Che-1 sustains autophagy and multiple myeloma cells growth by inhibiting mTOR pathway in response to cellular stress.

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Summary

Che-1 is a RNA polymerase II binding protein activated by the DNA damage response and involved in growth arrest through induction of specific genes. Mammalian target of rapamycin (mTOR) is a key protein kinase that regulates cell growth metabolism and autophagy to maintain cellular homeostasis. Its activity is inhibited by adverse conditions, including nutrient limitation, hypoxia and DNA damage. In this study, we demonstrate that Che-1 inhibits mTOR activity in response to stress conditions. We found that stress-activated Che-1 induces the expression of two important mTOR inhibitors, Redd1 and Deptor, and that this activity is required for sustaining autophagy. Strikingly, Che-1 expression correlates with Deptor expression and multiple myeloma progression, contributing to cell growth and survival in this disease.

Significance

The significance of this work is underscored by the presence of mTOR deregulation in a variety of human cancer and by the fact that mTOR is considered an important target in cancer therapy. Here, we provide evidence that Che-1 strongly controls mTOR inhibition in response to several cellular stresses by activating Redd1 and Deptor transcription. In addition, this new important link between cellular stress and mTOR activity regulates autophagy induction. Significantly, primary human multiple myelomas exhibit high levels of Che-1, Deptor and autophagy. In accordance, PI(3)K/mTORC2/Akt1 pathway and survival in multiple myeloma cells are dependent on Che-1 expression. These findings reinforce Che-1 as possible therapeutic target to increase drug efficacy in cancer therapy.

Introduction

Mammalian target of rapamycin (mTOR) is an evolutionary conserved serine/threonine kinase that regulates cellular homeostasis by coordinating anabolic and catabolic processes with nutrient, energy, oxygen availability and growth factor signaling (Sarbassov et al., 2005). mTOR forms two distinct signaling complexes called mTORC1 and mTORC2. The mTORC1 complex is responsible for control of cell growth and protein synthesis, whereas the mTORC2 controls the actin cytoskeleton and cell spreading (Guertin and Sabatini, 2007). The positive and negative control of mTORC1 activity is exerted through the tuberous sclerosis complex (TSC), a GTPase that negatively regulates the RHEB G-protein, which in turn positively regulates mTOR (Inoki et al., 2002). The 4EBP1 protein and the S6 kinase are two major substrates of mTOR (Ma and Blenis, 2009). The 4EBP1 protein binds and inactivates the translation initiation factor 4E, which is required for the translation of CAP-mRNAs. Phosphorylation of 4EBP1 by mTOR inactivates 4EBP1 and permits efficient translation. Phosphorylation and activation of S6 kinase enhances the translation of mRNAs involved in ribosomal and mitochondrial biogenesis, as well as oxygen delivery and consumption. mTORC2 modulates cell survival in response to growth factors by phosphorylating its substrates Akt and serum/glucocorticoid regulated kinase 1 (SGK1) (Guertin and Sabatini, 2007).

In all eukaryotes, mTOR is a master regulator that integrates the signals from nutrients and energy sensors with cell growth and proliferation. Nevertheless, mTOR not only controls the rate of protein synthesis, but also regulates transcriptional changes in response to a variety of conditions (Fingar and Blenis, 2004). In recent years, numerous stimuli have been shown to cause changes in the activity of mTOR cascade, and in addition, decreased mTOR signaling activity has been found to be associated with many types of stress, suggesting that this pathway plays an important role in the adaption to different stress conditions (Reiling and Sabatini, 2006). Moreover, several findings indicate that in presence of several types of cellular stress, the p53 and ATM pathways negatively regulates mTOR activity (Budanov and Karin, 2008; Feng et al., 2007; Cam et al., 2010).

Importantly, it is increasingly apparent that deregulation of the mTOR pathway occurs in common diseases including cancer (Sarbassov et al., 2005).

Che-1 (also named AATF and Traube) is an important RNA polymerase II binding protein involved in the regulation of gene transcription (Passananti et al, 2007), and its expression is essential for proliferation in early embryogenesis (Thomas et al., 2000). In addition to pro-proliferative function, Che-1 exhibits a strong anti-apoptotic activity (Page et al., 1999; Guo and Xie, 2004), and this protein is downregulated during apoptosis through its interaction with MDM2 (De Nicola et al., 2007) and NRAGE (Di Certo et al., 2007). Several findings support the hypothesis that Che-1 plays an important role in protecting cells from different kind of stress. Indeed, Che-1 inhibits aberrant production of amyloid beta peptide (Guo and Xie, 2004). In addition, in response to DNA damage, Che-1 is stabilized by ATM/Chk2 kinase and localizes to the *p53* promoter, increasing transcription of this gene and contributing to the increase in p53 protein levels after genotoxic stress (Bruno et al., 2006). Moreover, Che-1 results an antiapoptotic component of Endoplasmatic Reticulum (ER) stress (Ishigaki et al., 2010). It has been recently shown that Che-1 depletion strongly decreases mutant p53 expression in human cancer cells, activates DNA damage checkpoint, and induces p73 transcription and apoptosis in these cells (Bruno et al., 2010). Thus, we asked whether Che-1 is also activated by other cellular stresses, and whether Che-1 could be involved in the regulation of mTOR pathway.

