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Connexin 32 Is Involved in Mitosis

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KEY WORDS
peripheral neuropathy; connexin 32; Schwann cell differentiation

ABSTRACT
The X-linked form of Charcot-Marie-Tooth disorder (CMTX) is the second most frequent type (15% of CMT forms). It involves the GJB1 gene coding for connexin 32, a protein involved in gap junction formation and function. There is no curative treatment for CMTX. We present data on transgenic lines that was accomplished by inserting a human BAC carrying the *GJB1* gene, in which two different mutations in connexin 32 (Cx32) observed in patients were introduced. Investigation of these models implicated Cx32 in the control of mitotic stability. The model in which *Gjb1* has been invalidated had the same phenotype. This new function for Cx32 was recently confirmed by results from the Mitocheck program. Locomotor impediment was seen in the behavior of these animals, the severity of which correlated with transgene copy number and RNA expression.

INTRODUCTION

Charcot-Marie-Tooth disease is a heterogeneous inherited disorder affecting peripheral nerves (CMT, 40 loci having so far been described). Two forms, CMT1A and CMTX, account for 75% of patients with a clear familial transmission. CMT1A affects ~60% of patients and 15% suffer from CMTX (Boerkel et al., 2000).

X-linked Charcot-Marie-Tooth disease (CMTX) is an inherited disorder, presenting as peripheral neuropathy that affects males more severely than females (Martyn and Hughes, 1997). The average age of onset is ~16 years for males and 19 years for females. It presents with slow muscular atrophy and weakness, predominantly affecting distal muscles. Demyelinating and axonal anomalies are present, making it difficult to distinguish from other CMT forms by clinical evaluation alone.

CMTX is caused by mutations in the *GJB1* gene (Bergoffen et al., 1993; Ionascescu et al., 1996), located on the proximal long arm of the X chromosome. It encodes connexin 32 (Cx32), a gap junction protein present in myelin of the PNS (peripheral nervous system) and CNS (central nervous system) (Scherer et al., 1995). Cx32 is a membrane protein located in gap junctions, which forms hexameric hemichannels called connexons. The docking of two connexons across the intercellular gap triggers the formation of a channel that connects the cytoplasm of adjacent cells and allows the exchange of ions, small molecules (<1,000 Da) and signaling effectors (Bennett et al., 1991; Kumar et al., 1992; Liu et al., 2006; Mese et al., 2007). Pathogenic mutations of the protein affect the function of the channel (Abrams et al., 2001; Bicego et al., 2006, Martin et al., 2000; Oh et al., 1997; Wang et al., 2004).

Less than 10% of the mutations are null alleles, the majority corresponding to a loss of function and these can be classified into two categories:

1. Mutations affecting cell trafficking. The protein is observed in the endoplasmic reticulum and/or in the Golgi, but not on the cell membrane.
2. Mutations affecting connexon functions. Cx32 is present on the cell membrane, but connexons cannot be opened.

The only mouse model presenting with a neuropathy similar to that seen in patients is a null allele produced by *Gjb1* invalidation. This line presents a peripheral neuropathy (Scherer et al., 1998) and abnormalities in myelin (Anzini et al., 1997). In addition, expression of *Gjb1* in mice invalidated for this gene restores a normal phenotype (Scherer et al., 2005).

This model is useful for studying the pathophysiology of peripheral nerves linked to the absence of Cx32. However, it could not be used to identify drugs that may correct biochemical defects in the protein seen in experimental models and patients (cell trafficking and connexon deficiency). Furthermore, it cannot be used to test a candidate drug proposed to correct, even partly, Cx32 biochemical functioning as it is a null allele model.

Therefore, a mouse expressing a human mutated Cx32 was generated. The mutations G12S and S26L (Yoshimura et al., 1996), found in nonrelated CMTX families that affect trafficking of Cx32 (G12S, Wang et al., 2004) or connexon activity (S26L), have been introduced into a human BAC containing the *GJB1* gene to create mouse transgenic lines. Five transgenic lines that were generated have been investigated.

We found anomalies in these cell lines concerning centrosome duplication suggesting that Cx32 has a role in mitosis. These lines showed locomotor impairment, with the severity of at least the S lines being correlated with

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the number of copies of the transgene inserted into the murine genome.

MATERIALS AND METHODS

BAC Genetic Modification and Generation of Transgenic Lines

BAC modifications were generated by Gene Bridges GmbH Heidelberg using recombineering technology. Transgenic mice were produced using the standard technique of pronuclear injection (Huxley et al., 1996) with B6CBF1 mice as donors. Subsequent crosses were with the same F1 mice.

