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Ubiquitylation of the amino terminus of Myc by SCF$^{\beta\text{-TrCP}}$ antagonizes SCF^{Fbw7}-mediated turnover

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The SCF^{Fbw7} ubiquitin ligase mediates growth-factor-regulated turnover of the Myc oncoprotein. Here we show that SCF$^{\beta\text{-TrCP}}$ binds to Myc by means of a characteristic phosphodegron and ubiquitylates Myc; this results in enhanced Myc stability. SCF^{Fbw7} and SCF$^{\beta\text{-TrCP}}$ can exert these differential effects through polyubiquitylation of the amino terminus of Myc. Whereas SCF^{Fbw7} with the Cdc34 ubiquitin-conjugating enzyme specifically requires lysine 48 (K48) of ubiquitin, SCF$^{\beta\text{-TrCP}}$ uses the UbcH5 ubiquitin-conjugating enzyme to form heterotypic polyubiquitin chains on Myc. Ubiquitylation of Myc by SCF$^{\beta\text{-TrCP}}$ is required for Myc-dependent acceleration of cell cycle progression after release from an arrest in S phase. Therefore, alternative ubiquitylation events at the N terminus can lead to the ubiquitylation-dependent stabilization of Myc.

Deregulated expression of the c-*myc* proto-oncogene occurs in multiple human tumours, and many experiments with transgenic animals document the oncogenic potential of enhanced c-*myc* expression[1,2]. c-*myc* encodes a nuclear transcription factor, Myc, which can both activate and repress transcription[3]. One of the central functions of Myc is to enhance expression of a broad spectrum of genes involved in nucleotide biogenesis, ribosomal biogenesis and translation. In addition, Myc induces the expression of several cyclins and suppresses the transcription of cyclin-dependent kinase inhibitors. By means of both mechanisms, Myc promotes exit from quiescence and stimulates progression through G1 phase[4]. Elevated expression of Myc also accelerates progression through S phase of the cell cycle[5]. Conversely, depletion or loss of Myc delays cell cycle progression through S and G2 phases[6,7].

Myc protein is unstable and is subject to continuous ubiquitylation and degradation in the proteasome. At least four ubiquitin ligases have been identified that ubiquitylate Myc and regulate its turnover. The binding motif is known for one of them, SCF^{Fbw7} (SCF stands for Skp1/Cul1/F-box protein). The SCF^{Fbw7} complex recognizes Myc that is phosphorylated at threonine 58 (T58) by glycogen synthase kinase 3 (Gsk3)[8,9]. Because Gsk3 is inactivated by Akt-dependent phosphorylation, degradation by SCF^{Fbw7} links Myc turnover to growth-factor-dependent signalling[10]. Several mechanisms disrupt the Fbw7-dependent degradation of Myc in human tumours; for example, point mutations of T58 occur in plasmacytoma, and mutations in *FBW7* are found in multiple human tumours[11,12]. Less is known about the recognition of Myc by the other ubiquitin ligases: the F-box ubiquitin ligase SCF^{Skp2} and the Truss/Ddb1/Cul4 complex bind to the carboxy terminus of Myc; SCF^{Skp2} also binds to MycBoxII, a short sequence that is essential for all biological functions of Myc[13–15]. The Hect-domain ubiquitin ligase HectH9 (ARF-BP1, Huwe1) binds to both N-Myc and c-Myc and mediates the turnover of N-Myc[16,17]. Skp2 and HectH9 also positively affect Myc function, because they are required for the activation and repression of a subset of Myc target genes[13,14,16].

Degradation of Myc by SCF^{Fbw7} has been implicated in controlling Myc stability in G1 phase[10]. In contrast, little is known about the regulation of Myc stability in S and G2 phases. Because mammalian cells are less dependent on external growth factors after passage through the restriction point in late G1, it is possible that Myc is protected from SCF^{Fbw7}-mediated degradation during later phases of the cell cycle. For example, interaction of Aurora A with the N-Myc protein in neuroblastoma cells antagonizes its degradation by SCF^{Fbw7} in G2 phase[18]. We show here that the SCF^{Fbw7} and SCF$^{\beta\text{-TrCP}}$ ubiquitin ligases assemble functionally distinct polyubiquitin chains on the N terminus of Myc and that ubiquitylation by SCF$^{\beta\text{-TrCP}}$ thereby attenuates the degradation of Myc.

RESULTS

β-TrCP stabilizes Myc

We identified two different short hairpin RNAs (shRNAs) targeting the messenger RNA encoding one of the two isoforms of β-TrCP (*FBXW1B*) in a retroviral screen for genes required for Myc-induced apoptosis (Supplementary Information, Fig. S1a)[19]. To understand the mechanism underlying this observation, we expressed either a single shRNA that targets both β-TrCP isoforms or two different pairs of shRNAs targeting individual isoforms of β-TrCP in U2OS cells. Immunoblotting with a specific antibody confirmed that β-TrCP was efficiently depleted in each case (Fig. 1a). As expected, depletion of β-TrCP increased levels of Wee1 and IκBα, two substrates of β-TrCP (Fig. 1b)[20]. However, depletion of β-TrCP also led to a decrease in

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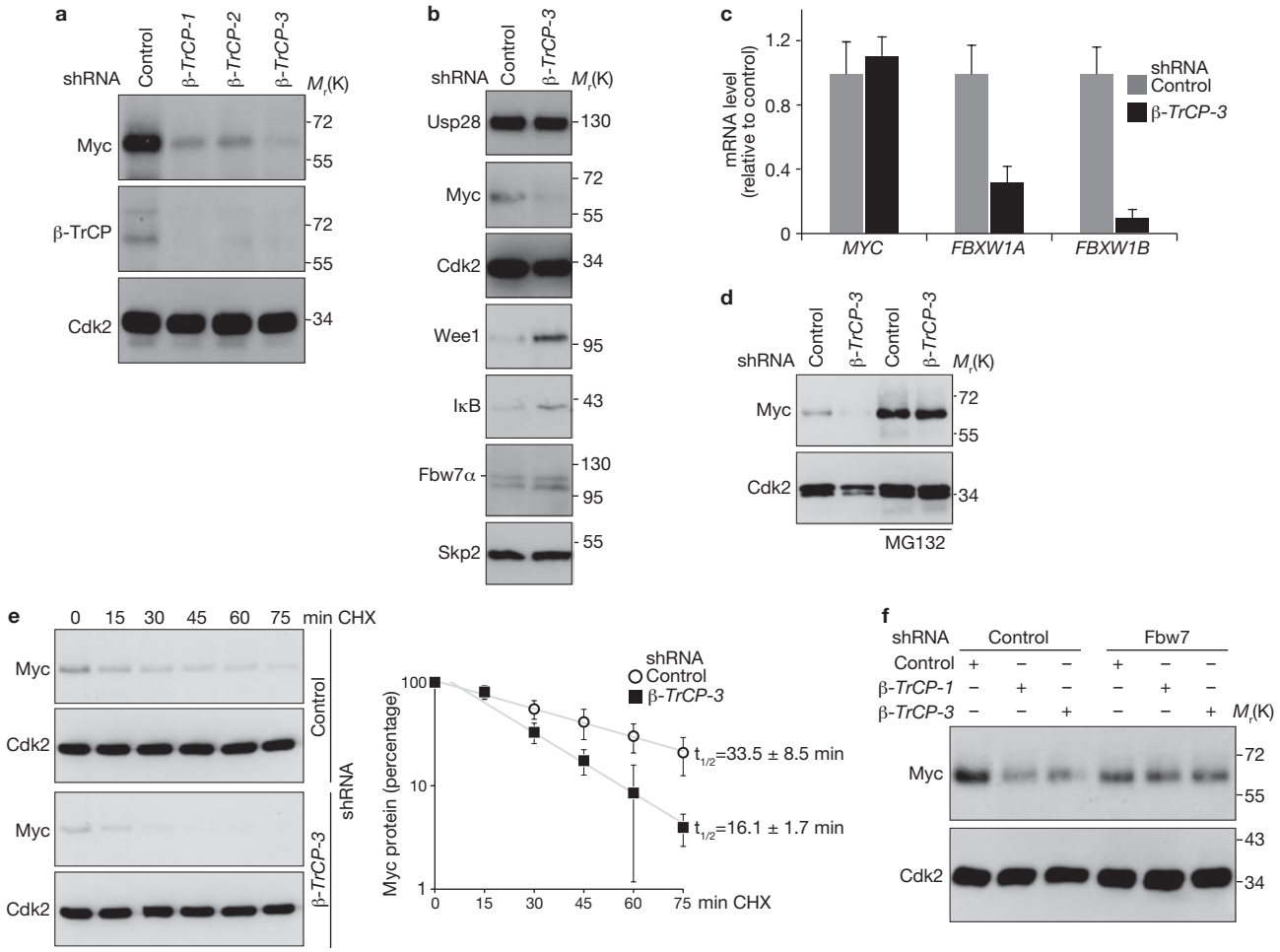

Figure 1 Depletion of β-TrCP stimulates Fbw7-dependent degradation of Myc. (**a**) Depletion of β-TrCP decreases steady-state levels of endogenous Myc. HeLa cells were transfected with pSuper-puro vectors encoding shRNAs against β-TrCP1 and β-TrCP2 (mixture of two different shRNA vectors each targeting one isoform for lanes labelled β-TrCP-1 and β-TrCP-2, and a single vector targeting a sequence common to β-TrCP1 and β-TrCP2 for the lane labelled β-TrCP-3) or a control sequence. Subsequently, cells were washed and selected with puromycin for 36 h, and cultured in puromycin-free medium for a further 24 h. Total cell extracts were prepared and levels of the indicated proteins were determined by immunoblotting. Uncropped images of all immunoblots are shown in Supplementary Information, Fig. S7. (**b**) Depletion of β-TrCP does not affect levels of proteins involved in Myc turnover. HeLa cells were transfected with shRNA vector β-TrCP-3 or control vector as described above. The panels show immunoblots of Myc, Usp28, Wee1 and IκBα, Fbw7, Skp2 and Cdk2. (**c**) Depletion of β-TrCP does not affect levels of *MYC* mRNA. Relative levels of *MYC*, *FBXW1A* (encoding β-TrCP1) and *FBXW1B* (encoding β-TrCP2) mRNAs were assessed by RQ-PCR and are shown on the right. Error bars show s.d. for technical triplicates. (**d**) Inhibition of proteasomal degradation abolishes the effect of β-TrCP depletion on Myc levels. HeLa cells transfected as in **b** were treated with the proteasome inhibitor MG132 as indicated and analysed by immunoblotting. (**e**) Depletion of β-TrCP stimulates turnover of Myc. Cells transfected as in **b** were treated with cycloheximide (CHX; 50 μg ml^{-1}) to block protein synthesis and collected at the indicated time points after addition of the drug. Total cell extracts were examined by immunoblotting. The panel on the right shows a quantification of three independent experiments; errors bars show s.d. (**f**) Co-depletion of Fbw7 alleviates the effect of β-TrCP depletion on Myc levels. HeLa cells were transfected with the indicated combinations of shRNA vectors, and total protein levels were analysed by immunoblotting. Depletion of Fbw7 leads to a roughly twofold decrease in c-*myc* mRNA levels in these cells; steady-state levels of Myc are therefore not enhanced after depletion of Fbw7 (not shown)[47].

levels of endogenous Myc protein, whereas it had no effect on *MYC* mRNA (Fig. 1a–c). The decrease in Myc levels in response to depletion of β-TrCP was reverted by the addition of the proteasome inhibitor MG132, suggesting that depletion of β-TrCP stimulates the proteasomal turnover of Myc (Fig. 1d). Consistently, depletion of β-TrCP led to a decrease in endogenous Myc stability and decreased the half-life ($t_{1/2}$) from 33.5 min to 16.1 min (Fig. 1e). Depletion of β-TrCP also decreased steady-state levels of Myc in non-transformed cells, such as MRC5 and WI38 human fibroblasts, arguing that control of Myc levels by β-TrCP is not restricted to tumour cells (Supplementary Information, Fig. S1b).

