Abnormal development of the cerebral cortex and cerebellum in the setting of lamin B2 deficiency

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Nuclear lamins are components of the nuclear lamina, a structural scaffolding for the cell nucleus. Defects in lamins A and C cause an array of human diseases, including muscular dystrophy, lipodystrophy, and progeria, but no diseases have been linked to the loss of lamins B1 or B2. To explore the functional relevance of lamin B2, we generated lamin B2-deficient mice and found that they have severe brain abnormalities resembling lissencephaly, with abnormal layering of neurons in the cerebral cortex and cerebellum. This neuronal layering abnormality is due to defective neuronal migration, a process that is dependent on the organized movement of the nucleus within the cell. These studies establish an essential function for lamin B2 in neuronal migration and brain development.

The nuclear lamina is an intermediate filament meshwork lying beneath the inner nuclear membrane that provides a structural scaffolding for the nucleus (1). The lamina is also important for other processes, including gene transcription, chromatin organization, nuclear pore distribution, nuclear envelope assembly, and tethering of the nucleus to the cytoskeleton (1, 2). The main components of the nuclear lamina are nuclear lamins, a class of intermediate filament proteins that is generally divided into two groups, A-type (lamins A and C) and B-type (lamins B1 and B2) (3, 4). Lamins A and C are produced from LMNA, whereas lamins B1 and B2 are encoded by distinct genes, Lmnb1 and Lmnb2, respectively. Lamins B1 and B2 are expressed in all cells and throughout development, whereas lamins A and C are expressed in differentiated cells, beginning at midgestation (3).

Interest in the nuclear lamins has intensified with the discovery that over a dozen human diseases, including muscular dystrophy, cardiomyopathy, lipodystrophy, and progeria, are caused by mutations in LMNA (5–7). To date, more than 340 missense, nonsense, frameshift, and splicing mutations have been identified (5). In contrast, no human diseases have been linked to these types of mutations in Lmnb1 and Lmnb2, and, so far, the only clear-cut association between B-type lamins and disease has been the finding of Lmnb1 gene duplications in autosomal-dominant leukodystrophy (8).

The paucity of “lamin B diseases” is probably not due to complete redundancy of lamins B1 and B2, as Lmnb1-deficient mice are small during embryonic development and die soon after birth with defects in lungs and bones (9). Also, Lmnb1-deficient fibroblasts display misshapen cell nuclei, aneu plyoidy, and early senescence (9). To further examine the functional importance of the B-type lamins, we generated Lmnb2-deficient mice.

We used gene targeting to insert a lacZ reporter into exon 1 of Lmnb2 (Fig. 1 A and B), abolishing the production of lamin B2 transcripts. As expected, homozygous Lmnb2 knockout (Lmnb2−/−) fibroblasts lacked lamin B2 (Fig. 1 C and D). The production of the lamin B3 transcript, a testis-specific transcript that uses an alternative first exon (located within intron 4 of Lmnb2), was not affected by our knockout mutation (Fig. S1). Lmnb2−/− fibroblasts did not have misshapen cell nuclei (Fig. 1E), unlike Lmnb1−/− fibroblasts (9). Also, primary Lmnb2−/− fibroblasts had a normal number of chromosomes (40.2 ± 0.5, n = 5 independent cell lines versus 39.2 ± 0.6 in WT cells, n = 2 independent cell lines). In parallel experiments, freshly isolated Lmnb1−/− fibroblasts exhibited polyploidy (57.8 ± 3.4 chromosomes), as previously reported (9).

