Dystrophin expression in the *mdx* mouse restored by stem cell transplantation

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The development of cell or gene therapies for diseases involving cells that are widely distributed throughout the body has been severely hampered by the inability to achieve the disseminated delivery of cells or genes to the affected tissues or organ[1]. Here we report the results of bone marrow transplantation studies in the *mdx* mouse, an animal model of Duchenne's muscular dystrophy[2], which indicate that the intravenous injection of either normal haematopoietic stem cells or a novel population of muscle-derived stem cells into irradiated animals results in the reconstitution of the haematopoietic compartment of the transplanted recipients, the incorporation of donor-derived nuclei into muscle, and the partial restoration of dystrophin expression in the affected muscle. These results suggest that the transplantation of different stem cell populations, using the procedures of bone marrow transplantation, might provide an unanticipated avenue for treating muscular dystrophy as well as other diseases where the systemic delivery of therapeutic cells to sites throughout the body is critical. Our studies also suggest that the inherent developmental potential of stem cells isolated from diverse tissues or organs may be more similar than previously anticipated.

To determine whether transplantation of bone marrow cells carrying a wild-type dystrophin gene could restore dystrophin expression within the myofibres of the *mdx* mouse, nine female *mdx* mice were lethally irradiated and subsequently injected via the tail vein with 5×10^5 or $1\text{–}5 \times 10^7$ bone marrow cells from normal male C57BL/10 mice (Table 1). Five weeks after transplantation, the haematopoietic cells of all transplanted animals were donor-derived (of male origin), as determined by fluorescence *in situ* hybridization (FISH) analysis for Y-chromosome-specific sequences (data not shown). At 5, 8 and 12 weeks after bone marrow transplantation, the tibialis anterior muscle from each transplanted animal was analysed for dystrophin expression by immunohistochemistry, followed by FISH analysis using a Y-chromosome-specific probe to detect donor-derived (male) cells (Table 1; Fig. 1). Because previous studies of nuclear domains in myofibres had shown that the expression of a protein can extend several micrometres away from the source nucleus[3–6], around 20 serial sections were analysed to attempt to associate a dystrophin-positive fibre with a donor-derived (male) nucleus.

At five weeks after bone marrow transplantation, less than 1% of the myofibres expressed dystrophin (Table 1), similar to background reversion levels described in other studies[7]. Eight weeks after bone marrow transplantation, however, about 1% of the muscle fibres expressed dystrophin in their normal sarcolemmal location, with 25–63% of dystrophin-expressing fibres containing detectable fused donor-derived nuclei (Table 1). By 12 weeks after bone marrow transplantation, as many as 10% of the muscle fibres within an individual mouse expressed dystrophin, with 10–30% of the dystrophin-positive myofibres containing detectable fused Y-chromosome-positive nuclei (Table 1; Fig. 1a, b).

Although other studies have documented the capacity of bone-marrow-derived cells to give rise to muscle[8,9], the identity of the cells with that potential had not been determined. To address this issue, we asked whether highly purified haematopoietic stem cells (HSCs) could give rise to dystrophin-positive myofibres after bone marrow transplantation. HSCs were isolated from the bone marrow of normal male C57BL/10 mice by fluorescence-activated cell sorting (FACS) purification of Hoechst 33342-stained (H0342) cells, as described[10]. These HSCs, termed SP cells, are Sca-1$^+$, c-Kit$^+$, CD43$^+$, CD45$^+$, lineage marker (B220, Mac-1, Gr-1, CD4, CD5, and CD8)-low/negative and CD34-negative, and can completely engraft recipients at very low cell numbers (100–500 cells per mouse)[10,11]. We injected 2,000–5,000 bone marrow SP cells of male origin into the tail veins of nine lethally irradiated female *mdx* mice (Table 1). Mice were killed 5, 8 and 12 weeks after stem cell injection and FISH analysis of the bone marrow confirmed that the female host was completely reconstituted with male donor cells (data not shown). The tibialis anterior muscle was examined for dystrophin expression and the presence of male donor cells by the analysis of serial sections, as described above (Table 1). As was the case with recipients engrafted with unfractionated bone marrow cells, less than 1% of the myofibres expressed dystrophin at 5 weeks (Table 1). At 8 weeks, up to 1% of the muscle fibres expressed dystrophin, with 20–40% of dystrophin-expressing fibres containing fused Y-chromosome-positive nuclei (Table 1). At 12 weeks, dystrophin was detected in up to 4% of the myofibres, with 10–30% of these containing fused donor nuclei (Table 1; Fig. 1c–f).

