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CK2 Phospho-Dependent Binding of R2TP Complex to TEL2 Is Essential for mTOR and SMG1 Stability

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SUMMARY

TEL2 interacts with and is essential for the stability of all phosphatidylinositol 3-kinase-related kinases (PIKKs), but its mechanism of action remains unclear. Here, we show that TEL2 is constitutively phosphorylated on conserved serines 487 and 491 by casein kinase 2 (CK2). Proteomic analyses establish that the CK2 phosphosite of TEL2 confers binding to the R2TP/prefoldin-like complex, which possesses chaperon/prefoldin activities required during protein complex assembly. The PIH1D1 subunit of the R2TP complex binds directly to the CK2 phosphosite of TEL2 in vitro and is required for the TEL2-R2TP/prefoldin-like complex interaction in vivo. Although the CK2 phosphosite mutant of TEL2 retains association with the PIKKs and HSP90 in cells, failure to interact with the R2TP/prefoldin-like complex results in instability of the PIKKs, principally mTOR and SMG1. We propose that TEL2 acts as a scaffold to coordinate the activities of R2TP/prefoldin-like and HSP90 chaperone complexes during the assembly of the PIKKs.

INTRODUCTION

TEL2 is an essential orphan protein that has been implicated in many distinct cellular processes, including DNA repair, DNA damage checkpoints, telomere maintenance, the biological clock, and signal transduction pathways (Ahmed et al., 2001; Collis et al., 2007; Kota and Runge, 1999; Rendtlew Danielsen et al., 2009; Shikata et al., 2007). The discovery that TEL2 binds to and is required for the stable expression of all six members of the phosphatidylinositol 3-kinase related protein kinase (PIKK) family (including ATM, ATR, DNA-PKcs, mTOR, SMG1, and TRRAP) raised the possibility that TEL2 affects such diverse cellular processes through its role as a universal regulator of the PIKKs (Takai et al., 2007). Indeed, within this family, ATM, ATR, and DNA-PKcs are essential for the DNA damage response (Shiloh, 2003); mTOR regulates translation, cell growth, autophagy, metabolism, and organization of the actin cytoskeleton (Wullschleger et al., 2006); TRRAP is an adaptor protein found in several multiprotein chromatin-remodeling complexes, with histone acetyltransferase activity responsible for transcription activation (McMahon et al., 2000); and SMG1 is involved in nonsense-mediated mRNA decay (Yamashita et al., 2005).

Analysis of Tel2-deficient mouse cells has excluded both the ubiquitin proteasome pathway and mRNA stability as contributing factors to PIKK instability (Takai et al., 2007). It would appear that Tel2 might cooperate with the Hsp90 chaperone pathway to ensure the stable expression of a subset of the PIKKs, as treating cells with the Hsp90 inhibitor 17-AAG results in loss of Atr, Atm, and DNA-Pkcs at the protein level. Nevertheless, this cannot fully explain the Tel2 loss-of-function phenotype, as mTor and Smg1 protein levels were largely unaffected (Takai et al., 2007). A possible role for TEL2 in PIKK complex assembly was also suggested by the observation that depletion of TEL2 or its binding partner TTI1 leads to the disassembly of mTOR complexes (Kaizuka et al., 2010). Therefore, it is possible that TEL2 might function to coordinate the activity of several chaperone pathways to facilitate the folding of PIKKs and the assembly and maintenance of their active complexes. Nevertheless, how this is achieved remains unclear.

PIKKs have been previously found in complex with RUVBL1 and RUVBL2 (also known as RVB1/RVB2, Pontin/Reptin, TIP49A/TIP49B, and TIH1/TIH2), two closely related ATPases of the AAA+ (*A*TPase *a*ssociated with diverse cellular *a*ctivities) family (Izumi et al., 2010). As part of chromatin-remodeling complexes, RUVBL1/2 are required for activities such as transcription and DNA damage repair. RUVBL1/2 are also found in complex with telomerase and are responsible for TERC (telomerase RNA component) stabilization and assembly of an active telomerase complex (Jha and Dutta, 2009). In budding yeast, Ruvbl1/2 exist in a complex with Tah1 and Pih1 (R2TP complex), which is required for the correct accumulation of box C/D small nucleolar ribonucleoproteins. Interaction of Tah1 with Hsp90 is essential for Pih1 stability and, therefore, for R2TP complex stabilization (Zhao et al., 2008). In mammalian cells, the R2TP complex has been shown to associate with prefoldin proteins, suggesting that the complex is involved in several chaperone pathways (Cloutier et al., 2009). Recently, RUVBL1/2 have

been implicated in PIKKs stability, possibly through affecting SMG1 function and/or through interaction with HSP90. It is currently unclear whether the impact of RUVBL1/2 depletion on PIKK stability is related to TEL2 function or whether it occurs via an unrelated mechanism (Izumi et al., 2010).

Here, we identify a highly conserved region of TEL2 that is constitutively phosphorylated on serines 487 and 491 by the serine/threonine kinase casein kinase 2 (CK2) in vitro and in vivo. Using phosphopeptide affinity chromatography and mass spectrometry, we establish that CK2 phosphorylation of this site in TEL2 generates an interaction surface for the R2TP/prefoldin-like complex, which contains chaperones and prefoldins implicated in protein complex assembly. Biochemical studies reveal that the PIH1D1 component of the R2TP complex binds directly to the phosphorylated CK2 site of TEL2 in vitro and is essential for the TEL2-R2TP complex interaction in vivo. Finally, we show that the CK2 phospho-dependent interaction between TEL2 and the R2TP complex affects PIKK functions and is essential for mTOR and SMG1 stability in vivo. Taken together, these findings describe a critical phospho-dependent interaction surface on TEL2 that biochemically connects the stability of a subset of PIKKs to the cochaperone R2TP/prefoldin-like complex.

RESULTS

TEL2 Is Phosphorylated by CK2 on Ser 487 and Ser 491 In Vitro

Despite the presence of several HEAT repeat motifs, which are believed to mediate protein-protein interactions, protein sequence alignments and secondary structure prediction algorithms have failed to identify other structural or functional motifs that could provide insight into the role of TEL2 in PIKK stabilization. Nevertheless, a number of highly conserved stretches of amino acids are apparent in the TEL2 protein sequence that could be of functional importance. In particular, the residues spanning 480–505 amino acids in human TEL2 contain four serine residues that exist within a primary consensus site for the serine/threonine protein kinase CK2 (Figure 1A). Given that this region in TEL2 is highly conserved across all eukaryotes and Cka1/Orb5 (*S. pombe* homolog of CK2) has been found in complex with Tel2/TORC1 and Tel2/TORC (Hayashi et al., 2007), we reasoned that the putative CK2 site may be of critical functional importance and that its analysis might provide insight into the function of TEL2 in PIKK regulation.

To investigate whether the 480–505 amino acid region of TEL2 is a bone fide target for CK2 phosphorylation, we generated peptide-scanning arrays for TEL2, which have previously been successfully employed to map CDK phosphorylation sites in DNA replication initiation proteins (Balestrini et al., 2010). The TEL2 peptide-scanning array comprises 18 amino acid peptides that encompass the full-length human TEL2 protein. TEL2 peptides were spotted onto a nitrocellulose membrane with each adjacent peptide on the array sharing 15 amino acids with its neighboring peptide, corresponding to a change of three amino acids per peptide. Incubation of the TEL2 peptide-scanning array with HEK293T whole-cell extracts (WCEs) and ^{32}P-ATP resulted in the phosphorylation of several distinct regions of TEL2, including the putative CK2 site (480–505 amino acids; Figure 1B). Moreover, peptides encompassing amino acids 480–505 were also phosphorylated when incubated with recombinant CK2 and ^{32}P-ATP. Additional signal was also detected at peptides covering amino acids 270–297 and 630–654. However, as neither region contained consensus CK2 sites and are not conserved across species, these sites were not pursued further (Figure 1B). To confirm that CK2 is the kinase responsible for phosphorylation of the 480–505 region of TEL2 in HEK293T WCEs, the CK2 inhibitor TBB (4,5,6,7-tetrabromo-benzimidazole) was added to the WCE prior to incubation with the peptide-scanning array. Robust phosphorylation of peptides containing the 480–505 region of TEL2, as well as positive control peptides containing the CK2 sites previously reported in MDC1, was observed in untreated WCEs (Figure 1C). In contrast, WCE treated with TBB failed to phosphorylate the putative CK2 site in TEL2 or the CK2 site in MDC1, whereas a CK2 unrelated site in TEL2 was efficiently phosphorylated (Figure 1C). Collectively, these results indicate that the 480–505 region of TEL2 can be phosphorylated by CK2 in vitro.

To investigate which of the four serine residues within the 480–505 region of human TEL2 are phosphorylated by recombinant CK2, we synthesized four different 38 amino acid biotinylated peptides to this regions, which were either: (1) wild-type, (2) serine 485 and 502 mutated to alanine, (3) serine 487 and 491 mutated to alanine, and (4) serines 485, 487, 491, and 503 mutated to alanine (Figure 1D). Each of the peptides was subjected to in vitro kinase assays with recombinant CK2 and spotted onto a nitrocellulose membrane, and the level of ^{32}P-ATP incorporation was measured by scintillation. Whereas mutation of serines 485 and 502 to alanines (S485/502A) had only a minor affect on peptide phosphorylation, mutation of serines 487 and 491 to alanines (S487/491A) or mutation of all four serine (485, 487, 491, and 502) residues to alanine (S4A) abolished CK2 phosphorylation to background levels. These data suggest that serines 487 and 491 in TEL2 are the major CK2 phosphorylation sites within the conserved 480–505 region (Figure 1D).

