Antisense oligonucleotides targeting exon 11 are able to partially rescue the Neurofibromatosis Type 2 phenotype *in vitro.*

N.Catasús[1], I.Rosas[1,2], S.Bonache[1,2], A.Negro[1,2], A.Plana[3], H.Salvador[4], E.Serra[5], I.Blanco[1,2] and E.Castellanos[1,2] on behalf of the NF2 Spanish National Reference Centre HUGTP-ICO-IGTP

1) Clinical Genomics Research Group, Germans Trias i Pujol Research Institute (IGTP); Can Ruti Campus, Badalona, Barcelona, 08916; Spain

2) Clinical Genomics Unit, Germans Trias i Pujol University Hospital (HUGTiP), Can Ruti Campus, Badalona, Barcelona, 08916; Spain

3) Dermatology Department, Germans Trias i Pujol University Hospital (HUGTiP), Can Ruti Campus, Badalona, Barcelona, 08916; Spain

4) Pediatric Oncology Unit, Hospital Sant Joan de Déu, Esplugues, Barcelona, Spain

5) Hereditary Cancer Group, Germans Trias i Pujol Research Institute (IGTP-PMPPC); Can Ruti Campus, Badalona, Barcelona, 08916; Spain

Correspondence should be addressed to Dr. Elisabeth Castellanos (ecastellanos@igtp.cat)

Corresponding author:

Dr. Elisabeth Castellanos

Clinical Genomics Unit - Genetics Service

Laboratori Clinic Metropolitana Nord (LCMN)

Hospital Universitari Germans Trias i Pujol

Crta. Canyet, s/n. Badalona, Barcelona, Spain

Email: ecastellanos@igtp.cat

Abstract

Neurofibromatosis type 2 (NF2) is an autosomal dominant condition characterized by the development of multiple tumours of the nervous system caused by loss of function variants in the *NF2* gene. The clinical presentation of the disease is variable and related to the type of the inherited constitutional mutation. Here, we evaluated the use of antisense oligonucleotides, specifically phosphorodiamidate morpholino oligomers (PMOs), to reduce the severity of the effects of *NF2* truncating variants by converting them to milder hypomorphic forms *in vitro*. Our results showed that the PMOs designed for variants located at +/- 13 from the intron-exon boundary region interfered with the correct splicing signalling of both *NF2* wild-type and mutated alleles. On the other hand, we were able to specifically induce the skipping of exons 4, 8 and 11 of both the mutated and the WT allele maintaining the *NF2* gene reading frame at cDNA level. Only the skipping of exon 11 produced a hypomorphic Merlin (Merlin-e11) able to partially rescue the phenotype observed in primary fibroblast cultures from NF2 patients harbouring a germline loss of function variant. This encouraging result is an *in vitro* proof of concept of the use of antisense therapy to develop personalized therapies for NF2.

INTRODUCTION

Neurofibromatosis type 2 (NF2) (OMIM 101000) is an autosomal dominant (AD) disease caused by loss-of-function (LOF) variants in the *NF2* tumour suppressor gene (22q12.2) [1,2]. The reported incidence is between 1 in 28,000-40,000 [3] and the hallmark of the disease is the presence of bilateral vestibular schwannomas (BVS) which clinically present with hearing loss, tinnitus and imbalance [4]. Common features of NF2 include cranial, spinal, peripheral nerve, and intradermal schwannomas; cranial and spinal meningiomas; and intrinsic central nervous system (CNS) tumours, usually spinal ependymomas [5,6]. Despite their benign nature, tumours are associated with high morbidity due to their multiplicity and anatomical location, which cause pain and nerve dysfunction leading to a reduced quality of life and life expectancy [7].

