

# The RET51/FKBP52 complex and its involvement in Parkinson disease

Daniela Fusco<sup>1</sup>, Manuela Vargiolu<sup>1</sup>, Michele Vidone<sup>1</sup>, Elisa Mariani<sup>1</sup>, Lucia Fiammetta Pennisi<sup>1</sup>, Elena Bonora<sup>1</sup>, Sabina Capellari<sup>2</sup>, Dietmar Dirnberger<sup>3,4,5</sup>, Ralf Baumeister<sup>3,4,5</sup>, Paolo Martinelli<sup>2</sup> and Giovanni Romeo<sup>1,\*</sup>

<sup>1</sup>Unità di Genetica Medica, Policlinico Universitario S. Orsola-Malpighi, Bologna, Italy, <sup>2</sup>Department of Neurological Sciences, University of Bologna, Bologna, Italy and <sup>3</sup>Bio3/Bioinformatics and Molecular Genetics, Faculty of Biology, <sup>4</sup>Center for Biochemistry and Molecular Cell Research, ZBMZ, Faculty of Medicine and <sup>5</sup>Center for Systems Biology (ZBSA), University of Freiburg, Martinsried, Germany

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**The tyrosine kinase receptor RET51 is expressed in distinct families of neurons where it promotes different functions. FKBP52 is an immunophilin with neuroprotective effects on different kinds of neurons. In this paper, we demonstrate that RET51 activation by both glial cell line-derived neurotrophic factor (GDNF) and NGF triggers the formation of RET51/FKBP52 complex. The substitution of the tyrosine 905 of RET51, a key residue phosphorylated by both GDNF and NGF, disrupts the RET51/FKBP52 complex. NGF and GDNF have a functional role in dopaminergic (DA) neurons where RET51 and FKBP52 are expressed with a yet undefined function. To clarify if RET51/FKBP52 complex should exert its function in DA neurons, we used an indirect approach by screening the genes encoding for RET51 and FKBP52 in a group of 30 Parkinson's disease patients. The degeneration of DA neurons is the main feature of PD, which is associated to a complex multifactorial aetiology combining environmental, age-related and genetic factors. We found a compound heterozygous carrying two mutations in *RET* and *FKBP52* that are sufficient to disrupt the RET51/FKBP52 complex, indicating its potential role in PD.**

## INTRODUCTION

*RET* proto-oncogene encodes one of the most important tyrosine kinase (TK) receptor involved in the development and maintenance of the nervous system. RET has a critical role in the development of the enteric nervous system (ENS), parasympathetic nervous system and kidney (1,2). In adult tissues, RET remains expressed in peripheral enteric, sympathetic and sensory neurons as well as in several brain regions including central motor, dopamine and noradrenaline neurons (3,4). The TK receptor RET51 is an isoform of *RET* composed of an extracellular binding domain, a hydrophobic transmembrane domain and a cytoplasmic portion with the TK and pro-apoptotic domains (5,6). Mutations of *RET* are spread all over the gene (7,8) affecting all the different domains of the protein with both gain of function (9) and loss of function effects (10). Loss-of-function mutation of *RET* are associated

with the Hirschsprung disease, an alteration of the ENS caused by failure of neural crest migration leading to defects in enteric innervations (10).

As a TK receptor, RET51 exerts its biological activity transducing extracellular signals. In the last decade, the novel concept that RET51 can transduce both negative and positive signals has emerged. In this context, this TK receptor is included in the family of the dependence receptor, a particular class of receptors able to transduce a positive signal in the presence of their ligand and a negative one, leading to apoptosis, in their absence (11). In the specific case of RET51, the absence of ligands induces its proteolytic cleavage by caspase-3 at D707 and D1017 amino acidic residues. This cleavage results in the release of the pro-apoptotic fragment, which, through a distinct series of not well-characterized events, leads to apoptosis (12). On the other hand, RET51 is the signalling receptor of a multi-molecular complex that

\*To whom correspondence should be addressed at: Unità di Genetica Medica, Policlinico Universitario S. Orsola-Malpighi pad.11, Via G. Massarenti, 9 40138 Bologna, Italy. Tel: +39 0512088420; Email: romeo@eurogene.org

binds to members of the glial cell line-derived neurotrophic factor (GDNF) family. The binding and activation of RET51 by GDNF-family ligands (GFLs) is enhanced by the co-receptors belonging to the GDNF-family receptor- $\alpha$  (13). RET51 activation involves diverse downstream targets within and outside lipid rafts, which have been proposed to serve as essential signalling compartments in the cell membrane (14) with important roles in cell adhesion, axon guidance and synaptic transmission. Interestingly, it has recently been observed that RET51 is activated by NGF in mature sympathetic neurons (15). NGF does not enhance RET51 phosphorylation by altering the availability of GFLs, but instead regulates RET51 through a GFL-independent mechanism that has slow kinetics, requiring hours to maximally induce RET51 phosphorylation (15). It is noteworthy that several populations of neurons respond synergistically to multiple neurotrophic factors or require several growth factors simultaneously (16). The mechanisms of cross-talk between signalling cascades remain largely unknown, and in particular, the pathway responsible for the NGF-dependent activation of RET51 has remained uncharacterized.

To better understand the signalling pathway of RET51 in response to NGF activation, we performed a split ubiquitin two-hybrid assay (17), a modified two-hybrid system that enables the interaction of proteins both in the cytoplasm and at the membrane levels to be detected.

Using this assay, we identified 10 novel potential interactors of RET. In the present work, the immunophilin FKBP52 (also known as FKBP4) was studied in depth. Our work shows that the novel molecular complex RET51/FKBP52 is formed in the context of RET51 phosphorylation mediated by both the neurotrophins GDNF and NGF. RET51 Y905A substitution is able to completely disrupt the interaction between the two proteins. Interestingly, this RET51 tyrosine residue is preferentially phosphorylated in response to NGF activation in mature neurons (18). Our findings prompted us to hypothesize a role for the RET51–FKBP52 complex in adult neurons of the central nervous system. Starting from the observation that *RET* conditional knock-out mice are characterized from a late and progressive degeneration of dopaminergic (DA) neurons (19), we hypothesized an active role for the RET51–FKBP52 complex in this specific class of neurons. The degeneration of midbrain DA neurons located in the substantia nigra, which project to the dorsal striatum (20), is the main histopathological feature of Parkinson's disease (PD), the most common movement disorder with an occurrence of 1–2% among people over 65 years of age (21).

Current theories suggest that a combination of age, genetic and non-genetic factors is involved in PD aetiology (22–24). Although environmental risks for PD have received significant interest, the importance of genetic factors underlying the likelihood of developing PD is increasingly recognized. Several genes have been associated with PD and in particular recessive mutations in *PARK2*, *PINK1* and *DJ1* genes are associated with early onset (EO)-PD (25–27). Parkin, PINK1 and DJ-1 proteins are involved in oxidative stress, mitochondrial function and ubiquitin–proteasome system (UPS) (27–29) but the molecular relationship among these three proteins remains to be defined. It is clear that to

explain the complex genetic component underlying the pathogenesis of EO-PD other genes remain to be found. Moreover, the cause of the selective loss of DA neurons in PD remains elusive. A growing line of evidence shows that both the environmental and genetic component of this multifactorial disease converge at least on two different molecular pathways in which, on the one hand, the energetic metabolism is impaired and, on the other, the UPS is altered (30). To clarify the contribution of the RET51–FKBP52 complex in DA neurons, we used an indirect approach looking for mutations in *RET* and *FKBP52* genes of patients affected by EO-PD. We report the case of an EO-PD patient who is double heterozygote for *FKBP52* and *RET* mutations. Finally we show that the two mutations are able to disrupt the RET51/FKBP52 molecular complex, which is suggestive for a possible role in DA neuron function and a putative causative effect in PD.

## RESULTS

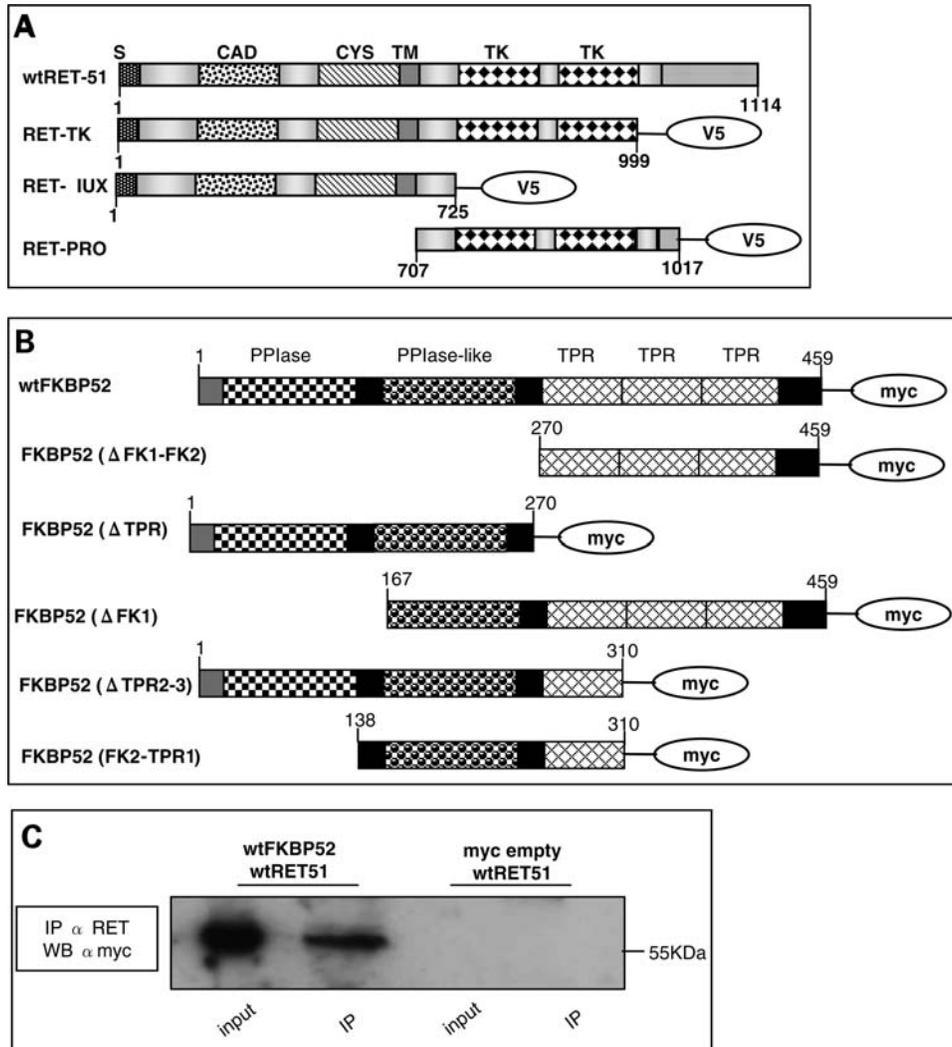
### FKBP52 is a binding partner of RET51 in human cell lines

In order to identify and characterize novel signalling pathways induced by the receptor RET51, we used the split ubiquitin two-hybrid assay (17). This technique is a modification of the classic yeast two-hybrid assay and enables to detect the interaction of proteins both in the cytoplasm and at the membrane level. By screening a human foetal brain cDNA library, we identified 10 novel potential interactors of RET51 including AIP (31) and FKBP52.