TEL2 Is Constitutively Phosphorylated In Vivo on Serines 487 and 491

To determine whether TEL2 serine 487 and 491 are phosphorylated by CK2 in vivo, we raised a rabbit polyclonal antibody against a synthetic peptide phosphorylated on serines 487 and 491. Following affinity purification, the antibody was able to recognize wild-type TEL2 overexpressed in HEK293T cells (Figure 2A). In contrast, an overexpressed form of TEL2 in which serines 487 and 491 are substituted for alanines (2A) was not detected with this antibody. To prove that the antibody recognizes exclusively the phosphorylated epitope, we treated a HEK293T WCE overexpressing TEL2 with λ phosphatase or incubated the WCE overnight in the absence of phosphatase inhibitors. Part of the lysate was subsequently rephosphorylated by incubation with recombinant CK2. The antibody failed to recognize dephosphorylated TEL2 on western blot but readily detected TEL2 that was rephosphorylated by CK2 (Figure 2B). To determine whether the antibody could also recognize endogenous TEL2, we performed immunoprecipitation of TEL2 from

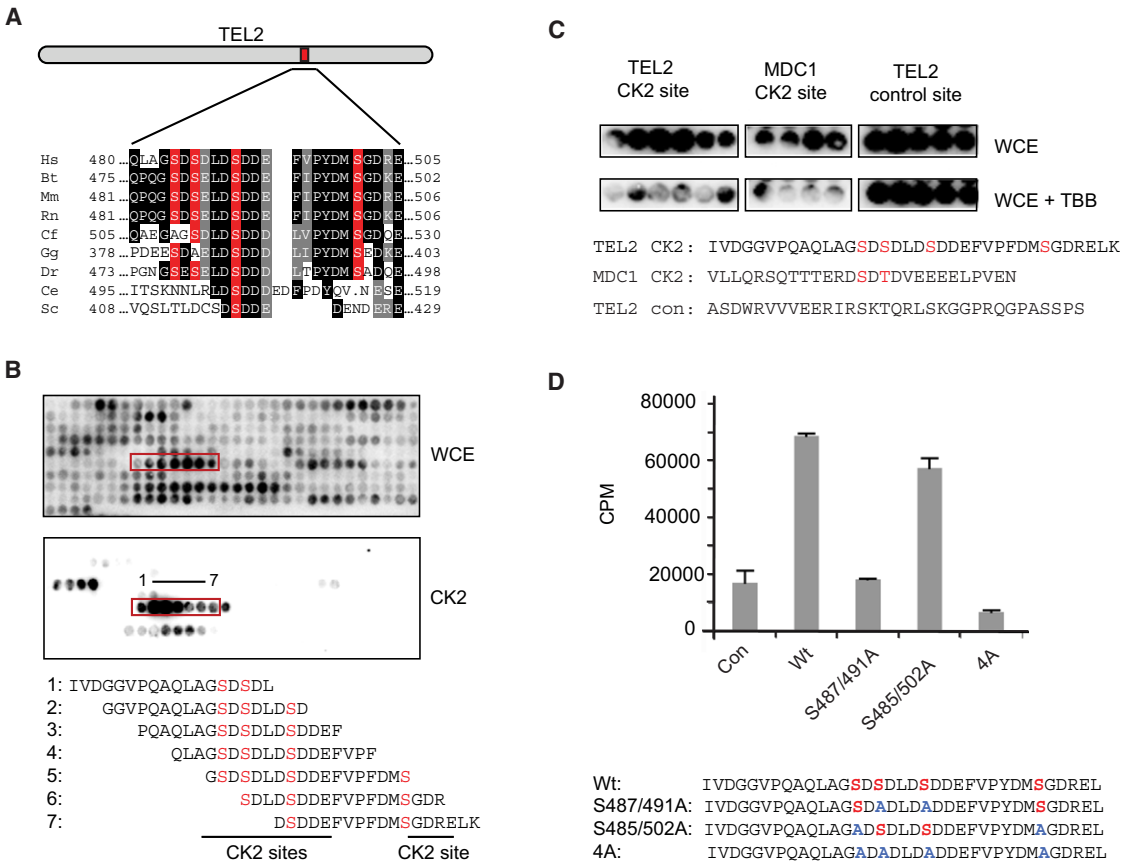

Figure 1. TEL2 Is Phosphorylated by CK2 on Conserved Residues Ser 485 and Ser 502 In Vitro

(A) Sequence alignment of the 480–505 amino acids region of Tel2 highlighting the conservation of CK2 consensus sites across species. Hs, human; Bt, cow; Mm, mouse; Rn, rat; Cf, dog; Gg, chicken; Dr, zebrafish; Ce, worm; and Sc, yeast. Serines that are part of CK2 consensus sites are boxed in red, identical residues in black, and similar in gray.

(B) Kinase assay on peptide-scanning arrays with HEK293T whole-cell extract (WCE) and recombinant casein kinase 2 (CK2). Peptides containing the conserved CK2 consensus sites are boxed in red, and their sequence is listed below the arrays.

(C) Kinase assay on peptide-scanning array containing peptides with the potential CK2 phosphorylation site in TEL2 (TEL2 CK2 site), control MDC1 peptides containing a known CK2 target site (MDC1 CK2 site), and a control TEL2 site that is phosphorylated in WCE, but not by CK2 (TEL2 con site). The assay was performed with WCE in the absence or presence of CK2 inhibitor 4,5,6,7-tetrabromo-benzimidazole (TBB; 75 μM).

(D) Kinase assay performed with recombinant CK2 and biotinylated peptides containing WT or mutated CK2 sequence on Ser 487 and 491 to Ala (S487, 491A), Ser 485 and 502 to Ala (S485, 502A), or all four Ser to Ala (4A).

Error bars represent standard deviations calculated from three separate experiments.

HEK293T whole-cell extract. The anti-TEL2-phospho-CK2 site antibody (pTEL2) efficiently immunoprecipitated a band corresponding in size to the endogenous TEL2 protein. Western blotting with a rabbit polyclonal antibody raised against the total TEL2 protein confirmed that this band corresponded to TEL2 (Figure 2C). The phosphorylation of TEL2 by CK2 on Ser 487 and 491 is likely constitutive, as the signal detected for wild-type TEL2 did not measurably change in cells treated with the replication inhibitor hydroxyurea (HU) for 30 min (Figure 2A). Furthermore, the levels of immunoprecipitated TEL2 did not measurably change after treatment with HU (Figure 2C).

To examine the subcellular localization of the CK2 phosphorylated form of TEL2, we expressed TEL2 wild-type (WT) or CK2 phosphosite mutant (2A) as N-terminal YFP fusions in HEK293T cells. The WT and 2A mutant of TEL2 exhibited similar subcellular localization, with higher abundance in the cytoplasm and nuclear periphery and a lower level of nuclear staining (Figure 2D). Immunostaining of TEL2 WT-expressing cells with the pTEL2 antibody gave predominantly cytoplasmic staining, with a low level of staining in the nucleus. In contrast, the pTEL2 staining was abolished to background levels in cells expressing the 2A mutant (Figure 2D). Based on these results, we conclude that TEL2 is constitutively phosphorylated on Ser 487 and 491 by CK2 in vivo, and the phospho form of overexpressed TEL2 is detected predominantly in the cytoplasm.

CK2 Phosphorylation of TEL2 on Serines 487 and 491 Confers Binding to Components of the R2TP Complex

Because CK2 phosphorylation of the DNA damage response proteins XRCC1 and MDC1 generates an interaction site on

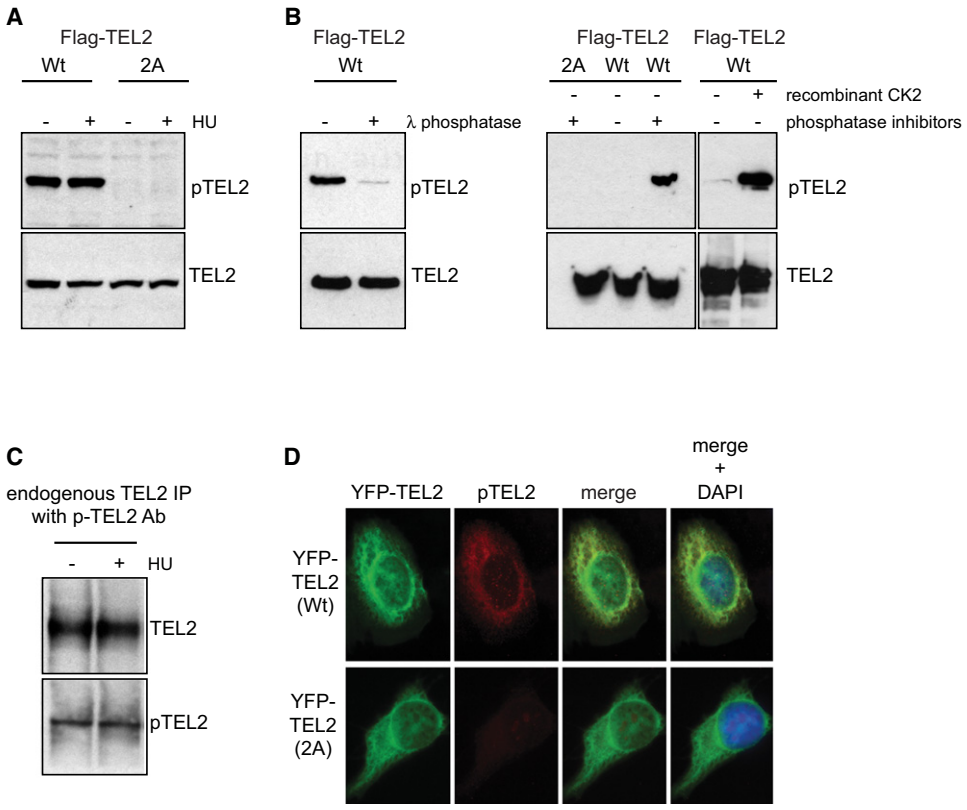

Figure 2. TEL2 Is Phosphorylated on Ser 487 and Ser 491 In Vivo

(A) Western blot of WCE from HEK293T cells expressing Flag-TEL2 WT or Flag-TEL2 Ser 487 and Ser 491 mutated to Ala (2A) with antibody raised against peptide containing phosphorylated Ser 487 and Ser 491 (pTEL2) and antibody recognizing the total protein (TEL2). The cells were either untreated or treated with 3 mM hydroxyurea (HU) for 30 min.

