LETTERS

14-3-3σ controls mitotic translation to facilitate cytokinesis

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14-3-3 proteins are crucial in a wide variety of cellular responses including cell cycle progression, DNA damage checkpoints and apoptosis. One particular 14-3-3 isoform, σ, is a p53-responsive gene, the function of which is frequently lost in human tumours, including breast and prostate cancers as a result of either hypermethylation of the 14-3-3σ promoter or induction of an oestrogen-responsive ubiquitin ligase that specifically targets 14-3-3σ for proteasomal degradation[1–9]. Loss of 14-3-3σ protein occurs not only within the tumours themselves but also in the surrounding pre-dysplastic tissue (so-called field cancerization), indicating that 14-3-3σ might have an important tumour suppressor function that becomes lost early in the process of tumour evolution[3,9]. The molecular basis for the tumour suppressor function of 14-3-3σ is unknown. Here we report a previously unknown function for 14-3-3σ as a regulator of mitotic translation through its direct mitosis-specific binding to a variety of translation/initiation factors, including eukaryotic initiation factor 4B in a stoichiometric manner. Cells lacking 14-3-3σ, in marked contrast to normal cells, cannot suppress cap-dependent translation and do not stimulate cap-independent translation during and immediately after mitosis. This defective switch in the mechanism of translation results in reduced mitotic-specific expression of the endogenous internal ribosomal entry site (IRES)-dependent form of the cyclin-dependent kinase Cdk11 (p58 PITSLRE), leading to impaired cytokinesis, loss of Polo-like kinase-1 at the midbody, and the accumulation of binucleate cells. The aberrant mitotic phenotype of 14-3-3σ-depleted cells can be rescued by forced expression of p58 PITSLRE or by extinguishing cap-dependent translation and increasing cap-independent translation during mitosis by using rapamycin. Our findings show how aberrant mitotic translation in the absence of 14-3-3σ impairs mitotic exit to generate binucleate cells and provides a potential explanation of how 14-3-3σ-deficient cells may progress on the path to aneuploidy and tumorigenesis.

Because many tumour suppressor proteins function during specific parts of the cell cycle, we examined whether the binding of ligands to 14-3-3σ showed a cell cycle dependence. U2OS cells were synchronized by a double thymidine block, and lysates were prepared at various times after release (Supplementary Fig. 1a). Immunoprecipitation of endogenous 14-3-3σ from these lysates showed a marked increase in 14-3-3σ-bound proteins in the mitotic and immediate post-mitotic periods (Fig. 1a, time points at 12 and 16 h after release). This same mitotic enrichment in ligand binding to 14-3-3σ was also observed in other cell types, including HCT116 cells and HeLa cells (Supplementary Fig. 1b–d), but was not observed when ligands of another endogenous 14-3-3 isoform, 14-3-3β, were analysed (Supplementary Fig. 1e) or in 14-3-3σ immunoprecipitations from asynchronous cells after DNA damage (Supplementary Fig. 2). The mitosis-dependent binding of ligands to 14-3-3σ seemed to be both dependent on phosphorylation, because binding was lost when lysates were treated with protein phosphatase 1 (Supplementary Fig. 1d), and direct, as shown with a two-dimensional 'far-western' assay (Fig. 1d).

The mitotic targets of 14-3-3σ were identified by mass spectrometry. This analysis revealed that a large proportion of 14-3-3σ ligands were proteins involved in the process of translation, including several initiation factors involved in mediating cap-dependent translation (Fig. 1b and Supplementary Table 1). Two-dimensional far-western blotting of mitotic 14-3-3σ immunoprecipitates showed that several of these proteins, including eukaryotic initiation factor 4B (eIF4B), eIF-2α and elongation factor 1α (EF1α), bound directly to 14-3-3σ, whereas other 14-3-3σ-associated proteins such as eIF4G seemed to be interacting indirectly (Fig. 1e).

