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Neurofibromin interacts with the cytoplasmic Dynein Heavy Chain 1 in melanosomes of human melanocytes

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ABSTRACT

Neurofibromin (NF1) is encoded by the *NF1* tumour suppressor gene. Mutations result in a disorder known as Neurofibromatosis Type 1 (NF-1), and patients are often diagnosed due to the presence of unusual pigmentary patterns that include Café au lait macules (CALMs). Little is known about how loss of NF1 results in pigmentary defects in melanocytes. We sought to identify novel NF1 interacting proteins and elucidate the molecular mechanisms underlying the pigmentary defects. The cytoplasmic Dynein Heavy Chain 1 (DHC) was found to interact with NF1 along microtubules in vesicular structures identified to be melanosomes. Our studies suggest that NF1 is involved in melanosomal localization, and that disruptions in NF1–DHC interactions may contribute to the abnormal pigmentary features commonly associated with this debilitating syndrome.

Structured summary of protein interactions:
NF1 physically interacts with **DHC** by anti bait coimmunoprecipitation (View interaction)

1. Introduction

The Neurofibromatosis Type 1 (NF-1) tumor predisposing syndrome has a birth incidence of 1:3000 and is the most common autosomal disorder of the nervous system in humans [1]. This syndrome is characterized by disorders that primarily affect cells of neural crest origin, with a number of pleiotropic manifestations that include learning disabilities, spinal abnormalities, Café au lait macules (CALMs), Lisch nodules, optic gliomas, benign tumours of the peripheral nervous system (neurofibromas) and malignant peripheral nerve sheath tumours (MPNSTs) [1]. This multisystem disorder occurs due to the deletions and loss of function mutations in the Neurofibromin tumour suppressor gene (NF1).The most extensively studied function of NF1 pertains to its Ras GTPase Activating Protein (RasGAP) function, mediated by the GAP Related Domain (GRD). The GRD accelerates the intrinsic activity of Ras-GTPase, resulting in the conversion of Ras-GTP to the inactive Ras-GDP state [2]. Loss of NF1 subsequently results in an accumulation of Ras-GTP that drives cell survival, proliferation and neoplastic transformation.

However, many of the manifestations of NF-1 cannot be attributed to its function through the GRD, and studies elucidating the role of other domains and their novel interacting proteins have enhanced our understanding of the pleiotropic effects of NF-1. The Tubulin Binding Domain (TBD) interacts with microtubules (MT), and this association has been demonstrated to impact the RasGAP activity of NF1 [3]. The Syndecan1 and Syndecan2 domains are proposed to function in the localization of NF1 to syndecan containing microdomains in the plasma membrane [4]. The C-terminal Domain interacts with 14-3-3η in a PKA phosphorylation dependent manner and inhibits NF1 GAP activity [5]. Kinesin-1 has been identified through size-exclusion chromatography experiments and mass spectrometry as a binding partner of a NF1 containing complex [6]. NF1 has also been reported to interact with the Amyloid Precursor Protein (APP) with a proposed functional relevance in the intracellular transport of melanosomes in melanocytes, which could be a potential pathological mechanism for CALM formation [7]. As we identify additional interacting partners and delineate their regulatory interplay with NF1, we will gain insight into novel functions and signaling mechanisms which may help us understand the non-neoplastic phenotypes associated with NF-1.

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To identify novel melanocyte-specific NF1 interacting proteins, we performed NF1 immunoprecipitations (IP) from melanocytes coupled with Mass Spectroscopy (MS). The cytoplasmic Dynein Heavy Chain1 (DHC) was found to interact with NF1, and to be involved in the correct localization of melanosomes along microtubules in melanocytes. This finding may provide valuable insight into the etiopathogenesis of CALMs, which are one of the most frequent manifestations of NF-1 [8].

2. Materials and methods

2.1. Cell culture, transfection and drug treatments

Normal human melanocytes (foreskin-derived) were grown in DMEM supplemented with 10% FBS and incubated at 37 °C with 5% CO_2. For transfections, Lipofectamine X (Invitrogen) was used in accordance with manufacturer's instructions. 0.5 μg of NF1 and scrambled shRNAs were used per 1.5×10^5 cells and maintained for 72 h prior to functional studies. For drug studies, cells were serum starved for 18 h and first treated with 2 μM MSH for 1 h, washed and then treated with 1 μM Melatonin for 30 min, and vice versa.

2.2. Expression and purification of GST and His fusion proteins

BL21(DE3) *Escherichia coli* cells were transformed with the NF1 GST constructs, grown overnight at 37 °C and induced with 0.5 mM IPTG for 3 h. The fusion proteins were purified by affinity chromatography with Glutathione Sepharose 4B (Amersham Biosciences) in accordance with the manufacturer's protocol.

2.3. Mass spectrometry (MS)

Protein-containing bands were in-gel digested with modified trypsin (Promega) to extract peptides from the gel. Trypsin was also used for gel-free digestion of IP protein products. Samples were subjected to LC/MS analysis at the Advanced Protein Technology Centre (Toronto, Canada).