In this study we demonstrate that in addiction to DNA damage, Che-1 is phosphorylated in response to hypoxia and glucose deprivation, and following these types of stress it regulates both mTORC1 and mTORC2 activity. Indeed, in Che-1 depleted cells failed to inhibit mTOR activity and cell growth upon several stress conditions. In addition, we determined that mTOR control by Che-1 is exerted by the induction of Redd1 and Deptor, two important repressors of mTOR activity (Brugarolas et al., 2004; Corradetti et al., 2005; Peterson et al, 2009). Furthermore, we found that Che-1, upon activation, regulates cell survival and autophagy Finally, we provide evidence that Che-1 expression correlates with multiple myeloma progression, sustaining Cell growth and

survival. Hence, our study revealed a new link between cellular stress and mTOR signaling, and suggesting Che-1 to be an attractive drug target for cancer therapy.

Results

Che-1 inhibits mTOR activity.

Che-1 was found to play an important role in DNA damage response and cell cycle checkpoint control (Bruno et al., 2006). To determine whether this protein also affects mTOR activity, we evaluated the effect of transient Che-1 overexpression on the phosphorylation of the key mTOR targets S6K, 4EBP1 and S6 in HCT116 cells. Ectopic expression of myc-tagged Che-1 dramatically inhibited endogenous T389 S6K and T37/T46 4EBP1 phosphorylation (fig. S1A). Similarly, Che-1 also inhibited the phosphorylation of S235/S236 S6 ribosomal protein, the downstream target of S6K (fig. S1A).

Further supporting the physiological relevance of Che-1 in the regulation of mTOR, we measured the activity of mTOR pathway in cells with RNAi mediated reductions of Che-1 expression. HCT116 cells expressing either of two siRNA targeting Che-1 showed an increase in S6K and 4EBP1 phosphorylation, and CCI-779 treatment, a rapamycin analog, completely inhibiting the effect of Che-1 RNAi (fig. 1A). The Che-1-mediated inhibition of mTOR activity was also observed in HeLa and MEF cells (fig. S1B), indicating that the effect is not specific to HCT116 cells. Notably, Che-1 depletion strongly activated mTORC2 activity as demonstrated by S473 Akt1 and S21/9 GSK-3α/β phosphorylation (fig. 1C). Consistent with these results, cells with reduced Che-1 expression exhibited a larger size than control cells, and rapamycin treatment reversed this phenotype (figs. 1B and S1C).

Among all of the upstream regulators of mTORC1 activity, TSC is considered to be the most important regulator, as it converges multiple upstream imputs in regulating mTORC1 activity (Sengupta et al, 2010). To examine the contribution of the TSC1/TSC2 complex to the regulation of mTOR function by Che-1, the effects of Che-1 transient overexpression on *TSC2* $^{-/-}$ MEFs were analyzed. As shown in fig. 1D, Che-1 inhibited S6 phosphorylation in *TSC2* $^{+/+}$ cells, whereas it failed to do so in TSC2 deficient cells. To further confirm these results in human cells, we

overexpressed Che-1 in an angiomyolipoma cell line carrying the methylation of the *TSC2* gene promoter (*TSC2* $^{-/Meth}$) (Lesma et al., 2009). In these cells Che-1 overexpression did not affect S6 phosphorylation (fig. 1E). In contrast, when the DNA methylase inhibitor 5-azacytidine reactivated TSC2 expression, Che-1 overexpression strongly inhibited mTOR activity (fig. 1E).

Altogether, these results indicate that Che-1 is an inhibitor of mTORC1 and mTORC2 activities.

Che-1 regulates mTOR activity in response to cellular stress.

In recent years, numerous stimuli have been shown to cause changes in the activity of mTOR cascade. In addition, decreased mTOR signaling activity has been found to be associated with many types of stress, suggesting that this pathway plays an important role in the adaption to different stress conditions (Reiling and Sabatini, 2006; Sengupta et al., 2010). We have previously demonstrated that DNA damage by different genotoxic agents is associated with Che-1 phosphorylation and extended half-life. These post-translational modifications are induced by ATM and Chk2 that phosphorylate Che-1 on specific residues and are functionally linked to DNA damage-induced G_2/M checkpoint (Bruno et al., 2006). Based on these observations, we hypothesized an involvement of Che-1 in the regulation of mTOR signaling in response to stress conditions. To test this, we evaluated the activation of Che-1 in response to different kinds of cellular stress (Ionizing Radiations (I.R.), hypoxia and glucose deprivation) by employing a specific antibody directed against the phosphorylated Ser 474 of Che-1 (Bruno et al., 2006). As is shown in fig. 2A, although to a different extent, Che-1 resulted activated by all stress conditions. In agreement with these results, histone H2AX phosphorylation was also observed in response to these treatments, indicating the induction of checkpoint kinases (fig.2A). Next, we tested whether Che-1 was requested for stress-induced down-regulation of mTOR activity. For this purpose, HCT116 cells transfected with siRNA Che-1 or siRNA GFP as negative control, were subjected to different cell stresses. In contrast to control cells, Che-1 depleted cells failed to down-regulate

phosphorylation of S6K and 4EBP1 in response to hypoxia (fig.2B), and similar results were observed when cells were treated with I.R. or 2-deoxy-glucose (2DOG) (fig. 2C). These findings were further confirmed when HeLa and MEF cells were treated as above (figs. S2A, S2B, and data not shown). Consistent with these results, the overexpression of a non-phosphorylable Che-1^{S4A} mutant (Bruno et al., 2006) showed an impaired ability to inhibit S6K1 and S6 phosphorylation when compared to Che-1 wild-type (fig. S2C).