FKBP52 belongs to the immunophilin super family, a highly conserved class of chaperone and adaptor proteins with immunosuppressive activity widely expressed in most tissues, particularly abundant in the brain and specifically in neurons (32,33). The binding of immunophilins to specific immunosuppressant molecules such as cyclosporine, FK506 and rapamycin has the overall effect of suppressing the immune reaction (34). This particular feature has been shown to have a protective effect on neurons (35). Moreover, recent studies have shown that immunophilins, particularly FKBP12 and FKBP52, have critical roles in nerve regeneration and a high level of these two proteins has been documented in neurons and nerve injuries (36).

This molecular and physiological background information made FKBP52 a good candidate to unravel the signalling pathways induced by NGF-RET51 activation.

To confirm the putative interaction between RET51 and FKBP52, we amplified the FKBP52 cDNA starting from SH-SY5Y human neuroblastoma cell line and cloned it into the mammalian expression vector pcDNA3.1myc/His. Vectors expressing wtRET51 (Fig. 1A) and wtFKBP52 (Fig. 1B) were co-transfected in the human cell line HEK 293. After 48 h of transfection, a co-immunoprecipitation (IP) experiment using the specific anti-RET51 antibody C20 was performed and the western blot (WB) analysis with an anti-myc antibody showed (Fig. 1C) that RET51 and FKBP52 interact in this human cell line. The same result was obtained in the complementary assay (Supplementary Material, Fig. S1).



**Figure 1.** FKBP52 binds RET51 in human cell lines. (A) Linear representation of RET51 and the deleted constructs produced. ■, signal sequence; ▨, cadherine-like domain; ▩, cysteine-rich region; ▧, transmembrane domain; ▣, TK domains. (B) Linear representation of FKBP52 and the deleted constructs produced. ▤, PPIase domain; ▥, PPIase-like domain; ▦, TPR domains. The drawings are not in scale. (C) Co-IP of FKBP52 with anti-RET51. Proteins extracted from HEK-293 human cell line co-transfected with wtFKBP52 and wtRET51 (left panel) or empty myc and wtRET51 (right panel) were immunoprecipitated with an anti-RET51 antibody. The WB analysis to reveal the presence of FKBP52 was performed with an anti-myc antibody. The co-IP and WB experiments were performed in duplicate.

**Mapping the domains involved in RET51/FKBP52 complex formation**

With the aim to understand the downstream effect of the RET51/FKBP52 complex, we mapped the domains responsible for the reciprocal interaction of the two proteins.

The RET protein is composed of three domains: an extra-cellular ligand-binding domain (four cadherin-like repeats and a cysteine-rich region), a hydrophobic transmembrane domain and a cytoplasmic portion (with the TK and the pro-apoptotic domains) (5,6). Four distinct domains constitute FKBP52: the first two domains include a functional site for peptidyl-prolyl *cis/trans* isomerase (PPIase) activity and a PPIase-like region. Three tetratricopeptide repeat (TPR) domains, which have a sequence usually recognized and bound by heat shock proteins, occupy the third structural domain, while the fourth

C-terminal domain contains a putative binding site for calmodulin (37).

Starting from the full-length cDNA of both RET51 and FKBP52, different portions were PCR-amplified and deleted constructs were produced: the cDNA of RET-TK containing amino acids from 1 to 999 of RET, RET-IUX (1–725) and RET-PRO (707–1017) (Fig. 1A) were cloned in the pcDNA3.1V5/His mammalian expression vector. The cDNA of FKBP52 (ΔTPRs) containing amino acids from 1 to 260, the cDNA of FKBP52 (ΔFK1-FK2) containing amino acids from 261 to stop codon, the cDNA of FKBP52 (ΔTPR2-3) containing amino acids from 1 to 300, the cDNA FKBP52 (ΔFK1) containing amino acids from 145 to stop codon and finally the cDNA of FKBP52 (FK2-TPR1) containing amino acids from 145 to 300 were all cloned in the pcDNA3.1myc/His mammalian expression vector (Fig. 1B). The plasmids containing the

wild-type cDNA of both proteins were co-transfected in combination with each of the deleted constructs in the HEK 293 cell line. After 48 h of transfection, the proteins were immunoprecipitated with C20 or anti-V5 antibodies. WB analysis performed with an anti-myc antibody showed that whereas RET-IUX (Fig. 2A) did not retain the ability of interacting with wtFKBP52, both RET-TK (Fig. 2B) and RET-PRO (Fig. 2B) maintained this interaction. Moreover, the WB analysis showed that neither FKBP52 ( $\Delta$ TPRs) (Fig. 2C) nor FKBP52 ( $\Delta$ FK1-FK2) (Fig. 2C) were able to interact with wtRET51, whereas FKBP52 ( $\Delta$ TPR2-3) (Fig. 2D), FKBP52 ( $\Delta$ FK1) (Fig. 2D) and FKBP52 (FK2-TPR1) (Fig. 2D) all retained the ability to bind RET51. These results indicate that the formation of the complex RET51/FKBP52 requires amino acids spanning residues 707–999 of RET51 and the FK2 and TPR1 domains of FKBP52. The same results were obtained in the complementary assay (Supplementary Material, Fig. S2).

### RET51 is not part of the ternary complex formed by HSP90, FKBP52 and its client proteins

FKBP52 is known to form ternary complexes in response to cellular stress with HSP90 and other client proteins (38). The substitution of the residue T143 disrupts the interaction between FKBP52 and HSP90 and the consequent formation of the ternary complexes (39). Since it has been shown that RET51 transduces some intracellular signals in response to stress (40), we investigated the implication of the RET51/FKBP52 complex formation in this context. Through a site-specific mutagenesis, the T143A (FKBP52-T143A) substitution was introduced in the FKBP52 cDNA previously produced (Fig. 3A). RET51 and FKBP52-T143A cDNAs were co-transfected in HEK 293 cells. After 48 h of transfection, proteins were co-immunoprecipitated with C20 antibody and WB analysis was performed with an anti-myc antibody. Figure 3A shows that the T143A substitution does not impair the formation of the RET51/FKBP52 complex, suggesting that this is not dependent on the presence of HSP90. This result was further confirmed by transfecting wtRET51 and wtFKBP52 alone or in combination in the HEK 293 cell line. After 48 h of transfection, the proteins were co-immunoprecipitated with C20 or anti-myc antibody, respectively, and a WB analysis with an antibody anti-HSP90 was performed. As shown in Fig. 3, FKBP52 interacts with HSP90 both in the presence (Fig. 3D) and in the absence (Fig. 3B) of RET51 but RET51 does not interact with HSP90 neither in the presence (Fig. 3D) nor in the absence (Fig. 3C-E) of FKBP52. This result demonstrates that RET51 is not part of the ternary complex formed by HSP90, FKBP52 and its client proteins.

