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Neurofibromatosis Type 1 Protein and Amyloid Precursor Protein Interact in Normal Human Melanocytes and Colocalize with Melanosomes

Sofie De Schepper[1,5], Joachim M.A. Boucneau[1,5], Wendy Westbroek[2], Mieke Mommaas[3], Jos Onderwater[3], Ludwine Messiaen[4], Jean-Marie A.D. Naeyaert[1] and Jo L.W. Lambert[1]

The neurofibromatosis type 1 (NF1) gene product, neurofibromin, is known to interact with Ras, thereby negatively regulating its growth-promoting function. Although this is a well-established interaction, the discovery of other neurofibromin interacting partners could reveal new functional properties of this large protein. Using yeast two-hybrid analysis against a brain cDNA library, we identified a novel interaction between the amyloid precursor protein and the GTPase activating protein-related domain of neurofibromin. This interaction was further analyzed in human melanocytes and confirmed by immunoprecipitation and colocalization studies. In addition, we observed a colocalization of amyloid precursor protein and neurofibromin with melanosomes. Amyloid precursor protein has been proposed to function as a vesicle cargo receptor for the motor protein kinesin-1 in neurons. This colocalization of amyloid precursor protein and neurofibromin with melanosomes was lost in melanocytes obtained from normal skin of a NF1 patient. We suggest that a complex between amyloid precursor protein, neurofibromin, and melanosomes might be important in melanosome transport, which could shed a new light on the etiopathogenesis of pigment-cell-related manifestations in NF1.

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INTRODUCTION

Neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant neurocutaneous disorders in man with a worldwide prevalence of roughly 1 in 3,500 individuals (Riccardi, 1981). The disorder primarily affects tissues of neural crest origin and exhibits a wide clinical expression spectrum, ranging from benign peripheral nervous system tumors (neurofibromas), optic pathway gliomas, skeletal dysplasia to pigmentary defects (café-au-lait spots, freckling, Lisch nodules). Apart from the fact that the disease is a heavy psychological burden, it is also accompanied by a number of complications such as an increased risk of developing malignancies and learning disabilities (De Schepper *et al.*, 2005). The disorder is caused by mutations of the *NF1* gene. This gene spans approximately 350 kb, is localized on chromosome 17q11.2, and has 60 exons. The transcript is 11–13 kb long and encodes a protein, called neurofibromin, of 2,818 amino acids (Viskochil *et al.*, 1993). Neurofibromin is a large protein expressed in numerous tissues with the highest expression levels being observed in neurons (Nordlund *et al.*, 1993). The only region of neurofibromin, which has been well characterized, is a central domain of about 360 amino acids showing a significant sequence and functional homology to the mammalian Ras-specific GTPase activating proteins (RasGAPs – eg $p120^{GAP}$). This domain is called the GAP-related domain (GRD) and increases the slow intrinsic GTPase activity of the growth regulator Ras up to 10^5-fold. A growing number of neurofibromin interacting or associating proteins and organelles have been described over the years. It has been shown that neurofibromin can bind to certain major cytoskeletal structures. Neurofibromin is able to associate with microtubules through its GRD and it has been proposed that this association is important for regulating the growth-promoting activity of Ras (Bollag *et al.*, 1993; Gregory *et al.*, 1993; Xu and Gutmann, 1997). An interaction switch between different cytoskeletal structures was described in telencephalic neurons, with neurofibromin exhibiting a biphasic differentiation-dependent expression pattern and a differential subcellular localization to the F-actin and microtubule cytoskeleton (Li *et al.*, 2001). In addition, a

[1]*Department of Dermatology, Ghent University Hospital, Ghent, Belgium;* [2]*Section on Human Biochemical Genetics, Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, USA;* [3]*Department of Molecular Cell Biology, Center for Electron Microscopy, Leiden University Medical Center, Leiden, The Netherlands and* [4]*Department of Genetics, University of Alabama at Birmingham, Birmingham, Alabama, USA*

[5]*These two authors contributed equally to this work.*

Correspondence: Dr Sofie De Schepper, Department of Dermatology, Ghent University, De Pintelaan 185, 9000 Ghent, Belgium.
E-mail: sofie.deschepper@ugent.be

Abbreviations: APP, β-Amyloid precursor protein; GAP, GTPase activating protein; GRD, GAP-related domain; NF1, neurofibromatosis type 1

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stable association between the kinesin-1 heavy chain and neurofibromin has recently been shown (Hakimi *et al.*, 2002). Interaction with extracellular matrix-binding proteins such as the transmembrane heparan sulfate proteoglycan syndecan-2 has been suggested as a mechanism for localizing neurofibromin to specific domains of the plasma membrane, perhaps contributing to synaptic RasGAP activity or adhesion signaling in the brain (Hsueh *et al.*, 2001). Colocalization of neurofibromin with the mitochondria in cultured cell lines (Roudebush *et al.*, 1997) has been described, whereas localization to the smooth endoplasmic reticulum was seen in neurons (Nordlund *et al.*, 1993).