Heterozygotes (Lmnb2+/−) were phenotypically normal. Intercrosses of Lmnb2+/− mice yielded Lmnb2−/−embryos at the expected Mendelian frequency [22.3% of 112 embryos collected between embryonic day 11 (E11) and E19.5]. At birth, Lmnb2−/− pups were normal in size and appearance, but they died within 15–60 min. Histopathological studies of E16.5–E19.5 Lmnb2−/− embryos did not uncover abnormalities in any organ system, except for the brain. The brain was nearly normal in size (reduced by only 5–10%). However, the forebrain exhibited a dramatic neuronal layering defect at E16.5 (Fig. 2 A and B), with an accumulation of cells in the intermediate zone (Fig. 2C). Similar findings were apparent in E18.5 embryos (Fig. 2D and S3 A and B) and newborns. At E18.5, the ventricular zone was virtually normal in size and with no noticeable accumulation of neurons; however, fewer neurons reached the cortical plate (Fig. S3 C and D).

At E13.5, before cortical neuronal migration gets underway, the neocortex of Lmnb2−/−embryos appeared similar to that of WT embryos, and the marginal zone was initially present at E16.5, as indicated by the expression of Reelin, a marker for Cajal-Retzius cells (10) (Fig. S4 A and B). However, Reelin expression was undetectable through E18.5 (Figs. S3 B and S4). Early stages of cortical plate formation were evident in Lmnb2−/− brains at E15.5 (with evidence of a marginal zone and a subplate) (Fig. S5 A and B). Thus, early development of the cortex appeared normal, and morphological defects appeared only when the second wave of neurons had begun their migration into the cortical plate (Fig. 2 and Fig. S5). These abnormalities are reminiscent of phenotypes in mice with defects in cortical neuron migration (11, 12).

At E8.5, β-galactosidase staining of Lmnb2−/−embryos revealed that Lmnb2 is expressed ubiquitously (Fig. 2E). At E11.5, β-galactosidase staining was prominent in the brain, limb buds, neural tube, and somites (Fig. 2F). In E16.5 embryos and newborns, staining was detected in the ventricular zone and upper layers of the cortex, the olfactory bulb, and cerebellum.
(Fig. 2 G–J). The in situ hybridization database GenePaint (www.
genepaint.org) (13) also documents strong Lmnb2 expression in the ventricular zone at E14.5. The presence of lamin B2 in the cortical neurons of adult rats had been previously documented by immunohistochemistry (14). Here, we report robust immunostaining for lamin B2 in the cortex of E17.5 WT embryos (Fig. S6 A and B). A similar staining pattern was observed with antibodies against lamin B1 (Fig. S6B). As expected, lamin B2 was absent in the cortex of E17.5 Lmnb2−/− embryos (Fig. S6C).

To determine if cortical neuron migration was perturbed in Lmnb2−/− embryos, we performed neuronal birthdating experiments by injecting BrdU. When embryos are pulse-labeled with BrdU, progenitors within the ventricular zone incorporate BrdU into their DNA. Newly born neurons do not reenter the cell cycle; therefore, their BrdU content remains constant as they migrate into the cortical plate. Meanwhile, progenitors continue to divide, diluting their BrdU content, and neurons born several days after the BrdU pulse contain less BrdU. To examine neuronal migration in Lmnb2-deficient mice, we injected BrdU at E13.5 and collected embryos at E18.5 and E19.5. In WT embryos, the neurons that stained most intensely for BrdU were found within the lower portion of the cortical plate (Fig. 3A, Fig. S7 A and B). Neurons born at later times normally migrate higher into the cortical plate; consequently, more superficial layers in WT brains stain less intensely for BrdU (Fig. 3A). In Lmnb2−/− embryos, the most intense BrdU staining was observed in superficial layers of the cortical plate (Fig. 3A, Fig. S7 A) (15) supported this interpretation (Fig. 3B). An injection of BrdU in WT mice at E15.5 labeled neurons in more superficial layers of the cortex. In contrast, the neurons that were labeled at E15.5 in Lmnb2−/− embryos were found in lower layers of the cortex, implying that those cells were defective in their ability to migrate into more superficial layers of the cortex (Fig. S7C).

