

A mutation in *GJB3* is associated with recessive erythrokeratoderma variabilis (EKV) and leads to defective trafficking of the connexin 31 protein

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Erythrokeratoderma variabilis (EKV) is a skin disorder characterized by variable (transient) erythemas and fixed keratosis. The disorder maps to chromosome 1p34–35, a location that contains the *GJB3* gene encoding the gap junction protein connexin 31. Until now, only heterozygote mutations in the form of dominant inheritance have been described in this gene associated with EKV. We report here a homozygote mutation in the connexin 31 gene, found in a family that shows recessive inheritance of the disorder, thus providing the first molecular support for a recessive variant of EKV. The entire *GJB3* coding sequence was scanned for mutations by sequencing. We detected a T→C transition at position 101 of the coding sequence, which replaces a leucine with a proline at residue 34 of the protein (L34P). Evolutionary analysis shows that this mutation is located at a highly conserved region of connexin in the first putative transmembrane helix (TMH). In transfected keratinocytes, L34P connexin 31 had a cytoplasmic distribution, suggesting that the mutant form of this protein will not form normal gap junctions between adjacent cells. The change of leucine to proline is likely to alter the structure of the first TMH of connexin by inducing a kink, thus influencing connexon structure and function.

INTRODUCTION

The erythrokeratodermas comprise a group of skin disorders characterized by erythematous hyperkeratotic plaques (1). Erythrokeratoderma variabilis (EKV) is a rare inherited form of these skin disorders characterized by the presence of transient erythematous and hyperkeratotic components. These lesions usually appear at birth or during the first years of life. Affected individuals present with sharply demarcated, relatively fixed hyperkeratotic plaques and migratory erythematous patches that appear and regress within minutes to hours. The development of these patches may be affected by various environmental factors, such as temperature changes, trauma or irritation. The inheritance pattern of EKV is primarily dominant, although there are reports of sporadic and recessive cases as well (2).

Autosomal dominant EKV was mapped to chromosome 1p34–35 (3), a location that is now known to contain four

genes encoding gap junction proteins, also known as connexins: *GJB3* (connexin 31, Cx31), *GJA4* (connexin 37, Cx37), *GJB5* (connexin 31.1, Cx31.1) and *GJB4* (connexin 30.3, Cx30.3) (4–6). Mutations associated with EKV were subsequently found in both connexin 31 and connexin 30.3 (4,5,7).

In this study, a novel mutation (L34P) in the connexin 31 gene was found in a homozygous form in affected individuals in a family that shows recessive inheritance of the disorder, thus providing molecular support for a recessive variant of EKV. Functional analysis of the expressed mutant L34P Cx31 protein suggests that it will not form gap junctions between keratinocytes.

RESULTS

We identified a novel mutation in the *GJB3* gene segregating with EKV. In addition, this is the first connexin mutation

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associated with a recessive skin disease. In the family in which the mutation was segregating, both parents are unaffected and have never suffered from EKV lesions throughout their lifetime. Three out of four children are affected (Fig. 1A). The course of the disease and clinical findings were similar in all the affected members. General medical history, including hearing disturbances, was unremarkable. The eruption began during the first year of life, spreading progressively until puberty, followed by gradual improvement. The eruption consisted of two components: transient erythematous patches, changing their configuration in course of weeks, and fixed erythrokeratotic plaques. Aggravation of lesions was noted during summers. On examination, figurate erythematous patches were seen on the abdomen (Fig. 1B) and well-defined, irregular brownish thickened skin was noted on the trunk, earlobes (Fig. 1C) and extensor aspects of the upper and lower limbs. Palms, soles, scalp, hair, nails and mucosae were normal. Biopsies were taken from all affected members, showing similar histological findings. These included marked, basket-weave orthohyperkeratosis, with a dense eosinophilic keratotic band just above the granular layer. The rest of the epidermis was markedly acanthotic, with fingerlike projections of the rete ridges. Mild lymphohistiocytic infiltrate was found in the upper dermis. We have ruled out progressive symmetric erythrokeratoderma (EPS), which may be inherited in a recessive mode (8), since members of the family have two types of lesions: variable erythema and fixed erythrokeratotic plaques. The distribution of lesions included the trunk, which is extremely rare in EPS. The clinical symptoms improved with age, while in EPS the rash constantly tends to worsen. Histological findings did not include foci of parakeratosis, symptoms that are associated with EPS.