It is well established that translation in mammalian cells is markedly altered during and immediately after mitosis[10], with a pronounced suppression of cap-dependent translation and a corresponding enhancement of cap-independent translation[11]. The biological mechanism and significance of this translational switch, however, are unclear. Given the abundance of proteins involved in translation in the 14-3-3σ immunoprecipitations, we investigated whether 14-3-3σ has a direct function in this process by using RNA interference (RNAi). Synchronous 14-3-3σ stable knockdown and control cells were pulse-labelled with [^{35}S]methionine, and newly translated proteins were analysed by SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography (Fig. 2a, d, and Supplementary Fig. 3a). As expected, in control short hairpin RNA (shRNA)-treated HeLa and U2OS cells, radiolabel incorporation into nascent polypeptides was suppressed at 12 and 16 h after release from the double thymidine block (Fig. 2a, e, lanes marked 'con')[11]. Remarkably, no suppression of mitotic translation was observed in either of these cell types when they were depleted of 14-3-3σ (lanes marked 'σ'). Identical results were also obtained with two other distinct 14-3-3σ shRNAs (data not shown). In contrast to 14-3-3σ depletion, RNAi-mediated knockdown of 14-3-3β had no effect on the suppression of translation during and immediately after mitosis (Fig. 2b, d). Furthermore, the aberrant mitotic translation seen in the 14-3-3σ knockdown cells did not result from a failure of these cells to enter mitosis (Fig. 2c). Although the global pattern of cell cycle progression did not change, we observed an increase in the population of 4*n* DNA-containing cells and a significant increase in mitotic index (Fig. 2c, and Supplementary Fig. 3b, c). Coexpression of an shRNA-resistant construct of 14-3-3σ together with 14-3-3σ shRNA restored the suppression of mitotic translation (Fig. 2e, lanes marked 'σ_r', and Fig. 2f, top). These findings indicate that 14-3-3σ is important

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in the physiological downregulation of new protein synthesis during and immediately after mitosis.

The inhibition of translation observed during mitosis has previously been shown to result from a global decrease in cap-dependent translation, whereas cap-independent translation is subsequently increased[12,13]. We used two different IRES-containing bi-cistronic vectors, a viral-based IRES from HIV[14], and a normal cellular IRES from $p27^{Kip1}$ (ref. 15), in a direct investigation of the relative effects of 14-3-3σ on cap-dependent versus cap-independent translation in synchronized U2OS and HeLa cells. As shown in Fig. 2g, 14-3-3σ-depleted cells failed to show the robust mitotic-specific increase in the ratio of cap-independent to cap-dependent translation that occurred in control cells. Thus, a loss of 14-3-3σ resulted in both aberrant persistence of cap-dependent translation and prevented the normal enhancement of cap-independent to cap-dependent translation during mitosis.

The relative amounts of different eukaryotic initiation factors in cells varies more than 100-fold[16], and they have distinct roles in the cap recognition process[17,18]. To investigate which eIF targets of 14-3-3σ are potentially responsible for the 14-3-3σ-mediated suppression of cap-dependent mitotic translation, we immunodepleted mitotic extracts with the use of anti-14-3-3σ antibodies and probed the depleted lysates for residual eIFs. Of the eIFs examined, the most pronounced result was obtained with eIF4B, which was completely absent from the 14-3-3σ-depleted mitotic lysates (Fig. 1c). eIF4B facilitates the ATP-dependent helicase activity of eIF4A to promote ribosome recruitment required for cap-dependent translation[19]. We further observed that transient overexpression of exogenous Flag-tagged eIF4B was able to overcome the ability of endogenous 14-3-3σ to suppress mitotic translation in normal cells but had no effect on the increased translation seen in 14-3-3σ knockdown cells (Fig. 2e, lanes marked '4B', and Fig. 2f, bottom). Taken together, the data in Figs 1 and 2 indicate that 14-3-3σ is important in the physiological downregulation of new protein synthesis during and immediately after mitosis by suppressing cap-dependent translation through binding to eIF4B.

Because loss of 14-3-3σ occurs early in the process of tumorigenesis, we also examined the 14-3-3σ knockdown cells for a corresponding cellular phenotype that might correlate with aberrant regulation of mitotic translation. In the 14-3-3σ knockdown cells we observed significantly increased numbers of cells displaying persistent cytokinetic bridges, binucleate cells (often containing midbody remnants), and cells that seemed to have 'fused' after mitosis with widely separated nuclei, probably reflecting a failure late in cytokinesis (Fig. 3a–c; $P < 0.05$ for 14-3-3σ shRNA versus control cells, Student's *t*-test, two-tailed; and Supplementary Fig. 3d–f). Intriguingly, these phenotypes seemed to correlate with a failure of Polo-like kinase-1 (Plk1), a critical mitotic kinase involved in the completion of cytokinesis[20,21], to localize to the midbody at the end of mitosis (Fig. 4a). Both the localization of Plk1 to the midbody and the mitotic phenotype of 14-3-3σ-depleted cells could be significantly restored by treatment of the cells with rapamycin, an inhibitor of cap-dependent, but not cap-independent, translation[22], immediately before mitotic entry (Figs 3b, c and 4a).

