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### ► To cite this version:

Wissem Deraredj Nadim, Shalina Hassanaly, H el ene B enedetti, Claudine Kieda, Catherine Grillon, et al.. The GTPase-activating protein-related domain of neurofibromin interacts with MC1R and regulates pigmentation-mediated signaling in human melanocytes. *Biochemical and Biophysical Research Communications*, 2021, 534, pp.758-764. 10.1016/j.bbrc.2020.11.003 . hal-03007433

**HAL Id: hal-03007433**

**<https://hal.science/hal-03007433>**

Submitted on 16 Nov 2020

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The GTPase-activating Protein-related Domain of neurofibromin interacts with MC1R  
and regulates pigmentation-mediated signaling in human melanocytes

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## **Abstract**

The melanocortin 1 receptor (MC1R) is a G-protein coupled receptor (GPCR) which plays a major role in controlling melanogenesis. A large body of evidence indicates that GPCRs are part of large protein complexes that are critical for their signal transduction properties. Among proteins which may affect MC1R signaling, neurofibromin (Nf1), a GTPase activating protein (GAP) for Ras, is of special interest as it regulates adenylyl cyclase activity and ERK signaling, two pathways involved in MC1R signaling. Moreover, mutations in this gene encoding Nf1 are responsible for neurofibromatosis type I, a disease inducing hyperpigmented flat skin lesions. Using co-immunoprecipitation and Bioluminescence Resonance Energy Transfer experiments we demonstrated a physical interaction of Nf1 with MC1R. In particular, the GAP domain of Nf1 directly and constitutively interacts with MC1R in melanocytes. Pharmacologic and genetic approaches revealed that the GAP activity of Nf1 is important to regulate intracellular signaling pathways involved in melanogenesis and, consequently, melanogenic enzyme expression and melanin production. These findings shed new light on the understanding and curing skin pigmentation disorders.

## **Keywords**

**BRET, MC1R, GPCR, neurofibromin, pigmentation, signaling**

## ***Acknowledgments***

The authors thank Fabienne Fasani and Alexia Perrin for cell culture. They also thank Anthony Guillemain, Marion Bonel and Fabienne Godin for their contribution to plasmids preparation and H  l  ne B  n  detti, Martine Decoville and Elodie Robin for all discussions.

## ***Funding***

This work was supported by Cosmetic Valley Pole of competitiveness, the French Ministry of Industry and the Conseil R  gional du Centre, FUI-AAP16 Glycoskin I., and by ANR (Contact-11-BSV-0008). WD

and SH are PhD students funded by the French Ministry of Research and FUI-AAP16 Glycoskin I Grant, respectively; CK is DR emeritus CNRS; CG and SM-L are CR CNRS.

## **INTRODUCTION**

Skin pigmentation is fundamental for protecting cutaneous cells from UV radiation damages. A defect in pigmentation is observed in several pathologies such as albinism, vitiligo, or neurofibromatosis type 1 [1]. Understanding the mechanism of pigmentation regulation is of great interest for elaborating strategies for both skin protection and cutaneous therapies. In melanocytes, the biosynthetic pathways for eumelanin and pheomelanin are determined by the signaling activity of melanocortin receptor MC1R, the best-known receptor involved in skin pigmentation [2]. Its activation by agonists,  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) or adrenocorticotrophic hormone ACTH, induces adenylate cyclase activation via the G $\alpha$ s protein, thus triggering cAMP synthesis and protein kinase A (PKA) activation, which in turn phosphorylates the cAMP Responsive Element Binding Protein (CREB) [3]. CREB is a transcription factor that regulates microphthalmia-associated transcription factor (MITF) expression which plays a fundamental role in melanin synthesis by controlling melanogenesis enzyme expression [4]. In human melanocytes, MC1R also activates, independently or dependently of cAMP pathway, the extracellular signal regulated kinases (ERK 1/2) which leads to transcriptional activation of MITF [2,3].

Besides neurofibromin (Nf1) is a GTPase activating protein and mutations in its gene (*NF1*) have been shown to induce changes in skin pigmentation [1,5]. In humans, mutations in the *NF1* gene cause neurofibromatosis type 1 (NF1), a common autosomal dominantly inherited disorder affecting about one in 3500 people worldwide. Among various symptoms, this disease is characterized by skin hyperpigmentation, multiple hyperpigmented patches of skin called "café-au-lait macules" (CALM) and skin-fold freckling [6]. Nf1 is a 2818 amino acid protein (220-280 kDa) organized in several functional domains which are well described [7]. Nf1 functions as a negative regulator of Ras protein by stimulating its low intrinsic GTPase activity. Consequently, Nf1 downregulates Ras signaling, including Raf/MEK/ERK and PI3K/Akt/mTOR pathways. In addition to inhibiting Ras signaling, Nf1 regulates adenylyl cyclase and thus cAMP formation through both a Ras-dependent and a Ras-independent manner [8]. Interestingly, using a global screen approach in mice, a novel class of pigmentation

mutants was identified as dark skin (Dsk) [9]. One of them, *Dark skin 9 (Dsk9)* mutant mice, displays a missense mutation in the GTPase-activating protein (GAP)-related domain (GRD) of Nf1 which leads to skin hyperpigmentation [10].

