mTOR under stress
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Chapter 3

Polo like kinase 1 inhibits mTOR complex 1 and promotes autophagy

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Abstract

Mammalian/ mechanistic target of rapamycin complex 1 (mTORC1) and polo like kinase 1 (PLK1) are major drivers of cancer cell growth and proliferation, and inhibitors of both protein kinases are currently being investigated in clinical studies. To date, mTORC1’s and PLK1’s functions are mostly studied separately, and reports on their mutual crosstalk are scarce. Here, we identify PLK1 as a physical mTORC1 interactor in human cancer cells. PLK1 inhibition enhances mTORC1 activity under nutrient sufficiency and in starved cells, and PLK1 directly phosphorylates the mTORC1 component raptor \textit{in vitro}. PLK1 and mTORC1 reside together at lysosomes, the subcellular site where mTORC1 is active. Consistent with an inhibitory role of PLK1 toward mTORC1, PLK1 overexpression inhibits lysosomal association of the PLK1-mTORC1 complex, whereas PLK1 inhibition promotes lysosomal localization of mTOR. PLK1-mTORC1 binding is enhanced by amino-acid starvation, a condition known to increase autophagy. mTORC1 inhibition is an important step in autophagy activation. Consistently, PLK1 inhibition mitigates autophagy in cancer cells both under nutrient starvation and sufficiency, and a role of PLK-1 in autophagy is also observed in the invertebrate model organism \textit{Caenorhabditis elegans}. In summary, PLK1 inhibits mTORC1 and thereby positively contributes to autophagy. Since autophagy is increasingly recognized to contribute to tumor cell survival and growth, we propose that cautious monitoring of mTORC1 and autophagy readouts in clinical trials with PLK1 inhibitors is needed to develop strategies for optimized (combinatorial) cancer therapies targeting mTORC1, PLK1, and autophagy.
Introduction

PLK1 (polo like kinase 1) is a ubiquitously expressed serine/threonine protein kinase, which is widely recognized as an oncogene that drives cellular proliferation by promoting mitosis and cytokinesis (Archambault et al., 2015; Strebhardt et al., 2015; Zitouni et al., 2014). The five polo like kinase (PLK) family members PLK1-5 all contain a polo-box domain that regulates their kinase activity and subcellular localization (Archambault et al., 2015; Strebhardt et al., 2015; Zitouni et al., 2014). PLK1 is the best described PLK protein, and is frequently used as a tumor marker, as high PLK1 expression correlates with poor prognosis in cancer (Craig et al., 2014). PLK1 inhibitors, such as BI2536, compete with adenosine triphosphate (ATP) for its binding to the catalytic domain of PLK1 (Steegmaier et al., 2007). Long-term PLK1 inhibition arrests cells in prometaphase, and thus PLK1 inhibitors are investigated as anti-mitotic agents for cancer treatment (Degenhardt and Lampkin, 2010; Lens et al., 2010; Strebhardt et al., 2015).

mTOR (mechanistic/mammalian target of rapamycin) is another serine/threonine protein kinase that promotes cellular growth and is also often targeted in cancer therapy (Cargnello et al., 2015; Chiarini et al., 2015). Although both PLK1 (Archambault et al., 2015; Zitouni et al., 2014) and mTOR (Shimobayashi and Hall, 2014) are conserved in invertebrates and mammals, only little is known about their crosstalk and mutual regulation of common downstream processes, as well as the implications thereof for cancer therapies.

The nutrient sensor mTOR is activated by metabolic stimuli, including amino acids, growth factors (e.g., insulin), and energy sufficiency (Bar-Peled and Sabatini, 2014; Laplante and Sabatini, 2012; Shimobayashi and Hall, 2014). mTOR acts in two structurally and functionally distinct multiprotein complexes, mTOR complex 1 (mTORC1) and mTORC2 (Laplante and Sabatini, 2012; Shimobayashi and Hall, 2014). Raptor (regulatory associated protein of mTOR complex 1) is a core component of mTORC1 (Laplante and Sabatini, 2012; Shimobayashi and Hall, 2014), which is a central controller of cellular growth and survival. Consistently, mTORC1 is dysregulated in many cancer types (Cargnello et al., 2015), and several compounds for pharmacological mTORC1 inhibition are investigated as cancer therapeutics (Cargnello et al., 2015; Chiarini et al., 2015). The mTORC1-specific allosteric inhibitor rapamycin and its analogues (rapalogs) are already approved for the treatment of several tumor entities (Chiarini et al., 2015). The more recently developed ATP-analogue mTOR inhibitors, such as Torin1 (Thoreen et al., 2009) and its derivatives, are currently tested in clinical
studies (Chiarini et al., 2015). They target both mTOR complexes, and also inhibit mTORC1 functions which are insensitive to rapamycin (Thoreen et al., 2009). Amino-acids and growth factor induced signaling pathways converge at the lysosomes to synergistically activate mTORC1 (Bar-Peled and Sabatini, 2014). mTORC1 activation by amino acids requires Rag GTPase-mediated mTORC1 translocation to lysosomes (Bar-Peled and Sabatini, 2014; Sancak et al., 2010; Sancak et al., 2008). Conversely, loss of lysosomal mTORC1 association mediates mTORC1 inhibition upon amino-acid withdrawal (Demetriades et al., 2014). At the lysosome, mTORC1 encounters the small GTPase Ras homolog enriched in brain (rheb) (Bar-Peled and Sabatini, 2014; Betz and Hall, 2013), which activates mTORC1 downstream of the insulin receptor - phosphatidylinositol 3-kinase (PI3K) - AKT signaling axis (Bar-Peled and Sabatini, 2014; Laplante and Sabatini, 2013; Shimobayashi and Hall, 2014). Rheb is inhibited by TSC1-TSC2 (tuberous sclerosis 1 and 2) complex, which acts as a GTPase-activating protein (GAP) on rheb. mTORC1 phosphorylates a number of substrates (Heberle et al., 2015) that mediate its anabolic outcomes. Among them is p70-S6K (ribosomal protein S6 kinase B 70 kDa) which is phosphorylated at threonine 389 (pT389) by mTORC1 (Howell et al., 2013; Laplante and Sabatini, 2013; Shimobayashi and Hall, 2014). In turn, p70-S6K activates protein synthesis by promoting expression of ribosomal components (Chauvin et al., 2014), and by phosphorylating translation initiation factors and components of the ribosomal machinery, including S6 (ribosomal protein S6) (Heberle et al., 2015). Only little is known about PLK1’s role in the mTORC1 pathway. Even though several studies correlate PLK1 inhibition with either decreased (Astrinidis et al., 2006; Li et al., 2014; Renner et al., 2010; Zhang et al., 2014) or increased (Spartà et al., 2014) p70-S6K or S6 phosphorylation, a clear functional interaction between PLK1 and mTORC1 has so far not been reported. Thus, it is unknown whether PLK1 regulates phosphorylation of mTORC1 substrates indirectly or directly, i.e., by physically acting on mTORC1. mTORC1 promotes cellular growth by inducing anabolic processes including protein synthesis, and by inhibiting catabolic processes (Heberle et al., 2015; Shimobayashi and Hall, 2014). Conversely, mTORC1 inhibition de-represses catabolic processes to promote cellular survival, e.g., when nutrients are scarce (Shimobayashi and Hall, 2014). The best described catabolic process inhibited by mTORC1 is autophagy, and this mTORC1 function is conserved from yeast and invertebrates such as Caenorhabditis elegans (Hansen et al., 2005) (C. elegans) to mammals (Feng et al., 2015). Autophagy is tightly balanced to maintain cellular homeostasis and fuel cells with nutrients and metabolite
intermediates under nutrient sufficiency and deprivation (Kaur and Debnath, 2015) via degradation of proteins, lipids, and organelles in the lysosomes (Feng et al., 2014; Feng et al., 2015). Macroautophagy (from here on referred to as autophagy) is to date the best characterized type of autophagy (Feng et al., 2014). During autophagy, double-membrane vesicles called autophagosomes are formed which fuse with late endosomes or lysosomes to form autolysosomes, in which the degradation of the sequestered material takes place (Feng et al., 2014; Yang and Klionsky, 2010). In the context of cancer, autophagy gains growing attention as autophagy contributes to the elimination of tumor cells, but also promotes tumor survival (Duffy et al., 2015; Kim and Guan, 2015; Rebecca and Amaravadi, 2015). Consequently, both autophagy inhibitors, such as chloroquine (Klionsky et al., 2012), and autophagy activators, e.g., proteasome and mTORC1 inhibitors (Duffy et al., 2015; Kim and Guan, 2015), are currently investigated in clinical trials. Of note, ATP analogue mTOR inhibitors such as Torin1 enhance autophagy more effectively than rapalogs, as ATP analogues block autophagy-inhibiting mTORC1 functions that are rapamycin resistant (Thoreen et al., 2009). Autophagy is also regulated by multiple mTORC1-independent cues (Feng et al., 2015). For example, during mitosis autophagy is inhibited in an mTORC1-independent manner (Eskelinen et al., 2002; Furuya et al., 2010). Links of PLK1 with autophagy are poorly explored. PLK1 is known to localize to centrosomes, kinetochores, and the mitotic spindle (Archambault et al., 2015), and PLK1 expression is increased during mitosis (Golsteyn et al., 1994). During this cell cycle phase PLK1 has been suggested to contribute to autophagy inhibition (Deeraksa et al., 2013; Valianou et al., 2015). As PLK1 research mostly focuses on mitotic cells, it is unknown whether PLK1 affects autophagy in interphase cells and which signaling networks might mediate such effects. Such knowledge would broaden the range of application of PLK1 inhibitors specifically to tumors that display low mitotic rates (Inwald et al., 2013), and/or require autophagy for cellular growth and survival (Palm et al., 2015). It would also reveal potential effects of PLK1 inhibitors on mTOR and autophagy networks that may be relevant for therapy outcome. Therefore, we analyzed in the present study whether and what type of crosstalk exists between PLK1, mTORC1, and autophagy in non-mitotic cancer cells.

We describe here a novel non-mitotic function of PLK1. We identify PLK1 as a physical interactor of mTORC1, whose scaffold component raptor is a direct PLK1 substrate in vitro. We find that PLK1 inhibition leads to hyperphosphorylation of the mTORC1 substrate p70-S6K. PLK1 resides with mTORC1
at lysosomes, a localization hitherto unknown for PLK1; and the PLK1-mTORC1 complex co-localizes with and physically binds LAMP2 (lysosomal associated membrane protein 2). Consistent with an inhibitory function of PLK1 toward mTORC1, overexpression of active PLK1 detaches the PLK1-mTORC1 complex from the lysosomes, and PLK1 inhibition increases mTOR localization at lysosomes. In keeping with this, PLK1 inhibition mitigates autophagy in both the invertebrate model organism C. elegans, and in mammalian cells, where autophagy is regulated in an mTORC1-dependent manner. In conclusion, PLK1 positively contributes to autophagy via inhibition of mTORC1 under nutrient sufficiency and starvation. Our findings highlight the importance of carefully monitoring PLK1-, mTOR-, and autophagy- activities in clinical studies, to identify leads for cancer therapy design.