In order to find other functional interactions of neurofibromin, we set up a yeast two-hybrid screening of the neurofibromin-GRD and a brain cDNA library. In this report, we demonstrate that β-amyloid precursor protein (APP) interacts with the GRD of neurofibromin. We show that in human melanocytes, neurofibromin and APP colocalize with melanosomes. This interaction complex could be part of a melanosome transport/biogenesis regulating mechanism or a signaling complex or might be a mechanism for sequestering neurofibromin from the plasma membrane, where it functions as a negative regulator of Ras.

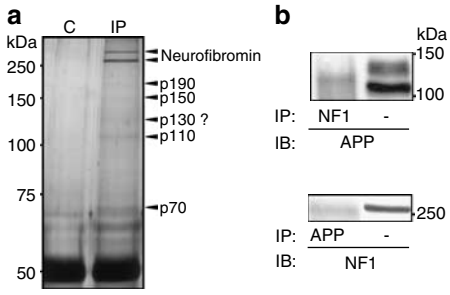

Figure 1. Identification of neurofibromin interacting proteins. (**a**) Neurofibromin in cell lysate of primary human epidermal melanocytes was immunoprecipitated with NF1 sc-67 antibody and the immunoprecipitate was analyzed by SDS-PAGE (6%) and silver stained. Lane C represents the rabbit polyclonal IgG isotype matched control, lane IP represents the neurofibromin immunoprecipitate. Molecular weight markers are shown on the left and on the right black arrows indicate the position of immunoprecipitated protein bands (p70, p110, p130, p150, p190). (**b**) Upper right figure: immunostaining with APP (CT15) antibody of NF1 sc-67 immunoprecipitate (left lane) compared to the total cell lysate (right lane). Lower right figure: immunoblotting with NF1 sc-67 antibody of APP (CT15) immunoprecipitate (left lane) compared to the total cell lysate (right lane). Molecular weight markers are shown in kDa.

RESULTS

Identification of APP as a neurofibromin-binding protein

Screening of possible direct interactions of neurofibromin with other proteins was performed using the GAL-4-based yeast two-hybrid vector system. The GRD of neurofibromin (bp 4,071–4,671) was screened against a commercially available brain cDNA library in the yeast strain *Saccharomyces cerevisiae* PJ-69-4A.

One of the interactions that remained after stringent selection with SD/-Leu-Trp-Ade, SD/-Leu-Trp-His, and the X-gal filter assay was identified as APP. Because of its important role in axonal transport in the brain (Kamal *et al.*, 2001) and its proposed role in melanosome transport and melanin release (Quast *et al.*, 2003), we decided to further investigate this interaction.

To further confirm and investigate the neurofibromin–APP interaction in human melanocytes, total melanocyte cell lysate was incubated with NF1(D) antibody to immunoprecipitate possible neurofibromin-containing protein complexes. As shown in Figure 1a, several bands with molecular masses between 70 and 200 kDa were obtained after silver staining of the gel (p70, p110, p130, p150, p190), but none of them were observed in the rabbit IgG isotype-matched control. The observed proteins p110 and p130 match the known molecular weights of immature (110 kDa) and mature (130 kDa) APP. Using the anti-APP antibody (CT15), APP was identified after Western blotting of a neurofibromin immunoprecipitate. The APP band, however, was located intermediate between the immature and mature form of APP. The reason for this remains unclear (Figure 1b, upper part). The reverse experiment was also performed to see whether neurofibromin co-immunoprecipitated with APP. This showed a 250 kDa band corresponding to the neurofibromin protein (Figure 1b, lower part).

Expression of *APP* and *NF1* gene (product) in cultured primary human epidermal melanocytes

We examined mRNA expression of APP and NF1 in normal human melanocytes using primers APP-F and APP-R (Quast *et al.*, 2003) and NF1-F and NF1-R. We saw the appropriate cDNA bands for the 751 and 770 isoforms of APP and for NF1 (94 bp) (Figure 2a). In addition, we checked the protein expression of APP using the polyclonal antiserum CT15 and of neurofibromin using the NF1(D) in normal human melanocytes by means of Western blot (Figure 2b). In human melanocyte protein extract a clear band representing neurofibromin was observed around 250 kDa. Two APP isoforms of around 110 and 130 kDa were detected, being the immature and mature isoforms of the protein, respectively.