Several studies have demonstrated that the oncosuppressor p53 inhibits mTORC1 in response to DNA damage, hypoxia or glucose deprivation by multiple mechanisms (Sengupta et al., 2010), and in response to DNA damage Che-1 is required to sustain p53 expression (Bruno et al., 2006; Bruno et al., 2010). Thus, to directly test whether endogenous p53 is required for mTOR regulation by Che-1, we investigated the effect of Che-1 inhibition in HCT116 p53 $^{-/-}$ cells. As is shown in fig. 2D, even in the absence of p53 expression, these cells down-regulated S6K and S6 phosphorylation and activity in response of hypoxia. Whereas hypoxia had a much weaker effect in Che-1 depleted cells, thus indicating that Che-1 controls mTOR functions by a p53-independent mechanism.

Che-1 induces Redd1 and Deptor expression.

The data described above demonstrate that Che-1 is activated and regulates mTOR signaling in response to stress conditions. Given that Che-1 is a RNA Pol II-binding protein involved in gene transcription, to shed light on the mechanism/s by which these effects are exerted, we took advance of a high-density Affimetrix microarray analysis performed using HCT116 transiently transfected with control siRNA or Che-1 siRNA (fig. S3A). Interestingly, among the downregulated genes in Che-1-depleted cells we identified *Redd1, Redd2* and *Deptor*, important genes involved in mTOR regulation (fig. S3B) (Brugarolas et al., 2004; Corradetti et al., 2005; Peterson et al, 2009). These microarray data were confirmed by other microarrays analyses previously performed in SKB3 and MDA-MB468 human mammary carcinoma cells (Bruno et al. 2010) and by reverse transcriptase-

polymerase chain reaction (RT-PCR) (fig. 3A). Furthermore, western blot analysis showed a significant reduction of Redd1 and Deptor protein levels in Che-1 depleted cells (fig. 3B). Consistent with these results, overexpression of Che-1 strongly induced mRNA and protein levels of both Redd1 and Deptor (figs. S3C and S3D). ChIP-seq analysis performed with anti-Che-1 and anti-phospho-S5 RNA Polymerase II demonstrated the co-localization of Che-1 and RNA Pol II onto *Redd1* and *Deptor* promoters (fig. 3C). Interestingly, ChIP assays confirmed that Che-1 is physically associated with *Redd1* and *Deptor* promoters in normal proliferating conditions and showed that its levels increase in response to genotoxic and energy stresses (Fig. 3D).

To assess whether the ability of Che-1 to activate the transcription of these genes depends on its phosphorylation state, we compared the effects of wild-type Che-1 and Che-1^{S4A} mutant on Redd1 and Deptor expression. As shown in fig. 3E, in response to hypoxia wild type Che-1 protein but not the mutant Che-1^{S4A} was detectable onto *Redd1* and *Deptor* promoters, thus indicating that the phosphorylation of Che-1 is required for its presence onto the promoter of these two genes.

Che-1 inhibits mTOR activity through Redd1 and Deptor induction.

The data presented above indicate that Che-1 regulates the expression of two important negative regulators of mTOR signaling, Redd1 and Deptor, thus we hypothesized that Che-1 might promote mTOR repression through activating these two genes. Several previous studies have shown that Redd1 transcription is highly induced by several types of stress (Ellisen et al., 2002; Brugarolas et al., 2004; Shoshani et al., 2002; Sofer et al., 2005). Whereas mTOR regulates Deptor mRNA expression (Peterson et al., 2009), however little is known about regulation of Deptor by stress. Western blot analysis from HCT116 cells treated with different kinds of stress confirmed Redd1 literature data and revealed that Deptor is also regulated in a similar manner (fig. 4A). In accordance with our hypothesis, both Redd1 and Deptor mRNA levels increased in response to hypoxia but this induction was not observed in Che-1 depleted

cells (fig. 4B). Consistent with these findings, Che-1, Redd1 or Deptor depletion showed similar effects on cell size and mTOR activity in response to hypoxia (figs. S4A and S4B). Strikingly, restoration of Redd1 and Deptor protein levels counteracted mTOR activation induced by Che-1 ablation (fig. 4D). Altogether, these results indicate that in response to cellular stress, Che-1 inhibits mTOR signaling by sustaining Redd1 and Deptor expression.