(B) Western blot analysis of WCE from HEK293T cells expressing TEL2 WT treated or untreated with λ phosphatase (left), incubated overnight in the presence or absence of phosphatase inhibitors (middle), or dephosphorylated TEL2 rephosphorylated by recombinant CK2 (right). Blots were stained with the pTEL2 and TEL2 antibodies.

(C) Western blot of pTEL2 immunoprecipitates from HEK293T WCE untreated or treated with 3 mM HU for 30 min.

(D) Localization of total and phosphorylated TEL2 WT and 2A. U2OS cells transiently transfected with YFP-TEL2 WT or YFP-TEL2 2A constructs (green signal) were stained with pTEL2 antibody (red signal).

these proteins for phospho-dependent binding by PNK and NBS1 (Chapman and Jackson, 2008; Loizou et al., 2004; Melander et al., 2008), respectively, we reasoned that CK2 phosphorylation of TEL2 on Ser 487 and 491 might also serve as a phospho-dependent protein-interaction surface. To test this possibility and to identify proteins that bind to TEL2 via the CK2 phosphosite, we designed biotinylated TEL2 peptides encompassing amino acids 480–505 that were either unphosphorylated (2S), phosphorylated (2pS on Ser 487 and 491), or in which serines 487 and 491 were mutated to alanines (2A). The biotinylated peptides were coupled to streptavidin beads and used to pull down proteins from cytoplasmic or nuclear extracts from HEK293T cells. Interacting proteins were then resolved on SDS-PAGE gels and stained with SyproRuby. Peptide pull-downs from nuclear extracts failed to identify any prominent bands with each of the three peptides (2S, 2A, or 2pS) (data not shown). In contrast, several prominent bands were specifically pulled down with the phosphorylated (2pS) peptide, but not with the 2S or 2A peptides (Figure 3A) from cytoplasmic extract. Similar results were obtained from peptide pull-down experiments with extracts derived from HeLa cells (Figure 3A) and also with TEL2 peptides phosphorylated on all four Serines (485, 487, 491, and 502) (data not shown).

Mass spectrometric analysis of the peptide pull-downs revealed that the prominent proteins retrieved from cytoplasmic extracts with the TEL2 2pS peptide were RUVBL1 (50 kDa), RUVBL2 (51 kDa), RPAP3 (76 kDa), and PIH1D1 (32 kDa), which together constitute the mammalian counterpart of the budding yeast R2TP complex (Ruvbl1, Ruvbl2, Tah1, and Pih1; Figure 3B). Intriguingly, the R2TP complex functions as a cofactor of Hsp90 in budding yeast (Zhao et al., 2008) and associates with a prefoldin-like complex in mammalian cells (Cloutier et al., 2009), which is composed of chaperones and is implicated in protein complex assembly. Although the R2TP complex components were the predominant proteins retrieved from extracts with the TEL2 2pS peptide, MS analysis also revealed the presence of

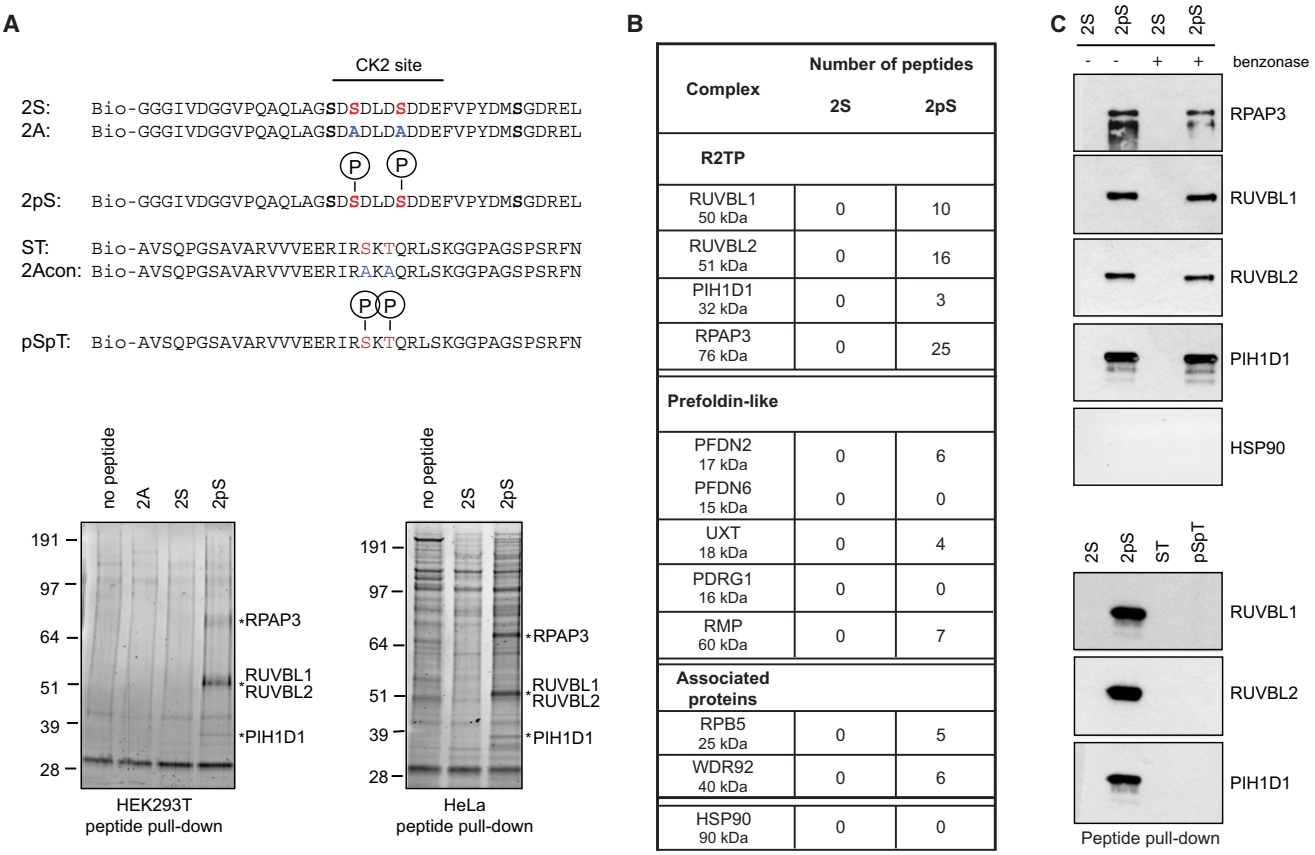

Figure 3. Phosphorylated Ser 487 and Ser 491 Interact with R2TP Complex In Vitro

(A) Sequence of biotinylated peptides used for peptide pull-down with cytoplasmic extract from HEK293T and HeLa cells. Sypro Ruby-stained SDS-polyacrylamide gels of peptide pull-downs.

(B) Number of peptides identified by mass spectrometry of members of R2TP and Prefoldin-like complex in pull-down with unphosphorylated (2S) and phosphorylated (2pS) peptides.

(C) Western blot analysis of peptide pull-downs. Cytoplasmic extract from HEK293T cells was untreated or treated with benzonase. The peptide pull-down was performed with unphosphorylated (2S) and phosphorylated (2pS) TEL2 peptides and with unrelated control unphosphorylated (ST) and phosphorylated (pSpT) peptides.

five of the seven prefoldin-like complex subunits (PFDN2, UXT, UR1, RPB5, and WDR92), but not the HSP90 chaperone (Figure 3B). Binding of the R2TP complex by the TEL2 2pS peptide was confirmed by western blotting (Figure 3C). Importantly, the TEL2 2pS peptide efficiently retrieved the R2TP complex from extracts treated with benzonase, excluding the possibility that this interaction occurs indirectly via DNA/RNA bridging (Figure 3C). Furthermore, a control peptide pull-down performed with an unrelated doubly phosphorylated peptide failed to pull down the RT2P complex from cytoplasmic HEK293T extracts (Figure 3C). These data suggest that the CK2 phosphosite in TEL2 binds to the R2TP complex in cytoplasmic extracts and does so independently of DNA/RNA bridging.

