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## **Germline mutation in the *NBRI* gene involved in autophagy detected in a family with renal tumors**

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## **Abstract**

Hereditary Renal Cell Carcinomas (RCC) are caused by mutations in predisposing genes, the major ones including *VHL*, *FLCN*, *FH* and *MET*. However, many families with inherited RCC have no germline mutation in these genes. Using Whole Exome Sequencing on germline DNA from a family presenting three different histological renal tumors (an angiomyolipoma, a clear-cell RCC and an oncocytic papillary RCC), we identified a frameshift mutation in the *neighbor of BRCA1 gene 1 (NBR1)*, segregating with the tumors. *NBR1* encodes a cargo receptor protein involved in autophagy. Genetic and functional analyses suggested a pathogenic impact of the mutation. Indeed, functional study performed in renal cell lines showed that the mutation alters NBR1 interactions with some of its partners (such as p62/SQSTM1), leading to a dominant-negative effect. This results in an altered autophagic process and an increased proliferative capacity in renal cell lines. Our study suggests that *NBR1* may be a new predisposing gene for RCC, however its characterization needs to be further investigated in order to confirm its role in renal carcinogenesis.

## **Keywords:**

Renal tumors, predisposition, NBR1, autophagy, dominant negative effect

## Introduction

With more than 400,000 new cases diagnosed and 175,000 deaths each year worldwide, kidney cancer is the 14<sup>th</sup> most common cancer [1]. Renal Cell Carcinomas (RCC), whose major histological subtypes are clear-cell (ccRCC 75%), papillary (pRCC 15%) and chromophobe (chRCC 5%) carcinomas, account for 90-95% of all kidney tumors. Among other subtypes of tumors, angiomyolipomas (AML) are the most frequent mesenchymal tumors [2][3]. Renal tumors can occur in a familial context, usually transmitted in an autosomal dominant mode. The discovery of predisposing genes has led to a better understanding of renal carcinogenesis [4]. Moreover, knowledge of these genes allows the identification of individuals at risk and early detection of tumors, often multifocal and bilateral in a familial context [5]. Today, a dozen of RCC predisposing genes have been identified, such as *VHL*, responsible for the von Hippel-Lindau disease and ccRCC development [6]. Overall, these RCC genes, also altered in renal sporadic cases, encode proteins involved in the mTOR proliferation or the hypoxia response pathways. The latter is regulated by the Hypoxia Inducible transcription Factors (HIF1, HIF2) that play a central role in carcinogenesis through their involvement in many cellular processes [7][8]. The characterization of both pathways in renal oncogenesis has led to the development of several targeted therapies [9].

However, many families with multiple cases of renal tumors still remain without any identified predisposing gene. We were particularly interested in a family comprising an AML, a ccRCC and an oncocytic pRCC. This family was tested negative for the main RCC genes (*VHL*, *FLCN*, *FH*, *MET*) and also for *TSC1* and *TSC2*, responsible for Tuberous Sclerosis in which AML frequently occur. In order to search for the predisposing gene in this family, we performed a Whole Exome Sequencing which allowed us to detect a germline mutation of the *neighbor of BRCA1 gene 1* (*NBRI*) encoding an autophagy cargo receptor.

## Materials and methods

### *Patient and tumor samples*

The family has given informed consent for research and blood samples. Study was first conducted on siblings II.1 and II.2 who were diagnosed with a renal tumor respectively at 44 and 58 years old (Fig.1A). In one of their sisters (II.4) an infracentimetric indeterminate renal lesion was fortuitously detected at 53 years old and is stable on last imaging at age 66. The last siblings (II.3 and II.5) had a normal renal imaging at age 63 and 68 respectively. Their mother I.2 was diagnosed with a ccRCC at 60 years old and deceased from metastases after 24 months of follow-up. Surgical specimens (renal tumor tissues and adjacent normal tissues) of the 3 patients were reviewed by two senior uropathologists (SF-VV). Two specimens were fixed in 10% formalin; one tumor was fixed in Bouin liquid, and one tumor was also frozen.