2.4. Immunoprecipitation immunoblotting and immunofluorescence analyses

Antibodies used were: NF1 (Rabbit polyclonal (sc-67), Santa Cruz Biotechnology) and NF1 (Mouse monoclonal (McNFn27b), Santa Cruz Biotechnology). α-tubulin (Rabbit monoclonal (11H10), Cell Signaling Technology), kinesin-1 (Rabbit monoclonal (K1014), Sigma), Dynein Heavy Chain (Mouse monoclonal, Sigma) and Normal rabbit IgG (sc-2027, Santa Cruz Biotechnology). The IPs and IFs were performed as described [9].

2.5. Proximity ligation assay

The assay was performed as described in Soderberg et al. [10]. Briefly, fixed (4% PFA) and permeabilized cells were incubated with primary NF1 and DHC, or NF1 and kinesin-1 antibodies overnight at 4 °C, washed, and further incubated with secondary antibodies with attached DNA strands as proximity probes for 1 h in a humidified 37 °C chamber. After hybridization and ligation of the DNA oligonucleotides, amplification solution along with polymerase was added which resulted in a rolling-circle-amplification (RCA) reaction. The concatemeric product was detected using complimentary fluorescently labeled oligonucleotides. Imaging was performed with a Zeiss Axiovert 200 microscope equipped with a Hamamatsu Orca AG CCD camera and a spinning disk confocal scan head. The brightness of images was increased for presentation. Details of statistical and image analysis have been described previously [9].

3. Results

3.1. Identification of DHC as a novel NF1–TBD interacting protein in human melanocytes

An immunoprecipitation–Mass spectroscopy (MS) screen using NF1 antibody incubated with normal human melanocyte lysate identified DHC, a component of the Dynein motor protein complex. This interaction was validated by NF1 immunoprecipitation followed by western blot analysis (WB) with DHC antibody. Immunoprecipitation with DHC antibody followed by western blotting with NF1 antibody confirmed that the two proteins formed complexes in vitro, while there was no non-specific interaction with the IgG negative control (Fig. 1A). To identify the domain of NF1 that mediates this interaction, we constructed GST-tagged NF1 domains (Cysteine/Serine Rich Domain – CSRD, Tubulin Binding Domain – TBD, Sec14p homology domain – Sec14 and Carboxy-Terminal Domain – CTD (Fig. 1B), and incubated these proteins with lysate from normal human melanocytes. The bound proteins were eluted and subjected to western blot analysis using DHC antibody. Only the NF1–TBD interacted with DHC, suggesting that this domain mediates the interaction (Fig. 1C).

The Dynein motor protein complex is involved in microtubule-dependent, retrograde transport in a number of cellular processes including mitosis, and transport of vesicles, melanosomes and mitochondria [11–13]. While the finding that DHC is a potential interacting protein is novel, kinesin-1, an evolutionarily unrelated anterograde microtubule-associated motor protein, has been reported to interact with a NF1 containing complex [6], which emphasizes a possible role of NF1 as part of a complex involved in the transport of cargo along MTs.

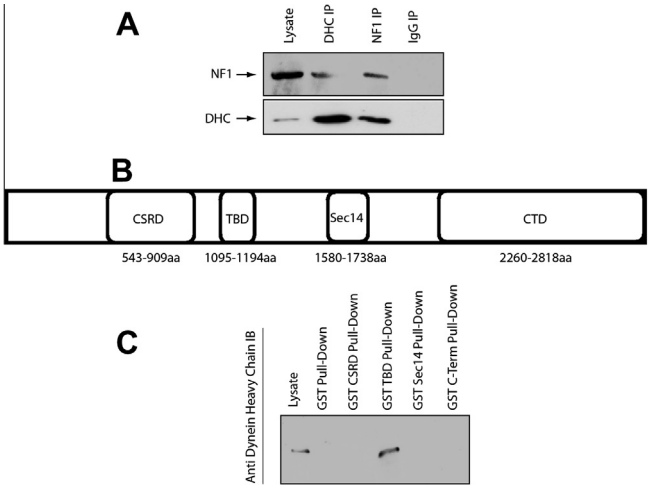

Fig. 1. DHC interacts with the Tubulin Binding Domain of NF1. (A) DHC forms a complex with NF1. Endogenous DHC from normal human melanocyte cell lysate was immunoprecipitated (IP), subjected to SDS PAGE and probed with anti-NF1 (lane 2). Immunoprecipitation with anti-NF1 and probe with anti-DHC (lane 3). No non-specific bands were observed when lysates were immunoprecipitated with IgG alone (lane 4). (B) Schema of NF1 domains used in the GST pull-down analysis. The CSRD: Cysteine/Serine Rich Domain, TBD: Tubulin Binding Domain, Sec14: Sec14p Homology Domain and CTD: C-terminal Domain of NF1 were cloned into GST-tagged vectors. (C) DHC interacts with only the TBD and not other NF1 domains. GST pull-down of CSRD, TBD, Sec14 and CTD after incubation with whole cell lysate, followed by western blot analysis with anti-DHC.