Depletion of β-TrCP had marginal effects on the stability of MycT58A, a mutant allele that is not recognized by Fbw7 (Supplementary Information, Fig. S1c, d) and on Myc levels when Fbw7 was simultaneously depleted (Fig. 1f). In contrast, β-TrCP was required to maintain Myc stability when Skp2, Trim32, HectH9 or E6AP was depleted (Supplementary Information, Fig. S1e, and data not shown). Ectopic expression of β-TrCP2 (*FBXW1B*; β-TrCP hereafter) inhibited Fbw7-mediated degradation of Myc (see Fig. 2d). In addition, stable expression of β-TrCP with recombinant retroviruses enhanced steady-state levels of endogenous Myc in U2OS and normal mammary epithelial (IMEC and MCF10A) cells (Supplementary Information, Fig. S1f). We

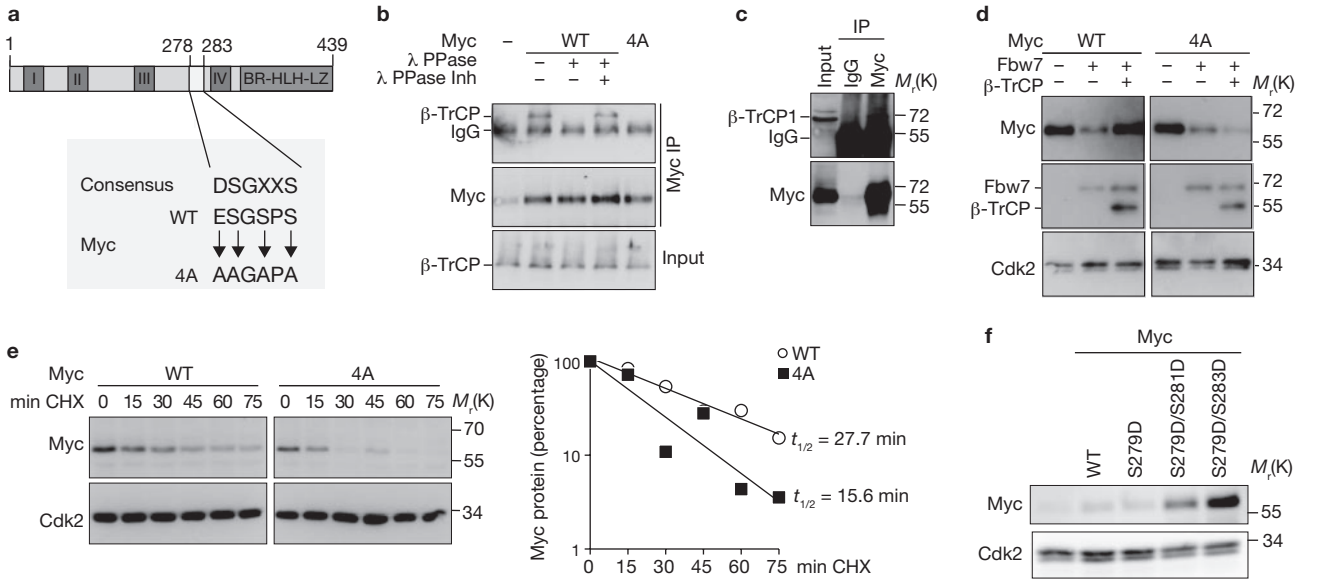

Figure 2 β-TrCP binds and regulates Myc stability through a consensus recognition motif. (**a**) Myc contains a consensus-binding motif for β-TrCP starting at E278. The indicated amino acids were mutated to alanine to generate the Myc4A mutant used in the following experiments. BR-HLH-LZ; basic region-helix-loop-helix-leucine zipper. (**b**) β-TrCP binds Myc by means of a consensus recognition motif in a phosphorylation-dependent manner. HeLa cells were transfected with cytomegalovirus (CMV)-driven expression vectors encoding β-TrCP, wtMyc or Myc4A as indicated, then lysed and immunoprecipitated with anti-Myc antibody. Immunoprecipitates were treated with the λ protein phosphatase (PPase) in the presence or absence of phosphatase inhibitor (Inh) as indicated, and analysed by immunoblotting. (**c**) Interaction of endogenous Myc and β-TrCP proteins. HEK293 cells were lysed and immunoprecipitated (IP) with anti-Myc (N-262) or control antibody. Immunoprecipitates were probed with antibodies directed against Myc and β-TrCP as indicated. The input lane corresponds to 2% of the material used for the immunoprecipitation. (**d**) β-TrCP antagonizes Fbw7-mediated degradation of wtMyc, but not that of Myc4A. HeLa cells were co-transfected with expression vectors encoding wtMyc or Myc4A, Fbw7 and β-TrCP as indicated. At 48 h after transfection, cells were harvested and protein levels were examined by immunoblotting. (**e**) HeLa cells transfected with expression vectors encoding wtMyc or Myc4A were treated with cycloheximide, harvested after the indicated times and analysed by immunoblotting with anti-Myc antibody. The panel on the right shows a quantification of the experiment. (**f**) HeLa cells were transfected with vectors encoding wtMyc or the indicated Myc mutants. At 48 h after transfection, cells were lysed and analysed by immunoblotting.

concluded that β-TrCP antagonizes the Fbw7-mediated degradation of Myc.

Depletion of β-TrCP had similar effects on Myc levels to those of depletion of Usp28, a de-ubiquitylating enzyme that antagonizes Fbw7-mediated degradation; simultaneous depletion of both proteins did not decrease Myc levels further (Supplementary Information, Fig. S2a). In addition, expression of a dominant-negative allele of Usp28 decreased the ability of β-TrCP to stabilize Myc (Supplementary Information, Fig. S2b), suggesting that both proteins act on the same pool of Myc proteins.

β-TrCP is a ubiquitin ligase for Myc

Depletion of endogenous β-TrCP or ectopic expression of β-TrCP had no effect, or only marginal effects, on the levels of several proteins involved in Myc turnover, including Usp28, Fbw7 and Skp2 (Fig. 1b; Supplementary Information, Fig. S2c). Sequence analysis showed that Myc itself contains a sequence ESGSPS at amino-acid residues 278–283 (Fig. 2a) that is highly similar to the phosphodegron (DpSGXXpS) recognized by β-TrCP[21]; replacement of aspartate by glutamate does not affect recognition by β-TrCP[22]. Indeed, β-TrCP was able to bind Myc in co-immunoprecipitation experiments when both proteins were ectopically expressed in HeLa or in non-transformed mammary epithelial cells (Fig. 2b; Supplementary Information, Fig. S2d). Incubation of the immunoprecipitate with λ protein phosphatase abolished binding of β-TrCP in the absence of a phosphatase inhibitor but not in its presence, arguing that β-TrCP binds to phosphorylated Myc (Fig. 2b). Furthermore, immunoblotting revealed the presence of endogenous β-TrCP in anti-Myc immunoprecipitates from HEK293 cells (Fig. 2c). Mutation of four of the amino acids constituting the phosphodegron (generating Myc4A) had no effect on the nuclear localization of Myc but abolished binding to β-TrCP, both in transformed cells and in non-transformed cells (Fig. 2b; Supplementary Information, Figs S2d and S3a). Similar results were obtained for all pairwise replacements of S279, S281 or S283 by alanine, whereas Myc with single amino-acid replacements still bound to β-TrCP (Supplementary Information, Fig. S3b). Expression of β-TrCP antagonized Fbw7-mediated degradation of wild-type Myc (wtMyc) but not that of Myc4A, demonstrating that binding of β-TrCP to Myc is required for stabilization (Fig. 2d). Consistent with a role for endogenous β-TrCP in stabilizing Myc, Myc4A was less stable than wtMyc (Fig. 2e; for wtMyc, $t_{1/2}$ = 27.7 min; for Myc4A, $t_{1/2}$ = 15.6 min). Steady-state levels of Myc4A were also consistently lower than those of wtMyc when both were expressed in several non-transformed cells (Supplementary Information, Fig. S3c, and data not shown). Conversely, mutating either S279/S281 or S279/S283 to the phosphomimetic aspartate led to enhanced levels of Myc (Fig. 2f).

β-TrCP is part of a ubiquitin ligase complex, $SCF^{β\text{-}TrCP}$, raising the possibility that $SCF^{β\text{-}TrCP}$-mediated ubiquitylation is required to attenuate the degradation of Myc. Consistent with this notion, expression of β-TrCP led to efficient ubiquitylation of wtMyc but not that of Myc4A (Fig. 3a). Furthermore, β-TrCP ubiquitylated Myc *in vitro* (Supplementary Information, Fig. S3d). We used a series of β-TrCP mutants to determine