Che-1 regulates autophagy through Deptor and Redd1.

mTOR signaling pathway is a critical negative regulator of autophagy and several kinds of stress such as glucose deprivation induce autophagy via inhibition of mTOR (Jung et al, 2010; Neufeld, 2010). Since recent studies reported that in response to certain kinds of stress Deptor and Redd1 induced the autophagy through suppression of mTOR activity (Molitoris et al., 2011; Gao et al., 2011; Zhao et al., 2011), we evaluated whether Che-1 was involved in autophagy induction. As shown in fig.5A, several stress conditions caused a time-dependent Deptor accumulation in HCT116 cells, as well as autophagy induction, as indicated by accumulation of LC3-II, a well-established hallmark of autophagy. Notably, in response to each treatment, Che-1 depletion strongly reduced Deptor accumulation and autophagy induction (fig. 5a). These results were confirmed when HCT116 cells were transiently transfected with a GFP-LC3 expression vector, control siRNA or Che-1 siRNA and treated with hypoxia or 2DOG. Cellular stresses strongly increased the percentage of cells with a punctate GFP-LC3 pattern in control siRNA cells, whereas Che-1 depletion significantly reduced the punctate GFP-LC3 pattern (figs. 5B and 5C). To evaluate whether the reduction of autophagy after Che-1 inhibition involves Deptor and Redd1 downregulation, we depleted Che-1 in HCT116 cells in the presence or in absence of Deptor and Redd1 overexpression and treated cells with hypoxia. As is shown in figs. 5D and 5E, overexpression of Deptor and Redd1 restored mTOR inhibition and the induction of autophagy in response to hypoxia. Altogether, these results indicate that in response to cellular stress, Che-1 regulates autophagy through the induction of Deptor and Redd1 expression.

Che-1 expression correlates with multiple myeloma progression.

The data described above indicate that Che-1 is required to inhibit mTOR activity and cell growth in response to several stresses by regulating Redd1 and Deptor expression. Recently, Sabatini's group described that Deptor is overexpressed in a subset of human multiple myelomas (MM) and this overexpression is necessary for Akt1 activation and cell survival (Peterson et al., 2009). To investigate the effect of Che-1 overexpression on Akt1 activity, HCT116 cells were transfected with myc-Che-1 expression vector. As is shown in fig S5A, overexpression of Che-1 led to an increase of mTORC2 signaling as monitored by Akt1 S473 and S308 and by and S21/9 GSK-3α/β phosphorylation. Interestingly, in agreement with the previous results, the activation of Akt1 was not observed in cells transfected with Che-1^{S4A} mutant (fig. S5B). These results prompted us to evaluate Che-1 expression in human MM cells. Oncomine database analyses suggested that Che-1 mRNA expression correlates with MM progression (figs. 6A and 6B) (Agnelli et al., 2009; Zhan et al., 2007). To further confirm these data, we analyzed Che-1 and Deptor expression in 49 human MM samples. These analyses revealed an almost complete positive correlation between Che-1 and Deptor expression in MM, in line with the indication that Che-1 regulates Deptor transcription (figs. 6C and 6D). Remarkably, high levels of Che-1 and Deptor were mainly observed in symptomatic myelomas together with a strong activation of autophagy, whereas the expression of these two genes was barely undetectable in all monoclonal gammopathies of undetermined clinical significance (MGUS) and in the majority of smoldering myelomas analyzed (figs. 6C and 6D). More importantly, the patients follow up performed until now revealed that three smoldering myelomas expressing high levels of Che-1, Deptor and autophagy (MM8, MM19 and MM33) (fig.6E) progressed to symptomatic myeloma six months later. Altogether, these findings strongly suggest that Che-1 might play a critical role in the in the progression of human MM by sustaining autophagy.

Che-1 sustains survival in multiple myeloma.

Next, we evaluated different MM cell lines and found that lines with high levels of Deptor (Kms18, Kms27 and RPMI-8226) showed high levels of Che-1 expression and of its phosphorylated form (fig. 7A). In agreement with these results, ChIP experiments demonstrated the presence of Che-1 onto Deptor promoter in Kms27 cells but not in U266 cells (fig. S5C). We therefore investigated whether Che-1 controls autophagy and survival in MM cells. Inactivation of Che-1 or Deptor in Kms18 and Kms27 cells produced similar effects, increasing mTORC1 activity as demonstrated by S6 phosphorylation and an inactivation of mTORC2 activity measured by Akt1 phosphorylation (fig. 7B). Accordingly, Che-1 depletion in these cells increased cell size (fig. S5D) and induced apoptosis (figs. 7B, 7D and S5E). Notably, overexpression of Deptor in Che-1 depleted Kms27 cells reversed mTORC1 activation, mTORC2 inhibition, cell growth and apoptosis (figs. 7C, 7D, S5D and S5E). Finally, we evaluated whether Che-1 is involved in the autophagy observed in MM cells. To this aim Kms27 cells were transfected with Che-1 or Deptor siRNa oligos. As is shown in fig. 7D, both Che-1 and Deptor depletion strongly reduced LC3-II levels in these cells. Consistently, the rescue of Deptor expression in Che-1 depleted cells restored high levels of LC3-II (fig. 7E). Together, these data strongly indicate a critical role of Che-1 in MM survival.

Discussion

The mTOR signaling pathway plays a central role in cell growth and survival control (Laplante and Sabatini, 2012). Therefore, it is not surprising that several stress conditions negatively regulate mTOR activity, thereby allowing cells to survive under non-optimal conditions (Sengupta et al. 2010). In particular, it has been demonstrated that the tumor suppressor p53 upon several kind of stresses can repress mTOR by multiple mechanisms (Ellisen et al., 2002; Feng et al., 2005; Budanov and Karin, 2008; Stambolic et al., 2011). We have previously reported that in response to DNA damage, Che-1 is stabilized and by promoting *p53* gene transcription, contributing to the maintenance of the G2/M checkpoint (Bruno et al., 2006).

