Deficiency in spliceosome-associated factor CTNNBL1 does not affect ongoing cell cycling but delays exit from quiescence and results in embryonic lethality in mice

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CTNNBL1 is an armadillo-repeat protein that associates with the CDC5L/Prp19 complex of the spliceosome. Unlike the majority of spliceosomal proteins (and despite having no obvious homologs), CTNNBL1 is inessential for cell viability as revealed by studies in both vertebrate B cell lines and in fission yeast. Here, however, we show that ablation of CTNNBL1 in the mouse germline results in mid-gestation embryonic lethality but that lineage-specific CTNNBL1 ablation in early B cell precursors does not affect the production and abundance of mature B lymphocytes. However, CTNNBL1-deficient resting B lymphocytes show sluggish exit from quiescence on cell activation, although once entry into cycle has initiated, proliferation and differentiation in response to mitogenic stimuli continue largely unaffected. A similar sluggish exit from quiescence is also observed on reprovision of nutrients to nitrogen-starved CTNNBL1-deficient yeast. The results indicate that, whereas other RNA splicing-associated factors have been connected to cell cycle progression, CTNNBL1 plays no essential role in cycling cells but does fulfill an evolutionarily conserved function in helping cells to undergo efficient exit from quiescence following activation.

Introduction

CTNNBL1 (catenin-β-like 1) is a widely expressed nuclear protein composed of multiple tandem armadillo domains that owes its name to the fact that it shows predicted structural homology (though little primary sequence homology) to β-catenin.1 CTNNBL1 likely functions in RNA splicing, since it is associated with the Prp19-CDC5L-PLRG1 complex (also known as the 19 or NTC complex) of the spliceosome, interacting directly with CDC5L.2-5 However, although present as a single-copy gene in vertebrates without any obvious sequence homologs, CTNNBL1 is inessential for cell viability since the Ctnnbl1 gene has been disrupted in both chicken and mouse B cell lines without notably affecting cell proliferation or viability.3,6

The fact that CTNNBL1 is inessential for the viability of mammalian cell-lines and yet the gene is widely-expressed and well conserved among most eukaryotes led us to ask whether more subtle effects of CTNNBL1 deficiency might be observed in intact animals, as opposed to in B cell-lines. Here we show that germline disruption of the mouse Ctnnbl1 gene leads to mid-term embryonic lethality, whereas lineage-specific ablation of Ctnnbl1 in primary B cells results in substantially delayed cell enlargement and exit from quiescence following mitogenic stimulation without having a major detectable effect on cell proliferation once cycling has been initiated.

Results

Germline ablation of CTNNBL1 results in midterm embryonic lethality. Gene targeting was used to generate clones of embryonic stem cells that carry, on one Ctnnbl1 allele, an insertion of a β-gal/neo cassette into the second Ctnnbl1 intron together with LoxP sites flanking the linked Ctnnbl1 exon 3 (Fig. 1A and B). This targeted allele is designated Ctnnbl1. The β-gal/neo cassette on this allele is itself flanked by flippase recognition target sequences and comprises (from 5' - to 3' -ends): an RNA splice acceptor site, an internal ribosomal entry site, a promoter-less β-galactosidase gene and a neomycin-resistance gene that is driven by a phosphoglycerate kinase promoter. It is therefore anticipated that transcription initiated from the Ctnnbl1 promoter on the Ctnnbl1 allele will give rise to a truncated N-terminal CTNNBL1 polypeptide that terminates at codon 82 together with β-galactosidase, whose translation will be initiated from the IRES. These Ctnnbl1−/− ES cells were injected into blastocysts isolated from C57BL/6 mice and the resultant chimaeras bred to obtain heterozygous mice carrying one targeted Ctnnbl1 allele in their germline.