### RET51/FKBP52 complex is induced by RET51 phosphorylation

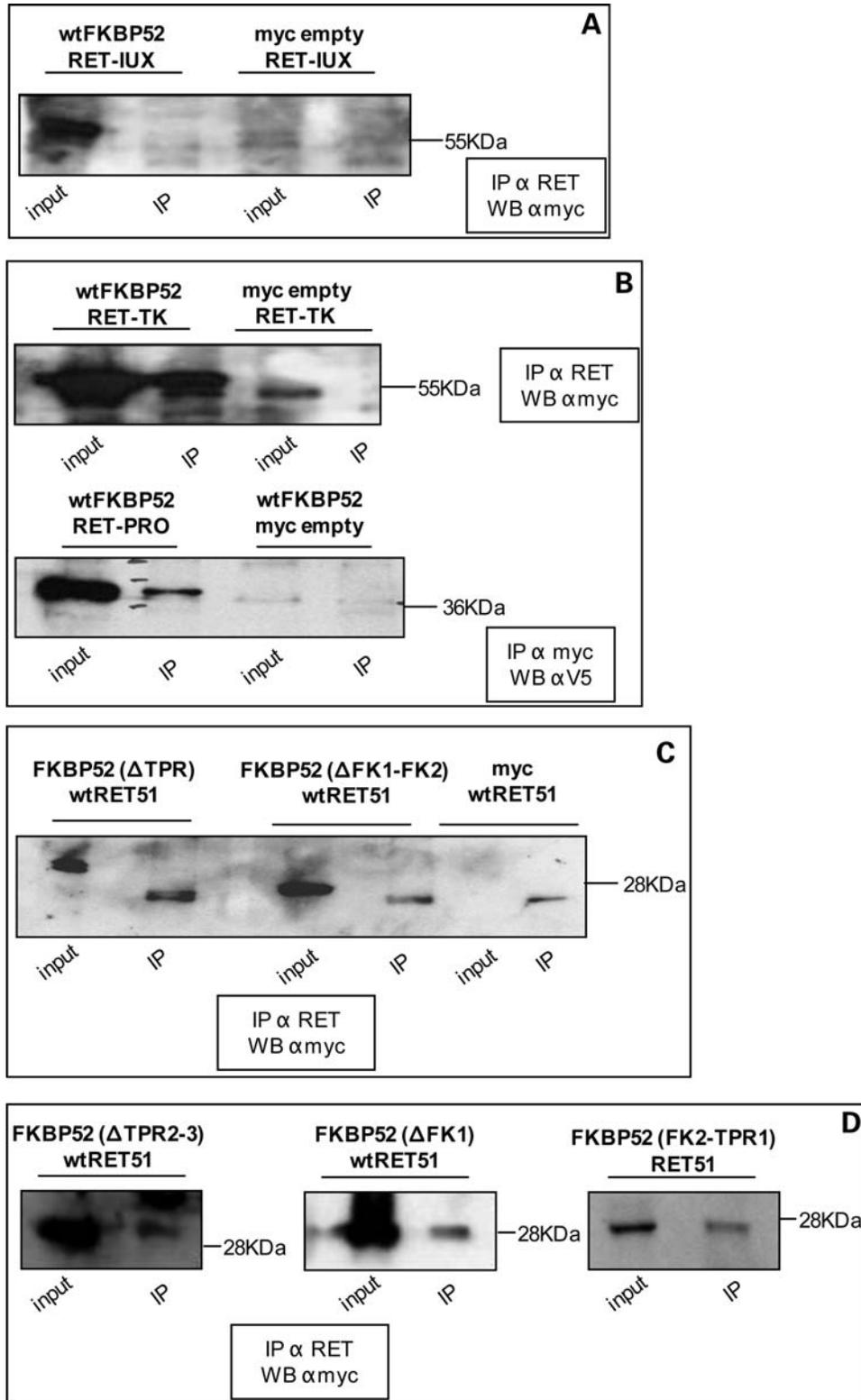
The phosphorylation of RET51 is the trigger for the transduction of all positive intracellular signalling mediated by the receptor. Starting from the hypothesis that RET51/FKBP52 complex may sustain the process of growth, trophism and differentiation of adult neurons, the effect of RET51 phosphorylation on the formation of the RET51/FKBP52 complex was investigated. To this purpose, we produced by site-specific mutagenesis the RET51 K758R mutant, namely RET51 dead kinase (RET51-DK) that, as previously shown (41), is unable to be phosphorylated. The co-IP assay performed with C20 antibody, after 48 h of transfection in the mammalian cell line HEK 293, showed that RET51-DK does not retain its interaction with FKBP52 (Fig. 3F), indicating a strong correlation between the status of phosphorylation of RET51 and the formation of the complex RET51/FKBP52. In order to avoid the RET51 autophosphorylation induced when the protein is overexpressed, we took advantage of a cell model that constitutively expresses RET51, i.e. the murine neuroblastoma cell line N2A. After 24 h of transfection of the wtFKBP52 cDNA, cells were either serum-starved or left in a medium containing serum. After 24 h, the proteins extracted from the cells grown in both conditions were co-immunoprecipitated with the C20 antibody and a WB analysis with an anti-myc antibody was performed. In N2A cell line, the RET51/FKBP52 complex is not able to form neither in a 10% fetal bovine serum (FBS)-containing medium (Fig. 3G) nor under starving conditions (Fig. 3G), confirming that the complex formation is induced by RET51 phosphorylation.

### The neurotrophins GDNF and NGF trigger specifically the formation of RET51/FKBP52 complex

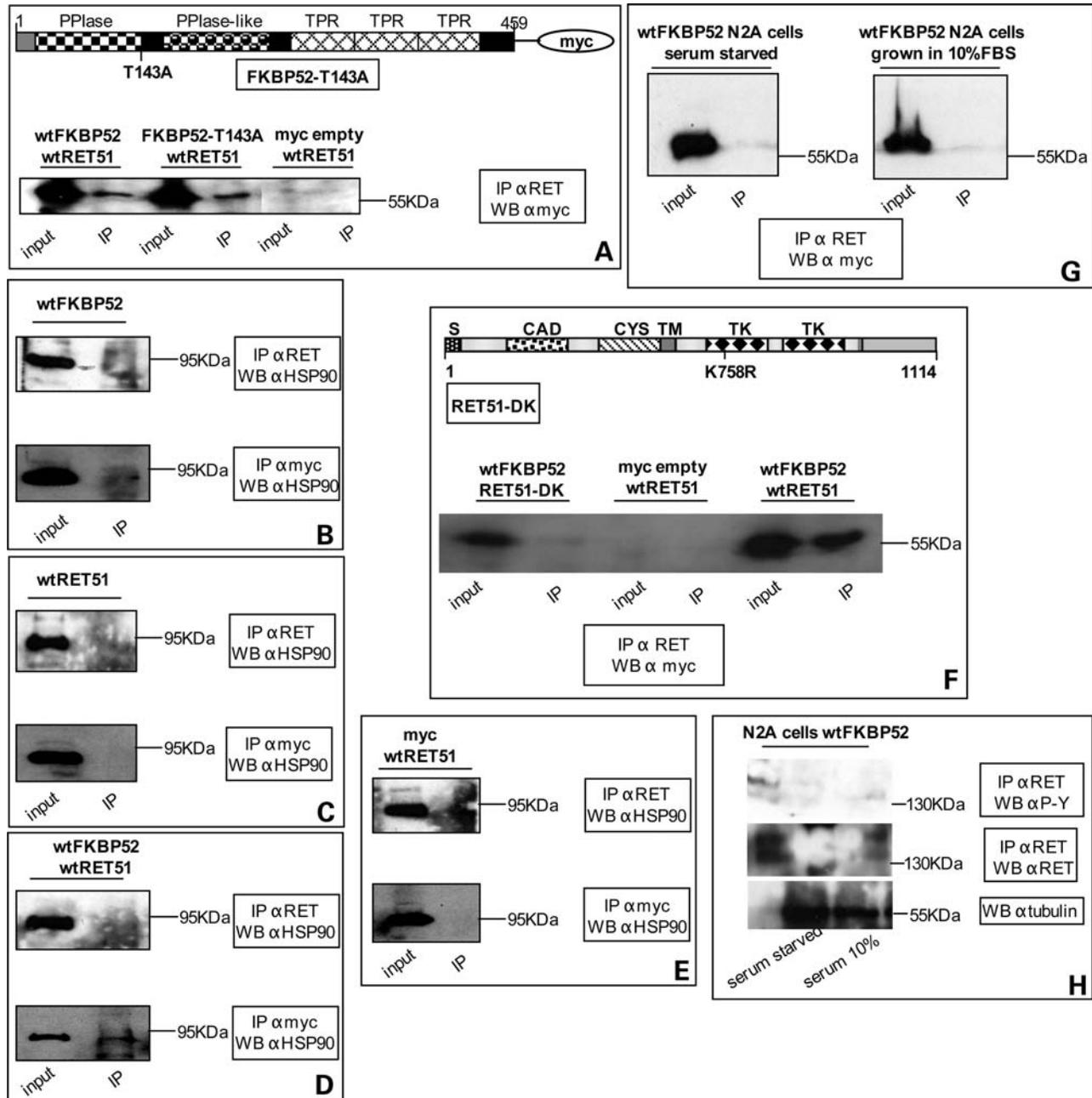
GFLs trigger RET51 phosphorylation (13). NGF promotes RET51 phosphorylation with a slower kinetic that does not alter the availability of GFLs, but instead regulates the receptor through a GFL-independent mechanism (15). It is unknown whether a cross-talk exists between the two signalling pathways or whether they act in two completely independent ways.

Since we showed that the formation of the RET51/FKBP52 complex is dependent on RET51 phosphorylation, we tested the hypothesis that individual neurotrophins may be sufficient by themselves to induce the interaction between the two proteins. To this aim, we performed a selective growth factors induction of RET51 in the N2A cell line. After 24 h of transfection of the wtFKBP52 cDNA, cells were either serum-starved or left in a medium containing serum. After 24 h, two starved cell samples were treated with GDNF or NGF; the proteins extracted from the cells were

**Figure 2.** Mapping the domains involved in RET51/FKBP52 complex formation. (A) co-IP of wtFKBP52 with anti-V5 antibody. Proteins extracted from the HEK-293 human cell line co-transfected with wtFKBP52 and RET51-IUX (left panel) or empty myc and RET51-IUX (right panel) were immunoprecipitated with an anti-V5 antibody. The WB analysis to reveal the presence of FKBP52 was performed with an anti-myc antibody. (B) co-IP of wtFKBP52 with anti-V5 antibody and co-IP of RET-PRO with anti-myc antibody. Proteins extracted from the HEK-293 human cell line co-transfected with wtFKBP52 and RET51-TK (left panel), empty myc and RET51-TK (right panel) were immunoprecipitated with an anti-RET51 antibody (upper panels); proteins extracted from the HEK-293 human cell line co-transfected with wtFKBP52 and RET-PRO (left panel) or empty V5 and wtFKBP52 (right panel) were immunoprecipitated with an anti-myc antibody (lower panels). The WB analysis to reveal the presence of FKBP52 was performed with an anti-myc antibody, whereas the WB analysis