Localization of APP and wild-type neurofibromin in cultured primary human epidermal melanocytes (healthy donor) and colocalization with melanosomes

As described earlier (Quast *et al.*, 2003) APP is mainly localized in a reticulum within the perinuclear region showing colocalization with the endoplasmic reticulum and premelanosomes and in granules at the tips of dendrites, representing mature melanosomes.

We investigated the distribution of APP and neurofibromin by indirect immunofluorescent labeling. In melanocytes, we observed the previously reported perinuclear staining pattern of APP associated with occasional staining of the dendrite tips (Figure 3b). Neurofibromin showed a granular staining pattern with perinuclear accentuation (Figure 3a).

Because both APP and neurofibromin displayed a similar organellar punctate staining pattern, a double labeling of neurofibromin and APP with the melanosomal marker NKI-beteb was performed.

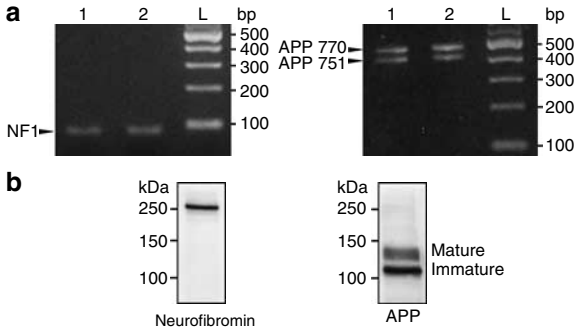

Figure 2. Expression of *NF1* gene (product) and *APP* gene (product) in primary human epidermal melanocytes. (**a**) Detection of NF1 (left) and APP (right) mRNA expression by RT-PCR analysis in primary human epidermal melanocytes (obtained from two different donors (1 and 2)), displaying the expression of both the 751 and 770 isoforms of APP. The same samples showed the appropriate 94 bp band (arrow) when analyzed with the exon 36–37 NF1 boundary primers. (**b**) Expression of neurofibromin and APP proteins was analyzed by SDS-PAGE (6%) followed by immunostaining with anti-neurofibromin (NF1 sc-67) and anti-APP (CT15) antibodies. Molecular weight markers are shown in kDa on the left. Note the immature and mature isoforms of APP.

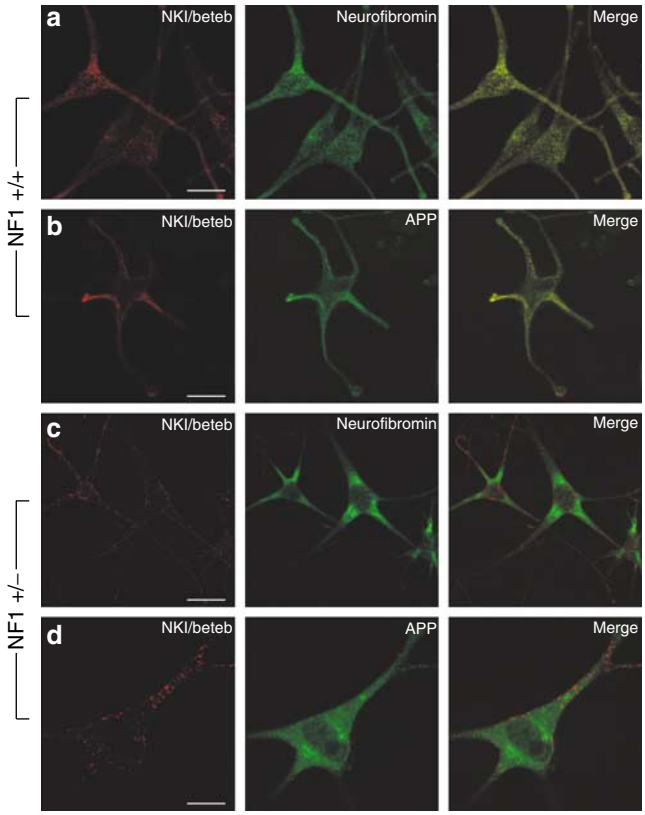

Figure 3. Subcellular distribution and localization of neurofibromin, APP and melanosomes in healthy donor ($NF1^{+/+}$) and NF1 patient ($NF1^{+/-}$) primary human epidermal melanocytes. Healthy donor melanocytes were cultured on coverslips for 24 hours, fixed and immunostained with (**a**) NKI-beteb and neurofibromin and (**b**) NKI-beteb and APP. The overlay panels show remarkable colocalization of neurofibromin especially in the perinuclear area and of APP in the perinuclear area and the dendrite tips. NF1 patient melanocytes were cultured on coverslips for 24 hours, fixed and immunostained with (**c**) NKI-beteb and neurofibromin and (**d**) NKI-beteb and APP. A clear absence of colocalization can be seen. Bars = 10 μm.