In the present study, we provide evidence that Che-1 negatively controls mTOR activity and this function requires TSC2 but not p53 expression. We show that Che-1 is activated by several kinds of stress and inhibits mTOR pathway by inducing *Redd1* and *Deptor* expression, two important inhibitors of mTOR. Furthermore, inhibition of Che-1 strongly decreases autophagy induction leading to apoptosis. We further report overexpression of Che-1 during MM progression and a positive correlation between Che-1 and Deptor expression in several human symptomatic myelomas, and we provide evidence that Che-1 sustains cell growth and survival in MM cells.

Our results report that not only DNA damage but also other kinds of stress lead to Che-1 phosphorylation and activation (fig.2A). Interestingly, we found histone H2AX phosphorylation in response to hypoxia and 2DOG treatments (fig. 2A), thus suggesting that these kinds of stress can activate DNA damage response. Several studies have demonstrated that hypoxia can activate ATM and Chk2 kinase activity (Cam et al., 2010; Gibson et al., 2005), whereas the effects of glucose deprivation on DNA integrity have not yet been clarified. Nevertheless, we can speculate that metabolic alterations generated by 2DOG treatment might produce oxidative DNA damage and activate checkpoint kinases.

We show that Che-1 sustains *Redd1* and *Deptor* gene expression (figs. 3A, 3B, S3A, S3C and S3D). We found that Che-1 is recruited onto their promoters together with RNA polymerase II (fig.3C) and that this recruitment is mediated by phosphorylation and increases in response to stress (figs. 3D and 3E). Strikingly, overexpression of Redd1 and Deptor in Che-1 depleted cells completely restored mTOR inhibition (figs. 4C and 4D).

As described above Redd1 and Deptor are two important inhibitors of mTOR activity (Brugarolas et al., 2004; Corradetti et al., 2005; Peterson et al, 2009). Redd1 functions in a TSC2-dependent manner to regulate mTORC1 activity (Brugarolas et al., 2004), whereas Deptor directly binds mTOR kinase and inhibits mTORC1 and mTORC2 activities (Peterson et al., 2009). Interestingly, although Deptor depletion strongly activates both mTORC1 and mTORC2 signaling, elevated expression of Deptor unexpectedly relieves mTORC1-mediated inhibition of PI3K and activates Akt1 (Peterson et al., 2009). The stability of Redd1 and Deptor are controlled by the $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase (Gao et al., 2011; Zhao et al., 2011; Duan et al., 2011; Katiyar et al., 2009). Furthermore, it has been described that mTOR generates a regulatory loop by controlling Deptor protein and mRNA expression (Peterson et al., 2009; Duan et al., 2011), thus highlighting the critical function of these proteins in mTOR pathway. Interestingly, a recent analysis of the mTOR-dependent phosphoproteome has identified the murine hortolog of Che-1 as a potential mTOR effector (Yu et al., 2011). Consistent with these findings, we found a direct interaction between Che-1 and mTOR (not shown), thus it is possible to speculate that Che-1 like Deptor might be a mTOR target and participate in the mTOR auto-amplification loop recently described (Duan et al., 2011). Che-1 inhibits mTORC1 in a TSC2-dependent manner (figs. 1D and 1E). While this finding is in agreement with Redd1 induction by Che-1, it is not easily reconcilable with Deptor activity, even if the mechanism/s by which Deptor regulates mTOR activity has not yet been completely described.

Our findings demonstrate that Che-1 inhibition decreases autophagic activation in response to cellular stress (figs. 5A and 5B), and this phenomenon is lost when Redd1 and Deptor expressions are restored (fig. 5D) This indicates that Che-1 plays a role in the autophagy induction, in part through regulating the activity of mTOR signaling, a central regulator of this pathway (Jung et al., 2010; Neufeld, 2010). Autophagy is a tightly regulated pathway involving the massive degradation of cellular organelles or cytosolic components (Kroemer et al., 2010). This pathway constitutes a major mechanism that allows cells to survive in response to several stressors and many molecular events support a mutual exclusion between autophagy and apoptosis (Kroemer et al., 2010). In agreement with this concept, our results suggest Che-1 as an important regulator of the balance between autophagy and apoptosis in response to cellular stress, promoting growth arrest and survival and inhibiting cell death.

We further report overexpression of Che-1 during MM progression (Agnelli et al., 2009; Zhan et al., 2007). This finding is intriguing, as Che-1 has been described to play a relevant role in protecting cells in ER stress/ unfolded protein response (UPR) (Ishigaki et al., 2010), which are able to render MM cells resistant to proapoptotic stresses (Shapiro-Shelef and Calame, 2004). Therefore, the identification of a new important mechanism that favors MM-specific survival might be critical in gaining further insights into the disease. Our results show that Che-1 and Deptor are poorly expressed in most monoclonal gammopathies and smoldering myelomas, whereas in about 65% of symptomatic diseases we found high levels of these proteins. Moreover, preliminary results showed that some smoldering myelomas expressing high levels of Che-1 and Deptor progressed to symptomatic disease six months later. Therefore, it is tempting to speculate that high levels of Che-1 and Deptor might be considered useful markers to monitor smoldering evolution. Further evaluations in a larger set of patients may possibly confirm or not this hypothesis.