DNA Isolation and Genomic qPCR

DNA was extracted from one mouse tail and 200 μM was used in a qPCR medium containing primers specific to GJB1, designed in-house (GAT CAA ACG CCC TGA CTT CCC; ACA GAA CAG GTG GGT GCA G) and cybergreen. Amplicon size was 87 bp. Primers specific to the gene Factor 8 (linked to chromosome X, ACT AAC CTG GGT TTT CCA; TCA AAC ACA AAA TGG TCT ATG AAG) and DNA from males were used. Amplicon size was 89 bp. PCR mixtures were amplified with a ROCHE 480 light cycler. ΔΔCt methods were used to evaluate the number of copies integrated in the measure, using *F8* amplification (one copy in males) as a reference.

Cell Culture Conditions

For the isolation of fibroblasts, a small fragment was removed from a mouse ear, dipped in alcohol solution, cut into small pieces in a sterile Petri dish in PBS containing fungicide (fonigizon) diluted 1/250, and transferred to 2 mL tubes containing 1 mL dissociation buffer (DMEM plus 20% FBS, 1 mg/mL BSA, 0.5 mg/mL collagenase, 0.75 mg/mL trypsin, and penicillin/streptomycin). The tubes were incubated in a water bath with agitation for 1 h at 37°C. Fibroblast medium (DMEM, 10% FBS, 2 mM Gln, 100 U/mL penicillin, 100 μg/mL streptomycin) was added to each tube and the samples centrifuged at 150*g* for 10 min. The cells were resuspended in fibroblast medium, seeded into Petri dishes and incubated at 37°C in air with 5% CO_2. The culture medium was replaced every two days. When subconfluent, the cells were trypsinized and expanded into tissue culture flasks. All experiments were performed using cells between Passages 4 and 8.

Chromosome Spreading and Nuclear Volume Evaluation

Interphase and metaphase spreads from *PKD2*-Y fibroblasts were prepared by standard cytogenetic protocols. Nuclei were stained with DAPI and nuclear volume was measured with *ImageJ* software.

Centrosome Labeling

Cells were grown on glass coverslips for 24 h to allow cultures to reach 80% confluence. To measure the number of centrosomes, cells were fixed with 4% PFA, permeabilized with methanol at −20°C for 8 min, and blocked with 0.5% Triton X-100 in PBS milk 1% for 30 min at RT. To detect centrosomes, cells were incubated overnight at 4°C with a mouse anti-γ-tubulin antibody (GTU-88; Sigma) diluted 1/1,000 in PBS milk, 0.1% Triton 100 0.05% After washing, cells were incubated for 1 h at RT with Cy3-conjugated goat anti-mouse IgG secondary antibody (Caltag Laboratories) diluted 1/2,000 in PBS milk, 0.1% Triton 100–0.05%. The preparations were counterstained with DAPI in Vectashield mounting medium (Vector Laboratories). Fluorescence images were acquired with a microscope (Leica DMR) equipped with a PL APO objective.

Extraction of RNA and Semiquantitative RT-PCR

RNA was extracted from 2×10^6 cells using Trizol. cDNA was synthesized using an Invitrogen kit and resuspended in water. To compare the levels of human and mouse mRNA, cDNA was amplified using two primers: F: TCA TGG TGC TGG TGG TGG CT, and R: CCA CCA CAG TGT CCC TGA GA, which were homologous to the mouse and the human cDNAs. The PCR product crosses an intron and does not amplify from genomic DNA. The 302 bp human product cuts with *Msc1* to give two fragments of 128 and 174 bp. The mouse product of the same size is not cut by this enzyme. Amplification was for 30 cycles.

Products were analyzed on 2% agarose gels. Pictures were taken using a digital imager. The surface was evaluated using the *ImageJ* package.

Locomotor Test

Locomotor ability was tested using the rotarod procedure, requiring motor coordination and balance control (Dunham et al., 1957). A machine manufactured by Bioseb was used for this purpose. A previously used procedure (Norreel et al., 2001) was adapted and two velocities (15 and 25) were used.

Statistical Analysis

Statistical analysis was involved Prism v5.0 software. Mann-Whitney and chi-square tests were used for trend analysis. The significance threshold was $P < 0.05$.