In melanocytes, NKI-beteb stains the melanocyte-specific glycoprotein Pmel17 or gp100 and displays a punctate staining pattern throughout the cell body and dendrites with accentuation around the nucleus and in the dendrite tips. APP shows a similar staining pattern especially around the perinuclear area, which colocalizes with premelanosomes, and in some of the dendrite tips, marking mature melanosomes. The staining was less intense in the cell periphery. Figure 3b shows high levels of colocalization between APP and NKI-beteb. Neurofibromin shows a perinuclear staining pattern with no obvious staining of the dendrite tips (Figure 3a). To study colocalization of neurofibromin and APP with melanosomes on an ultra-structural level, immuno-electron microscopy was performed. Figure 4a shows APP as 15 nm gold particles situated on organelles resembling melanosomes. Figure 4b shows neurofibromin as 15 nm gold particles situated on melanosomes. To make sure that the observed organelles are in fact melanosomes and not lysosomes or other organelles, a double immunostaining of neurofibromin (10 nm gold) and NKI-beteb (15 nm gold) was performed (Figure 4d). The same was performed for APP (Figure 4c). The results show remarkable colocalization on melanosomes. Owing to technical limitations, we were not able to perform double labelings of APP with neurofibromin.

An *NF1* gene mutation deranges the colocalization of both APP and neurofibromin with melanosomes

Double immunofluorescent staining was performed on cultured $NF1^{+/-}$ primary human epidermal melanocytes obtained from the skin of a severely affected NF1 patient with a deletion of two nucleotides of the *NF1* gene (3,525_3,526del2). Figure 3c shows almost complete loss of the overlap between neurofibromin and NKI-beteb, which implicates that mutant neurofibromin is unable to colocalize on melanosomes. Interestingly, Figure 3d indicates that this *NF1* gene deletion also affects the colocalization between APP and melanosomes.

Ultrastrastructural study with immuno-electron microscopy displayed less staining of neurofibromin (15 nm gold) (Figure 4f). The observed decrease in neurofibromin expression can be explained by the haploinsufficiency of the *NF1* gene locus due to NF1 heterozygosity. In the double immunostainings of APP (15 nm gold) and NKI-beteb (10 nm gold) (Figure 4 g), and neurofibromin (15 nm gold) and NKI-beteb (10 nm gold) (Figure 4 h), a specific and clear staining of melanosomes is visible. Neither APP nor neurofibromin localize to melanosome structures nor to any other identifiable structure.

DISCUSSION

Using yeast two-hybrid analysis against a brain cDNA library, we found a direct binding of APP with the GRD of neurofibromin. As our primary interest is in the pigment cell-related manifestations in NF1, we further investigated the APP–neurofibromin interaction in primary human epidermal melanocytes.

APP is a type I transmembrane cell surface protein with a large N-terminal extracellular part. Being known as the