Moreover, we previously demonstrated that the serotonin 5-HT<sub>6</sub> receptor (5-HT<sub>6</sub>R), a transmembrane receptor belonging to the GPCR family, like MC1R, directly interacts with Nf1 and that this interaction regulates 5-HT<sub>6</sub>R constitutive activity [11].

Based on these results and on the role of both Nf1 and MC1R in pigmentation, we undertook to explore a potential physical interaction between these two proteins and to investigate the role of Nf1 on MC1R-mediated signaling and the melanogenesis pathways.

## **MATERIALS AND METHODS**

### **Reagents**

For reagents, antibodies and plasmid constructs, see supplementary material and methods

### **Cell culture and transfections**

HEK-293 cells were grown in Dulbecco modified Eagle medium supplemented with 10% (v/v) foetal bovine serum. Human normal melanocyte cell line (HNMi) was established in our laboratory (CNRS patent No. 99–1616) and was grown in Opti-MEM-1 with Glutamax-1 (Invitrogen™) supplemented with 0.5 µg/mL fungizone, 40 µg/mL gentamycin and 2% heat inactivated MycoPlex (PAA, Les Mureaux, France) as described [24]. Cells were cultured at 37°C in a 5% CO<sub>2</sub>/95% air atmosphere.

For BRET experiments cells were transfected with the calcium phosphate-DNA coprecipitation method as described previously [25]. In others experiments, cells were transfected with 50 mM of siRNA or 10 µg of plasmid/100-mm dish using Lipofectamine 2000 according to manufacturer's recommendations. For treatment, FTI 277 was used at 10 µM. All experiments were performed 48 h after transfection or treatment.

### **BRET measurements**

HEK-293 were resuspended in Hank's balanced salt solution (HBSS) and incubated for 15 min at 25°C in the absence or presence of  $\alpha$ -MSH (1  $\mu$ M). After addition of Coelenterazine H (5  $\mu$ M), fluorescence, luminescence and BRET measurements were performed with a Mithras LB 940 Multireader (Berthold, Bad Wildbad, Germany) as previously described [25].

### **Co-immunoprecipitation assays**

HNMI were co-transfected with HA-MC1R and C-terminal YFP-fused proteins. Forty-eight hours after transfection, cells were lysed in buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Triton X-100 plus protease cocktail inhibitor. The lysates were then centrifuged at 10,000 x g for 10 min. The supernatants were incubated with EZview Red anti-HA (Sigma) or anti-Nf1 (Santa Cruz) affinity gel for 3 hours at 4°C. Beads were then washed five times with lysis buffer and resuspended in Laemmli sample buffer.

### **Western blotting**

The cell lysates and immunoprecipitates from transfected HEK-293 cells or HNMI were analyzed by electrophoresis on SDS/PAGE, transferred to PVDF membranes and immunostained with various antibodies and HRP-coupled secondary antibodies. Immunoreactive bands were detected using the Dura detection kit. Acquisition was made using SynGene Pxi gel imaging system (Ozyme). For quantification, blots were analyzed using Quantity One software (Biorad).

### **Fontana-Masson staining**

Cells were stained with Fontana ammoniacal silver solution as previously described [26].

## **Determination of melanin production**

Melanin production was assessed according to previous publications with some modifications [27]. Forty-eight hours after transfection, culture supernatants were collected and melanocytes harvested by trypsinization and counted using a hemocytometer. After centrifugation, cells were resuspended in 1N NaOH; 6N NaOH was added in culture supernatant and both were incubated at 80°C during 30 minutes. The absorbance was measured at 420 nm and quantification of melanin determined using a standard curve with commercial melanin, after normalization to the cell number.

## **RESULTS**

### **Biochemical and biophysical characterization of the interaction between Nf1 and MC1R**

Using co-immunoprecipitation-immunoblotting assays, we identified Nf1 as a new partner of MC1R (Figure 1A). Immunoreactive bands corresponding to MC1R were only detected in the anti-Nf1 antibody precipitate, supporting the existence of a specific interaction between Nf1 and MC1R. Then, in order to characterize the domains of Nf1 involved in this association, five domains of Nf1 were tested: the GRD, the C-terminal domain of Nf1 (CTD), the Sec14p homologous domain (Sec), the Pleckstrin Homology domain (PH) and the Sec and PH domains (SecPH). As shown in Figure 1B, MC1R showed a strong interaction only with GRD, PH and SecPH domains.