### *Genetic analysis*

Whole Exome Sequencing (WES) was performed by Gustave Roussy plateforme ([as described in supplementary material and methods](#)). Sanger sequencing was used to confirm the *NBR1* mutation in DNA extracted from blood or tumor samples, and to analyze RNA from the frozen pRCC.

### *Immunohistochemistry*

Immunohistochemical staining (IHC) was performed using the automated system BOND RX (Leica Biosystems) on the 3 tumor tissues, adjacent normal renal parenchyma and one normal renal tissue without adjacent tumor. Sections were cut at 4µm and deparaffinized. Endogenous peroxidase activity was blocked with H<sub>2</sub>O<sub>2</sub> solution. Then, samples were incubated with the NBR1 antibody (Atlas Antibodies) for 30min at RT. Antibody detection was done with the Bond Polymer Refine Detection kit as recommended (Leica Biosystems).

### *Functional studies*

Normal (HKC8) and tumor (786-O, ACHN) renal cell lines were used (as described in [supplementary material and methods](#)). Briefly, these cells were transiently transfected with pcDNA3, pcDNA3-HA-NBR1 (courtesy by Terje Johansen) and pcDNA3-HA-NBR1 E528fs\*17 to analyze wild-type and mutated NBR1 expression by Western Blot and to quantify the effect of NBR1 mutation on cell proliferation by MTT proliferation and colony formation assays. To analyze wild-type and mutated NBR1 protein interaction and localization, renal cells were transfected with pMXspuro-GFP-NBR1 and pMXspuro-mCherry-NBR1 (Addgene), pMXspuro-GFP-NBR1 E528fs\*17 and pMXspuro-mCherry-NBR1 E528fs\*17. For autophagy study, cells were cultured for 2h in DMEM (control condition), HBSS (starvation condition) or HBSS and Chloroquine (lysosome inhibitor).

### *Statistical analysis*

All data were generated from at least 3 independent experiments and are presented as means  $\pm$  s.d. or s.e.m. Statistical differences were assessed thanks to the Student's paired t-test or ANOVA with Tukey's post-hoc test with the GraphPad Prism software. \*  $p < 0.05$  was considered significant.

## Results and Discussion

The family studied includes three cases of renal tumors with the particularity to be associated with distinct histological subtypes (Fig.1A). The patient II.1 developed an AML without epithelioid component, and her brother (II.2) an oncocytic papillary carcinoma: this is a rare variant of pRCC, defined by papillary structures lined by large cells with abundant granular eosinophilic cytoplasm resembling oncocytes. Their mother (I.2) developed a typical ccRCC. The coexistence of several histological subtypes within the same family is rare since in general a specific histological subtype is associated with a predisposing gene, and is only seen in syndromes such as Birt-Hogg-Dubé or Tuberous Sclerosis [4].

We searched by WES for the presence of potentially pathogenic variants in genes involved in kidney biogenesis or carcinogenesis. No mutation in genes known to predispose to renal tumors was detected. However, these analyses allowed us to identify a frameshift mutation in the *NBR1* gene, encoding a cargo receptor protein involved in macroautophagy [10]. The presence of the c.1581dupA (p.E528fs\*17) mutation was verified by Sanger sequencing in the three affected individuals (I.2, II.1 and II.2), and was not found in II.3 and II.5 who are unaffected and in II.4 with the small indeterminate renal lesion (Fig.1B). This frameshift was never described in databases (gnomAD, COSMIC). However, somatic mutations of *NBR1* have been reported in ccRCC and pRCC at 2.08% and 1.78% respectively in the TCGA database.

We observed by immunohistochemistry in normal renal tissues a weak cytoplasmic staining for NBR1 of tubules and podocytes (Fig.1C). We detected NBR1 expression in smooth muscle cells and adipocytes in AML (II.1), and in oncocytic cells in the pRCC (II.2). No expression was noted in the ccRCC (I.2). It seems that there is a conservation of NBR1 expression in tumor cells except the ccRCC case. However, lack of NBR1 expression in this tumor should be interpreted with caution because of Bouin's fixation.