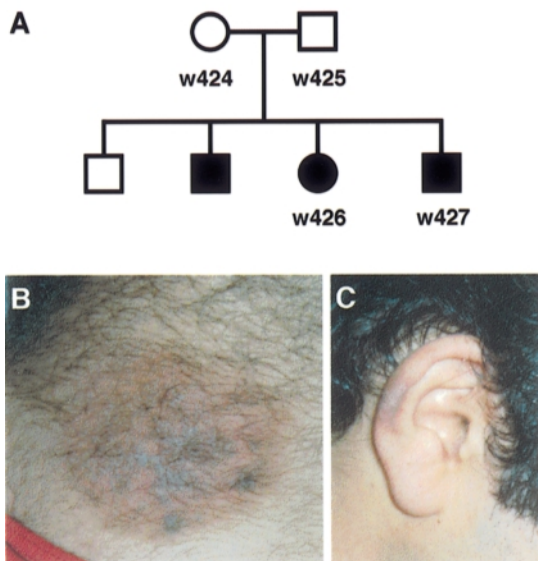


Figure 1. Family with recessive EKV. (A) Pedigree of the Israeli family (Jewish-Buchari origin), displaying recessive EKV. Both parents are healthy, while three out of their four children are affected with EKV. Filled shapes represent EKV-affected individuals. Numbered individuals participated in this research. (B, C) Erythematous and hyperkeratotic patches on the abdomen and ear, respectively, in an affected individual.

Initially, sequencing of both connexin 31 and connexin 30.3 open reading frames was performed for w427, an affected child. One silent change was found in connexin 30.3 (C420T), while a homozygous substitution, T101C, was found in the connexin 31 gene (Fig. 2A). The healthy father (w425) was found to be heterozygous for the T101C nucleotide substitution in GJB3 by sequencing (Fig. 2A). The nucleotide change causes a missense mutation, changing codon 34 from leucine to proline (L34P), and adds a BanI restriction site. The change in restriction pattern was used to test the rest of the family and the controls (Fig. 2B). Digestion of the wild-type product gave three observed fragments (64 bp, 266 and 271 bp comigrate, and 316 bp) while the homozygote mutation gives four observed fragments (64 bp, 101 bp, 215 bp, 266 and 271 bp comigrate). The mother and another affected child were found to be heterozygous and homozygous for the mutation, respectively.

The mutation was not found in 208 control chromosomes, examined by restriction enzyme analysis, derived from the Jewish non-Ashkenazi population (including 30 Buchari chromosomes) (data not shown).