3.2. NF1 interacts with DHC along microtubules in normal human melanocytes

To confirm that NF1 colocalizes with DHC in situ, we performed immunoflourescence and confocal microscopy studies with antibodies to NF1 and DHC. NF1 and DHC colocalized significantly throughout the cells with high intensity staining in the perinuclear area and with punctate distribution in the tips of melanocytes (Fig. 2A). A similar distribution and overlap was found for NF1 and kinesin-1 (Supplementary Fig. 1A).

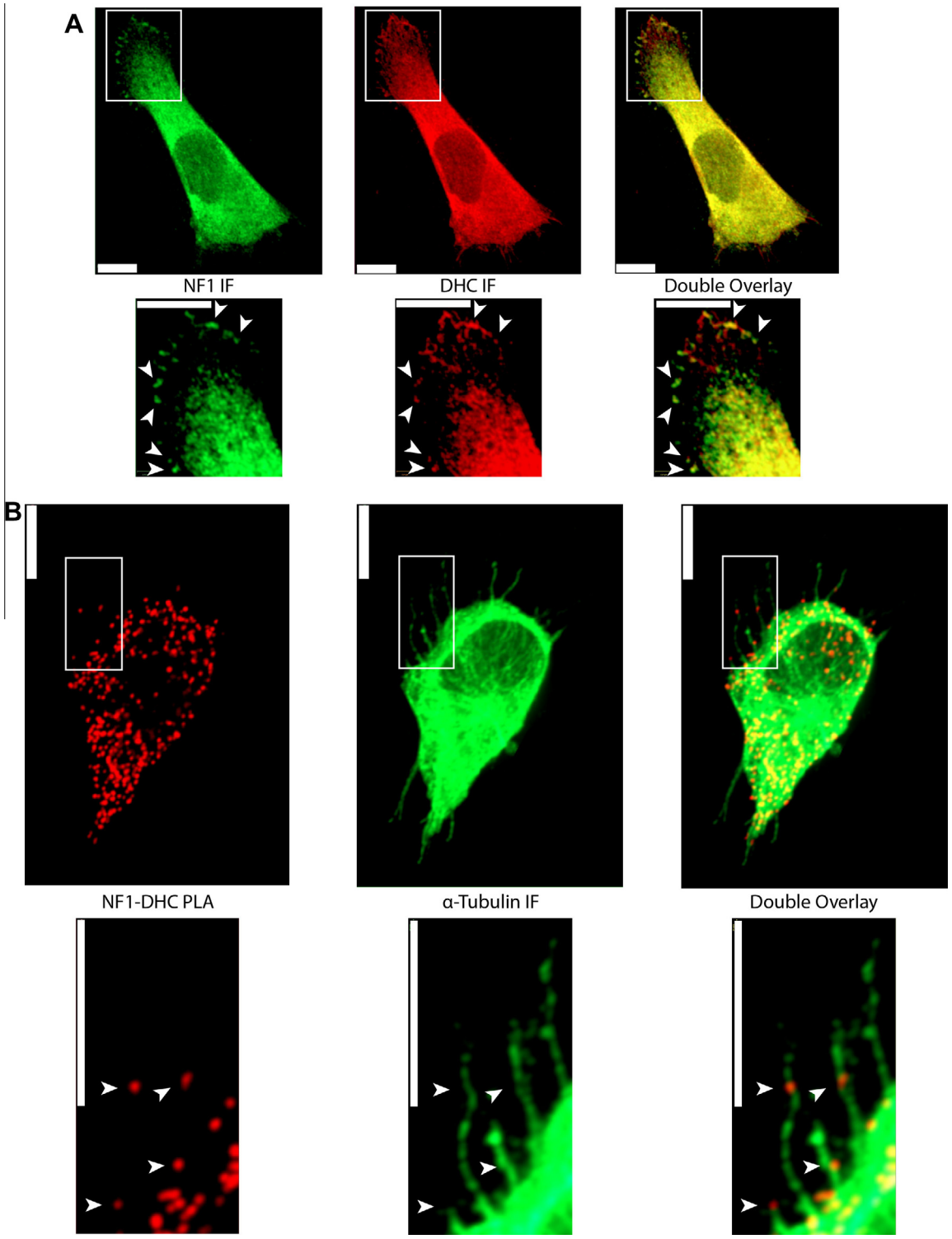

Fig. 2. NF1 interacts with DHC along microtubules in normal human melanocytes. (A) Confocal fluorescence images showing intracellular immunostaining with anti-NF1 and anti-DHC of endogenous NF1 and DHC in normal human melanocytes in the perinuclear region and cell periphery. Arrowheads indicate vesicular co-immunostaining at distal tips. Mander's correlation coefficient (R) for NF1-DHC double immunoflourescence = 0.95 ± 0.02, $n = 20$. (B) PLA for NF1 and DHC coupled with immunofluorescence for α-tubulin reveals that the NF1–DHC interaction sites, as indicated by the red PLA signals, occurs predominantly along MTs as indicated by arrowheads in the magnified images. $R = 0.74 \pm 0.02$, $n = 20$.