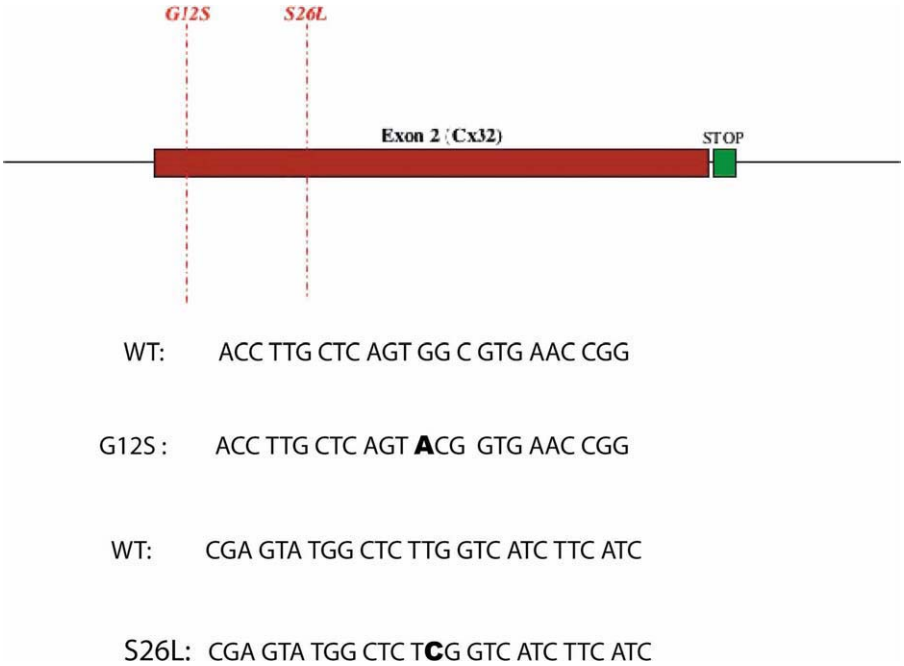
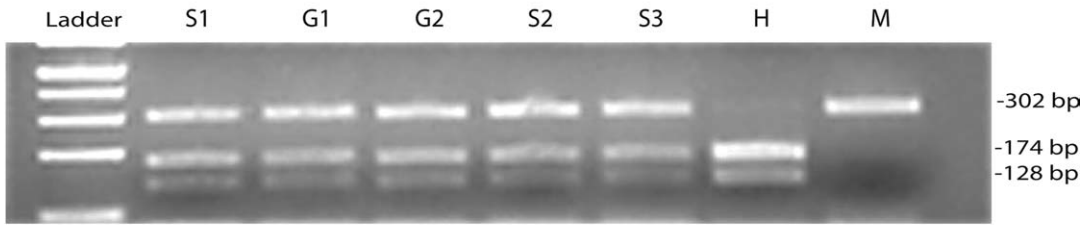


Fig. 1. Genetic engineering of *GJB1*. Location of inserted mutations, as well as sequencing of recombinant BAC and inserted BACs are indicated. Mutated nucleotides are in bold. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

A

Transgenic lines	WT	G1	G2	S1	S2	S3
Nb copies of transgene	0	1	2	1	2	3

B



C

Transgenic lines	G1	G2	S1	S2	S3
Cx 32 transgene/endogene expression	0,4	0,5	0,4	0,5	0,55

Fig. 2. Copy number of integrated transgene and Expression of *GJB1*. **A:** Genomic qPCR. Primers amplifying *GJB1* (mouse as well as human) or F8 gene were used to perform amplify these genes by qPCR. ΔCt between *F8* and *GJB1* was used to determine the number of copies of the transgene integrated in transgenic lines. **B:** Semiquantitative evaluation of expression of the human *GJB1* gene. Human as well as murine *GJB1* transcripts have been amplified (see Methods). The human transcript was discriminated from mouse transcript by digestion with Msc1 (digesting only the human amplicon). S and G indicate the different transgenic lines. H indicates a human cDNA and M a mouse WT cDNA. Digested material was analyzed on 2% agarose gels, stained with ethidium bromide. **C**: Bands from gel pictures have been scanned using the Image J package and surface evaluated. Ratio between transgenic and wild type provided a semiquantitative evaluation of expression of the transgene.

RESULTS

Generation of Transgenic Lines

The mutations G12S and S26L (Yoshimura et al., 1996) have been defined as documented examples of mutations in non-related CMTX families affecting trafficking of Cx32 (G12S, Wang et al., 2004) or connexon activity (S26L) (Fig. 1).They were introduced into the *GJB1* gene contained in a human BAC (RP11-485H3) using homologous recombination in *E. coli* (Warning et al., 2005).