To confirm these results and because artifactual protein aggregation can arise in denaturing detergents, we examined whether Nf1-domains/MC1R complexes could occur in living HEK-293 cells by using BRET assays. The extent of energy transfer between constructs encoding MC1R and Nf1 domains, fused respectively to RLuc and YFP at their C-terminal, was plotted as a function of increasing acceptor-donor ratios estimated by the ratio between fluorescence and luminescence (Figure 1C). Interestingly, the curve obtained for MC1R-RLuc with GRD-YFP was saturating and best fitted by a non-linear regression equation ( $BRET_{max} = 21.4 \pm 1.8$  mBU;  $BRET_{50} = 3.0 \pm 0.6$ ). In contrast, when MC1R was



transfected with SecPH, or PH domain, the BRET signal increased as a linear regression consistent with non-specific interactions between these BRET pairs (Figure 1C). Altogether, these data suggest a direct constitutive interaction of MC1R with Nf1 through its GRD domain. After stimulation by  $\alpha$ -MSH, no significant change in the BRET parameters could be detected ( $BRET_{max}$   $20.0 \pm 2.9$  mBU and  $BRET_{50}$   $4.7 \pm 1.6$ ) when compared to unstimulated cells ( $BRET_{max}$   $20.9 \pm 1.6$  mBU and  $BRET_{50}$   $2.7 \pm 0.6$ ) (Figure 1D). These observations suggest that the interaction of MC1R with Nf1 through its GRD domain is constitutive and not regulated by receptor activation.

#### **Analysis of the impact of GRD mutations on GRD/MC1R interaction.**

We then explored whether this interaction might be affected in pathologic conditions. We assessed whether mutations in GRD, i.e. R1276P identified in NF1 patient and known to completely abolish its Ras-GAP activity (GRD-Dead) [12], and N1430K (GRD-Dsk9) from Dsk9 mutant mice, were able to induce a differential effect on BRET parameters (Figure 2A). When transfected in HEK-293 cells, these two GRD mutants were well expressed (Figure 2B). We observed saturable and specific BRET signals for both mutants (Figure 2C) with a significant increase in the BRET<sub>max</sub> values ( $BRET_{max}$   $31.9 \pm 1.1$  mBU and  $BRET_{max}$   $36.1 \pm 3.7$  mBU for GRD-dead and GRD-Dsk9, respectively) compared to that obtained with wild type GRD ( $BRET_{max}$   $20.4 \pm 1.7$  mBU). These BRET changes may reflect a conformational change of a pre-existing interaction or recruitment between the two partners. In order to explore these two possibilities, the BRET values were expressed as percentage of BRET<sub>max</sub> (Figure 2D). The curves obtained were very similar and BRET<sub>50</sub> values were not changed by GRD mutations, suggesting that the enhanced BRET signal results from conformational rearrangement induced by mutations in GRD domain. Altogether, these data suggest that MC1R interacts with GRD regardless of the GRD mutational status.

#### **Role of Nf1 on MC1R-operated signaling and downstream signaling pathway**

Based on results indicating that Nf1 is involved in several cellular pathways by acting on Ras-MAPK pathway and cAMP cascade, we also explored whether MC1R signaling downstream to Ras and involved in melanogenesis, namely ERK1/2 and CREB activation, may be altered by Nf1 depletion. We demonstrated that Nf1 silencing (Figure 3A) induced a strong increase in ERK and CREB phosphorylation which is inhibited by addition of Ras inhibitor or GRD overexpression. GRD mutants were unable to counteract this effect (Figure 3B, C).

### **Nf1 and GRD regulate melanogenesis pathway in melanocytes**

To investigate the functional crosstalk between Nf1 and melanogenic pathways, we investigated the effect of Nf1 depletion on the expression of various melanogenesis players. Silencing of Nf1 induced an increase in MITF expression (+52% *versus* siRNA control), the key transcription factor in melanogenesis pathway (Figure 3D, E). Similarly, the expression of melanogenic enzymes, tyrosinase, TRP1 and TRP2, was upregulated after transfection with NF1 siRNA (+64%, +150% and +96% *versus* siRNA control, respectively).

We further specified the role of GRD and Ras pathway in melanogenic response. Ras inhibition in Nf1 deficient cells, achieved either by GRD transfection or by Ras inhibitor treatment, led to the decrease of the expression of all melanogenic factors which returned to the level displayed by siRNA control-transfected cells. However, both GRD mutants were unable to counteract the effect of NF1 siRNA on the expression of these proteins (Figure 3D, E).

### **The GAP activity of Nf1 controls melanin production inside melanocytes and in their supernatant**

Due to the important role of Nf1 GAP activity on the expression of melanogenic enzymes, we studied its effect on melanin production. Nf1 depleted HNMi showed a hyperpigmented phenotype compared to siRNA control-transfected HNMi (Figure 4A). To confirm these results, we quantified both the intracellular and secreted melanin (Figure 4B, C). When Nf1 expression was decreased, melanin production was greater inside HNMi and in culture supernatants (+348% and +215% *versus* control

siRNA-expressing cells, respectively). The expression of GRD-WT or cells treatment with Ras inhibitor fully reversed this increase in melanin level both in cell and in medium. Conversely, transfection with GRD-Dead or GRD-Dsk9 mutants was not effective to reduce melanin production as GRD-WT did and melanin production remained in the same range as that with siRNA control (+277% and +319% inside the cells and +175% and +213% in the supernatants, respectively).