The mutation, located in exon 13/21, leads to a premature stop codon in the middle of the NBR1 protein (Fig.1D). As RNA quality is finely controlled by the nonsense-mediated mRNA decay

(NMD), mRNAs comprising a premature stop codon are generally eliminated [11]. The efficiency of this system can be modulated by cellular stress, such as hypoxia or reactive oxygen species (ROS), and may lead to the stabilization of NMD-targeted mRNA [12]. We hypothesized that the NMD might be inefficient, leading to the presence of a stable truncated NBR1 protein. Indeed, sequencing of RT-PCR products from the brother II.2 frozen tumor revealed the presence of mRNA carrying the mutation (Fig.1E), suggesting that the mutation does not alter mRNA stability and may allow the synthesis of NBR1 truncated form.

The role of NBR1 in autophagy is to send damaged organelles, in particular peroxisomes, and protein aggregates towards autophagosomes, by its interaction with the LC3 membrane component, where they will be sequestered and then degraded following fusion with lysosomes [13][14]. Previous studies have shown the involvement of autophagy in renal carcinogenesis, with the inhibition of genes essential for autophagosome expansion, the over-expression of p62 (encoded by *SQSTM1* in 5q chromosome often gained in RCC), or with the deregulation of the mTOR pathway [15][16][17]. The *NBR1* mutation induces the loss of important interacting domains (Fig.1D), such as LIR (LC3) and ubiquitin residues (UBA) domains suggesting a pathogenic impact of this mutation. Thus, we evaluated the possible deleterious impact of this mutation in autophagy in renal cell lines. As NBR1 and its partner p62 function as homo- and/or hetero-oligomers to bring cellular components to phagophores [10], we explored the capacity of the truncated NBR1 protein to interact with itself and some of its partners. By immunoprecipitation, we showed that the mutated NBR1 is able to interact with its wild-type isoform, as well as with p62 (Fig.2A). This result suggests that during autophagy activation, mutated NBR1 can interact with some of its partners. However, the lack of its C-terminal domains could alter its function and allow the mutant form of NBR1 to act as a dominant-negative. Thus, we compared p62 degradation (as a marker of autophagy) in renal cells overexpressing wild-type and mutated NBR1 after starvation-induced autophagy. With empty vector and wild-type NBR1, p62 level decreases after 2h of starvation while it increases with mutated NBR1. This decrease is only transient in presence of the empty vector,

since p62 level returns to basal rate for longer starvation times. By contrast, in cells expressing wild-type NBR1, p62 level remains low and does not return to basal rate. In presence of mutated NBR1, a transient decrease is detected after 8h, but p62 level is always higher than in control or wild-type NBR1 cells (Fig.2B-2C). Therefore, it seems that p62 turnover in cells expressing mutated NBR1 is delayed and reduced compared to cells with empty vector or expressing wild-type NBR1. Moreover, in tumor patient samples, we observed by IHC a granular p62 staining, and with a specific PMP70 staining, a peroxisome condensation, suggesting an altered p62 degradation and pexophagy (Fig.S1-S2, [in supplementary data](#)). As control, chloroquine treatment (an inhibitor of late autophagy) also prevented the degradation of p62 in presence of wild-type and mutated NBR1, indicating that autophagosome degradation is not affected (Fig.S3). Furthermore, we analyzed by immunofluorescence NBR1 shipment to autophagosomes. During starvation-induced autophagy, we observed that mutated NBR1 seems to be able to colocalize with LC3B (Fig.S4) and p62 (Fig.S5) like wild-type NBR1. Our results suggest that despite its colocalization with autophagosomes, it seems that mutated NBR1 could prevent the cellular components delivery to autophagosomes during autophagy.