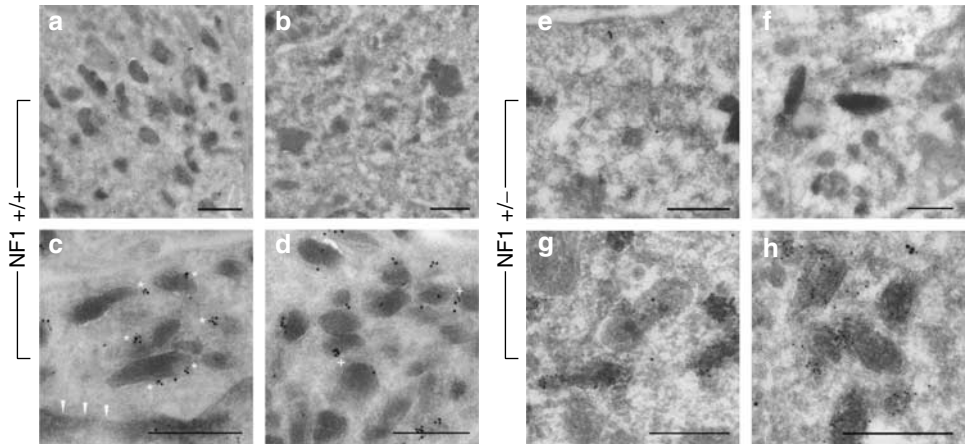

Figure 4. Ultrastructural distribution and localization of neurofibromin, APP and melanosomes in healthy donor (NF1$^{+/+}$) and NF1 patient (NF1$^{+/-}$) primary human epidermal melanocytes. Paraformaldehyde-fixed ultrathin cryosections of healthy donor primary human epidermal melanocytes were labeled with (**a**) β-amyloid antibody (15 nm gold) and (**b**) neurofibromin antibody (15 nm gold). Both APP and neurofibromin showed melanosomal localization. (**c**) A double labeling with NKI-beteb antibody (15 nm gold) and β-amyloid antibody (10 nm gold) revealed complexes associated on melanosomes (asterisk). The plasmamembrane of the dendrite is marked with arrows. (**d**) A double labeling with NKI-beteb antibody (15 nm gold) and anti-neurofibromin (10 nm gold) showed several melanosome-associated colocalization complexes (plus signs). Paraformaldehyde-fixed ultrathin cryosections of NF1 patient primary human epidermal melanocytes were labeled with (**e**) APP antibody (15 nm gold) and (**f**) neurofibromin antibody (15 nm gold). No melanosomal localization was seen. (**g**) The double labeling with NKI-beteb (10 nm gold) and APP (15 nm gold) and with (**h**) NKI-beteb (10 nm gold) and neurofibromin (15 nm gold) only showed clear labeling of the melanosomal marker NKI-beteb but no colocalization of (**g**) APP or (**h**) neurofibromin. Bars = 500 nm.

precursor of the amyloid-beta (Aβ) peptides involved in the pathogenesis of Alzheimer's disease, APP consists of different isoforms generated by alternative splicing of the *APP* gene. APP isoforms 751 and 770 (numbers referring to their length in amino acids) are found as the major translation products in, among others, the epidermis, whereas isoform 695 (lacking exon 7 and 8) is most abundant in the brain (Quast *et al.*, 2003). APP expression is especially high in epidermal melanocytes and therefore it has been suggested as an immunocytochemical marker for this cell type (Quast *et al.*, 2003). β-Amyloid, a neurotoxic peptide, is generated from APP through the action of β- and γ-secretases. However, the α-secretase pathway splits APP within the Aβ domain, producing a large amino-terminal non-amyloidogenic sAPPα, which functions as a regulator of dendrite motility and melanin release in epidermal melanocytes and melanoma cells (Quast *et al.*, 2003). Several interacting partners of APP have been recently described. The Aβ peptide binds to cyclin B1 and increases human cyclin-dependent kinase-1 activity (Milton, 2002). In addition, two-hybrid screening assays have detected three proteins that interact with the specific GYENPTY domain in the cytoplasmic tail of APP: Fe65, X11, and mDab1 (mammalian homologue of *Drosophila* disabled). Together with PAT1 (protein interacting with APP tail 1), they may provide a link between APP and the cytoskeleton (Herzog *et al.*, 2004). APP also functions as a matrix-binding protein interacting with perlecan, laminin, collagen type IV and endactin, and as a copper-binding protein important in neuronal copper homeostasis (Herzog *et al.*, 2004). Recent studies have demonstrated that the C-terminal part of APP forms a complex with neuronal kinesin-1 by direct binding to the tetratricopeptide repeat domain of the kinesin light chain (Kamal *et al.*, 2000) and that the JNK signaling scaffold protein JIP1b mediates this association (Inomata *et al.*, 2003). Interestingly, neurofibromin has also been shown to interact with kinesin-1, with cytoskeletal structures such as microtubules and with extracellular matrix-binding proteins such as syndecan-2 (Hsueh *et al.*, 2001; Li *et al.*, 2001). The direct association between neurofibromin and kinesin-1 was established by conventional and affinity chromatography, Western blot, and immunoprecipitation in the soluble and particulate fraction of HeLa extract and calf brain (Hakimi *et al.*, 2002). For the first time, we show a direct interaction between APP and the GRD of neurofibromin in the brain and in melanocytes, which is not surprising considering that both are linked to kinesin-1 and to the microtubular cytoskeleton and therefore must exhibit a close spatial relationship. In addition, we confirm the previously described colocalization of APP with melanosomes (Quast *et al.*, 2003) and show that neurofibromin is also present on the melanosomal membrane and forms a complex with APP. In neurons, APP is transported from the neuronal cell bodies towards the distal nerve terminals by kinesin-1-mediated axonal transport (Koo *et al.*, 1990; Sisodia *et al.*, 1993). As has already been suggested in a recent publication (Herzog *et al.*, 2004), we also believe that full-length APP aids the kinesin-mediated microtubular transport of melanosomes along the dendrites.