## **DISCUSSION**

MC1R belongs to the GPCR family and to the subfamily of melanocortin receptors. Its activity is largely regulated by extracellular signals:  $\alpha$ -MSH and ACTH as agonists and agouti-signaling protein and  $\beta$ -defensin-3 as antagonists [3]. On the other hand, MC1R signaling is also regulated by various intracellular factors, such as  $\beta$ -arrestins, which are known to regulate the amount of GPCRs localized at the plasma membrane and their functional coupling to signal transduction pathways. [13]. More recently, it was shown that ubiquitinated arrestin2 can also stably interact with MC1R [14]. In addition MC1R can interact with other accessory proteins like melanocortin-2-receptor accessory proteins (MRAP1 and MRAP2) or the E3 ubiquitin ligase Mahogunin Ring Finger-1 (MGRN1) which are all involved in the regulation of its membrane expression and its functional coupling [15,16].

Our study reveals a new level of complexity in MC1R signaling as we demonstrated the interaction of MC1R with Nf1, a modular protein composed of several functional domains, including the central GRD domain acting as a Ras-specific GTPase activating protein down-regulating Ras function [7]. Here we showed that MC1R directly interacts with the GRD domain in an agonist-independent manner and that the GRD domain displaying the R1276P mutation, inducing a lower GAP activity, still interacts with the receptor. These results suggest that Nf1, endogenously expressed in melanocytes, constitutively interacts with MC1R regardless of the level of the GAP activity of Nf1 or the stimulation of the receptor. Interestingly, MC1R was shown to interact with two other cytosolic tumor suppressors, the Merlin, which is encoded by the gene neurofibromine 2 [17] and the phosphatase and tensin homolog (PTEN) [18]. PTEN is recruited by  $\alpha$ MSH-MC1R complex only after stimulation by UVB irradiation, avoiding the

premature UV-induced senescence of melanocytes [18]. Besides, Nf1 loss was reported to induce senescence in adult melanocytes [19]. Here, we demonstrate that, unlike PTEN, Nf1 interacts constitutively with MC1R which leads us to postulate that this interaction may therefore contribute to melanocyte senescence prevention.

Nf1 which has been detected in melanocytes [20] has been shown to be involved in skin pigmentation and in carcinogen-induced pigmentation [21]. Several works show that Nf1 can regulate MITF activity and melanogenesis process via a Ras-ERK dependant pathway [5]. In addition, a single mutation in its GAP domain (L1425D), which decreases GAP activity, was reported to abrogate TRP-2 expression [22]. In the present paper, we demonstrated that the regulation of melanogenesis enzyme expression by Nf1 is effectively controlled by its GRD domain by interacting directly with MC1R and by modulating intracellular pathways upstream TRP2.

The physical interaction of MC1R with Nf1 together with their common role in skin pigmentation led us to further characterize the role of Nf1 in MC1R downstream signaling events involved in skin melanogenesis. In agreement with studies performed on melanocyte-derived induced-pluripotent stem cells [19,23], our results show that Nf1 depletion in cultured human melanocytes induces a significant upregulation of melanogenic proteins which finally turns to the synthesis of melanin and promotes the extracellular release of the pigment. We observed that overexpression of GRD or incubation with a Ras inhibitor, which mimics GRD function of Nf1, rescues the signaling alterations and hyperpigmentation induced by cellular depletion of Nf1. In contrast, GRD dead overexpression did not restore normal phenotype pigmentation. These data suggest that Nf1 GAP activity is crucial to control skin pigmentation. The role of Nf1 in melanogenesis was further supported by the discovery of the Nf1 Dsk9/+ mouse which exhibit hyperpigmentation of both dermis and epidermis. The Dsk9 mutation is located within the GRD, therefore impairing NF1's Ras regulation function [10]. Altogether, we propose that, by affecting their GRD function and/or their ability to interact with protein partners like Ras or MC1R, mutations within the GRD may induce a dysregulation of melanogenic signaling, as observed in this study.

Interestingly, the hyperpigmentation phenotype associated with NF1 was investigated and demonstrated that Nf1 inactivation affects melanogenesis by increasing cAMP production and ERK phosphorylation [23]. Consistent with these findings, our study sheds light on a new molecular mechanism that could be at the origin of this signaling alteration. Indeed, we demonstrated here that Nf1, by constitutively interacting with MC1R, plays a key part of its signaling network and is essential to control Ras pathway activation and pigmentation in melanocytes. This interaction opens new unexplored strategies to correct pigmentation not only in NF1 patients but also in other pigmentation disorders.

### Legends to figures

**Figure 1: Biochemical and biophysical characterization of the interaction of Nf1 and its domains with MC1R.** (A) Biochemical analysis of the interaction between Nf1 and MC1R. HNMI were transfected with HA-MC1R or empty vector pCDNA3 (Mock). Anti-Nf1 immunoprecipitates from cell lysates were analyzed by immunoblotting. (B) Top: Schematic representation of Nf1 and its various domains used in this study. Bottom: HNMI were co-transfected with YFP-tagged Nf1 domains and HA-MC1R. Cell lysates and anti-HA immunoprecipitates were analyzed by immunoblotting. (C) BRET analysis of the interaction of Nf1 domains with MC1R. BRET titration curves were generated in HEK-293 cells co-transfected with a constant amount of MC1R-RLuc (25 ng) and increasing concentrations of YFP-tagged Nf1 domains (GRD, SecPH, PH) (from 0 to 8  $\mu$ g), stimulated or not with 1  $\mu$ M of  $\alpha$ -MSH (D). Plotted results are from three independent experiments using GraphPad Prism 4 software for Windows (GraphPad Software Inc, San Diego, CA, USA).