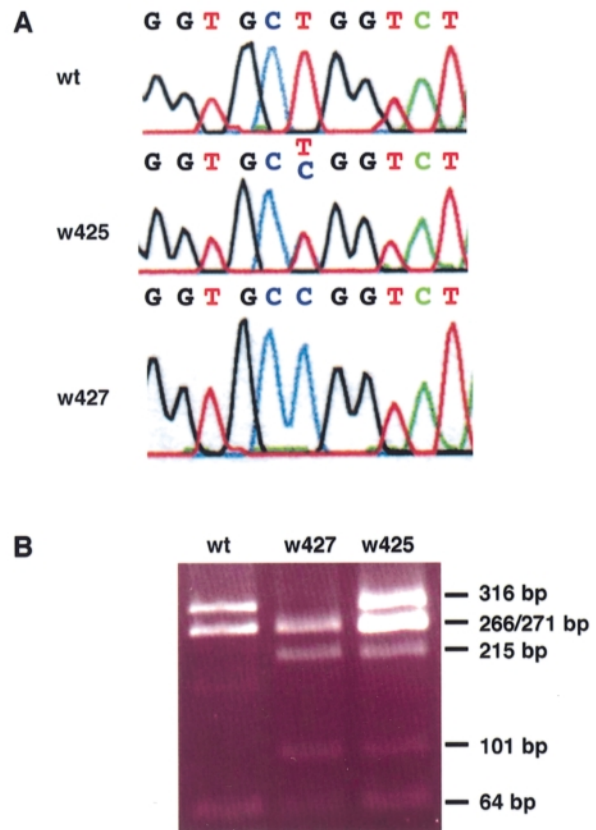


Figure 2. Mutation analysis of T101C. (A) Sequence chromatograms derived from a control, heterozygote individual w425 and homozygote individual w427. A T→C substitution at nucleotide 101 converts a leucine to a proline at codon 34. (B) BanI restriction analysis of the T101C mutation. Digestion of the normal PCR product gives four fragments (seen as three bands): 64 bp, 266 + 271 bp and 316 bp, while digestion of the mutant gives five fragments, seen as four: 64 bp, 101 bp, 215 bp and 266 + 271 bp. The heterozygote will include all six fragments.

The leucine residue is present at codon 34 in all connexin 31, 30.3 and 31.1 species, while in connexin 26, 30 and 32 a conservative substitution to another hydrophobic residue, methionine, is found. The degree of conservation of this mutated residue in connexin 31 was analyzed using ConSurf (9), a program that assigns amino acid conservation grades in the range 0–1, where 1 is maximal conservation. A score of 0.97 was obtained for Leu34, while the whole protein has an average score of 0.80. It is important to note that all the

residues of the first helix have a score higher than 0.9, that is, the first TM domain is highly conserved (Fig. 3).

As the human Cx31 antibody (10) does not detect Cx31 protein particularly well on paraffin-embedded material, the effect of the L34P mutation on the localization of the Cx31 protein was investigated by transfection studies of different cell types. Following transfection of both HeLa and DT cell lines with wild-type and L34P connexin 31, the cellular localization of the chimeric proteins were compared. The wild-type