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Interbreeding of the heterozygous mice failed to yield any weaned *Ctnnb1<sup>−/−</sup>* homozygous offspring (Fig. 1C). A similar failure to obtain animals homozygous for an inactivated *Ctnnb1* allele was observed when interbreeding a different line of mice that carry a βgal/neo genetrap insertion into the first intron of *Ctnnb1* (Fig. 1C). Thus, germline deficiency in CTNNBL1 appears to be embryonically lethal. Analysis of embryos generated by intercrossing *Ctnnb1<sup>−/−</sup>* heterozygous mice reveals that although *Ctnnb1<sup>−/−</sup>* embryos can be obtained at day 8.5, their viability is already compromised by mid-gestation (Fig. 1D). Thus, CTNNBL1 deficiency is lethal around the embryonic midterm. We have not identified any specific lineage that is responsible for this effect: staining of *Ctnnb1<sup>−/−</sup>* heterozygous embryos (which carry the βgal/neo genetrap insertion on one *Ctnnb1* allele) for β-galactosidase activity indicates that *Ctnnb1* exhibits a broad expression pattern (Fig. 1E).

**B cells develop in the absence of CTNNBL1.** The embryonic lethality resulting from germline CTNNBL1 deficiency contrasts with the healthiness of CTNNBL1-deficient B lymphoid cell-lines. We therefore wondered if it would be possible to obtain primary B cells lacking CTNNBL1. Mice bearing the βgal/neo targeting on one *Ctnnb1* allele (*Ctnnb1<sup>−/−</sup>* in Fig. 2A) were crossed with mice that express Flip recombinase in the germline in order to yield offspring in which the βgal/neo cassette had been deleted through Flip-mediated recombination. The resulting *Ctnnb1<sup>flx</sup>* allele is functional (in that *Ctnnb1<sup>flx/flx</sup>* mice are viable

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**Figure 1.** Targeted inactivation of *Ctnnb1* results in mid-term embryonic lethality. (A) Targeting the mouse *Ctnnb1* locus. The top line depicts the mouse *Ctnnb1* locus (three exons: E1, E2 and E3 are depicted) aligned with the targeting construct which contains a promoterless β-galactosidase/pgk-neo cassette flanked by flippase recognition sites (oval shapes) integrated into intron 2 with LoxP sites (open triangles) flanking E3. The left and right homology arms are 4.6 and 4.8 kb, respectively. Restriction endonuclease sites Ase I (A) and Sap I (S) are indicated as are the locations of the left- and right-hand probes (LHP and RHP) used for the Southern blot analysis. (B) Southern blot of tail DNA from mice carrying the targeted β-gal/neo cassette insertion into *Ctnnb1* E3 on one allele as well as controls hybridized with probes LHP and RHP. (C) Genotypes of weaned animals born to *Ctnnb1<sup>−/−</sup>* intercrosses. A similar analysis is shown for the progeny born to intercrosses of AX0016 mice that carry a gene trap insertion into the first *Ctnnb1* intron. (D) Genotypes of embryos obtained at day 8.5 or 11.5 of gestation from *Ctnnb1<sup>−/−</sup>* intercrosses. (E) Broad expression of the *Ctnnb1* locus as revealed by staining of a day 14.5 *Ctnnb1<sup>−/−</sup>* embryo for β-galactosidase activity.
and express CTNNBL1) but retains LoxP sites flanking Ctnnbl1 exon 3, meaning that the locus can then be inactivated by Cre-mediated recombination. Indeed, crossing Ctnnbl1flx mice with animals expressing Cre in the germline yielded a Ctnnbl1$^{flx/Δ}$ allele that, in homozygous form, resulted in embryonic lethality (five litters of Ctnnbl1$^{flx/Δ}$ intercrosses yielded 20 Ctnnbl1$^{flx/Δ}$ heterozygotes and seven Ctnnbl1$^{flx/Δ}$ homozygotes but no Ctnnbl1$^{flx/Δ}$ homozygotes).

Mice were generated that carried a targeted inactivation of Ctnnbl1 on one allele, a flx-Ctnnbl1 locus on the other allele and which also expressed the Cre recombinase under control of the early B lymphocyte-specific mb1 promotor. Analysis of DNA, RNA and protein in the splenic B cells from these mb1-Cre Ctnnbl1$^{flx/Δ}$ mice revealed that the Cre-mediated deletion of Ctnnbl1 had been effective with the cells lacking CTNNBL1 RNA and protein (Fig. 2B and C). Splenic B cells were nevertheless present in normal (indeed, very slightly increased) numbers and were similarly distributed between follicular, marginal and B1 subsets as found in controls as judged by cell surface marker analysis (Fig. 2D). There was also no obvious perturbation of earlier stages of B cell differentiation as judged by analysis of B cell populations in the bone marrow (Fig. 2E). Thus, although CTNNBL1 is essential for embryonic development, it is dispensable for the generation and maturation of B cells.