**Figure 2: Expression of GRD mutants and study of their interaction with MC1R in HEK-293 cells.** (A) Mutations in the GRD region of Nf1. (B) Expression of GRD mutants was evaluated by western blot analysis. (C-D) BRET saturation curves analysis of the interaction of MC1R with wild type (WT) or

mutated GRD domains. Cells were transfected with a constant amount of MC1R-RLuc (25 ng) and increasing concentrations of GRD-tagged YFP constructs (from 2 to 8  $\mu$ g). BRET signals were plotted as a function of the ratio between total fluorescence and luminescence. The BRETmax, the maximal BRET ratio obtained for a given pair was calculated from the fitted curves. The BRET max obtained with wild type GDR was  $20.4 \pm 1.7$  mBU and was statistically different from those obtained for GRD mutants (BRETmax= $31.9 \pm 1.1$  mBU for GRD dead, \*\*p<0.05 vs wt GRD and BRET max=  $36.1 \pm 3.7$  mBU for GRD Dsk9, \*\*p<0.01 vs wt GRD) (C) and data shown in figure C were expressed as percent of the BRETmax obtained for each BRET pairs (D). Plotted results are from 3 independent experiments.

**Figure 3: The GAP activity of Nf1 controls melanogenesis pathway in melanocytes.** Cells were transfected with control siRNA or NF1 siRNA in the presence of indicated GRD domains or treated with FTI 277. Lysates were analyzed by immunoblotting. (A) Expression of Nf1 in HNMI, 48h after transfection with NF1 siRNA or control siRNA (B) Expression of CREB, p-CREB, ERK1/2 and p-ERK1/2 evaluated by Western blot analysis . (C) The histograms represent the results of densitometric analyses, means  $\pm$  SEM from 3 independent experiments. (D) Effect of mutated GRD domains of Nf1 and Ras inhibitor (FTI 277) on the expression of various proteins involved in melanogenesis (MITF, Tyrosinase, TRP1, TRP2) after silencing of Nf1 expression. (E) Histograms represent the results of densitometric analyses, means  $\pm$  SEM from 3 independent experiments. \*\*\*\*p<0.0001 vs basal conditions; ##### p<0.0001 vs Nf1 deficient cells.

**Figure 4: The GAP activity of Nf1 controls melanin production inside melanocytes and in their supernatant.** (A) Microscopic image of HNMI transfected with control siRNA or NF1 siRNA. Fontana-Masson staining was realized 48 hours after transfection. Effect of mutated GRD domains of Nf1 and of a Ras inhibitor (FTI 277) on melanin production in HNMI supernatants (B) or in HNMI cell lysates (C). HNMI were transfected with control siRNA or NF1 siRNA. Nf1 deficient cells were also transfected with

GRD domains or treated with FTI 277. Melanin production was assayed 48 hours after transfection in cell supernatants and in cell lysates as indicated. Presented results are from one representative experiment out of three, means  $\pm$  SEM, n=4. \*\*\*\*p<0.0001 vs basal conditions; #####p<0.0001 vs Nf1 deficient cells.

