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An absence of both lamin B1 and lamin B2 in keratinocytes has no effect on cell proliferation or the development of skin and hair

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Nuclear lamins are usually classified as A-type (lamins A and C) or B-type (lamins B1 and B2). A-type lamins have been implicated in multiple genetic diseases but are not required for cell growth or development. In contrast, B-type lamins have been considered essential in eukaryotic cells, with crucial roles in DNA replication and in the formation of the mitotic spindle. Knocking down the genes for B-type lamins (*LMNB1*, *LMNB2)* in HeLa cells has been reported to cause apoptosis. In the current study, we created conditional knockout alleles for mouse *Lmnb1* and *Lmnb2*, with the goal of testing the hypothesis that B-type lamins are crucial for the growth and viability of mammalian cells *in vivo*. Using the keratin 14-*Cre* transgene, we bred mice lacking the expression of both *Lmnb1* and *Lmnb2* in skin keratinocytes (*Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$). *Lmnb1* and *Lmnb2* transcripts were absent in keratinocytes of *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ mice, and lamin B1 and lamin B2 proteins were undetectable. But despite an absence of B-type lamins in keratinocytes, the skin and hair of *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ mice developed normally and were free of histological abnormalities, even in 2-year-old mice. After an intraperitoneal injection of bromodeoxyuridine (BrdU), similar numbers of BrdU-positive keratinocytes were observed in the skin of wild-type and *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ mice. *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ keratinocytes did not exhibit aneuploidy, and their growth rate was normal in culture. These studies challenge the concept that B-type lamins are essential for proliferation and vitality of eukaryotic cells.

INTRODUCTION

The nuclear lamina, an intermediate filament meshwork lying beneath the inner nuclear membrane, is composed mainly of four proteins—lamins A, C, B1 and B2 (1,2). Lamins A and C are alternatively spliced products of a single gene (*LMNA*) (3,4), whereas lamin B1 and lamin B2 are products of distinct genes, *LMNB1* and *LMNB2*, respectively (5,6). The lamins were initially divided into two groups, A-type (lamins A and C) and B-type (lamins B1 and B2), on the basis of different isoelectric points, but other considerations have strengthened the rationale for this categorization (2,7). For example, the sequences of lamins B1 and B2 are more similar to each other than to lamins A and C (5). Both lamin B1 and lamin B2 contain a farnesyl lipid anchor, whereas lamin C and mature lamin A do not (8). Also, lamins A and C are expressed late in embryonic development, primarily in differentiated cells, whereas lamins B1 and B2 are expressed in all somatic cell types, including undifferentiated cells at the earliest stages of embryonic development (9,10). In addition, hundreds of missense, non-sense and frameshift mutations in *LMNA* have been linked to a panoply of genetic diseases (including muscular dystrophy, cardiomyopathy, lipodystrophy, neuropathy and progeria), but these classes of mutations have not been reported for *LMNB1* or *LMNB2* (8).

The A-and B-type lamins have been reported to differ in terms of their importance for the growth and vitality of mammalian cells (11–13). A knockdown of *LMNA* in HeLa cells had no effects on cell viability, whereas a knockdown of *LMNB1* or *LMNB2* arrested growth and led to apoptosis

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(11). These findings seemed consistent with reports that B-type lamins have unique and important functions in the cell nucleus. For example, lamin B1, but not A-type lamins, was reported to localize to areas of DNA replication (14), and depleting a B-type lamin in *Xenopus* blocked DNA replication (15). In addition, Tsai *et al*. (16) reported that lamin B1 has a crucial role in the assembly of the mitotic spindle, and that the spindle complex was disrupted by a dominant-negative lamin B1 mutant. In addition, B-type lamins are thought to be important for chromatin organization and gene expression at the nuclear periphery (7,17).

To assess the functional relevance of the B-type lamins, our laboratory began by creating *Lmnb1* and *Lmnb2* knockout mice (12,13). Both knockout mice died shortly after birth. *Lmnb1*- and *Lmnb2*-deficient fibroblasts were able to grow in culture, although the absence of lamin B1 was associated with misshapen cell nuclei. The single-knockout models did not, of course, permit firm conclusions about the importance of the B-type lamins, given that each knockout model retained the other B-type lamin.

In the current study, we took the next step and examined the consequences of inactivating both *Lmnb1* and *Lmnb2* in the same cell. We generated conditional knockout alleles for *Lmnb1* and *Lmnb2* and then bred mice lacking both lamin B1 and lamin B2 in skin keratinocytes. We chose skin keratinocytes, rather than some other cell type, for several reasons. First, the skin and hair express large amounts of lamin B1 and lamin B2 (18). Second, on a practical level, the keratin 14-*Cre* transgene (*K14Cre*) is extremely effective for inactivating genes in keratinocytes (19,20). Third, the turnover of skin keratinocytes is high (21), and we reasoned that any effect of the B-type lamins on cell proliferation would be readily detectable in those cells. Fourth, the formation of the skin epidermis and hair involves complex developmental programs, and it seemed reasonable that these programs would be disrupted by disturbances in cell growth or gene expression.