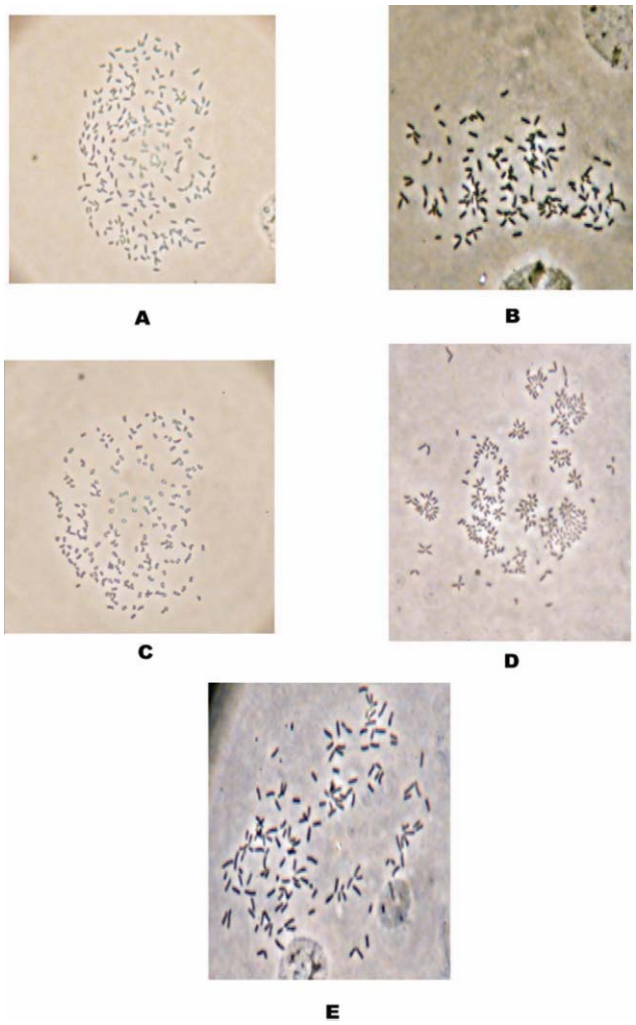


Fig. 3. Mitotic analysis of the 5 transgenic lines. Fibroblasts from young transgenic mouse from different lines were cultured for mitotic spreads, as indicated in methods. Chromosomes have been stained viewed microscopally. **A**: G1, **B**: G2, **C**: S1, **D**: S2, **E**:S3. [Color figure can be viewed in the online issue, which is available at wileyonline library.com.]