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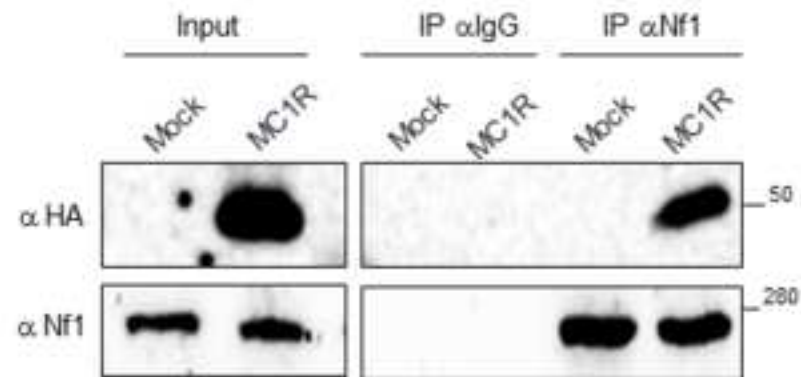
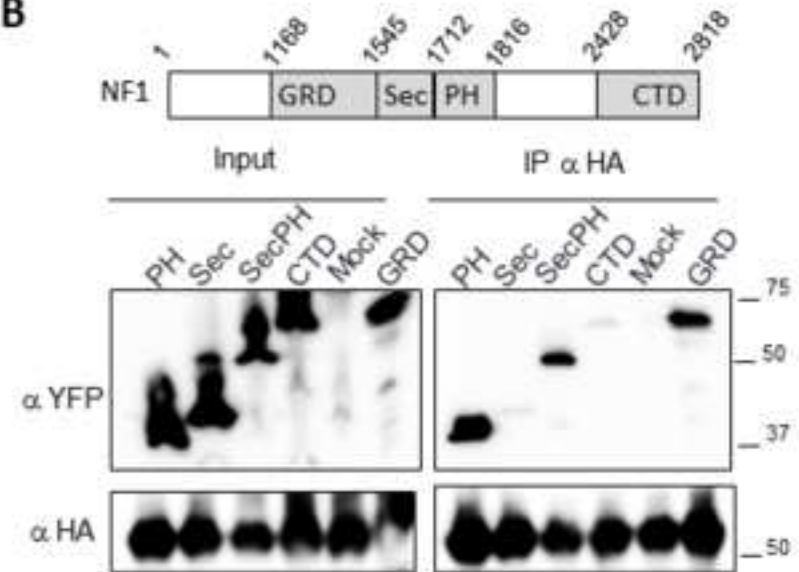
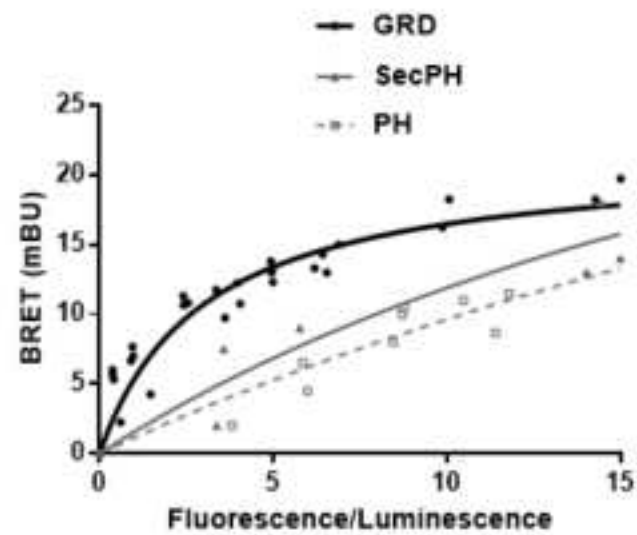
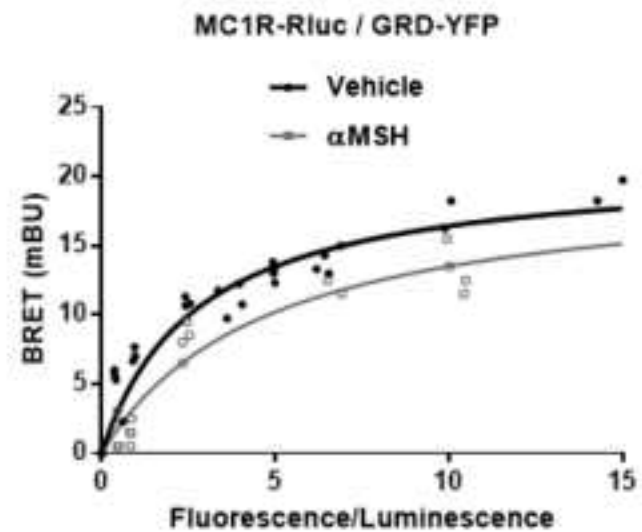
**A****B****C****D**