Donor-derived nuclei were associated with both individual dystrophin-positive fibres and clusters of dystrophin-positive

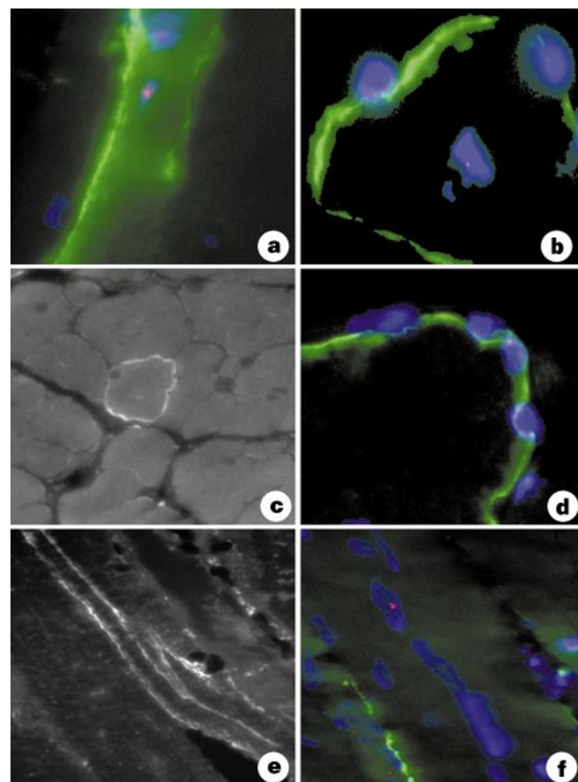

Figure 1 Dystrophin expression and detection of Y-chromosome-positive nuclei in the tibialis anterior muscle 12 weeks after whole bone marrow or haematopoietic stem cell transplantation into lethally irradiated female *mdx* recipients. Donor nuclei (nuclei counterstained with DAPI in blue and Y chromosomes shown as red hybridization signals) were found fused to dystrophin-positive myofibres (in green) at 12 weeks after either whole bone marrow (**a**, **b**) or highly purified haematopoietic stem cell transplantation (**c–f**). In **f**, the fused donor nucleus was detected further upstream of the dystrophin-positive fibre shown in **e**.

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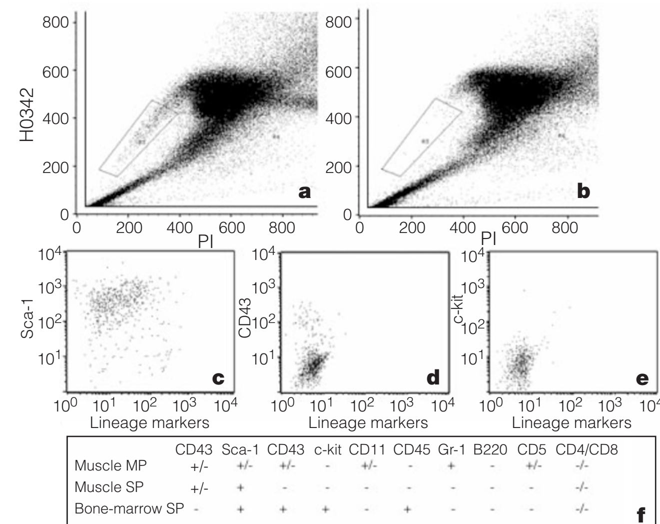
Table 1 Detection of donor-derived dystrophin-positive muscle fibres after transplantation of whole bone marrow or bone marrow SP cells