Results

PLK1 physically interacts with mTOR and raptor

We have recently analyzed the mTOR interactome by quantitative proteomics (Schwarz et al., 2015). In this study (Schwarz et al., 2015), we purified endogenous mTOR kinase by immunoprecipitation (IP) from the cervical cancer cell line HeLa, and analyzed mTOR IPs versus mock IPs, conducted with an unspecific control IgG. We reanalyzed those data here, and found that PLK1 was specifically identified by tandem mass spectrometry in mTOR IPs for two out of three biological replicates ((Schwarz et al., 2015), Table S4) with six peptides and a sequence coverage of 11 % (Supplementary Fig. 1A). Annotated MS1 and fragment spectra for one of the PLK1 peptides are shown in Supplementary Fig. 1B,C. Physical interaction of PLK1 with mTOR has not been reported previously. To validate this finding, we performed PLK1 and mock IPs and analyzed them by immunoblotting (Fig. 1A, Supplementary Fig. 1D). TSC2 and the mTORC2 component rictor (raptor-independent companion of mTOR complex 2) were specifically detected in PLK1 IPs, serving as positive controls, as interaction of TSC2 and rictor with PLK1 has been shown earlier (Astrinidis et al., 2006; Shao and Liu, 2015). Of note, we also specifically detected mTOR and the mTORC1 component raptor in the PLK1 IP, but not in the mock IP (Fig. 1A). To test if raptor is required for PLK1-mTORC1 binding, we immunoprecipitated PLK1 from lysates of stably transduced HeLa cells with doxycycline-inducible expression constructs for short hairpin RNAs targeting raptor (shRaptor) (Dalle Pezze et al.,
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2012), or harboring a non-targeting sequence (shControl). PLK1 bound mTOR to the same extent in shRaptor or shControl knockdown cells (Supplementary Fig. 1E,F), suggesting that PLK1 physically binds mTORC1 via mTOR.

PLK1 inhibits mTORC1 in non-mitotic cells

Next, we investigated whether PLK1 influences mTORC1 activity. We tested this first upon mTORC1 activation with amino acids and insulin. To inhibit PLK1, we treated HeLa cells for 30 minutes with the ATP-competitive PLK1 inhibitor BI2536 (Steegmaier et al., 2007). We combined the PLK1 inhibitor treatment with amino-acid/insulin stimulation, and analyzed phosphorylation of p70-S6K-T389 as a bona fide readout for mTORC1 activity. As expected, immunoblotting showed that amino-acid/insulin stimulation increased p70-S6K-T389 phosphorylation, consistent with mTORC1 activation (Fig. 1B, first versus third lane). Treatment with the PLK1 inhibitor BI2536 further enhanced p70-S6K-T389 phosphorylation significantly (Fig. 1B, third versus fourth lane; Fig. 1C). Thus, PLK1 inhibition leads to p70-S6K-T389 hyper-phosphorylation upon stimulation with amino acids and insulin, suggesting that PLK1 inhibits mTORC1.

To confirm this result by another mode of PLK1 inhibition and to control for possible off-target effects of the PLK1 inhibitor BI2536, we next inhibited PLK1 by RNA interference (RNAi). To this end, we stably transduced HeLa cells with doxycycline-inducible expression constructs for shRNAs targeting PLK1 (shPLK1), or a non-targeting sequence (shControl). Knockdown was induced by doxycycline treatment for two days. Surprisingly, we observed no change in p70-S6K-T389 phosphorylation in shPLK1 as compared to shControl cells (Fig. 1D,E). This seemed contradictory to the increase in p70-S6K-T389 phosphorylation that we observed upon BI2536 treatment (Fig. 1B,C).

A main difference between BI2536- versus shPLK1-treated cells was that the treatment with the inhibitor was done for a brief interval (i.e., 30 minutes), whereas shPLK1 treatment was carried out for two days, which was required to achieve efficient PLK1 knockdown. During these two days, we observed an increasing amount of rounded and detached cells, probably due to elevated numbers of mitotic cells, as long term PLK1 inhibition leads to mitotic arrest (Sumara et al., 2004; van Vugt et al., 2004). We thus hypothesized that the difference in p70-S6K-T389 phosphorylation in shPLK1 versus BI2536-treated cells could result from a larger fraction of mitotic cells in shPLK1 cultures, or from differing (off-target) effects during shPLK1 or BI2536 treatment. To test the
Figure 1. PLK1 binds and phosphorylates mTORC1, and PLK1 inhibition activates mTORC1 in interphase cells.

(A) HeLa cells were cultured in full medium. Immunoprecipitation (IP) was performed with PLK1 and control (mock) antibodies. Samples were analyzed by immunoblotting. Data shown are representative of n=4 independent experiments. (B) HeLa cells were starved for 1 h for amino acids and growth factors, stimulated with amino acids and insulin for 35 minutes and treated with the PLK1 inhibitor BI2536 for 30 minutes, as indicated. Samples were analyzed by immunoblotting. Data shown are representative of n=3 independent experiments. (Figure legend continued on next page)
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(C) Quantitation of data shown in (B). Ratio of p70-S6K-pT389 / p70-S6K is calculated for n=3 independent experiments. Data are normalized to 1 for the amino-acids/insulin stimulated control condition, and represented as mean ± SEM. A one-way ANOVA followed by Bonferroni’s multiple comparison test was applied; ns, non-significant; **, p≤0.01. (D) PLK1 (shPLK1) or control (shControl) shRNA HeLa cells were starved for amino acids and growth factors for 1 h, and stimulated with amino acids/insulin for 30 minutes. Samples were analyzed by immunoblotting without removal of the mitotic cells. Data shown are representative of n=3 independent experiments. (E) Quantitation of data shown in (D). Ratio of p70-S6K-pT389 / p70-S6K is calculated for n=3 independent experiments. Data are normalized to 1 for amino-acids/insulin stimulated shControl condition, and represented as mean ± SEM. A one-way ANOVA followed by Bonferroni’s multiple comparison test was applied; ns, non-significant. (F) raptor shRNA (shRaptor) or shControl HeLa cells were arrested in mitosis by nocodazole treatment. Samples were analyzed by immunoblotting. Data shown are representative of n=3 independent experiments. (G) shPLK1 or shControl HeLa cells were starved for amino acids and growth factors for 16 h and stimulated with amino acids/insulin for 35 minutes. Mitotic cells were removed by shake-off. Samples were analyzed by immunoblotting. Data are representative of n=4 independent experiments. (H) Quantitation of data shown in (G). Ratio of p70-S6K-pT389 / p70-S6K is calculated for n=4 independent experiments. Data are normalized to 1 for the amino-acids/insulin stimulated shControl condition and represented as mean ± SEM. A one-way ANOVA followed by Bonferroni’s multiple comparison test was applied; ns, non-significant; **, p≤0.01. (I) HeLa cells were treated with BI2536 and/or Torin1 as indicated, and stimulated as described in (B). Samples were analyzed by immunoblotting. Data shown are representative of n=3 independent experiments. (J) Quantitation of data shown in (I). Ratio of p70-S6K-pT389 / p70-S6K is calculated for n=3 independent experiments. Data are normalized to 1 for control condition (no Torin1, no BI2536), and represented as mean ± SEM. A one-way ANOVA followed by Bonferroni’s multiple comparison test was applied; ns, non-significant; **, p≤0.01. (K) PLK1 kinase assay. HA-Raptor was immunopurified from HeLa cells. An unspecific IgG antibody was used as negative control. All samples were dephosphorylated before adding them to the kinase reaction with recombinant PLK1. Data shown are representative of n=3 independent experiments. (L) Quantitation of data shown in (K) for n=3 independent experiments. Data are normalized to 1 for HA Raptor phosphorylation by PLK1. Data are represented as mean ± SEM. A one-way ANOVA followed by Bonferroni’s multiple comparison test was applied; ns, non-significant; **, p≤0.01; IB, immunoblot; KA, kinase assay.

first possibility directly, we analyzed if mitotic markers were increased in shPLK1- and/or BI2536-treated cells. In shPLK1-treated cells, we observed increased phosphorylation of the mitotic marker Histone H3 at serine 10, and decreased levels of the G1/S phase marker cyclin E1, indicative of an increased mitotic cell fraction in shPLK1 cultures (Fig. 1D). In contrast, short-term treatment with the PLK1 inhibitor BI2536 did not lead to an apparent increase in Histone H3-S10 phosphorylation (Supplementary Fig. 2A). As a positive control, the Histone H3-pS10 antibody was in parallel used to detect a cell lysate of mitotic cells (Supplementary Fig. 2A), and showed a strong signal for Histone H3-pS10. In agreement with earlier studies (Sumara et al., 2004; van Vugt et al., 2004; Zitouni et al., 2014), long-term overnight BI2536 treatment enhanced Histone H3-S10 phosphorylation (Supplementary Fig. 2B). Thus, we conclude that short-term BI2536 treatment failed to cause a detectable shift in cell cycle distribution, whereas long-term shPLK1 induction did. This may be the reason for
the observed differences in mTORC1 signaling between these two experimental setups.

To further test this, we aimed to separate effects directly mediated by PLK1 from its indirect, mitotic arrest-related effects. For this purpose, we first analyzed p70-S6K phosphorylation in mitotic versus asynchronous cell cultures, with or without mTORC1 inhibition by shRNA-mediated raptor knockdown (shRaptor, Fig. 1F). We arrested cells in prometaphase by nocodazole treatment, followed by a mitotic shake-off to enrich for mitotic cells. Immunoblot analysis showed that PLK1 levels were increased in nocodazole plus shake-off treated cells, indicative of a mitotic arrest (Golsteyn et al., 1994). Phosphorylation of the p70 isoform p70-S6K at T389 was observed in asynchronous cells, but not in cells with mitotic arrest, indicating that mTORC1 is inactive in mitotic cells (Fig. 1F). Interestingly, phosphorylation of the p85 isoform p85-S6K at T412 (Magnuson et al., 2012) (p85-S6K-T412, which is detected by the same antibody as p70-S6K-T389 and thus appears at a higher molecular weight in the same blot) was enhanced in mitotically arrested cells compared to non-arrested cells (Fig. 1F, first versus second lane). This p85-S6K-pT412 induction possibly explains earlier reports on mTORC1 activation in mitosis (Ramírez-Valle et al., 2010). In contrast, phosphorylation of p85-S6K-T412 in nocodazole-arrested cells was not inhibited by shRNA knockdown of the mTORC1 component raptor (Fig. 1F, fourth versus second lane), indicating that a kinase other than mTOR as member of mTORC1 mediates this event. In contrast, shRaptor did reduce the signals for p70-S6K-pT389 in asynchronous cells (Fig. 1F, first versus third lane). Thus, the absence of p70-S6K-pT389 signals in prometaphase-arrested cells suggests that mTORC1 is inhibited in mitosis (Fig. 1F, second and fourth lane), which is in line with previous findings (Shah et al., 2003). This supports our hypothesis that an increase in the amount of mitotic cells in a culture, as observed after PLK1 knockdown, may mask mTORC1 activation in the non-mitotic cell fraction in the same culture.