Figure 1

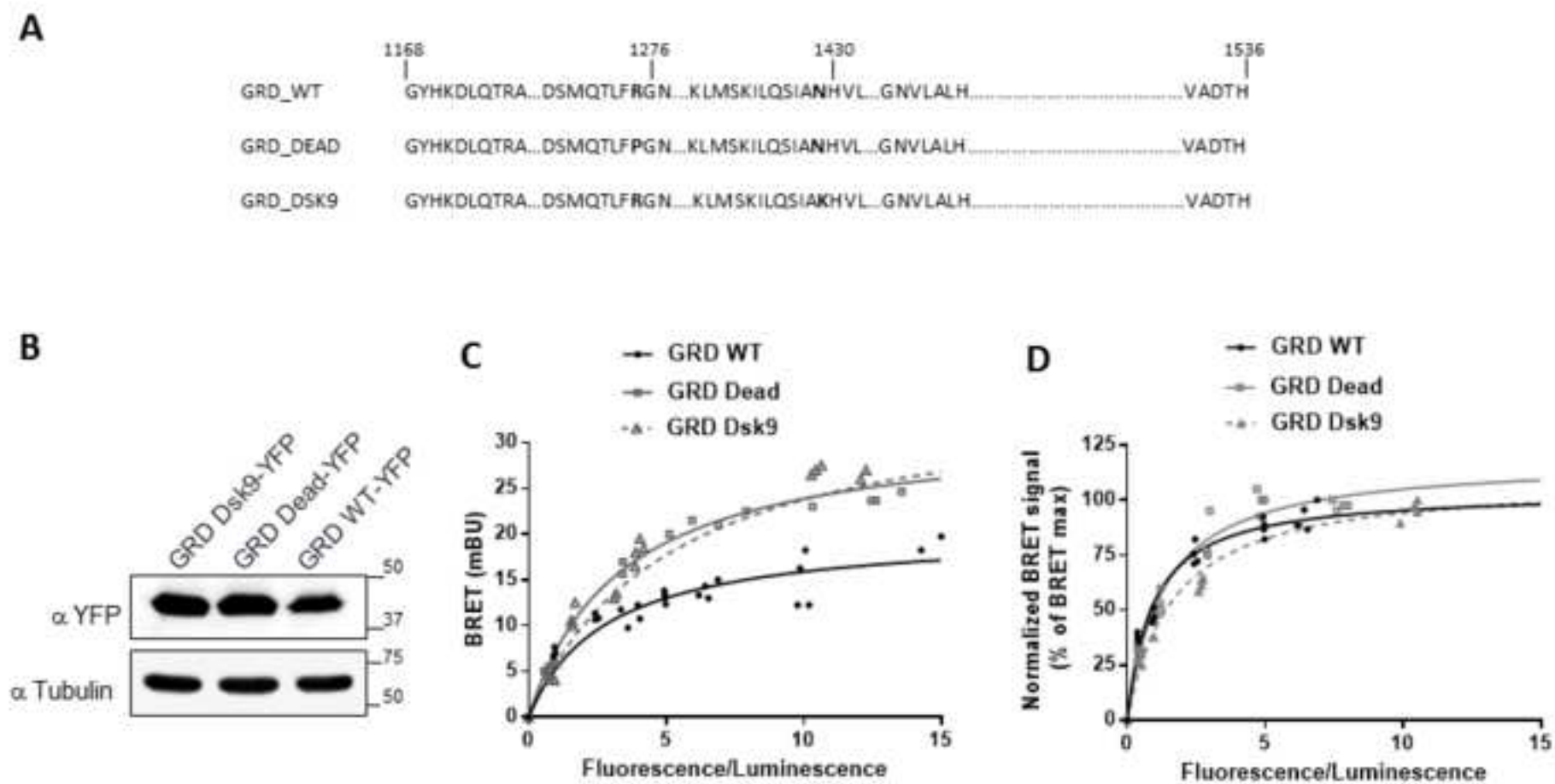


Figure 2

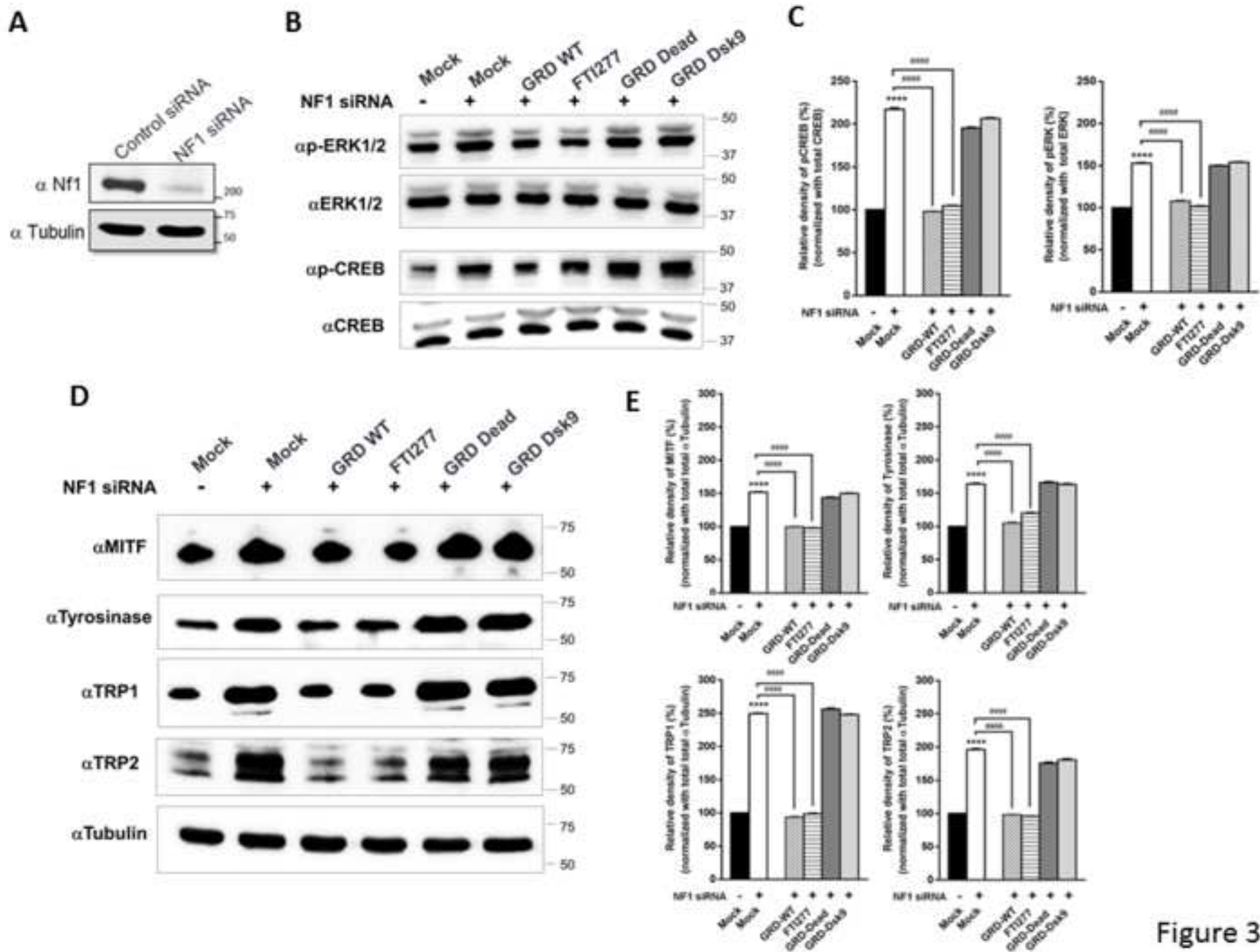