There are limited therapies available for these patients and radiotherapy and surgery for tumour resection are the classical treatments [8]. Currently, medical therapy is also an option since Bevacizumab, an anti-angiogenic monoclonal antibody, has been demonstrated to be an effective treatment for vestibular schwannomas (VS) inducing the reduction of tumour growth and an improvement in hearing in adult NF2 patients [6,9]. However, Bevacizumab does not seem to be equally effective in all NF2 patients and, in addition, it has no effect on other types of tumours present due to the disease, such as meningiomas [10]. Efforts have been made to develop other therapies for NF2-related tumours, such as Losartan (antihypertensive), Lapatinib (tyrosine kinase inhibitor) or AR-42 (Histone deacetylase (HDAC) inhibitor) [11–14], but there still remains a need to identify clinically relevant therapeutic targets to treat NF2 patients.

The *NF2* gene consists of 17 exons [15,16] with a wide mutational spectrum, 85-90% are germline point mutations and 10-15% are large deletions distributed throughout the entire gene [17,18]. *NF2* encodes for Merlin (Moesin-Ezrin-Radixin-Like protein), a 595aa scaffold protein involved in membrane protein organization, regulation of cell-cell adhesion and with a regulatory role in the cytoskeleton architecture [19,20]. Merlin is comprised of three structural

regions: a N-terminal FERM domain which is subdivided into three globular domains (F1, F2 and F3), an α-helical coiled-coil domain and a C-terminal hydrophilic tail [21].

The genotype-phenotype correlation of NF2 has long been studied [22–30]. For NF2, it is well reported that nonsense variants and large deletions, which account for around 30% of cases, are associated with severe manifestations of the disease, whereas missense, or in-frame variants present a milder form of NF2. Besides, about 25% of the point mutations affect the correct splicing of the *NF2* gene, and this mechanism is associated with a variable severity of the disease [27,29,31]. The well-known association between the genetic variant type and disease severity may constitute an opportunity to develop personalized gene therapies based on the specific variant of the *NF2* gene.

Antisense gene therapy has been successfully used to regulate gene expression in some pathologies. Most of the target diseases are inherited in an autosomal recessive manner, such as Spinal Muscular Atrophy (SMA) [32], homozygous familial hypercholesterolemia (HoFH) [33], or Duchenne muscular dystrophy (DMD) [34] the latter two have been approved by the FDA and are amongst others currently in the advanced stages of clinical trials, [35,36]. Furthermore, there are also examples for AD diseases, such as Retinitis pigmentosa (RP) [37], amyotrophic lateral sclerosis [38] or Huntington's disease [39]. In all cases, antisense oligonucleotides (ASOs) have been used. These are single-stranded modified DNA-like molecules of 15 to 30 nucleotides that act at RNA level by specifically correcting or changing the expression of the target protein to a less deleterious form [40,41]. ASOs have been therapeutically used to reduce protein production whether via RNase H cleavage or mRNA-ASO complex to block mRNA-ribosome interaction. It is also possible to modulate the splice signalling and among the different types of ASOs, Phosphorodiamidate Morpholino Oligomers (PMOs) have highly desirable molecular properties that make them suitable for this last approach. In PMOs the deoxyribose sugar element is replaced by a six-member morpholino ring, and the phosphodiester inter-subunit bonds are

replaced by phosphorodiamidate linkages; all of which produce a structure that provides high-targeting specificity, stability, low toxicity and resistance to nucleases, assuring the maintenance of a good long-term activity within the cell [42].

In this study we assessed two different approaches to test *in vitro* antisense therapy for NF2: the first consisted of modulating and correcting the aberrant splicing of the *NF2* gene originating from different point-mutations located near the canonical splice site; the second consisted of forcing the skipping of those exons that carry a frameshift or a nonsense variant, while preserving the reading frame. Thus, taking into consideration the NF2 genotype-phenotype association, nonsense variants that generate a more severe phenotype could be altered at RNA level to generate less deleterious protein forms.