Since DHC transports cargo along MTs, and NF1 is also known to colocalize with tubulin, we performed the Proximity Ligation Assay (PLA) for NF1 and DHC to evaluate whether these two proteins interact in situ. The NF1–DHC PLA reaction was coupled with an IF for α-tubulin to ascertain whether NF1 and DHC interact along MTs as this would provide further insight into the functional relevance of this interaction. As shown in Fig. 2B, NF1 and DHC interact along MTs as represented by the red PLA signals. To further query whether the NF1–kinesin-1 interaction also occurs in melanocytes along MTs, we repeated the PLA to visualize sites of NF1–kinesin-1 interaction, and coupled this with an IF for α-tubulin (Supplementary Fig. 1B). As previously reported [6], NF1 and kinesin-1 interact along MTs. The interaction of NF1 with the two MT-dependent motor proteins supports our hypothesis of a role of NF1 in intracellular trafficking of cargo.

3.3. NF1 interacts with DHC in melanosomes

To identify the structures that NF1 and DHC colocalize with, we performed immunofluorescence with a monoclonal antibody against NK1/beteb, a melanosome marker and antibody to NF1. NF1 immunofluorescence colocalized with the melanosome mar-

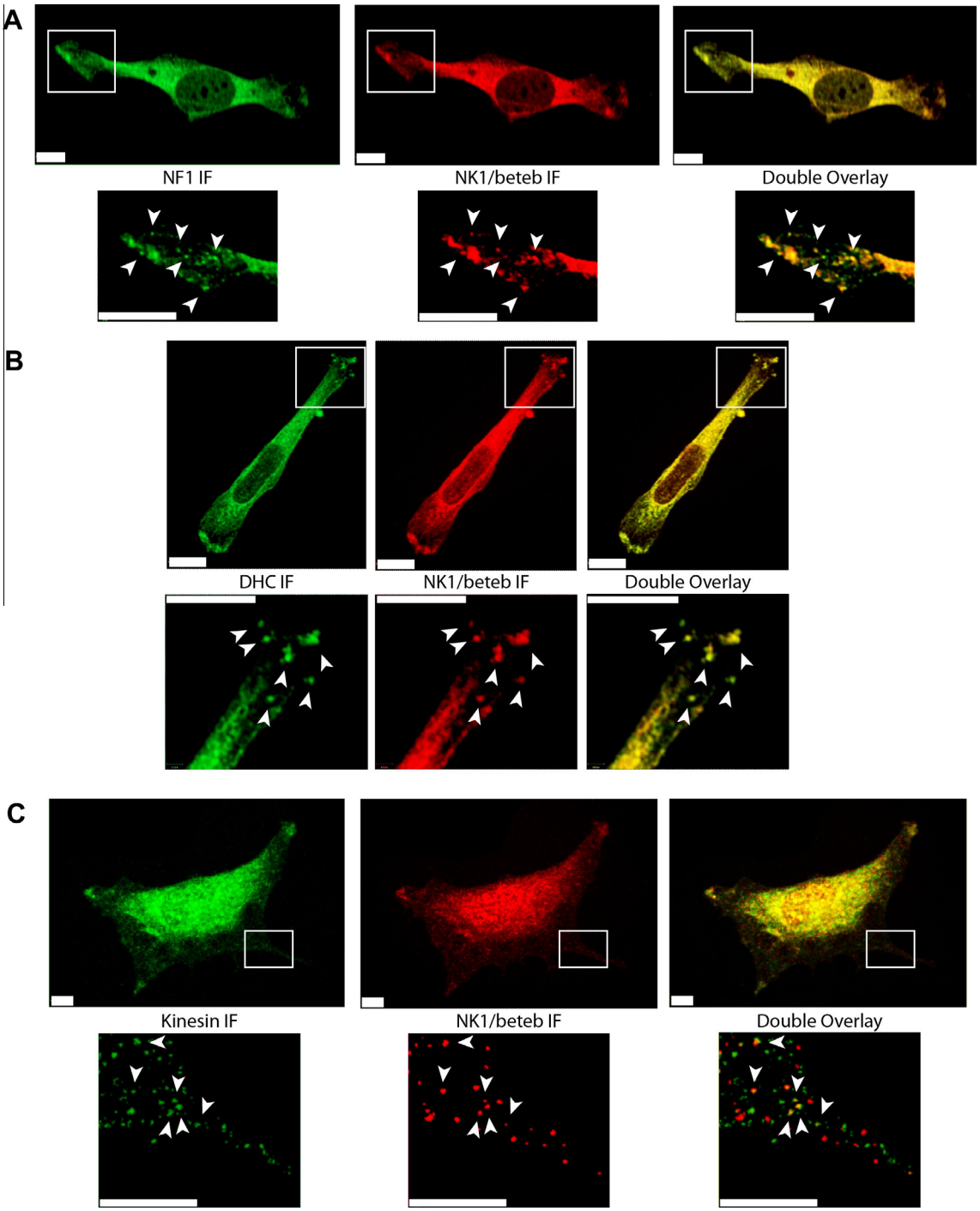

Fig. 3. NF1, DHC and kinesin-1 colocalize with melanosomes. (A) NF1 immunoflourescence coupled with IF for NK1/beteb reveals the NF1-stained vesicular structures to be melanosome. $R = 0.92 \pm 0.04$, $n = 20$. (B) A double IF for DHC and NK1/beteb also demonstrates that this retrograde motor protein colocalizes with melanosomes. (C) Kinesin-1 also significantly overlaps with melanosomes. $R = 0.94 \pm 0.04$, $n = 20$.

ker in the perinuclear area and more distinctly in the distal tips, with both antibodies showing a punctate, granular pattern (Fig. 3A). Since DHC functions in retrograde cargo transport, we co-stained melanocytes with DHC and NK1/beteb, and observed overlapping signals (Fig. 3B). A similar overlap was also observed using antibodies to kinesin-1 and NK1/beteb, confirming that the vesicular structures were melanosomes (Fig. 3C) [14].

Having demonstrated the colocalization of NF1, kinesin-1 and DHC in melanosomes, we further performed the PLA to confirm whether NF1 specifically interacts with the two motor proteins in melanosomes. A combination of NF1–DHC PLA along with NK1/beteb immunoflouresence revealed significant overlap of the two signals in the perinuclear area and more discretely in the distal tips, confirming that melanosomes are the structures wherein NF1 interacts with DHC (Fig. 4A). A lower number of NF1–kinesin-1 PLA signals colocalized with melanosomes, but nonetheless confirmed that NF1 and kinesin-1 also interact in this organelle (Fig. 4B).