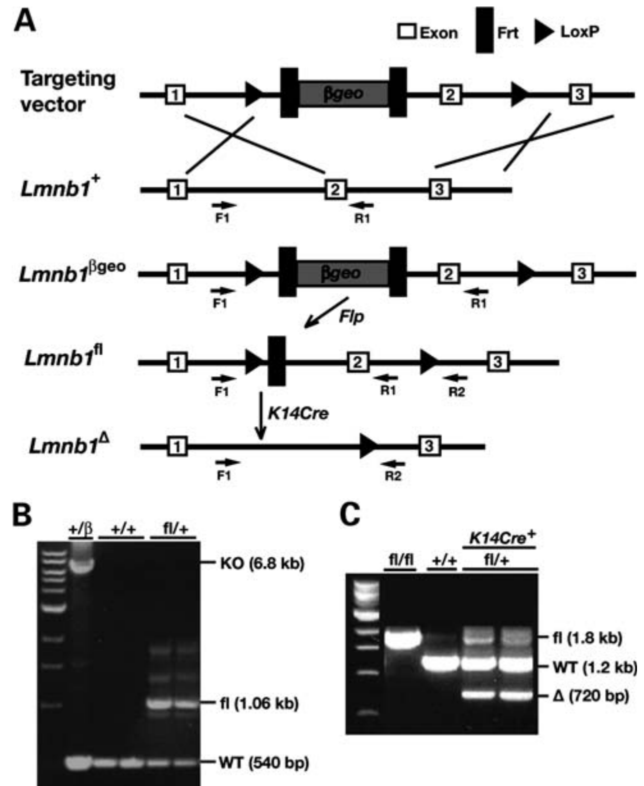

Figure 1. Generation of keratinocyte-specific *Lmnb1* knockout mice. (**A**) Schematic of the *Lmnb1* targeting vector containing a *βgeo* gene-trapping cassette flanked by FRT sites in intron 1 and a pair of *loxP* sites flanking exon 2. The gene-trapping cassette in the targeted allele ($Lmnb1^{\beta geo}$) was removed with *Flp* recombinase, creating a floxed allele ($Lmnb1^{fl}$). With expression of *Cre* recombinase, the floxed allele is converted into a null allele ($Lmnb1^{\Delta}$). Open squares represent exons; *loxP* and FRT sites are depicted as black triangles and black rectangles, respectively. PCR primers (F1, R1, LoxpR2) are indicated with arrows. (**B**) PCR of genomic DNA from $Lmnb1^{+/+}$, $Lmnb1^{+/\beta geo}$ and $Lmnb1^{fl/+}$ ES cells with primers F1 and R1. WT (wild-type), 540 bp; $Lmnb1^{\beta geo}$ (KO), 6.8 kb; $Lmnb1^{fl}$, 1.06 kb. (**C**) PCR of genomic DNA from $Lmnb1^{fl/fl}$, $Lmnb1^{+/+}$ and $Lmnb1^{fl/+}K14Cre$ mice with primers F1 and LoxpR2. WT, 1.2 kb; $Lmnb1^{fl}$ (fl), 1.8 kb; $Lmnb1^{\Delta}$ (Δ), 720 bp.

RESULTS

Inactivating *Lmnb1* and *Lmnb2* in keratinocytes using conditional alleles

The targeting vectors for *Lmnb1* and *Lmnb2* (Figs 1 and 2) were designed to introduce an FRT-flanked *βgeo* cassette into intron 1, along with *loxP* sites flanking exon 2 (Figs 1 and 2). After identifying targeted cell lines, the *βgeo* cassette was excised with *Flp* recombinase, generating conditional knockout or 'floxed' alleles ($Lmnb1^{fl}$ and $Lmnb2^{fl}$) (Figs 1 and 2).

We used the *K14Cre* transgene to delete exon 2 and thereby inactivate *Lmnb1* and *Lmnb2* in skin keratinocytes. Keratinocyte-specific *Lmnb1* ($Lmnb1^{fl/fl}K14Cre$ or $Lmnb1^{\Delta/\Delta}$), *Lmnb2* ($Lmnb2^{fl/fl}K14Cre$ or $Lmnb2^{\Delta/\Delta}$) and double-knockout mice ($Lmnb1^{fl/fl}Lmnb2^{fl/fl}K14Cre$ or $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$) were born at the expected Mendelian frequency. The mice survived for >2 years and were indistinguishable from wild-type littermates.

Skin keratinocytes from newborn $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice contained only trace amounts of *Lmnb1* and *Lmnb2* transcripts, as judged by qRT-PCR (Fig. 3A and B). There was no effect of the keratinocytes knockouts on gene expression in other tissues (Supplementary Material, Fig. S1). Lamin B1 and lamin B2 proteins were undetectable in extracts from primary $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ keratinocytes (Fig. 3C), and immunofluorescence microscopy revealed a complete absence of lamins B1 and B2 in freshly isolated $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ keratinocytes (Fig. 3D; Supplementary Material, Fig. S2A). Microscopy also showed an absence of lamins B1 and B2 in keratinocytes of the ear skin (Fig. 3E) and tongue (Supplementary Material, Fig. S2B and C) from $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice.

$Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ keratinocytes proliferate normally

Bromodeoxyuridine (BrdU) labeling studies revealed that mitotically active skin keratinocytes (i.e. BrdU-positive) were equally abundant in wild-type and $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice (Fig. 4A and B). Similarly, there were similar numbers of keratinocytes expressing the phosphorylated form of histone H3 (a marker of mitosis) in wild-type and $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice (Fig. 4C and D). Primary $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$

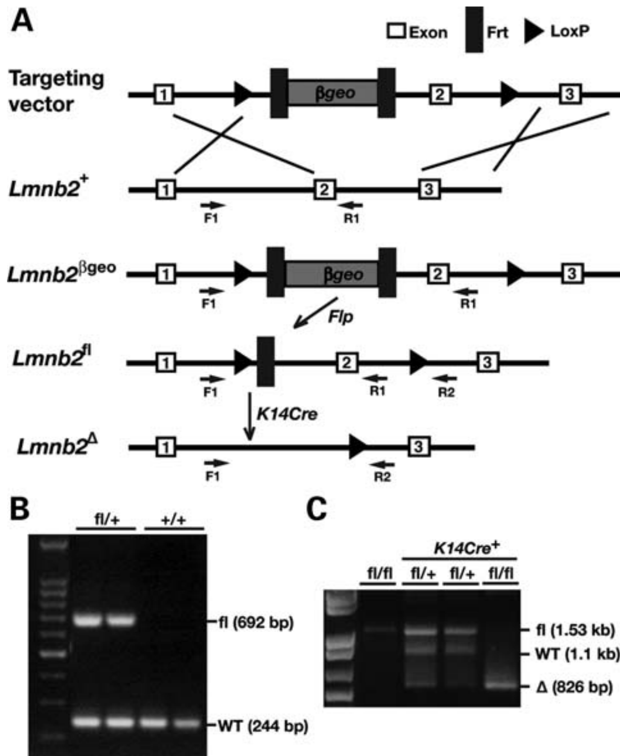

Figure 2. Generation of keratinocyte-specific *Lmnb2* knockout mice. (**A**) Schematic of the *Lmnb2* targeting vector containing a β*geo* gene-trapping cassette flanked by FRT sites in intron 1 and a pair of *loxP* sites flanking exon 2. The gene-trapping cassette in the $Lmnb2^{\beta geo}$ allele was removed with *Flp* recombinase, creating a floxed allele ($Lmnb2^{fl}$). With the expression of *Cre* recombinase, the floxed allele is converted into a null allele ($Lmnb2^{\Delta}$). Open squares represent exons; *loxP* and FRT sites are depicted as black triangles and black rectangles, respectively. PCR primers (F1, R1 and R2) are indicated by arrows. (**B**) PCR of genomic DNA from $Lmnb2^{+/+}$ and $Lmnb2^{fl/+}$ ES cells with primers F1 and R1. WT (wild-type), 244 bp; $Lmnb2^{fl}$ (fl), 692 bp. (**C**) PCR of genomic DNA from $Lmnb2^{fl/fl}$, $Lmnb1^{fl/+}K14Cre$ and $Lmnb2^{fl/fl}K14Cre$ ($Lmnb2^{\Delta/\Delta}$) mice with primers F1 and R2. WT, 1.1 kb; $Lmnb2^{fl}$ (fl), 1.53 kb; $Lmnb2^{\Delta}$ (Δ), 826 bp.