We searched for proteins that were translated in mitosis in a cap-independent manner and were postulated to be involved in the mitotic process. One of the candidates, PITSLRE/Cdk11, is a member of the Cdc2-like protein kinase family that undergoes cap-independent translation from an internal ribosome entry site during mitosis to produce a 58-kDa isoform that facilitates proper mitotic progression and termination[23–25]. In both U2OS cells and HeLa cells we observed that 14-3-3σ knockdown cells, but not control cells, failed to synthesize p58 PITSLRE in mitosis (Fig. 4b, c, 16 h lanes). Depletion of PITSLRE/Cdk11 from U2OS cells caused an increase in mitotic content as well as the number of binucleate cells, exactly as observed in 14-3-3σ-depleted cells (Supplementary Figs 3 and 4). Addition of rapamycin to 14-3-3σ knockdown cells restored the mitotic translation of p58 PITSLRE (Fig. 4b, c). Furthermore, transient transfection of a complementary DNA encoding the 58-kDa isoform of PITSLRE into the 14-3-3σ knockdown cells was sufficient to relocalize Plk1 at the midbody and partly rescue the mitosis-defective cell phenotype, reducing the number of binucleate and fused cells by slightly more

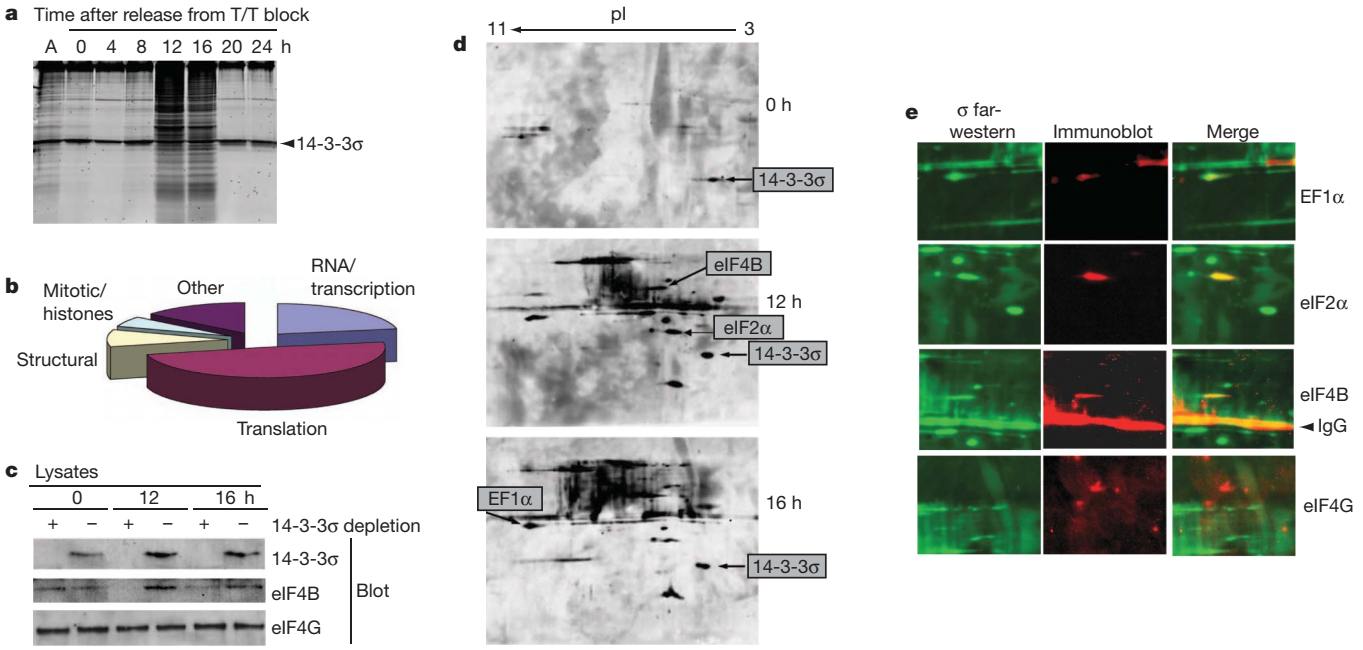

Figure 1 | 14-3-3σ binds to its targets during mitosis. **a**, SDS–PAGE analysis of 14-3-3σ immunoprecipitates from synchronized U2OS cells. Sypro-Ruby stain. T/T, double thymidine; A, asynchronous cells. **b**, Mitotic ligands of 14-3-3σ identified by mass spectrometry categorized into five major groups. **c**, 14-3-3σ quantitatively immunodepletes eIF4B but not