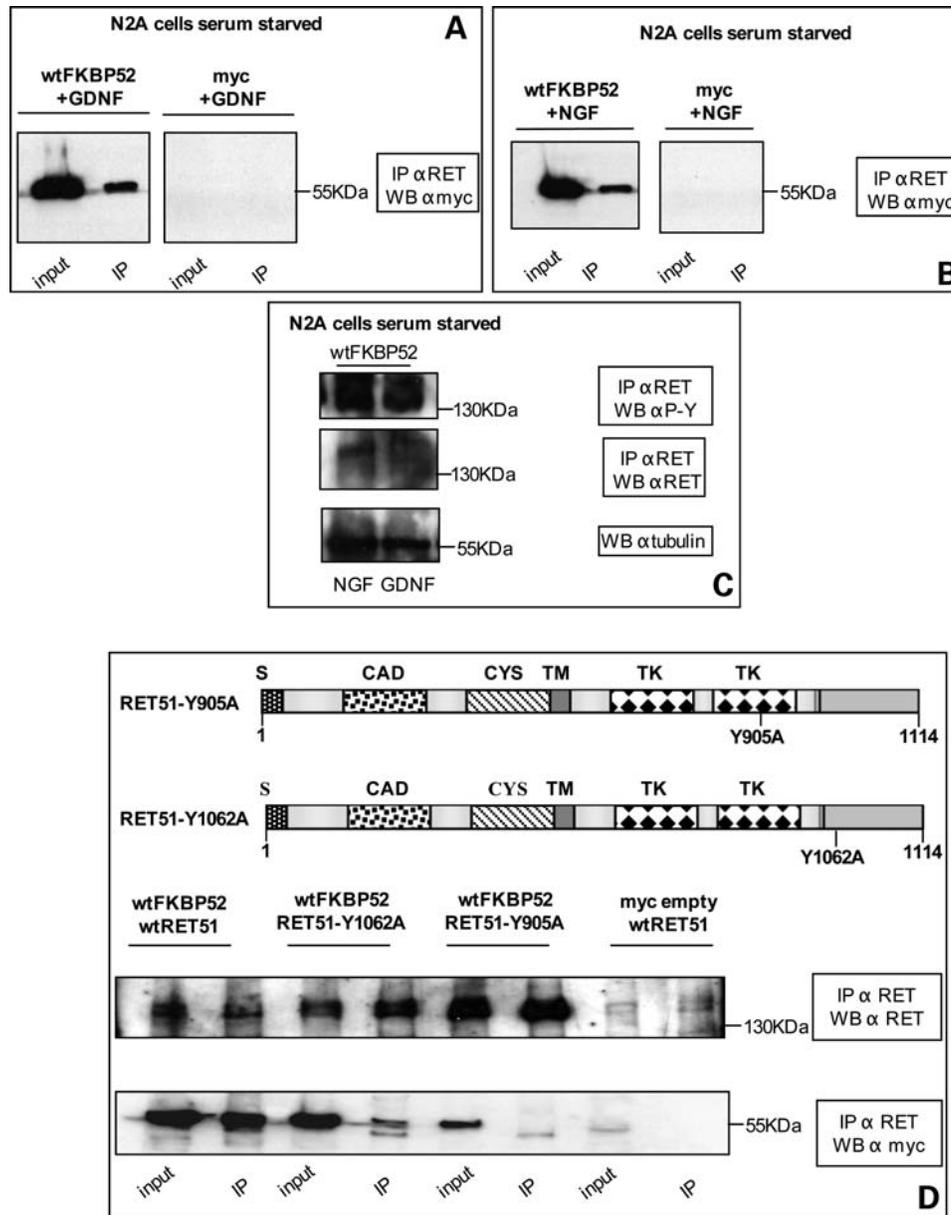
to reveal the presence of RET-PRO was performed with an anti-V5 antibody. (C) co-IP of FKBP52 deleted constructs with anti-RET51 antibody. Proteins extracted from HEK-293 human cell line co-transfected with wtRET51 and FKBP52 ( $\Delta$ TPRs), wtRET51 and FKBP52 ( $\Delta$ FK1-FK2) or wtRET51 and empty myc were immunoprecipitated with an anti-RET51 antibody. The WB analysis to reveal the presence of the FKBP52 deleted constructs was performed with an anti-myc antibody. (D) co-IP of FKBP52 deleted constructs with anti-RET51 antibody. Proteins extracted from the HEK-293 human cell line co-transfected with wtRET51 and FKBP52 ( $\Delta$ TPR2-3), wtRET51 and FKBP52 ( $\Delta$ FK1) or wtRET51 and FKBP52 (FK2-TPR1) immunoprecipitated with an anti-RET51 antibody. The WB analysis to reveal the presence of the FKBP52 deleted constructs was performed with an anti-myc antibody. All the co-IP and WB experiments were performed in duplicate.



**Figure 3.** The RET51/FKBP52 is not formed in the context of the FKBP52/HSP90/client proteins ternary complex and is induced by RET51 phosphorylation. (A) Linear representation of FKBP52-T143A and co-IP with anti-RET51 antibody. The drawing is not in scale. Proteins extracted from HEK-293 human cell line co-transfected with wtFKBP52 and wtRET51, FKBP52-T143A and wtRET51 or empty myc and wtRET51 were immunoprecipitated with an anti-RET51 antibody. The WB analysis to reveal the presence of both FKBP52 and its mutated form was performed with an anti-myc antibody. The co-IP and WB experiments were performed in duplicate. (B–E) Proteins extracted from the HEK-293 human cell line transfected with wtFKBP52 (B) or wtRET51 (C) or co-transfected with wtFKBP52 and wtRET51 (D) or empty myc and wtRET51 (E) were immunoprecipitated with an anti-RET51 antibody (upper panels) or with an anti-myc antibody (lower panels). The WB analysis to reveal the presence of the HSP90 was performed with an anti-HSP90 antibody (B–E). (F) Linear representation of RET51-DK and co-IP with anti-RET51 antibody. The drawing is not in scale. Proteins extracted from the HEK-293 human cell line co-transfected with wtFKBP52 and RET51-DK, empty myc and wtRET51 or wtFKBP52 and wtRET51 were immunoprecipitated with an anti-RET51 antibody. The WB analysis to reveal the presence of FKBP52 was performed with an anti-myc antibody. The co-IP and WB experiments were performed in duplicate. (G) Co-IP experiment in N2A cells. After 24 h of transfection with wtFKBP52, cells were serum-starved (left panel) or not (right panel). After an overnight starvation, the proteins extracted from the cells were immunoprecipitated with an anti-myc antibody. The WB analysis to reveal the presence of FKBP52 was performed with an anti-myc antibody. All the co-IP and WB experiments were performed in duplicate. (H) Control immunoblotting performed with anti-phospho-Y, anti-RET and anti-tubulin, respectively, of the samples deriving from the experiment described in (G).

co-immunoprecipitated with the C20 antibody and a WB analysis with an anti-myc antibody was performed. As shown in Fig. 4, both GDNF (Fig. 4A) and NGF (Fig. 4B)

triggered the formation of RET51/FKBP52 complex, suggesting that the complex responds specifically to these two neurotrophins.



**Figure 4.** The neurotrophins GDNF and NGF trigger specifically the formation of RET51/FKBP52 complex through the Y905 of RET51. **(A)** GDNF treatment of N2A cells. After 24 h of transfection with wtFKBP52, cells were serum-starved and the next day were treated for 15 min with 50 ng/ml GDNF. After the treatment, the proteins extracted from the cells were immunoprecipitated with an anti-RET51 antibody. The WB analysis to reveal the presence of FKBP52 was performed with an anti-myc antibody. **(B)** NGF treatment of N2A cells. After 24 h of transfection with wtFKBP52, cells were serum-starved and the next day were treated overnight with 100 ng/ml NGF. After the treatment, the proteins extracted from the cells were immunoprecipitated with an anti-RET51 antibody. The WB analysis to reveal the presence of FKBP52 was performed with an anti-myc antibody. **(C)** Control immunoblotting performed with anti-phospho-Y, anti-RET and anti-tubulin, respectively, of the samples deriving from the experiment described in (A) and (B). **(D)** Linear representation of RET51-Y905A and RET51-Y1062A. The drawings are not in scale. Proteins extracted from HEK-293 human cell line co-transfected with wtFKBP52 and wtRET51, wtFKBP52 and RET51-Y1062A, wtFKBP52 and RET51-Y905A or empty myc and wtRET51 were immunoprecipitated with an anti-RET51 antibody. The WB analysis to reveal the presence of both FKBP52 and its mutated form was performed with an anti-myc antibody (bottom panel). As a control, a WB with an anti-RET antibody was performed (middle panel). All the co-IP and WB experiments were performed in duplicate.

#### The tyrosine 905 of RET51 is a key residue for the formation of RET51/FKBP52 complex

Since we showed that the TK domain of RET51 is necessary for complexing with FKBP52, in close dependence on RET51 phosphorylation, the effective contribution of the tyrosines to the formation of RET51/FKBP52 complex was studied.

Interestingly, within the TK domain of RET51, two tyrosines, namely 905 and 1062, become phosphorylated in the presence of both GDNF and NGF (13,15). In particular, these two tyrosines are the preferential substrates for NGF activation of RET51 (18).

We hence introduced the Y905A and the Y1062A substitution in the expression vector containing RET51 by site-specific

mutagenesis. The RET51–Y905A and RET51–Y1062A constructs were co-transfected, respectively, with wtFKBP52 cDNA in HEK 293 cells. After 48 h of transfection, the proteins were immunoprecipitated with the C20 antibody and a WB analysis with an anti-myc antibody was performed. The results shown in Fig. 4D demonstrate that whereas the substitution Y1062A does not affect the formation of the complex, Y905A substitution induces the loss of binding to FKBP52 (Fig. 4D). The same result was obtained in the complementary assay (Supplementary Material, Fig. S3).

### Mutation screening in EO-PD patients

Several studies have indicated that RET and its signalling pathway may play a critical role in the development and maintenance of DA neurons. It is well established that RET is involved in the development of DA neurons (42) but the molecular mechanisms underlying this process remain to be clarified. Kramer *et al.* (19) produced a transgenic mouse with the selective ablation of RET observing the loss of DA neurons in substantia nigra pars compacta. It is clear that this TK receptor is a critical regulator of long-term maintenance of the nigrostriatal DA system but it remains still unclear whether this protein actively participates in those processes whose alteration leads to CNS disorders such as PD. Furthermore, both GDNF and NGF promote the survival and function of DA neurons in the midbrain. Clinical trials based on GDNF, NGF and FK506 have been developed for PD treatment but to date no meaningful findings have pointed out these molecules and their effectors as real players in PD pathogenesis. Since we showed that the formation of RET51/FKBP52 molecular complex occurs in response to neuronal growth factors likely transducing a positive intracellular signal, it is plausible to suppose that its disruption should alter some physiological neuronal function. On the basis of these assumptions, we hypothesized a functional role of RET51/FKBP52 complex in DA neurons and possibly in the pathogenesis of PD.