The CK2 Phosphosite of TEL2 Is Required for Binding to the R2TP/Prefoldin-like Complex but Is Dispensable for Interaction with ATM, ATR, mTOR, and HSP90

Because an interaction between TEL2 and the R2TP complex might provide a molecular explanation for the role of TEL2 in PIKK stability, we next sought to confirm these interactions within the context of full-length TEL2 protein (Figure 4A). To this end, we generated HEK293 Flp-In cell lines that stably express Flag-TEL2 (WT) or Flag-TEL2 (2A) mutant. Western blotting of Flag immunoprecipitates from Flag-TEL2 (WT)-expressing cells revealed the presence of each of the four subunits of the R2TP complex (Figure 4B). In contrast, binding of the R2TP complex was abolished in the Flag-TEL2 (2A) mutant (Figure 4B). Because the CK2 site in TEL2 lies within the region (291–603 amino acids) that confers binding to ATR (Rendtlew Danielsen et al., 2009) (Figure 4A), we also examined the Flag-TEL2 immunoprecipitates for binding to the PIKK family kinases ATM, ATR, TOR, and SMG1. Western blotting revealed comparable interactions with ATM, ATR, TOR, and SMG1 with the Flag-TEL2 (WT) and Flag-TEL2 (2A) mutant (Figures 4C and Figure S1 available online). Given the potential connection of R2TP complex to the HSP90 chaperone pathway and/or the prefoldin-like complex, we also examined interactions between TEL2 and HSP90 and TEL2 and RMP, one of the members of the prefoldin-like complex found by mass spectrometry. Both Flag-TEL2 (WT) and Flag-TEL2 (2A) mutant exhibited

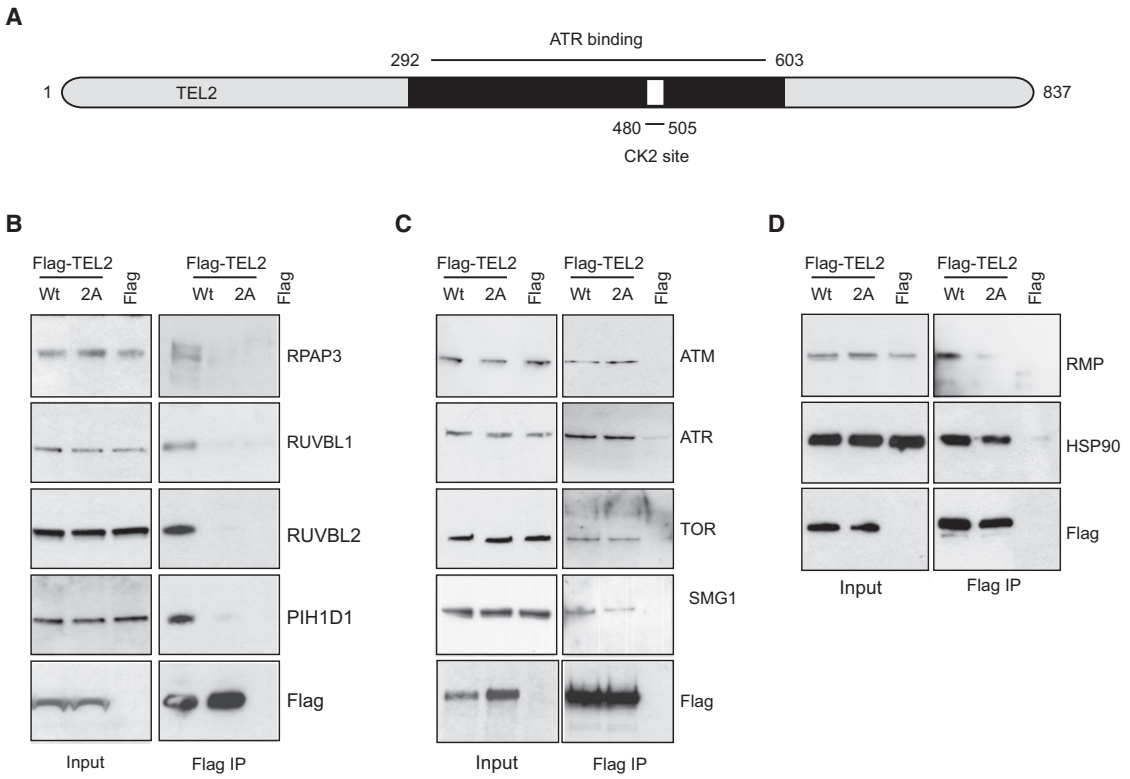

Figure 4. The CK2 Phosphosite Is Essential for Interaction with R2TP Complex and Members of Prefoldin-like Complex, but Not with ATM, ATR, TOR, SMG1, or HSP90

(A) Schematic representation of TEL2. The area responsible for binding of ATR is boxed black, and the area containing the CK2 sites is boxed red.

(B–D) Western blots of M2 anti-Flag agarose immunoprecipitates of extracts from HEK293 Flp-in cells stably transfected with Flag-TEL2 WT, Flag-TEL2 2A, or empty vector (Flag). Blots were stained with antibodies recognizing members of (B) R2TP complex; (C) ATM, ATR, TOR, and SMG1; and (D) HSP90 and Prefoldin-like complex component RMP.

comparable binding to HSP90α, whereas RMP binding was compromised with the Flag-TEL2 (2A) mutant (Figure 4D). Immunoprecipitation of endogenous TEL2 also efficiently pulled down HSP90α (Figure S2), whereas the Tel2 phosphopeptide did not (Figures 3B and 3C). These data indicate that the CK2 phosphosite in TEL2 is required for interaction with the R2TP and prefoldin-like complex in vivo but is dispensable for interaction with HSP90 and the PIKK family of kinases.

PIH1D1 Binds Directly to the CK2 Phosphosite of TEL2 In Vitro

To determine which of the subunits of the R2TP complex is responsible for binding to the CK2 phosphosite in TEL2, we purified Flag-TEL2 and the individual components of the RT2P complex as GST fusions for in vitro binding experiments. Full-length Flag-TEL2 (WT) and the Flag-TEL2 (2A) mutant were purified to near homogeneity from HEK293 Flp-In cells using high-salt conditions (Figure 5A). The purified Flag-TEL2 (WT) was either maintained with its CK2 site phosphorylated (p-WT) in the presence of phosphatase inhibitors or dephosphorylated (dep-WT) by overnight incubation in the absence of phosphatase inhibitors (Figure 5A). Whereas GST-PIH1D1, GST-RUVBL1, and GST-RUVBL2 could be expressed and purified to near homogeneity from *E. coli* (Figure 5A), GST-RPAP3 was insoluble and could not be studied further (data not shown). For in vitro binding experiments, anti-Flag M2 agarose beads with bound purified Flag-TEL2 (pWT, dep-WT, and 2A) proteins were incubated with GST-PIH1D1, GST-RUVBL1, or GST-RUVBL2. GST-RUVBL1 and GST-RUVBL2 failed to bind directly to TEL2. In contrast, GST-PIH1D1 efficiently bound to the phosphorylated form of Flag-TEL2 (p-WT) but exhibited severely reduced binding to both the dephosphorylated Flag-TEL2 (dep-WT) and the Flag-TEL2 (2A) mutant (Figure 5B). These data indicate that PIH1D1 binds directly to the CK2 phosphosite in TEL2 in vitro.

If PIH1D1 is also responsible for binding of the R2TP/prefoldin-like complex to the CK2 phosphosite of TEL2 in vivo, we reasoned that the interaction between RUVBL1, RUVBL2, RPAP3, and TEL2 should be dependent on PIH1D1. To test this hypothesis, we performed peptide pull-downs from extracts of HEK293T cells treated with control or PIH1D1 siRNA (Figure 5C). As expected, components of the R2TP/prefoldin

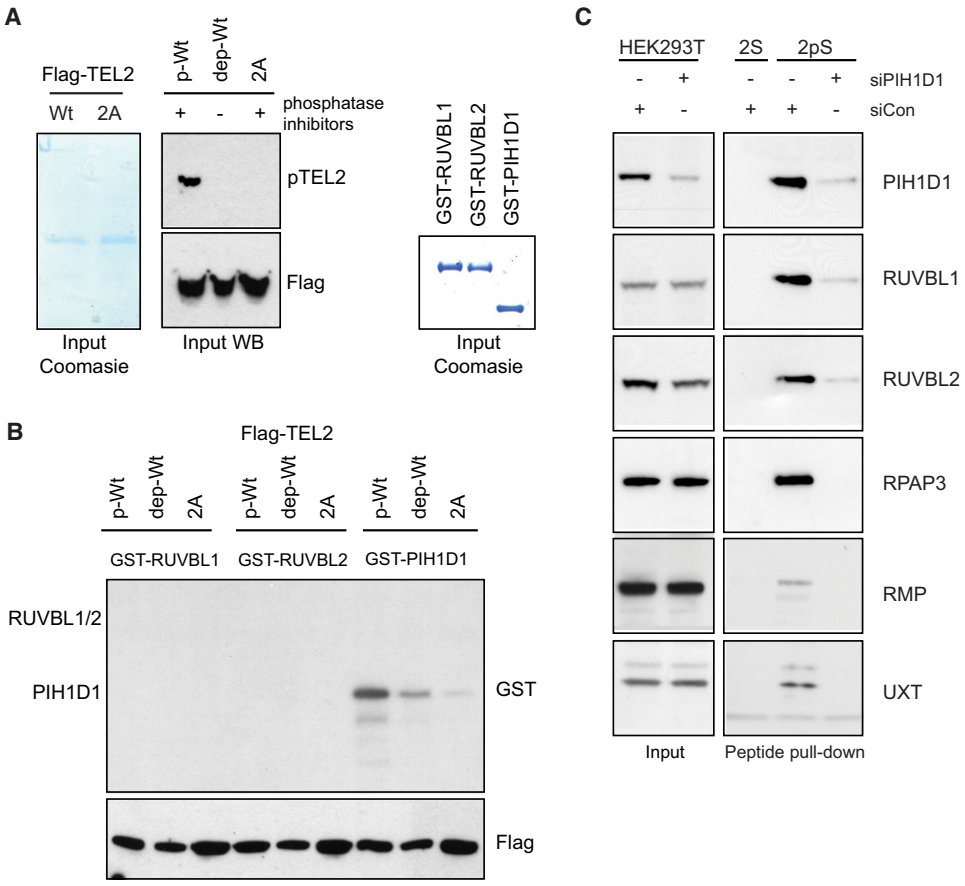

Figure 5. PIH1D1 Binds Directly to the CK2 Phosphosite of Tel2 and Is Required for Binding of the R2TP/Prefoldin-like Complex to This Site

(A) Coomasie staining of Flag-TEL2 WT and Flag-TEL2 2A purified from HEK293 Flp-in cell lines (left). Western blot of purified Flag-TEL2 WT and 2A mutant incubated overnight in presence or absence of phosphatase inhibitors with pTEL2 and Flag antibodies (middle). Coomassie staining of GST-RUVBL1, GST-RUVBL2, and GST-PIH1D1 purified from *E. coli* (right).