RESULTS

ANTISENSE THERAPY FOR *NF2* SPLICING VARIANTS

The experimental approach for *NF2* splicing variants consisted of designing specific PMOs for each variant to be tested in order to mask the aberrant splice signalling triggered by the pathogenic variant and promote the spliceosome to properly recognize the correct splice site without altering the wild type (WT) allele (Figure 1). Two different PMOs were designed for each variant. The first design was a 25-mer variant-specific facilitated by Gene Tools according to their guidelines for intron and exon binding subunits and self-complementary moieties. We made a second design using splice site predictors to study the variant surrounding sequence (acceptor splicing sites, exonic splicing enhancers (ESEs) or exonic splicing silencers (ESSs)) (Human Splice Finder) in order to avoid the induction of possible new splicing signals due to the effect of the PMO. In addition, the design avoided masking the canonical splicing GT-AG sites and branch points (YNYYRAY) when possible (Table 1, Figure 2A).

We tested the PMOs' efficacy in primary fibroblasts from NF2 patients harbouring heterozygous germline variants located near the canonical splice site of exons 3, 8 and 15 (Table S1) from the Spanish National Reference Centre (CSUR) on Phakomatoses. A dose-response study was performed to test the effect on splicing of different concentrations of PMOs after 24h of treatment and the efficiency was assessed at cDNA level by RT-PCR. The effect of PMOs on the splicing of *NF2* was analyzed by electrophoresis and confirmed by Sanger sequencing, revealing a maximum effect at 20µM. A time course was carried out treating fibroblasts with 20µM PMO during 24h, 48h and 72h. PMO treatment in Patient_Spl_1 and Patient_Spl_2 increased the skipping of exons 15 and 8 respectively in both of the designs, in contrast to the expected effect of blocking only the germline variant and restoring the NF2 correct splicing without altering the transcription of the WT allele (Figure 2B, B.I and B.II). Similarly, the treatment of PMOs of patients Patient_Spl_3 and Patient_Spl_4, both with nearby variants near the canonical splice site of exon 2, revealed that the variant-specific PMO caused the skipping of both exons 2 and 3 of the NF2 gene instead of correcting the aberrant NF2 splicing due to germline variants (Figure 2B, B.III and B.IV). The same effect was observed with the two different designs and the effect was confirmed by Sanger sequencing (Figure S1). Fibroblasts from healthy donors (FibCtrl) were also treated with each designed variant-specific PMO and they showed the same effect as fibroblasts derived from patients. The effect of the treatment on Merlin was assayed by Western Blot and no improvement in Merlin levels was detected after the treatment at the studied time points (Figure S2).

ANTISENSE THERAPY FOR *NF2* TRUNCATING VARIANTS

Differently from the previous approach, this aimed to test if PMOs could be used to specifically force the skipping of in-frame exons carrying a frameshift or nonsense variant, thus maintaining the *NF2* gene reading frame, and test if the generated Merlin protein could act as an hypomorphic form, still preserving partial function and to some extent able to rescue an *in vitro* phenotype. In this way, taking into account the genotype-phenotype association in which

nonsense or truncating variants are related to more severe phenotypes than in-frame variants, those that generate a more severe phenotype could be modified to less deleterious forms (Figure 3), assessing if the effect of the exon skipping on both alleles was more beneficial than a truncating variant in heterozygosis. A pair of PMOs were designed to be complementary to both 5'- and 3'- intron-exon boundaries to induce skipping of exons 4, 8 or 11 of the *NF2* gene (Exon-Specific PMOs) (Table 2).

Fibroblasts from healthy donors were treated with a pair of PMOs designed to target the acceptor and the donor sites of each exon. A dose response study confirmed the efficiency of the treatment showing full skipping of exons 4 and 8 at 20µM (Figure S3 A and B). For exon 11, two different designs were tested (Table 2; PMO_ES11_v1 and PMO_ES11_v2), while the first (PMO_ES11_v1) failed to induce skipping (data not shown), the second design (PMO_ES11_v2) showed a maximum effect when inducing the skipping of exon 11 at 40µM, and the cells showed an expression of more than 50% of the exon-less form (Figure S3.C). As for the splicing variants, a time course experiment was performed to test the effect at distinct time points (24h, 48h and 72h). In all cases, no differences were observed over time, indicating that the maximum effect of the treatment at RNA level was reached at 24h and maintained at 72h (Figure S3).