CTNNBL1-deficient B cells showed reduced immunoglobulin class switching. Previous results have revealed that CTNNBL1 deficiency reduces the frequency of immunoglobulin gene conversion in the chicken DT40 B cell line whereas no effect of CTNNBL1 deficiency on immunoglobulin class switch recombination was observed in the mouse CH12 B cell line.3,6 We were therefore interested in ascertaining whether CTNNBL1 deficiency had any effect on immunoglobulin class switching in primary mouse B cells. Comparison of switching to IgG1 in splenic B cells from mb1-Cre, Ctnnbl1$^{flx/Δ}$ and mb1-Cre, Ctnnbl1$^{flx/Δ}$ mice that had been cultured for 3 d in the presence of LPS IL4 revealed that the CTNNBL1-deficient B cells gave roughly one-third less switching (Fig. 3A). This reduced switching does not correlate with any change in the abundance of AID (Fig. 3B). Similar results regarding diminished class switching were obtained using B cells stimulated with anti-CD40 and IL4 (Fig. S1) as well with regard to switching to IgG3 (not shown).

We were, however, concerned to find out whether the diminished switching correlated with any alteration in the rate of B cell proliferation and therefore performed the switching assay on B cells that had been labeled with CFSE, allowing cellular proliferation to be monitored by way of CFSE dilution. The results (Fig. 3Ci) reveal that the CTNNBL1-deficient B cells do indeed exhibit a perturbation in clonal expansion in that their CFSE fluorescence curves as analyzed at day 3 are shifted to the right as compared with the controls. The CTNNBL1-deficient B cells had, on average, undergone 1–2 fewer cell divisions than their wild type counterparts. Nevertheless, even when this reduced proliferation is taken into account, the CTNNBL1-deficient B cells show a significant (averaging 30%) reduction in the extent of switching to IgG1 that is observed in each cycle of clonal expansion (Fig. 3Cii).
Figure 2. For figure legend, see page 736.
in the ratio of total exonic to intronic reads (Fig. 6A). However, not only is there no evident difference in the extent of this overall increase when comparing control and CTNNBL1-deficient B cells, we have also not been able to discern any individual transcripts whose splicing is affected by CTNNBL1 deficiency (Fig. 6B). Thus, although there is some sample-to-sample variation in the read ratios in individual genes, the results do not reveal any individual gene whose read ratio is consistently altered as a consequence of CTNNBL1 deficiency by an amount beyond the level of variability attributable to the observed noise of the data set (i.e., by an amount greater than 2 standard deviations, where the standard deviation is deduced from sample data with similar read ratios and assuming a normal distribution). Furthermore, the B cells from control and knockout mice show a remarkably similar pattern of gene activation at the 12 h time point. The most notable difference (apart from those in CTNNBL1 itself) is an increase in the reads for haem oxygenase (Hmox1) in the activated CTNNBL1-deficient sample, which likely reflects small differences in the efficiency of red blood cell lysis during sample preparation since the inductive form of Hmox1 is very sensitive to byproducts of erythrocyte clearance9 (Fig. S2). Thus, although we cannot exclude the possibility that CTNNBL1 deficiency has a small effect on the splicing of one or several specific RNAs, the RNaseq data do not reveal any genes that have suffered a major discernible splicing perturbation.

**Ctnnbl1** S. pombe exhibit delayed exit from starvation-induced quiescence. CTNNBL1 is conserved as a single-copy gene from *Schizosaccharomyces pombe* through to man. If potentiating timely exit from quiescence is a core function of CTNNBL1, then this function might well be preserved through evolution. Following extended nitrogen starvation, *S. pombe* exhibits a quiescence that is in many respects similar to that observed in mammalian cells.10,11 We therefore asked whether inactivation of the *ctnnbl1* locus in *S. pombe* affected the speed of exit from quiescence. Although deletion of the *ctnnbl1* locus did not have any detectable effect on the doubling time of *S. pombe* during log-phase growth in rich medium as monitored in multiple experiments, deficiency in CTNNBL1 led to a considerable (3–4 h) and reproducible delay in the initiation of cell proliferation following nitrogen starvation which correlated with delayed exit from G1 (Fig. 7).