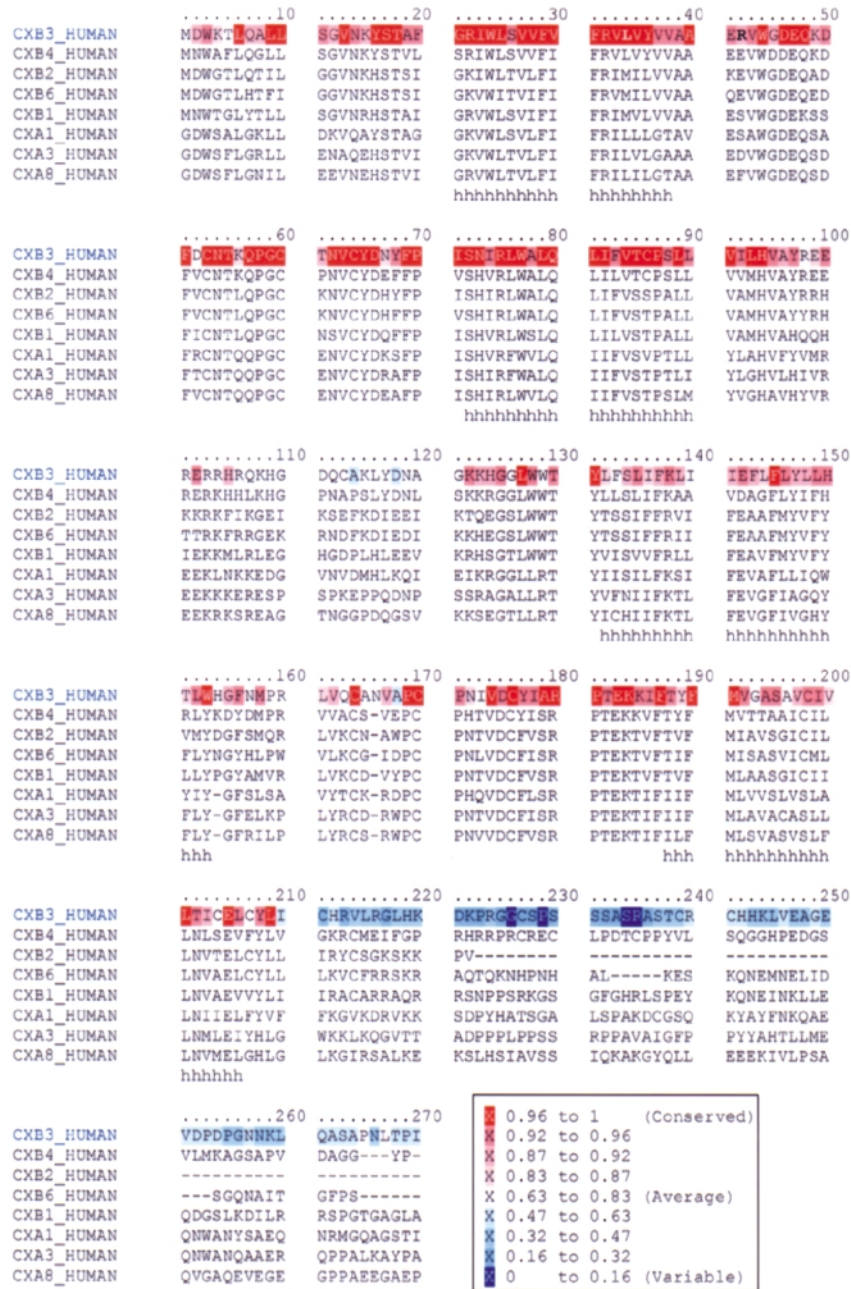


Figure 3. Multiple sequence alignment of connexin 31 (CXB3_HUMAN) and seven other human connexins associated with human disease. Residue conservation scores, calculated using ConSurf (9), from 53 homologue sequences in the SwissProt database (26) are color-coded onto the sequence of connexin 31 (see key). Leu34 and Arg42 of connexin 31, both of which are associated with EKV, are in bold fonts and the location of the four putative TM helices, predicted using the PredictProtein server (<http://maple.bioc.columbia.edu/predictprotein/>) (27), are indicated as 'h' at the bottom line of the alignment.

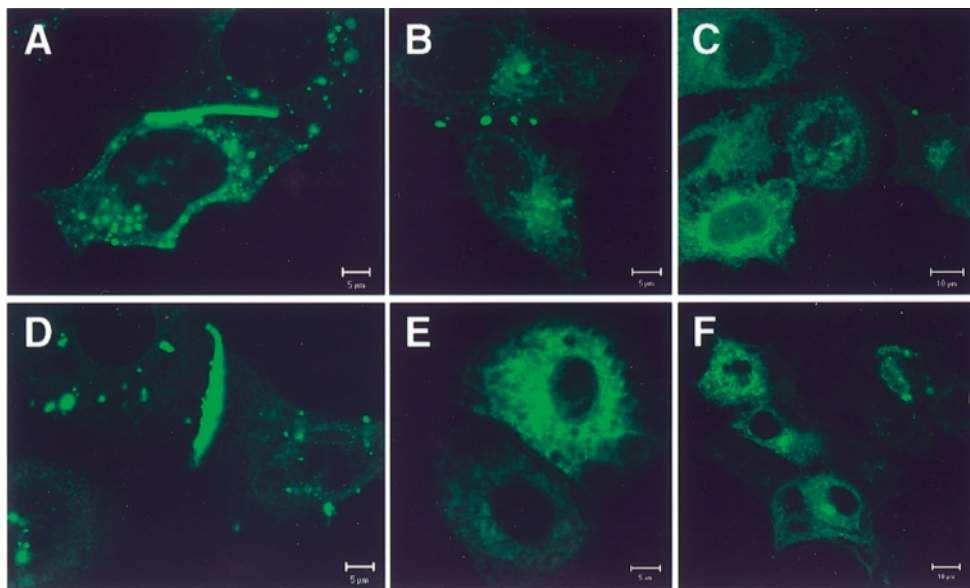


Figure 4. Connexin 31 localization studies. HeLa cells transfected with (A) pCx31WT/EGFP, (B,C) pCx31L34P/EGFP; keratinocyte-derived DT cells transfected with (D) pCx31WT/EGFP and (E,F) pCx31L34P/EGFP. Note localization of wild-type GJB3 along the membrane between two cells, where functional gap junctions will form. In a small proportion of HeLa cells, there is some localization of the mutant protein along the membrane, although most is localized in the cytoplasm. In the keratinocyte cells, there is complete mislocalization of the L34P protein.