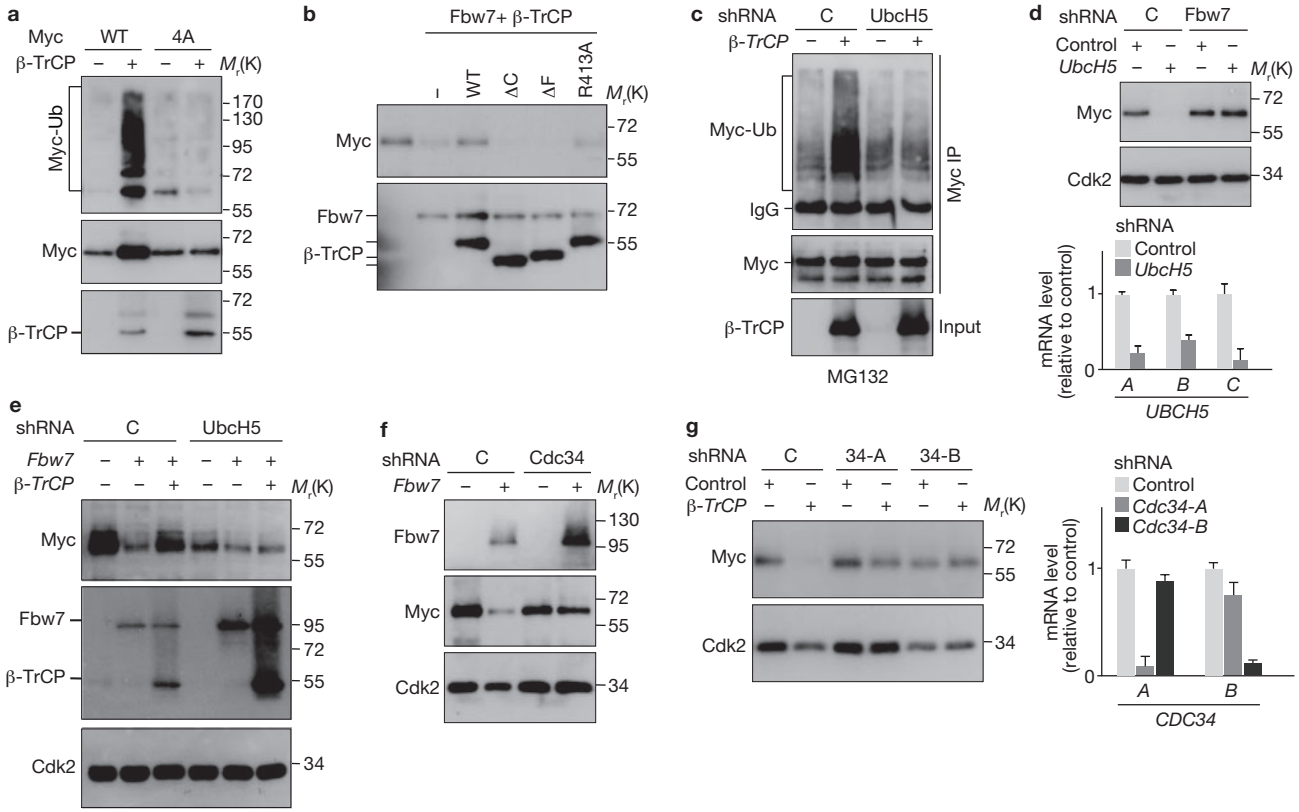

Figure 3 Fbw7 and β-TrCP regulate Myc levels by means of Cdc34-dependent and UbcH5-dependent ubiquitylation, respectively. (**a**) β-TrCP ubiquitylates wtMyc, but not Myc4A, *in vivo*. HeLa cells were transfected with expression vectors encoding wtMyc or Myc4A, His-tagged ubiquitin and β-TrCP as indicated; they were lysed and proteins were collected with Ni^{2+}-nitrilotriacetate (Ni^{2+}-NTA) resin followed by immunoblotting. Ub, ubiquitin. (**b**) Ubiquitylation-deficient mutants of β-TrCP fail to stabilize Myc. HeLa cells were transfected with expression vectors encoding Myc, Flag-tagged Fbw7 and either wild-type β-TrCP (WT) or the indicated mutant alleles (ΔC, deletion of the WD40 repeat domain; ΔF, deletion of the F-box domain; R413A, ubiquitylation-deficient point mutant). At 48 h after transfection, cells were harvested and analysed by immunoblotting. (**c**) β-TrCP requires UbcH5 to ubiquitylate Myc *in vivo*. HeLa cells were transfected with expression vectors encoding wtMyc, haemagglutinin-tagged ubiquitin, β-TrCP and control (C) or UbcH5-targeting shRNA vectors, lysed under denaturing conditions, and precipitated with anti-Myc antibody. Immunoprecipitates were examined by immunoblotting. (**d**) Depletion of UbcH5 decreases steady-state levels of endogenous Myc. HeLa cells were transiently co-transfected with pSuper-puro shRNA vectors targeting UbcH5, Fbw7 or control vector, selected with puromycin and analysed by immunoblotting. The bottom panel shows a RQ-PCR analysis documenting the mRNA levels of all three *UBCH5* isoforms in cells transfected with the shRNA vectors targeting *UBCH5*. Error bars show s.d. for technical triplicates. (**e**) β-TrCP requires UbcH5 to antagonize Fbw7-mediated degradation of Myc. HeLa cells were transfected with expression vectors encoding Flag-tagged Fbw7 and β-TrCP, and pSuper-puro vectors expressing either control or UbcH5-targeting shRNAs. At 72 h after transfection, cells were harvested and lysates were examined for levels of the endogenous Myc protein. (**f**) Fbw7 requires Cdc34 to degrade Myc. HeLa cells were transfected with expression vectors encoding wtMyc, Flag-tagged Fbw7 and pSuper vectors expressing shRNAs targeting Cdc34, and analysed as before. (**g**) Co-depletion of Cdc34 stabilizes Myc on depletion of β-TrCP. HeLa cells transfected with the indicated combinations of pSuper-puro vectors targeting Cdc34A, Cdc34B, β-TrCP and a control sequence were analysed for protein levels by immunoblotting 72 h after transfection. The graph on the right documents the efficiency of knockdown with the indicated Cdc34 shRNAs. Error bars show s.d. for technical triplicates.

whether domains involved in $SCF^{β\text{-}TrCP}$ function were required to stabilize Myc (Fig. 3b). Deletion of the WD40 domain that mediates binding of β-TrCP to its substrate (ΔC) as well as deletion of the F-box that binds Skp1 and recruits β-TrCP into the $SCF^{β\text{-}TrCP}$ complex (ΔF) abrogated the ability of β-TrCP to antagonize Fbw7-mediated degradation (Fig. 3b). Furthermore, mutation of arginine 413 of β-TrCP2 (equivalent to R474 of β-TrCP1), which contacts the aspartate residue of the DSG motif, to alanine caused a similar loss of the ability of β-TrCP to stabilize Myc (Fig. 3b)[23]. Similarly, β-TrCP-LI65EE, a mutant of β-TrCP that is deficient in dimerization, led to a decrease in steady-state levels of Myc, arguing that stabilization of Myc is mediated by dimeric β-TrCP (Supplementary Information, Fig. S3e)[24].

To rigorously demonstrate a requirement for ubiquitylation in the β-TrCP-mediated stabilization of Myc, we made use of the observation that β-TrCP uses UbcH5 to ubiquitylate IκB[25]. Consistently, shRNA-mediated depletion of UbcH5 abrogated the ubiquitylation of Myc by β-TrCP (Fig. 3c). Depletion of UbcH5 also decreased steady-state levels of endogenous Myc in HeLa cells (Fig. 3d, e). This effect was abrogated by co-depletion of Fbw7, demonstrating that depletion of UbcH5 promotes the degradation of Myc (Fig. 3d). Furthermore, ectopic expression of β-TrCP antagonized Fbw7-mediated degradation of Myc in control cells but not in cells depleted of UbcH5, demonstrating that stabilization of Myc by β-TrCP requires UbcH5 (Fig. 3e). This experiment also shows that Fbw7 degrades Myc in the absence of UbcH5, raising the question of which Ubc is used by Fbw7. Consistent with the finding that the yeast orthologue of Fbw7, Cdc4, requires Cdc34 for the ubiquitylation of its substrates, depletion of Cdc34 abrogated the Fbw7-mediated degradation of Myc (Fig. 3f)[26]. Furthermore, depletion of Cdc34 inhibited the destabilization of Myc observed in the absence of β-TrCP (Fig. 3g). The data show that

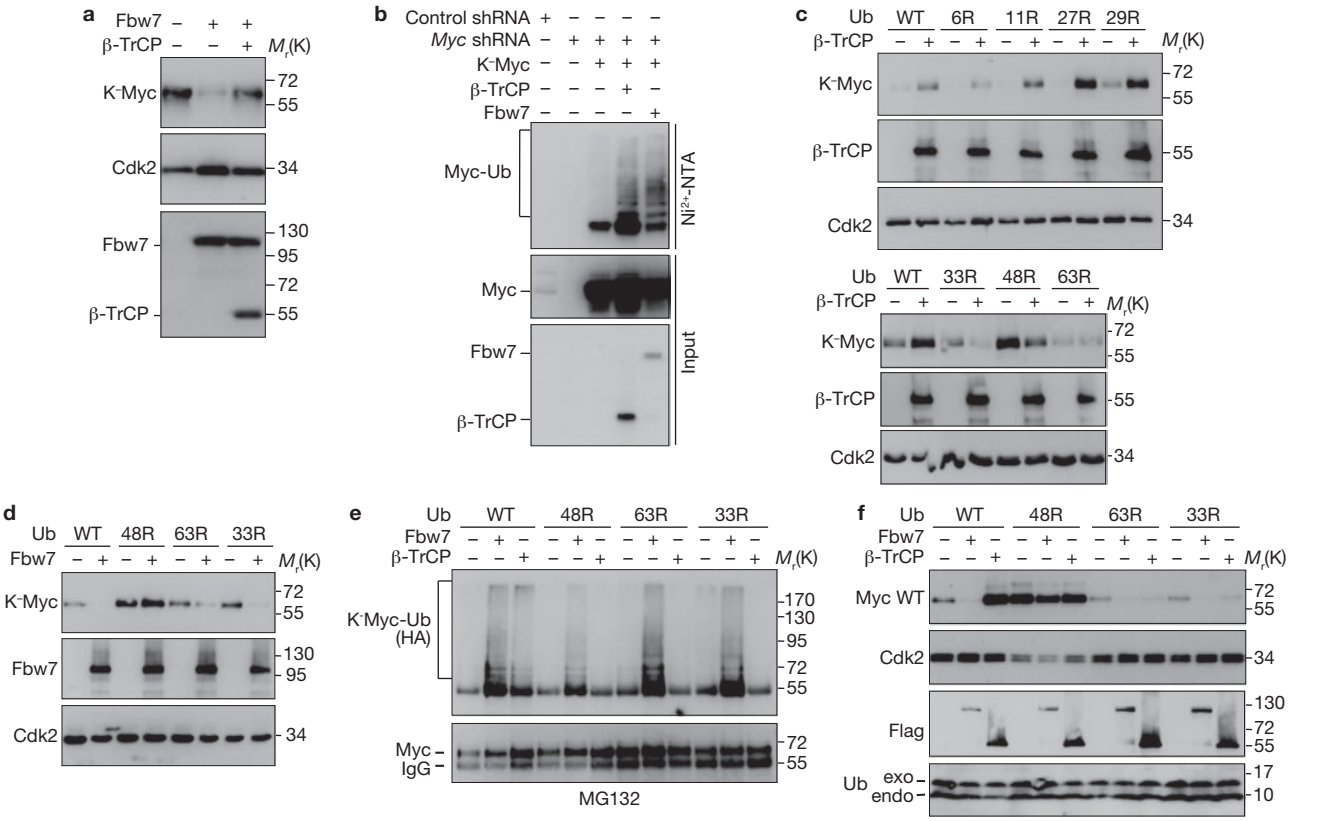

Figure 4 Fbw7 and β-TrCP regulate Myc turnover through the assembly of polyubiquitin chains with different linkages on Myc. (**a**) β-TrCP antagonizes the degradation of K-Myc by Fbw7. HeLa cells were transfected with expression vectors encoding K-Myc, Flag-tagged Fbw7 and β-TrCP, and analysed as before. (**b**) Both Fbw7 and β-TrCP assemble polyubiquitin chains on K-Myc *in vivo*. HeLa cells were co-transfected with shRNA targeting the 3′ untranslated region (UTR) of the endogenous Myc mRNA and expression vectors encoding K-Myc, His-tagged ubiquitin and the indicated F-box proteins. One day after transfection, cells were washed and selected with puromycin for 36 h. Cells were lysed, and ubiquitylated proteins were recovered with Ni^{2+}-NTA resin, followed by immunoblotting with anti-Myc antibodies. (**c**) β-TrCP requires K33, K48 and K63 of ubiquitin to stabilize K-Myc. HeLa cells were transfected with expression vectors encoding Myc, Flag-tagged β-TrCP and either WT ubiquitin or mutant alleles in which the indicated lysine had been replaced by arginine. Cells were harvested and Myc levels were assessed by immunoblotting. (**d**) Fbw7 requires K48, but not K33 or K63, of ubiquitin to degrade K-Myc. The experiment was performed as in **c**. (**e**) Fbw7 requires K48 of ubiquitin, whereas β-TrCP requires K33, K48 and K63, to ubiquitylate K-Myc. HeLa cells were transfected with expression vectors encoding K-Myc, Fbw7 or β-TrCP, and either WT or mutant alleles of haemagglutinin-tagged ubiquitin. Cells were harvested after 48 h and ubiquitylated Myc was recovered by immunoprecipitation. (**f**) β-TrCP and Fbw7 require different lysine residues of ubiquitin to regulate levels of wtMyc. HeLa cells were transfected with expression vectors encoding wtMyc, Flag-tagged Fbw7 or β-TrCP, and either WT or mutant alleles of haemagglutinin-tagged ubiquitin as indicated. Total protein levels were analysed by immunoblotting.

β-TrCP requires UbcH5 to ubiquitylate and attenuate the degradation of Myc, whereas Fbw7 uses Cdc34 to degrade Myc.

β-TrCP and Fbw7 ubiquitylate the N terminus of Myc

We considered several hypotheses about how β-TrCP might stabilize Myc. β-TrCP antagonized the Fbw7-mediated degradation of Myc but not that of N-Myc or of cyclin E, arguing that both F-box proteins do not compete for a limiting pool of central Skp1/Cul1/Rbx1 modules (Supplementary Information, Fig. S4a). Furthermore, Myc did not induce heterodimerization of both F-box proteins (not shown). Next, we set out to identify the lysine residues in Myc that are ubiquitylated by Fbw7 and β-TrCP. A series of mutant alleles of Myc in which individual or multiple lysine residues were replaced by arginine were all ubiquitylated and degraded by Fbw7 (not shown). We therefore generated lysine-free (K$^-$)Myc, in which all lysine residues were replaced by arginine. Fbw7 degraded K-Myc and this was reverted by co-expression of β-TrCP (Fig. 4a). Furthermore, both Fbw7 and β-TrCP ubiquitylated K-Myc *in vitro* almost as efficiently as wtMyc (Supplementary Information, Fig. S3c). Therefore, Fbw7 and β-TrCP can ubiquitylate Myc and regulate its stability through the N terminus. K-Myc bound both Max and Miz1, arguing that it is not an unfolded protein (Supplementary Information, Fig. S4b). This is supported by the finding that K-Myc induced apoptosis with a similar efficiency to that of wtMyc; in contrast, K-Myc was less efficient in inducing cell proliferation, which is consistent with observations that ubiquitylation of Myc is required for its mitogenic properties[13,14,16] (Supplementary Information, Fig. S4c). Furthermore, ubiquitylation of K-Myc by Fbw7 and β-TrCP depended on the integrity of their respective recognition motifs, demonstrating that recognition of K-Myc by both ligases is identical to that of wtMyc (Supplementary Fig. 4d, e). Both Fbw7 and β-TrCP assembled extended polyubiquitin chains on K-Myc *in vivo* and *in vitro*, making it unlikely that models in which different chain lengths account for the different effects on Myc stability are correct (Fig. 4b; Supplementary Information, Fig. S3d)[27].