A defect in neuronal migration was further supported by immunohistochemical studies at E19.5 with cortical layer–specific markers (Fig. 3 C–F). In WT cortex, the newer neurons, positive for NeuN, migrated past older Ctip2-positive neurons.
and into more superficial layers of the cortex (Fig. 3C). In Lmnb2−/− embryos, most NeuN-positive neurons tended to accumulate in lower levels of the cortex, below the Ctip2-positive cells (Fig. 3C). Similar findings were observed in sections stained for FoxP1, a marker for superficial layers of the cortical plate (layers III–V) (Fig. 3D) (16). In Lmnb2−/− embryos, FoxP1-positive neurons accumulated in lower levels of the cortical plate and did not reach their normal position (Fig. 3D). Similar abnormalities of cortical layering were observed with other pairs of layer specific markers: Cux1 (layers II–IV) and FoxP1 (Fig. 3E), and FoxP2 (layer VI) and Ctip2 (Fig. 3F). In each case, neuronal populations were intermixed in the Lmnb2−/− brains.

Although neuron layering was dramatically perturbed in Lmnb2−/− mice, the size of the brain was minimally reduced and the overall number of cells in the cortex did not appear to be altered. However, the number of cells positive for the mitotic antigen Ki-67 in the ventricular zone at E15.5 was somewhat altered. However, the number of cells positive for the mitotic marker (Fig. 4A) was somewhat reduced at E18.5, and the mobility of neuronal progenitors proliferate in the VZ, postmitotic cells leave the VZ and migrate along glial fibers to the CP. (D) Sections from E18.5 embryos showing similar defects. (E and F) Whole-mount β-galactosidase staining of Lmnb2−/− embryos at E8.5 (E) and E11.5 (F). Staining was ubiquitous at E8.5; at E11.5, staining was prominent in the forebrain (fb), midbrain, hindbrain, limb buds (lb), tailbud, somites, neural tube (nt), and retina. heart (h). (G) Whole-mount staining of an E16.5 Lmnb2−/− brain cut sagittally; β-galactosidase expression is found in the cortex (cx), olfactory bulb (ob), midbrain (mb), brainstem (bs), inferior colliculus (ic) and superficial layer of the cerebellum (ce); ventricle (vt); hypothalamus (th). (H) 40-μm section of the cortex of an E16.5 Lmnb2−/− embryo and (I) 20-μm section of the cortex of a newborn Lmnb2−/− pup, after β-galactosidase staining, revealing Lmnb2 expression in the VZ. (J) β-Galactosidase staining of the brain of a newborn Lmnb2−/− mouse, revealing Lmnb2 expression in the ob, cx, and vt.

**Discussion**

Human geneticists have made remarkable progress in uncovering diseases associated with defects in lamins A and C, and additional studies with cultured cells and mouse models are beginning to define the functions of these proteins in health and disease (5–7). Meanwhile, the functional relevance of the other nuclear lamins, and in particular lamin B2, has remained obscure. In the current study, we shed light on this issue. We report that mice lacking lamin B2 are entirely normal in size at birth but die shortly thereafter. Detectable pathology is confined to the brain, and the most striking finding is a defect in the layering of neurons in the cerebral cortex. The cortical abnormality was striking at E16.5 and remained obvious in newborn mice. BrdU birthdating experiments and immunohistochemical studies revealed that the neuronal layering defect was due to defective migration of neurons from the ventricular zone to the cortical plate.