The existence of a complex between APP, neurofibromin, and melanosomes is especially interesting in light of the many pigment-cell-related manifestations seen in NF1, with café-au-lait macules being the major hallmark. Our experiments on NF1$^{+/-}$ melanocytes obtained from the skin of a severely affected NF1 patient shows that the colocalization of both APP and neurofibromin with melanosomes is disturbed by the *NF1* gene mutation (Figures 3c, d and 4e–h).

NF1 melanocytes, carrying an *NF1* gene defect, contain many large melanosomal complexes and increased amounts

of melanin compared to normal human melanocytes (Martuza *et al.*, 1985; Kaufmann *et al.*, 1991). Melanin macroglobuli originate from the fusion of melanosome complexes with phagolysosomes. They are aggregates of melanosomes at various stages of melanization (De Schepper *et al.*, 2005). In this viewpoint, a mutated *NF1* gene product could, via the interaction with kinesin and APP, cause problems in melanosome biogenesis or transport or could even impair the interchange between melanosomes and lysosomes, leading to macromelanosome formation.

In addition, an interesting notion considering NF1 and Alzheimer's disease (both being common neurological disorders) is that neurofibromin and APP share their interaction with the molecular motor protein kinesin-1. In neuronal cells, processing of APP to β-amyloid can occur in an axonal membrane compartment, containing β-secretase and presenilin-1, transported by kinesin-1 (Kamal *et al.*, 2000, 2001). By demonstrating an interaction between neurofibromin and APP and localizing both constituents to the melanosomes of normal human melanocytes, we might expand the insight into vesicular trafficking. In light of the frequent cognitive defects in NF1 (mental retardation, learning disabilities, etc), it could be that mutation of the *NF1* gene product leads to an impaired kinesin-1-mediated protein and vesicle trafficking in neurons, perhaps distorting neurotransmitter transport or synaptic RasGAP activity in the brain (Hsueh *et al.*, 2001).

In summary, we provide new evidence for the existence of a complex consisting of melanosomes, neurofibromin, and APP, which might be relevant for the etiopathogenesis of several symptoms found in NF1, such as pigmentation or cognitive disorders.

MATERIALS AND METHODS

Cell culture

Primary human epidermal melanocyte cultures used for yeast two-hybrid screening, co-immunoprecipitation, indirect immunofluorescence and immuno-electron microscopy were obtained from neonatal foreskin of a healthy control individual or from an NF1 patient normal skin biopsy. Written informed consent was obtained from all patients and all described protocols/studies were approved by the medical ethical committee of the University of Ghent. The study was conducted in accordance with institutional guidelines on the Declaration of Helsinki Principles. Melanocyte cultures were established as described previously (Naeyaert *et al.*, 1991; Smit *et al.*, 1998). Briefly, the cells were cultured in Ham F10 (Gibco, Invitrogen Ltd, Paisley, UK) medium supplemented with 2.5% fetal calf serum, 1% Ultroser-G, 5 ng/ml basic fibroblast growth factor, 10 ng/ml endothelin-1, 0.33 nM cholera toxin, 5.3 nM D12-*O*-tetradecanoylphorbol-13-acetate and 0.033 mM 3-isobutyl-1-methyl-xanthine until subconfluency (80%).

Antibodies and reagents

Rabbit polyclonal antibodies NF1(D) (sc-67) (1/500) directed against the C-terminus of neurofibromin and NF1(N) (sc-68) (1/500) directed against the N-terminus were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody NKI-beteb aimed against the (pre)melanosomal silver protein was obtained from Monosan (Uden, The Netherlands). Rabbit polyclonal antiserum CT15 directed against the C-terminus of APP (1/500) was kindly provided by Dr E. Koo (UCSD, San Diego, CA). Goat polyclonal amyloid antibody (sc-5399) (Santa Cruz Biotechnology, Santa Cruz, CA) was directed against the C-terminus and recommended for detection of β-amyloid and amyloid A4. Secondary rabbit anti-mouse and rabbit anti-goat immunoglobulins used for immuno-electronmicroscopy were obtained from Dako (DakoCytomation, Heverlee, Belgium). Horse radish peroxidase-conjugated anti-rabbit IgG antibody (1/4,000) and HRP-conjugated anti-mouse IgG antibody (1/3,000) were from Amersham Biosciences (Orsay, France).