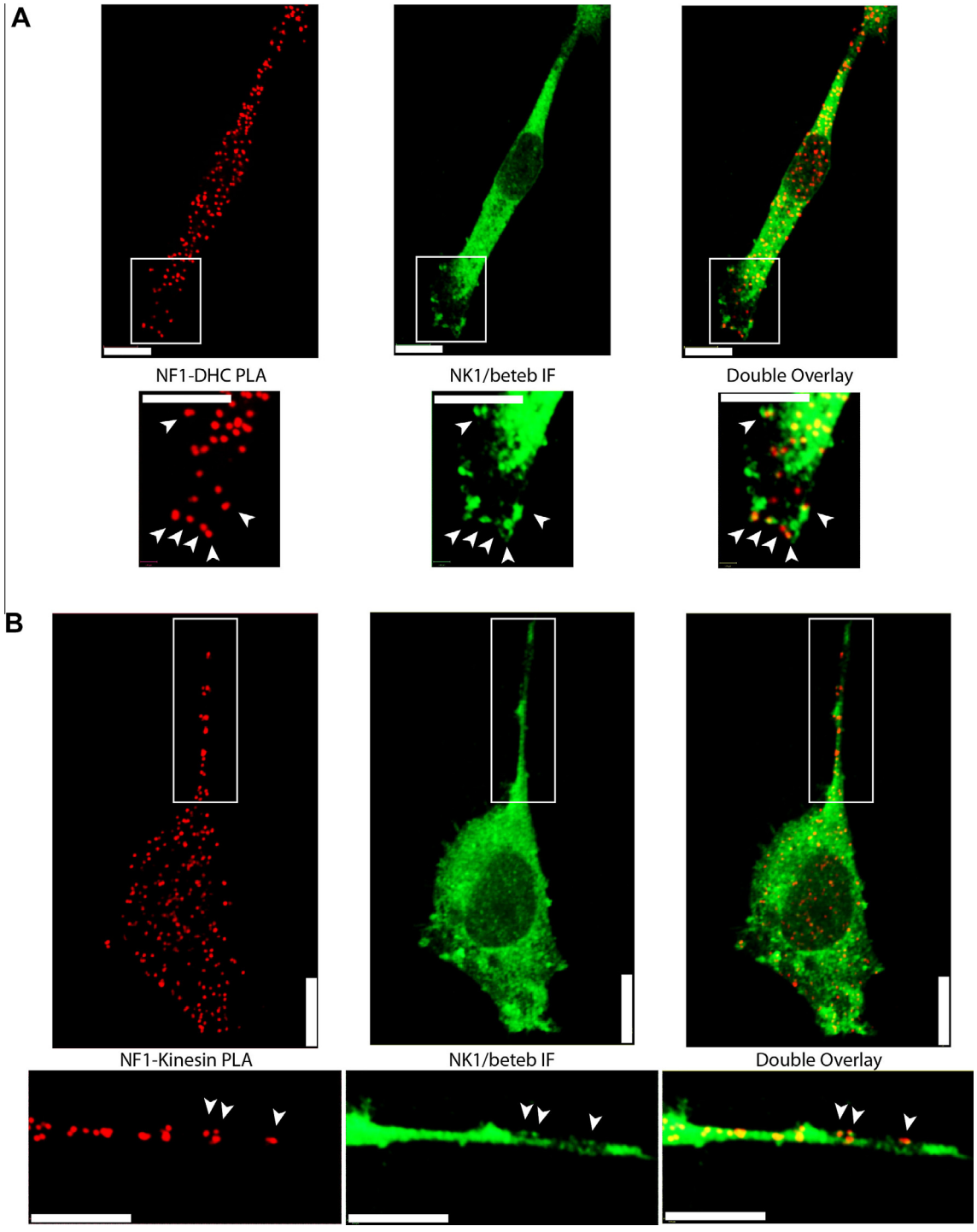

Fig. 4. NF1 interacts with DHC and kinesin-1 in melanosomes. (A) PLA for NF1 and DHC coupled with immunofluorescence with anti-NK1/beteb demonstrates that these two proteins interact in melanosomes. $R = 0.81 \pm 0.05$, $n = 20$. (B) NF1 and kinesin-1 also interact in melanosomes as visualized by the presence of the PLA signals in NK1/beteb-positive vesicular structures. $R = 0.78 \pm 0.04$, $n = 20$. All scale bars correspond to 10 μm.

3.4. NF1 plays a functional role in melanosome localization

NF1 has been suggested to play a role in melanosome distribution [7]. We therefore asked about the functional importance of the NF1-DHC interaction in human melanocytes in the context of melanosome localization. Since NF1 has been reported to be present on the melanosomal membrane [7,15], our findings suggest NF1 may be an adaptor between melanosomes and motor proteins, and that loss of NF1 may result in aberrant formation of the melanosome transport complex. To investigate the functional relevance of the loss of NF1 on melanosome localization we used an NF1-specific shRNA approach to knockdown (KD) NF1 in normal human melanocytes. Scrambled shRNA was used as a negative control (Supplementary Fig. 2). The shRNA constructs also encode Red Fluorescent Protein (RFP) that allowed for visualization of shRNA-transfected cells. NF1 KD and control cells were treated with melatonin which induces perinuclear aggregation of melanosomes, and with Melanocyte Stimulating Hormone (MSH) which induces anterograde