To test this, we combined PLK1 knockdown with removal of mitotic cells by shake-off. The removal of the mitotic cells was efficient, as evidenced by the decline in Histone H3-S10 phosphorylation in cultures after shake-off, compared to those without shake-off (Supplementary Fig. 2C, fourth versus third lane). In the non-mitotic cells that remained in the culture after shake-off, PLK1 knockdown did significantly increase p70-S6K-T389 phosphorylation in response to amino-acid/insulin stimulation (Fig. 1G,H) to a similar extent as BI2536 (Fig. 1B,C). Thus, both BI2536 and shPLK1 treatments in non-mitotic cells yielded qualitatively and
quantitatively similar results, namely p70-S6K-pT389 induction. This suggests that PLK1 acts to inhibit mTORC1 in non-mitotic cells. To test whether enhanced p70-S6K-pT389 in PLK1-inhibited cells is consistent with mTORC1 activation, we combined PLK1 inhibition by BI2536 with mTOR inhibition by Torin1 (Thoreen et al., 2009). Torin1 reduced p70-S6K-T389 phosphorylation both in control and BI2536-treated cells (Fig. 1I,J), consistent with the notion that increased p70-S6K-T389 phosphorylation in PLK1-inhibited cells is mediated by mTOR.

Taken together, p70-S6K-T389 was hyper-phosphorylated when PLK1 was blocked pharmacologically or through shRNA in non-mitotic cells. This suggests that PLK1 inhibits mTORC1 and limits the extent of p70-S6K-T389 phosphorylation in response to nutrients and insulin in interphase cells.

**PLK1 phosphorylates the mTORC1 component raptor *in vitro***

We found that PLK1 physically interacts with mTOR and its specific binding partner raptor (Fig. 1A), and that PLK1 inhibition activates mTORC1 in amino-acid/insulin-stimulated cells (Fig. 1B,G). Therefore, we next tested whether mTORC1 can function as a direct PLK1 substrate *in vitro*. The mTORC1 component raptor acts as a scaffold for the binding of mTORC1’s substrates (Shimobayashi and Hall, 2014) and is required for mTORC1 activity (Hara et al., 2002; Kim et al., 2002). Raptor is targeted by a number of kinases that signal to mTORC1 (Shimobayashi and Hall, 2014), for example AMPK (AMP-activated protein kinase) (Shimobayashi and Hall, 2014) and RPS6KA1/RSK (ribosomal protein S6 kinase A1) (Carrière et al., 2008). To test whether PLK1 is also capable of phosphorylating raptor, we overexpressed and immunopurified HA-tagged raptor from HeLa cells and used it as a substrate for *in vitro* kinase assays with recombinant PLK1 and 33P-labeled ATP (Fig. 1K). We detected incorporation of 33P at the molecular weight of HA-raptor, and this signal was reduced by the PLK1 inhibitor BI2536 (Fig. 1K,L). Thus, the observed HA-raptor phosphorylation was PLK1-specific. The mTOR inhibitor Torin1 did not significantly reduce the radioactive HA-raptor signal (Fig. 1K,L), suggesting that mTOR background activity does not contribute to the signal. As a negative control we omitted either PLK1 or HA-raptor from the in vitro kinase reaction. In both cases, no radioactive signal was detected at the molecular weight of HA-raptor (Fig. 1K, first and third lane), showing that the signal is raptor specific and requires the presence of PLK1. Thus, we conclude that PLK1 can directly phosphorylate raptor *in vitro*. 
PLK1 resides with mTORC1 at lysosomes, and active PLK1 decreases lysosomal association of the PLK1-mTORC1 complex

Since PLK1 binds and can directly phosphorylate mTORC1, at least in vitro, we next asked in which common subcellular compartment they reside. In line with its function as a mitotic regulator, PLK1 localizes to multiple mitosis-specific structures, including centrosomes, kinetochores, and the spindle midzone (Archambault et al., 2015; Zitouni et al., 2014), but also to the Golgi (Preisinger et al., 2005). Lysosomal localization is well described to be required for mTORC1 activation by amino acids and insulin (Sancak et al., 2010; Sancak et al., 2008), although mTOR localizes also to various other compartments (Betz and Hall, 2013; Thomas et al., 2014; Zhou et al.). Localization of PLK1 to the lysosome has to the best of our knowledge so far not been reported. In order to test whether in non-mitotic cells PLK1 resides with mTORC1 at lysosomes, we first analyzed the localization of PLK1, mTOR and the lysosomal marker LAMP2 by immunofluorescence (IF) in unsynchronized HeLa cells (Fig. 2A,B). Consistent with mTOR’s known localization at lysosomes (Sancak et al., 2010), there was strong overlap of mTOR and LAMP2 stainings (Fig. 2A). In addition, PLK1 and mTOR co-localized with each other in a lysosomal pattern (Fig. 2B), suggesting that they reside together at a common subcellular site. We tested the specificity of the PLK1 antibody in mitotic metaphase and anaphase cells, where it detected PLK1 at the mitotic spindle, as reported (Strebhardt, 2010) (Fig. 2C). It was experimentally not possible to perform PLK1-LAMP2 co-stainings as the antibodies against both PLK1 and LAMP2 were raised in mice and antibodies suitable for IF from other species were not available. To further test whether PLK1 localizes to lysosomes, we used sucrose gradients to fractionate cell lysates from unsynchronized HeLa cell cultures. The mitotic marker Histone H3-pS10 was undetectable in these cultures, as compared to lysates from mitotically arrested HeLa cells (Fig. 2D), suggesting that mitotic cells in the unsynchronized cultures were below the detection threshold. As expected, distribution of endogenous PLK1 partially overlapped with fractions that contained the nuclear markers lamin A/C and Histone H3. However, much stronger signals for PLK1 were found in fractions that were positive for the lysosomal marker LAMP2, mTOR, and raptor (Fig. 2E,F). Thus, PLK1 co-resided with mTOR and raptor in the lysosomal fractions, suggesting that PLK1 may bind to lysosomes. To test whether PLK1 indeed physically interacts with lysosomal components, we analyzed PLK1 IPs
with a LAMP2 antibody. Indeed, the lysosomal marker LAMP2 and the mTORC1 component raptor (positive control, see also Fig. 1A) were specifically detected in PLK1 IPs, but not in mock IPs (Fig. 2G), suggesting that PLK1 resides together with mTORC1 at lysosomes.

As our previous data (Fig. 1B,C,G-J) suggested that PLK1 inhibits mTORC1, and lysosomal relocalization is an important mode of mTORC1 regulation (Demetriades et al., 2014; Menon et al., 2014; Sancak et al., 2010; Sancak et al., 2008), we next tested whether PLK1 induction alters LAMP2-association of the PLK1-mTORC1 complex. To this end, we transfected cells with myc-tagged, wild type PLK1 (myc-PLK1wt) (Golsteyn et al., 1994) or an empty-control vector, performed PLK1 IPs, and detected LAMP2, mTOR and raptor by immunoblotting (Fig. 2H). Myc-PLK1wt overexpression did not alter the endogenous mTOR, raptor, or LAMP2 levels in the lysates. Of note, myc-PLK1wt overexpression strongly enhanced mTOR and raptor signals in PLK1 IPs, whereas the LAMP2 signal was strongly decreased (Fig. 2H). As endogenous mTOR and raptor levels were unaltered in the lysates (Fig. 2H), our data suggest that there is an increase in the amount of PLK1-mTORC1 complexes upon myc-PLK1wt expression, and these complexes do not physically bind the lysosomal marker LAMP2. Thus, our data are consistent with a model in which active PLK1 dissociates mTORC1 from lysosomes. To test whether its kinase activity is required for overexpressed PLK1 to detach mTORC1 from LAMP2, we transfected cells either with myc-PLK1wt, or with a dominant-negative lysine 82 to arginine mutated PLK1 variant (myc-PLK1K82R) (Smits et al., 2000), and performed PLK1 IPs (Fig. 2I). Endogenous mTOR, raptor, and LAMP2 levels were similar in lysates from myc-PLK1wt or myc-PLK1K82R transfected cells. In PLK1 IPs, the amounts of co-immunoprecipitated mTOR and raptor were similar for overexpression of myc-PLK1wt or dominant-negative myc-PLK1K82R. In contrast, LAMP2 signals were stronger in PLK1 IPs from cells overexpressing dominant negative myc-PLK1K82R, as compared to myc-PLK1wt (Fig. 2I). This suggests that inactive myc-PLK1K82R binds mTORC1 and the lysosomal protein LAMP2. Active myc-PLK1wt loses LAMP2 association while it binds mTORC1 with the same efficiency as inactive myc-PLK1K82R. In summary, these data are consistent with the notion that PLK1 binds mTORC1 at lysosomes, and that active PLK1 dissociates the PLK1-mTORC1 complex from the lysosomes, thereby mediating mTORC1 inhibition. If this was the case, a decrease in mTORC1 activity would be expected following overexpression of wild type PLK1 as compared to inactive PLK1. To test this, we analyzed p70-S6K-T389 phosphorylation in starved or
Chapter 3

Figure 2. PLK1 resides with mTORC1 at lysosomes, and overexpression of active PLK1 decreases lysosomal association of the PLK1-mTORC1 complex.

(A) Immunofluorescence analysis of HeLa cells that were cultured in full medium and stained with LAMP2 and mTOR antibodies. White regions in merged image (right) of LAMP2 (green) and mTOR (magenta) indicate co-localization. Nuclei were stained with Hoechst 33342. Scale bar 20 µm. Representative images are shown for n=3 independent experiments. (Figure legend continued on next page)
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(B) Immunofluorescence analysis of HeLa cells that were cultured in full medium and stained with PLK1 and mTOR antibodies. White regions in merged image (right) of PLK1 (green) and mTOR (magenta) indicate co-localization. Nuclei were stained with Hoechst 33342. Scale bar 20 µm. Representative images are shown for n=3 independent experiments. (C) Immunofluorescence analysis of HeLa cells that were synchronized in prometaphase with nocodazole for 16 h and released for 30 minutes in full medium. Cells were stained with PLK1 antibody. Nuclei were stained with Hoechst 33342. Scale bar 10 µm. Representative images of cells in metaphase (left) and anaphase (right) are shown for n=3 independent experiments. (D) Analysis of input sample taken before fractionation in sucrose gradient (E). The mitotic cell lysate was collected from HeLa shPLK1 knockdown cultures without mitotic shake-off. Samples were analyzed by immunoblotting. Data shown are representative of n=2 independent experiments. (E) HeLa cells were starved for 1 h for amino acids and growth factors and stimulated with amino acids and insulin for 35 minutes. Samples were separated in a 10-40 % sucrose gradient and analyzed by immunoblotting. Data shown are representative of n=3 independent experiments. (F) Quantitation of data shown in (E) for n=3 independent experiments. (G) HeLa cells were cultured in full medium. Immunoprecipitation (IP) was performed with PLK1 and control (mock) antibodies. Samples were analyzed by immunoblotting. Data shown are representative of n=3 independent experiments. (H) HeLa cells overexpressing wild type myc-PLK1wt or empty vector were cultured in full medium. Immunoprecipitation (IP) was performed with PLK1 and control (mock) antibodies. Samples were analyzed by immunoblotting. Data shown are representative of n=3 independent experiments. (I) HeLa cells overexpressing myc-PLK1wt or kinase-defective, dominant negative myc-PLK1K82R were cultured in full medium. Immunoprecipitation (IP) was performed with PLK1 and control (mock) antibodies. Samples were analyzed by immunoblotting. Data shown are representative of n=3 independent experiments. (J) HeLa cells overexpressing myc-PLK1wt or kinase-defective, dominant negative myc-PLK1K82R were starved for 1 h for amino acids and growth factors, and stimulated with amino acids and insulin for 35 minutes. Cells were then starved for amino acids for 10 minutes as indicated, and samples were analyzed by immunoblotting. Data shown are representative of n=3 independent experiments.