Then, we compared the proliferative capacity of normal and tumor renal cells after transfection with wild-type or mutated *NBR1* expression vectors. Proliferation assays showed an increased proliferation capacity for mutated NBR1 overexpressing 786-O and ACHN cells (Fig.2D), and a higher number of colonies compared to control or NBR1 wild-type transfected cells (Fig.2E and data not shown). By contrast, no difference in cell proliferation and colony formation was observed in HKC8 cells transfected with wild-type or mutated *NBR1* vectors (data not shown). Thus, the increased proliferation capacity of the mutated NBR1 seems to be specific for tumor cells. Furthermore, Marsh *et al.* recently reported that aberrant NBR1 accumulation promotes metastasis growth in a mouse model of breast cancer [18]. We can speculate that mutated NBR1 is not degraded in patient II.2, leading to its accumulation and its partners and targets, conferring a stress that could initiate the oncogenesis.

Interestingly, it has been reported that following cleavage by an enterovirus, the NBR1 C-terminal form possesses a dominant negative function because of its ability to compete with wild-type isoforms of NBR1 and p62 for binding to ubiquitinated proteins and LC3 [19], while the N-terminal form stabilizes p62 and blocks its degradation by autophagy [20]. Moreover, NBR1, by its Pex5 binding domain, is particularly involved in pexophagy [10][14][21]. Interestingly, in normal kidney, peroxisomes are abundant and decrease in HIF2-positive RCCs [14][22][23]. Our results suggest that the ability of the mutated NBR1 to interact with itself and with p62, is associated with a delay in p62 turnover, that may lead to a defect in autophagy and pexophagy.

Finally, in order to evaluate the frequency of *NBR1* germline mutations, we performed targeted sequencing in 28 probands from families with inherited ccRCC. No mutation was detected in these patients (data not shown). As our series was relatively small and these families have developed only ccRCC without AML, it would be interesting to search for *NBR1* mutations in families with isolated AML and patients with clinical manifestations of Tuberous Sclerosis without mutation in *TSC* genes.

Regarding the sibling in the family, who presented a small indeterminate lesion in a kidney and does not carry the *NBR1* mutation, there is no indication to biopsy nor surgery as the lesion has not evolved since 13 years. We cannot therefore determine the exact nature of this lesion, but it could be linked to another event, not discovered yet, or to multifactorial transmission, leading to renal lesion.

Several laboratories are conducting research for new predisposing genes, as many families with RCC are not mutated in the known genes. However, since the discovery of *PBRM1* or *CDKN2B* [24][25], no new candidate gene has been reported [26], suggesting that the major RCC predisposing genes have been discovered. However, the number of families without identified genes is large suggesting that new RCC candidate genes may explain predisposition in only one or a few families. Given the involvement of autophagy and the deregulation of autophagy genes in RCC,

*NBR1* may be a new predisposing gene for RCC. However, its characterization needs to be further investigated in order to confirm its role in renal carcinogenesis.

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## **Author’s contribution**

FA performed the experiments, data acquisition, analysis and interpretation, and participated to write the manuscript. SC and PG: sample collection and Sanger sequencing. KP performed IHC. SF and VV: sample collection, interpretation of HE & IHC, and participated to write the manuscript. ND performed the WES; MD & BJ the bioinformatic analyses. SGi & BBP: sample collection, participation in the sequencing analysis and writing of the manuscript. BG critically reviewed and edited the manuscript. SR managed patient and clinical data acquisition, and revised the manuscript. FR conceptualized the study and critically reviewed the manuscript. SGa conceptualized the study, participated in data analysis and interpretation, and wrote the manuscript. All authors read and approved the final manuscript.

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## Captions

### **Figure 1. Identification of a germline mutation in the *NBR1* gene within a particular family.**

**A** Pedigree of a French family with familial renal tumors, followed by PREDIR center. Affected patients are in black. Birth date and age of diagnosis are indicated below symbols, with *NBR1* status after sequencing of blood DNA: wild-type (WT) or mutated (MUT).

**B** Chromatograms showing *NBR1* sequence of affected individuals from pedigree showed in A. The arrows show the frameshift mutation (c.1581dupA; p.E528fs\*17) in patient samples. The chromatograms correspond to germline (left panel) and tumor (right panel) DNA Sanger sequencing.