**Discussion**

The results presented here reveal that although CTNNBL1 is not needed for the maturation or ongoing proliferation of primary mouse B cells, it is required for their timely exit from quiescence as well as for mouse embryonic development. A similar requirement for CTNNBL1 for timely exit from quiescence is also observed in fission yeast.

The embryonic lethality of CTNNBL1 deficiency manifests itself around the mid-term of pregnancy, by which time most of the major cell lineages have been established. Although gross inspection of mid-term embryos has not revealed any organ that is specifically affected by CTNNBL1 deficiency, it remains possible that there is a particular cell type whose function or development is critically dependent on CTNNBL1 and that which accounts for the lethality of CTNNBL1 deficiency. Alternatively it may be that the phenotype observed in the B cells (sluggish exit from quiescence) has a severe effect on embryonic viability when recapitulated in multiple lineages during synchronized embryonic development and lineage expansion. It will therefore be interesting to cross our floxed CTNNBL1 allele into mouse lines carrying Cre recombinases active in multiple lineages to ascertain whether there is any specific lineage or developmental stage at which loss of CTNNBL1 leads to a more severe phenotype than that observed in lymphocytes.

The molecular associations of CTNNBL1 suggest that it likely functions in RNA splicing. Although clearly not essential for splicing, a possibility suggested by this work is that CTNNBL1 may function in linking RNA splicing with timely exit from quiescence. This would be by no means the first occasion on which a link has been proposed between deficiency in an RNA splicing factor and the control of cell cycle progression.12-15 However, the suggestion here is that CTNNBL1 may function in connection with the timely “bulking up” of RNA splicing that must occur on exit from quiescence and allow progression through to S phase. Thus although CTNNBL1 deficiency does not affect the expression of early activation markers, CTNNBL1 deficiency does manifest itself in a delay to the subsequent cell enlargement/blasting. Such blasting must be accompanied by a substantial increase in the RNA processing and protein translation machinery of the cell. In contrast, a requirement for
CTNNBL1 deficiency on delaying cell blasting is especially marked with respect to the small follicular B cells as compared with their larger marginal zone counterparts. The phenotype revealed by CTNNBL1 deficiency is an intriguing one and suggests that there may be other genes whose primary function is to assist cell blasting and exit from quiescence. Several studies have been performed to identify pathways associated with cell quiescence—in budding and fission yeast, in nematode worms as well as in mammalian lymphocytes.

Such studies have identified genes and pathways functioning in the maintenance of the quiescent state but have not, so far as we are aware, revealed any genes whose deficiency results in a phenotype similar to that seen with CTNNBL1. We suspect that this does not reflect that CTNNBL1 is unique with respect to functioning specifically in the blasting/“bulking-up” phase of cell activation without being needed for immediate early gene activation or subsequent ongoing cell proliferation. Rather we suspect that such a phenotype might not have been easily scored in previous work, especially if deficiency in such genes leads to embryonic lethality, since it would only be readily apparent by following the blasting of resting cells obtained from animals carrying a lineage-specific gene ablation. It will be interesting therefore in future work not only to establish the molecular mechanism by which CTNNBL1 deficiency leads to sluggish exit from quiescence without affecting initial cell activation but also, by interrogation of mouse lines carrying other conditional gene ablations, to ascertain whether a similar phenotype is obtained with deficiencies in other genes.