Amino-acid/insulin-stimulated cells that were transfected with myc-PLK1wt or inactive myc-PLK1K82R (Fig. 2J). Consistent with an inhibitory function of active PLK1 toward mTORC1, p70S6K-pT389 induction by amino acids plus insulin was lower in myc-PLK1wt transfected cells compared to myc-PLK1K82R transfected cells. Thus, we conclude that active myc-PLK1wt reduces lysosomal association of the PLK1-mTORC1 complex, which correlates with decreased p70S6K-T389 phosphorylation. This indicates that decreased lysosomal association contributes to mTORC1 inhibition by PLK1.

**PLK1 inhibition reduces autophagy in an mTORC1-dependent manner in interphase cells**

As amino-acid starvation is a condition that inhibits mTORC1 and increases autophagy, we used this condition to test if PLK1 inhibition activates mTORC1 and thereby inhibits autophagy. We first analyzed whether PLK1 contributes to mTORC1 inhibition upon amino-acid starvation. To this end, HeLa cells were
Figure 3. PLK1 inhibition hyperactivates mTORC1 and increases lysosomal mTORC1 localization in amino-acid starved interphase cells.

(A) HeLa cells were starved for 1 h for amino acids and growth factors, and stimulated with amino acids and insulin for 35 minutes. Cells were then starved for amino acids for 5, 10, 15 and 30 minutes and treated with BI2536 or carrier, as indicated. Samples were analyzed by immunoblotting. Data shown are representative of n=4 independent experiments. (Figure legend continued on next page)
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(B) Quantitation of data shown in (A). Ratio of p70-S6K-pT389 / p70-S6K is calculated for n=4 (5 minutes starvation and 15 minutes starvation); n=3 (10 minutes starvation) independent experiments. Data are normalized to 1 for starvation control condition and represented as mean ± SEM. A non-parametric two-tailed Student’s t test was applied; *, p≤0.05. (C) Immunofluorescence analysis of HeLa cells that were starved for 1 h for amino acids and growth factors, stimulated with amino acids and insulin for 35 minutes, followed by 30 minutes amino-acids starvation, with or without the PLK1 inhibitor BI2536. Staining was performed with mTOR and LAMP2 antibodies. White regions in merged image (right) of mTOR (green) and LAMP2 (magenta) indicate co-localization. Nuclei were stained with Hoechst 33342. Scale bar 20 µm. Representative images are shown for n=3 independent experiments. (D) Analysis of mTOR-LAMP2 co-localization by Pearson’s correlation coefficient for experiment shown in (C). Data are represented as mean ± SEM, and are representative of n=3 independent experiments. A non-parametric two-tailed Student’s t test was applied; *, p≤0.05. (E) Immunofluorescence analysis of HeLa cells that were treated as described in (C). Staining was performed with RagC and LAMP2 antibodies. White regions in merged image (right) of RagC (green) and LAMP2 (magenta) indicate co-localization. Nuclei were stained with Hoechst 33342. Scale bar 20 µm. Representative images are shown for n=3 independent experiments. (F) Analysis of RagC-LAMP2 co-localization by Pearson’s correlation coefficient for experiment shown in (E). Data are represented as mean ± SEM, and are representative of n=3 independent experiments. A non-parametric two-tailed Student’s t test was applied; ns, non-significant. (G) HeLa cells were either cultured in full medium or starved for amino acids/ growth factors for 16 h. IP was performed with PLK1 and mock antibodies. Samples were analyzed by immunoblotting. Data shown are representative of n=3 independent experiments. (H) Quantitation of IP samples shown in (G). Ratio mTOR/ PLK1 is calculated for n=3 independent experiments. Data are normalized to 1 for control condition and represented as mean ± SEM. A non-parametric two-tailed Student’s t test was applied; *, p≤0.05. (I) HeLa cells were treated as described in (G). IP was performed with PLK1 and mock antibodies. Samples were analyzed by immunoblotting. Data shown are representative of n=4 independent experiments. (J) Quantitation of IP samples shown in (I). Ratio raptor/ PLK1 is calculated for n=4 independent experiments. Data are normalized to 1 for control condition and represented as mean ± SEM. A non-parametric two-tailed Student’s t test was applied; *, p≤0.05.

starved for amino acids, with or without PLK1 inhibition by short term (30 minutes) BI2536 treatment. The cells were harvested at 5 – 30 minutes after onset of amino-acid starvation (Fig. 3A). Consistent with mTORC1 inhibition, p70-S6K-pT389 declined over time and was fully inhibited at 30 minutes after onset of amino-acid starvation. Notably, p70-S6K-T389 phosphorylation remained higher when PLK1 was inhibited by BI2536, as compared to the control cells (Fig. 3A,B). As the inhibitory effect of PLK1 toward mTORC1 is restricted to interphase cells, we analyzed the mitotic marker Histone H3-pS10 in amino-acid starved and BI2536-treated cells (Supplementary Fig. 2A). Histone H3 phosphorylation was high in mitotic control cells but not detectable in amino-acid starved and BI2536 treated cells, suggesting that these cultures were non-synchronized. Thus, PLK1 inhibition led to p70-S6K-T389 hyper-phosphorylation in amino-acid starved interphase cells. This suggests that PLK1 restricts mTORC1 activity not only upon amino-acid/ insulin stimulation (Fig. 1B), but also contributes to mTORC1 inhibition in response to amino-acid starvation (Fig. 3A,B).
As our data suggested that PLK1 inhibits mTORC1 by reducing its lysosomal binding (Fig. 2H,I), we next tested if PLK1 inhibition altered mTOR co-localization with the lysosomal marker LAMP2 in amino-acid starved cells. Indeed, IF analysis showed that PLK1 inhibition by BI2536 significantly increased mTOR-LAMP2 co-localization (Fig. 3C) as tested by the Pearson’s correlation coefficient (r mTOR-LAMP2, control = 0.33, SEM = 0.02; r mTOR-LAMP2, BI2536 = 0.47, SEM = 0.04; p mTOR-LAMP2, control, BI2536 = 0.006. A non-parametric two-tailed Student’s t test was applied.) (Fig. 3D). Localization of RagC (Ras related GTP binding C), a known mediator of lysosomal mTOR localization (Sancak et al., 2010), was not altered by BI2536 treatment (Fig. 3E,F; r RagC LAMP2, control = 0.53, SEM = 0.06; r RagC LAMP2, BI2536 = 0.49, SEM = 0.04; p RagC LAMP2, control, BI2536 = 0.72). This is in agreement with earlier reports that RagC localization remains unaltered upon changes in extracellular amino-acid concentrations (Sancak et al., 2010). Thus, PLK1 inhibition aberrantly enhanced mTOR co-localization with LAMP2 in amino-acid starved cells, suggesting that PLK1 inhibits mTORC1 by decreasing its association with RagC-positive lysosomes.

As our earlier data indicated that myc-PLK1wt overexpression inhibits mTORC1 (Fig. 2I,J), and the extent of interaction between them may contribute to PLK1-mediated mTORC1 inhibition, we next tested whether endogenous PLK1-mTORC1 binding was altered by amino-acid starvation. Therefore, we performed PLK1 IPs from amino-acid starved or full medium-cultivated cells. We found that the signals for both mTOR (Fig. 3G,H) and raptor (Fig. 3I,J) were increased in PLK1 IPs from amino-acid starved cells. We consistently immunoprecipitated less PLK1 from amino-acid starved cells, which led to a decrease in PLK1 signals (Fig. 3G,I). Nevertheless, the signals for co-immunoprecipitated mTOR and raptor were stronger for PLK1 IPs from amino-acid starved cells as compared to full medium-cultivated cells (Fig. 3G,I), indicating an increase in PLK1-mTOR/ raptor binding under amino-acid starvation. To quantify the relative amount of raptor or mTOR bound to PLK1 in non-starved versus amino-acid starved cells, we normalized the raptor and mTOR signals to the PLK1 levels in each respective IP. We found that physical PLK1 interaction with raptor and mTOR significantly increased upon amino-acid withdrawal (Fig. 3H,J). We conclude that increased mTORC1-PLK1 binding occurs when mTORC1 is inhibited by amino-acid starvation. This is consistent with our earlier finding that overexpression of active PLK1 led to increased PLK1-mTORC1 binding and reduced lysosomal association of the PLK1-mTORC1 complex, correlating with reduced mTORC1 activity (Fig. 2H-
J). As amino-acid deprivation inhibits mTORC1, we tested if mTOR inhibition by Torin1 could phenocopy the observed increase in PLK1-mTOR binding in amino-acid starved cells (Fig. 4A,B). Therefore, we performed mTOR IPs from HeLa cells cultivated in full medium and treated for 30 minutes with Torin1 or carrier (Fig. 4A, 4B). Torin1 inhibited p70-S6K T389 phosphorylation but did not alter PLK1-mTOR binding, suggesting that mTORC1 kinase activity does not control its own binding to PLK1. Next we tested if PLK1 activity affected the induction of PLK1-mTOR binding by amino-acid starvation. Therefore, we performed PLK1 IPs from HeLa cells that were treated with the PLK1 inhibitor BI2536 or carrier, and starved for amino acids or cultivated in full medium. Amino-acid withdrawal enhanced endogenous PLK1-mTOR binding 4-fold to the same extent in the presence or absence of BI2536 (Fig. 4C,D), suggesting that PLK1 kinase activity does not mediate enhanced PLK1-mTOR binding upon amino-acid deprivation. Thus, amino-acid deprivation may represent an input that is separate from mTORC1 and PLK1, as inhibition of mTOR or PLK1 did not alter increased PLK1-mTOR binding in amino-acid starved cells. Of note, we observed that acute amino-acid starvation not only significantly enhanced PLK1-mTOR binding (Fig. 4E,F) but also cytoplasmic co-localization of PLK1 and mTOR (Fig. 4G,H). This suggests that enhanced PLK1-mTOR association in amino-acid deprived cells may contribute to mTORC1 inhibition, via PLK1-mediated mTORC1 localization away from lysosomes.