We found a strong increase of autophagy induction in symptomatic myelomas (figs. 7C, 7D and 7E). This result is in complete agreement with the evidence that ER stress triggers

autophagy to sustain cell viability, expecially in cancer cells (Suh et al., 2012; Yorimitsu et al., 2006). In addition, a recent study has demonstrated that autophagy is specifically required for plasma cell homeostasis by limiting ER expansion (Pengo et al., 2012). Therefore, it is possible to hypothesize a scenario in which increasing levels of ER stress/UPR during MM progression induce Che-1 and Deptor expression to inhibits mTORC1 activity, thereby activating autophagy and promoting cell survival by Akt1 activation.

In conclusion, these findings highlight the important role that Che-1 has in response to many stress conditions, regulating processes such as cell growth, autophagy and apoptosis, and further strengthens the notion that Che-1 could be considered a valid target for novel therapeutic approaches.

EXPERIMENTAL PROCEDURES

Cell lines, constructs and transfections

HCT116 wild type and *p53*-/-, Hela, MEF *TSC2*+/+ *p53-/-* and MEF *TSC2*-/- *p53-/-* cells (kindly provided from Dr. D. Kwitakowski, Harvard Medical School) were cultured in DMEM supplemented with 10% FBS Angiomiolipoma cells TSC2-/meth ASM (9C) were cultered in DMEM/F12 supplemented with 15% FBS, EGF 10ng/ml and Hydrocortisone 0,1nM (Lesma , et al 2009). U266, KMS18, KMS27, RPMI-8826, ARH77, JJN3, MOLP8 multiple myeloma cell lines, were cultered in RPMI1640 supplemented with 10% FBS. For hypoxia exposure, culture dishes were placed in a hypoxia chamber (Fisher Scientific) allowing the formation of a hypoxic environment of 5% CO2, 95% N2. Unless stated otherwise, these hypoxic levels (1% of oxygen concentration, 24 h) was used in all experiment. Transfections were carried out by Lipofectamine 2000 (Life Technologies) following the manufacturer's instructions. The following plasmids were used in transfection experiments: pRK5 human Flag-Deptor (Addgene ID, 21334), Myc-tagged Che-1 and its mutant Myc-Che-1^{S4A} were already described (Fanciulli et al., 2006). Myc-tagged Redd1 was generated by cloning Redd1 cDNA into the pCS-MT vector. GFP-LC3 expression vector was kindly provided from Dr. Hovard (Martens et al., 2005). The mTOR inhibitor CCI-779 was purchased from Chemocare, whereas 2DOG was purchased from Sigma.

Cell extracts and Western blot analysis

Cells were rinsed three times with ice-cold PBS, harvested, centrifuged at 4°C and cell pellets lysed by incubation at 0°C for 30 minutes in 30 μl lysis buffer (50 mM Tris, pH 8.0, 250 mM NaCl, 0,5 mM EDTA, 50 mM NaF, 1mM $NaOV_4$, 1mM PMSF, 10 mg/ml leupeptin) supplemented with 0,5 % NP40. Solubilized proteins (25 μg) were resolved on Mops NuPAGE precast 4%–12% gels (Life Technologies). Western blotting analyses were performed using the following rabbit polyclonal antibodies: anti-Che-1 (Fanciulli et al., 2010), PARP-1 p85 fragment

(Promega), anti- Deptor (Upstate/Millipore); Anti-Redd1 (Proteintechgroup); antibodies anti- S6K1 phospho-T389, 4E-BP1 phospho-T37/T46, Akt1/ phospho-S473, Akt1 phospho-T308, GSK α/β phospho-S21/9, TSC2, 4E-BP1, Akt1, S6K, Cleaved Caspase-7 and LC3 from Cell Signaling Technology; mouse monoclonal antibodies anti- Myc 9e10 and β actin from Life Techonologies, Flag M2 (1804, Sigma) and g-H2AX phospho-S139, clone JBW301 (Merck-Millipore) were also used. Secondary antibodies used were goat anti-mouse and goat anti-rabbit, conjugated to horseradish peroxidase (Biorad). Immunostained bands were detected by the chemiluminescent method (Amersham).