RT-PCR

Using the RNeasy method (Qiagen, Leusden, The Netherlands) total RNA was extracted from normal human melanocytes. cDNA was prepared from total RNA using random primers (Invitrogen Ltd, Paisley, UK) and the Superscript II RT enzyme (Invitrogen Ltd, Paisley, UK). PCR amplification reaction was performed using normal human melanocyte cDNA, the Extaq enzyme (Takara Shuzo Co, Otsy, Shiga, Japan) and primers APP-F 5′-AAGCCACAGAGA GAACCACCAGCATT-3′ and APP-R 5′-GCTTGACGTTCTGCCTCTT CCCATT-3′ (Quast *et al.*, 2003) for APP and NF1-F 5′-ACGAGTGTC TCATGGGCAGAT-3′ and NF1-R 5′-ACTGTTGTAAGTGTCAGGTC CTTTTAAG-3′ for NF1. The thermal cycling conditions for APP were 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, and conditions for NF1 were 35 cycles at 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 15 seconds. PCR products were size-separated on a 2% agarose gel and visualized by ethidium bromide staining.

Yeast two-hybrid analysis

Based on the full-length cDNA sequence of neurofibromin (GenBank accession number: NM_000267), the region of interest was PCR amplified using normal human melanocyte cDNA prepared from total RNA. This region is referred to as ''bait'' (major part of the GRD: bp 4,071–4,671). Primers used for amplification were baitF 5′-ATC AGTTCCTCCTCAGAATTC-3′ and baitR 5′-TACCTGATGCCTAGTCA TAAA-3′. The bait plasmid was constructed by directional insertion of PCR product in the cloning site (*Eco*RI/*Sma*I) of the pBD-Gal 4 vector (Stratagene, La Jolla, CA).

As an additional positive control syndecan-2 (Genbank accession number: XM_040582) was used (Hsueh *et al.*, 2001). This plasmid was constructed by *Bam*HI-*Eco*RI directional cloning into the pAD-Gal4-2.1 vector (Stratagene, La Jolla, CA) using the following primers: SynF 5′ GCGGAGTCGAGAGCAGAG-3′ and SynR 5′ TTAC GCATAAAACTCCTTAGTAG-3′. The inserts were sequence verified by Baseclear (Leiden, The Netherlands). The human brain cDNA library (''prey'') was inserted in the pAD-Gal4 vector. All constructs were tested for auto-activation. Internal positive and negative controls from Stratagene, being p53–pSV40 and pLamin–pSV40, respectively, were used. Briefly, the NF1-GRD-pBD-Gal4 construct and the commercial brain library construct (Clontech, BD Biosciences, Palo Alto, CA) were sequentially transformed in the PJ-69-4A yeast strain. Potential interactions were scored on selective SD/-Leu-Trp-Ade, SD/-Leu-Trp-His growth medium and the β-galactosidase filter assay. Only in cases where all three assays were positive, yeast DNA was prepared for further analysis. After transformation of the yeast DNA in supercompetent *E. coli*, the interacting proteins were sequence verified by Baseclear (Leiden, The Netherlands).

Immunoprecipitation

Normal human melanocytes were grown until subconfluency (80%) in D12-*O*-tetradecanoylphorbol-13-acetate supplemented Ham F10 medium (Gibco, Invitrogen Ltd, Paisley, UK). After washing the cells with dextrose supplemented phosphate-buffered saline, the cells were lysed for 30 minutes at 4°C with a mild lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.7% NP40, pH 7.4), containing protease inhibitors (10 μl/ml phenylmethylsulfonyl fluoride, 10 μl/ml Leupeptin, 10 μl/ml Aprotinin) and phosphatase inhibitors (20 μl/ml $NaVO_3$, 50 μl/ml $Na_4P_2O_7$, 10 μl/ml NaF 100 ×). Insoluble cell material was pelleted by centrifugation at 14,000 r.p.m. at 4°C for 10 minutes. The supernatant was collected and protein concentration was determined with the DC Protein Assay (BioRad, Hercules, CA). The supernatant was precleared with 25 μl protein A sepharose beads (Amersham Biosciences, Orsay, France) under shaking for 30 minutes at 4°C. After centrifugation, immunoprecipitation was performed by incubating precleared supernatant with either NF1(D) (sc-67) or APP antiserum CT15 under shaking for minimum 3 hours at 4°C, followed by the addition of 50 μl protein A sepharose beads and incubation for 1 hour at 4°C under shaking. Unbound proteins were removed by extensive washing. Immunoprecipitated proteins were extracted in 38 μl 1.5 × Laemmli Sample Buffer and boiled for 5 minutes. The immunoprecipitates were analyzed by Western blotting.