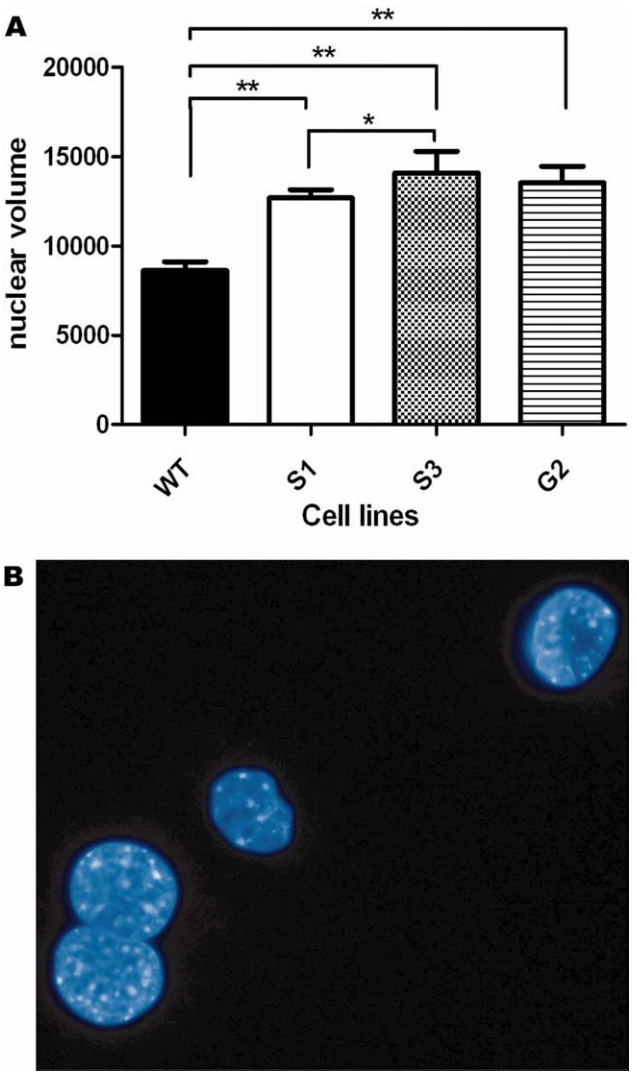


Fig. 4. Nuclear volume of WT and transgenic lines**. A**, Nuclei of WT, S1, S3 and G2 transgenic line from spreads on glassslides stained with DAPI. Nuclei volumes of ~100 nuclei per cell line were analyzed from pictures of nuclei (**B**), using the Image j software. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Recombinant BACs DNA has been injected into the oocytes of B6CBF1 mice (Huxley et al., 1996). Five transgenic founders were born; two contained the G12S mutation (G1, G2) and three the S26L mutation (S1, S2, S3), confirmed by sequencing the inserted transgene (Fig. 1).

The number of copies of *GJB1* inserted into the mouse genome was measured by a genomic qPCR approach (see Methods). There was one inserted copy for S1 and G1, two for S2 and G2, and three for S3 (Fig. 2A).

In addition, expression of the transgene was assessed by semiquantitative RT-PCR (see Methods). All lines expressed the human *GJB1* gene (Fig. 2B) at the level that correlated with the transgene copy number (Fig. 2C).

Transgenic Line Investigation

Mitotic chromosomes were spread on glass slides and analyzed by microscopy. All mitoses proved unexpectedly to be polyploidy (Fig. 3). This was confirmed by measuring the nuclear volume of cells from WT and transgenic animals (Fig. 4). This was observed in each of the five transgenic lines and therefore could not be attributed to disruption of a gene involved in mitotic stability.

Mitotic instability frequently occurs in cancer (reviewed in Marx, 2001) and has been associated with centrosome over-duplication (Matsomuto and Maller, 2001). Therefore, whether mitotic instability in the transgenic lines is associated with centrosome over-duplication was investigated.

Centrosome numbers increased as multicentrosomal cells were present in transgenic fibroblasts (Fig. 5A,B, and Table 1). BAC used for the transgenic lines contained genes other than *GJB1*: MED12, NLGN3, ZMYM3 and NONO. MED12 and NONO were truncated in BAC, and not expressed. The first two genes are predominantly