Recently, different linkages between the individual ubiquitin moieties in a polyubiquitin chain have been shown to cause differences in rates

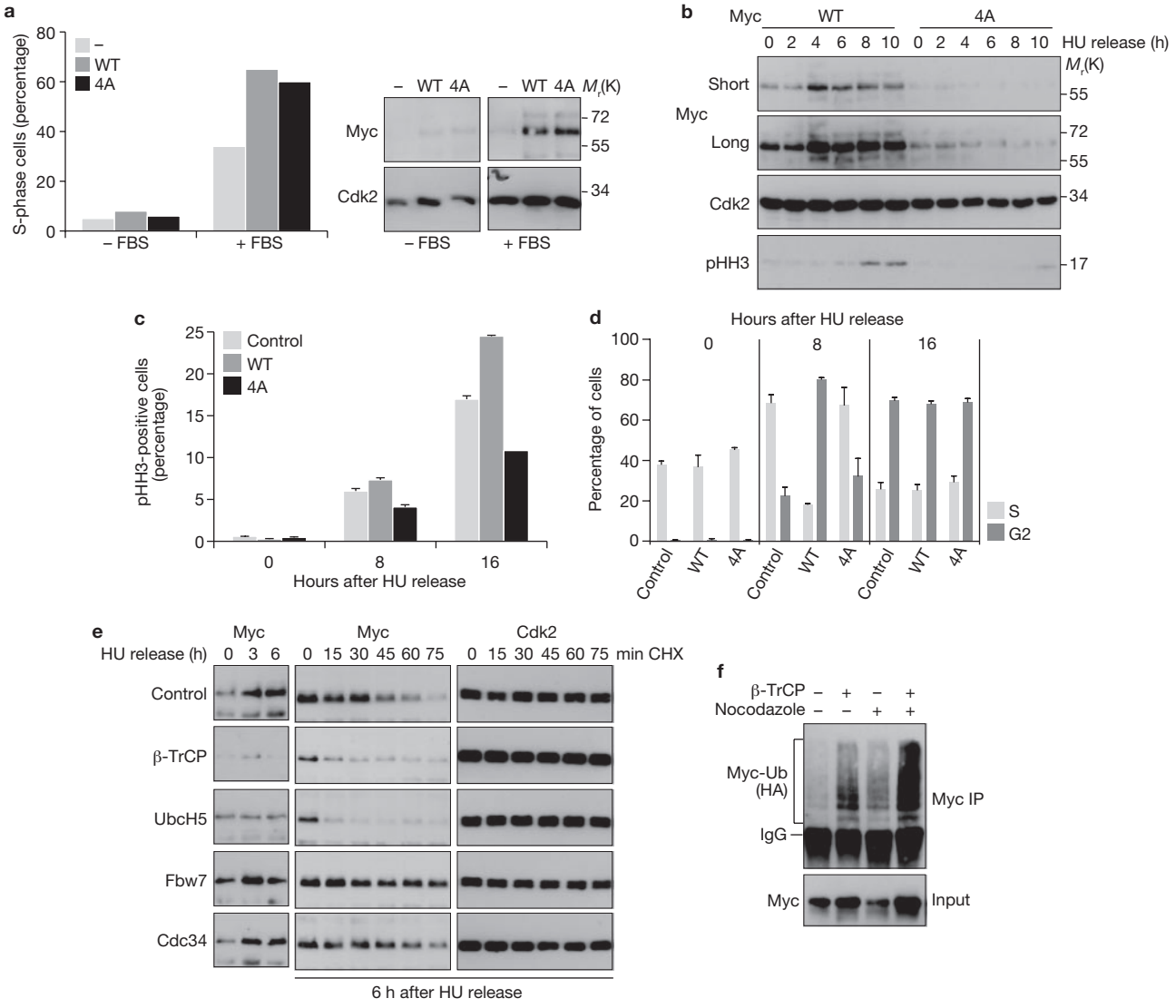

Figure 5 β-TrCP-dependent ubiquitylation is required for Myc-dependent cell cycle progression during S and G2 phases. (**a**) wtMyc and Myc4A do not differ in their ability to stimulate G1 progression. NIH/3T3 mouse fibroblasts were transduced with retroviruses encoding wtMyc or Myc4A along with a control vector; cells were selected and pools were arrested by serum deprivation for 48 h and released in 10% FBS. The percentage of cells in S phase was determined by staining with propidium iodide followed by FACS analysis. Protein levels were examined by immunoblotting. (**b**) wtMyc and Myc4A differ in their ability to stimulate G2 progression after release from a block in S phase. U2OS cells stably expressing wtMyc or Myc4A were arrested at the G1/S boundary with hydroxyurea (HU) for 24 h, and then released into nocodazole-containing medium for the indicated times. Total protein levels were analysed by immunoblotting. (**c**) FACS analysis documenting stimulation of mitotic entry by wtMyc but not by Myc4A. the experiment was performed as in **b**, but cells were fixed overnight with 70% ethanol, stained with an antibody that recognizes phosphorylated histone H3 (pHH3) and propidium iodide, and analysed by flow cytometry. The graph shows the percentage of pHH3-positive cells. Error bars show s.d. for technical triplicates. (**d**) Quantification of the propidium iodide–FACS analysis described in **c**. Error bars show s.d. for technical replicates. (**e**) β-TrCP and UbcH5 are required for the stability and accumulation of endogenous Myc after release from a hydroxyurea-mediated arrest. U2OS cells were infected with retroviruses expressing shRNA targeting the indicated proteins, and were subsequently arrested and released as described in **c**. The left panels document total Myc levels, and the middle panels show the stability of Myc protein after the addition of cycloheximide; these assays were performed at 6 h after HU release. (**f**) β-TrCP-dependent ubiquitylation of Myc is enhanced in nocodazole-arrested cells. HeLa cells were transiently transfected with wtMyc, haemagglutinin (HA)-tagged ubiquitin, and β-TrCP. At 24 h after transfection, cells were washed with PBS, and supplemented with fresh medium containing nocodazole where indicated. After 16 h, cells were then lysed under denaturing conditions, ubiquitylated Myc was recovered as described above, and proteins were analysed by immunoblotting.

of degradation, suggesting that this might cause the different effects of Fbw7 and β-TrCP[28–30]. Consistent with such a view, single substitutions of K33, K48 or K63 of ubiquitin diminished or abolished the ability of β-TrCP to stabilize Myc, demonstrating that the formation of heterotypic chains is required for the stabilization of K⁻Myc by β-TrCP (Fig. 4c). In contrast, only the replacement of K48 of ubiquitin abrogated the ability of Fbw7 to degrade K⁻Myc, arguing that Fbw7 forms homotypic polyubiquitin chains for degradation (Fig. 4d and data not shown). This is supported by ubiquitylation assays demonstrating that replacement of K33, K48 or K63 of ubiquitin all abolished the ability of β-TrCP to ubiquitylate K⁻Myc (Fig. 4e). In contrast, only replacement of K48 impaired the formation of high-molecular-mass ubiquitin conjugates on K⁻Myc by

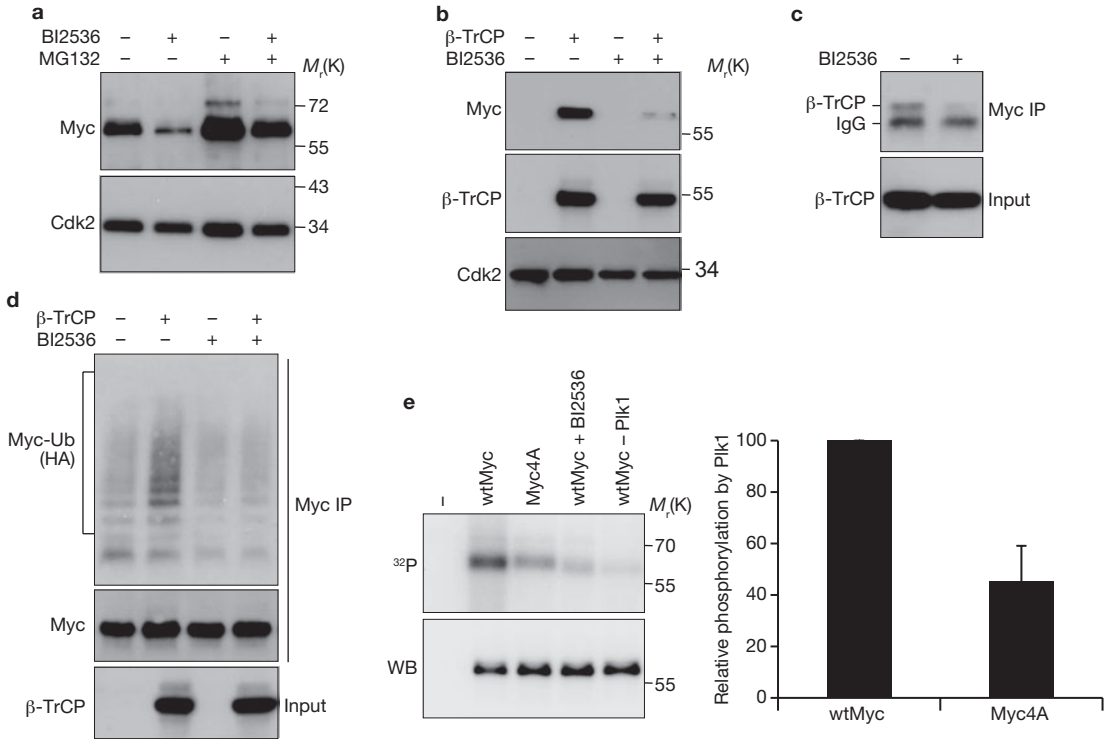

Figure 6 Polo-like kinase 1 (Plk1) regulates β-TrCP-dependent ubiquitylation and Myc stability in G2 phase. (**a**) Inhibition of Plk1 triggers the proteasome-dependent degradation of Myc. U2OS cells stably expressing wtMyc were arrested by the addition of hydroxyurea and treated with the Plk1 inhibitor BI2536 (50 nM) for 3 h during the release from the arrest in the absence or presence of MG132, and protein levels were assessed by immunoblotting. (**b**) β-TrCP-dependent stabilization of Myc requires Plk1 activity. HeLa cells were transfected with wtMyc and Flag-tagged β-TrCP. At 24 h after transfection, cells were treated for 20 h with hydroxyurea, washed and released into fresh medium in the presence or absence of BI2536 for 6 h, and analysed by immunoblotting. (**c**) Binding of β-TrCP to Myc depends on Plk1 activity. HeLa cells were transfected with Flag-tagged β-TrCP, treated with MG132 in the presence or absence of BI2536 for 16 h, lysed and then immunoprecipitated with anti-Myc antibody. Protein complexes were analysed by immunoblotting. (**d**) Plk1 stimulates the ubiquitylation of Myc by β-TrCP. HeLa cells that had been transfected with expression vectors encoding wtMyc, Flag-tagged β-TrCP and HA-tagged ubiquitin were treated with MG132 in the presence or absence of BI2536 as indicated, and ubiquitylation of Myc was analysed as described above. (**e**) Plk1 phosphorylates Myc *in vitro*. HEK293 cells were transfected with expression vectors for wtMyc or Myc4A. At 48 h after transfection, cells were lysed and immunoprecipitated with anti-Myc antibody. Immunopurified proteins were incubated with Plk1 and ^{32}Pγ-ATP, reactions were boiled in SDS sample buffer, resolved by SDS–PAGE, and gels were dried and analysed by autoradiography. In parallel, immunoprecipitates were assayed by immunoblotting with anti-Myc antibody; where indicated, 100 nM BI2536 was added. WB, western blot. The right panel shows a quantification of five independent experiments; error bars show s.d.

Fbw7 (Fig. 4e). Fbw7 also required K48 to degrade wtMyc, and β-TrCP required K63 and K33 of ubiquitin to stabilize it (Fig. 4f); finally, mutation of K48 blocked the ubiquitylation of wtMyc by Fbw7 but not by β-TrCP (Supplementary Information, Fig. S5a). The data show that the polyubiquitin chains assembled by Fbw7 and β-TrCP on both wtMyc and K$^-$Myc differ in the linkages between the ubiquitin moieties. In particular, β-TrCP efficiently ubiquitylated T58AMyc and T58AK$^-$Myc yet had no detectable effect on the turnover of these proteins (Supplementary Information, Figs S1c, d and S5b, and data not shown), arguing that the polyubiquitin chains assembled by β-TrCP do not target Myc to the proteasome *in vivo*.