We analyzed whether the skipping of both the in-frame *NF2* exon harbouring a truncating variant and the wild-type *NF2* allele, was less deleterious than harbouring a non-functional allele in primary NF2 patients' derived fibroblasts. First, we studied the capacity of restoring Merlin levels *in vitro* after the PMO treatment (Figure 4). Unexpectedly, Merlin western blot showed progressive loss of protein signal when targeting exons 4 (Merlin-e4) or 8 (Merlin-e8), studied in two and five samples respectively at the 24h, 48h and 72h time points (Figure 4, A and B). In order to elucidate if the induced skipping of exons 4 or 8 caused a reduction of Merlin levels; or if there was a loss in the detection of Merlin by the antibody used, possibly due to a misfolded protein; the exon-less NF2 cDNAs were cloned to a N-terminal FLAG® Tag Expression Vector and transfected to HeLa cells. Flag-Merlin Western Blot showed that there was little or no expression

of Merlin-e4 or Merlin-e8, indicating that these new Merlin forms could not be synthesized or were degraded shortly after synthesis (Figure S4).

The skipping of exon 11 generated a shorter form of Merlin, indicating that a potential hypomorphic Merlin could be resulting from the skipping of this exon (Merlin-e11) (Figure 4C).

E11 PMO treatment of *NF2*$^{+/-}$ fibroblasts partially rescued the phenotype *in vitro*

Functionality of Merlin-e11 was studied in primary fibroblasts harbouring a heterozygous truncating variant in exon 11: one from an adult patient (Patient_ES11_1) and another from a paediatric patient (Patient_ES11_2) (Table S1). Given the complexity of determining differences in severity in the phenotype from studying the signalling pathways in which Merlin is involved [29], physiological read outs were assessed. Due to the role of Merlin in cytoskeletal organization [38,39], Merlin-e11's functional activity was first tested through actin immunofluorescence. Phalloidin staining revealed an improvement of the organizational capacity of the cytoskeleton after PMO treatment, although the phenotype observed in both samples was slightly different: patient_ES11_1 showed prominent membrane ruffles that decreased in response to treatment, (Figure 5A) while in patient_ES11_2 we observed an improvement in the cell-cell contact organization (Figure 5B). Specifically, paediatric derived NF2+/- fibroblasts showed an improvement in cell culture, as it seemed that the treatment and expression of Merlin-e11 allowed fibroblasts to recover part of the cell-cell contact inhibition and grow as a monolayer instead of growing overlapped.

In addition, proliferation rates were tested after 72h of treatment by flow cytometry. patient_ES11_1 showed a statistically significant depletion of proliferation when treated with PMOs (65.6%, $p<0.05$) (Figure 6A) while patient_ES11_2 showed a less pronounced decrease (25.1%) (Figure 6B). Considering both samples, primary fibroblasts with a truncating variant in exon 11 showed a 51% reduction in proliferation when compared with the vehicle (Endo-Porter,

p<0.01) and 39.7% compared to treatment control (p<0.01), on average (Figure 6C, Figure S5). PMO treatment did not induce significant differences in cell viability (Figure S6).

DISCUSSION

Neurofibromatosis type 2 (NF2) is a multisystem genetic disorder for which the development of effective therapeutic options with no adverse consequences is needed. The clinical presentation of the disease is variable and related to the type of germline variant inherited in the *NF2* gene [8]. In this context, the development of personalized therapies based on the type of *NF2* variant constitutes an opportunity. In this study, we tested the use of PMOs as antisense therapy to reduce the severity of the effects of *NF2* truncating and splicing variants by changing them to milder hypomorphic forms *in vitro* by two different approaches.