As mTORC1 inhibition de-represses autophagy (Shimobayashi and Hall, 2014), we next tested if PLK1 via mTORC1 inhibition enhances autophagy. To this end, we inhibited PLK1 by BI2536 in amino-acid starved and control cells and detected LC3 (microtubule associated protein 1 light chain 3 alpha) (Klionsky et al., 2012) which is a widely used autophagy marker (Klionsky et al., 2012; Mizushima et al., 2010). Unprocessed LC3 (LC3-I) is soluble and resides in the cytoplasm. Upon autophagy induction, LC3-I is processed at its C-terminus and conjugated with phoshatidylethanolamine (referred to as LC3-II). LC3-II associates with autophagosomal inner and outer membranes (Mizushima et al., 2010), and becomes degraded upon fusion with lysosomes (Feng et al., 2014; Feng et al., 2015). Yet, dual processing of LC3 renders the interpretation of LC3-II signals challenging (Klionsky et al., 2012). On the one hand, LC3 is lipidated and integrated into the autophagosomal membrane, leading to an increase in LC3-II signal in immunoblots. On the other hand, LC3-II is degraded by lysosomal proteases upon autophagosomal-lysosomal fusion, decreasing the LC3-II signal. Thus, LC3-II degradation can mask the increase in LC3-II.
Figure 4. Starvation enhances PLK1-mTOR binding and cytoplasmic PLK1-mTOR co-localization. (A) HeLa cells were cultured in full medium and treated for 30 minutes with Torin1 or carrier, respectively. Immunoprecipitation (IP) was performed with PLK1 and control (mock) antibodies. Samples were analyzed by immunoblotting. Data shown are representative of n=3 independent experiments. (B) Quantitation of IP samples shown in (A). Ratio mTOR/PLK1 is calculated for n=3 independent experiments. Data are normalized to 1 for control condition and represented as mean ± SEM. A non-parametric two-tailed Student’s t test was applied; ns, non-significant. (Figure legend continued on next page)
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Upon autophagy induction. To prevent LC3-II degradation and enable detection of LC3-II accumulation, we supplemented all media for autophagy assays with the v-ATPase inhibitor Bafilomycin A1 (BafA). BafA inhibits autophagosomal-lysosomal fusion, a late step in the autophagy process. Thus, LC3-II can still be integrated into the autophagosomal membrane, but it is no longer degraded by lysosomal proteases, and LC3-II accumulation can be reliably detected. In keeping with this, BafA strongly induced LC3-II levels in HeLa cells (Supplementary Fig. 2D).

Upon amino-acid starvation for 30 minutes, we observed that PLK1 inhibition by BI2536 caused a significant decrease in LC3-II levels (Fig. 5A,B), indicating that PLK1 plays a positive role in autophagy. Next, we tested whether LC3-II reduction by PLK1 inhibition required mTOR activity. To this end, we combined PLK1 inhibition by BI2536 with mTOR inhibition by Torin1, and starved cells for amino acids (Fig. 5C,D). Whereas PLK1 inhibition by BI2536 significantly reduced LC3-II levels, BI2536 had no significant effect on LC3-II levels when combined with the mTOR inhibitor Torin1 (Fig. 5C,D). We also analyzed LC3-II levels in shControl and shPLK1 knockdown cells, without and with Torin1 treatment. In these experiments, mitotic cells were removed by shake off. PLK1 knockdown significantly reduced LC3-II, and this effect was suppressed by Torin1...
Figure 5. PLK1 inhibition reduces the autophagy marker LC3-II in interphase cells.

(A) HeLa cells were starved for 1 h for amino acids and growth factors, stimulated with amino acids and insulin for 35 minutes, followed by 30 minutes amino-acids starvation. All media were supplemented with Bafilomycin A1. BI2536 was added as indicated for 30 minutes. Data shown are representative of n=3 independent experiments. (B) Quantitation of data shown in (A). Ratio LC3-II/ GAPDH is calculated for n=3 independent experiments. Data are normalized to 1 for the control condition and represented as mean ± SEM. A non-parametric two-tailed Student’s t test was applied; *, p≤0.05. (C) HeLa cells were treated with BI2536 and/or Torin1 as indicated, and stimulated as described in (A). Samples were analyzed by immunoblotting. Data shown are representative of n=3 independent experiments. (D) Quantitation of data shown in (C). Ratio LC3-II/ GAPDH is calculated for n=3 independent experiments. Data are normalized to 1 for the control condition (no Torin1, no BI2536) and represented as mean ± SEM. A non-parametric two-tailed Student’s t test was applied; ns, non-significant; *, p≤0.05. (E) shPLK1 or shControl HeLa cells were starved for 1 h for amino acids and growth factors, stimulated with amino acids and insulin for 35 minutes, followed by 20 minutes amino-acids starvation. All media were supplemented with Bafilomycin A1. Cells were treated with Torin1 as indicated. Mitotic cells were removed by shake-off. Hence, only interphase cells were analyzed. Data shown are representative of n=4 independent experiments. (F) Quantitation of data shown in (E). Ratio LC3-II/ GAPDH is calculated for n=4 independent experiments. Data are normalized to 1 for the shControl condition (no Torin1) and represented as mean ± SEM. A non-parametric two-tailed Student’s t test was applied; ns, non-significant; *, p≤0.05.
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Thus, LC3-II reduction by PLK1 inhibition or knockdown required mTOR activity, suggesting that PLK1 positively contributes to autophagy by inhibiting mTORC1.

To further validate a role for PLK1 in autophagy regulation, we employed a tandem mRFP-GFP-LC3 reporter, which allows monitoring of autophagic flux (Kimura et al., 2007; Klionsky et al., 2012; Mizushima et al., 2010) and provides information about the status of the autophagy process. GFP (green fluorescent protein) displays higher sensitivity to low pH than mRFP (Kimura et al., 2007) (monomeric red fluorescent protein). Therefore, the tandem mRFP-GFP-LC3 reporter allows tracking of acidification of autolysosomes by providing a readout for autophagosome and autolysosome numbers (Kimura et al., 2007; Klionsky et al., 2012; Mizushima et al., 2010). HeLa cells were transiently transfected with the reporter construct in combination with PLK1 or control siRNA knockdown, and subjected to full-medium conditions or amino-acid starvation. Mitotic cells were removed by shake-off. Fixed cells were stained with Hoechst, imaged by wide-field microscopy (Fig. 6A), and deconvoluted images were analyzed as described previously (Szyniarowski et al., 2011). The few remaining mitotic cells, as determined by chromatin condensation state detected by Hoechst DNA staining, were omitted from the analysis. GFP puncta, representing autophagosomes, and mRFP puncta, representing autolysosomes plus autophagosomes, were counted. To determine the percentage of autolysosomes, we subsequently calculated the difference between mRFP and GFP puncta, which we expressed as the percentage of all mRFP positive puncta per cell (Fig. 6B). As expected, starvation increased the percentage of autolysosomes consistent with enhanced autophagic flux. PLK1 knockdown reduced the percentage of autolysosomes under full medium conditions and upon amino-acid starvation (Fig. 6B). This is in agreement with the decline in LC3-II levels upon PLK1 inhibition detected by immunoblotting (Fig. 5A-F).

We further consolidated this finding by analyzing the autophagy substrate SQSTM1/p62 (sequestosome 1). p62 is recruited by LC3 into autophagosomes, and thus p62 punctate structures represent LC3-positive autophagosome-associating p62. When autophagy is blocked, p62 foci accumulate due to inefficient autophagosome turnover (Klionsky et al., 2012). We detected p62 foci by IF in amino-acid starved cells that were treated with the PLK1 inhibitor BI2536 or carrier (Fig. 6C,D). In agreement with the reduced LC3-II levels (Fig. 5A-F) and decreased percentage of autolysosomes (Fig. 6B), we found that p62 foci numbers were significantly increased in PLK1 inhibited cells, compared to the
Figure 6. PLK1 inhibition impairs autophagy in non-mitotic cells and in *C. elegans* dauers. (A) HeLa cells were transfected with mRFP-GFP-LC3 tandem reporter, followed by PLK1 siRNA transfection on the next day. Cells were either kept in full medium, or starved for serum and amino acids for 16 h. Mitotic cells were removed by shake-off before fixation of cells 24 h after siRNA transfection. Representative images are shown for each condition. Scale bar 10 µm. Data shown are representative of n=2 independent experiments. (Figure legend continued on next page)
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control cells, thus providing further evidence that PLK1 is a positive regulator of autophagy.

The autophagy analyses reported above were performed in interphase cells. However, we could not rule out that PLK1 may regulate autophagy in mitotic cells as well. To test this, we analyzed LC3-II during mitosis. We arrested cells in mitosis by a consecutive aphidicolin-nocodazole block, released them for different times as indicated (Supplementary Fig. 2E), and detected cell cycle markers and LC3-II by immunoblotting. Consistent with an increased amount of mitotic cells in the culture, we observed increased phosphorylation of Histone H3 at serine 10 and decreased levels of the G1/S phase marker cyclin E1. Autophagy, as monitored by LC3-II levels, was low in mitotic cells as compared to control cells from an asynchronous culture (Supplementary Fig. 2E), which is consistent with previous reports (Eskelinen et al., 2002; Furuya et al., 2010). Thus, we conclude that mitotic cells display low autophagy, suggesting that PLK1 may promote autophagy primarily in non-mitotic cells. This is also in agreement with our finding that autophagic control by PLK1 depends on mTOR activity (Fig. 5C-F), which is inhibited during mitosis (Fig. 1F).

We next aimed to determine whether PLK1’s role in autophagy in non-mitotic cells is conserved from invertebrates to mammals. For this purpose, we used the genetic model organism C. elegans. We analyzed the role of plc-1, the C. elegans PLK1 ortholog, in dauer larvae, a developmentally arrested stage of C. elegans in which the animals display cell cycle quiescence and therefore...
consist of non-mitotic cells. Dauer entry and G1 cell cycle arrest in C. elegans larvae occur in response to environmental stresses, including starvation (Hong et al., 1998). Specifically, we utilized animals carrying a thermosensitive, mutant allele for the insulin/IGF-1 receptor *daf-2(e1370)*, and stably expressing the transgene GFP::LGG-1 (Egan et al., 2011) (orthologous to mammalian LC3; scheme of the experimental setup provided in Supplementary Fig. 2F). *daf-2(e1370)* mutants enter the dauer stage upon shift to the restrictive temperature (25°C), during which markers of autophagy are increased (Melendez et al., 2003). Moreover, RNAi knockdown of genes involved in the autophagic process changes the subcellular localization of GFP::LGG-1 in hypodermal cells of *daf-2(e1370)* mutants, while causing an enhanced GFP::LGG-1 signal in these cells (Melendez et al., 2003). Consistent with these observations, we found a 3-fold increase in GFP::LGG-1 intensity in the body of *daf-2(e1370)* dauers subjected to RNAi against the autophagy WIPI protein *atg-18* (Lu et al., 2011), compared to control RNAi (Fig. 6E,F). This indicates that inhibition of autophagy causes increased GFP::LGG-1 levels in *daf-2* dauer larvae. When we quantified GFP::LGG-1 intensity in *daf-2* dauers subjected to *plk-1* RNAi, we observed that inhibition of *plk-1*, like inhibition of *atg-18*, significantly increased GFP::LGG-1 levels compared to dauers treated with control RNAi (Fig. 6E,F). Thus, the effect of *plk-1* RNAi on autophagy recapitulated the effect of *atg-18* RNAi, suggesting that PLK1/PLK-1 is a conserved regulator of autophagy. As *C. elegans* dauer larvae consist of G1/S interphase cells (Hong et al., 1998), our data further suggest that similarly to mammalian cells, *C. elegans* PLK-1 positively regulates autophagy in non-mitotic cells.

**Discussion**

In the present study, we show that PLK1 physically binds and phosphorylates mTORC1. In interphase cells, inhibition of PLK1 increases mTORC1 activity, as measured by p70-S6K-T389 phosphorylation. Consistently, mTORC1’s lysosomal association (Fig. 2I) and activity (Fig. 2J) are decreased in cells overexpressing active PLK1, as compared to the inactive protein. In line with this, PLK1 inhibition mitigates autophagy in an mTOR-dependent manner (Fig. 5C-F).