Lmnb2−/− mice had a smaller cerebellum with a complete absence of foliation, and the hippocampus was also abnormal. Defective migration of cortical neurons is a hallmark of lissencephaly, a human brain disease characterized by a smooth brain devoid of folds (19, 20), and some forms of lissencephaly are also accompanied by cerebellar hypoplasia or dysplasia (11). A deficiency in Reelin also causes severe abnormalities in the cortex, cerebellum, and hippocampus (21), and we did observe markedly reduced levels of Reelin expression at later stages of development.
**Fig. 3.** Lmnb2 deficiency causes defective neuronal migration in the brain. (A) Birthdating experiment demonstrating defective neuronal migration in the cortex of Lmnb2^{-/-} embryos. Pregnant females were injected with BrdU at E13.5; brains were collected at E18.5, sectioned, and stained with DAPI and a rat monoclonal antibody against BrdU. Neurons born later (therefore containing less BrdU) migrate to more superficial layers of the cortex. In Lmnb2^{-/-} embryos, intense BrdU staining is noted in the most superficial layers of the cortical plate, consistent with an inability of the neurons born later to migrate past older BrdU-positive neurons. Arrow indicates the orientation of neuronal migration. (B) Sections from the same experiment stained with a sheep polyclonal antibody against BrdU (red) and an antibody against calbindin (Bottom). Calbindin stains the layer of Purkinje cells (arrow) and the external granule layer (arrowhead). Cp, choroid plexus. (C) Immunostaining of a cerebellar section from an E16.5 Lmnb2^{-/-} embryo and a newborn Lmnb2^{-/-} mouse (P0) after whole-mount staining for p-galactosidase. Lmnb2 expression is noted in the superficial layer of the cerebellum, the external granule layer (arrowhead) and in the vicinity of the Purkinje cell layer (arrow). (D) Immunostaining of cortical sections of E17.5 Lmnb2^{-/-} embryos with antibodies against lamin B1 (green) and lamin B2 (red). Nuclear DNA was stained with DAPI. Lamins B1 and B2 are detected in all cells, with a stronger signal in the Purkinje layer (arrow).

**Fig. 4.** Abnormal cerebellar morphology in Lmnb2^{-/-} mice. (A) Cross-sections of cerebellum in newborn WT and Lmnb2^{-/-} mice. The cerebellum of Lmnb2^{-/-} embryos was smooth and smaller in size; asterisks show fissions in the WT cerebellum (absent in the Lmnb2^{-/-} cerebellum). (B) Cerebellar sections of E16.5 WT and Lmnb2^{-/-} embryos stained with H&E (Top) and with an antibody against calbindin (Bottom). Calbindin stains the layer of Purkinje cells (arrow in WT section; layer is also visible in the H&E-stained section). Note the reduced thickness of the external granule layer in Lmnb2^{-/-} cerebellum (arrowhead). Cp, choroid plexus. (C) Cross-section of the cerebellum from an E16.5 Lmnb2^{-/-} embryo and a newborn Lmnb2^{-/-} mouse (P0) after whole-mount staining for p-galactosidase. Lmnb2 expression is noted in the superficial layer of the cerebellum, the external granule layer (arrowhead) and in the vicinity of the Purkinje cell layer (arrow). (D) Immunostaining of a cerebellar section of an E17.5 Lmnb2^{-/-} embryo with antibodies against lamin B1 (green) and lamin B2 (red). Nuclear DNA was stained with DAPI. Lamins B1 and B2 are detected in all cells, with a stronger signal in the Purkinje layer (arrow).

in Lmnb2^{-/-} embryos. Although we cannot exclude a significant role for Reelin in the Lmnb2 phenotype, we do not believe that altered Reelin expression is the entire story. First, Lmnb2^{-/-} mice exhibit a perinatal lethal phenotype, whereas Reelin-deficient mice do not (21). Second, the subplate was separated from the marginal zone in Lmnb2^{-/-} mice at E15.5—a process known to be blocked in the setting of Reelin deficiency (21).

The neuronal migration defect in Lmnb2^{-/-} mice was initially surprising to us because diseases caused by lamin A and lamin C defects are confined largely to mesenchymal tissues (skeletal muscle, heart, adipose tissue, connective tissue, and bone). In hindsight, however, the involvement of a nuclear lamin in neuronal migration makes sense, for the simple reason that cortical neuron migration is utterly dependent on the ability of the neurons to move their own nucleus (12, 22). Migration of neurons from the ventricular zone to the cortical plate is a saltatory process that involves the coupled translocation of the centrosome and the nucleus (22). The initial step is a forward movement of the centrosome toward the leading process of the neuron. Next, cytoplasmic motors pull the cell nucleus along microtubules toward the centrosome. These cytoplasmic motors have been noted to associate with the nuclear envelope (23). After the nucleus is translocated forward, the trailing process of the cell body is remodeled, resulting in net forward movement of the soma. Repeated cycles of this process—extension of the centrosome followed by nucleokinesis—make it possible for cortical neurons to migrate over large distances (22).