**C** *NBR1* immunostaining observed by immunohistochemistry for the three patient tumors and one normal kidney tissue. In the II.2 patient, we observed a weak *NBR1* cytoplasmic staining of proximal tubules and podocytes in the normal kidney and a cytoplasmic expression in tumor oncocyte cells in the oncocyte papillary Renal Cell Carcinoma. Immunohistochemistry was similar for all normal kidney tissue analyzed. In the II.1 patient angiomyolipoma, *NBR1* expression is present in smooth muscle cells and adipocytes. No expression was noted in the clear-cell Renal Cell Carcinoma in the patient I.2.

**D** Schematic representation of *NBR1* protein modified from [13]. The 966 amino acids isoform contains Phox and Bem1 domain (PB1), zinc finger (ZZ), 4 tryptophan (FW), glutamic acid rich region (polyE), LC3-interacting motif (LIR in red) and ubiquitin-binding domain (UBA in blue). The arrow indicates the location of the frameshift mutation leading to a truncated isoform of 544 amino acids lacking the poly E, LIR and UBA domains.

**E** Chromatograms showing *NBR1* sequence of tumor cDNA (after reverse transcription of tumor mRNA) by Sanger sequencing from frozen oncocyte pRCC diagnosed in II.2 patient.

**Figure 2. Functional study of the *NBR1* mutation.**

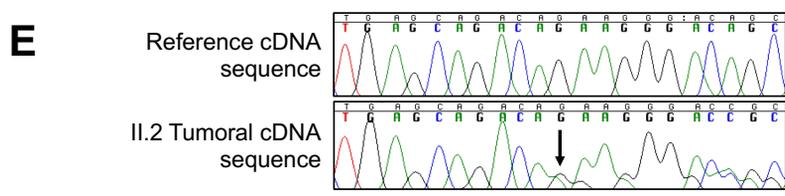
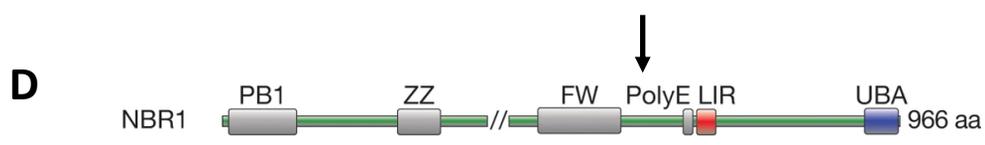
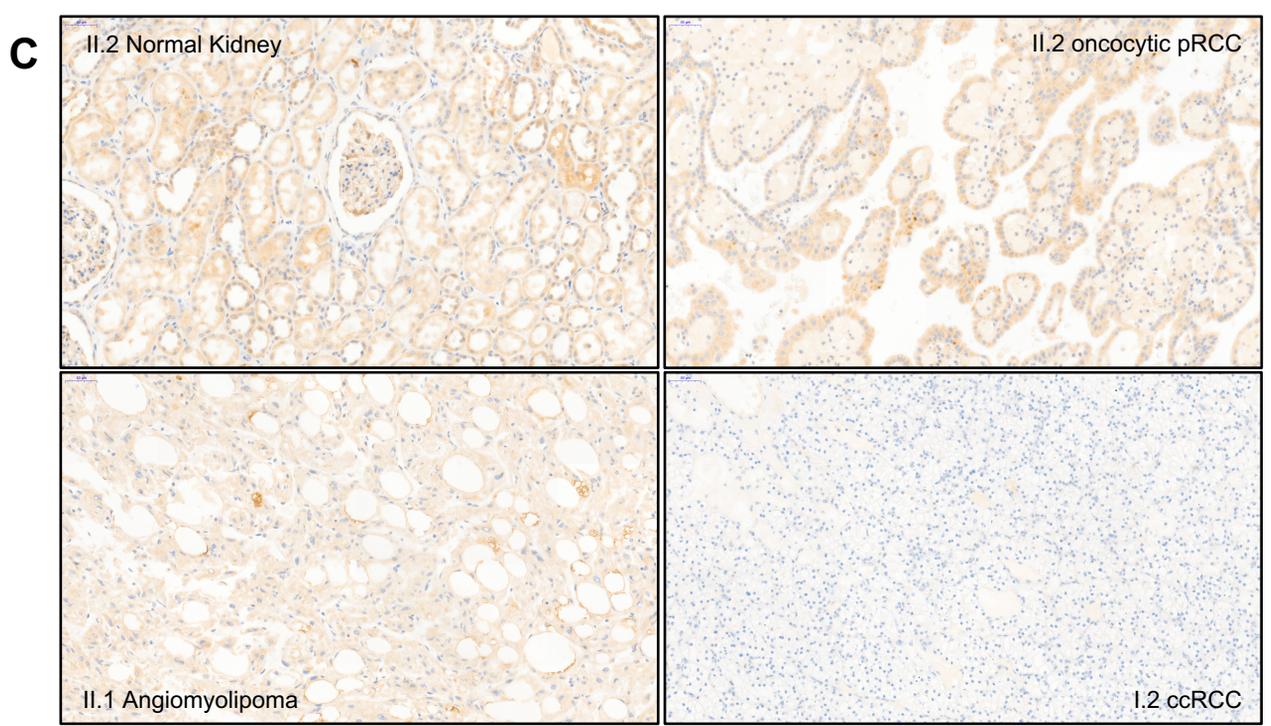
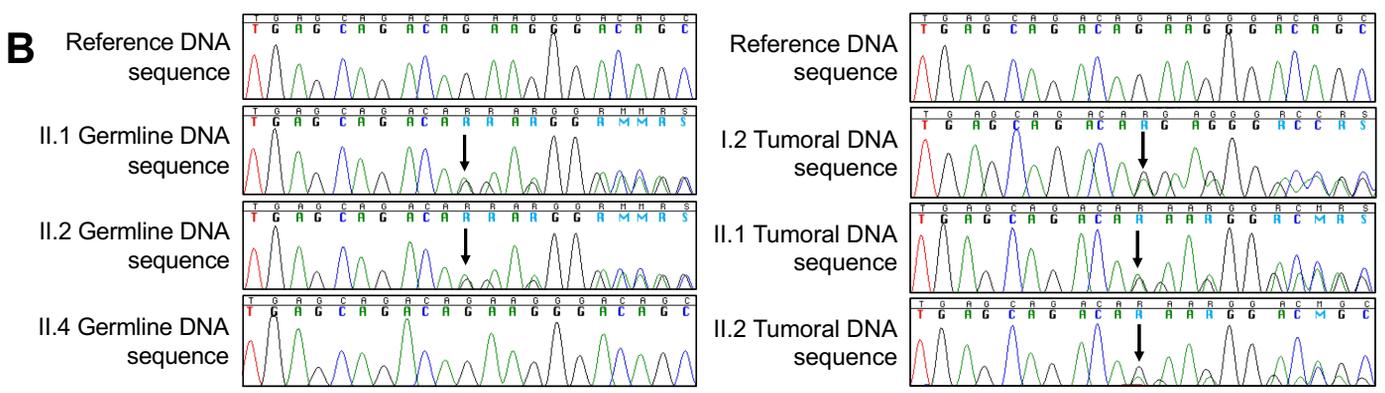
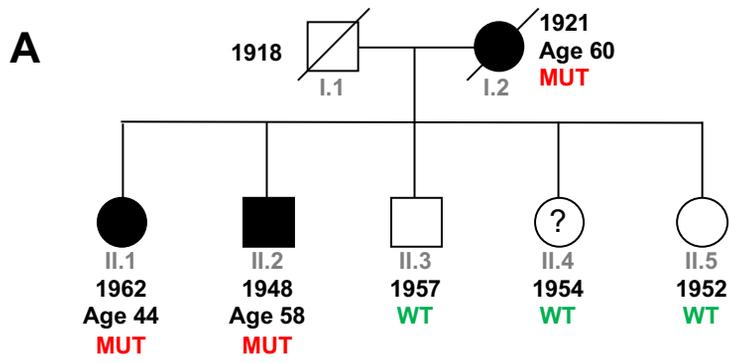
**A** Co-immunoprecipitation of mCherry-NBR1 WT, mCherry-NBR1 E528fs\*17 and endogenous p62 with GFP-NBR1 or GFP-NBR1 E528fs\*17. Protein extracts are from HKC8 transfected with the indicated plasmids above immunoblot for 24h.