Ubiquitylation of Myc by β-TrCP is required for release from S-phase arrest

To understand the physiological significance of these findings, we compared the response of cells to retroviral expression of wtMyc or Myc4A. We observed no difference in the ability of either protein to induce apoptosis in serum-starved cells (not shown). Furthermore, both wtMyc and Myc4A accelerated the progression of NIH/3T3 fibroblasts and U2OS cells through G1 phase to a similar extent (Fig. 5a and data not shown). In contrast, wtMyc and Myc4A differed when we arrested U2OS cells in early S phase by hydroxyurea and measured their progression into mitosis after release from the arrest. Addition of hydroxyurea lowered steady-state levels of both wtMyc and Myc4A (Supplementary Information, Fig. S6a). On release from arrest, levels of wtMyc increased, whereas those of Myc4A remained low (Fig. 5b)[31]. Whereas expression of wtMyc accelerated progression into mitosis relative to control cells, as indicated by the percentage of cells that stained positive for phosphorylated histone H3, Myc4A was unable to do so (Fig. 5c). Fluorescence-activated cell sorting (FACS) analysis of the DNA content after release from hydroxyurea showed that the difference between cells expressing wtMyc and those expressing Myc4A was detectable as early as late S phase (Fig. 5d; see time point at 8 h). Furthermore, depletion of β-TrCP or of UbcH5, but not of Cdc34 or Fbw7, prevented the accumulation of endogenous Myc and stimulated its turnover after release from S-phase arrest (Fig. 5e). Depletion of β-TrCP or UbcH5 delayed progression into mitosis, whereas knockdown of Cdc34 stimulated it (Supplementary Information, Fig. S6b). Consistently, *in vivo* ubiquitylation assays showed that synchronization of cells in mitosis by the addition of nocodazole enhanced the ability of β-TrCP to ubiquitylate Myc and attenuate Myc

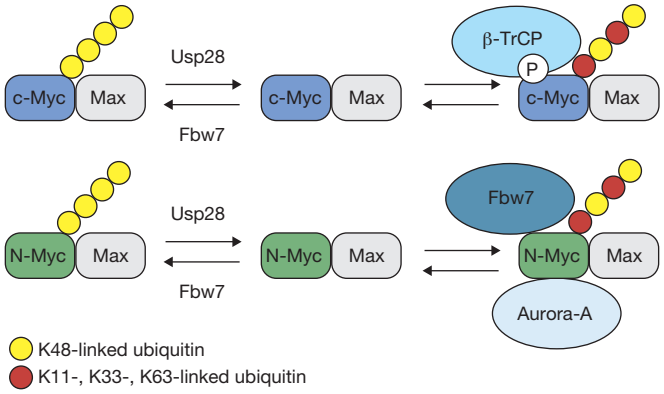

Figure 7 Model describing our findings. Myc protein levels are dynamically controlled by different ubiquitin ligases and de-ubiquitylating enzymes. Fbw7-dependent assembly of K48-linked ubiquitin chains induces rapid Myc degradation; ubiquitylation can be reversed by the Usp28 de-ubiquitylating enzyme[19]. Phosphorylation of c-Myc by the Plk1 kinase during recovery from replicative stress triggers ubiquitylation by the β-TrCP ubiquitin ligase, which assembles atypical chains on c-Myc and attenuates proteasome-dependent turnover. Fbw7-mediated ubiquitylation of the N-Myc protein can be altered by the Aurora A kinase in G2, leading to the assembly of heterotypic chains and the transient stabilization of N-Myc[17].

degradation (Fig. 5f). The data show that factors that are present in S and G2/M promote the ubiquitylation of Myc by β-TrCP and that this is required for Myc to facilitate recovery from an S-phase arrest.

Progression through G2 is promoted by Aurora A and Polo-like kinase 1 (Plk1)[32,33]. Because depletion of Aurora A has little effect on (c-)Myc stability, we used a specific inhibitor of Plk1, namely BI2536, to test whether inhibition of Plk1 affects Myc turnover[34,35]. Incubation of cells with BI2536 led to a decrease in levels of endogenous Myc, which was reverted by the addition of MG132, arguing that inhibition of Plk1 promotes the turnover of Myc (Fig. 6a). Furthermore, incubation with BI2536 attenuated the ability of β-TrCP to stabilize Myc, inhibited the association of β-TrCP with Myc, and abolished the ability of β-TrCP to promote the ubiquitylation of Myc (Figs 6b–d). These findings suggest a model in which phosphorylation of Myc by Plk1 promotes association with β-TrCP. Consistently, Plk1 phosphorylated Myc *in vitro* (Fig. 6e). Relative to wtMyc, phosphorylation of Myc4A by Plk1 was decreased, demonstrating that at least one of the sites in Myc that are phosphorylated by Plk1 is contained in the phosphodegron recognized by β-TrCP.

DISCUSSION

We have shown here that the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase stabilizes the Myc protein on recovery from an S-phase arrest in a ubiquitylation-dependent manner. The ability of $SCF^{\beta\text{-}TrCP}$ to stabilize Myc depends on UbcH5. Previous work had shown that UbcH5 mediates the $SCF^{\beta\text{-}TrCP}$-dependent ubiquitylation of several substrate proteins. In contrast, degradation of Myc by SCF^{Fbw7} depends on Cdc34. Cdc34 and UbcH5 differ in the linkage that they catalyse: Cdc34 promotes the synthesis of K48-linked chains because the enzyme contains an acidic loop that orients the substrate-bound ubiquitin in a position that is incompatible with other linkages[36]. In contrast, chains assembled by UbcH5 can carry different linkages because UbcH5 self-assembles, by means of binding to ubiquitin on the neighbouring activated UbcH5, allowing more flexible linkages[37,38]. Consistent with these data, ubiquitylation of Myc by SCF^{Fbw7} is impaired by mutation of K48 of ubiquitin, but not by mutation of other residues. In contrast, individual mutations of several lysine residues abolish the ability of $SCF^{\beta\text{-}TrCP}$ to ubiquitylate and stabilize Myc, demonstrating that $SCF^{\beta\text{-}TrCP}$ assembles heterotypic chains that carry different linkages in a single chain.

The differently linked chains assembled by $SCF^{\beta\text{-}TrCP}$ and SCF^{Fbw7} correlate closely with different effects of both ligases on Myc stability: whereas SCF^{Fbw7} degrades Myc, $SCF^{\beta\text{-}TrCP}$ stabilizes Myc. A mutant of Myc that does not bind $SCF^{\beta\text{-}TrCP}$ is ubiquitylated by SCF^{Fbw7} and vice versa; therefore both SCF complexes bind Myc independently of each other. Both SCF complexes can ubiquitylate and regulate Myc stability through the N terminus of Myc. The simplest explanation of our findings is that stabilization of Myc by $SCF^{\beta\text{-}TrCP}$ results from the ubiquitylation of the same acceptor site — the N terminus and potentially additional residues — that is used by SCF^{Fbw7} and reflects the difference in efficiencies by which different polyubiquitin chains are degraded by the proteasome. This model is supported by the observation that stabilization of Myc by $SCF^{\beta\text{-}TrCP}$ requires Usp28, a de-ubiquitylating enzyme that is associated with SCF^{Fbw7} and removes ubiquitin chains that are assembled by SCF^{Fbw7} (see Fig. 7)[19]. Furthermore, β-TrCP efficiently ubiquitylates T58A (a mutant of Myc that does not bind Fbw7) but does not regulate the stability of this protein, demonstrating that the ubiquitin chains assembled by β-TrCP do not target Myc to the proteasome. A similar model has recently been proposed for the Ring1B ubiquitin ligase: here, auto-ubiquitylation at a cluster of lysine residues activates the enzyme, whereas ubiquitylation of the same residues by E6AP leads to its degradation[30]. Exactly how the topology of polyubiquitin chains influences degradation by the proteasome is under intensive investigation[28]. For example, our data do not discriminate whether the chains assembled by $SCF^{\beta\text{-}TrCP}$ are linear or branched; the latter have recently been shown to be non-degradable[29].

Ubiquitylation by $SCF^{\beta\text{-}TrCP}$ occurs preferentially in G2 phase and is required for the ability of Myc to facilitate recovery from an S-phase arrest. The residues in Myc that constitute the binding site for $SCF^{\beta\text{-}TrCP}$ are phosphorylated by Plk1, which is most active in G2 and M phases[33]. Our observations suggest a model in which phosphorylation of Myc by Plk1 recruits $SCF^{\beta\text{-}TrCP}$.

Two models could account for the requirement of Plk1 and $SCF^{\beta\text{-}TrCP}$ for Myc function in S and G2 phases. First, Myc acts upstream of FoxM1, a transcription factor that coordinates the expression of a large program of genes required for progression through G2/M and that itself requires phosphorylation by Plk1 for its activity[39]. Attenuation of Myc degradation may therefore be required to establish a specific pattern of gene expression in G2. However, real-time quantitative PCR (RQ-PCR) experiments failed to reveal differences in expression of *FOXM1* or other proposed targets of Myc between cells expressing wtMyc and Myc4A, suggesting that this is not a critical function of Myc regulation by Plk1 and $SCF^{\beta\text{-}TrCP}$. Furthermore, neither the level of Myc nor its degradation by Fbw7 fluctuate during an unperturbed cell cycle[31,40].

Levels of total Myc and Myc phosphorylated at T58 increase after release from a checkpoint-mediated arrest in S phase, arguing that Fbw7-mediated degradation of Myc is attenuated under these conditions[31]. Plk1 is dispensable for progression through the G2 phase of an unperturbed cell cycle but has essential functions on recovery from checkpoint arrest, suggesting that Myc and Plk1 act in common pathways that promote DNA replication and recovery from checkpoint-mediated

arrest[33,41,42]. During S phase, Plk1 binds to the Mcm2 and Mcm7 helicases and antagonizes the inactivation of replication origins by the Atm and Atr kinases[42]. Similarly, Myc binds to Mcm7 and promotes DNA replication in a transcription-independent manner[43,44]. Stabilization of Myc may therefore be a critical function of Plk1 after release from a hydroxyurea-mediated arrest in S phase. During G2, phosphorylation of the checkpoint protein claspin by Plk1 allows its ubiquitylation by $SCF^{\beta\text{-}TrCP}$ and its subsequent proteasomal degradation, ending Atr-dependent signalling[45,46]. Similarly, Myc displaces the TopBP1 protein, which activates the Atr kinase, from its binding site on Miz1, thereby facilitating its ubiquitylation by HectH9 and its subsequent degradation, arguing that attenuation of Myc degradation contributes to the termination of checkpoint responses in G2 (ref. 47).

The binding site for $SCF^{\beta\text{-}TrCP}$ is not conserved in the related N-Myc and L-Myc proteins. We have recently found that Aurora A binds to a complex of N-Myc and Fbw7 and stabilizes N-Myc, but not c-Myc, in G2 phase[35]. Potentially, therefore, N-Myc and c-Myc have undergone convergent evolution to allow cells to attenuate Fbw7-mediated degradation in response to specific intracellular signals (Fig. 7).

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/

Note: Supplementary Information is available on the Nature Cell Biology website.