keratinocytes exhibited normal cell growth curves (Supplementary Material, Fig. S3A) and had normal numbers of chromosomes (Supplementary Material, Fig. S3B). Lamin A/C expression was not upregulated, either in $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ keratinocytes (Fig. 3C, Supplementary Material, Fig. S4) or in immortalized $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ fibroblasts (Supplementary Material, Fig. S5).

$Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ keratinocytes have an increased frequency of misshapen cell nuclei

To determine whether the absence of B-type lamins affects nuclear shape, we assessed the frequency of misshapen cell nuclei in primary keratinocytes from wild-type, $Lmnb1^{\Delta/\Delta}$, $Lmnb2^{\Delta/\Delta}$ and $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice ($n = 3$ cell lines/genotype; 300–900 cells/genotype). Keratincytes from wild-type mice exhibited a relatively high frequency of misshapen cell nuclei (blebs and folds) when compared with our earlier studies with cultured fibroblasts (22,23). When we examined the mutant keratinocytes, we found more misshapen nuclei (mainly nuclear blebs) in $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ than in wild-type keratinocytes ($P < 0.0005$), and there was a trend toward more misshapen nuclei in $Lmnb1^{\Delta/\Delta}$ and $Lmnb2^{\Delta/\Delta}$ keratinocytes (Fig. 5A and B). These differences were consistent in keratinocytes prepared from different mice (Fig. 5B). While nuclear blebs were relatively common in cultured $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ keratinocytes (Fig. 5A and B), we never observed blebs in the skin keratinocytes of $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice by immunofluorescence microscopy (with a lamin A/C antibody) or by routine histology (Supplementary Material, Figs S2, S6).

Normal skin and hair in $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice

The skin and hair appeared entirely normal in $Lmnb1^{\Delta/\Delta}$ $Lmnb2^{\Delta/\Delta}$ mice up to 24 months of age (through all stages of the hair cycle). Also, we observed no histological abnormalities in the skin or hair of $Lmnb1^{\Delta/\Delta}$, $Lmnb2^{\Delta/\Delta}$ and $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice, and skin thickness was similar (Supplementary Material, Fig. S6). Finally, electron microscopy of skin keratinocytes from wild-type and $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice ($n > 60$ micrographs/genotype) uncovered no abnormalities (Fig. 6). Specifically, the nuclear membrane, nuclear pores and heterochromatin were indistinguishable in wild-type and $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ keratinocytes (Fig. 6).

DISCUSSION

The B-type lamins, lamins B1 and B2, are fundamental building blocks of the nuclear lamina and are thought, based on cell biology studies, to have unique and important roles in cell growth and mitosis, DNA replication, chromatin organization and gene expression (2,7,24). Genetic studies support this view. When *Lmnb1* and *Lmnb2* were knocked down in cultured cells with siRNAs (11), the cells stopped proliferating and died. Also, *Lmnb1* and *Lmnb2* knockout mice die shortly after birth (12,13). In addition, the absence—at least thus far—of loss-of-function mutations in human *LMNB1* and *LMNB2* could be viewed as being consistent with an essential function for the B-type lamins in eukaryotic cells. In the current study, we explored the functional importance of lamins B1 and B2 in adult mouse tissues. We generated conditional knockout alleles for *Lmnb1* and *Lmnb2* and went on to create keratinocyte-specific knockout mice, reasoning that any unique and vital function for B-type lamins in the nucleus would likely manifest itself in that rapidly proliferating cell type. The knockout of *Lmnb1* and *Lmnb2* in keratinocytes was efficient; transcript levels for those genes were virtually abolished, and lamin B1 and lamin B2 proteins were undetectable in skin keratinocytes. Remarkably, there were no apparent consequences from the loss of B-type lamins. The skin and hair were normal, even at 2 years of age. The chromosome number in freshly isolated keratinocytes was normal, and the proliferation of keratinocytes in $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice was no different than in wild-type mice. By electron microscopy, there were no abnormalities in the nuclear envelope or in the distribution of heterochromatin within the cell nucleus. These observations suggest that the B-type lamins are dispensable in keratinocytes and argue against an essential role for the B-type lamins in DNA replication or cell proliferation.