eIF4G from mitotic (12 h), but not from interphase (0 h), U2OS cell extracts. **d**, Two-dimensional far-western blotting of 14-3-3σ immunoprecipitates, with purified 14-3-3σ as a probe for direct binding. pI, isoelectric point. **e**, eIF4B, eIF2α and EF1α as direct mitotic ligands of 14-3-3σ. Two-dimensional far-western blots (green) re-probed with antibodies against the indicated proteins (red). eIF4G in the 14-3-3σ immunoprecipitates does not directly bind to 14-3-3σ.

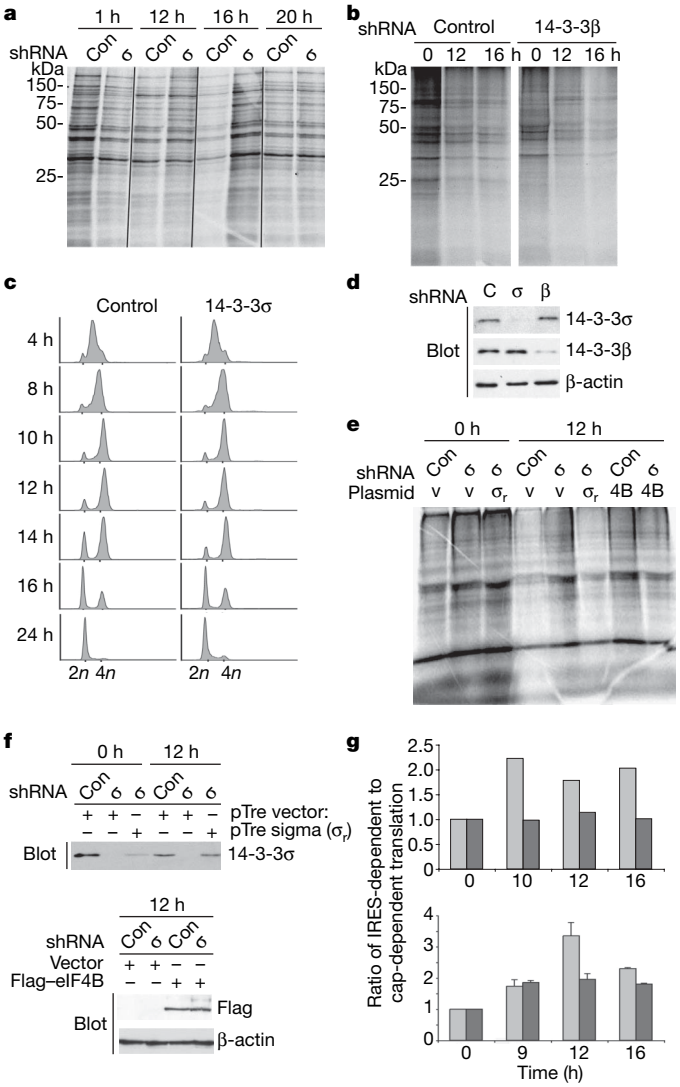

Figure 2 | 14-3-3σ knockdown cells fail to suppress cap-dependent translation during and immediately after mitosis. **a**, **b**, Control, 14-3-3σ (**a**) and 14-3-3β (**b**) shRNA HeLa cells were pulse-labelled with [^{35}S]methionine. New protein synthesis was assayed by SDS–PAGE and autoradiography. Numbers at the left indicate molecular masses. **c**, Cell cycle distribution of control and 14-3-3σ shRNA-treated cells. **d**, Efficiency of 14-3-3 knockdown. C, control. **e**, Overexpression of eIF4B overcomes 14-3-3σ-dependent suppression of translation in mitosis. V, vector; 4B, Flag-eIF4B; σ_r, RNAi-resistant 14-3-3σ. Synchronized cells pulse-labelled as in **b** at indicated times. **f**, Expression of σ_r and Flag–eIF4B in **e**. **g**, Ratio of cap-independent to cap-dependent translation in synchronized control (light grey bars) and 14-3-3σ (dark grey bars) knockdown cells assayed by firefly/*Renilla* luciferase activity at the indicated times. Top, U2OS cells and HIV IRES; bottom, HeLa cells and p27^{Kip1} IRES. Results are means and s.e.m. from duplicate experiments.