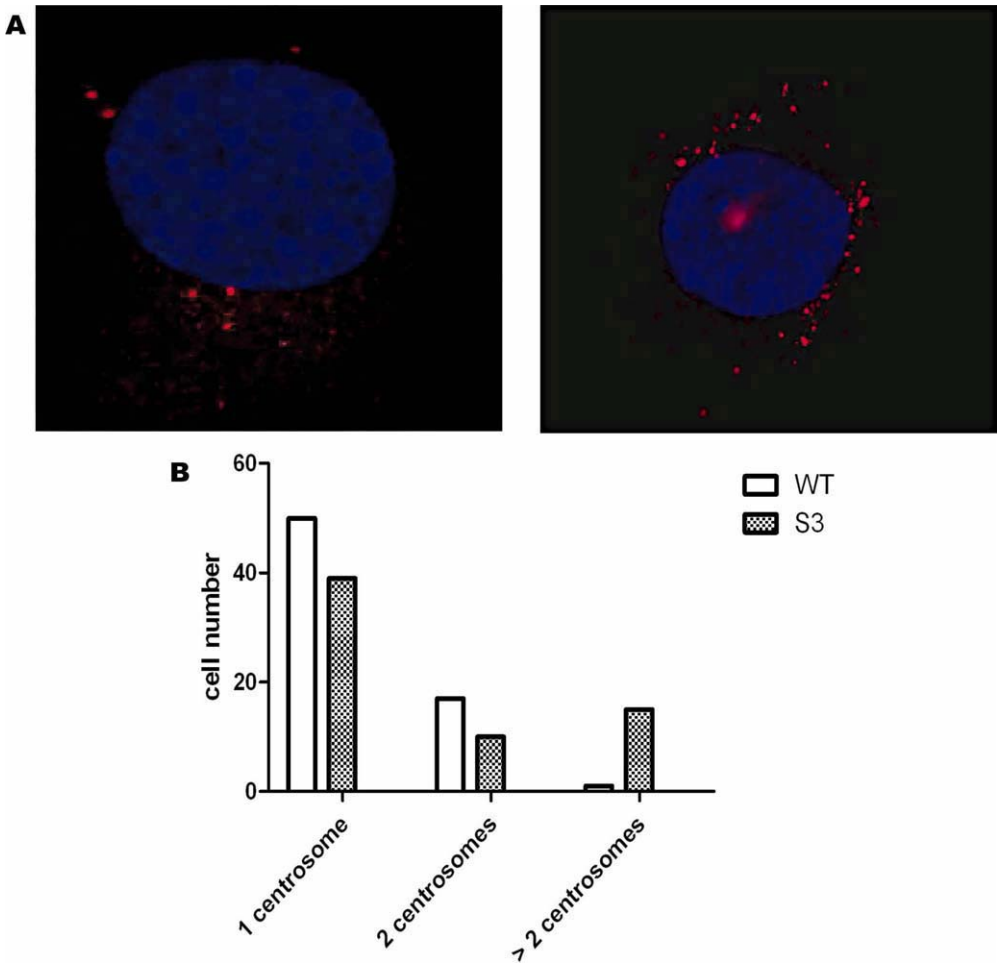


Fig. 5. Centrosome duplication in WT and transgenic lines **A:** Fibroblasts from the S3 line were cultured of glass coverslips and their centrosomes stained with anti γ-tubulin antibody. Nuclei have been stained with DAPI. **B**: The number of centrosomes per cell in 150 cells was estimated for WT fibroblasts and the S3 transgenic line of either WT or S3 have been analyzed. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1. Number of Centrosomes Per Cell in WT and Transgenic

Mouse line	WT%	G2%	S1%	S3%
1 centro	72.5	61	62	64
2 centro	25.5	30	18	16
> 2 centro	2	9	20	20

About 150 cells have been analyzed in different lines. WT was compared with transgenic lines using a chi-square test for trend, with a P of <0.0001. There is no statistically significant difference between the three transgenic lines.

expressed in the brain, but do not seem to be expressed in fibroblasts (Philibert et al., 2000; Van der Marel S, 1996). However, to confirm that the anomalies in the centrosome numbers in the transgenic lines are due to anomalies in Cx32, the numbers were determined in cells isolated from embryonic mesenchyme cells in which Cx32 is also not expressed (data not shown), and in adult fibroblasts originating from KO animals have invalid *Gjb1* genes. Embryonic fibroblasts (Fig. 6A) had no abnormal centrosome duplication or nuclear volume. However, adult fibroblasts had abnormal centrosome overduplication (Fig. 6B) and an increase in nuclear volume (Fig. 6D), with were abnormal shapes, frequently being multilobed, Fig. 6C).

To confirm the role of Cx32 in mitotic stability through a search carried out on the web site of the mitocheck project (www.mitocheck.org), GJB1 was one of the genes found to be involved in nuclear division.

Locomotor Phenotype

The locomotor behavior of the animals (aged one year) was investigated using the rotarod test (Fig. 7A). All tested lines (3) showed locomotor impairment. The severity of the impairment in the S lines was associated with copy number. S3 (three copies) could only stay 1 min on the rotarod (25 rpm), whereas the S1 line (only one copy) stayed 4 min. In checking the age at which the phenotype became apparent, the G2 line developed it between 6 and 7 months, with the severity increasing with age (Fig. 7B).

DISCUSSION

Investigating the transgenic lines we have produced lines, each were polyploidy, ruling out the possibility that