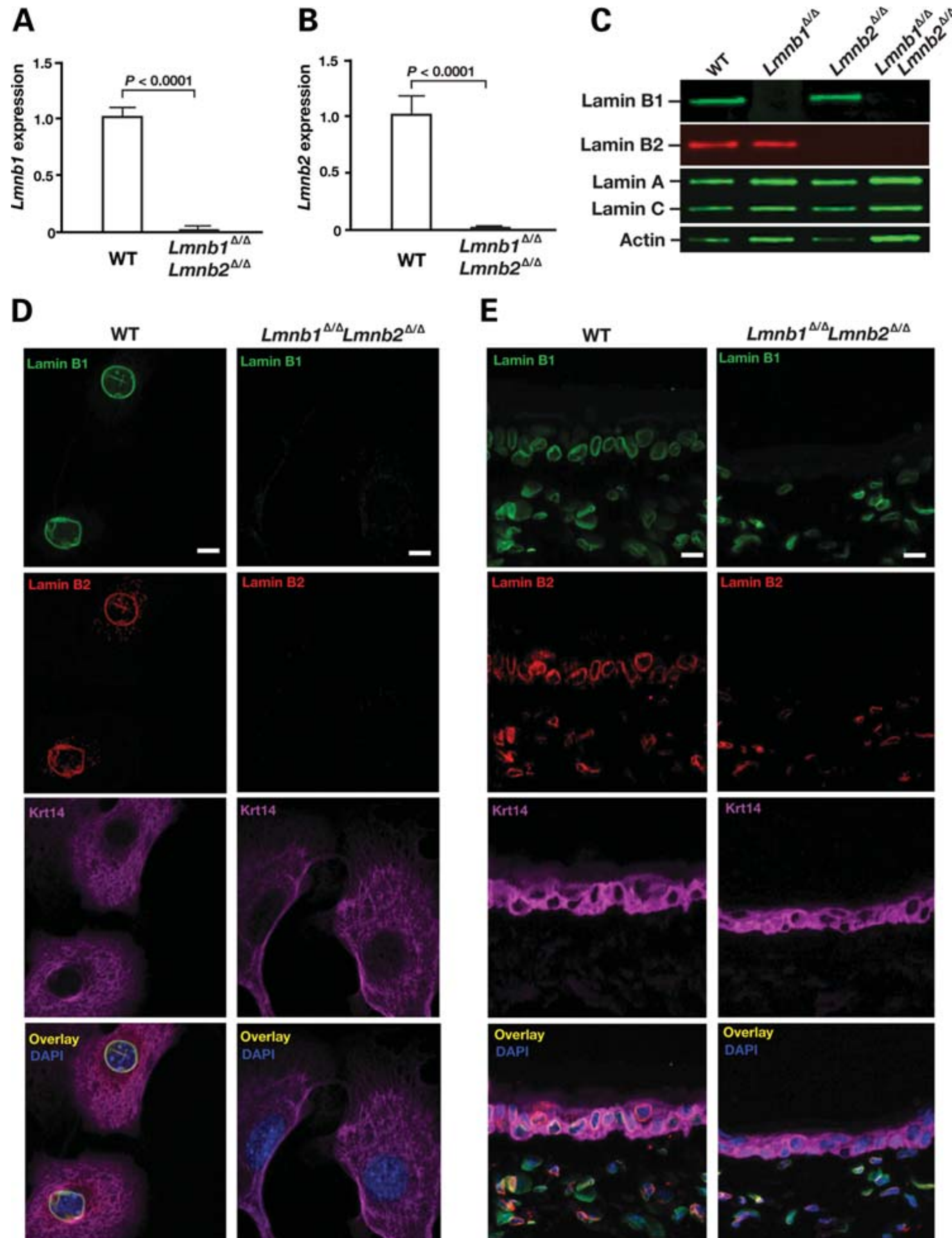

Figure 3. Inactivation of *Lmnb1* and *Lmnb2* in skin keratinocytes. (**A** and **B**) qRT-PCR studies of *Lmnb1* (A) and *Lmnb2* (B) expression in keratinocytes from $Lmnb1^{+/+}$ and $Lmnb1^{fl/fl}Lmnb2^{fl/fl}K14Cre$ ($Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$) mice ($n = 4$/genotype). Data were normalized to β2-microglobulin and gene expression was compared with that in the $Lmnb1^{+/+}$ mice (which was set at 1.0). (**C**) Western blots of extracts from $Lmnb1^{+/+}$, $Lmnb1^{\Delta/\Delta}$, $Lmnb2^{\Delta/\Delta}$ and $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ keratinocytes with antibodies against lamin B1, lamin B2 and lamin A/C. Actin was used as a loading control. (**D** and **E**) Immunofluorescence microscopy of primary keratinocytes (**D**) and ear skin (**E**) from wild-type (WT) and $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice (four mice/group were examined) with antibodies against lamin B1 (green), lamin B2 (red) and keratin 14 (magenta). DNA was visualized with DAPI (blue). Scale bar, 20 μm.

Cultured $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ skin keratinocytes had significantly more misshapen cell nuclei than wild-type keratinocytes, but misshapen nuclei were never observed in the skin keratinocytes from $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice (either by routine histology or immunohistochemistry). In the case of some mutations—although perhaps not all (25)—we suspect that cultured cells are particularly susceptible to the development of nuclear blebs, perhaps because the pancake-like shape of cells grown on plastic renders nuclei more susceptible to shape abnormalities (26). Previously, we examined nuclear shape abnormalities in a *Lmna* knock-in model ($Lmna^{LAO/LAO}$) that synthesizes exclusively *mature* lamin A (bypassing prelamin A synthesis and processing) (26). By immunofluorescence microscopy, there were many misshapen nuclei in cultured $Lmna^{LAO/LAO}$ fibroblasts but none was found in tissues of $Lmna^{LAO/LAO}$ mice.

Our findings do not mean that the B-type lamins are devoid of purpose. In fact, we know that this is not the case—simply because *Lmnb1* and *Lmnb2* knockout mice cannot survive after birth. However, we can conclude that lamins B1 and