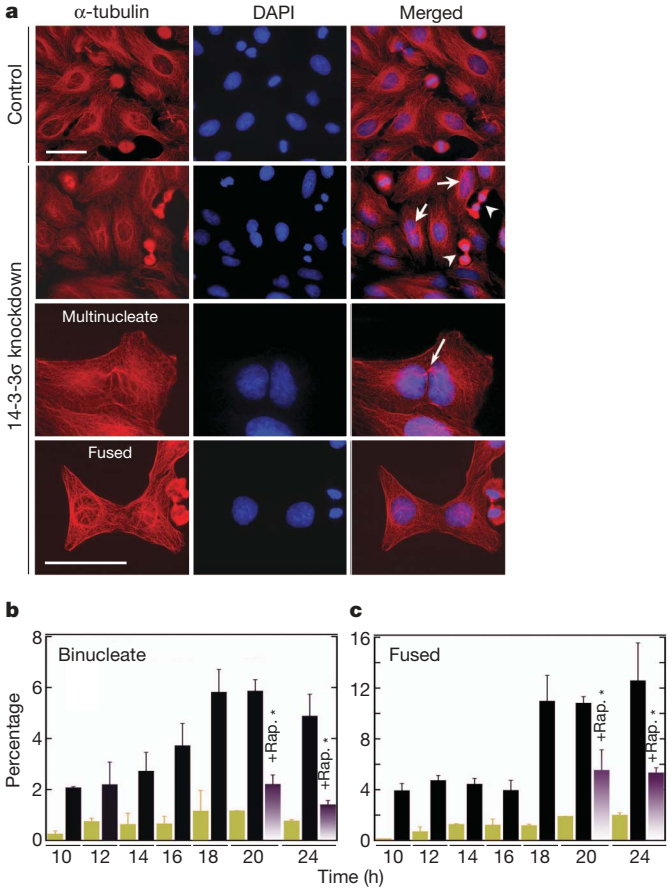

Figure 3 | Depletion of 14-3-3σ results in impaired cytokinesis. **a**, 14-3-3σ knockdown U2OS cells, stained as indicated, show increased numbers of binucleate cells (short arrows) and cells in terminal cytokinesis (arrowheads). Close-up views demonstrate persistent midbody structures (long arrow), and a 'fused' post-mitotic cell lacking distinct intercellular boundaries. Scale bars, 10 μm. DAPI, 4,6-diamidino-2-phenylindole. **b**, **c**, Rapamycin treatment before mitotic entry suppresses binucleate (**b**) and fused-cell (**c**) formation in 14-3-3σ knockdown cells. Synchronized U2OS shRNA cells were scored at the indicated times after release. Green bars, control shRNA; black bars, 14-3-3σ shRNA; +Rap., 14-3-3σ shRNA cells treated with 20 ng ml^{-1} rapamycin at 9 h. Asterisk, $P < 0.02$ for rapamycin-treated versus untreated 14-3-3σ knockdown cells. Results are means and s.d. from three independent experiments.

than 50% (Fig. 4d, e, and Supplementary Fig. 4i). Thus, 14-3-3σ is required in normal mitosis to suppress cap-dependent translation through binding eIF4B, allowing the cap-independent translation of critical mitotic regulators including p58 PITSLRE kinase.

Two recent reports[26,27] have suggested that the formation of binucleate cells as a result of cytokinesis failure is an early event in tumour formation and underlies the subsequent development of genomic instability. We have observed that 14-3-3σ, an important tumour suppressor protein whose expression is lost in a variety of epithelial tumours, has a critical function in regulating protein synthesis during and immediately after mitosis and is required for the IRES-dependent translation of p58 PITSLRE, a protein kinase critical to the proper completion of cytokinesis. These findings therefore establish a connection between the aberrant regulation of mitotic translation and improper cytokinesis resulting in a phenotype that is associated with early stages of human oncogenesis.

METHODS

Immunoprecipitation studies and mass spectrometry analysis. U2OS, HeLa and HCT116 cell lysates were prepared and immunoprecipitated overnight at 4 °C with an anti-14-3-3σ monoclonal antibody (CS112)[28,29]. Immunoprecipitates were digested with trypsin and analysed on a QSTAR XL Pro quadrupole time-of-flight mass spectrometer (Applied Biosystems).

