biological replicates from a single experiment; identical results were obtained in n=3 experiments.

Fig. S5. AZD8055-dependent loss of cyclin D1 (CCND1) is rescued by eIF4E expression.
HEK293 TR cells, HEK293 TO-eIF4E cells or HEK293 TO-4EBP1AA cells were treated over time course of 6 days with tetracycline to induce the expression of eIF4E (TO-eIF4E) or 4EBP1 R13A/F113A (TO-4EBP1AA). Cells were then either treated with DMSO or AZD8055 for the final 24hrs and cell lysates were prepared, fractionated by SDS-PAGE and immunoblotted for the indicated antibodies (CCND1 = cyclin D1).
Supp Figure 1

A

<table>
<thead>
<tr>
<th>CO115</th>
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<tr>
<td>P-ERK1/2</td>
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<tr>
<td>P-S6K</td>
<td>S6K</td>
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<tr>
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0 0.1 1 10 0 0.1 1 10 AZD8055 (µM)

B

SW620 cells

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0 1 2 4 0 1 2 4 Time (hrs)
Supp Figure 3

A

PP242

SW620: 8055R

SW620

[\text{[H]} \text{Thym Inc (%)}] vs. [PP242] [\mu\text{M}]

B

WYE-125132

SW620: 8055R

SW620

[\text{[H]} \text{Thym Inc (%)}] vs. [WYE-125132] [\mu\text{M}]

C

Doxorubicin

[\text{[H]} \text{Thym Inc (%)}] vs. [Doxorubicin] [\mu\text{M}]

D

Paclitaxel

[\text{[H]} \text{Thym Inc (%)}] vs. [Paclitaxel] [\text{nM}]

E

Etoposide

[\text{[H]} \text{Thym Inc (%)}] vs. [Etoposide] [\mu\text{M}]

SW620

SW620: 8055R
Supp Figure 4

A

[PI-103] μM

[3H] Thym Inc (%)

0 25 50 75 100 125 150

0.001 0.01 0.1 1 10

PI-103

SW620: 8055R

SW620

B

[ZSTK474] μM

[3H] Thym Inc (%)

0 25 50 75 100 125 150 175

0.001 0.01 0.1 1 10

ZSTK474

SW620: 8055R

SW620

C

[Rapamycin] (nM)

[3H] Thym Inc (%)

0 25 50 75 100 125

0.001 0.1 1 10 1000

Rapamycin

SW620

SW620 8055R
Supp Figure 5

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- 4EBP1
- eIF4E
- P-eIF4E
- CCND1
- P-H3
- Actin