No. of animals	Weeks after injection	No. cells injected*	% Dys$^+$ myofibres (total)†	% Y$^+$ nuclei in myofibres‡
3	5	5×10^5 per animal (WBM)	0.5; 0.5; 1 (795)	0; 0; 0
3	8	5×10^5; 5×10^7; 5×10^7 (WBM)	1; 1; 1 (919)	63; 36; 25
3	12	1×10^7; 5×10^7; 5×10^7 (WBM)	10; 5; 1 (552)	12; 8; 33
4	5	2,000 per animal (BM-SP)	0.5; 0.5; 0.6; 0.7 (1,016)	0; 0; 0; 0
2	8	2,000 per animal (BM-SP)	1; 0.5 (1,715)	39; 22
3	12	2,000; 5,000; 5,000 (BM-SP)	4; 1; 3 (1,214)	12; 28; 14

* WBM: whole bone marrow; BM-SP: bone-marrow SP cells.
† Percentage of dystrophin-positive fibres in each animal. (total) represents the average total number of myofibres.
‡; Percentage of dystrophin-positive fibres for which a Y-chromosome-positive nucleus was found in the skeletal muscle of each animal for all sections analysed (on average, one or none per section).

fibres. Among 39 photographed sections, 4–8 Y$^+$ nuclei were centrally located, 5–6 nuclei were peripherally located within myofibres and 7–9 nuclei were clearly fused to host myofibres but their location (central or peripheral) was uncertain. However, in some cases where clusters of dystrophin-positive fibres were analysed, no more than a single male nucleus was found. This finding could be due to either preferential fusion of Y$^+$ donor cells to clusters of revertant fibres or, alternatively, to an underestimate of the actual proportion of dystrophin-positive fibres associated with donor-derived nuclei. In fact, only a portion of each myofibre was analysed by immunohistochemistry and FISH, and control experiments showed that the FISH analysis does not identify 100% of male nuclei. In addition, although the percentage of fibres associated with a donor-derived nucleus appeared to decrease at longer times after transplantation of bone marrow cells (Table 1), this may be simply because the domain of dystrophin expression from an individual donor nucleus increasing with time[3–6].

As the Hoechst 33342 staining/FACS method for purification of haematopoietic stem cells appears to depend upon a set of physical properties that are common to haematopoietic stem cells from many species, including humans, in a manner independent of antibodies[11], we next asked whether this method could be used to isolate putative stem cells from skeletal muscle. Mononuclear cells were isolated from 3–5-week-old mouse skeletal muscle[12], stained with 12.5 $\mu g\,ml^{-1}$ of H0342, and analysed as described[10]. As observed after the staining of bone marrow cells, FACS analysis of muscle cells revealed a side population (SP) displaying low staining with H0342 and a main population (MP) of cells that were more brightly stained with the dye (Fig. 2a). As was the case for bone marrow SP cells, the muscle SP cell population disappeared upon addition of verapamil (Fig. 2b), a drug that blocks the efflux of



	CD43	Sca-1	CD43	c-kit	CD11	CD45	Gr-1	B220	CD5	CD4/CD8
Muscle MP	+/-	+/-	+/-	-	+/-	-	+	-	+/-	-/-
Muscle SP	+/-	+	-	-	-	-	-	-	-	-/-
Bone-marrow SP	-	+	+	+	-	+	-	-	-	-/-

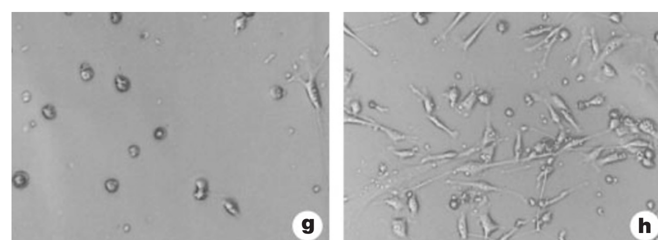


Figure 2 Isolation and characterization of muscle SP cells. A side population (SP) of cells was identified by FACS analysis (**a**, boxed area). In the presence of verapamil, this population was not visible (**b**). Over 80% of muscle SP cells express the antigen Sca-1 and are negative for lineage markers (**c**), CD43 (**d**) and c-Kit (**e**). The antigens expressed on muscle SP and MP cells compared to bone marrow SP cells are shown in **f**. +/–, mixture of positive and negative cells; +, cells positive for the marker; –, cells negative for the marker. Muscle SP cells (**g**) and MP cells (**h**) in culture appear morphologically different.