Cx31-EGFP was trafficked to the plasma membrane, localizing in areas of cell-cell contact (Fig. 4A,D). In contrast, the majority of L34P-Cx31 had a cytoplasmic distribution (Fig. 4B,C,E,F). In a few transfected HeLa cells (< 5% of transfected cells, e.g. Fig. 4B,C), a small proportion of the mutant protein was seen to have a membranous localization. However, no membranous localization of the mutant protein was observed in the keratinocyte-derived cell line (Fig. 4E,F).

DISCUSSION

Mutations described so far in relation to EKV are heterozygous and are associated with dominant inheritance (3–5,7). In this paper, we report a novel GJB3 mutation that segregates in a family with a recessive pattern of inheritance of EKV.

Gap junction proteins are molecules that form intercellular channels connecting adjacent cells, and are widely expressed in the human body. However, a mutation in a specific connexin will usually affect only one organ, probably because of its specific role (11). At least 10 different types of connexins are expressed in different keratinocyte populations of human skin (10). So far, mutations in four connexins have been associated with skin disorders (reviewed in 12): connexin 31 with EKV (5), connexin 30.3 with EKV associated with erythema gyratum repens-like features (4), connexin 26 with Vohwinkel's syndrome (13,14) and connexin 30 with hidrotic ectodermal dysplasia (15). Interestingly, mutations in three of these connexins are also associated with hearing loss (16–18). Until now, mutations in connexins have only been identified in dominantly inherited forms of EKV. We can only speculate as to why the L34P mutation is required in the recessive form in order to lead to this phenotype in keratinocyte cells. In the

heterozygote form, this missense mutation may not be able to exert a dominant-negative effect, and thus will only manifest itself clinically in the homozygote. It is unclear if this is a function of the location of the missense mutation or of the particular amino acid change.

Connexins share a common structure of four transmembrane (TM) helices connected by two extracellular loops and one cytoplasmic loop; thus, both their C and N termini are cytoplasmic. The mutated leucine residue identified in this study lies in the first TM helix of the protein. Different functions are predicted for each domain of the protein (reviewed in 19). Initially, it had been suggested that the location of the dominant mutation in connexin 31 could determine which organ will be affected – skin or inner ear. Mutations in the extracellular loops, implicated in the specificity of connexin-connexin interactions, would result in hearing loss, whereas mutations in other domains, such as those associated with voltage gating, would result in EKV (19). However, as the number of EKV-associated mutations has risen, this correlation has proven to be untrue. From this study, it is also evident that distinct recessive GJB3 mutations can also cause skin disease or deafness.

ConSurf evolutionary analysis shows that the leucine residue is highly conserved throughout different species and different connexins. The change of this residue to proline may change the structure of the TM helix, thus influencing the connexin structure and function. The change from a leucine to a proline is particularly significant; proline provides a unique conformational property not found with any other residues (20). The replacement of a leucine by a proline may introduce a kink in the α helix that will compromise connexin function.

Further support for the likely deleterious effect of the L34P mutation in connexin 31 comes from the functional and genetic

analysis of the M34T mutation in connexin 26. Recent genetic studies support M34T in connexin 26 acting as a recessive mutation associated with mild to moderate hearing loss (21,22). Functional studies indicate that M34T connexin 26 oligomerizes poorly and has limited trafficking to the plasma membrane (23; W-L. Di and D.P. Kelsell, unpublished data).

The wild-type Cx31-EGFP was concentrated in areas of cell-cell contact, suggesting that they were forming gap junctions between adjacent transfected cells. In HeLa cells, while the majority of L34P-Cx31 had a cytoplasmic distribution, there was some membranous localization in a minority of cells, suggesting that it is capable of forming some gap-junction-plaque-like structures under certain cellular conditions. In a keratinocyte-derived cell line, the L34P-Cx31 protein was only expressed in the cytoplasm.

The high conservation of the residue, the location of the mutation, the previous association of connexin 31 with EKV and the functional data all strongly support that L34P is the basis of recessive EKV in this family.

MATERIALS AND METHODS

Patients and clinical examination

A Jewish family originating from Buchara, Uzbekistan was studied. Both parents were healthy, whereas three out of four siblings were affected (Fig. 1A). The parents and three affected children were personally examined by two of the authors (M.L. and B.M.) on two different occasions, while the unaffected child was reluctant to cooperate with the study and therefore was not included. This project was approved by the Helsinki Committee at the Edith Wolfson Medical Center. After informed consent, blood samples were taken from the parents and two affected children, while skin biopsies were taken for histological examination from all the affected members. Human control DNA samples were obtained from The National Laboratory for the Genetics of Israeli Populations at Tel Aviv University, Israel (<http://www.tau.ac.il/medicine/NLGIP/nlgip.htm>).