Immunofluorescence assays

Immunofluorescence analyses of anti-Che1, anti-Deptor or anti-LC3 on cd138+ positive cell were performed using cytospin preparations of cells mounted on glass slides using a Thermo Shandon cytospin 2 (Thermo Fisher Scientific, Waltham, MA, USA), whereas transfected HCT116 cells were subjected to immunofluorescence assays 48 hours following transfection. Briefly, cells were fixed in 4% formaldehyde for 15 minutes and then permeabilised with 0.1% Triton X100 in phosphate-buffered saline (PBS) for 5 minutes. Primary antibodies were used for immunostaining, followed by Alexa-Fluor-594-conjugated and Alexa-Fluor-488-conjugated anti-rabbit IgG (Life Technologies). Nuclei were visualized by staining with 1 μg/ml Hoechst dye 33258 (Sigma). As control, the primary antibodies were omitted (data not shown). Immunofluorescence analysis was performed using the microscope Axioskop 2 plus and fluorescence signals were analysed by recording images using a CCD camera (Zeiss, Oberkochen, Germany).

siRNA

The 22-nucleotide siRNA duplexes corresponded to nucleotides 1062–1083 and 1473–1492 of human Che-1 sequence, and to nucleotides 122–143 of the negative control green fluorescent protein (GFP). Sequence were in vitro synthesized by Silencer siRNA construction kit (Ambion)

following manufacturer's instruction. RNA interference was performed as previously described (Bruno et al., 2002). siRNA-mediated interference experiments of Deptor and Redd1 expression were performed by transfecting specific pool of double-stranded RNA oligonucleotides (Stealth, Life Technologies) using Lipofectamine 2000 (Life Technologies).

Chromatin Immunoprecipitation assays (ChIP)

Chromatin immunoprecipitation assays in HCT116, KMS18 and U266 cells were performed as previously described (Bruno et al., 2002) by using the rabbit polyclonal antibody anti-Che-1. In each experiment, signal linearity was ensured by amplifying increasing amounts of template DNA. Generally, DNA representing from 0.005% to 0.01% of the total chromatin sample (input) or from 1% to 10% of the immunoprecipitated was amplified using promoter-specific primers. Immunoprecipitations with no specific immunoglobulins (Santa Cruz) were performed as negative control.

ChIP-Sequencing analysis

See the Supplemental Experimental Procedures.

RNA Isolation and RT-PCR Analysis

Cells were harvested 36 hr after transfection and total RNA isolated using TRIZOL reagent (Life Technologies) in accordance with the manufacturer's instructions, and the first-strand cDNA was synthesized with the Thermo Script RT-PCR kit (Life Technologies).

For semiquantitative RT-PCR analysis, RT-PCR was performed with a Platinum quantitative RT-PCR kit (Life Technologies) in accordance with the manufacturer's instructions. PCR products were separated onto 1.5% agarose gel.

Patients material

MGUS and MM patient samples were collected as part of routine clinical examination. Written informed consent to participate in this study was provided by all subjects in accordance with the Declaration of Helsinki and the Mayo Clinic Institutional Review Board. MGUS and MM patients were distinct according to the international working group classification based on the level/concentration of serum M-protein and percentage of bone marrow plasma cells. The distinction between symptomatic and asymptomatic myeloma depends on the presence or absence of myeloma-related organ or tissue impairment (The International Myeloma Working Group, 2003). MGUS patients had <10% BM clonal PCs and < 3 g/dl of serum M-protein while patients diagnosed with MM had 10% or greater clonal BM-PCs and/or > 3 g/dl of serum M-protein. Bone Marrow aspirates were enriched for plasma cells by magnetic cell separation using a human CD138 positive selection kit (Miltenyi-Biotec - Germany) and Macs Separator (Macs Miltenyi Biotec - Germany).

Accession number

All microarray raw data tables have been deposited at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE20622 (submitter M. Fanciulli).

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Legends to figures

Figure 1: Che-1 inhibits mTOR activity. A: Western blot (WB) analysis of total cell extracts (TCEs) from HCT116 cells transiently transfected with siRNA GFP (siControl) or two different siRNA Che-1 (siChe-1-1 and siChe-1-2) and treated where indicated with the mTOR inhibitor CCI-779. **B**: Cell size analysis of HCT116 cells transiently transfected as in **A** and treated where indicated with CCI-779. Seventy-two hours after transfection, cell size was measured with a Coulter counter. The data represent the mean ± SD from three independent experiments performed in duplicate. **C**: WB analysis performed with the indicated antibodies (Abs) of HCT116 cells tranfected with siRNA GFP (siControl) or siRNA Che-1 (siChe-1). **D**: WB with the indicated Abs of TCEs from TSC2 +/+ and TSC2-/- cells transiently transfected with Myc-Che-1 or pCS-MT empty vector. **E:** WB with the indicated Abs of TCEs from ASM cells transiently transfected as in D and treated where indicated with 5-Azacytidine (5-Aza).

Figure 2: Che-1 regulates mTOR activity in response to cellular stress. A: WB analysis with the indicated Abs of TCEs from HCT116 treated with I. R. (10Gy) (left), hypoxia (center) or 25 mM 2DOG (right). **B** and **C**: WB with the indicated Abs of TCEs from HCT116 cells with siRNA GFP (siControl) or siRNA Che-1 (siChe-1)and treated with hypoxia (**B**), I.R. (**C**, left) or 2DOG (**C**, Rigth). **D**: WB analysis of TCEs from HCT116 p53-/- cells treated as in **B**.