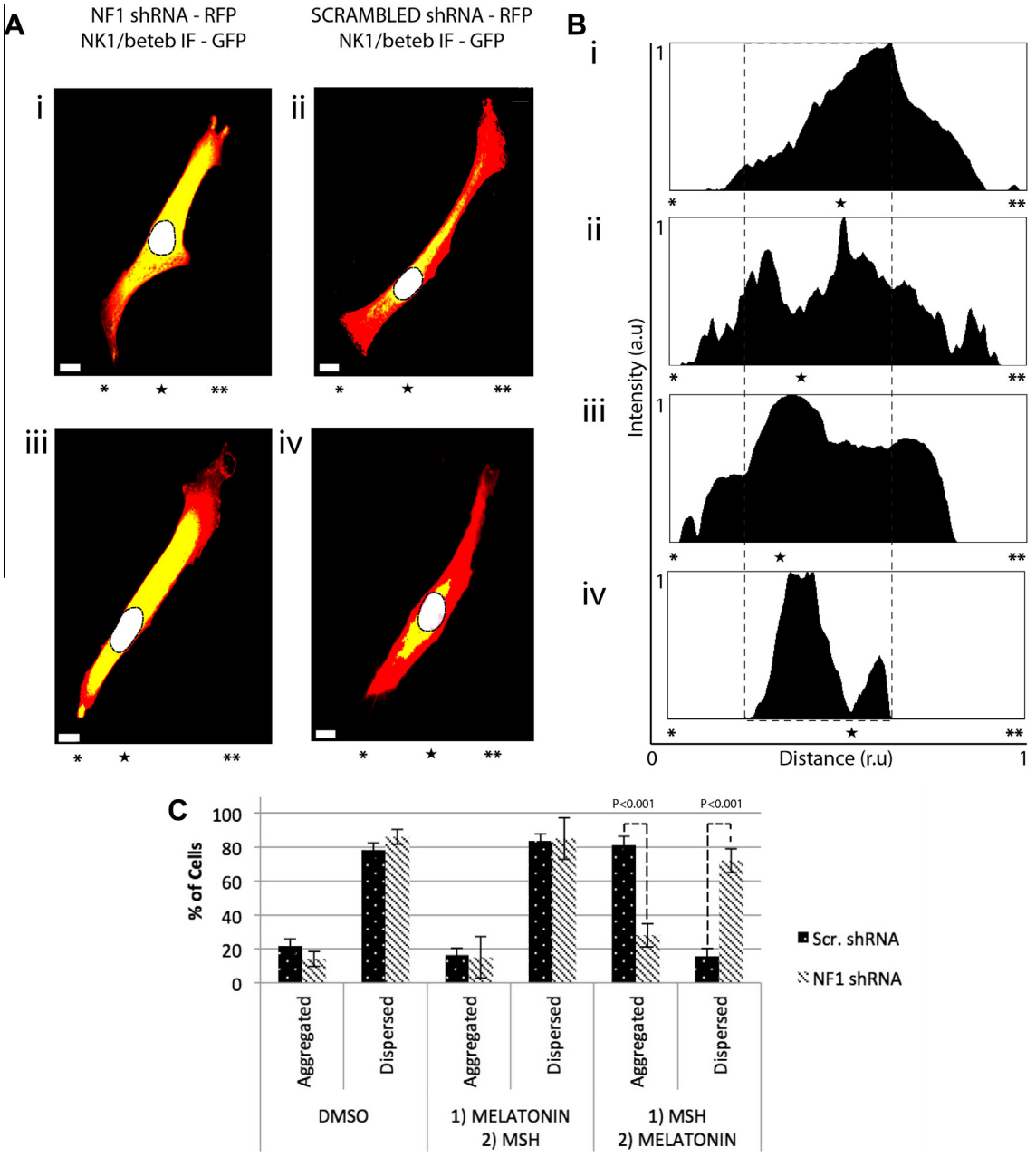

Fig. 5. Loss of NF1 results in altered melanosome localization. (A) Cells were transfected with NF1 shRNA or scrambled shRNA, stimulated with 1 μM melatonin for 30 min followed by 2 μM MSH for 1 h (i and ii), or incubated with 2 μM MSH for 1 h followed by 1 μM melatonin for 30 min (iii and iv). The cells were then fixed and stained with NK1/beteb to visualize melanosome distribution. The green signal of melanosomes overlays with the RFP to produce a strong yellow signal indicative of the localization of melanosomes within individual melanocytes.The nucleus is pseudo-coloured white. '*' indicates the left-most tip of the cell, '**' indicates the right-most extremity and '★' indicates the nucleus of the cell. (B)The Intensity Plot Profile (IPP) quantifies the vertically averaged (*y*-axis column average plot) pixel intensity along the horizontal distance of the image (*x*-axis). The IPP of (i) and(ii) reflects melanosome distribution in the perinuclear area and the cell periphery. NF1 loss did not affect the kinesin-1-dependent, distal localization of melanosomes. In (iii) and (iv), the final treatment is with melatonin which normally results in perinuclear aggregation as seen in (iv), but in NF1 KD cells (iii), melanosomes remain dispersed. (C) Quantification of the percentage of cells with perinuclear aggregation vs. dispersed distribution of melanosomes from the two different drug treatment strategies in (A and B). Error bars represent the S.D. Three biological replicates were performed of which the average is plotted in the graph. The paired two-tailed *t* test significance value for NF1 shRNA compared to Scrambled shRNAis 0.01. Scale bars correspond to 10 μm.