Two-dimensional gel electrophoresis. 14-3-3σ immunoprecipitates were resolved by isoelectric focusing with IPGphor strips as described previously[29]. Separation in the second dimension was performed with 12% Anderson gels and used for far-western blot analyses with bacterially purified 14-3-3σ (about 50 μg ml^{-1}) as a probe, followed by immunoblotting with CS112.

Lentiviral shRNA constructs. 14-3-3σ, 14-3-3β and p58 PITSLRE shRNAs were subcloned into pLentilox 3.7 for lentiviral production as described[30]. Heterogeneous populations stably expressing shRNA were established in HeLa and U2OS cells.

[^{35}S]Methionine pulse-labelling. Cells were labelled with 100 μCi of [^{35}S]methionine for 20 min, lysed, and precipitated with acetone. Resuspended

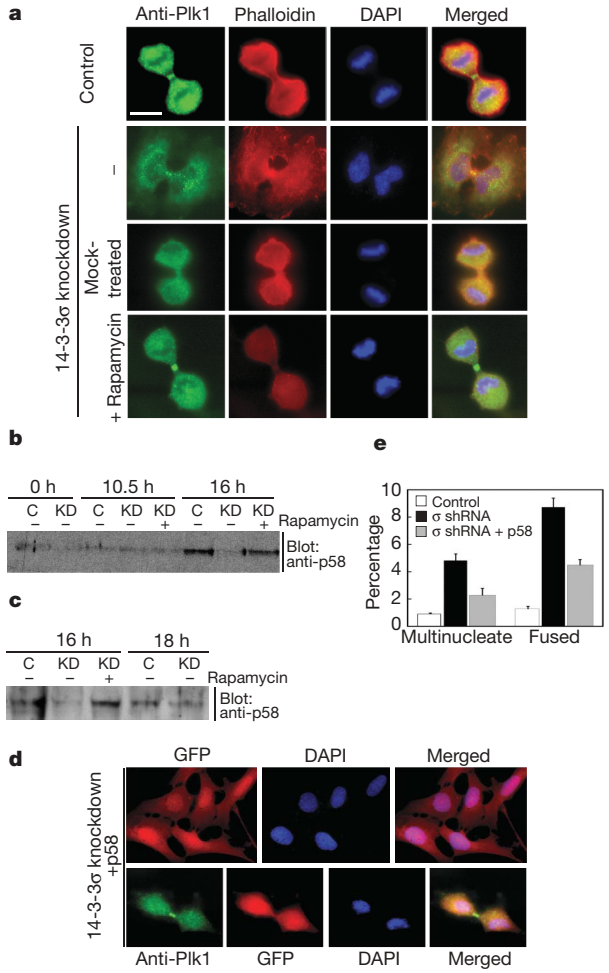

Figure 4 | Depletion of 14-3-3σ blocks IRES-dependent mitotic translation of p58 PITSLRE kinase and results in failure of Plk1 to localize at the midbody. **a**, Lack of localization of Plk1 to the midbody and cell fusion with failure to complete cytokinesis in 14-3-3σ knockdown U2OS cells is reversed by rapamycin before mitotic entry. Scale bar, 5 μm. **b**, **c**, Synchronized control (C) or 14-3-3σ shRNA-treated (KD) U2OS (**b**) and HeLa (**c**) cells, with and without rapamycin treatment, lysed at the indicated times and immunoblotted for endogenous p58 PITSLRE expression[25]. IRES-dependent translation of p58 PITSLRE in 14-3-3σ knockdown cells was restored by rapamycin. **d**, **e**, Forced expression of p58 PITSLRE reverts the mitotic phenotype of 14-3-3σ knockdown cells. GFP-expressing 14-3-3σ knockdown U2OS cells were transfected with p58 PITSLRE or vector control, then imaged (**d**) and scored (**e**) 18 h after release from a double-thymidine block. Results in **e** are means and s.d. from three independent experiments.

proteins were separated on 12% SDS–PAGE gels and analysed by autoradiography. Where indicated, cells were treated with rapamycin at a final concentration of 20 ng ml^{-1} 9 h after release from a double thymidine block.

Additional information and detailed protocols are provided in Supplementary Methods.

Received 13 July 2006; accepted 9 January 2007; corrected 10 April 2007.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank P. Stern, D. Lowery and W. Merrick for reagents and technical assistance. This work was supported by postdoctoral fellowships from the Anna Fuller Fund and the NIH to E.W.W., an EMBO long-term fellowship to M.A.T.M.v.V., the David H. Koch Cancer Research Fund, NIH grants, and a Burroughs-Wellcome Career Development Award to M.B.Y.

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