PLK1 is mainly perceived as a regulator of mitotic progression (Archambault et al., 2015; Zitouni et al., 2014). Here we describe a novel function of PLK1 in interphase cells where it inhibits mTORC1 and activates autophagy under nutrient sufficiency and amino-acid deprivation. Our data suggest that
the functions of PLK1 in mitotic and interphase cells are mediated by distinct mechanisms since (i) PLK1 inhibition increases mTORC1 activity in interphase cells (Fig. 1G,H) but not in mitotic cells (Fig. 1D,E); (ii) PLK1 inhibition reduces autophagy in interphase cells (Fig. 5 and 6). In contrast, mitotic cells display high PLK1 levels (Sumara et al., 2004; van Vugt et al., 2004) (Fig. 1F) but low autophagy (Supplementary Fig. 2E), suggesting that PLK1 promotes autophagy primarily in non-mitotic cells; (iii) autophagy inhibition by PLK1 inhibition depends on mTOR activity (Fig. 5C-F). mTORC1 in mitotic cells is inhibited (Fig. 1F) and cannot be activated by PLK1 inhibition (Fig. 1D,E). Thus, we conclude that PLK1 inhibits mTORC1 and activates autophagy in interphase but not in mitotic cells. Which mechanisms may coordinate PLK1’s mitotic and interphase functions is currently unknown and will require further investigation.

Our finding that PLK1 inhibits mTORC1 in interphase but not in mitotic cells helps to resolve seemingly contradictory reports on the effects of PLK1 inhibition on mTORC1. For example, our findings are in agreement with Spartà et al. (2014) who reported that the PLK1 inhibitor BI6727 increases p70-S6K and S6 phosphorylation. Yet, this has been so far debatable as four other studies (Astrinidis et al., 2006; Li et al., 2014; Renner et al., 2010; Zhang et al., 2014) reported that PLK1 inhibition suppresses the phosphorylation of mTORC1 substrates. At first glance this seems to be at odds with our findings and those of Spartà et al. (2014) However, Renner et al. (2010), Astrinidis et al. (2006), Zhang et al. (2014) and Li et al. (2014) used long term treatments with PLK1 inhibitors or siRNA, increasing the amounts of mitotic cells in the cultures. In some studies, long-term PLK1 inhibition was even combined with a mitotic block (Astrinidis et al., 2006; Renner et al., 2010). Thus, the reduced mTORC1 activity reported in those studies was measured in mitotic cells. In agreement with those data, we also show that mTORC1 is inhibited in mitotic cells (Fig. 1F). However, after removal of mitotic cells, our data reveal that PLK1 inhibition activates mTORC1 in interphase cells (Fig. 1G), which corresponds with data from Spartà et al. (2014). Thus, our findings resolve and unify earlier - seemingly paradoxical - findings on PLK1 inhibitor effects on the mTORC1 substrate p70-S6K. Our results also complement previous studies on PLK1 inhibitor effects on autophagy. We observed here that PLK1 inhibition causes a decline in autophagy in interphase cells, as determined by the reduction in LC3-II accumulation and autolysosome numbers (Fig. 5, 6A,B). In agreement, Valianou et al. (2015) showed in TSC1- or TSC2-deficient lymphangioleiomyomatosis patient derived cells that BI2536 moderately inhibits autophagy. Our finding that PLK1 regulates
mTORC1 adds to the interpretation of these data. As loss of TSC1 or TSC2 leads to massive mTORC1 hyperactivation, mTORC1 can most probably not be much further activated by PLK1 inhibition in a TSC1- or TSC2-deficient background. This may explain the only moderate effect of BI2536 on autophagy observed in that study. Another study in LNCaP cells reported that long-term treatment with BI2536 for five days leads to mitotic arrest and necroptosis, correlating with cell-death related autophagy activation (Deeraksa et al., 2013). In our hands, autophagy was decreased in HeLa cells upon a 38 hours mitotic block (Supplementary Fig. 2E). Thus, autophagic activity during mitosis may vary depending on the length of cell cycle arrest and the cell type studied.

We find here that the mTORC1 component raptor is directly phosphorylated in vitro by PLK1. Which raptor residues may be PLK1 substrate sites? We analyzed the raptor sequence for known consensus phosphorylation motifs of PLK1 (Bibi et al., 2013), but did not find any. Thus, PLK1 substrate sites in raptor cannot be theoretically predicted. Three raptor phosphorylation sites at S722, S863, and S877 have been previously identified by two studies (Grosstessner-Hain et al., 2011; Santamaria et al., 2011), which reported on PLK1-dependent mitotic phosphoproteomes. We did not observe changes in phosphorylation of raptor-S722 and S863 upon BI2536 treatment in non-mitotic cells (Supplementary Fig. 2G). For raptor-pS877, we did not detect a specific signal with the available antibody (data not shown). Also other reported raptor phosphorylation sites (Foster et al., 2010) at S859 and T706 remained unchanged by BI2536 (Supplementary Fig. 2G). Thus, further studies are needed to gain insight into raptor residues that are phosphorylated by PLK1 in interphase cells. Likewise, discovery proteomic studies are required to identify other interphase substrates and thereby more generally delineate the interphase response of the PLK1 phosphoproteome to changes in nutrient supply.

The central platform for mTORC1 signaling is the lysosome, which is also the essential compartment for autophagy. Consistent with a role for PLK1 in mTORC1 regulation and autophagy, we report that PLK1 co-localizes with mTOR in a lysosomal pattern (Fig. 2A,B) and the lysosomal marker LAMP2 co-immunoprecipitates with PLK1 (Fig. 2G). Furthermore, in sucrose gradients PLK1 is detected in the lysosomal fraction, jointly with mTOR and raptor, and the mTORC1 regulator TSC2 (Fig. 2E,F). This finding is intriguing as there is so far no other report on lysosomal targeting of PLK1. Consistent with PLK1’s lysosomal association reported here, PLK1 contains a GY motif which is a lysosomal targeting signal (Ono et al., 1997).
Under which physiological conditions and in response to which stimuli does PLK1 inhibit mTORC1? We find here that PLK1 inhibition increases mTORC1 activity both under nutrient sufficiency (Fig. 1B,C,G-J) and amino-acid withdrawal (Fig. 3A,B), and the extent of p70-S6K-pT389 induction by PLK1 inhibition is equally strong in nutrient-induced (Fig. 1C,H) and starved cells (Fig. 3B). This suggests that PLK1 is active and inhibits mTORC1 in both conditions. Of note, short and long term starvation enhances binding of endogenous PLK1 with mTORC1 (Fig. 3G-J, 4E,F), and this is independent of mTORC1 or PLK1 kinase activity (Fig. 4A-D). This suggests that amino-acid starvation regulates upstream cues which cause enhanced PLK1-mTOR association. The molecular mediators which control starvation-induced PLK1-mTOR binding remain to be determined. Whereas PLK1 kinase activity does not affect starvation-induced PLK1-mTOR binding (Fig. 4C,D), PLK1 inhibition does lead to aberrant lysosomal localization of the PLK1-mTOR complex (Fig. 3C,D). This suggests that the increased PLK1-mTOR interaction in response to amino acid starvation does not happen at the lysosome, but another localization, e.g., in the cytoplasm. This is in agreement with the enhanced cytoplasmic co-localization of mTOR and PLK1 upon amino acid starvation (Fig. 4G,H). This implies that enhanced PLK1-mTOR binding in starved cells is a separate mechanism that indirectly adds to PLK1’s inhibitory effect on mTORC1 via localization away from lysosomes (scheme on the two separate mechanisms provided in Supplementary Fig. 2H).

Similar to mTORC1 activity, its localization as well as autophagy are altered by PLK1 inhibition both under nutrient starvation and sufficiency. Increased lysosomal localization or binding of mTOR upon PLK1 inactivation can be detected in starved (Fig. 3C,D) and full medium cultivated cells (Fig. 2I). Hence, PLK1 contributes under both conditions to mTOR re-localization away from lysosomes. In keeping with this, PLK1 inhibition mitigates autophagy both under full medium conditions and in starved cells (Fig. 6A,B). We conclude that mTORC1 inhibition by PLK1 is a general mechanism, which is not restricted to starved cells only, and consequently PLK1 positively contributes to autophagy both under starvation and nutrient-replete conditions (see scheme Supplementary Fig. 2H). Of note autophagy does not only occur in starved cells, but is also a critical house-keeping and pro-survival pathway under nutrient sufficiency. Basal autophagy maintains, for example, protein homeostasis by removing misfolded proteins, and mobilizes cellular energy and nutrient stores to maintain a stable pool of metabolite intermediates (reviewed by Kaur et al. (2015)).

Our finding that PLK1, next to mitotic progression, promotes autophagy...
in interphase cells suggests that for therapies of low grade tumors, which typically contain only 5-10% mitotic cells, PLK1 inhibitors may perform better than other purely antimitotic agents. As novel therapeutics are often tested first in advanced tumors, this point may have been missed so far, and clinical studies are needed to address performance of PLK1 inhibitors versus other antimitotics such as paclitaxel in low grade tumors. Beyond this, our findings suggest that combinatorial targeting of mTORC1 and PLK1 may hold promise for cancer treatment. PLK1 (Craig et al., 2014; Strebhardt et al., 2015; Yim, 2013) and mTOR (Cargnello et al., 2015; Chiarini et al., 2015) are common drug targets in cancer therapy, but combinatorial treatments are rarely tested even in preclinical studies. It seems promising that combination of the dual PI3K/mTOR inhibitor BEZ235 and the PLK1 inhibitor BI2536 in xenograft models of colorectal cancer shows that either inhibitor alone fails to enhance apoptosis, but combinatorial treatment inhibits mTORC1 readouts and leads to massive tumor cell death (Tan et al., 2013). We show here that PLK1 inhibition can activate mTORC1 and suppress autophagy. As this may affect tumor cell survival and growth, we advocate cautious monitoring of mTORC1 and autophagy readouts in clinical trials with PLK1 inhibitors. Correlation of such data with clinical outcome may allow development of strategies for optimized (combinatorial) cancer therapies, to simultaneously target PLK1 and mTOR in tumors where mTORC1 is activated by PLK1 inhibition.
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Chapter 3

Materials and Methods

Cell culture and cell treatments
HeLa α Kyoto cells were cultivated in full medium DMEM (Dulbecco’s Modified Eagle Medium, PAN Biotech, P04-03600) supplemented with 10 % FCS (PAA, Cat A15-102, Lot A10208-0991), 3 mM L-glutamine (Gibco, Life Technologies, 25030-024) at 37 °C, 7.5 % CO2. For amino-acids/insulin stimulation, cells were cultivated in DMEM, supplemented with 3 mM L-glutamine and 100 nM insulin (Sigma-Aldrich, I1882), for the indicated time points. Prior to starvation experiments, cells were washed twice with PBS (phosphate-buffered saline; PAN Biotech, P04-36500). Starvation was either performed for amino acids/growth factors in HBSS (Hank’s buffered salt solution; PAN Biotech, P04-32505), or for amino acids in amino acid free DMEM (PAN Biotech, P04-01507) supplemented with 4.5 g/l glucose (Sigma-Aldrich, G7021) and 100 nM insulin, as indicated. Mitotic shake-off was performed where indicated to remove the mitotic cells. Prior to the mitotic shake-off, cells were starved 16 h for amino acids/growth factors in HBSS. Nocodazole or consecutive aphidicolin-nocodazole treatment were performed as described before.(Thein et al., 2007) In brief, for consecutive aphidicolin-nocodazole treatment cells were treated 16 h with 1.6 μg/mL aphidicolin (Sigma-Aldrich, A0781), followed by a release into the cell cycle using full medium for 7 h and subsequently treated with 100 ng/mL nocodazole (Sigma-Aldrich, M1404) for 15 h, followed by release for the indicated times.

siRNA knockdown of PLK1 was induced using ON-TARGET plus SMARTpool siRNA, final concentration 10 nM (Dharmacon, L-003290-00). siRNA transfection was performed using Lipofectamine 2000, (Life Technologies, 11668-019) and DNA transfection was performed with JetPEI (PolyPlus, 101-40) according to the manufacturers’ protocol.