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AUTHOR CONTRIBUTIONS

N.P., C.S. and L.A.J. performed the experimental work. N.P. and M.E. planned the experiments. M.E. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Plasmids. To construct shRNA vectors, 60 bp hairpin oligonucleotides were designed and subcloned into pSuper-puro vectors (Oligoengine), as previously described[19]. The following 19-mer sequences were targeted: *MYC* 3′ UTR, 5′-ACACAATGTTTCTCTGTAA-3′; *FBXW1A*-1, 5′-GGAACTGTGTGTCAAATAC-3′; *FBXW1A*-2, 5′-GAGAAGGCACTCAAGTTTA-3′; *FBWX1B*-1, 5′-ACTCGGTGATTGAGGACAA-3′; *FBWX1B*-2, 5′-CGTCAATGTAGTAGACTTT-3′; *FBXW1A/FBWX1B*, 5′-GTGGAATTTGTGGAACATC-3′; *FBXW7*-1, 5′-GAATGGAACTCAAAGACAA-3′; *FBXW7*-2, 5′-GGGAAAGAAACCATGCAAA-3′; *USP28*-1, 5′-GTGGCATGAAGATTATAGT-3′; *USP28*-2, 5′-GGAGTGAGATTGAACAAGA-3′; *UBCH5A* (*UBE2D1*), 5′-GAGAATGGACTCAGAAATA-3′; *UBCH5B* (*UBE2D2*), 5′-CAGCATTTGTCTTGATATT-3′; *UBCH5C* (*UBE2D3*), 5′-AGAGATAAGTACAACAGAA-3′; *CDC34A* (*UBE2R1*), 5′-GGAAGTGGAAAGAGAGCAA-3′; *CDC34B* (*UBE2R1*), 5′-GGAAATGGAGAGACAGTAA-3′; *SKP2*-1, 5′-CAAATTTAGTGCGACTTAA-3′; *SKP2*-2, 5′-GAGCAAAGGGAGTGACAAA-3′; *HECTH9*-1, 5′-CTACAATGGTGCAGGTTAA-3′; *HECTH9*-2, 5′-GCAGCAGTACAGACTTTAA-3′; *E6AP*-1, 5′-CCATATTGTGATAAGGTAA-3′; *E6AP*-2, 5′-CCATTTGTAGACCACGTAA-3′; *TRIM32*-1, 5′-GCACTATTATGTAAAGATA-3′; *TRIM32*-2, 5′-CGTCTTTACCAGTATGAAA-3′; *FBXW8*-1, 5′-CGTAGTTGTTCTAATATTA-3′; *FBXW8*-2, 5′-GAATTTACTTGGATTTAAA-3′.

The shRNA sequence targeting *FBXW1A/B* has been published[48]. CMV-driven expression vectors encoding Fbw7 and β-TrCP2 were a gift from Markus Welcker and Bruce Clurman. Complementary DNAs encoding Cul1, Rbx1, Skp1, Uba1, UbcH5 and Cdc34 were cloned by RT–PCR from HeLa-cell mRNA into pcDNA3, pcDNA3–haemagglutinin (HA) or pRSET vectors (Invitrogen). Expression vectors for His-tagged ubiquitin and wtMyc have been described previously[19,35]. HA-tagged ubiquitin expression vectors were generated by PCR. All mutations were introduced by using PCR and confirmed by sequencing.

Cell culture. HeLa, U2OS and NIH/3T3 cells were cultured in DMEM medium (Lonza) containing 10% FBS (Sigma). Immortalized mammary epithelial cells (IMECs) were cultured in DMEM/F12 medium (Gibco) with 0.5 μg ml^{-1} hydrocortisone, 10 ng ml^{-1} epidermal growth factor and 5 μg ml^{-1} insulin (all from Sigma) as described[49]. Hydroxyurea was used at 1.5 mM final concentration.

Immunoprecipitation and immunoblotting. For immunoprecipitation, cells were lysed for 30 min in IP buffer containing 50 mM Tris-HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Nonidet P40 and protease inhibitor cocktail (Calbiochem) on ice. Cell lysates were cleared by centrifugation and immunoprecipitated with the indicated antibodies for 2 h to overnight at 4 °C. Protein complexes were collected by incubation for 2 h with Protein A-Sepharose or protein G-Sepharose beads (Sigma). Immunoprecipitates were washed three times with IP buffer, boiled in SDS sample buffer and analysed by immunoblotting as described previously[35].

The following antibodies were used: anti-Myc N-262 and C-33, anti-Cdk2 M2, anti-ubiquitin P4D1, anti-Skp2 H-435, anti-Wee1 C-20, anti-IκB C-21, anti-Miz1 H-190, anti-Max C-17 (Santa Cruz), anti-HA HA.11 (Covance), anti-Flag M2 (Sigma), anti-β-TrCP1 (Zymed/Invitrogen) and anti-Fbw7 (a gift from Axel Behrens).

***In vivo* ubiquitylation assays.** HeLa cells were co-transfected with CMV-driven expression vectors encoding Myc, Fbw7, β-TrCP and HA-tagged ubiquitin, and lysed by boiling for 10 min in buffer containing 20 mM Tris-HCl pH 7.4, 5 mM EDTA, 10 mM dithiothreitol (DTT) and 2% SDS. Lysates were diluted 1:10 with IP buffer, centrifuged for 10 min at 16,000*g* and immunoprecipitated with anti-Myc antibodies. Precipitates were analysed by immunoblotting with anti-HA antibodies. Ubiquitylation assays with His-tagged Myc or His-tagged ubiquitin were performed by affinity purification on Ni^{2+}-NTA resin (Qiagen) as described previously[19].

***In vitro* ubiquitylation assay.** To obtain SCF complexes, HeLa cells were transfected with expression vectors encoding Cul1, Rbx1, Skp1, and Flag-tagged Fbw7 or β-TrCP. At 48 h after transfection, cells were lysed and immunoprecipitated with anti-Flag antibodies. Protein complexes were eluted in Ub buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) containing 0.5 mg ml^{-1} Flag peptide (Sigma). E1 (His-tagged Uba1) and E2 enzymes (His-tagged Cdc34b and UbcH5c) were produced in *Escherichia coli* strain BL21 and purified on Ni^{2+}-NTA resin (Qiagen) in accordance with the manufacturer's instructions. To generate substrate, HeLa cells were transfected with HA-tagged wtMyc or K^-Myc expression vectors, lysed 48 h later in IP buffer, and immunoprecipitated with anti-Myc antibodies. Immunoprecipitates were used in ubiquitylation reactions, performed for 1 h at 25 °C in Ub buffer supplemented with 100 ng of E1, 100 ng of E2, immunopurified SCF complex, 5 μg of ubiquitin (Sigma), in the presence or absence of 2 mM ATP. Reactions were stopped by boiling in SDS sample buffer, and proteins were analysed by immunoblotting.

RT–PCR. RNA was extracted with Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. cDNA was synthesized with Moloney-murine-leukaemia virus reverse transcriptase as described[19]. Quantitative PCR **[AU: OK?]** was performed with Absolute qPCR SYBR Green Mix (Thermo Scientific) reagent in accordance with the manufacturer's instructions on the MX3000 cycler (Stratagene/Agilent Technologies). Primer sequences were as follows: *MYC*, 5′-CCTACCCTCTCAACGACAGC-3′ (forward) and 5′-CTCTGACCTTTTGCCAGGAG-3′ (reverse); *FBXW1A*, 5′-TGCTCTATGCCCAGGTCTCT-3′ (forward) and 5′-AGGGGGTTCGCCATTATTAC-3′ (reverse); *FBXW1B*, 5′-AAACCAGCCTGGAATGTTTG-3′ (forward) and 5′-CAGTCCATTGCTGAAGCGTA-3′ (reverse); *FBXW7*, 5′-CAGCAGTCACAGGCAAATGT-3′ (forward) and 5′-CAGCAGTCACAGGCAAATGT-3′ (reverse); *UBCH5A* (*UBE2D1*), 5′-AGCGCATATCAAGGTGGAGT-3′ (forward) and 5′-GTCAGAGCTGGTGACCATTG-3′ (reverse); *UBCH5B* (*UBE2D2*), 5′-GATCACAGTGGTCTCCAGCA-3′ (forward) and 5′-TCCATTCCCGAGCTATTCTG-3′ (reverse); *UBCH5C* (*UBE2D3*), 5′-TCATTGGCAAGCCACAATTA-3′ (forward) and 5′-CGAGACAAATGCTGCCATTA-3′ (reverse); *CDC34A* (*UBE2R1*), 5′-TGGACGAGGGCGATCTATAC-3′ (forward) and 5′-CCCCCGTCTCGTAGATGTTA-3′ (reverse); *CDC34B* (*UBE2R2*), 5′-ACTGCCTTCTGAAAGGTGGA-3′ (forward) and 5′-CTCCATCCTTTTCTGCTTCG-3′ (reverse); *SKP2*, 5′-ACATTTCAGCCCTTTTCGTG-3′ (forward) and 5′-CAGGACACCCAGGAAGGTTA-3′ (reverse); *HECTH9*, 5′-AGTGACTACCCCCACGACTG-3′ (forward) and 5′-GTTGGCTGCATCCTCTAAGC-3′ (reverse); *E6AP*, 5′-TTGCCACCATTTGTAGACCA-3′ (forward) and 5′-CCAATTTCTCCCTTCCTTCC-3′ (reverse); *TRIM32*, 5′-GCCAACTTCCCTTCCCTTAG-3′ (forward) and 5′-GTCCTATTTGGGCAGTGCAT-3′ (reverse); *FBXW8*, 5′-CTCCCAAAGTGCTGGGATTA-3′ (forward) and 5′-ACACGTGTGCAACTGAGAGG-3′ (reverse).

***In vitro* kinase assay.** HEK 293 cells were transfected with expression vectors encoding wtMyc or Myc4A, treated with 50 ng ml^{-1} nocodazole for 16 h, lysed in IP buffer and immunoprecipitated for 3 h with rabbit anti-Myc antibody. Immunoprecipitates were washed three times in IP buffer and once in kinase assay buffer containing 20 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM ATP. Reactions were performed for 1 h in kinase buffer in the presence of 12.5 μCi of [γ-^{32}P]ATP (Hartmann Analytic) and 0.2 μg of purified Plk1 (NEB) at room temperature.

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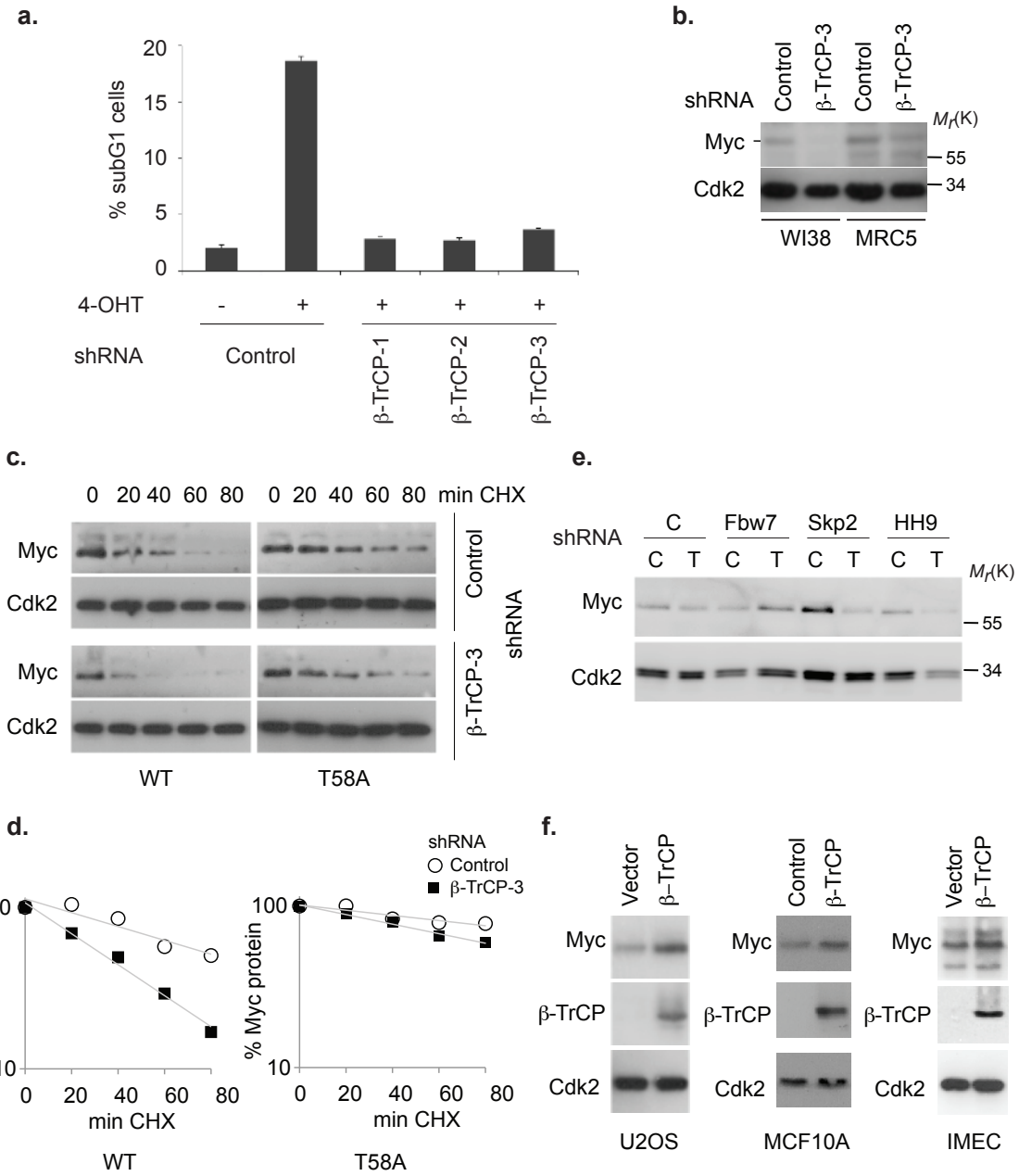