Starting from this consideration, we selected a group of 30 patients with PD with onset before 50 years or a positive family history for the disease for the screening of *RET* and *FKBP52* genes. The group comprises 26 patients with EO-PD either sporadic or familial, 18 males and 8 females, with an average age at onset of 41 years (20–50) and four patients with late onset (LO)-PD and a family history of autosomal dominant PD (all males, age at onset ranging from 53 to 61 years).

In this cohort of patients, we found a compound heterozygote carrying the point mutation c.25934 G > A (p.Arg144His) in *RET* and the c.6962\_6964delGAG (p.Glu422del) deletion in *FKBP52* (Fig. 5A–D), validated by allele separation (data not shown). The RET51 amino acidic substitution R144H was tagged as possibly damaging by *in silico* prediction performed with the PolyPhen software (43).

The patient reported a ‘subjective mild tremor’ since his 20s. Clinical diagnosis was performed at the age of 39 years when a motor impairment of the right hand was reported followed 1 year later by a resting tremor at the same hand and later at the right leg. The symptoms responded well to

L-dopa therapy. At 46 years of age, he underwent left stereotactic subthalamic surgery. The disease evolved with the involvement of the left hemibody (3.5 years after the onset), facial hypomimia, hypokinetic dysarthria, diffuse arm and legs rigidity with bradykinesia, decreased gait velocity and stride length with freezing of gait and dyskinesias. The patient reported that he has a long-time constipation problem. He does not have a familial history of parkinsonism but it is not possible to exclude familiarity because his mother died of diabetes at the age of 56 years.

As controls, 100 cases selected by neurologists among the partners of affected patients and clinically analysed for PD were screened and no mutations in *RET* and *FKBP52* genes were found.

### Genotype–phenotype correlation for the novel *RET* and *FKBP52* mutations

Both loss-of-function and gain-of-function mutations are associated with *RET* proto-oncogene in different pathologies (7). Most of the *RET* mutations map within the extracellular cysteine-rich domain of the protein: the overall effect produced by these mutations is a constitutive dimerization of the receptor that in some cases leads to its constitutive activation (gain of function) (9), whereas in other they produce loss-of-function effects (8). The majority of the extracellular domain mutations of *RET* are associated with a loss-of-function effect and they are distributed along the entire external portion of the protein (7).

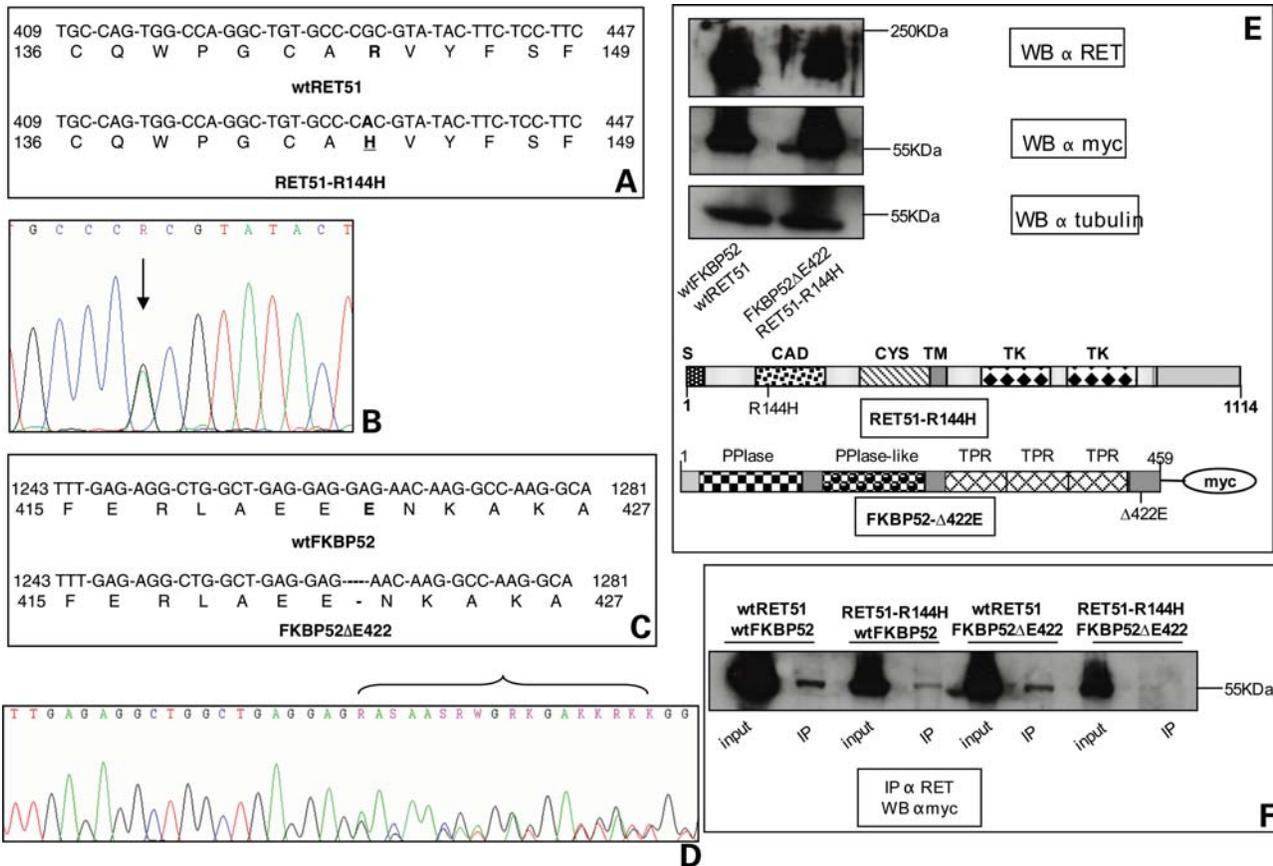
Since the newly identified mutation of *RET* maps within the extracellular portion of the protein as a first step, we verified whether the expression level of the mutated protein coincided with the wild-type.

We produced an RET51–R144H mutant construct through site-specific mutagenesis. After 48 h of transfection, the WB analysis performed with an anti-extracellular portion of RET-specific antibody (PAN-RET) confirmed that the mutant protein had an expression level comparable with wt RET51 in the HEK 293 human cell line (Fig. 5E).

Since no pathogenic variations of *FKBP52* have been reported to date, we tested the effect of the Glu422del on the FKBP52 protein expression level. We hence introduced, by site direct mutagenesis, the E422 deletion in the FKBP52 expressing vector (FKBP52ΔE422). The WB analysis performed with an anti-myc antibody confirmed that the FKBP52ΔE422 protein was correctly expressed and that its expression level was comparable with wtFKBP52 in the HEK 293 human cell line (Fig. 5E).

Since both proteins were correctly expressed, we then studied the effect of the two mutations on the ability of RET51 and FKBP52 to form a complex.

To this end, the HEK 293 human cell line was transfected with the following combination of plasmids: wtFKBP52–RET51R144H, FKBP52ΔE422–wtRET51 and FKBP52ΔE422–RET51R144H. After IP with a RET51-specific antibody, the WB analysis showed that the simultaneous occurrence of both mutated proteins impairs their ability to form the RET51/FKBP52 complex (Fig. 5F). The same result was obtained in the complementary assay (Supplementary Material, Fig. S1).



**Figure 5.** Identification and characterization of *RET* and *FKBP52* mutations in an EO-PD patient. (A) Comparison between the amino acid and cDNA sequences of wtRET51 and RET51-R144H. (B) Chromatogram of the genomic sequence of *RET*, derived from an EO-PD patient. The arrow indicates the heterozygous c.25934 G > A. (C) Comparison between the amino acid and cDNA sequences of wtFKBP52 and FKBP52ΔE422. (D) Chromatogram of the genomic sequence of *FKBP52*, derived from an EO-PD patient. The curly bracket indicates the mismatched pairing caused by c.6962\_6964delGAG. The PCRs and the sequencing analysis were performed in duplicate. (E) Linear representation of RET51-R144H and FKBP52ΔE422 (bottom panel). The drawings are not in scale. Expression analysis of mutated constructs. A WB analysis of proteins extracted from the HEK-293 human cell line transfected with wtRET51, RET51-R144H, wtFKBP52 and FKBP52ΔE422 was performed with the following antibodies: anti-RET to compare the expression level of wtRET51 and RET51-R144H; anti-myc to compare the expression level of wtFKBP52 and FKBP52ΔE422; anti-tubulin to normalize. The WB analyses were performed in duplicate. (F) Proteins extracted from the HEK-293 human cell line co-transfected with wtFKBP52 and wtRET51, wtFKBP52 and RET51-R144H, FKBP52ΔE422 and wtRET51 or RET51-R144H and FKBP52ΔE422 were immunoprecipitated with an anti-RET51 antibody. The WB analysis to reveal the presence of both FKBP52 and FKBP52ΔE422 was performed with an anti-myc antibody. The co-IP and WB experiments were performed in duplicate.