**Materials and Methods**

**Generation of Ctnnb1-targeted mice.** The targeting construct for conditional Ctnnb1 inactivation (Fig. 1A) was obtained from EUCOMM (construct PG00008_A_1_E07). E14(129/Ola) ES cells were transfected with AslI-digested linear construct and 84 G418-resistant clones tested for targeted integration by Southern analysis using PCR-generated probes (5′-GGA AAG GTG ATA ACC CTA GAC ACT TTT G and 5′-TCC CAC CCA TCC TCG GCC TCA GTG C for probe LHP; 5′-TTC CCA GAA TCA GTT TCT TCT CGT C and 5′-TCT CAC CTG AAA GGA AGG CCA TCT for probe RHP). From 26 correctly targeted clones, two were used to generate blastocyst chimeras that led to germ line transmission. The selection cassette on the CTNNBL1 might not be manifest in cells already undergoing rapid division, since such cells will inherit a substantial endowment of transcription and translation factors from their parents. Indeed, the need for bulking up may especially apply to lymphoid cells. The adaptive immune system is notable in comprising very long-lived quiescent cells with little cytoplasm, which, following antigen triggering, take time to blast and then undergo multiple sequential rounds of rapid division. It is notable that the effect of
targeted allele in these Cnml+/- mice was removed in vivo by crossing with a FLPase-expressing transgenic mouse line9 to produce Cnml-/- animals. Breeding with mice in which Cre recombinase expression was driven from within the B cell-specific mb1 gene9 (kindly provided by Michael Reth) was used to generate CnmlPβGal/mb1Cre/- experimental animals that carry a B cell-specific deletion of Cnml exon3 on one allele and a null Cnml knockout on the other, as well as CnmlPβGal/mb1Cre/- control littermates which retain one intact Cnml allele with the other allele carrying a B cell-specific exon3 deletion. Mice expressing Cre from within the cd19 gene21 were also used for B-cell specific gene deletion (Fig. S1).

Mice in which the Cnml locus on one allele had been inactivated by integration of a gene-trapped promoterless β-Gal/neomycin cassette into the first Cnml intron were generated using ES cell line AX0016 obtained from the Sanger Institute Gene Trap Resource (GenBank accession number CZ259087).

Embryos (d14.5 post-mating) were dissected, fixed in 4% paraformaldehyde and stained with 2 ml of X-gal (2.25 mg/ml), 1 mM MgCl2, 45 ml PBS, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide for 48 h at 37°C prior to washing in PBS and refixing in 4% paraformaldehyde.

Cellular analyses. For analysis of lymphoid subpopulations, cell suspensions from bone marrow and spleen were depleted of erythrocytes by incubation in RBC lysis buffer (Ebioscience) and stained using fluorescent rat mAbs to mouse cell surface markers (CD8-FITC, CD4-PE, CD19-Pacific Blue, PE-CD45R(B220), CD21-FITC, CD23-PE, CD43-FITC and Ig-M-FITC from BD Targeted allele in these Cnml+/- mice was removed in vivo by crossing with a FLPase-expressing transgenic mouse line9 to produce Cnml-/- animals. Breeding with mice in which Cre recombinase expression was driven from within the B cell-specific mb1 gene9 (kindly provided by Michael Reth) was used to generate CnmlPβGal/mb1Cre/- experimental animals that carry a B cell-specific deletion of Cnml exon3 on one allele and a null Cnml knockout on the other, as well as CnmlPβGal/mb1Cre/- control littermates which retain one intact Cnml allele with the other allele carrying a B cell-specific exon3 deletion. Mice expressing Cre from within the cd19 gene21 were also used for B-cell specific gene deletion (Fig. S1).