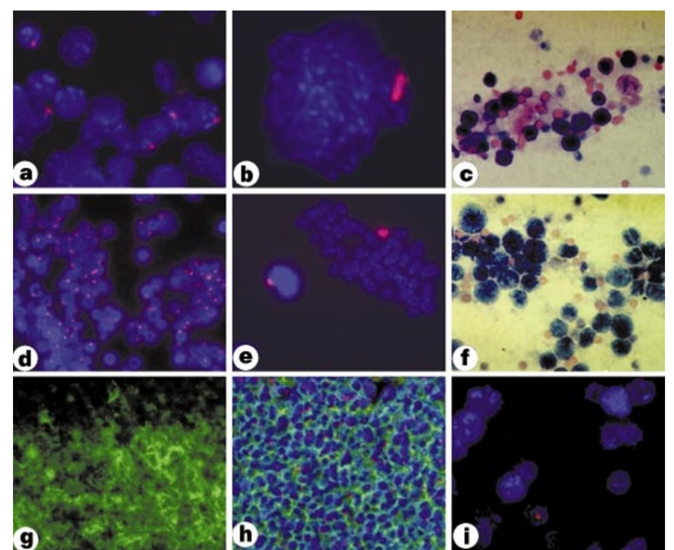


Figure 3 Detection of donor nuclei in the bone marrow and spleen of *mdx* recipients after injection of muscle SP cells. **a–c**, Bone marrow, animal 4; **d–f**, bone marrow, animal 3. Y-chromosomes are detected as red hybridization signals over the nuclei (**a**, **d**, 60× original magnification). Y-chromosome positive metaphases were detected at high magnification (**b**, **e**, 100×). Giemsa stain of bone marrow samples shows the presence of several cell types (**c**, **f**, 100×). Expression of CD43 (**g**) or CD45 (**h**) antigens in spleen tissue sections from cells of donor origin (**h**); animal 3. **i**, Detection of few male nuclei in the bone marrow of animals co-injected with muscle and bone marrow SP cells.

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Table 2 Detection of donor-derived dystrophin-positive muscle fibres after transplantation of muscle SP cells

Animal	Days after injection	No. cells injected	% Dys$^+$ myofibres (total)*	% Y$^+$ nuclei in myofibres†	% Y$^+$ nuclei in bone marrow‡
1	17	7,000	9 (254)	3	41
2	21	10,000	3 (276)	7	75
3	30	20,000	6 (820)	9	91
4	30	13,000	5 (800)	6	30
5	28	19,000	ND	ND	80

* Dystrophin-positive fibres as a percentage of total number of fibres (total).
† Percentage of dystrophin-positive fibres for which a Y-chromosome-positive nucleus was detected in one given section (on average, 1 or 2 per section).
‡ Percentage of Y$^+$ nuclei in the bone marrow samples detected by FISH. As a positive control for these experiments, the FISH probe was hybridized in parallel to interphase nuclei from male cells, and the hybridization efficiency was >95%.
ND: not determined.

Hoechst dye[10]. In contrast, the MP myoblasts were unaffected by verapamil (Fig. 2b).

Characterization of muscle SP cells revealed several unique features that distinguished them from bone marrow SP and muscle MP cells. First, isolation of muscle SP cells required a concentration of H0342 dye that was 2.5 times greater than that used to purify bone marrow SP cells[10]. In addition, although both muscle and bone marrow SP cells were Sca-1$^+$ lin$^-$, as predicted for early progenitor cells, c-Kit and CD45, two surface markers expressed on bone marrow SP cells[11], were not present on muscle SP cells (Fig. 2e, f). Similarly, over 90% of muscle SP cells were negative for CD43 (Fig. 2d), another marker detected on bone marrow SP cells. In contrast, muscle MP cells expressed several lineage markers (lin$^+$), such as CD11, Gr-1 and CD5 (Fig. 2f). The two muscle-cell populations were also different in appearance. *In vitro* culture experiments revealed that, after one week, nearly all MP cells adhered to the culture dish and were fully differentiated into myoblasts (Fig. 2h), with a few intervening fibroblasts as determined by desmin staining (data not shown). In contrast, most SP cells maintained a spherical shape and failed to settle on the plate (Fig. 2g). Only after 2 weeks in culture did muscle SP cells differentiate as a mixture of myoblasts and fibroblasts.