The cytoplasmic molecules involved in nuclear movement and neuronal migration have been studied intensively. For example,
Lamins are nuclear proteins, but are known to be mechanically linked to the cytoskeleton via nuclear-envelope-spanning complexes of SUN- and KASH-domain proteins (27–29). Because Klarsicht, a KASH protein, interacts with the Drosophila B-type lamin and is required for nuclear movement in photoreceptor cells (26), it seems possible that neuronal migration in mammals could also involve SUN- and KASH-domain proteins. In the future, defining molecular partners for lamin B2 could uncover other nuclear proteins involved in neuronal migration, just as screened for LIs1 interacting proteins provided additional cytoskeletal players in nuclear migration (12).

Other studies have linked the nuclear lamins to elements of the cytoskeleton (30). For example, Lammerding and coworkers (31) showed that the nuclei of cells spin in the absence of lamin B1, strongly suggesting that lamin B1 helps to anchor the nucleus to the cytoskeleton. Although the current studies focused entirely on Lmnb2-deficient mice, the involvement of lamin B1 in nuclear–cytoskeletal interactions raises the question of whether lamin B1 might also be important for neuronal migration in the brain. This issue needs to be investigated.

The discovery of neuronal migration defects in the setting of Lmnb2 deficiency could explain the absence of reported associations between LMNB2 mutations and the types of human diseases closely associated with LMNA mutations (e.g., muscular dystrophy, cardiomyopathy). LIs1 mutations cause devastating developmental brain abnormalities, and we suspect that consequences of Lmnb2 deficiency will be similar. Over the next few years, we predict that Lmnb2 mutations will be identified in children with severe developmental brain disorders. If this prediction is borne out, the spectrum of diseases associated with the nuclear envelope would be significantly expanded (5–7).

Materials and Methods

Generation of Lmnb2-Deficient Mice. Arms for a sequence-replacement gene-targeting vector were amplified from genomic DNA of E14Tq2A ES cells derived from 129/Ola mice (32) and cloned into the Topo vector (Invitrogen). The 5′ arm (containing sequences upstream of the ATG in exon 1) was amplified with primers 5′-TGG CGG CGG CGG AGT CCT GCA GGG CAG TGT GAA GCA TAT AGG GGG-3′ and 5′-GAG CTA GCA TGA CGG AGG TGG CGG CGG CGG CAG GAT ACT CTC-3′; the 3′ arm was amplified with primers 5′-ATA AGA ATG CGG CGG CGG ATT CGG TGC GTC GGC ACC GGC GAC GC-3′ and 5′-ATA GAT TAG CGG CGG CGG CTC TGC TCC AGG AAT GCA ATG GG-3′. The 5′ arm was excised with NotI and Nhel and cloned upstream of a promoterless lacZ cassette (to insert lacZ at the site of the Lmnb2 ATG). An EcoRI fragment spanning the entire 5′ arm and lacZ was subcloned into pSK+PNT (33) (obtained from P.G.-tk testes). The 3′ arm was inserted into the NotI site downstream from the neo cassette. The vector was linearized with Xhol and electroeluted into E14Tq2A ES cells. After selection with G418 (125 μg/ml), G418, Invitrogen) and ganciclovir (2 μM; Sigma), ES cell colonies were picked and screened for recombination with Southern blots on EcoRV digests with 5′ and 3′ flanking probes. The 5′ probe was amplified from E14Tq2A genomic DNA with primers 5′-GAA GCC CGG CGG AGG TGG GAC TGG TTA CTG TAA CAC ACC CAC AAC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC ACC AC
[PBS, 20 mM potassium ferricyanate, 20 mM potassium ferrocyanate, 2 mM MgCl$_2$, 0.2% Nonidet P-40, 0.1% sodium deoxycholate, and 0.8 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xglu)](36). Staining specificity was established by parallel studies with WT tissues, which did not stain for β-galactosidase activity. After staining, tissues were washed in PBS and post-fixed in 4% paraformaldehyde in PBS. Thick sections of brain tissues were cut manually with double-edge stainless steel blades; 40-μm thin sections were cut from stained tissues after embedding in O.C.T. (Optimal Cutting Temperature; Tissue-Tek, Sakura Finetek). Sections were counterstained with eosin, dehydrated and mounted in Permount (Fisher Scientific). Images were recorded with a Leica MZ6 dissecting microscope and a Leica DFC290 digital camera.