(B) M2-Flag agarose pull-down of phosphorylated Flag-TEL2 WT (p-Wt), dephosphorylated Flag-TEL2 WT (dep-Wt), and Flag-TEL2 2A in the presence of GST-RUVBL1, GST-RUVBL2, or GST-PIH1D1.

(C) Western blot analysis of peptide pull-down of unphosphorylated (2S) and phosphorylated (2pS) TEL2 peptides with HEK293T whole-cell extract from cells treated either with control siRNA (siCon) or with siRNA targeting PIH1D1 (siPIH1D1). The blot was stained with antibodies recognizing members of R2TP complex and two members of the prefoldin-like complex (RMP and UXT).

complex and two members of the prefoldin-like complex, RMP and UXT, were efficiently retrieved from extracts derived from cells treated with control siRNA with the phosphorylated TEL2 (2pS) peptide, but not with the unphosphorylated TEL2 (2S) peptide. In contrast, the TEL2 (2pS) peptide failed to retrieve the R2TP complex components PIH1D1, RUVBL1, or RUVBL2 from extracts depleted for PIH1D1 by siRNA (Figure 5C). Furthermore, PIH1D1 was efficiently retrieved from extracts depleted for RUVBL1/2 by siRNA (Figure S3). Although we observed greater than 95% knockdown of RUVBL1/2 by siRNA, the remaining RUVBL1/2 proteins also came down with the PIH1D1 (Figure S3). Thus, depletion of RUVBL1/2 had no significant effect on the binding of PIH1D1 to the phosphopeptide, whereas loss of PIH1D1 results in loss of R2TP complex binding to the phosphopeptide. We propose that the association of the R2TP complex with TEL2 is mediated by direct binding of PIH1D1 to the CK2 phosphosite in TEL2.

The CK2 Phosphosite in Tel2 Is Important for mTor and Smg1 Stability in Mouse Cells

It has been previously shown that conditional deletion of Tel2 in $Tel2^{F/-}$ mouse embryonic fibroblasts (MEFs) leads to loss of PIKK proteins (Takai et al., 2007). To examine the functional importance of the CK2 phosphosite in Tel2 in vivo, we generated: (1) a 2A substitution mutant (Ser488/492 to Ala) and (2) a 4A substitution mutant (Ser486/488/492/503 to Ala) in mouse Tel2 and examined their ability to rescue the instability of PIKKs in conditional Tel2 knockout MEFs (Takai et al., 2007). Consistent with our results with the human Tel2 protein, western blotting of Flag immunoprecipitates of Tel2 (WT) confirmed the presence of the R2TP complex components Ruvbl2 and Pih1d1, whereas Tel2 (2A) and (4A) mutants were compromised for R2TP complex binding (Figure 6A). Expression of Tel2 (WT) in Tel2-deficient MEFs was sufficient to rescue the instability of the PIKKs (Figure 6B). In contrast, complete reduction of Smg1, significant

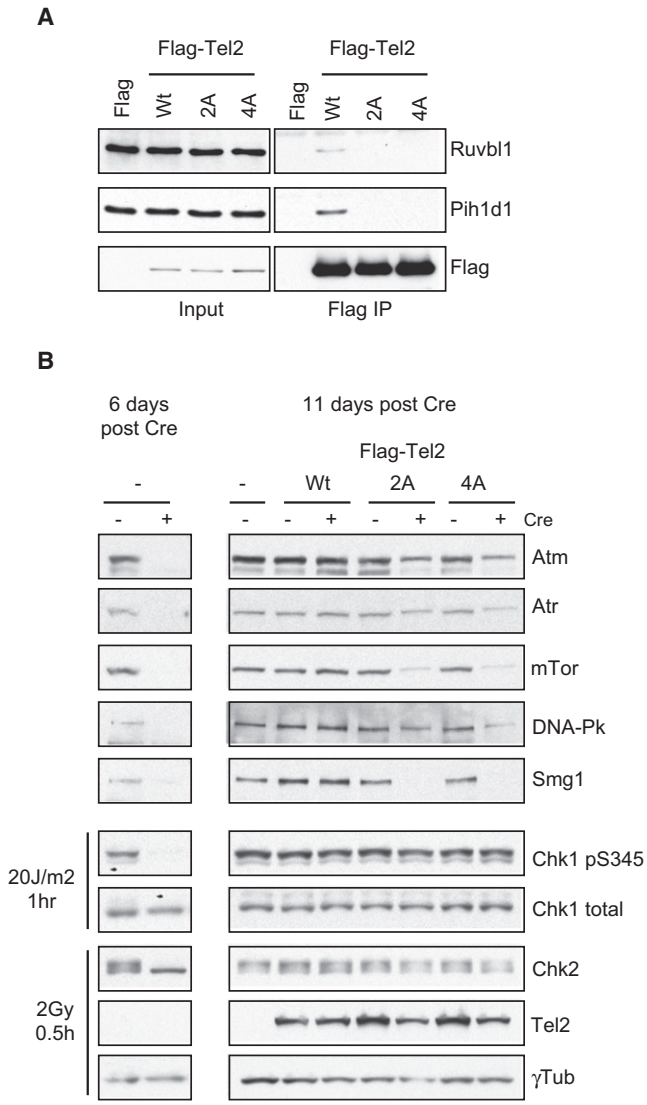

Figure 6. The CK2 Phosphosite in Tel2 Is Required for Ruvbl2 and Pih1d1 Binding and mTor and Smg1 Stability in Mouse Cells

(A) Western blot analysis of anti-Flag immunoprecipitation of WCE from Tel2$^{F/-}$ mouse embryonic fibroblasts (MEFs) reconstituted with Flag-Tel2 WT. Flag-Tel2 wild-type or mutant (2A and 4A) alleles.

(B) Tel2$^{F/-}$ MEFs were stably transfected with empty vector (–), Flag-Tel2 WT, or mutant (2A, 4A), and the endogenous Tel2 was deleted by Cre induction with 4-hydroxytamoxifen. Lysates were analyzed 6 or 11 days after Cre induction before or after treatment with UV (20 J/m^2, 1 hr) or γIR (2 Gy, 0.5 hr). Western blot analysis is shown for Atm, Atr, mTor, Smg1, and DNA-Pk and loading controls (Nbs1 and Tubulin). Checkpoint activation was assessed by western blotting for Chk1 (pSer345) and Chk2 phosphorylation.

reduction of mTor, and minor reduction of Atm, Atr, and DNA-Pkcs were observed in Tel2-deficient MEFs expressing either the Tel2 (2A) or (4A) mutants (Figure 6B).

In addition to promoting PIKKs stabilization, it has been demonstrated that Tel2 functions to promote Atm and Atr activation (Anderson et al., 2008; Collis et al., 2007; Rendtlew Danielsen et al., 2009). Although Atm and Atr levels were not significantly reduced in the Tel2 (2A) and (4A) mutants, it remained possible that these mutants were compromised for kinase activation. To investigate this possibility, we examined Chk1 and Chk2 phosphorylation in response to UV and IR (targets of Atr and Atm activation, respectively). Similar to Tel2 (WT), Tel2-deficient MEFs expressing either the Tel2 (2A) or (4A) mutants exhibited robust Chk1 Ser345 phosphorylation and Chk2 phosphorylation in response to UV and IR, respectively (Figure 6B). We also examined the affect of Tel2 loss on the phosphorylation of p70S6K by mTOR. Loss of Tel2 abolished p70S6K phosphorylation, and this was rescued by complementation with Tel2 wild-type (Figure S4). In contrast to loss of Tel2 that completely eliminates mTOR, the residual levels of mTOR remaining in the Tel2 2A mutant were sufficient to allow p70S6K phosphorylation. Collectively, these data indicate that the interaction of Tel2 with the R2TP/prefoldin-like complex via the CK2 phosphosite is essential for mTor and Smg1 stability but is less critical for Atm, Atr, and DNA-Pkcs stability and activation.

DISCUSSION

Here, we report that TEL2 is constitutively phosphorylated by CK2 on conserved serine residues 487 and 491. We present several lines of evidence suggesting that phosphorylation of this site in TEL2 creates a binding surface that confers interaction with the R2TP/prefoldin-like complex. First, TEL2 peptides phosphorylated on serine residues 487 and 491 efficiently retrieve the R2TP/ prefoldin-like complex from cell extracts, whereas the unphosphorylated or serine-to-alanine mutant peptides do not. Second, TEL2 coimmunoprecipitates with the R2TP/prefoldin-like complex, and this interaction is abolished by mutation of serines 487 and 491 to alanines.

Intriguingly, the R2TP (Rvb1-Rvb2-Tah1-Pih1) complex was first identified as a cofactor of Hsp90 in budding yeast (Zhao et al., 2008). Hsp90 binds directly to the R2TP complex subunit Tah1 and its vertebrate homolog RPAP3 through its tetratricopeptide repeat domains (Zhao and Houry, 2007). Studies of the R2TP complex in yeast are suggestive of a function in the maturation and assembly of ribonucleoprotein (RNP) complexes, including snoRNPs (Zhao and Houry, 2007; Zhao et al., 2008). A role in protein complex assembly is likely conserved, as the vertebrate R2TP complex components also associate with various RNP complexes (snoRNP, snRNP, L7Ae RNPs, and selenoprotein-coding mRNPs), and inhibition of Hsp90 compromises L7Ae RNP assembly and reduces L7Ae protein levels (Boulon et al., 2008). Such a function is also supported by the fact that the R2TP complex associates with a prefoldin-like module to form a larger 11 subunit R2TP/prefoldin-like complex, which has been proposed to be involved in the assembly of large protein complexes such as RNA polymerases (Cloutier et al., 2009). RUVBL1 and RUVBL2 have been also shown to be essential for telomerase holoenzyme assembly (Venteicher et al., 2008). A role for TEL2 in PIKK complex assembly is also supported by a recent study showing that Tel2 only binds to newly synthesized PIKKs (Takai et al., 2010).