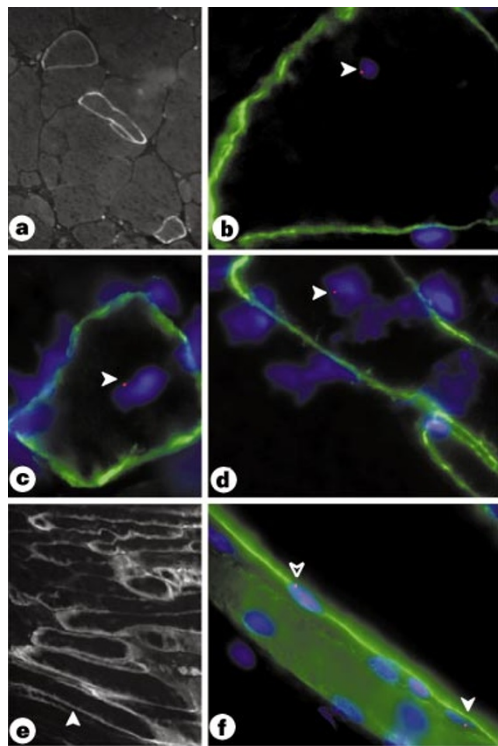

Figure 4 Dystrophin-positive fibres were detected in skeletal muscle tissue sections of animals 3 and 1. **a**, **e**, Low-magnification fields (original magnification (25×) showing the dystrophin-positive myofibres seen in **b–d**, respectively. **a**, Animal 3; **e**, animal 1. Solid arrowheads in **b–d**, **f** indicate at high magnification (100×) donor male nuclei fused to host dystrophin-positive myofibres. A donor nucleus appears to be juxtaposed to the sarcolemma of the myofibre, implying that it may be a satellite cell (**f**, open arrowhead).

To evaluate the potential of muscle SP cells to contribute to muscle and/or haematopoietic compartments, we prepared muscle SP cells from normal C57BL/10 male mice and injected them into the tail veins of lethally irradiated female *mdx* mice. Preliminary bone marrow transplantation experiments using muscle SP cells indicated that at least ten-fold more muscle SP cells than bone marrow SP cells were needed for reliable radioprotection (data not shown). Based on those studies, 7,000–20,000 male muscle SP cells were injected into five lethally irradiated *mdx* females (Table 2). At day 17, one mouse injected with 7,000 muscle SP cells (Table 2, animal 1) appeared weak and was killed. The other animals were killed at 28–30 days, when all seemed in good health (Table 2, animals 2–5). Analysis of the bone marrow cells of the recipients by FISH indicated variable engraftment by donor cells, ranging from 30–91% (Fig. 3a, d; Table 2). Metaphase spreads containing the Y chromosome were also detected by FISH (Fig. 3b, e), indicating that the introduced male muscle SP cells could divide *in vivo*. Giemsa staining of bone marrow samples of these animals revealed diverse types of haematopoietic cell (Fig. 3c, f). In addition to bone marrow, spleen tissue sections of animals 3 and 4 were immunostained with either anti-CD43 or anti-CD45 antibodies, two surface markers that are expressed by haematopoietic cells but not by muscle SP cells (Fig. 2f). Both antibodies revealed the presence of immunoreactive cells (Fig. 3g, h). Co-detection of donor nuclei by FISH showed that over 90% of the spleen cells, including those expressing CD43 or CD45, were positive for the Y chromosome and thus of donor origin (Fig. 3h). Collectively, these findings showed that muscle SP cells can reconstitute the haematopoietic compartment of lethally irradiated recipients, albeit less effectively than bone marrow SP cells. In fact, in competitive repopulation studies of lethally irradiated *mdx* females injected with a mixture of 200 bone marrow SP cells derived from *mdx* females and 6,000 muscle SP cells derived from normal males, less than 1% of the bone marrow nuclei were positive for the Y chromosome by FISH at 4 and 8 weeks (Fig. 3i).