Western blotting

Immunoprecipitates or total cell lysate were solubilized and denatured by boiling in 1.5 × or 4 × Laemmli sample buffer respectively, containing 5% β-mercapto-ethanol and 0.25% bromophenol blue. Proteins were separated by SDS-PAGE (6% gel) and electro-blotted on to polyvinylidine diflouride membranes (Amersham Biosciences, Orsay, France) or silver stained using the Silver Stain Plus kit (Biorad, Hercules, CA). Membranes were blocked for 1 hour at room temperature in 5% non-fat dry milk in Tris buffered saline with 0.1% Tween (137 mM NaCl, 20 mM Tris, pH 7.6 with 0.1% Tween-20), followed by incubation with the appropriate primary antibody. Following several washing steps with Tris buffered saline with 0.1% Tween, membranes were incubated with the appropriate secondary antibody for 1 hour at room temperature. After final washing steps with Tris buffered saline with 0.1% Tween, bound antibodies were detected using the enhanced chemiluminescence detection system ECL + Plus (Amersham Biosciences, Orsay, France) according to the manufacturer's protocol.

Indirect immunofluorescence and confocal microscopy

Melanocytes were grown on coverslips and fixed for 20 minutes at room temperature with 3% paraformaldehyde in phosphate-buffered saline. After three washes in tris-buffered saline, cells were submerged for 10 minutes with 50 mM NH_4Cl in phosphate-buffered saline[E] (Eisen formulation) followed by three washes in tris-buffered saline. For permeabilization, cells were treated for 5 minutes with 0.2% Triton X-100 in phosphate-buffered saline. Double staining for neurofibromin and melanosomes was performed by first incubating for 2 hours at room temperature in a 1/40 NKI-beteb and a 1/50 NF1(D) dilution. After three washes in tris-buffered saline, the coverslips were incubated for 1 hour with an FITC-labeled rabbit anti-mouse (1/20) and a biotinylated donkey anti-rabbit (1/50) secondary antibody, respectively. Double staining for APP and melanosomes was performed by first incubating for 2 hours at room temperature in a 1/40 NKI-beteb and a 1/100 APP (CT15) dilution. After three washes in tris-buffered saline, the coverslips were incubated for 1 hour at room temperature in a biotinylated donkey anti-rabbit (1/50) and a FITC-labeled rabbit anti-mouse (1/20) antibody dilution. Slides were coverslipped in Prosan fluorescence mounting fluid and confocal image (1 μm) sections were obtained with a BioRad Confocal Laser Scanning Microscope (Radiance 2100 blue laser diode).

Immunogold transmission electron microscopy

Melanocytes were fixed for 24 hours at room temperature in 2% paraformaldehyde in 0.1 M PHEM buffer (60 mM Pipes/NaOH, 25 mM Hepes, 10 mM EGTA and 2 mM $MgSO_4$, pH 6.9–7.0) and processed for immunogold labeling as described elsewhere (Mommaas *et al.*, 1992a, b). Briefly, cells were pelleted and embedded in 12% gelatin, cut into 1 mm³ cubes, cryoprotected in 2.3 M sucrose and snapfrozen in liquid nitrogen. For localization of neurofibromin and APP, ultrathin cryosections were incubated with the anti-neurofibromin antibody NF1(D) diluted 1/500 or anti-goat β-amyloid/amyloid A4 (1/500) antibody (with intermediate rabbit-anti goat step) or anti-APP (1/500) antibody (CT15), followed by 15 nm protein A-gold incubation (1/200). For colocalization with melanosomes a double labeling with the NKI-beteb antibody (dilution 1/300), after an intermediate incubation step with a secondary rabbit anti-mouse bridging antibody (1/200) and the NF1(D) (1/500) antibody was performed. For double labeling with NKI-beteb and anti-goat β-amyloid antibody, both an intermediate step with a secondary rabbit anti-mouse (1/200) and rabbit anti-goat (1/200) antibody, respectively, were necessary. All antibodies were incubated with 10 or 15 nm Protein A gold particles (1/200) for visualization. As negative controls the primary antibodies were omitted, solely including the secondary antibodies and 10 and 15 nm Protein A gold particles. After immunolabeling, sections were embedded and contrasted in methylcellulose/uranyl acetate and viewed with a Philips EM 410 electron microscope (Eindhoven, The Netherlands).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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