Because the CK2 phosphosite peptide retrieved nine of eleven subunits of the R2TP/prefoldin-like complex, we propose that the interaction between TEL2 and the R2TP complex

represents a critical physical connection linking several chaperone complexes implicated in protein complex assembly. The importance of the TEL2 CK2 phosphosite is reinforced by our findings that 2A (S487/491A) and 4A (S485/487/491/502A) substitution mutants failed to rescue Tel2-deficient mouse cells. Of interest, the 2A and 4A substitution mutants would appear to confer a separation of function phenotype when compared to the null mutant; in contrast to complete loss of Tel2, which affects the stability of all PIKKs, the Tel2 CK2 phosphosite mutant had a clear impact on the stability of mTor and Smg1 but had only a minor effect on the stability and activation of Atm, Atr, and DNA-Pkcs. This result raises the possibility that the PIKKs may differ in their reliance on specific chaperones for their assembly/stability. In support of this possibility, we find that the TEL2 CK2 phosphosite mutant still retains the ability to bind to HSP90, which may be sufficient for the assembly/stability of ATM, ATR, and DNA-PKcs, but not mTOR and SMG1. This is in agreement with the fact that treatment of cells with HSP90 inhibitors results in complete loss of ATM and DNA-PKcs within 24 hr, whereas mTOR and SMG1 protein levels remained largely unaffected (Takai et al., 2007). Furthermore, the 2A mutation in human cells abolishes binding of TEL2 to RMP, one of the seven subunits of the prefoldin-like complex. Thus, TEL2 interactions with both HSP90 and the R2TP/prefoldin-like complex may be critical for assembly/stability of ATM, ATR, and DNA-PKcs and mTOR and SMG1, respectively. How TEL2 and HSP90 interact independently of the R2TP/prefoldin complex remains to be defined. Nevertheless, our data are consistent with a model in which Tel2 functions to coordinate the activity of Hsp90 and RT2P/prefoldin-like chaperone complexes during the assembly/stabilization of the PIKKs (Figure 7).

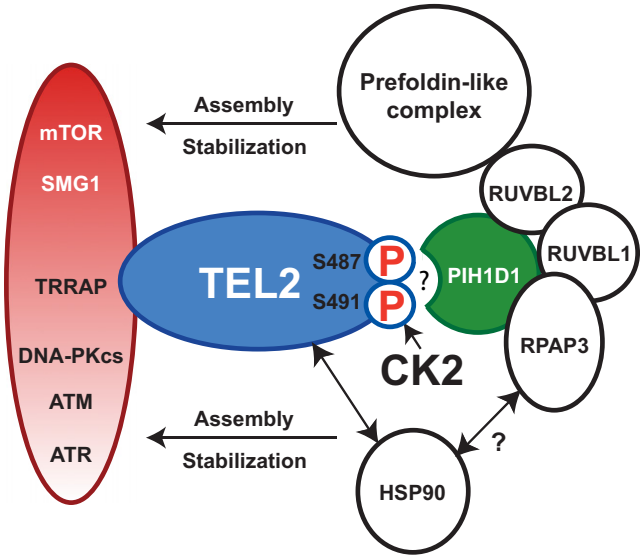

Figure 7. TEL2 Acts as a Scaffold to Coordinate the Activities of R2TP/Prefoldin-like and HSP90 Chaperone Complexes during the Assembly/Stabilization of PIKKs

Shown is a schematic of the interactions mediated by TEL2 with the PIKKs (ATM, ATR, DNA-PK, TTRAP, mTOR, and SMG1), the R2TP/prefoldin-like complex, and the HSP90 chaperone. TEL2 is constitutively phosphorylated on Serine 487 and 491 by CK2. PIH1D1 binds specifically to the CK2 phosphosite in TEL2 by an unknown mechanism (?), thus recruiting the R2TP/prefoldin-like complex. HSP90 may bind to the RPAP3 subunit via its tetratricopeptide repeat motifs but also binds to TEL2 independently of the R2TP complex. The combined chaperone activities of R2TP/prefoldin-like complex and HSP90 facilitate the assembly and/or stabilization of the PIKKs.

The importance of the TEL2-R2TP complex interaction in relation to PIKK stability is also supported by a recent study showing that depletion of RUVBL1/2 from cells leads to instability of the PIKKs. Whether the effect of RUVBL1/2 on PIKKs protein stability is related to Tel2 function or occurs via alternate mechanisms had not been addressed (Izumi et al., 2010). Our findings establish the molecular basis for this interaction; RUVBL1/2, as part of the R2TP complex, associate with the CK2 phosphosite of TEL2, which, in turn, binds directly to the PIKKs (Figure 7). Although the PIKK interaction region in TEL2 overlaps with the CK2 phosphosite, the phosphorylation of this site is dispensable for PIKK binding. Furthermore, interaction between RUVBL2 and RPAP3 has been previously shown to regulate DNA damage responses (Parusel et al., 2006), and RMP (URI), which is a part of the prefoldin-like complex, is involved in the transcriptional response to nutrient signaling controlled by mTOR, as well as in the maintenance of DNA integrity (Gstaiger et al., 2003; Parusel et al., 2006). The connections between TEL2, PIKKs, and chaperone complexes may provide a plausible explanation for these phenotypes.

Biochemical analysis of the interaction between TEL2 and the R2TP complex revealed that the PIH1D1 subunit is required for recognition and binding of the CK2 phosphosite in TEL2. In support of this conclusion, we found that recombinant PIH1D1 binds directly to purified TEL2 in a CK2 phosphosite-dependent manner. Furthermore, depletion of PIH1D1 in cells abolished the interaction between the CK2 phosphosite in TEL2 and the R2TP complex. Thus, PIH1D1 is a phosphopeptide-binding protein that is able to specifically recognize and bind to a region of TEL2 that is constitutively phosphorylated by CK2.

Constitutive CK2 phosphorylation of XRCC1 and MDC1 has been previously shown to create phosphosites that are recognized and bound by PNK and NBS1, respectively. For the case of PNK, phospho-recognition/binding of the CK2 phosphosite in XRCC1 is mediated by an FHA domain (Clements et al., 2004), whereas Nbs1 utilizes both FHA and twin BRCT modules for phospho-recognition/binding of the CK2 phosphosite in MDC1 (Chapman and Jackson, 2008). In contrast to PNK and NBS1, PIH1D1 lacks any recognizable phospho-recognition/binding motifs, such as FHA or BRCT domains. PIH1D1 would therefore appear to possess a distinct mode of phospho-recognition/binding that warrants further structure and functional analysis. Because the binding of the PIH1D1 (and the R2TP complex) to TEL2 is dependent on CK2 phosphorylation, it is possible that CK2 phosphosite recognition may be a common mechanism by which the R2TP complex is recruited to other key targets, such as RNPs and RNA polymerases. If so, maintenance of phosphoprotein interactions and/or comparative proteomic studies with CK2 inhibitors may facilitate the identification of new R2TP/prefoldin-like complex substrates.

EXPERIMENTAL PROCEDURES

Cell Culture, siRNA, and Drug Treatments

U2OS, HEK293T (Cancer Research UK Cell Services), and HEK293 Flp-In cells (Invitrogen) were maintained as adherent monolayer in DMEM media containing 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% carbon dioxide. Stable HEK293 Flp-In cell lines were created by transfection of empty pDEST-Flag/FRT/TO and pDEST-Flag/FRT/TO -TEL2 WT, 2A, or 4A according to the Flp-In cell lines manual and selected in media containing 150 mg/ml Hygromycin B (Invitrogen). Transient transfection was done using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The Tel2-inducible knockout mice were described previously and cultured in DMEM media without pyruvate containing 10% FCS. Media were supplemented 4-OHT (Sigma) as indicated (Takai et al., 2007). (Stealth siRNA for PIH1D1 (Invitrogen) mix of HSS123721 [CCUUCCACCGGAAGAGAAAGCAAUU] and HSS12372 [GACGUAGCUGU CAAC AGCGACUUCU]). Cells were transfected with 30 nM siRNA using Dharmafect 3 (Dharmacon). The transfection was repeated after 48 hr, and cells were collected 72 hr after the first transfection. For replication stress induction, the cells were treated with 3 mM hydroxyurea (Sigma) for 30 min before analysis.

Analysis of Tel2 CK2 Phosphosite Mutations in MEFs

Tel2-inducible knockout mouse embryonic fibroblasts (MEFs), SV40LT-immortalized $Tel2^{FLOX/-}$ R26Cre-ER^{T} ($Tel2^{F/-}$) MEFs were generated and maintained as described previously (Takai et al., 2007). Tel2 immunoprecipitations from MEFs were performed using one 15 cm plate of MEFs expressing Flag-tagged WT, 2A, or 4A Tel2. The cells were rinsed in cold PBS, scraped in 1 ml lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 10% glycerol, 1 mM EDTA, 5 mM β-mercaptoethanol, 1 × complete protease inhibitor mix, and 1 × PhosSTOP phosphatase inhibitor mix [Roche]), and then disrupted by sonication. The lysate was cleared by centrifugation at 16,000 × g for 15 min at 4°C. Twenty-five ul of anti-Flag M2 agarose beads (Sigma) was added to the lysate and incubated at 4°C for at least 2 hr. The beads were washed four times with lysis buffer. After removal of the buffer, the beads were suspended in 60 ul of 2 × Laemmli buffer. The effect of Tel2 2A and 4A mutations on ATM, ATR, and mTOR signaling in MEFs was determined as described previously (Takai et al., 2007, 2010).