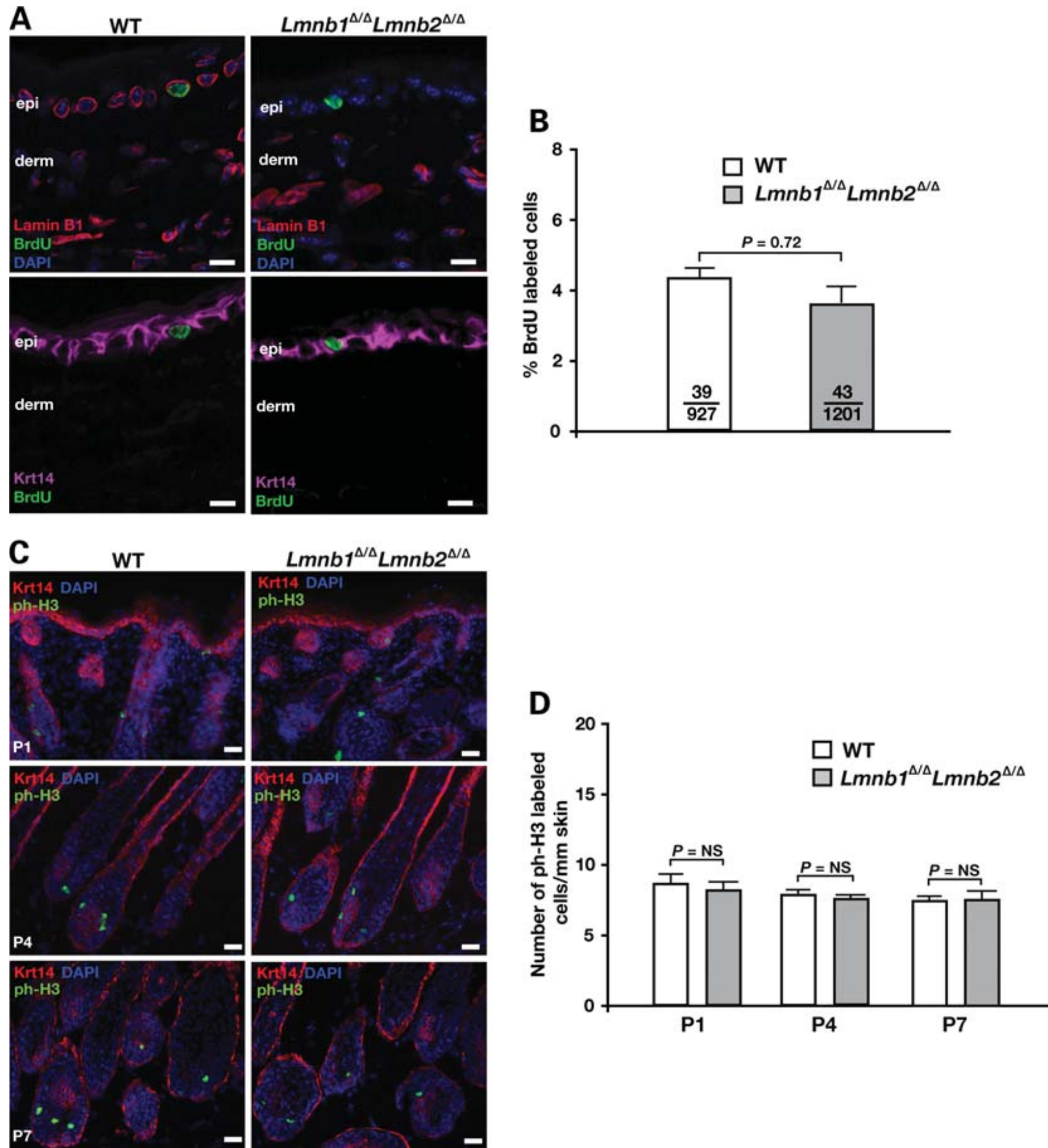

Figure 4. Normal proliferation of keratinocytes from *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ mice. (**A**) BrdU labeling studies in 40-day-old wild-type (WT) and *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ mice. Mice were given an intraperitoneal injection of BrdU and sacrificed 1 h later. Ear skin was examined by immunofluorescence microscopy with antibodies against BrdU (green), lamin B1 (red) and keratin 14 (Krt14; magenta). DNA was visualized with DAPI (blue). epi, epidermis; derm, dermis. Scale bar, 50 μm. (**B**) Percentage of BrdU-positive keratinocytes in ear skin from wild-type and *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ mice. Numbers of BrdU-positive cells (numerator) and total cells (denominator) examined are recorded within each bar ($P = 0.72$, *t*-test). (**C**) Immunofluorescence microscopy studies of the skin of wild-type and *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ mice at P1, P4 and P7 with antibodies against phosphohistone H3 (ph-H3; green) and keratin 14 (Krt14; red). DNA was visualized with DAPI (blue). Scale bar, 50 μm. (**D**) Number of ph-H3-positive keratinocytes/mm of skin from wild-type and *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ mice at P1, P4 and P7. A total of 10 mm of skin was analyzed for each genotype. No differences were observed.

B2 are dispensable for skin development and proliferation (and that A-type lamins appear to be sufficient for the integrity of the cell nucleus *in vivo*). Ultimately, we suspect that the B-type lamins will turn out to be dispensable in other tissues as well; indeed, we already know that mice lacking both *Lmnb1* and *Lmnb2* in hepatocytes (*Lmnb1*$^{fl/fl}$*Lmnb2*$^{fl/fl}$ mice harboring an albumin–*Cre* transgene) are free of hepatocellular disease (S.H.Y., L.G.F., S.G.Y., unpublished data).

If B-type lamins are not uniquely important in proliferating cells such as keratinocytes, then what is their purpose and why have they been highly conserved through evolution? A likely clue, we believe, has emerged from studies of *Lmnb2*-deficient mice (13). Lamin B2 deficiency causes defective neuronal migration in the developing brain, leading to disordered layering of neurons in the cerebral cortex. Migration of cortical neurons during development is a saltatory process that involves repeated cycles of pulling the cell nucleus forward into the leading edge of the cell (13). The 'tugging' on the cell nucleus, applied by cytoplasmic dynein motors, exerts deformational forces on the cell nucleus and likely places a premium on the scaffolding provided by the nuclear lamina. The B-type lamins may be well suited for holding the