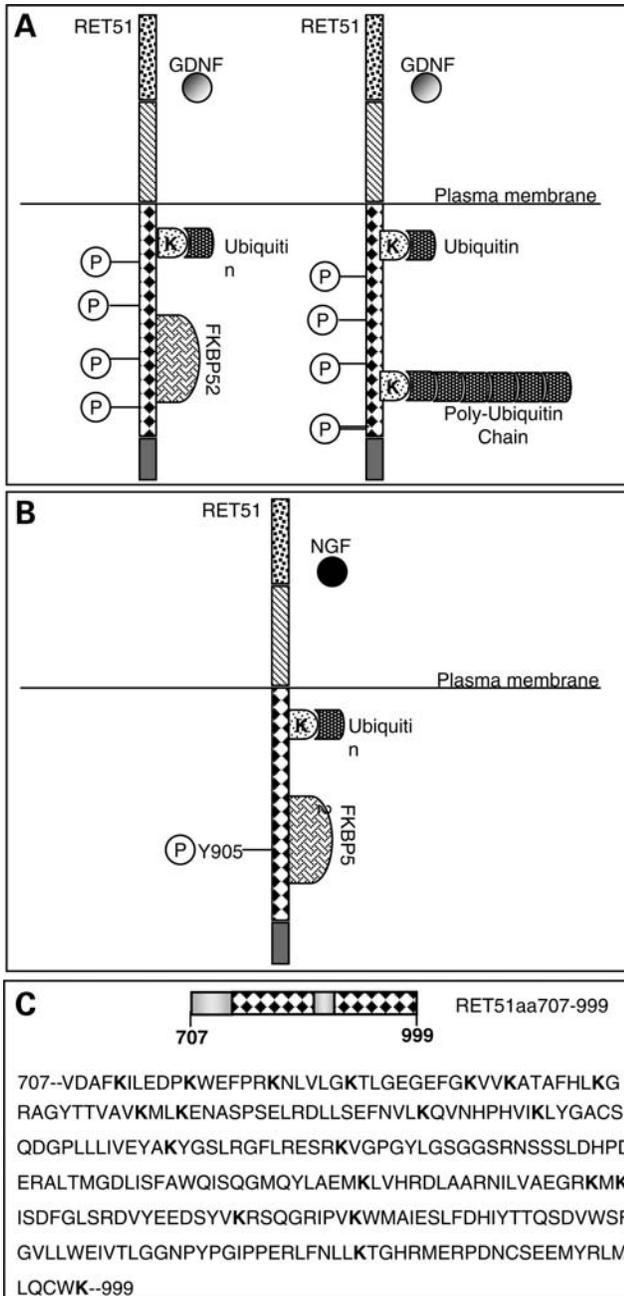
## DISCUSSION

In the present study, we identified and characterized the RET51/FKBP52 molecular complex defining its involvement in EO-PD.

In our study, we show that RET51 interacts with the neuroprotective immunophilin FKBP52 (Fig. 1C) and that this interaction is strictly dependent on the status of phosphorylation of RET51 (Fig. 3F and G). FKBP52 contributes to a variety of CNS function mainly mediating the neurotrophic and neuroprotective effect of its ligand FK506 (35); furthermore, it has been speculated that FKBP52 participates in the retrograde signalling pathway of the neurons by regulating microtubules dynamics (44).

The characterization of the RET51/FKBP52 complex has shown that both GDNF (Fig. 4A) and NGF (Fig. 4B) trigger its formation and that the substitution of Y905 of RET51 hampers the interaction of the two proteins (Fig. 4C). Pierchala *et al.* showed that tyrosine 905 of RET51 is

preferentially phosphorylated by NGF with a slow kinetic in adult sympathetic neurons. It has been proposed that NGF-mediated phosphorylation of RET51 stabilizes the protein promoting its mono-ubiquitination in contrast to the usual poly-ubiquitination promoted upon RET51 GDNF-mediated phosphorylation (18). It has been reported that some immunophilins and immunophilin-like proteins can exert their chaperone activity by protecting their client proteins from ubiquitination (45). We hence propose a model (Fig. 6A and B) by which FKBP52, interacting with RET51 upon NGF activation, physically blocks by steric hindrance the poly-ubiquitination site of RET51. According to our model, in the presence of GDNF (Fig. 6A), RET51 can be both mono- and poly-ubiquitinated since the hyper-phosphorylation of RET51 weakens its interaction with FKBP52 and not all the RET51 molecules will be complexed with FKBP52. On the other hand, in the presence of NGF (Fig. 6B), the phosphorylation level of RET51 is lower (compared with the one induced by GDNF) and strictly associated with tyrosine 905, so as the RET51/FKBP52



**Figure 6.** FKBP52 should modify the availability of RET51 poly-ubiquitination sites. Schematic representation of the model proposed. According to this model, the presence of FKBP52 hinders the poly-ubiquitination of RET51 in favour of its mono-ubiquitination. (C) Linear representation and amino acidic sequence of the RET51 domain responsible for the interaction of FKBP52. The lysine residues are in bold. The drawings are not in scale.

complex formation. We propose that, in this context, the great majority of RET51 molecules are complexed with FKBP52 whose presence hides the poly-ubiquitination lysine residue of RET51. The ubiquitination consensus sequence remains to be defined, but it is known that ubiquitin is attached to a lysine residue (46,47) and it is very frequent that the mono- and poly-ubiquitination lysines do not overlap in proteins (46). It is noteworthy that the amino acidic sequence of RET51 responsible of

the interaction with FKBP52 contains nearly 20 lysine residues (Fig. 6C), all putative substrates for ubiquitin ligation.

In order to define the functional role of the newly identified RET51/FKBP52 molecular complex, we hypothesized that the complex should exert its signalling pathway in the adult neurons of the CNS. To prove this hypothesis, through an indirect approach, we provided genetic and molecular evidence for a direct involvement of the RET51/FKBP52 complex in EO-PD, indicating a role for the complex in adult DA neurons. The growing line of evidence connecting both RET51 and FKBP52 with the maintenance CNS and neuroprotection, combined with our new findings, prompted us to screen a group of 30 patients EO-PD for *RET* and *FKBP52* genes. We here report ever the first case of a compound heterozygous carrying the point mutation c.25934 G > A (p.Arg144His) in *RET* gene and the c.6962\_6964delGAG (p.Glu422del) deletion in the *FKBP52* gene.

The RET51 R144H substitution maps within the extracellular domain of the receptor (6). Both loss-of-function and gain-of-function mutations have been described in the *RET* gene and most of them map within the extracellular domain of the protein (7). The majority of the extracellular mutations of *RET* associated with a loss of function produces aberrant form of the protein (47). On this basis, we tested the effect of the R144H amino acidic substitution on the expression and phosphorylation level of RET51-R144H protein in comparison with wtRET51. RET51-R144H protein expression level was comparable with wt protein (Fig. 5E).

To date, no pathogenic mutations of *FKBP52* have been reported. We tested the effect of the FKBP52 E422 deletion on the expression level of FKBP52 $\Delta$ E422 protein in comparison with wtFKBP52. The FKBP52 $\Delta$ E422 expression level was comparable with the wild-type protein. The FKBP52 $\Delta$ E422 deletion maps within the putative calmodulin-binding site of the protein that, according to our results, should not be required for the interaction with RET51 (Fig. 2C and D). Different studies have shown that functional mutations of FKBP52 alter its ability to bind molecular partners (i.e. HSP90) even though these mutations map outside the interaction domain (34). Starting from this assumption, we tested the effect of the newly identified *RET* and *FKBP52* mutations on the formation of RET51/FKBP52 complex. Our results showed that only the presence of both the RET51 R144H substitution and FKBP52 E422 deletion alters the formation of the RET51/FKBP52 complex (Fig. 5F). This molecular finding may provide the explanation for the PD phenotype in our compound heterozygous patient. Hence, it indicates that only the presence of both mutations may determine a pathogenic effect disrupting the RET51/FKBP52 complex and its downstream signalling pathway.

In conclusion, in this study, we show and characterize the novel molecular complex RET51/FKBP52, and for the first time, we suggest a putative causative effect of RET51 in PD. Moreover, we propose a novel molecular pathway that may be responsible for the onset, progression and development of the disease and should contribute to solving the pathogenetic architecture of PD. Screening of a larger cohort of patients, not only displaying an EO phenotype, is ongoing in order to both support the evidence of the involvement of the RET51/FKBP52 molecular complex in PD and then to

clarify if its derangement is restricted to a specific group of patients.

## MATERIALS AND METHODS

### Split ubiquitin yeast two-hybrid assay

The construct used as bait is based on the previously described vector pCUP1-ubc9-CRU (17), which is the low-copy yeast–*Escherichia coli* shuttle vector that carries a CRU locus, a copper-dependent promoter (PCUP1) for bait expression and an HIS3 marker. The human coding sequence corresponding to the RET51 intracellular domain excluding transmembrane segment was amplified by PCR from a human whole-brain cDNA preparation (Clontech, Palo Alto, CA, USA) using primers RET51-FW (5'-AATTGTGCGACATGCACTGCTAC CACAAGTTTGCCC-3') and RET51-RV (5'-AGCGGCCGC ACTATCAAACGTGTCCATTA-3'), introducing *SalI* and *NotI* restriction sites, respectively. The RET51 PCR product was used to replace the Ubc9 insert in pCUP1-ubc9-CRU using *SalI*–*NotI* restriction sites, resulting in pCUP1-Fz1-CRU. The employed human fetal brain *Nubi*–split ubiquitin cDNA library was kindly provided by GPC Biotech AG (Munich, Germany). The cDNA library screening and the selection of positive clones were performed as previously described (17).

### Cell cultures and transfections

HEK293 and NEURO2A cells were grown at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in DMEM (Celbio, Milan, Italy) supplemented with 10% FBS (Euroclone, Wetherby, West York, UK), 2 mM L-glutamine (Euroclone), 100 U/ml penicillin and 100 µg/ml streptomycin (Euroclone). HEK 293 and NEURO2A cells were plated on 60 mm Petri dishes 1 day before transfection performed using Lipofectamine™ Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. In single and double transfections, 5 and 8 mg of plasmid DNA, respectively, were used. The cells were harvested 48 h after transfection and subsequently lysated.