DNA extraction, sequencing and restriction enzyme analysis

DNA was extracted from blood using a DNA isolation kit for mammalian blood (Boehringer-Mannheim). A 917 bp fragment containing the connexin 31 open reading frame (ORF) (GenBank XM_018110) was amplified using the primers 1F: TCACCTATTCATTCATACGATGG and 2R: GGCAGCCCGCATGTTGCC, in an MJ Research Thermocycler (PTC 200), under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 0.5 min, 60°C for 0.5 min, 72°C for 1 min; and 72°C for 5 min. The connexin 30.3 ORF (GenBank AL121988) was amplified as an 801 bp fragment under the same conditions, using primers 1F: 5'-CAATCGCACCAGCATTAAAGGG-3' and R: 5'-TGATCTTATCTGCTGATCTCGCAG-3' (4). Sequencing was performed using the same primers as those used for PCR, the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems) and an ABI 377 DNA sequencer. Mutations were confirmed by

sequencing in both directions. The mutation that we detected causes a replacement of a single nucleotide, forming an additional BanI restriction site in the connexin 31 gene. The PCR product generated as described above was digested with BanI (New England Biolabs), and the restriction products were run on a 4% agarose gel.

Evolutionary analysis

The degree of conservation of the mutated residue was analyzed using ConSurf (<http://bioinfo.tau.ac.il/ConSurf/>) (9). This software examines the closest homologues of a given protein and assigns a score to each of the protein's residues according to its conservation amongst the homologues. The scores range between 0 and 1; the higher the conservation level, the higher the score.

Transfection with chimeric Cx31-EGFP plasmids

A full-length clone of the connexin 31 wild-type gene was fused inframe to the N terminus of enhanced green fluorescent protein (EGFP) by a two-step cloning procedure. First, the authentic connexin stop codon was changed from TGA to GGA by PCR using the primers with a restriction site sequence SacII, 5'-GCCCCGCGGATGGACTGGAAGACACTC-3' for forward and 5'-GCCCCGCGGCTCCGATGGGGGTCAGGTT-3' for reverse, and was cloned directly into a TA cloning vector, pGEM-T (Promega). Second, the SacII fragment of connexin 31 from pGEM-T/Cx31 was further cloned into a pEGFP-N3 vector (Clontech). In this orientation, the EGFP is at the C terminus of the connexin 31 protein, and the stop codon of connexin 31 was altered to allow 'read through' and expression of the EGFP protein. All positive clones were identified by restriction enzyme analysis and automated DNA sequencing to determine that there were no 'introduced' nucleotide errors in the cloned connexin 31. The L34P was manufactured by a site-directed mutagenesis kit (Stratagene) using the mutagenic forward primer 5'-GTCTCCGGGTGCCGGTATACGTGGTGG-3' and reverse primer, 5'-CCACCACGTATACCGGCACCCGGAAGAC-3' (mutation site is bold and underlined). The supercoiled double-stranded DNA from pCx31WT/EGFP was used as a template. The nucleotide T was substituted for a C, resulting in a substitution of a leucine for a proline at residue 34. DNA sequence analysis indicated that this was the only nucleotide alteration.

HeLa Ohio cells (ECACC, CRUK) were cultured in DMEM supplemented with 10% fetal calf serum, penicillin/streptomycin and L-glutamine. DT cells, which were immortalized from normal keratinocytes with SV40 T antigen (24), were cultured as described by Leigh and Watt (25). Both cell lines were transfected with the pCx31WT/EGFP or pCx31L34P/EGFP using Transfast (Promega). Briefly, the cells ($7 \times 10^5/60$ mm dish) were transfected with 7 µg of plasmid DNA in 1:2 Transfast reagent. Twenty-four hours post transfection, the cells were fixed with 4% buffered paraformaldehyde, and the morphologies of Cx31-EGFP fusion proteins were visualized by virtue of the inherent fluorescence of EGFP and recorded using a confocal microscope (Carl Zeiss).

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