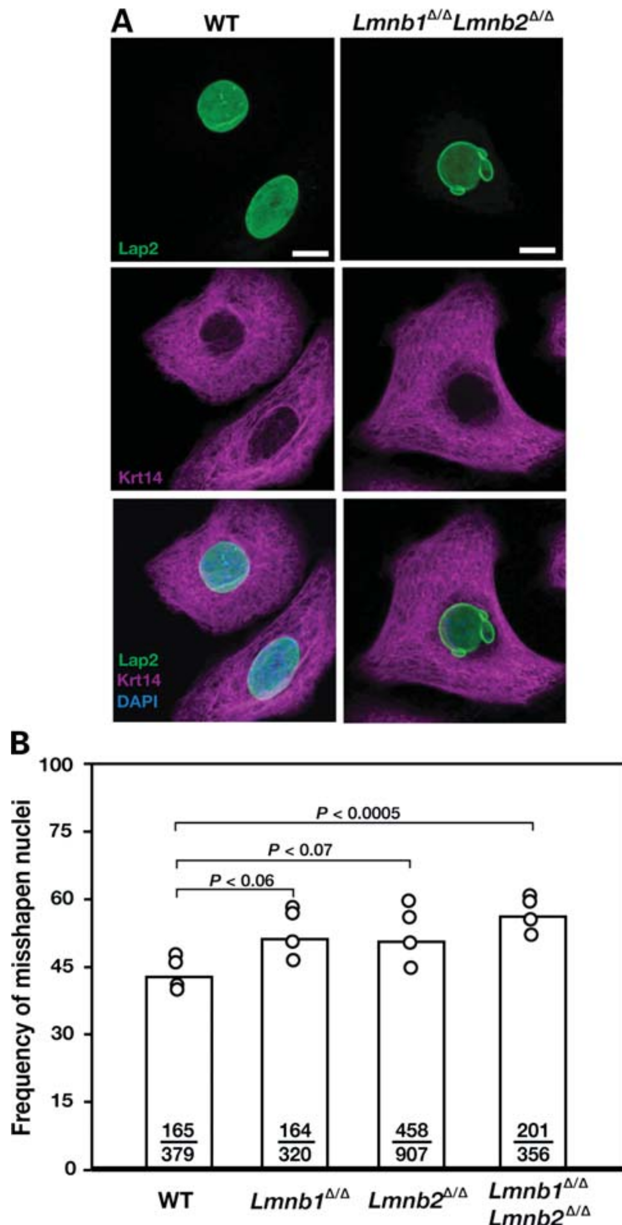

Figure 5. Increased frequency of nuclear blebbing in *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ keratinocytes. (**A**) Immunohistochemical staining of keratinocytes from wild-type and *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ mice with antibodies against Lap2 (green) and keratin 14 (Krt14; magenta). DNA was visualized with DAPI (blue). (**B**) Frequency of misshapen nuclei in wild-type, *Lmnb1*$^{\Delta/\Delta}$, *Lmnb2*$^{\Delta/\Delta}$ and *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ keratinocytes ($n = 4$/genotype tested). Bars indicate the frequency of misshapen nuclei; the number of cells with nuclear blebs and the total number of cells examined are provided within each bar. *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ keratinocytes had more nuclear blebs, as judged by a χ^2 test. Significant differences between wild-type and *Lmnb1*$^{\Delta/\Delta}$*Lmnb2*$^{\Delta/\Delta}$ keratinocytes were also observed by a *t*-test, by comparing the percentages of misshapen nuclei in keratinocytes prepared from different mice ($P < 0.001$). Slides were scored by two reviewers blinded to genotype. Circles represent the frequency of misshapen nuclei of each individual mouse.

nuclear envelope together, given that these lamins are anchored to the inner nuclear membrane by a farnesyl lipid. In any case, B-type lamins are clearly essential for neuronal migration in the brain but not for the development of skin or the proliferation of keratinocytes.

The human genetics of A- and B-type lamins are markedly different. There are hundreds of missense, frameshift, nonsense and splicing mutations in *LMNA* that elicit a panoply of human diseases (8). But for lamins B1 and B2, there are no diseases resulting from these classes of mutations, and the only bona fide disease association is autosomal-dominant leukodystrophy with a *LMNB2* gene duplication (27). This discrepancy in disease associations remains somewhat enigmatic, but mouse models are starting to provide a framework of understanding. In light of the current studies, it would be difficult to claim that the absence of human mutations is due to a requirement for the B-type lamins in the proliferation and survival of eukaryotic cells. The developmental biology studies by Coffinier *et al*. (13) also provide important insights; those studies suggested that mutations in *LMNB1* and *LMNB2* might ultimately be found in association with neurodevelopmental diseases rather than with the usual spectrum of *LMNA* diseases (e.g. lipodystrophy, progeria, cardiomyopathy, muscular dystrophy).

MATERIALS AND METHODS

Generation of keratinocyte-specific *Lmnb1* and *Lmnb2* knockout mice

To create conditional knockout alleles for *Lmnb1* and *Lmnb2*, we used ‘targeted trapping/conditional-ready’ gene-targeting vectors (28). Bacterial artificial chromosome (BAC) clones for *Lmnb1* and *Lmnb2* were isolated from a mouse BAC library (strain 129OlaHsd). Second, a *βgeo* gene-trapping cassette was inserted into the targeted BAC vector clones with the λ-Red recombineering system and Gateway cloning (Invitrogen). Both *Lmnb1* and *Lmnb2* targeting vectors were designed to insert one *loxP* site upstream of the *βgeo* cassette in intron 1, and a second *loxP* site in intron 2. The targeting vectors were linearized with *AsiS*I and electroporated into strain 129/OlaHsd ES cells; targeted ES cell clones were identified by Southern blotting with a flanking probe. Next, the targeted ES cells were transfected with a supercoiled *Flp* recombinase plasmid to excise the *βgeo* cassette, creating ES cells harboring a conditional knockout allele (exon 2 is flanked by *loxP* sites). Those ES cells were used to produce germline-transmitting chimeric mice. The ‘floxed’ alleles were not hypomorphic alleles; *Lmnb1* and *Lmnb2* expression levels in *Lmnb1*$^{fl/fl}$ and *Lmnb2*$^{fl/fl}$ mice were equivalent to those in wild-type mice (Supplementary Material, Fig. S7). A more detailed description of screening, genotyping and breeding is provided in the Supplementary Materials.

Studies with epidermal keratinocytes

Primary keratinocytes were isolated from mouse skin on postnatal Day 1 or 4 (P1 or P4) and seeded into 6-well tissue culture plates with mitotically inactivated 3T3 fibroblasts. The cells were cultured in Williams E medium with 0.07 mM $CaCl_2$ in 5% CO_2 at 37°C. For growth assays, equal numbers of cells were seeded into each well of a 6-well tissue culture plate along with mitotically inactivated 3T3 fibroblasts. Numbers of keratinocytes were counted with a Coulter counter at Days 1, 5, 8 and 11. To assess