### cDNA constructs

Total RNA was extracted from SH-SY5Y neuroblastoma cell line using Trizol™ Reagent (Invitrogen) and retrotranscribed using SuperScript™ III Reverse Transcriptase (Invitrogen). The human FKBP52 coding sequence was amplified from the cDNA obtained by from SH-SY5Y cell line PCR using primers *EcoRV* FKBP52-F (5'-CCCCAGATATCGGATAA ACGGCGCGC-3') and *XhoI* FKBP52-R (5'-GGACTCG AGCTGCTTCTGTCTCCACCT-3'), introducing *EcoRV* and *XhoI* restriction sites, respectively. The PCR product double-digested with *EcoRV* and *XhoI* was cloned into pcDNA3.1-myc/His expression vector (Invitrogen). One-base mutation T143A was introduced into wild-type FKBP52 clones using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using the primers: FKBP52\_T143A\_F (5'-GGGAGAAGATCTGgCGGAAGAGGAAGATGGC-3') and FKBP52\_T143A\_R (5'-GCCATCTTCTGTTCCGcC

AGATCTTCTCCC-3'). The construct containing tag-free FKBP52 was produced by subcloning FKBP52 coding sequence from pcDNA3.1myc/His expression vector (Invitrogen) into pcDNA3.1(+) (Invitrogen) using the primers *EcoRV* FKBP52-F (5'-CCCCAGATATCGGATAA ACGGCGCGC-3') and *XhoI* ext FKBP52 (5'-GGTGACCTCGAGTCATG CTTCTGTCTCCACC-3').

The deleted constructs of FKBP52 were generated by PCR amplification using the primers specified below in the following combination: FKBP52(ΔTPR3) containing amino acids 1–348 using primers 1 and 3, FKBP52(Δ TPR2-3) containing amino acids 1–300 using primers 1 and 4, FKBP52(ΔTPR1-2-3) containing amino acids 1–260 using primers 6 and 7, FKBP52(FK2-TPR1) containing amino acids 145–300 using primers 5 and 4, FKBP52(FK2-TPR1-2) containing amino acids 145–348 using primers 5 and 3, FKBP52(ΔFK1) containing amino acids from 145 to stop codon using primers 5 and 2 and FKBP52(ΔFK1-FK2) containing amino acids from 261 to stop codon using primers 8 and 9.

- (1) *EcoRV*\_FKBP52-F (5'-CCCCAGATATCGGATAA ACG GCGCGC-3')
- (2) *XhoI*\_FKBP52-R (5'-GGACTCGAGCTGCTTCTGTCTC CACCT-3')
- (3) FKΔTPR3\_ *XhoI*\_R (5'-CGTTGTctcgCCAGTTCTAG-3')
- (4) FKΔTPR2-3\_ *XhoI*\_R (5'-GCTctcgagCCAGCCAAGACA CG-3')
- (5) FKc-ter\_ *EcoRV*\_F (5'-GAGAAGATCGATATCATGGA GG-3')
- (6) FKBP52\_1\_ *EcoRV*\_F (5'-GGGATATCCCATGACAGC CGAGGAGATG-3')
- (7) FKBP52\_2\_ *XhoI*\_R (5'-GGC TCG AGC CTCCCAAGA CTCCTTGGC-3')
- (8) FKBP52\_3\_ *EcoRV*\_F (5'-GGGATATCCCATGAATTCA GAAGAGAAG-3')
- (9) FKBP52\_4\_ *XhoI*\_R (5'-GGC TCG AGC TGCTTCTGT CTCCACCTG-3')

The PCR product double-digested with *EcoRI* and *XhoI* was cloned into pcDNA3.1myc/His expression vector (Invitrogen). The plasmids pJ7Ω-RET51 (RET51-FL), pJ7Ω-RET9 (RET9-FL) and pJ7Ω-RET51-D707N (RET51-D707N) were kindly provided by P. Mehlen [Apoptosis Cancer and Development Laboratory, Lyon, France (12)]. One-base mutations were introduced into wild-type RET51 and FKBP52 clones using the QuikChange site-directed mutagenesis kit (Stratagene).

The deleted constructs RET-TK containing amino acids 1–999 of RET and RET-IUX (1-725) were generated by PCR amplification using as forward primer a unique primer for both the constructs RET\_ *HindIII*\_F (5'-GGGATATCCCATG GCGAAGGCGACG-3') and as reverse primers RET\_IUX-TA\_ *XbaI*\_R (5'-GGTCTAGAAGAACCAAGTTCTTCCGAG G-3'), RET\_TK\_ *XbaI*\_R (5'-GGTCTAGAGCCGCAAACAC CGGCCTTTTGTCCG-3'), respectively. The PCR products double-digested with *HindIII* and *XbaI* were cloned in frame into pcDNA3.1/V5-His© TOPO® TA Expression Vector (Invitrogen). RET-PRO (708-1017) was generated using primers RET\_ *HindIII*\_F (5'-GGGATATCCCATGGCGAA GGCGACG-3') and *NotI*\_PRORET\_R (5'-GCGGCCGCGTC

CAAGTAGTCTCTCCT-3') and cloned into pcDNA3.1myc/His expression vector (Invitrogen).

All plasmid sequences were verified by direct sequencing using Big Dye v3.1 kit according to the manufacturer's instruction and loaded onto a 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA).

### Immunoprecipitation assays and WB analysis

The cells were lysed in 100  $\mu$ l of IP buffer 1 $\times$  (Sigma-Aldrich, St Louis, MO, USA) plus protease Inhibitors (Roche Diagnostics, Mannheim, Germany).

Fifty microlitres of the cell lysates were incubated at 4°C for 4 h mixing the sample with 1 mg of primary antibody. After 4 h incubation, Protein G beads equilibrate in IP buffer (Sigma-Aldrich) were added to the cell lysate and incubated overnight at 4°C, mixing the sample. The bead pellets were washed three times with 500  $\mu$ l of IP buffer. After the last wash, 40  $\mu$ l of 1 $\times$  Laemmli (8% SDS; 20% glycerol; 125 mM Tris-HCl, pH 6.8; 0.0025% Bromophenol blue; 10% 2-mercaptoethanol) sample buffer was added to the bead pellet. After boiling for 5 min and centrifuging at a maximum speed, the supernatant was collected. Immunoprecipitates and total cell lysates were separated by SDS-PAGE and transferred to the nitrocellulose membrane. Immunodetection and IP were performed using specific primary antibodies for RET [Ret (C-20) sc-1290; Ret (H-300) sc-13104] and HSP90 [HSP 90 $\alpha$ / $\beta$  (H-114) sc-7947], from Santa Cruz Biotechnology and for the myc and V5 epitopes R950-25 and R960-25 from Invitrogen. The secondary antibodies used were HRP-conjugate anti-mouse or anti-rabbit (Sigma-Aldrich) HRP-conjugate and AP-conjugate anti-mouse (Invitrogen). Development was performed using Millipore ECL system.

### Mutation screening

A total of 30 PD patients with onset before 50 years or a positive family history for the disease were studied. The group comprised 26 patients with EO-PD either sporadic or familial, 13 males and two females, with an average age at onset of 41 years (20–50) and four patients with LO-PD and a family history of autosomal dominant PD (all males, age at onset ranging from 53 to 61 years).

All patients gave written informed consent and the relevant ethical committees have approved the study. The DNAs were extracted from blood using following the protocol previously described (44). The whole *RET* and *FKBP52* coding sequences were analysed for mutations by direct sequencing using Big Dye v3.1 and 3730 automated sequencer (Applied Biosystems). The PCR conditions are available under request. *In silico* analysis was performed using PolyPhen (43). The allele separation for the validation of *FKBP52* deletion was obtained by cloning the PCR product in the pcDNA3.1/V5-His $\text{\textcircled{C}}$  TOPO $\text{\textcircled{R}}$  TA Expression Vector (Invitrogen).

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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*Conflict of Interest statement.* None declared.

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### REFERENCES

- Durbec, P.L., Larsson-Blomberg, L.B., Schuchardt, A., Costantini, F. and Pachnis, V. (1996) Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. *Development*, **122**, 349–358.
- Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F. and Pachnis, V. (1994) Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature*, **367**, 380–383.
- Trupp, M., Belluardo, N., Funakoshi, H. and Ibanez, C.F. (1997) Complementary and overlapping expression of glial cell line-derived neurotrophic factor (GDNF), c-ret proto-oncogene, and GDNF receptor-alpha indicates multiple mechanisms of trophic actions in the adult rat CNS. *J. Neurosci.*, **17**, 3554–3567.
- Tsuzuki, T., Takahashi, M., Asai, N., Iwashita, T., Matsuyama, M. and Asai, J. (1995) Spatial and temporal expression of the ret proto-oncogene product in embryonic, infant and adult rat tissues. *Oncogene*, **10**, 191–198.
- Anders, J., Kjar, S. and Ibanez, C.F. (2001) Molecular modeling of the extracellular domain of the RET receptor tyrosine kinase reveals multiple cadherin-like domains and a calcium-binding site. *J. Biol. Chem.*, **276**, 35808–35817.
- Takahashi, M., Buma, Y. and Hiai, H. (1989) Isolation of ret proto-oncogene cDNA with an amino-terminal signal sequence. *Oncogene*, **4**, 805–806.
- Arighi, E., Borrello, M.G. and Sariola, H. (2005) RET tyrosine kinase signaling in development and cancer. *Cytokine Growth Factor Rev.*, **16**, 441–467.
- Pasini, B., Ceccherini, I. and Romeo, G. (1996) RET mutations in human disease. *Trends Genet.*, **12**, 138–144.
- Mulligan, L.M., Marsh, D.J., Robinson, B.G., Schuffenecker, I., Zedenius, J., Lips, C.J., Gagel, R.F., Takai, S.I., Noll, W.W., Fink, M. *et al.* (1995) Genotype-phenotype correlation in multiple endocrine neoplasia type 2: report of the International RET Mutation Consortium. *J. Intern. Med.*, **238**, 343–346.
- Romeo, G., Ronchetto, P., Luo, Y., Barone, V., Seri, M., Ceccherini, I., Pasini, B., Boccardi, R., Lerone, M., Kaariainen, H. *et al.* (1994) Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. *Nature*, **367**, 377–378.
- Mehlen, P. and Bredesen, D.E. (2004) The dependence receptor hypothesis. *Apoptosis*, **9**, 37–49.
- Bordeaux, M.C., Forcet, C., Granger, L., Corset, V., Bidaud, C., Billaud, M., Bredesen, D.E., Edery, P. and Mehlen, P. (2000) The RET