To evaluate the ability of muscle SP cells to differentiate into muscle, bone marrow transplant recipients engrafted with muscle SP cells were analysed by immunohistochemistry combined with FISH (Table 2; Fig. 4). Analysis of 15–30 skeletal muscle tissue sections from each of four recipients revealed donor-derived dystrophin-positive myofibres, with on average one or two Y$^+$ nuclei fused to dystrophin-positive fibres in each section (Fig. 4). For animals injected with muscle SP cells, analysis of 18 photographed muscle tissue sections from three different animals revealed a pattern of nuclear localization similar to that seen in animals injected with bone marrow cells. Of a total of 28 donor male nuclei within dystrophin-positive myofibres, 12 nuclei were centrally located in the dystrophin-positive myofibres (Fig. 4b–d), 9 were peripherally located (Fig. 4f, solid arrowhead) and 7 were clearly fused to the myofibres but whether they were centrally or

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peripherally located was unclear. Furthermore, a few donor nuclei were detected juxtaposed to a myofibre (Fig. 4f, open arrowhead), at a position consistent with that of satellite cells[13]. Analysis of serial muscle tissue sections for the expression of dystrophin and laminin showed that donor male nuclei that were juxtaposed but not fused to dystrophin-positive myofibres were encompassed by laminin staining in the adjacent sections, implying that satellite cells arise after transplantation of muscle SP cells (data not shown). In contrast, analysis of more than 200 tissue sections from animals injected with either whole bone marrow or highly purified haematopoietic cells failed to reveal the presence of Y^+ nuclei at this position. Although these findings indicate that muscle-derived stem cells may differ from bone marrow derived stem cells in their capacity to give rise to satellite cells, more detailed studies of the precise anatomical position and molecular characteristics of these cells associated with donor-derived nuclei will be necessary to definitely address this issue.

Previous cell transplantation strategies aimed at the delivery of dystrophin have been quite successful[4,14], but have required multiple, local intramuscular injections, as systemic delivery of cells to muscle through myoblast transplantation does not appear to occur[15]. Similarly, the delivery of dystrophin to muscle by *in vivo* gene transfer with viral vectors has resulted in only local restoration of dystrophin[16–19]. The most important practical conclusion of our studies is that bone marrow or muscle SP cells appear to provide a means for the systemic, rather than local, repair of muscle, as a consequence of the delivery of the cells throughout the vascular system. Although, in general, the proportion of dystrophin-positive myofibres that resulted from stem cell transplantation was below the levels that would be likely to be needed to provide clinical benefit in patients with muscular dystrophy, it is possible that, in the future, the procedures for stem cell transplantation that we have employed could be optimized to provide levels of engraftment of muscle that would be clinically useful[20,21].

Our studies also shed important new light on the biological properties of different stem cell populations. First, the experiments involving the transplantation of highly purified haematopoietic stem cells provide the first direct evidence that haematopoietic stem cells with the capacity for the complete reconstitution of lethally irradiated recipients have the potential to differentiate into muscle. Previous *in vitro* studies[22–24] had indicated that mesenchymal stem cells of the bone marrow stroma might be the relevant source of the cells with muscle potential revealed by the earlier bone marrow transplantation studies of Ferrari *et al.* and Bittner *et al.*[8,9]. A second important biological finding from our studies is that, using purification methods similar to those used previously to isolate haematopoietic stem cells, a population of cells from muscle can be isolated with similar functional and phenotypic properties to those of haematopoietic stem cells isolated from bone marrow. These results raise the possibility that there may be some direct relationship between bone marrow derived stem cells and other tissue- or organ-specific stem cells. One possibility that needs to be explored further is that stem cells resident in specific tissues or organs originate from a common set of cells in the bone marrow, yet adopt tissue- or organ-specific characteristics upon seeding within a specific local environment. Alternatively, organ- and tissue-specific stem cell populations may arise solely as a consequence of the normal development of that specific tissue or organ, but in general share similar phenotypic and functional characteristics. In this regard, it is interesting that we have found that SP-like cells can be isolated from a number of other differentiated tissues and organs (unpublished results). Several studies have also shown that certain bone marrow derived cells have the potential to differentiate into other non-haematopoietic cells, such as endothelial cells[25,26], and that some CNS-derived stem cell lines have the potential to differentiate into haematopoietic cells[27]. Clearly, more studies to assess the origin and functional properties of different stem cell populations and their relationship to bone marrow derived stem cells are urgently needed. □

Methods

Mouse strains

C57BL/10 mice and C57BL/10ScSn-*Dmd*^{mdx}/J (X-linked muscular dystrophy) mice were obtained from the Jackson Laboratory.