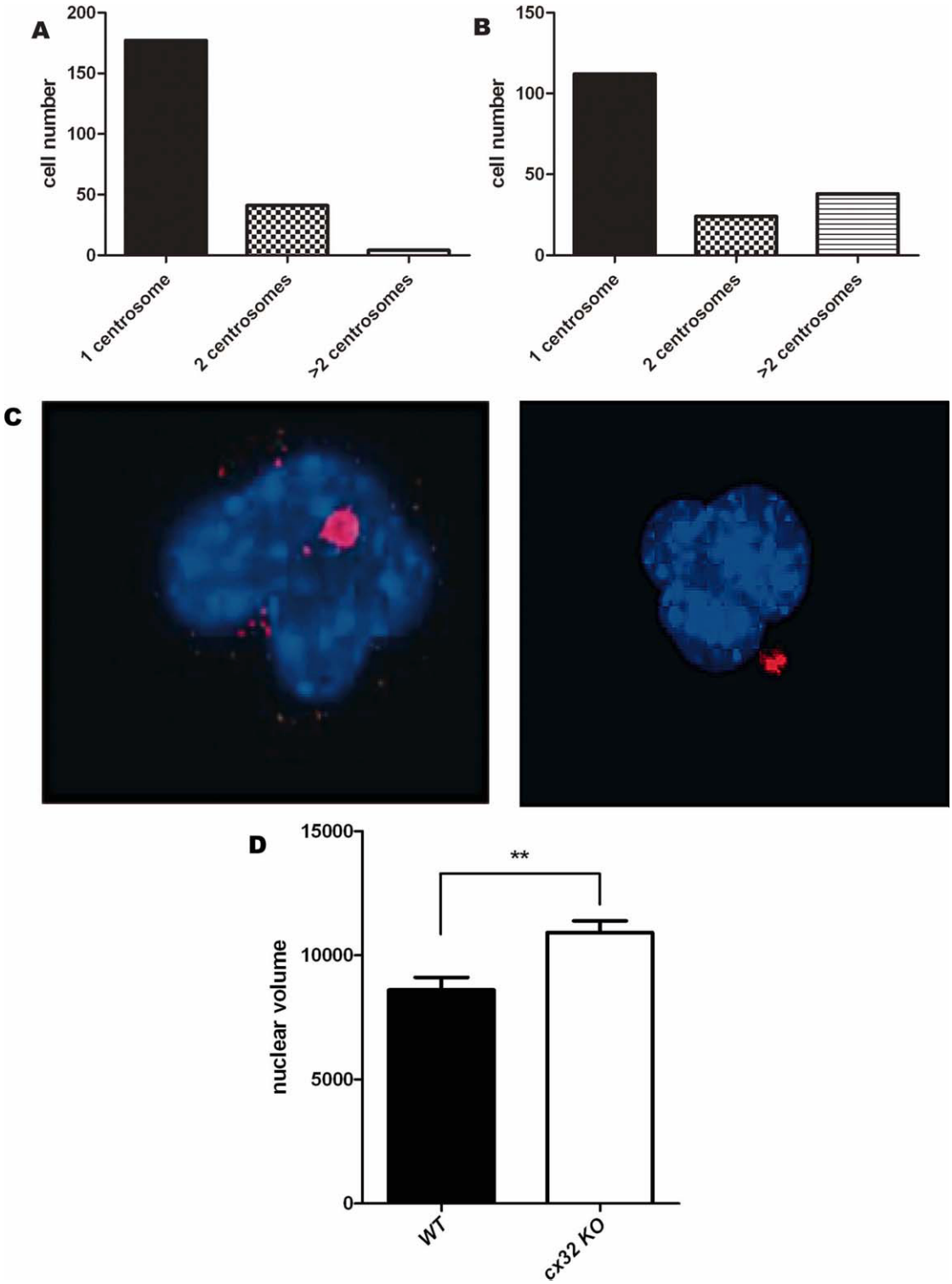


Fig. 6. Centrosome duplication in embryonic and adult KO cells**.** Centrosome per cell of embryonic (**A**) or adult fibroblasts (**B**) from a KO model were estimated, as in Fig. 5. **C**: Morphology of nuclei from adult fibroblasts isolated from KO adults animals. **D**: Volume of nuclei of embryonic or adult fibroblasts as in figure 4, after DAPI staining. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

this anomaly was caused by interruption by the transgene of a gene involved in mitotic stability. To confirm this, mitosis from animals where *Gjb1* was invalidated (Scherrer et al., 1998) showed anomalies and large nuclear volumes, indicating that these are probably caused by anomalies in *GJB1* expression and/or Cx32 mutations.