- proto-oncogene induces apoptosis: a novel mechanism for Hirschsprung disease. *EMBO J.*, **19**, 4056–4063.
13. Tahira, T., Ishizaka, Y., Itoh, F., Sugimura, T. and Nagao, M. (1990) Characterization of ret proto-oncogene mRNAs encoding two isoforms of the protein product in a human neuroblastoma cell line. *Oncogene*, **5**, 97–102.
  14. Tansey, M.G., Baloh, R.H., Milbrandt, J. and Johnson, E.M. Jr (2000) GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. *Neuron*, **25**, 611–623.
  15. Tsui-Pierchala, B.A., Milbrandt, J. and Johnson, E.M. Jr (2002) NGF utilizes c-Ret via a novel GFL-independent, inter-RTK signaling mechanism to maintain the trophic status of mature sympathetic neurons. *Neuron*, **33**, 261–273.
  16. Trupp, M., Raynoschek, C., Belluardo, N. and Ibanez, C.F. (1998) Multiple GPI-anchored receptors control GDNF-dependent and independent activation of the c-Ret receptor tyrosine kinase. *Mol. Cell. Neurosci.*, **11**, 47–63.
  17. Dimberger, D., Messerschmid, M. and Baumeister, R. (2008) An optimized split-ubiquitin cDNA-library screening system to identify novel interactors of the human Frizzled 1 receptor. *Nucleic Acids Res.*, **36**, e37.
  18. Pierchala, B.A., Milbrandt, J. and Johnson, E.M. Jr (2006) Glial cell line-derived neurotrophic factor-dependent recruitment of Ret into lipid rafts enhances signaling by partitioning Ret from proteasome-dependent degradation. *J. Neurosci.*, **26**, 2777–2787.
  19. Kramer, E.R., Aron, L., Ramakers, G.M., Seitz, S., Zhuang, X., Beyer, K., Smidt, M.P. and Klein, R. (2007) Absence of Ret signaling in mice causes progressive and late degeneration of the nigrostriatal system. *PLoS Biol.*, **5**, e39.
  20. Kish, S.J., Shannak, K. and Hornykiewicz, O. (1988) Uneven pattern of dopamine loss in the striatum of patients with idiopathic Parkinson's disease. Pathophysiologic and clinical implications. *N. Engl. J. Med.*, **318**, 876–880.
  21. de Rijk, M.C., Launer, L.J., Berger, K., Breteler, M.M., Dartigues, J.F., Baldereschi, M., Fratiglioni, L., Lobo, A., Martinez-Lage, J., Trenkwalder, C. *et al.* (2000) Prevalence of Parkinson's disease in Europe: a collaborative study of population-based cohorts. Neurologic Diseases in the Elderly Research Group. *Neurology*, **54**, S21–S23.
  22. Priyadarshi, A., Khuder, S.A., Schaub, E.A. and Priyadarshi, S.S. (2001) Environmental risk factors and Parkinson's disease: a metaanalysis. *Environ. Res.*, **86**, 122–127.
  23. Rocca, W.A., McDonnell, S.K., Strain, K.J., Bower, J.H., Ahlskog, J.E., Elbaz, A., Schaid, D.J. and Maraganore, D.M. (2004) Familial aggregation of Parkinson's disease: The Mayo Clinic family study. *Ann. Neurol.*, **56**, 495–502.
  24. Simon, D.K., Lin, M.T. and Pascual-Leone, A. (2002) 'Nature versus nurture' and incompletely penetrant mutations. *J. Neurol. Neurosurg. Psychiatry*, **72**, 686–689.
  25. Bonifati, V., Dekker, M.C., Vanacore, N., Fabbrini, G., Squitieri, F., Marconi, R., Antonini, A., Brustenghi, P., Dalla Libera, A., De Mari, M. *et al.* (2002) Autosomal recessive early onset parkinsonism is linked to three loci: PARK2, PARK6 and PARK7. *Neurol. Sci.*, **23** (Suppl. 2), S59–S60.
  26. Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. and Shimizu, N. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature*, **392**, 605–608.
  27. Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G. *et al.* (2004) Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science*, **304**, 1158–1160.
  28. Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K. *et al.* (2000) Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.*, **25**, 302–305.
  29. Zhang, L., Shimoji, M., Thomas, B., Moore, D.J., Yu, S.W., Marupudi, N.I., Torp, R., Torgner, I.A., Ottersen, O.P., Dawson, T.M. *et al.* (2005) Mitochondrial localization of the Parkinson's disease related protein DJ-1: implications for pathogenesis. *Hum. Mol. Genet.*, **14**, 2063–2073.
  30. Eriksen, J.L., Wszolek, Z. and Petrucelli, L. (2005) Molecular pathogenesis of Parkinson disease. *Arch. Neurol.*, **62**, 353–357.
  31. Vargiolu, M., Fusco, D., Kurelac, I., Dimberger, D., Baumeister, R., Morra, I., Melcarne, A., Rimondini, R., Romeo, G. and Bonora, E. (2009) The tyrosine kinase receptor RET interacts *in vivo* with aryl hydrocarbon receptor-interacting protein to alter survivin availability. *J. Clin. Endocrinol. Metab.*, **94**, 2571–2578.
  32. Sinars, C.R., Cheung-Flynn, J., Rimerman, R.A., Scammell, J.G., Smith, D.F. and Clardy, J. (2003) Structure of the large FK506-binding protein FKBP51, an Hsp90-binding protein and a component of steroid receptor complexes. *Proc. Natl Acad. Sci. USA*, **100**, 868–873.
  33. Steiner, J.P., Dawson, T.M., Fotuhi, M., Glatt, C.E., Snowman, A.M., Cohen, N. and Snyder, S.H. (1992) High brain densities of the immunophilin FKBP colocalized with calcineurin. *Nature*, **358**, 584–587.
  34. Davies, T.H. and Sanchez, E.R. (2005) Fkbp52. *Int. J. Biochem. Cell Biol.*, **37**, 42–47.
  35. Gold, B.G. (1999) FK506 and the role of the immunophilin FKBP-52 in nerve regeneration. *Drug Metab. Rev.*, **31**, 649–663.
  36. Ruan, B., Pong, K., Jow, F., Bowlby, M., Crozier, R.A., Liu, D., Liang, S., Chen, Y., Mercado, M.L., Feng, X. *et al.* (2008) Binding of rapamycin analogs to calcium channels and FKBP52 contributes to their neuroprotective activities. *Proc. Natl Acad. Sci. USA*, **105**, 33–38.
  37. Wu, B., Li, P., Liu, Y., Lou, Z., Ding, Y., Shu, C., Ye, S., Bartlam, M., Shen, B. and Rao, Z. (2004) 3D structure of human FK506-binding protein 52: implications for the assembly of the glucocorticoid receptor/Hsp90/immunophilin heterocomplex. *Proc. Natl Acad. Sci. USA*, **101**, 8348–8353.
  38. Carrello, A., Allan, R.K., Morgan, S.L., Owen, B.A., Mok, D., Ward, B.K., Minchin, R.F., Toft, D.O. and Ratajczak, T. (2004) Interaction of the Hsp90 cochaperone cyclophilin 40 with Hsc70. *Cell. Stress Chaperones*, **9**, 167–181.
  39. Miyata, Y., Chambraud, B., Radanyi, C., Leclerc, J., Lebeau, M.C., Renoir, J.M., Shirai, R., Catelli, M.G., Yahara, I. and Baulieu, E.E. (1997) Phosphorylation of the immunosuppressant FK506-binding protein FKBP52 by casein kinase II: regulation of HSP90-binding activity of FKBP52. *Proc. Natl Acad. Sci. USA*, **94**, 14500–14505.
  40. Myers, S.M. and Mulligan, L.M. (2004) The RET receptor is linked to stress response pathways. *Cancer Res.*, **64**, 4453–4463.
  41. Arighi, E., Popsueva, A., Degl'Innocenti, D., Borrello, M.G., Carniti, C., Perala, N.M., Pierotti, M.A. and Sariola, H. (2004) Biological effects of the dual phenotypic Janus mutation of ret cosegregating with both multiple endocrine neoplasia type 2 and Hirschsprung's disease. *Mol. Endocrinol.*, **18**, 1004–1017.
  42. Li, L., Su, Y., Zhao, C., Zhao, H., Liu, G., Wang, J. and Xu, Q. (2006) The role of Ret receptor tyrosine kinase in dopaminergic neuron development. *Neuroscience*, **142**, 391–400.
  43. Sunyaev, S., Ramensky, V. and Bork, P. (2000) Towards a structural basis of human non-synonymous single nucleotide polymorphisms. *Trends Genet.*, **16**, 198–200.
  44. Chambraud, B., Belabes, H., Fontaine-Lenoir, V., Fellous, A. and Baulieu, E.E. (2007) The immunophilin FKBP52 specifically binds to tubulin and prevents microtubule formation. *Faseb J.*, **21**, 2787–2797.
  45. Kazlauskas, A., Poellinger, L. and Pongratz, I. (2000) The immunophilin-like protein XAP2 regulates ubiquitination and subcellular localization of the diosin receptor. *J. Biol. Chem.*, **275**, 41317–41324.
  46. Hicke, L., Scubert, H.L. and Hill, C.P. (2005) Ubiquitin-binding domains. *Nat. Rev. Mol. Cell Biol.*, **6**, 610–621.
  47. Weissman, A.M. (2001) Themes and variations on ubiquitylation. *Nat. Rev. Mol. Cell Biol.*, **2**, 169–178.
  48. Lahiri, D.K. and Schnabel, B. (1993) DNA isolation by a rapid method from human blood samples: effects of MgCl<sub>2</sub>, EDTA, storage time, and temperature on DNA yield and quality. *Biochem. Genet.*, **31**, 321–328.