movement of melanosomes away from the perinuclear area to the distal tips [16]. NF1 shRNA or scrambled shRNA-transfected cells were first treated with 1 μM Melatonin for 30 min, and then with 2 μM MSH for 1 h. This treatment resulted in an expected melanosome distribution in the perinuclear area and also in the cell periphery (Fig. 5A, i and ii). However, a significant difference was noted in melanosome distribution in the NF1 KD cells when they were first treated with MSH and then treated with melatonin (Fig. 5A, iii and iv). While scrambled shRNA-transfected cells exhibited an aggregation of melanosomes in the perinuclear region and not towards the distal tips (Fig. 5B, iv), NF1 shRNA-transfected melanocytes showed high central melanosomal concentration while retaining distal distribution (Fig. 5B iii). This difference was significant compared to scrambled shRNA-transfected controls ($P = 0.010$) (Fig. 5C).Our findings suggest that melanosomes in NF1−/− cells are unable to translocate away from the cell periphery even upon high concentrations of Melatonin treatment, possibly due to their inability to associate with the dynein motor protein complex in the absence of NF1.

4. Discussion

There are reports of distinct aberrations in melanosome size and distribution in melanocytes derived from NF-1 patients, but the underlying pathophysiological mechanisms are as yet unclear. One study has reported that melanocytes from NF-1 patients contain enlarged melanin granules called macromelanosomes [17] which are present in both CALM and non-CALM derived melanocytes [18,19]. Kaufmann et al. [20] have also shown by means of in vitro studies that NF1 defects affect melanogenesis in NF-1-derived epidermal melanocytes. While the presence of these melanin macroglobules [21] explain the observation that the skin of NF-1 patients is more pigmented than that of their unaffected siblings [22], it does not explain the hyperpigmentation present only in CALM melanocytes.