Purification of bone marrow and haematopoietic stem cells

Bone marrow was extracted from the femurs and tibias of 6–8-week-old animals and red blood cells were removed with ammonium chloride solution (Sigma). To purify haematopoietic stem cells, bone marrow cells were resuspended at 10^6 cells per ml and stained with 5 μg ml^{-1} Hoechst 33342 (Sigma) as described[10]. Cells were then magnetically pre-enriched for Sca-1-positive cells using the MACS (Miltenyi Biotec) and streptavidin microbeads. Before cell sorting, cells were resuspended in HBSS containing 2% FCS and 2 μg ml^{-1} propidium iodide (PI). Flow cytometric analysis and cell sorting were performed on a dual-laser FACSVantage flow cytometer (Becton Dickinson). Both the Hoechst and propidium iodide dyes were excited at 350 nm and their fluorescence was measured at 450 and 600 nm. The sorting gate for the haematopoietic stem cell population was established as described[10].

Isolation of muscle SP cells

We isolated skeletal muscle myoblasts from 3–5-week-old donor male tissue as described[12]. Before H0342 staining, red cells were lysed[28]. Primary myoblasts were resuspended at 10^6 cells per ml and stained with 12.5 μg ml^{-1} of H0342 in PBS-0.5%BSA for 90 min at 37 °C. In parallel, as a negative control for SP cells, we stained 10^6 cells in the presence of 50 μM verapamil and used them to set the gate for isolation of SP cells by FACS in the test sample[10]. Cells were washed once in cold PBS-0.5%BSA, resuspended at 10^8 cells per ml and incubated for 10 min on ice with 10 μg ml^{-1} of biotinylated anti-Sca-1 antibody (Pharmingen). For cell-lineage marker analysis, muscle SP and MP cells were stained as described[10]. Before FACS analysis and sorting, samples were enriched for Sca-1$^+$ cells and stained with 2 μg ml^{-1} PI[10]. Before being injected into animals, muscle SP cells were washed once in PBS-0.5%BSA and resuspended in 200 μl of PBS-0.5%BSA. For cell culture, muscle SP and MP myoblasts were maintained as described[12].

Bone marrow, haematopoietic or muscle stem cell transplantation

Female *mdx* recipients were lethally irradiated with 1200 rad given in two doses (600 rad each), 3 h apart. Bone marrow, haematopoietic or muscle stem cells were given intravenously into the tail vein. Mice were maintained on acidified water after transplantation. All animal care was in accordance with institutional guidelines.

Tissue collection and FISH analysis

Recipient animals were killed and skeletal muscle and spleen were snap-frozen in cold isopentane and stored at −80 °C. The bone marrow was isolated from the hind leg bones. Cells were washed in 1 × PBS and filtered through a 70 μM filter. For Giemsa staining, bone marrow cells were spread on a glass slide, fixed in methanol for 3 min and stained according to the manufacturer's instructions (Sigma). For FISH analysis of bone marrow nuclei, cells were treated with hypotonic solution and fixed in methanol and acetic acid before slide preparation as described[29].

We prepared the Y-chromosome FISH probe (a gift from E. Snyder) by labelling 1 μg of plasmid DNA with digoxigenin-11-dUTP as described[29,30]. FISH was standardized on whole nuclei isolated from a male murine muscle cell line and on male muscle tissue sections. The hybridization efficiency was >90% on whole nuclei and 70–80% on tissue sections.

Immunohistochemistry and *in situ* hybridization were performed on the same tissue sections as described[30]. Slides were examined using a Zeiss Axiophot microscope and images were collected using a CCD camera (Photometrics) as described[30].