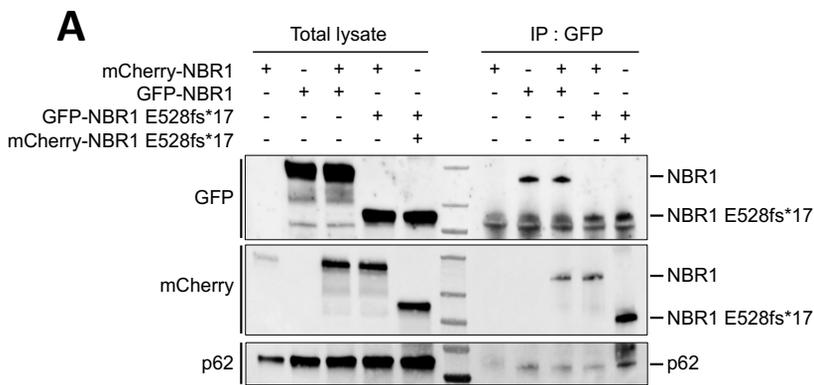
**B** Overexpression of NBR1 protein carrying the E528fs\*17 mutation in HKC8 normal renal cell line leads to a delay in p62 degradation induced by starvation. Cells were transfected with empty vector, HA-NBR1 or HA-NBR1 E528fs\*17. After 24h of transfection, cells were treated with DMEM (control condition), HBSS (Hanks' Balanced Salt Solution) for different times.

**C** p62 turnover quantification. p62 turnover was analysed by electrophoresis and quantified using Image Quant TL 8.2 software. Relative quantity of p62 was normalized to  $\beta$ -actin (mean  $\pm$  s.e.m., n=3). \*\*\*p<0.005. \*\*p<0.01. \*p<0.05. compared to wild-type NBR1.

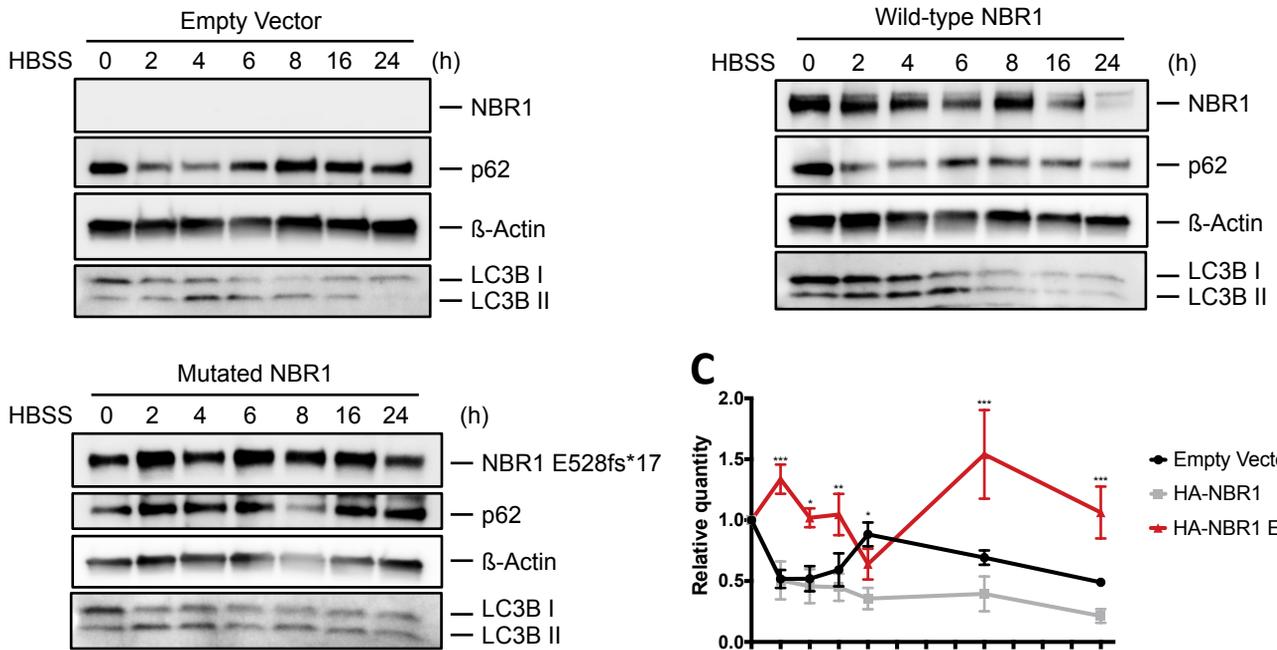
**D** Cell proliferation experiments using tetrazolium-based MTT assay with cells expressing the empty vector, HA-NBR1 or HA-NBR1 E528fs\*17. The experiment was performed in 786-O (left panel) and ACHN (right panel) cell lines (mean  $\pm$  s.d., n=3). \*\*\*\* p<0.0005. \*\*\*p<0.005.