Overexpression of PLK1 was performed using the following constructs: empty vector pRcCMV (Invitrogen V75020), pRcCMV myc-PLK1K82R (Smits et al., 2000) (Erich Nigg, Addgene plasmid 41157), and pRcCMV myc-PLK1wt (Golsteyn et al., 1994) (Erich Nigg, Addgene plasmid 41160). The medium was exchanged 6 h post transfection. Cells were harvested after removal of the mitotic cells by mitotic shake-off 24 h or 48 h post transfection, with similar results.

The shPLK1 HeLa cell line was generated using the pTRIPZ system (Dharmacon). For virus generation HEK293T cells were co-transfected using jetPEI with the PLK1 shRNA construct, (target sequence shPLK1: CTGTCTGAAGCATCTTCTG; Dharmacon, RHS4740-EG5347) or a non-targeting
control sequence, respectively, with the Trans-Lentiviral shRNA Packaging system. The virus supernatant was harvested 72 h after transfection. HeLa cells were seeded in the morning and the infection with the virus supernatant was performed for 16 h. The transduction step was repeated twice. Selection of successfully transduced cells was achieved by adding puromycin (final concentration 2 µg/mL; Sigma-Aldrich, P8833). Stably transduced doxycycline-inducible HeLa shRPTOR cell line and a control cell line (shControl) harboring a non-targeting control sequence were described previously (Dalle Pezze et al., 2012). Knockdown was induced with doxycycline for 3 days (final concentration 2 µg/mL; Sigma-Aldrich, D3447). Doxycycline was removed for 16 h before the start of all experiments in this study.

**Antibodies and reagents**

The following antibodies were purchased from Cell Signaling Technology, Inc: raptor (2280), nTOR (2983), p70-S6K-pT389 (9206), p70-S6K (9202), LC3 (2775), ULK1-pS757 (6888), ULK1 (4773), lamin A/C (2032), cyclin E1 (4129). GAPDH antibody was bought at Abcam (ab8245). PLK1 (sc-55504), LAMP2 (sc-18822), raptor-pS663 (sc-130214), and c-Myc (sc-40) antibodies for immunoblotting and normal mouse IgG (sc-2025) and normal rat IgG (sc-2026) for immunoprecipitation were obtained from Santa Cruz, Biotechnology, Inc. Histone H3-pS10 (A301-844A) and Histone H3 (A300-822A) antibodies were bought from Bethyl Laboratories, Inc. The HA antibody (11867423001) was obtained from Roche. ACTIN (MAB1501) and raptor-pS722 (09-104) antibodies were purchased from Merck Millipore. raptor-pT706, raptor-pS859 and raptor-pS877 (Foster et al., 2010) were a kind gift from Diane C. Fingar, University of Michigan Medical School, Ann Arbor, MI, USA. HRP (horseradish peroxidase)-conjugated goat anti-mouse (31430) and goat anti-rabbit IgG (31460) were ordered from Thermo Scientific Pierce, HRP-conjugated light chain specific antibody for blotting after IP was obtained from Jackson ImmunoReseach Laboratories, Inc. (115-035-174). For immunofluorescence experiments, the RagC (9480) and mTOR (2983) antibodies were purchased from Cell Signaling Technology, Inc., LAMP2 (sc-18822) and PLK1 (sc-17783) were bought from Santa Cruz, Biotechnology, Inc. The p62 antibody was obtained from Progen Biotechnik (GP62-C). All secondary antibodies for immunofluorescence experiments were bought from Thermo Fisher Scientific: Goat anti-Guinea Pig IgG (H+L), Alexa Fluor® 568 conjugate (A-11075), Goat anti-Rabbit IgG (H+L), Alexa Fluor® 568 conjugate (A-11036), Goat anti-Rabbit IgG (H+L), Alexa Fluor® 488 conjugate (A-11008), Goat anti-
Mouse IgG (H+L), Alexa Fluor® 488 conjugate (A-11001), and Goat anti-Mouse IgG (H+L), Alexa Fluor® 568 (A-11004). Bafilomycin A1 was bought at Tebu-Bio (BIA-B1012) and throughout the study used at a final concentration of 100 nM. PLK1 inhibitor BI2536 (Axon Medchem, 1129) was used at 100 nM final concentration and added 30 minutes before lysis throughout the study unless otherwise stated and mTOR inhibitor Torin1 (Axon MedChem, 1833), was used at 250 nM and added 30 minutes before stimulation with amino acids/insulin.

Cell lysis and immunoblotting.

HeLa cells were washed twice with PBS before lysis in RIPA lysis buffer (1 % NP40, 0.1 % sodiumdodecylsulfate, 0.5 % sodiumdeoxycholate in PBS) supplemented with Complete Protease Inhibitor Cocktail (Roche, 11836145001), Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich, P5726) and Cocktail 3 (Sigma-Aldrich, P0044). Adjustment of the protein concentration, SDS PAGE and immunoblot were performed as described before (Dalle Pezze et al., 2012). Pierce ECL western blotting substrate (32209) or SuperSignal West FEMTO (34095), both Thermo Scientific Pierce were used to detect chemiluminescence using a LAS-4000 mini camera system (Fujifilm Life Science Systems) or LAS-4000 mini camera system (GE Healthcare).

Quantification of raw image files was performed using ImageQuant TL Version 8.1, GE Healthcare. Background subtraction was performed using the rolling ball method with a defined radius of 200 for all images. For graphical representation, raw images from the Fujifilm camera were exported as Color TIFF files using the Fujifilm software Multi Gauge version 3.0, Fujifilm Life Science Systems, and further processed with Adobe Photoshop version CS2. Raw images taken with the LAS-4000 mini, GE Healthcare system were exported as RGB color TIFF files using ImageJ, and further processed with Adobe Photoshop version CS5.1.

Immunoprecipitation (IP)

HeLa cells were washed 3x with ice-cold PBS and harvested in IP lysis buffer (40 mM HEPES, 120 mM NaCl and 0.3 % CHAPS, pH 7.5) supplemented with Complete (Roche, 11836145001), Phosphatase Inhibitor Cocktail 2 and Cocktail 3 (Sigma-Aldrich, P5726, P0044). Lysates were pre-cleaned by adding 10 µL/mL magnetic Dynabeads® Protein G (Life Technologies, 10009D), pre-washed in lysis buffer, for 30 minutes at 4 °C with end over end rotation. A lysate sample was taken up in 5x SDS sample buffer (50 % glycerol, 5 % β-mercaptoethanol, 0.3 M SDS, 0.03 M Tris (pH 6.8), 0.2 µM bromphenol blue) for subsequent analysis by
immunoblot. IP was performed by adding 7.5 µg of a specific antibody or control IgG (“mock”, negative control) per mL lysate for 30 minutes at 4 °C. Subsequently, 37.5 µL pre-washed Dynabeads® Protein G per mL lysate were added to the IP reactions for 1.5 h at 4 °C. Beads were washed three times briefly and twice for 10 minutes in IP lysis buffer and resuspended in 1x SDS sample buffer.

**PLK1 kinase assay**

HeLa cells were transfected with pRK5-HA-raptor (David Sabatini, Addgene plasmid 8513,(Kim et al., 2002) gift from David Sabatini) 48 h prior to the experiment. HA-raptor was immunopurified using an HA antibody. A control (mock) IP was performed with rat IgG. The immunoprecipitates were dephosphorylated with alkaline phosphatase (10 U; Thermo Scientific, EF0652 ) for 1 h at 37 °C, and washed with IP lysis buffer, followed by a washing step with kinase assay buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 10 mM MgCl2). Recombinant PLK1 (0.1 µg; Enzo Life Sciences, BML-SE466-0005) was added to raptor and mock IPs as indicated. The kinase – substrate mixture was pre-incubated on ice for 15 minutes with BI2536 (100 nM), Torin1 (250 nM) or carrier, respectively. An ATP mix with 1 mM cold ATP (GE Healthcare, 27-1006-01) and 5-10 µCi [γ-33P] ATP (PerkinElmer) was added and incubated for 30 minutes at 30 °C with gentle shaking. Samples were washed once with kinase assay buffer before resuspension in 1x SDS sample buffer and heated for 15 minutes at 68 °C. Samples were separated by SDS-PAGE and phosphorylation was analyzed by autoradiography. For quantitation the signal which was measured for the condition without PLK1 was considered as background and thus subtracted. For non-radioactive assay the same protocol was performed, 0.4 µM cold ATP was added and samples were analyzed by immunoblotting.

**Sucrose gradient**

HeLa cells were treated as indicated and lysed in homogenization buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 25 mM KCl, 5 mM MgCl2, 3 mM imidazole), supplemented with Complete (Roche, 11836145001) and Phosphatase Inhibitor Cocktail 2 and Cocktail 3 (Sigma-Aldrich, P5726, P0044). Plates were incubated for 30 minutes on a rocking platform at 4 °C. Subsequently, cells were scraped and centrifuged at 12,000 g for 10 minutes at 4 °C. The supernatant was transferred to a new tube and the protein concentration was determined using Bradford assay. 1.5 mg protein was loaded on a sucrose gradient. A continuous gradient was prepared from 10 % - 40 % sucrose using an ultracentrifuge tube
with a total volume of 4 mL. Lysates were distributed in the sucrose gradient using ultracentrifugation (194.000 g, at least 16 h, Beckman SW55 Ti rotor). After centrifugation, each sample was divided into 12 fractions and taken up in 5x loading buffer. Samples were analyzed by immunoblot. Quantitation was performed by determination of the relative intensities of PLK1 positive signals. The percentage of PLK1 in a certain fraction was calculated by building the ratio between the relative intensity in a single lane and the relative intensity of the sum of all PLK1 positive signals. The percentage of PLK1 in either the lysosomal or the nuclear fraction was calculated by addition of normalized PLK1 in LAMP2 or Histone H3 and lamin A/C positive lanes, respectively.