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Evidence that a free-running oscillator drives G1 events in the budding yeast cell cycle

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In yeast and somatic cells, mechanisms ensure cell-cycle events are initiated only when preceding events have been completed[1]. In contrast, interruption of specific cell-cycle processes in early embryonic cells of many organisms does not affect the timing of subsequent events[2], indicating that cell-cycle events are triggered by a free-running cell-cycle oscillator. Here we present evidence for an independent cell-cycle oscillator in the budding yeast *Saccharomyces cerevisiae*. We observed periodic activation of events normally restricted to the G1 phase of the cell cycle, in cells lacking mitotic cyclin-dependent kinase activities that are essential for cell-cycle progression. As in embryonic cells, G1 events cycled on schedule, in the absence of S phase or mitosis, with a period similar to the cell-cycle time of wild-type cells. Oscillations of similar periodicity were observed in cells responding to mating pheromone in the absence of G1 cyclin (Cln)- and mitotic cyclin (Clb)-associated kinase activity, indicating that the oscillator may function independently of cyclin-dependent kinase dynamics. We also show that Clb-associated kinase activity is essential for ensuring dependencies by preventing the initiation of new G1 events when cell-cycle progression is delayed.

In wild-type budding yeast, completion of cell-cycle events in one cell-cycle phase is essential for the transition to the next phase[1], although mutants have been identified that can initiate synchronous, periodic rounds of budding in the absence of DNA replication and mitosis[3]. Strains bearing temperature-sensitive mutations in genes involved in protein degradation, *cdc4*, *cdc34* or *cdc53*, cannot enter S phase or mitosis but do initiate synchronous rounds of budding at the restrictive temperature[4]. The molecular basis for the inability of these mutants to replicate DNA or complete mitosis is related to the stabilization of the Clb/Cdc28-specific inhibitor, Sic1p, and the consequent loss of Clb-kinase activity[5]. That *cdc4*, *cdc34* and *cdc53* cells produce multiple buds indicates that the initiation of G1 events may be uncoupled from the completion of S phase or mitosis by the loss of Clb-associated kinase activity.

To investigate this idea, we analysed events normally restricted to G1 in synchronous populations of cells bearing the temperature-sensitive mutation *cdc4-3*, or cells disrupted for all six B-type cyclin genes. Synchronous populations of G1 cells were collected by centrifugal elutriation, and then shifted to the restrictive temperature (36 °C). Budding and transcript levels were analysed at 10-min intervals. Synchronous oscillations of *CLN2* transcript levels were reproducibly observed with periods coinciding with the budding cycles (Fig. 1d–f, g–i). The cycling of G1 events indicates the existence of an oscillator that cycles independently of B-type cyclin activity and the completion of S phase or mitosis. The period of budding and transcription cycles in cells lacking B-type cyclin activity is very similar to the period observed in normally dividing wild-type cells (Fig. 1a–c), indicating that G1 events in normally dividing cells may be entrained to the same independent oscillator.

Activation of Cln/Cdc28 kinase is a critical G1 event that is required for the initiation of budding and the transition from G1 to S phase[6]. Therefore, we investigated whether the oscillations in *CLN2* transcript levels in cells lacking Clb activity led to functional oscillations in Cln2-associated kinase. Indeed, Cln2-associated kinase did oscillate synchronously with the appearance of new buds in *cdc4-3* cells (Fig. 1j, k). However, it is likely that the oscillation of Cln2-associated kinase activity is not essential for driving budding cycles, as constitutive expression of Cln2 from the *GAL1* promoter did not prevent rebudding in *cdc4-3* cells (data not shown). Other events essential for rebudding cycles may be entrained to the oscillator independently of Cln2-associated kinase activity (see below).

As Clns can auto-activate their own transcription[7,8] and also appear to stimulate their own proteolysis[9,10], they could conceivably define an oscillator that drives G1 events in the absence of cell-cycle progression. Our finding that *cdc4-3 cdc28-4* double mutant cells do not exhibit budding or G1-specific transcription cycles indicates that Cln/Cdc28 kinase activity may be essential for these events (data not shown). However, our experiments do not distinguish whether Clns are components of an oscillator or are simply entrained to a Cln-independent oscillator, as the outputs we measured (budding and transcription) are themselves dependent on Cln/Cdc28-kinase activity.

Previous observations indicate that measurable oscillations may occur independent of Cln/Clb-associated kinase activity. In response to mating pheromone, budding yeast adopt a unique morphology, termed 'shmoo', by polarizing growth to form a

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