Owing to the important role of NF1 in the regulation of Ras signaling, a key pathway in cells that mediates mitogenic signaling, there has been speculation that aberrant Ras activation in melanocytes may underlie the increased melanogenesis and melanosome density observed in CALM-derived melanocytes. It was found, however, that the Ras–GTP levels in NF-1 melanocytes were similar to those of normal skin from healthy donors, excluding aberrant Ras signaling as a causative factor [23]. Since formation of these hyperpigmented, localized skin lesions must be attributed to regulatory mechanisms of NF1 unrelated to Ras, we asked whether we could identify novel NF1–protein interactions in melanocytes as it may relate to pigmentation.

For the first time we demonstrate an interaction between NF1 and DHC in melanocytes. Using a combination of techniques such as PLA combined with immunofluorescence, we showed that this interaction occurred along MTs and colocalized with melanosomes. Pigmentation is dependent on melanosome relocalization from the area of synthesis in the perinucleus to the distal extremities, and melanosomes are trafficked by motor proteins along MTs in a bidirectional manner. The anterograde movement of melanosomes has been shown to be driven by members of the kinesin motor protein family [14,24], while retrograde movement is carried out by the Dynein motor protein complex. Dynein is essential in regulating centripetal movement of melanosomes by countering the activity of kinesin-1, thus preventing an aberrant accumulation of melanosomes in the distal extremities of the melanocytes [25]. Drawing upon this important function of dynein, we hypothesize that loss of NF1, and subsequently loss of the NF1–DHC interaction, results in aberrant retrograde trafficking of melanosomes resulting in mislocalization to the distal tips.

Functional studies revealed that suppression of NF1 levels in melanocytes results in increased intensity of NK1/beteb immunostaining, particularly in the nuclear region of the cell, possibly owing to aberrant melanogenesis and macromelanosome formation. This was coupled with a dispersed distribution of melanosomes even upon treatment with Melatonin, which aggregated melanosomes towards the perinuclear area in untransfected and scrambled shRNA-transfected cells. Skin pigmentation is dictated by the density of melanosomes present in melanocytic dendrites which are then transferred to neighbouring keratinocytes for distribution to the upper layers of the epidermis [17,26]. This is a key mechanism that is thought to underlie complexion colouration in a number of hypopigmentation abnormalities including nevus depigmentosis [27] and Griscelli–Prunieras disease [28] where the melanocytes have irregular or short dendritic formation, and reduced melanosomes in keratinocytes. It is also suggested that the hypopigmented lesions in tuberous sclerosis occur due to disrupted melanosome transfer to keratinocytes [29].

Regarding the hyperpigmentation end of the spectrum, Nevus spilus is a skin lesion that resembles CALMs and electron microscopic observations by Takahasi [19] revealed a higher number of melanosomes in keratinocytes possibly due to increased transfer from melanocytes and/or decreased degradation in keratinocytes. Similar studies were conducted in NF-1 CALMs with the supposition being that melanocytes transfer a higher number of melanosomes into keratinocytes [19]. Findings from our studies add to the proposed model by suggesting thatNF1 loss results in increased aberrant localization of melanosomes to the distal tips of melanocytes, thus increasing their availability for exocytosis into kertinocytes, where they accumulate to cause increased pigmentation of the skin.

We further speculate this novel function of NF1 in DHC-mediated trafficking of melanosomes to be haplo-sufficient for NF1 as the hyperpigmentation defects are observed only in CALMs and not in non-lesional melanocytes. While previous findings by De Schepper et al. [30] led to the conclusion that CALM melanocytes possess the second NF1 allele in all NF-1 patients in their study, an important caveat was that somatic intragenic mutations were not queried. In light of the findings by Eisenbarth et al. [31] who demonstrated that CALMs do indeed have a higher frequency of second hit mutations for NF1 in melanocytes compared to keratinocytes ($P = 0.018$) or fibroblasts ($P = 0.000$), we are able to conclude that CALM melanocytes are indeed NF1−/−, and hence manifest the pigmentary aberrancies while the NF1+/− non-CALM cells do not.

We further speculate that loss of function of NF1 in microtubule-dependent motor protein-driven localization of cargo may underlie some of the other phenotypic manifestations observed in Neurofibromatosis Type 1. Arun et al. [9]demonstrate that NF1 is also part of RNA granule complexes, which are ribonucleoprotein particles that are transported by motor proteins along microtubules, and that regulate protein synthesis in a temporal and spatial manner. Aberrant localization of mRNAs contained within these granules in neurons and Schwann cells have been shown to underlie the cognitive deficits in the Fragile X mental retardation syndrome and peripheral neuropathy in a mouse model of motor dysfunction, respectively [32,33].

Thus, our demonstration of a novel NF1–DHC interaction reveals a novel function of NF1. Experimental findings indicate a functional role in melanosome localization which sheds insight into a possible molecular mechanism underlying CALM formation. Further exploration of this new avenue will yield fruitful in our understanding of the etiopathogenesis of other manifestations associated with Neurofibromatosis Type 1 including motor deficits and learning disabilities.

Conflict of interest

The authors state no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.03.035.

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