Immunofluorescence microscopy
For all immunofluorescence experiments, cells were washed in PBS and fixed with 4 % paraformaldehyde in PBS for 20 minutes at room temperature. After washing the cells three times with PBS, permeabilization was performed as indicated. Cells were washed in PBS and blocked with 0.3 % BSA (bovine serum albumin) or 0.3 % FCS in PBS, as indicated. Hoechst 33342 (end concentration 1 µg/mL; Invitrogen, H3570) was added and incubated for 30 minutes in the dark at room temperature. Cells were mounted with Mowiol 4-88 (Carl Roth, 07131) solution, which was prepared according to the manufacturer’s instruction including DABCO (1,4-Diazabicyclo[2.2.2]octane, Sigma-Aldrich, D27802) supplemented with 10 % NPG (n-propyl-gallate), and analyzed using fluorescence microscopy. For co-localization analysis of mTOR-LAMP2, mTOR-PLK1, and RagC-LAMP2 permeabilization was performed with 0.1% Triton-X-100 in PBS for 1 minute and cells were blocked with 0.3 % FCS in PBS. Z stack images were taken with an AxioObserver Z1 compound microscope with Apotome from Zeiss, 63x objective, AxioCam MRM3 CCD camera. For quantitative analysis, 4-5 representative fields of view were captured for each condition with identical exposure times and the same magnification. The Pearson’s correlation coefficient was calculated across raw files, without any image processing, using the co-localization module of the Zen software (Zen2012 blue edition software, Zeiss) after automatically setting the threshold with the Costes method. For presentation in figures, single layers of representative raw Z stacks were exported as TIFF with no compression using Zen2012 blue edition software (Zeiss) and brightness or contrast were adjusted, for better visibility. Brightness or contrast adjustment was not performed prior to quantitation, and thus did not influence image quantitation.

PLK1 staining in mitotic cells was performed after prometaphase arrest.
and a release of 30 minutes in full medium. Mitotic cells were collected by centrifugation (500 g, 4 minutes). PFA-fixed cells were permeabilized with 0.1% Triton-X-100 in PBS for 1 minute and cells were blocked with 0.3 % FCS in PBS.

To monitor autophagosomes and autolysosomes an mRFP-GPF-LC3 tandem construct (Tamotsu Yoshimori, Addgene, plasmid 21074, (Kimura et al., 2007) gift from Tamotsu Yoshimori). Cells were grown on coverslips, and transfected with mRFP-GFP-LC3 plasmid. After 48 h cells were fixed as described above. Permeabilization was performed with 0.1 % Triton-X-100 in PBS for 30-45 s and 0.3 % BSA was used for blocking. Images were taken with an AxioImager Z1 compound microscope from Zeiss, 63x objective, AxioCam MRm3 CCD camera. Prior to quantification images were deconvoluted using Huygens software, Huygens remote manager v3.0.3 (Scientific Volume Imaging). For image parameters a pixel size of 60 nm was assumed. For processing parameters the classic maximum likelihood estimation deconvolution algorithm was chosen and the signal/ noise ratio was set to 90 for all channels. The number of green and red puncta was counted using the spot detection function of Imaris Version 7.7.2 (BITPLANE AG). The background subtraction was ticked. As filter type, quality above threshold was chosen. Within one experiment the threshold and the estimated xy diameter were kept equal for all analyzed images. To determine the percentage of autolysosomes per cell, we counted red and green puncta, and subsequently calculated the difference between mRFP and GFP puncta which we expressed as the percentage of all red puncta per cell. At least 25 non- mitotic cells, as judged by Hoechst staining, were counted per condition. For presentation in figures, representative raw images were exported as TIFF with no compression using Zen2012 blue edition software (Zeiss) and brightness or contrast were adjusted, for better visibility. Images are shown without prior deconvolution. Brightness or contrast adjustment was not performed prior to quantitation, and thus did not alter the numbers of quantified green and red puncta.

For the p62 staining, permeabilization was performed with 1 % Saponin for 15 minutes and cells were blocked with 0.3 % FCS in PBS. Z stack images were taken with an AxioObserver Z1 compound microscope with Apotome from Zeiss, 63x objective, AxioCam MRm3 CCD camera. For quantitative analysis, at least five representative fields of view were captured for each condition with identical exposure times and the same magnification. The total area of p62 positive foci was calculated using ImageJ 1.47v. The threshold was set manually and kept identical for comparative analysis before applying the “Analyze Particles”
function. The total area was normalized to the number of nuclei.

For presentation in figures, maximum intensity projections of representative raw images were exported as TIFF with no compression using Zen2012 blue edition software (Zeiss) and brightness or contrast were adjusted, for better visibility. Brightness or contrast adjustment was not performed prior to quantitation, and thus did not influence image quantitation.

C. elegans experiments

The C. elegans strain MAH14 (daf-2(e1370); adIs2122[lgg-1p::gfp::lgg-1 +rol-6](Egan et al., 2011)) was used for this study. The strain was maintained at 20°C and raised on NGM plates seeded with Escherichia coli strain OP50 as previously described (Brenner, 1974). To investigate autophagy, eggs from MAH14 animals were transferred to RNAi plates (atg-18 RNAi clone was from Vidal library (Rual et al., 2004), and plk-1 RNAi clone was from Ahringer library (Kamath et al., 2003)) and incubated at 25°C to induce dauers. Following incubation for 6 days, dauers were anesthetized with sodium azide, arranged vertically on agar plates and imaged using an AxioImager Z1 compound microscope fitted with an AxioCam MRm3 CCD camera. GFP intensity was quantified using Image J software and normalized to the size of the animals.

Statistics

Quantitations of experiments were displayed and statistically analyzed using GraphPad Prism Version 5.00. For all experiments in human cells, the mean and the standard error of the mean (SEM) were plotted. For quantitation of GFP::LGG-1 fluorescence in C. elegans the mean and the standard deviation (s. d.) were plotted. Two groups were compared using a non-parametric two-tailed Student’s t-test assuming unequal variances. For comparison of multiple groups, a one-way ANOVA followed by Bonferroni’s multiple comparison test was used. P values below 0.05 were considered significant.
PLK1 inhibits mTORC1 and promotes autophagy

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PLK1 inhibits mTORC1 and promotes autophagy


Supplementary Information for
Chapter 3

Polo-like kinase 1 inhibits mTOR complex 1 and promotes autophagy
PLK1 inhibits mTORC1 and promotes autophagy

MSAAVTAGKLARAPADPGKAGVPGVAAPGAPAAAPPAKEIPEVLVDPRSSRRYVRGRLGKGFAKCEFISDADTKVEFAGKIVPKSSLLKLKHQREKMSMISHRSLAHQHVGFGHGEDDFDVFV
VLELCRRSSLLEHLHRRKALTEPEARARYLQIVLGCYLLHRNVRIHRDLKGLNLNEDLEVKIGDFGLATKVEYDGERKKTLCGTPNYIPEEVSLSKGHGFSFEVDSWVSICIMTLLVGKPFPETSCLE
KTYLRIKKNYESIPKHINPVAASLILQMQLTDPTAPINELNDEFTSGYIPARLPITCLTIPFRFSIAPSSLDPSNRKPLTVLANKGLENFLPERPRKEEFVPVRGVEVVDCHLSMDLQLQLHSV
NASKFESERGLVRQEEADEPACIFNWSKVDYSKGYGLQGLCDNSVGVLFNDSTLILYNGDLSQYIERDGTEYTLYTSHPNSLMMKKITLLYYFRNYMESHILKANGAMIIFREGDELRFLYLRTWFRRTSAILIHLNSGVSQINFQDHHTKLILCPHAAVYIDERDFRTYRSLLEEEYGCCKELSRLYARTMVDKLLSSASNRKAS

Supplementary Fig. 1. PLK1 interacts with mTOR.

(A, B, C) MS data were extracted from Table S4 and dataset PXD001244 of the ProteomeXchange database (http://proteomecentral.proteomexchange.org) of (Schwarz et al., 2015), for detailed analysis of PLK1-specific SILAC peptides in mTOR immunoprecipitations (IP) versus mock IP. (A) PLK1 identification by MS-based analysis of mTOR interactors in HeLa cells. Shown is the full-length amino acid sequence of PLK1 with six identified peptides in red. Two peptides are overlapping (FSIAPSSL DPSNR and KPLTVLNK). Sequence coverage: 11%. (B) Zoomed MS1 spectrum showing the protonated peptide IGDFGLATK of PLK1 in a SILAC experiment. Observed is the "heavy"-labeled (mTOR IP) peptide, while no distinctive isotopic pattern is present for the corresponding "light"-labeled (mock IP) peptides. Thus, PLK1 was found to be specifically enriched with mTOR. The star indicates the peptide peak which was subjected to collision-induced dissociation (CID) for identification. (C) Annotated MS2 spectrum of the peptide IGDFGLATK of PLK1 in a SILAC experiment. Observed is the "heavy"-labeled (mTOR IP) peptide, while no distinctive isotopic pattern is present for the corresponding "light"-labeled (mock IP) peptides. Thus, PLK1 was found to be specifically enriched with mTOR. The star indicates the peptide peak which was subjected to collision-induced dissociation (CID) for identification. (C) Annotated MS2 spectrum of the peptide IGDFGLATK of PLK1. (D) HeLa cells were cultured in full medium. Immunoprecipitation (IP) was performed with PLK1 and control (mock) antibodies. Samples were analyzed by immunoblotting. Data shown are representative of n=3 independent experiments. (E) HeLa shRaptor or shControl cells were cultured in full medium. Immunoprecipitation (IP) was performed with PLK1 and control (mock) antibodies. Samples were analyzed by immunoblotting. Data shown are representative of n=4 independent experiments. (F) Quantitation of IP samples shown in (E). Ratio mTOR/PLK1 is calculated for n=4 independent experiments. Data are normalized to 1 for shControl condition and represented as mean ± SEM. A non-parametric two-tailed Student’s t test was applied; ns, non-significant.
Supplementary Figure 2. PLK1 regulates autophagy in interphase cells.

(A) HeLa cells were starved for 1 h for amino acids (aa) and growth factors, treated with BI2536 for 30 minutes prior to lysis, and stimulated with amino acids and insulin (ins) for 35 minutes. Mitotic cells were collected from shPLK1 knockdown cells. Samples were analyzed by immunoblotting. n=3 independent experiments.

(B) HeLa cells were treated with BI2536 for 16 h. Samples were analyzed by immunoblotting. n=3 independent experiments.

(C) HeLa shPLK1 cells were induced for 48 h with doxycycline and mitotic cells removed by shake-off. Samples were analyzed by immunoblotting. n=3 independent experiments.

(D) HeLa cells were starved for 1 h for amino acids and growth factors, stimulated with amino acids and insulin for 35 minutes, followed by 30 minutes amino acids starvation. Media were supplemented with Bafilomycin A1 (BafA) as indicated. n=3 independent experiments.

(E) HeLa cells were arrested in mitosis by consecutive aphidicolin and nocodazole treatment and released for the indicated times, up to 4 hours. Samples were analyzed by immunoblotting. n=3 independent experiments.

(F) Schematic overview of the autophagy assay in C. elegans. (G) HeLa cells were treated and kinase reaction was performed as described in Fig. 1K. Samples were analyzed by immunoblotting. n=2 independent experiments.

(H) Model of the two separate mechanisms of PLK1-mTORC1 (mTOR-raptor) crosstalk in autophagy: 1.) Active PLK1 translocates mTORC1 away from the lysosome to inhibit mTORC1 and activate autophagy. 2.) Amino-acid starvation increases PLK1-mTORC1 interaction in the cytoplasm, and this is independent of mTOR or PLK1 kinase activity. Increased cytoplasmic PLK1-mTORC1 binding may indirectly contribute to PLK1’s inhibitory effect on mTORC1.
PLK1 inhibits mTORC1 and promotes autophagy