Mass Spectrometry Analysis and Protein Identification

Whole lanes on the SDS-polyacrylamide gel stained with Sypro-Ruby were cut into slices (1–2 mm) using a scalpel and were robotically processed for mass spectrometry using the Janus liquid handling system (PerkinElmer, UK) as previously described (Collis et al., 2008).

Plasmids

pDEST-Flag/FRT/TO-TEL2 WT (HCLK2) was previously described (Collis et al., 2007). The 2A and 4A mutations were introduced using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). For immunofluorescence, the TEL2 constructs were cloned by Gateway LR reaction to pDEST-YFP/FRT/TO. cDNAs for RPAP3, PIH1D1, RUVBL1, and RUVBL2 were purchased from Origene and cloned to pDONR221 (Invitrogen), from which they were cloned by Gateway LR reaction to pDEST-GST plasmid for bacterial expression.

Protein Extracts and Immunoprecipitation

For whole-cell extracts, the cells were solubilized on ice in lysis buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 1 mM EDTA, and 10% glycerol) supplemented with 50 U/μl benzonase (Novagen), protease inhibitors (11836170001, Roche), and phosphatase inhibitors (P2850 and P5726, Sigma). Cleared lysates were produced by centrifugation of the resulting samples at 16,000 × g for 15 min at 4°C. Treatment with λ phosphatase (NEB) was performed with lysates with no added phosphatase inhibitors using 4000 U of λ phosphatase per 1 mg of the total lysate. The reaction was incubated for 30 min at 30°C. For the HEK293T cytoplasmic and nuclear extracts, cells from 32–150 mm 80% confluent dishes were collected, resuspended, and 2× washed in 2 ml of NP-40buffer (10 mM Tris-HCl [pH 7], 10 mM NaCl, 3 mM MgCl2, 30 mM sucrose, and 0.5% NP-40). The supernatant was kept as the cytoplasmic fraction, and the pellet was resuspended and washed twice with $CaCl_2$ buffer (10 mM Tris-HCl [pH 7], 10 mM NaCl, 3 mM $MgCl_2$, 30 mM sucrose, and 100 μM $CaCl_2$). The pellet was resuspended in Buffer C (20 mM Tris-HCl [pH 7.9], 20% glycerol, 0.1 M KCl, and 0.2 mM EDTA), and the lysate was pelleted at 25,000 × g for 30 min. The supernatant was used as nuclear extract. The HeLa nuclear and cytoplasmic extracts were purchased from CilBioech, Belgium. For benzonase treatment, we used 50 U/μl benzonase (Novagen). For Flag immunoprecipitations, 1 mg of the whole-cell extract was incubated with 25 μl of M2-anti Flag beads (Sigma) for 2 hr at 4°C. Beads were then pelleted and washed three times in 20 × bed volume of the lysis buffer (excluding benzonase). The bound protein was eluted with Flag peptide according to Sigma instructions, and the resulting eluates were passed through a BioRad column to remove traces of contaminating beads. Inputs represent ~1/20th of the extract used for the immunoprecipitation, and for the purpose of clarity, some immunoprecipitates shown in the figures are from longer exposures than those shown for their corresponding inputs. SYPRO Ruby (Invitrogen) staining of polyacrylamide gels was performed as described in the manufacturer's protocol. For pTEL2 immunoprecipitations, 1 mg of the whole-cell extract was incubated with 25 μl of protein A beads coupled with 5 μl of pTEL2 antibody for 2 hr at 4°C. Beads were then pelleted, washed three times in 20 × bed volume of the lysis buffer (excluding benzonase), resuspended in 2 × SDS loading sample buffer, and boiled for 5 min.

Peptide Synthesis and Pull-Down Experiments

The peptide pull-down was carried out using the following biotinylated peptides (LRI protein chemistry facility): TEL2 2S, Bio-GGGIVDGGVPQAQLAG SDSDLDSDDEFVPYDMSGDREL; TEL2 2A, Bio-GGGIVDGGVPQAQLAGSDA DLDADDEFVPYDMSGDREL; TEL2 2pS, Bio-GGGIVDGGVPQAQLAGSD[pS] DLD[pS]DDEFVPYDMSGDREL; TEL2 4A, Bio-GGGIVDGGVPQAQLAGADAD LDADDEFVPYDMAGDREL; TEL2 4pS, Bio-GGGIVDGGVPQAQLAG[pS]D [pS]DLD[pS]DDEFVPYDM[pS]GDREL. 10 μg of each of the peptides was coupled to 40 μl of Streptavidin-coated magnetic beads (Invitrogen) and added to 4 mg of nuclear extract precleared by incubation for 30 min room temperature with uncoupled beads. The coupled beads and the lysates were incubated for 2 hr at 4°C. The beads were 4× washed with TBST (Tris buffer saline, 0.1% Tween20), resuspended in 2 × SDS loading sample buffer, and boiled for 5 min. Immunoprecipitation from MEFs was as described previously (Takai et al., 2007).

Recombinant Proteins and Pull-Down Assay

Recombinant proteins were expressed in *E. coli* strain BL21. Fusion proteins were purified on glutathione Sepharose 4B according to the batch method described in the GST manual (GE Healthcare). Tel2-purified proteins on anti-Flag M2 agarose were isolated from stably transfected HEK293 Flp-In cells expressing Flag-TEL2 WT or Flag-TEL2 4A. The cells were solubilized in lysis buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1mM DTT, 1mM EDTA, and 10% glycerol) supplemented with 50 U/μl benzonase (Novagen), protease inhibitors (11836170001, Roche) and phosphatase inhibitors (P2850 and P5726, Sigma). The whole-cell extracts were obtained by centrifugation and incubated with anti-FLAG M2 agarose beads (Sigma) for 2 hr at 4°C (25 ul of the beads for 1 mg proteins of the whole-cell extract). For purification of the dephosphorylated proteins, the beads were incubated overnight with whole-cell extract without addition of the phosphatase inhibitors. Beads were successively washed in lysis buffer (1×) and wash buffer (4×) (20 mM HEPES [pH 7.5], 400 mM NaCl, 1% Triton X-100, 1 mM DTT, and 1 mM EDTA). Rephosphorylation of Tel2 isolated from 10 mg of total lysate by CK2 was performed with 1 μg of recombinant CK2 alpha 1 (Invitrogen) in 500 μl of kinase buffer (10 mM HEPES [pH 7.6], 50 mM NaCl, 10 mM MgCl2, 10 mM MnCl2, 2.5 uM ATP, and 1 mM DTT) for 30 min at 30°C. Beads were washed 4× in wash buffer. For pull-down assay, 50 μl of the M2 beads with bound TEL2 and 30 μg of purified GST proteins were used per reaction. The beads and proteins were diluted in 500 μl of peptide pull-down buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 1 mM EDTA, and 10% glycerol) and incubated for 2 hr at 4°C. Proteins were eluted

from the M2-Flag agarose beads using Flag peptide according to manufacturer's instructions.

Peptide Arrays and Kinase Assays

Peptide arrays for phosphorylation site screens were made from 18 mer peptides derived from the TEL2. Starting from the N terminus, each 18 mer peptide in the array was advanced from the previous by 3 residues in the C-terminal direction. Peptides were arrayed on nitrocellulose membranes (LRI protein chemistry facility). TEL2 peptide arrays were incubated with 6 ml of kinase buffer (50 mM TrisHCl [pH 7.5], 10 mM $MgCl_2$, and 5 mM DTT), containing 27 mg of whole-cell extract from HEK293T cells or 2 μg of recombinant CK2 (Invitrogen) and 80 μCi of γ-^{32}P-ATP at 30°C for 30 min. Kinase assay was stopped by addition of EDTA, and the membranes were subsequently washed for 20 min with 1 M NaCl and 1% SDS and 10–20 times with 0.5% phosphoric acid before exposing for autoradiography. For kinase assay on peptides, we used 15 μg of the following peptides synthesized at LRI protein chemistry facility: WT, IVDGGVPQAQLAGSDSDLDSDDEFVPYDMSGDREL; S487, 491A, IVDGGVPQAQLAGSDADLDADDEFVPYDMSGDREL; S485, 502A, IVDGGVPQAQLAGADSDLDSDDEFVPYDMAGDREL; 4A, IVDGGVPQAQLAGADADLDADDEFVPYDMAGDREL. The kinase assay was performed in 30 μl of kinase buffer (50 mM TrisHCl [pH 7.5], 10 mM $MgCl_2$, 5 mM DTT) with 0.2 μg of recombinant CK2 (Invitrogen) and 0.25 μCi of γ-^{32}P-ATP for 10 min at room temperature. The reaction was stopped by addition of 5 μl 0.5M EDTA (pH 8.0), and the reactions were spotted onto 2.1 cm diameter Whatman P81 cellulose phosphate filter circles. The circles were washed 3× in cold 0.5% phosphoric acid and 1× with acetone, dried at room temperature, put into scintillation vials with 5 ml of scintillation liquid (Ecoscint A, National diagnostics), and the scintillation measured.

Immunofluorescence

U2OS cells were grown on glass coverslips and transfected with YFP constructs. At 48 hr after transfection, they were fixed in 2% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. The anti-phospho TEL2 antibody was used as primary antibody and AlexaFluor 594 coupled antibody as secondary (Molecular Probes). Samples were analyzed using a Deltavision system.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.molcel.2010.08.037.