Figure S1 Regulation of Myc levels and function by β-TrCP. (**a**) β-TrCP is required for Myc-induced apoptosis. U2OS-MycER cells were infected with retroviruses expressing sh-β-TrCP and stimulated by addition of 200nM 4-OHT. The percentage of cells with a subG1 content of DNA was determined 48 hours later. Error bars show standard deviation (SD) of biological triplicates. (**b**) β-TrCP is required to maintain expression levels of Myc in non-tumorigenic MRC5 and WI38 fibroblasts. Both cell types were transfected with the indicated shRNA vectors ("C"=control, "T"=sh-β-TrCP) and levels of endogenous Myc analyzed by immunoblotting 48 hours after transfection. (**c**) β-TrCP is required to stabilize wtMyc, but not MycT58A. U2OS cells were infected with retroviruses expressing either wtMyc or MycT58A and superinfected with either control retroviruses or retroviruses expressing sh-β-TrCP. Cycloheximide was added for the indicated times and lysates probed with anti-Myc and anti-Cdk2 antibodies as indicated. (**d**) Quantification of the experiment shown in panel (**c**). (**e**) Depletion of Fbw7, but not of Skp2 or HectH9 relieves the requirement for β-TrCP. HeLa cells were transfected with the indicated combinations of shRNA vectors ("C"=control, "T"=sh-β-TrCP). Cells were harvested 48 hours after transfection. (**f**) Retroviral transduction of β-TrCP enhances Myc levels in U2OS, MCF10A and IMEC cells. The indicated cells were transduced with β-TrCP-expressing retroviruses, selected with hygromycin, and assayed for protein levels using immunoblotting.

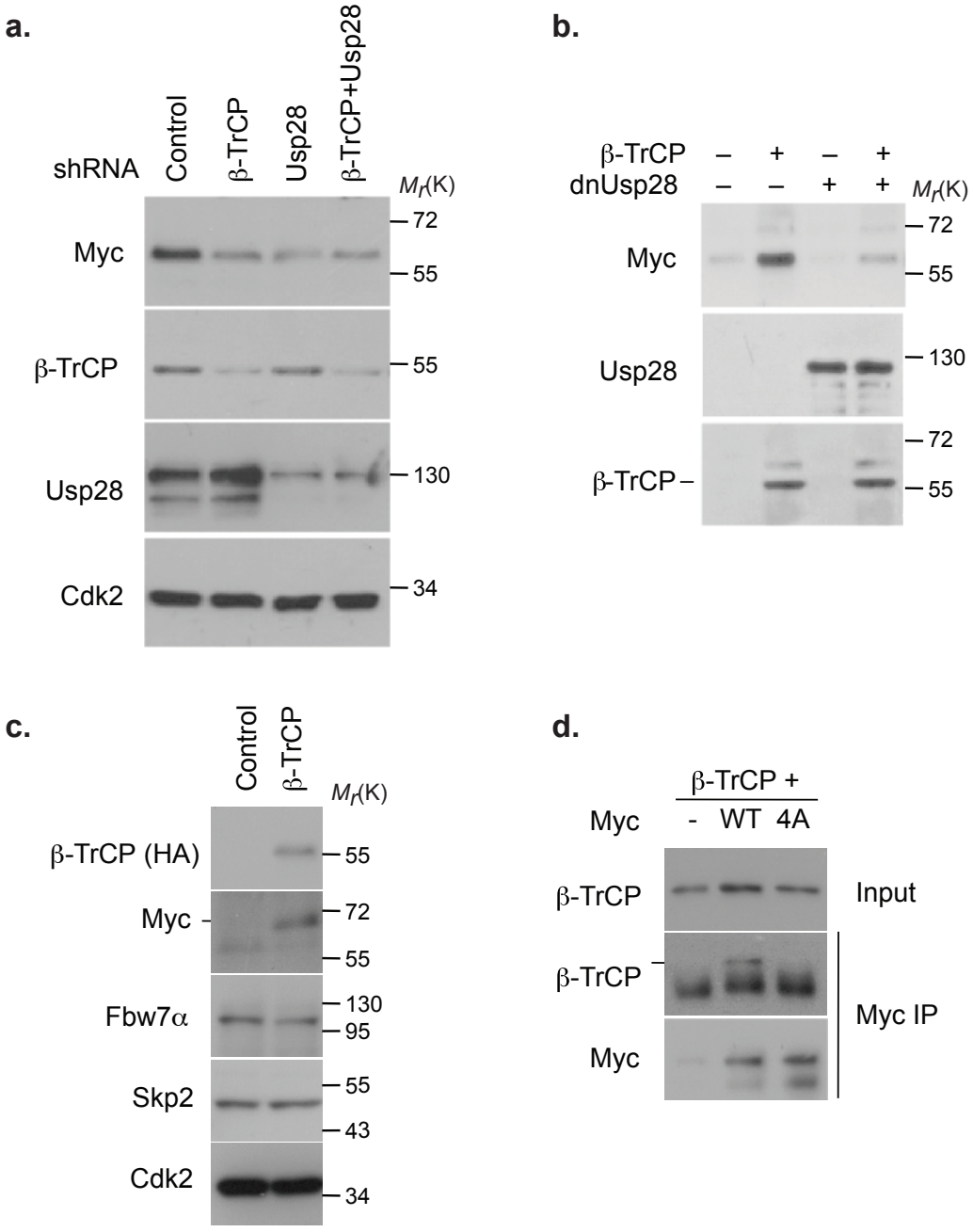

Figure S2 Functional interaction of Usp28 and β-TrCP in regulating Myc stability. (**a**) Co-depletion of Usp28 and β-TrCP. HeLa cells were transfected with pSUPER vectors expressing the indicated shRNAs. Cells were harvested 48 hours after transfection and lysates analyzed by immunoblotting. (**b**) Expression of dominant-negative Usp28 inhibits stabilization of Myc by β-TrCP. HeLa cells were transfected with CMV-driven expression vectors encoding Myc, β-TrCP and Usp28C171A, a mutant allele in which the catalytic cysteine is replaced by alanine[18]. Cells were analyzed as described before. (**c**) Ectopic expression of β-TrCP has marginal or no effects on Skp2 and Fbw7 expression levels. U2OS cells were infected with either control or retroviruses expressing β-TrCP and immunoblots probed with the indicated antibodies after selection. (**d**) Myc and β-TrCP can form a complex in non-transformed cells. Human mammary epithelial (IMEC) cells were transfected with expression vectors encoding β-TrCP and either wildtype Myc or Myc4A, respectively; after transfection, complex formation was assayed by immunoprecipitation using α-Myc antibodies.

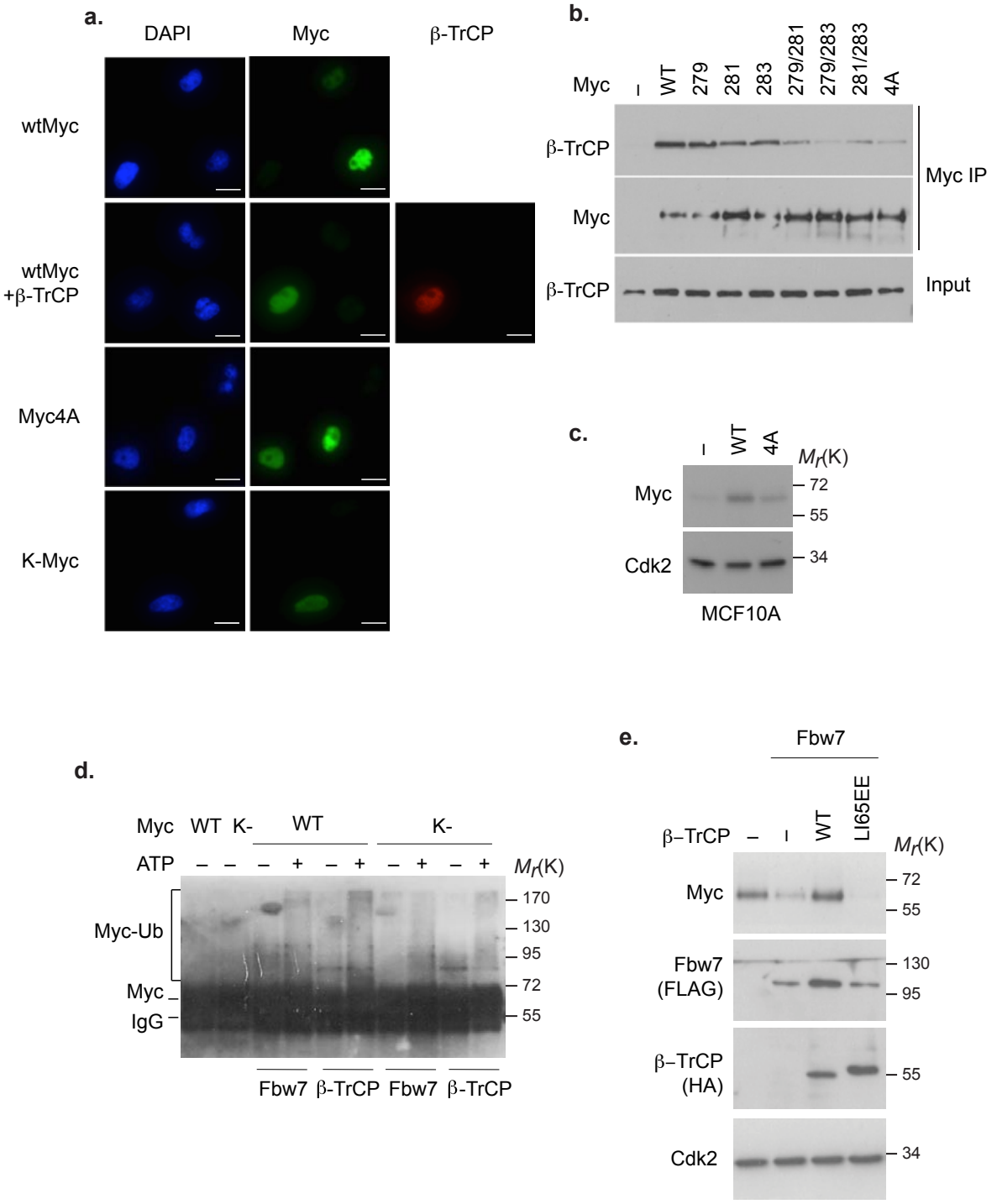

Figure S3 Recognition of Myc by β-TrCP. (**a**) Nuclear localization of wtMyc and of Myc4A. HeLa cells were transfected with CMV-driven vectors expressing the indicated proteins and analyzed by indirect immunofluorescence using antibodies against Myc and HA (β-TrCP); nuclei were counterstained using 4',6-diamidino-2-phenylindole (DAPI). Scale bar, 15 μm. (**b**) Complex formation of Myc with β-TrCP requires an intact phosphodegron motif. HeLa cells were transfected with expression vectors encoding β-TrCP, wtMyc or mutant alleles of Myc carrying alanine residues at the indicated amino acid positions. 48 hours after transfection, cell lysates were immunoprecipitated with anti-Myc antibodies and lysates probed with anti-β-TrCP antibodies. The input corresponds to 2% of the material used for immunoprecipitation. (**c**) Myc4A displays lower steady-state levels than wtMyc when expressed in mammary epithelial cells. MCF10A were transfected with expression vectors encoding wtMyc and Myc4A as indicated and probed 48hrs after transfection. RQ-PCR data documented that mRNA expression levels of wtMyc and Myc4A were identical (not shown). (**d**) Fbw7 and β-TrCP ubiquitinate wtMyc and K$^-$Myc *in vitro*. *In vitro* ubiquitination reactions were performed as described in detail in Materials and Methods. Cdc34B and UbcH5c were used as ubiquitin-conjugating enzymes for SCF^{Fbw7} and $SCF^{beta\text{-}TrCP}$ complexes, respectively. (**e**) Dimerization of β-TrCP is required to inhibit Fbw7-induced turnover of Myc. HeLa cells were transfected with expression vectors encoding either wtMyc, FLAG-tagged Fbw7 and either wt or dimerization-deficient HA-tagged β-TrCP as indicated. 48 hours after transfection cells were lysed and protein levels were determined by immunoblotting.