Figure 3

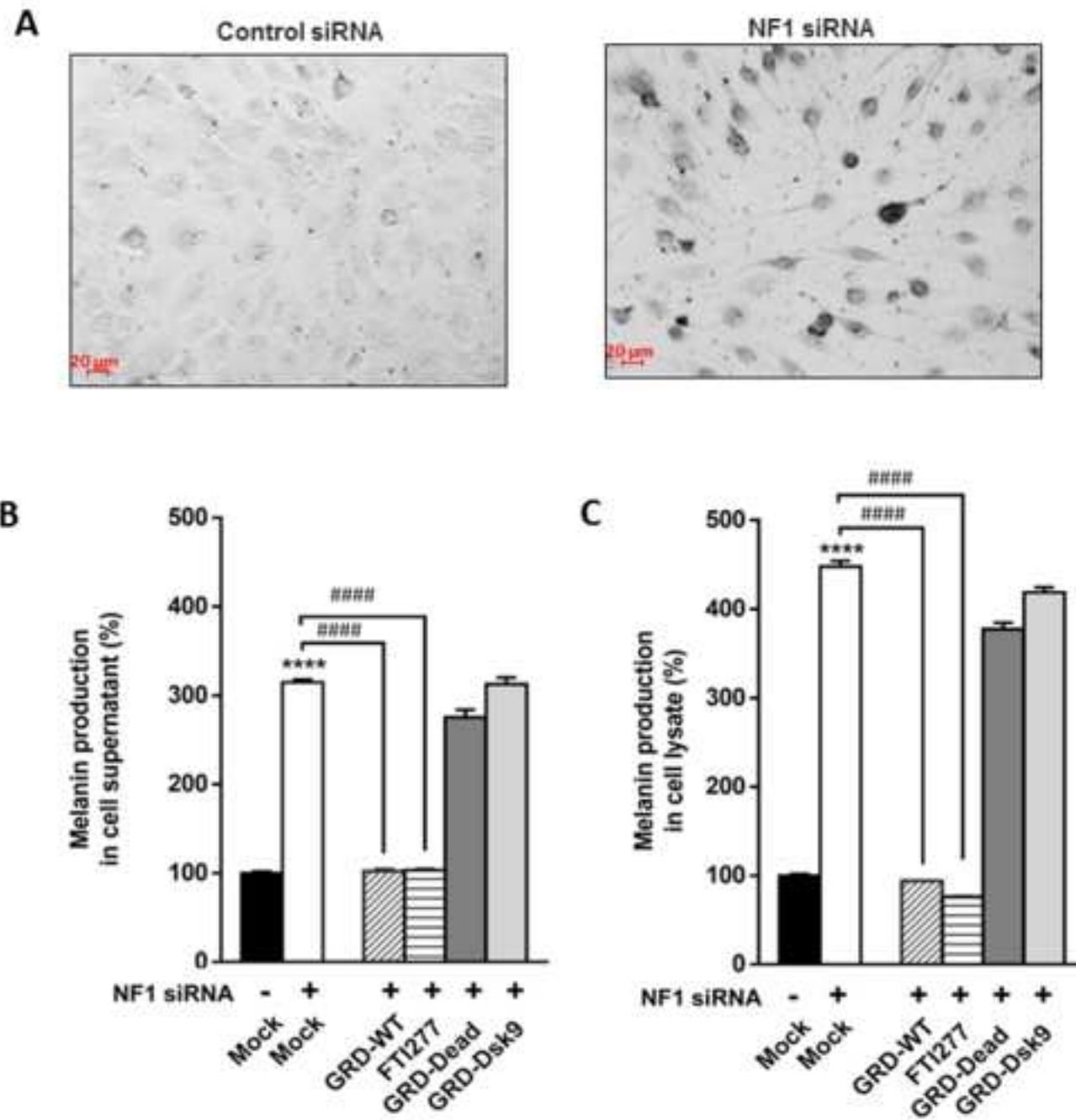


Figure 4