**Histological and Immunohistochemical Staining of Mouse Tissues.** Paraffin-embedded sections of mouse embryos (5-μm thick) were stained with hematoxylin and eosin according to ref. 37. For immunostaining, sections were rehydrated and boiled for 10 min in 10 mM sodium citrate, pH 6; endogenous peroxidase activities were quenched by incubating with 0.3% H$_2$O$_2$ for 30 min. After permeabilization with 0.1% Tween-20 and blocking for 20 min in 2.5% horse serum (Vector Laboratories), sections were incubated overnight at 4 °C with primary antibodies diluted as indicated in Table S1. Mouse monoclonal antibodies were detected with VECTASTAIN Elite ABC Kits, and rabbit polyclonal antibodies were detected with IMPRESS Reagent, (both Vector Laboratories) and DAB substrate (Roche). Sections were counterstained with 0.5% methyl green (Sigma) in PBS, 20 mM potassium ferricyanate, 20 mM potassium ferrocyanate, 2 mM MgCl$_2$, 0.2% Nonidet P-40, 0.1% sodium deoxycholate, and 0.8 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xglu)](36). Staining specificity was established by parallel studies with WT tissues, which did not stain for β-galactosidase activity. After staining, tissues were washed in PBS and post-fixed in 4% paraformaldehyde in PBS. Thick sections of brain tissues were cut manually with double-edge stainless steel blades; 40-μm thin sections were cut from stained tissues after embedding in O.C.T. (Optimal Cutting Temperature; Tissue-Tek, Sakura Finetek). Sections were counterstained with eosin, dehydrated and mounted in Permount (Fisher Scientific). Images were recorded with a Leica MZ6 dissecting microscope and a Leica DFC290 digital camera.


For most of the immunochemical studies, mouse tissues were frozen in O.C.T.; 10-μm thick sections were fixed for 5 min with ice-cold acetone or 0.3% bovine serum albumin followed by five dips in acetone, and permeabilized with 0.1% TWEEN-20. Background staining for mouse antibodies was minimized with the Mouse-on-Mouse kit (Vector Laboratories). To detect BrDU, sections 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. Otherwise, sections were blocked with 2.5% horse serum for 1 h at room temperature and incubated overnight at 4 °C with primary antibodies at the dilutions indicated in Table S1. Alexa Fluor 488- and Alexa Fluor 568-conjugated secondary antibodies were used at a 1:200 dilution, and Alexa Fluor 555–conjugated streptavidin was diluted at 20 μg/mL (all from Molecular Probes, Invitrogen). After counterstaining with DAPI, sections were mounted with Prolong Gold antifade (Invitrogen), and images were recorded with an Axiovert 200M microscope using a 10× objective with an AxioCam MRm and an ApoTome (all from Zeiss).

**Note Added in Proofs.** While the present manuscript was under revision, a report by Zhang et al. revealed that indeed both SUN1/2 and Nesprin 1/2 proteins are essential for neurogenesis and neuronal migration in mice (Neuron 64:173–187; October 29 2009).

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