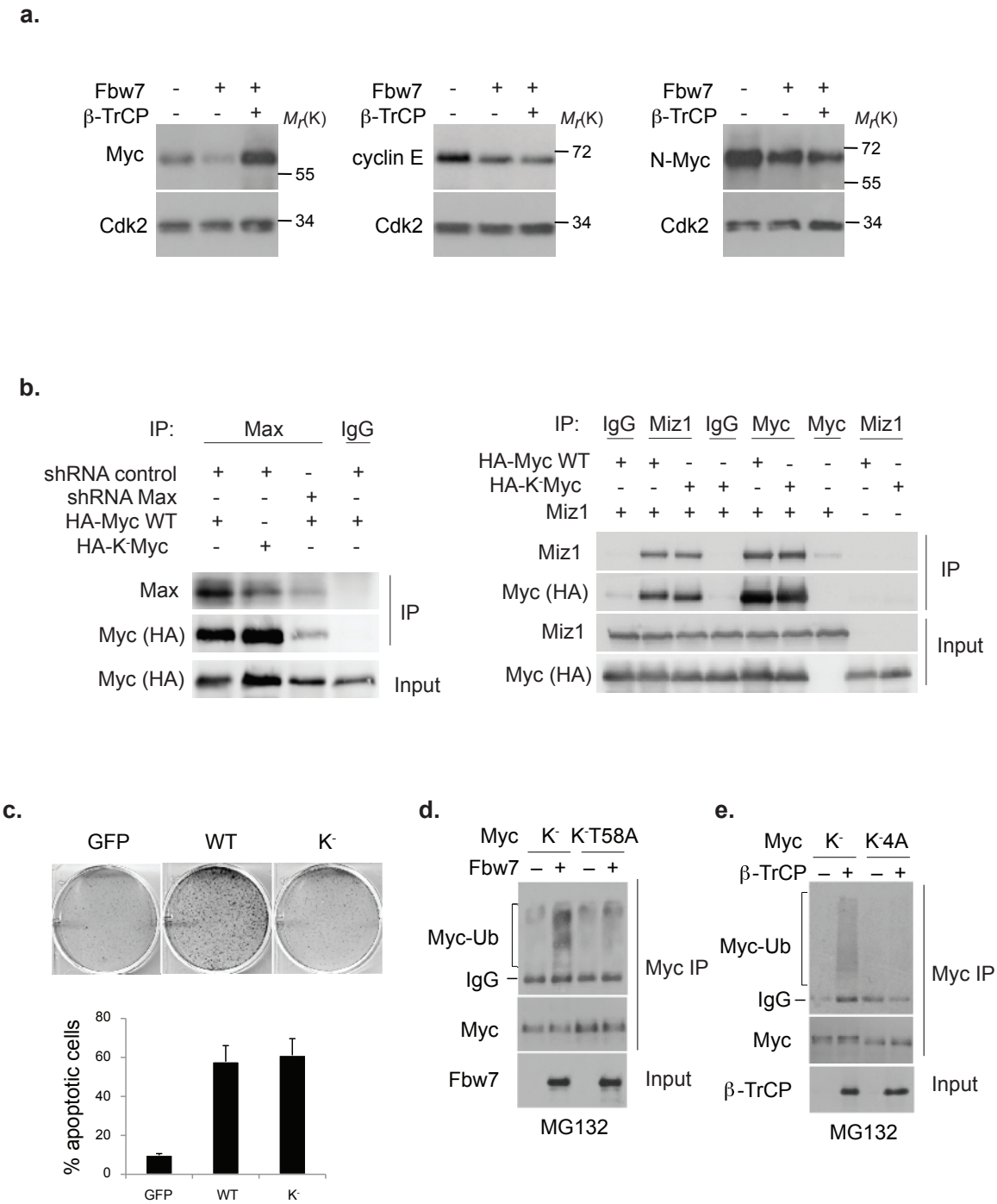

Figure S4 Characterization of K-Myc. (**a**) β-TrCP prevents degradation of Myc, but not of N-Myc or cyclin E, by Fbw7. HeLa cells were transfected with CMV-driven expression vectors expressing the indicated proteins. 48 hours after transfection, cells were harvested and lysates analyzed by immunoblotting using the indicated antibodies. (**b**) K-Myc binds to Max and Miz1. HeLa cells were transfected with vectors expressing the indicated shRNAs and expression vectors encoding wtMyc or K-Myc as indicated. 48 hours after transfection, cells were lysed and immunoprecipitated using either control, anti-Max or anti-Miz1 antibodies. Precipitates were analyzed by immunoblotting. (**c**) K-Myc efficiently induces apoptosis and is compromised in its mitogenic properties. Immortalized mammary epithelial cells were transduced with retroviruses expressing GFP, wtMyc or K-Myc, selected, and seeded at $1^{x}10^{4}$ in 6-well plates. 10 days later cell colonies were stained with crystal violet (upper panel). To measure apoptosis, U2OS cells expressing GFP, wtMyc or K-Myc were incubated under low serum conditions (0.05% FBS) for 96 hours. Cells were fixed with ethanol and analyzed using PI-FACS (lower panel). Error bars show standard deviation (SD) of biological triplicates.(**d**) Ubiquitination of K-Myc by Fbw7 depends on T58 *in vivo*. HeLa cells were co-transfected with expression vectors encoding K-Myc or K-T58AMyc, HA-tagged ubiquitin and Fbw7 as indicated. 48 hours later, cells were lysed and ubiquitinated Myc was recovered using immunoprecipitation. (**e**) Ubiquitination of K-Myc by β-TrCP requires an intact β-TrCP recognition motif. HeLa cells were co-transfected with expression vectors encoding K-Myc or K-4AMyc, HA-tagged ubiquitin and β-TrCP as indicated. 48 hours later cells were lysed and immunoprecipitated with anti-Myc antibodies.

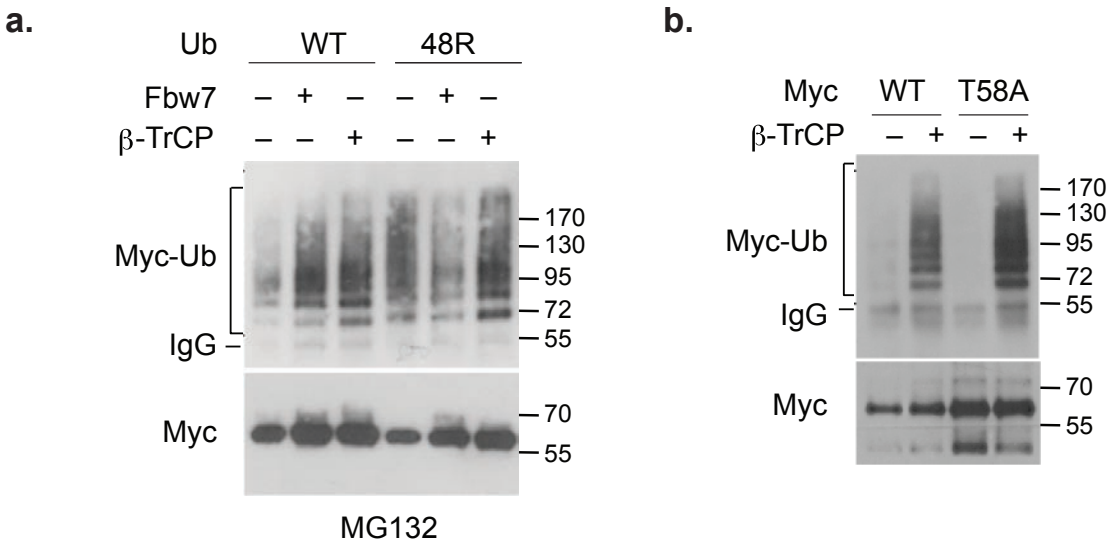

Figure S5 Ubiquitination of Myc by β-TrCP and Fbw7. (**a**) Ubiquitination of Myc by Fbw7, but not by β-TrCP requires K48 of ubiquitin. HeLa cells were transfected with CMV-driven expression vectors encoding His-tagged ubiquitin or ubiquitin K48R, Myc, β-TrCP of Fbw7 as indicated. Cells were analyzed as described before. (**b**) β-TrCP ubiquitinates T58AMyc. HeLa cells were co-transfected with expression vectors encoding wtMyc or T58AMyc, HA-tagged ubiquitin and β-TrCP as indicated. 48 hours later, cells were lysed and Myc ubiquitination was analyzed using immunoprecipitation and immunoblotting.

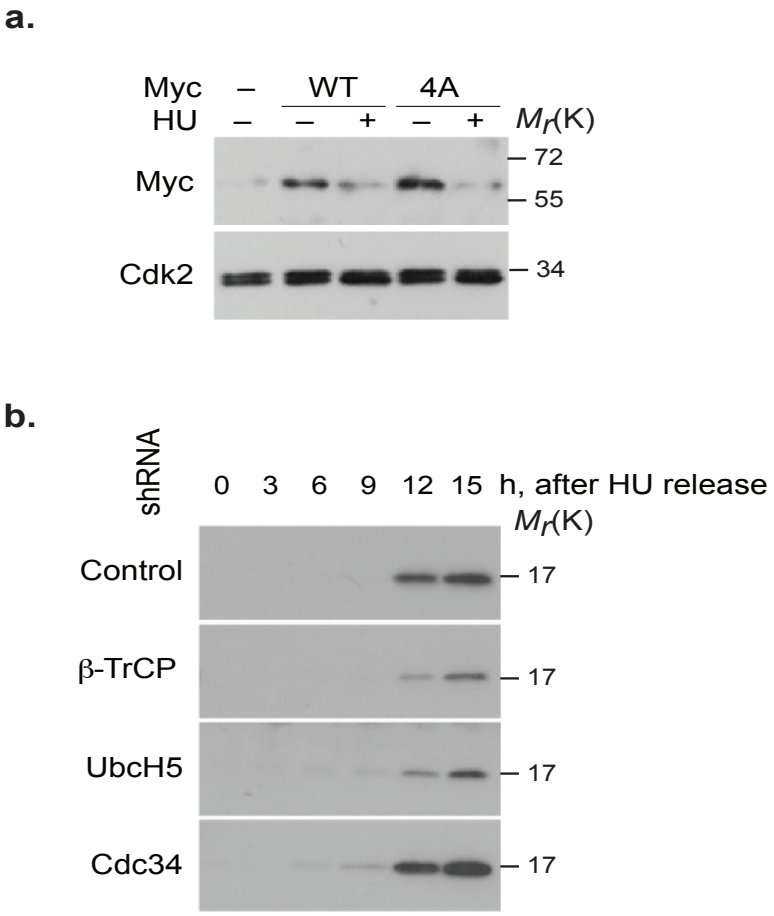

Figure S6 β-TrCP/UbcH5-dependent mitotic entry after hydroxyurea-induced arrest. (**a**) Arrest in S phase lowers steady-state levels of Myc and Myc4A. U2OS cells were left untreated or arrested in S phase by addition of hydroxyurea for 16 hours. Cells were harvested and lysates were analyzed by immunoblotting with anti-Myc or anti-Cdk2 antibodies. (**b**) β-TrCP and UbcH5 are required for mitotic entry after hydroxyurea-induced arrest. U2OS cells were infected with retroviruses expressing shRNA targeting the indicated proteins, arrested with hydroxyurea for 16 hours and released for the indicated times in the nocodazole-supplemented medium. Cell lysates were analyzed by immunoblotting using anti-phospho histone H3 antibody.

Figure S7 Uncropped images of all immunoblots shown in the individual figures.