**E** Colony Formation Assay for the growth of 786-O cells transfected with empty vector, HA-NBR1 or HA-NBR1 E528fs\*17. The colonies (left panel) were counted and compared between groups (right panel) (mean  $\pm$  s.d., n=3). \*\*\*\* p<0.0005.

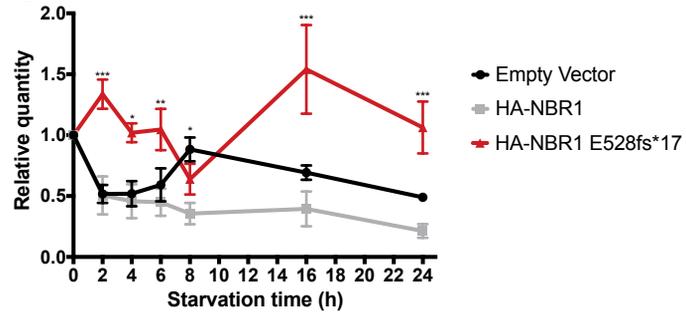




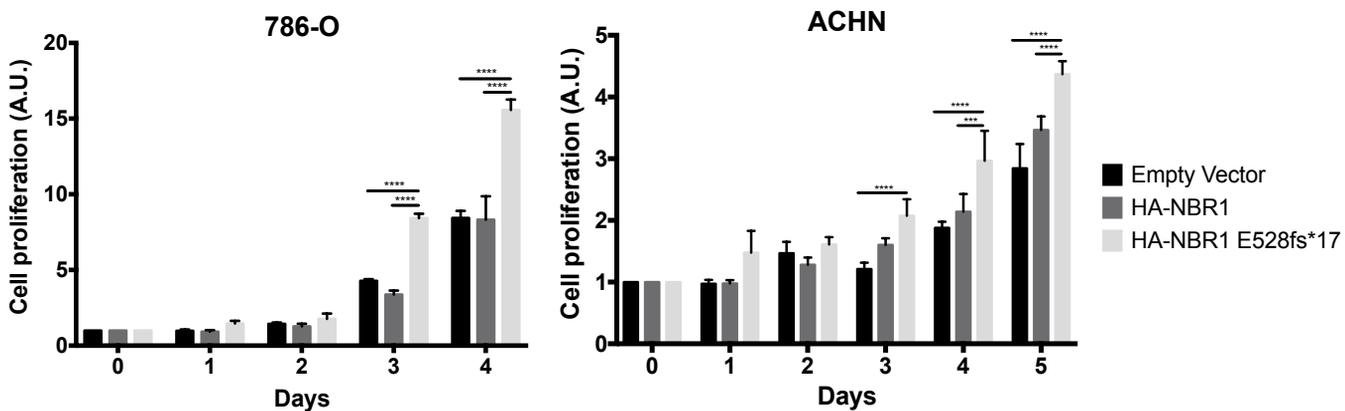
**B**



**C**



**D**



**E**