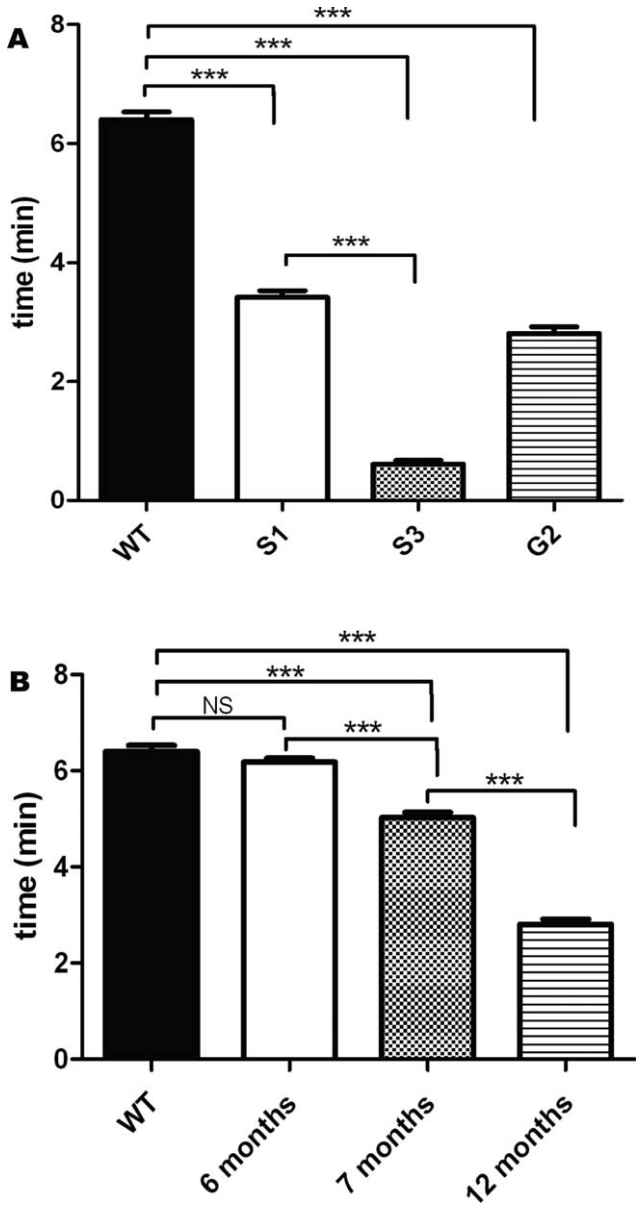


Fig. 7. Locomotor behavior of WT and transgenic lines**.** Locomotor behavior of different transgenic lines was measured using the rotarod test (rotation speed 25 rpm). An average of four animals were analyzed and ~20 estimates made per animal. **A**: Locomotor ability of WT and three different transgenic lines (S1, S3, and G2). **B**: Development of rotarod skill with age for the G2 line.

The results were compared with data in the data-base of the Mitocheck program. The aim of this project was to define genes involved in cell division (Hutchins et al., 2010; Newman et al., 2010). Four hundred genes producing anomalies in mitosis were selected, one being GJB1. Cells transfected with GJB1 siRNA had an abnormal nuclear shape (multilobed, unusual nuclear shapes) similar to that of KO fibroblasts. In addition, abnormal mitotic alignment was also recorded, indicating anomalies in mitotic spindle formation and/or chromosome segregation. The latter, giving mitotic instability that leads to polyploidy, is usually due to anomalies in mitotic spindle assembly linked to centrosome overduplication, and is frequently associated with anomalies in nuclear shape (reviewed in Newman et al., 2010). We found this in our transgenic lines, and in fibroblasts from KO animals, a result confirmed by the Mitocheck program, describing abnormal nuclear shape, multilobed nuclei and anomalies in mitotic alignment in cells invalidated or expression of *Gjb1,* thereby leading to the conclusion that Cx32 is involved in mitosis, and more precisely in mitotic stability.

It may appear surprising that a null allele and point mutations occur with the same phenotype. However, this has been described for other systems involving a defect in the control of centrosome duplication (Burtey et al., 2008). In addition, a few patients with a deletion in *GJB1* present with the CMTX phenotype (Gonzaga-Jauregi et al., 2010).

Cx32 has been implicated in the control of cell–cell interactions and homeostasis. However, the impact of these functions in cellular or tissue functioning is poorly understood. GJB1 is involved in cancer as a tumor suppressor gene (reviewed in Yano et al., 2006), but the mechanism is not understood. Several hypotheses related to cell cycle sensing and cycling have been proposed (reviewed in Vinken et al., 2011). However, the majority of data describe variations in connexin expression or modifications during the cell cycle, but no evidence that there is a direct effect on the control of these processes. Therefore, it is important to investigate the mechanisms underlying the role of Cx32 in mitotic stability.

Finally, it is also important to assess the implication of this function in CMTX disorder. From the locomotor tests carried out, the transgenic lines produced locomotor impairment, the severity being correlated in the S lines with the number of copies of the transgene inserted in the murine genome.

Molecular mechanism remain unknown. It could be due to anomalies of cell division, and also by the toxicity due to anomalies in cell trafficking. Interaction between cell division and myelin formation can occur, but the majority of the reports concern *PMP22* and CMT1A (Atanososki et al., 2000; Sancho et al., 2001). Therefore, it is proposed that anomalies in mitosis observed in our transgenic lines could be associated with the disorder, and that the CMTX phenotype is associated, like the CMT1A phenotype, with perturbation of cell cycling in Schwann cells.

This requires confirmation using genetic rescue and/or using drugs that interact with the described function. It will also be worth analyzing the cell cycle of primary Schwann cells in the systems we have described.

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