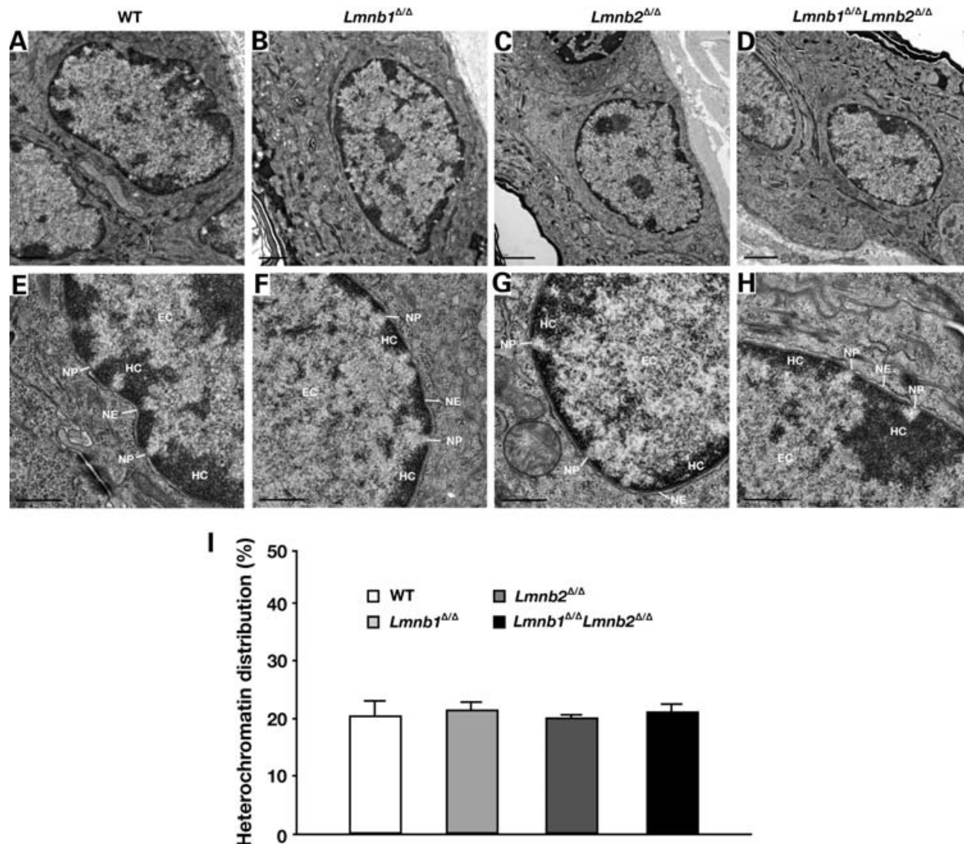

Figure 6. Electron micrographs of keratinocyte nuclei in the skin from $Lmnb1^{+/+}$, $Lmnb1^{\Delta/\Delta}$, $Lmnb2^{\Delta/\Delta}$ and $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ mice, showing heterochromatin (HC), euchromatin (EC), nuclear pores (NP) and nuclear envelope (NE). Magnification in (**A**)–(**D**): ×22, 700. Scale bar, 1.0 μm. Magnification in (**E**)–(**H**): ×74, 200. Scale bar, 0.5 μm. (**I**) Percentage of heterochromatin in the nuclei of $Lmnb1^{+/+}$, $Lmnb1^{\Delta/\Delta}$, $Lmnb2^{\Delta/\Delta}$ and $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ skin keratinocytes. We examined, by electron microscopy, a total of 26 WT nuclei, 28 $Lmnb1^{\Delta/\Delta}$ nuclei, 31 $Lmnb2^{\Delta/\Delta}$ nuclei and 35 $Lmnb1^{\Delta/\Delta}Lmnb2^{\Delta/\Delta}$ nuclei.

chromosome number, primary keratinocytes were incubated for 4 h with 10 μg/ml colcemid (Sigma). The cells were then centrifuged and treated with 0.075 M KCl and fixed in a mixture of glacial acetic acid and methanol (1:2.5). After spreading on slides, DNA was stained with Giemsa (Gibco). Images were obtained on a Nikon Eclipse E600 microscope with a 40×/0.75 objective and processed with SPOT software (Diagnostic Instruments).

Histological studies and immunofluorescence microscopy

Mouse skin was fixed for 4 h in 10% paraformaldehyde (PFA), dehydrated for 24 h in 70% ethanol and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin. For immunofluorescence microscopy studies, skin and tongue were embedded in Sakura Tissue-Tec OCT compound, frozen on dry ice, and 8 μm thick sections were cut with a cryostat. Slides were fixed in 4% formalin for 10 min, permeabilized with PBS containing 0.1% Tween-20 and incubated in blocking buffer (PBS containing 10% donkey or goat serum, 0.2% BSA, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) for 1 h. Slides were then incubated overnight at 4°C with primary antibodies diluted in blocking buffer containing 0.1% Triton X-100. Secondary antibodies were incubated in a similar fashion for 0.5–1 h. After washing the slides, they were fixed with 4% PFA for 10 min, incubated in PBS/DAPI for 10 min and mounted with Prolong-Gold Antifade reagent (Invitrogen). The following antibodies were used for the immunochemical studies: lamin B1 (goat, 1:200, Santa Cruz), lamin B2 (mouse, 1:200, Invitrogen), lamin A (mouse, 1:200, Millipore), keratin 14 (rabbit, 1:800, Covance), phosphohistone H3 (rat, 1:1000, Sigma), Lap2 (mouse, 1:400, BD Laboratories) and BrdU (rat, 1:200, Abcam). Alexa Fluor 488- and Alexa Fluor 568-conjugated secondary antibodies were used at a 1:200 dilution. Lamin B2- and lamin A-specific monoclonal antibodies were labeled with Alexa Fluor 555 and Alexa Fluor 488 Antibody Labeling Kits (Invitrogen), respectively. Images were recorded with an Axiovert 200M microscope equipped with an AxioCam MRm and an ApoTome (all from Zeiss).

BrdU labeling

Forty-day-old mice were given an intraperitoneal injection of BrdU (40 mg/kg of body weight; Sigma) and euthanized 1 h later. Ear tissue biopsies were pretreated with 1 N HCl for 10 min on ice, 2 N HCl for 10 min at room temperature, followed by 10 min at 37°C and 0.1 M sodium borate (pH 8.5) for 12 min. Sections (8 μm) were cut and processed for immunofluorescence microscopy as described earlier.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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REFERENCES