Figure 3: Che-1 induces Redd1 and Deptor expression. A: Equal amounts of RNA (Total RNA inpu) from from HCT116 cells transiently transfected with siRNA GFP (siControl) or siRNA Che-1 (siChe-1) were analyzed by RT-PCR (25-30 cycles) for the expression of the indicated genes. The negative control lanes represent RT-PCR in the absence of cDNA. **B**: WB analysis with the indicated Abs of TCEs from HCT116 cells transiently transfected as in **A**. **C:** a representative

snapshot showing Che-1 and phospho-S5 RNA Polymerase II recruitment to *Redd1* (upper panel) and *Deptor* (lower panel) promoters. **D:** total cell extracts (TCEs) from HCT116 cells were subjected to Chromatin immunoprecipitation (ChIP) using anti-Che-1 antibody. Immunoprecipitates from each sample were analyzed by PCR and a sample representing linear amplification (0.2-0.4μl) of the total input chromatin (Input) was included in the PCRs as a control. Additional control included a precipitation performed with nonspecific IgGs. **E**: HCT116 cells were transiently transfected with Myc-Che-1 Wild Type or Myc-Che-1^{S4A}. Then, cells were subjected to ChIP using anti-Myc Ab or no IgGs.

Figure 4: Che-1 inhibits mTOR activity through Redd1 ad Deptor induction. A: WB analysis with the indicated Abs of TCEs from HCT116 treated with I. R. (10Gy) (left), hypoxia (center) or 25 mM 2DOG (right). **B:** RT-PCR analysis from from HCT116 cells transiently transfected with siRNA GFP (siControl) or siRNA Che-1 (siChe-1) and exposed to hypoxia for the indicated times. **C:** Cell size analysis of HCT116 cells transiently transfected with siRNA GFP (siControl), siRNA Che-1 (siChe-1) and where indicated Flag-Deptor and Myc-Redd1 expression vectors. Seventy-two hours after transfection, cell size was measured with a Coulter counter. The data represent the mean ± SD from three independent experiments performed in duplicate. **D:** WB analysis with the indicated Abs of TCEs from HCT116 cells transiently transfected as in **C** and exposed to hypoxia for 16 hours.

Figure 5: Che-1 regulates autophagy through Deptor and Redd1. A: WB analysis with the indicated Abs of TCEs from HCT116 transiently transfected with siRNA GFP (siControl) or siRNA Che-1 (siChe-1) and treated with I. R. (10Gy) (left), hypoxia (center) or 25 mM 2DOG (Right). **B:** HCT116 cells transiently transfected with siRNA GFP (siControl) or siRNA Che-1 (siChe-1) and GFP-LC3 expression vector were treated where indicated with hypoxia or 2DOG.

Cells were fixed with 4% formaldehyde and analyzed by fluorescence microscopy. Scale bar 10 μm. **C:** HCT116 cells were transiently transfected and treated as in B. Twenty-four hours later cells were fixed and GFP-LC3 distribution was assessed by fluorescence microscopy and percentages represent punctate-LC3 expressing cells. The data represent the mean ± SD from three independent experiments performed in triplicate. **D:** WB analysis with the indicated Abs of TCEs from HCT116 cells transiently transfected with siRNA GFP (siControl), siRNA Che-1 (siChe-1) and where indicated expression vectors. **E:** HCT116 cells transiently transfected with siRNA GFP (siControl) or siRNA Che-1 (siChe-1) and GFP-LC3, Flag-Deptor and Myc-Redd1 expression vectors were treated where indicated with hypoxia. Cells were fixed with 4% formaldehyde and analyzed by fluorescence microscopy. Scale bar 10 μm.

Figure 6: Che-1 expression correlates with multiple myeloma progression. A and **B:** All data was provided by the Oncomine database. The associated p values are shown for each study. A: Data from Agnelli et al. (2009) reanalyzed to show expression levels of Che-1 in normal bone marrow, monoclonal gammopathy of undetermined significance (MGUS), MM and plasma cell leukemia (n=158). **B:** Data from Zhan et al. (2007) reanalyzed to show expression levels of Che-1 in MGUS and smoldering myelomas (n=56). Both box-and-whisker plots show the upper and lower quartiles (25-75%) with a line at the median, whiskers extend from the 10^{th} to the 90^{th} percentile, and dots correspond to the minimal and maximal values. **C:** Table illustrating positive Che-1 and Deptor protein expression in plasma cells isolated from the bone marrow of 49 MM patient samples using CD138 magnetic beads. **D:** Representative WB with the indicated Abs of TCEs from CD138 purified MM primary tumors. **E:** Plasma cells purified from primary smoldering myelomas were fixed with 4% formaldehyde and analyzed by fluorescence microscopy with the indicated Abs. Nuclei were visualised by staining with Hoechst dye. Scale bar 10 μm.

Figure 7: Che-1 sustains survival in multiple myeloma. A: WB analysis with the indicated Abs of TCEs from the indicated human MM cell lines. B: Kms18 and Kms27 cells were transiently transfected with siRNA GFP (siControl), siRNA Che-1 (siChe-1) or siRNA Deptor (siDeptor). **C, D and E:** Kms27 cells were transiently transiently transfected with siRNA GFP (siControl), siRNA Che-1 (siChe-1) and where indicated Flag-Deptor expression vector. WB analysis of TCEs from these cells was performed with the indicated Abs. (**C** and **E**). Cell death was assayed by trypan blue staining twenty-hours after the transfection, and percentages represent trypan blue incorporating cells. Data are presented as the mean ± SD from three independent experiments performed in duplicate (**D**).

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