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Figure 5. CTNNBL1 deficiency delays cell enlargement and S-phase entry but not the upregulation of early activation markers. (A) Comparison of blasting of splenic B cells from littermate pairs of mb1-Cre Ctnnbl1^−/flx and control mice after 24 h of incubation with LPS as monitored by cell scatter analysis. (i) Individual contour plots of live cells from two of the littermates pairs with the gating for blasts vs. resting cells indicated. (ii) Percentage of large cells (blasts) gate in multiple B cell cultures 24 h post-activation (mean and sd are indicated). (B) Induction of expression of cyclin D3, CDK6 and phosphorylated Rb after 24 h of incubation with LPS as monitored by western blot analysis. The abundance of lamin and α-tubulin served as loading controls. (C) Surface expression of CD69, CD86 and MHC class II on splenic B cells from littermate pairs of mb1-Cre Ctnnbl1^−/flx and mb1-Cre Ctnnbl1^+/flx control mice as analyzed after various times of incubation with LPS. (i) Histogram plots from a representative experiment and (ii) line graphs depicting the median fluorescence intensity at each time point derived from eight experiments. (Averages and sds are indicated). (D) Comparison of the blasting of splenic follicular and marginal zone B cells following LPS activation. The B cells were obtained from CD19-Cre Ctnnbl1^−/flx and CD19-Cre Ctnnbl1^+/flx control mice (with the CD19-Cre giving, like the mb1-Cre, efficient B cell-specific deletion of Ctnnbl1 [Fig. S1]). (i) Histogram plots depicting the electronic volumes of sorted follicular and mantle zone B cells. The purity (assessed by flow cytometry of surface markers) of the sorted populations at the start of the cultures is indicated. (ii) The proportion of marginal zone B cells with diameter > 9 μm at different times post-stimulation in multiple samples (means and S.E.M. shown).
PharMingen; B220-APC and IgM-APC from Invitrogen; CD3-PECY7 from Ebioscience). For analysis of cell activation, resti
spenic B cells were magnetically separated from CD43+ cells using MACS/LD columns (Miltenyi) and splenic marginal zone
and follicular B cells sorted from (B200+, CD19)-gated splenocytes on the basis of their CD23CD21high or CD23CD21-
phenotype. Cells in RPMI/10%FBS/0.05 mM 2-mercaptoethanol were cultured in the presence of either 50 μg/ml E. coli LPS
or 1 μg/ml anti-CD40 (HM40–3; PharMingen) together with 50 ng/ml IL-4 (R&D Systems). Cell activation and immuno-
globulin class switching were then monitored by staining with antibodies to CD69, CD86 or MHC class II or with bionyt-
lated anti-mouse IgG3 or IgG1 and APC-streptavidin together with PE-conjugated anti-mouse CD45R(B220). CFSE labeling
(Invitrogen) was performed according to manufacturer’s instructions. To monitor cellular DNA synthesis, cells were cultured
in the presence of 10 μM BrdU, fixed, permeabilized, treated with DNase and stained with anti-FITC-conjugated anti-BrdU
antibody according to manufacturer instructions (FITC BrdU flow kit, BD PharMingen). For cell cycle analysis, ethanol-fixed
cells were permeabilized on 0.1% Triton in the presence of propidium iodide. Flow cytometry analysis was done on LSRII (BD) or Eclipse (i-Cyt) (for six
color analysis or electronic volume measurements, respectively).

**Biochemical analyses.** For RNA analyses, total RNA was extracted with PureLink RNA purification kit (Ambion) using a DNase treatment step. For Q-PCR, 200 ng total RNA was reverse-transcribed using random primers and the cDNA amplified using SYBR Green qPCR SuperMix Universal (Invitrogen) according to manufacturer’s instructions. Expression of *Ctnnbl1* exon 3 was monitored using primers 5′-TCT CCG GAT TGT CTG GAA AC and 5′-GCT GGA TGA AAG CTC AGT GAA. HPRT and β2-microglobulin served as controls.

For protein expression analysis, cells (2 × 106) were lysed in RIPA buffer containing Protease and PhosStop inhibitors
(Roche) and the clarified Benzonase- (Novagen) treated lysate then subjected to SDS-PAGE. Western blot analysis was
performed using a rabbit antisera to α-tubulin and lamin-B (Abcam). Loading controls were provided by staining with rabbit antiser...
plates containing G418. Integration was confirmed by PCR using primers whose locations are indicated. (B) Δctnnb1 S. pombe grow similarly to controls after spotting on to YES plates. Viability of cells in log-phase growth in either yeast extract (YE) or minimal medium (EMM) from serial 10-fold dilutions spotted onto YE plates containing 5 mg/ml phloxine B. (C) Nitrogen-starved Δctnnb1 S. pombe showed delayed initiation of growth compared with controls after transfer into rich medium. Growth curves after release from nitrogen starvation (time zero) are shown for wild type S. pombe (wt; black line), two-independent Δctnnb1 mutants (red and orange lines) in which the ctbb1 locus had been replaced by a kan’ cassette as well as of S. pombe carrying a control ctbb1 targeting in which the ctbb1 locus had been replaced by 3HA-tagged CTNNBL1 driven from the nmt1 promoter (blue dashed line). Similar results were obtained in four independent experiments. Growth of serial 10-fold dilutions of 3 week-starved S. pombe cultures on YE plates revealed that CTNNBL1 deficiency did not affect their viability. (D) Starved CTNNBL1-deficient S. pombe exhibit delayed exit from G0, following release from nitrogen starvation. Quiescent S. pombe, which adopt a small, round shape on nitrogen starvation, elongate prior to their first cell division on release into rich medium.21 Cells were fixed in 70% ethanol at the times indicated and visualized by phase-contrast microscopy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be downloaded here:
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