1. Stewart, C.L., Roux, K.J. and Burke, B. (2007) Blurring the boundary: the nuclear envelope extends its reach. *Science*, **318**, 1408–1412.
2. Dechat, T., Adam, S.A., Taimen, P., Shimi, T. and Goldman, R.D. (2010) Nuclear lamins. *Cold Spring Harb. Perspect. Biol.*, **2**, a000547.
3. Lin, F. and Worman, H.J. (1993) Structural organization of the human gene encoding nuclear lamin A and nuclear lamin C. *J. Biol. Chem.*, **268**, 16321–16326.
4. Fisher, D.Z., Chaudhary, N. and Blobel, G. (1986) cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. *Proc. Natl Acad. Sci. USA*, **83**, 6450–6454.
5. Hoger, T.H., Zatloukal, K., Waizenegger, I. and Krohne, G. (1990) Characterization of a second highly conserved B-type lamin present in cells previously thought to contain only a single B-type lamin. *Chromosoma*, **99**, 379–390.
6. Vorburger, K., Kitten, G.T. and Nigg, E.A. (1989) Modification of nuclear lamin proteins by a mevalonic acid derivative occurs in reticulocyte lysates and requires the cysteine residue of the C-terminal CXXM motif. *EMBO. J.*, **8**, 4007–4013.
7. Hutchison, C.J. (2002) Lamins: building blocks or regulators of gene expression? *Nat. Rev. Mol. Cell Biol.*, **3**, 848–858.
8. Worman, H.J., Fong, L.G., Muchir, A. and Young, S.G. (2009) Laminopathies and the long strange trip from basic cell biology to therapy. *J. Clin. Invest.*, **119**, 1825–1836.
9. Rober, R.A., Weber, K. and Osborn, M. (1989) Differential timing of nuclear lamin A/C expression in the various organs of the mouse embryo and the young animal: a developmental study. *Development*, **105**, 365–378.
10. Stewart, C. and Burke, B. (1987) Teratocarcinoma stem cells and early mouse embryos contain only a single major lamin polypeptide closely resembling lamin B. *Cell*, **51**, 383–392.
11. Harborth, J., Elbashir, S.M., Bechert, K., Tuschl, T. and Weber, K. (2001) Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J. Cell Sci.*, **114**, 4557–4565.
12. Vergnes, L., Peterfy, M., Bergo, M.O., Young, S.G. and Reue, K. (2004) Lamin B1 is required for mouse development and nuclear integrity. *Proc. Natl Acad. Sci. USA*, **101**, 10428–10433.
13. Coffinier, C., Chang, S.Y., Nobumori, C., Tu, Y., Farber, E.A., Toth, J.I., Fong, L.G. and Young, S.G. (2010) Abnormal development of the cerebral cortex and cerebellum in the setting of lamin B2 deficiency. *Proc. Natl Acad. Sci. USA*, **107**, 5076–5081.
14. Moir, R.D., Montag-Lowy, M. and Goldman, R.D. (1994) Dynamic properties of nuclear lamins: lamin B is associated with sites of DNA replication. *J. Cell Biol.*, **125**, 1201–1212.
15. Lopez-Soler, R., Moir, R., Spann, T., Stick, R. and Goldman, R. (2001) A role for nuclear lamins in nuclear envelope assembly. *J. Cell Biol.*, **154**, 61–70.
16. Tsai, M.Y., Wang, S., Heidinger, J.M., Shumaker, D.K., Adam, S.A., Goldman, R.D. and Zheng, Y. (2006) A mitotic lamin B matrix induced by RanGTP required for spindle assembly. *Science*, **311**, 1887–1893.
17. Dechat, T., Adam, S.A. and Goldman, R.D. (2009) Nuclear lamins and chromatin: when structure meets function. *Adv. Enzyme Regul.*, **49**, 157–166.
18. Hanif, M., Rosengardten, Y., Sagelius, H., Rozell, B. and Eriksson, M. (2009) Differential expression of A-type and B-type lamins during hair cycling. *PLoS ONE*, **4**, e4114.
19. Hafner, M., Wenk, J., Nenci, A., Pasparakis, M., Scharffetter-Kochanek, K., Smyth, N., Peters, T., Kess, D., Holtkotter, O., Shephard, P. *et al.* (2004) Keratin 14 Cre transgenic mice authenticate keratin 14 as an oocyte-expressed protein. *Genesis*, **38**, 176–181.
20. Lee, R., Chang, S.Y., Trinh, H., Tu, Y., White, A.C., Davies, B.S., Bergo, M.O., Fong, L.G., Lowry, W.E. and Young, S.G. (2010) Genetic studies on the functional relevance of the protein prenyltransferases in skin keratinocytes. *Hum. Mol. Genet.*, **19**, 1603–1617.
21. Fuchs, E. and Raghavan, S. (2002) Getting under the skin of epidermal morphogenesis. *Nat. Rev. Genet.*, **3**, 199–209.
22. Toth, J.I., Yang, S.H., Qiao, X., Beigneux, A.P., Gelb, M.H., Moulson, C.L., Miner, J.H., Young, S.G. and Fong, L.G. (2005) Blocking protein farnesyltransferase improves nuclear shape in fibroblasts from humans with progeroid syndromes. *Proc. Natl Acad. Sci. USA*, **102**, 12873–12878.
23. Yang, S.H., Bergo, M.O., Toth, J.I., Qiao, X., Hu, Y., Sandoval, S., Meta, M., Bendale, P., Gelb, M.H., Young, S.G. *et al.* (2005) Blocking protein farnesyltransferase improves nuclear blebbing in mouse fibroblasts with a targeted Hutchinson-Gilford progeria syndrome mutation. *Proc. Natl Acad. Sci. USA*, **102**, 10291–10296.
24. Broers, J.L., Ramaekers, F.C., Bonne, G., Yaou, R.B. and Hutchison, C.J. (2006) Nuclear lamins: laminopathies and their role in premature ageing. *Physiol. Rev.*, **86**, 967–1008.
25. Wang, Y., Ostlund, C. and Worman, H. (2010) Blocking protein farnesylation improves nuclear shape abnormalities in keratinocytes of mice expressing the prelamin A variant in Hutchinson-Gilford progeria syndrome. *Nucleus*, **1**, 432–439.
26. Coffinier, C., Jung, H.J., Li, Z., Nobumori, C., Yun, U.J., Farber, E.A., Davies, B.S., Weinstein, M.M., Yang, S.H., Lammerding, J. *et al.* (2010) Direct synthesis of lamin A, bypassing prelamin A processing, causes misshapen Nuclei in fibroblasts but No detectable pathology in mice. *J. Biol. Chem.*, **285**, 20818–20826.
27. Padiath, Q.S., Saigoh, K., Schiffmann, R., Asahara, H., Yamada, T., Koeppen, A., Hogan, K., Ptacek, L.J. and Fu, Y.H. (2006) Lamin B1 duplications cause autosomal dominant leukodystrophy. *Nat. Genet.*, **38**, 1114–1123.
28. Fu, J., Teucher, M., Anastassiadis, K., Skarnes, W. and Stewart, A. (2010) A recombineering pipeline to make conditional targeting constructs. *Methods Enzymol.*, **477**, 125–144.