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Supplemental Information

CK2 Phospho-Dependent Binding of R2TP Complex to TEL2 Is Essential for mTOR and SMG1 Stability

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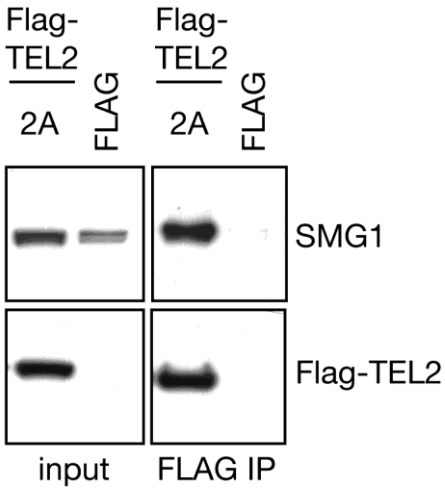

Figure S1. The CK2 phospho-site in TEL2 is not required for binding to SMG1. Western blots of M2 anti-Flag agarose immunoprecipitates of extracts from HEK293 Flp-in cells stably transfected with Flag-TEL2 2A or empty vector (Flag). Blots were stained with antibodies to SMG1.

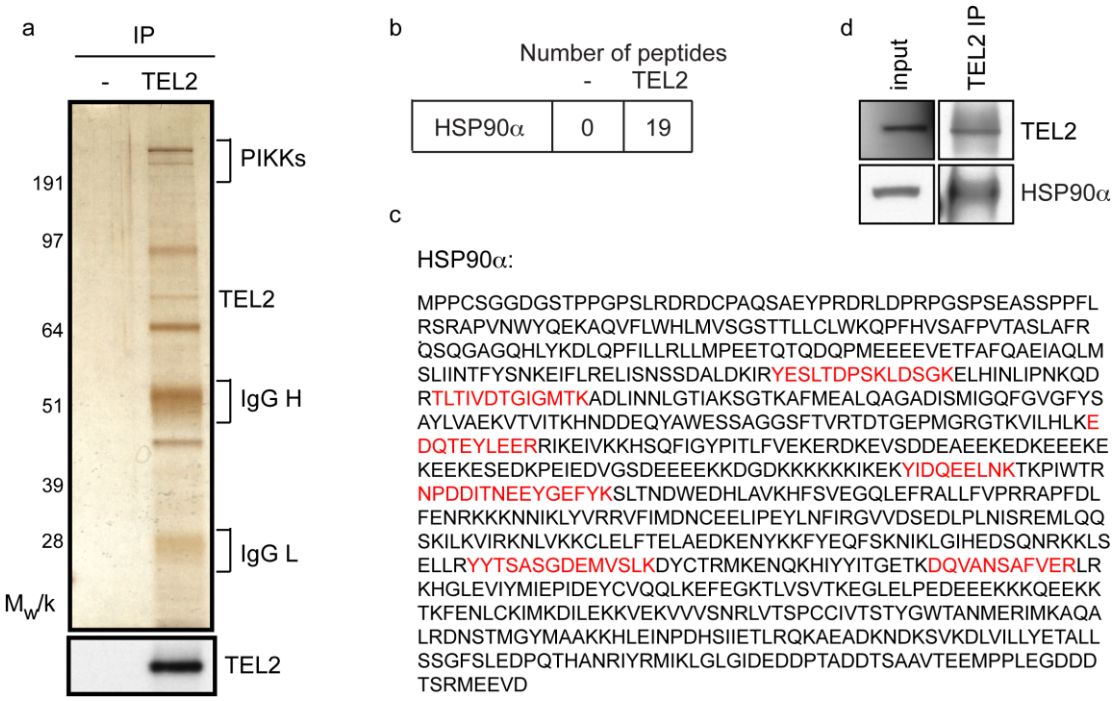

Figure S2. TEL2 interacts with the HSP90α chaperone. **a**. Silver stained gel showing the proteins that immunoprecipitate with endogenous TEL2 from HEK293T whole cell extracts. **b**. Number of HSP90α identified by mass-spectrometry (MS) with control (-) and TEL2 antibodies. **c**. HSP90α protein sequence with the peptides identified by MS shown in red. **d**. Immunoprecipitation of endogenous TEL2 from HEK293T cells and western blot for HSP90α.

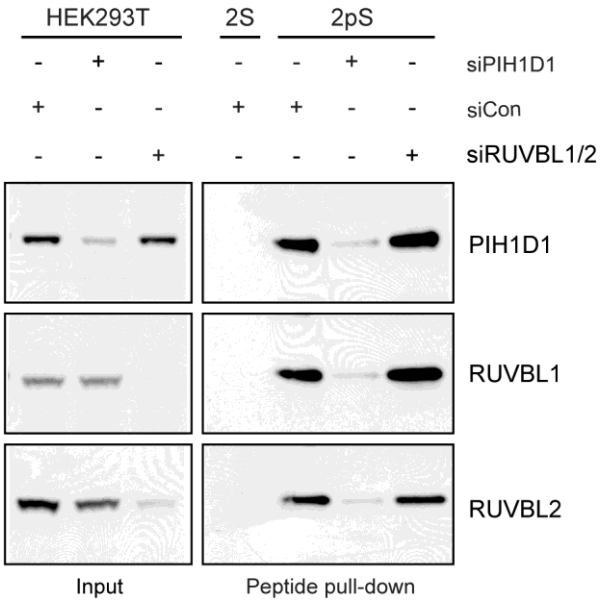

Figure S3. RUVBL1/2 are dispensable for PIH1D1 binding to the CK2 phospho-site in human cell extracts. Western blot analysis of peptide pull-downs of unphosphorylated (2S) and phosphorylated (2pS) TEL2 peptides with HEK293T whole cell extract from cells treated either with control siRNA (siCon), siRNA targeting PIH1D1 (siPIH1D1) or with siRNA targeting RUVBL1/2 (siRUVBL1/2). The blot was stained with antibodies recognizing members of R2TP complex.).

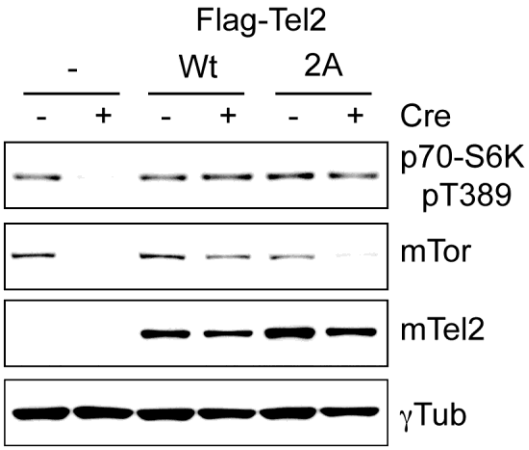

Figure S4. The CK2 phospho-site in Tel2 is required for mTOR stability but is dispensable for mTOR activity in mouse cells. Western blot analysis of mTOR activity in MEFs after the starvation and re-stimulation with serum were shown. MEFs were stably transfected with vector (-), wild type Tel2 (Wt) or the CK2 sites mutant Tel2 (2A) and then deleted endogenous Tel2. Phosphorylation of p70-S6 kinase (T389) was used as readout of mTOR activity. Expression of Tel2-Wt rescued mTOR level and phosphorylation of p70-S6 kinase phosphorylation. Tel2-2A mutant failed to fully restore mTOR level, though it was sufficient to restore mTOR activity to phosphorylate p70-S6 kinase.

SUPPLEMENTAL MATERIALS AND METHODS

Antibodies

The anti-phospho TEL2 antibody was raised and purified by Bigenes, Berlin against phosphorylated peptide CLAGSD[pS]DLD[pS]DDEFV-amide (Biosyntan, Berlin). TEL2 (HCLK2) antibody has been described previously (Collis et al., 2007). Antibodies against ATM (ab91, rabbit polyclonal), mTOR (ab51089, rabbit polyclonal), RMP (ab72134, rabbit polyclonal), UXT (ab77483, goat polyclonal), PIH1D1 (ab57512, mouse monoclonal), HSP90 (ab13492, mouse monoclonal), GST (ab6613, goat polyclonal) RUVBL1 (ab51500, mouse monoclonal and ab75826, rabbit polyclonal) and RUVBL2 (ab36569, rabbit polyclonal) were purchased from Abcam. Antibodies against RPAP3 (WH0079657M1, mouse monoclonal anti-FLJ21908), γ-tubulin GTU-88 (T6557, mouse monoclonal) and antiFlag M2-peroxidase conjugate (A 8592, mouse monoclonal) were purchased from Sigma. ATR (N-19) antibody (sc-1887, goat polyclonal) and Chk1 (sc-8408, rabbit polyclonal) were purchased from Santa Cruz biotechnology, Chk1 pS345 (#2348, rabbit polyclonal) and Chk2 (611570) were purchased from Cell Signaling Technology and from BD Biosciences, respectively. Anti-mTel2 mouse polyclonal antibody was described previously (Takai et al., 2007). ATM (MAT3, Sigma), ATR (N-19, Santa Cruz Biotechnology), DNA-PKcs (Ab-4, Lab Vision), mTOR (Cell Signaling Technology), SMG1 (Bethyl Laboratories), Chk1 (Santa Cruz Biotechnology), Chk1 pS345 (Cell Signaling Technology), Chk2 (BD Biosciences), γ-tubulin (GTU-88, Sigma), PIH1D1 (Abcam), Reptin (TIP49b, BD Biosciences), and Tel2 (mouse polyclonal antibody, Takai *et al*. 2007). Phospho-p70 S6 kinase (Thr389) (108D2, Cell Signaling Technology).