The first approach targeted the splicing variants with the aim of concealing the erroneous splice signal caused by the pathogenic variant and restoring levels of Merlin, in a similar way to the approach that our group used for a *NF2* deep intronic variant [43]. In this study it has not been possible to design PMOs that permit correction of the splicing signalling and furthermore, it was not possible to achieve specificity to target the mutated allele as the same effect was observed in both patient-derived fibroblasts and control fibroblasts. We hypothesize that when the splicing variant is within +/- 13 nucleotides of the acceptor or donor splice site, the PMO could allosterically interfere with the branch point and the U2 snRNA incorporation into the spliceosome [44]. The spatiotemporal localization of the two alleles during splicing could prevent the PMO from specifically targeting the mutated allele and thus it would interfere in the cis-regulation of RNA elements of both alleles equally, resulting in an increased pathogenic effect [45]. Since the approach requires the PMO to be specific for the allele harbouring the pathogenic variant, there is little room for variation in the PMO design.

As far as we know, there are no studies in the literature that have been able to repair the LOF variant effect and recover gene function through the use of ASOs for a splicing mutation located in the canonical splice regions in genes causing autosomal dominant diseases. It has only been possible through CRISPR/Cas technology by the induction of single nucleotide substitutions [46,47]. Allele-specific approaches have been successfully tested for intronic variants through splice modulation ASOs that mask the erroneous signalling and given their intronic location and distance to splicing regulatory regions, proper protein synthesis can be recovered [48–52].

Furthermore, the approach for the truncating variants tried to benefit from the fact that these variants are associated with more severe phenotypes in comparison to in-frame variants in *NF2* [28,30,53]. Thus, we proposed inducing skipping of the exon carrying the variant with the hypothesis of generating potential homozygous hypomorphic forms of Merlin, still aiming to ameliorate the pathogenicity of the heterozygous truncating variant *in vitro*. The skipping of exons 4 or 8 harbouring *NF2* LOF variants was not able to generate an exon-less Merlin, because Merlin-e4 and Merlin-48 were degraded. Exons 4 and 8 of the *NF2* gene code for the subdomains B and C respectively of the FERM domain, where the majority of reported pathogenic variants occur; and which has the most potential for anti-mitogenic signalling and tumour suppressor function [17,21]. The observed results could be due to the fact that the lack of an exon coding for the FERM domain would cause an unbearable aberrant folding of the protein and its synthesis would be prevented or it would be forcibly degraded. In addition, the FERM domain is responsible for the intramolecular interaction with the C-terminal domain, which results in Merlin being in a closed and active conformation [54–56], thus, if this folding fails, it is possible that the resulting protein would not be able to execute the suppressor activity.

For autosomal recessive diseases, such as SMA or DMD [57,58], an autosomal dominant with a dominant negative effect, Frontotemporal dementia with parkinsonism-17 (FTDP-17) or RP, allele specific ASOs have been used to induce degradation of the disease-causing allele via RNAse

H activity and for this purpose, the native DNA backbone is not modified [44,59–61]. Recent evidence suggests that Merlin could act in a negative dominant manner through its self-dimerization through FERM-FERM interactions [62], so the use of RNAse H dependent ASOs could be a possible antisense approach for mutations located in the FERM domain, in both in-frame and out of frame exons. In addition, in contrast to the approach tested in this study, we hypothesize that the degradation of the mutant transcript could prevent interference with the spliceosome during the splicing of the wild-type allele, and therefore, the use of RNAse H dependent ASOs should be also taken into consideration for patients harbouring splicing variants in the *NF2* gene. In the light of our findings, the degradation of the *NF2* mutated allele could bring an improvement in the *in vitro* phenotype and open the door for future gene therapy approaches.

When targeting exon 11 of the *NF2* gene, an exon-less Merlin was expressed (Merlin-e11). Merlin, as a member of the ERM protein family, is involved in the link between actin cytoskeleton and adherent junctions with a relevant role in dynamic cytoskeleton remodelling, such as in membrane ruffling or in the formation of actin micro-spikes. Consistent with other studies which found that Merlin loss leads to a dramatic increase of membrane ruffles [63], here we showed a decrease in membrane ruffle formation in response to the expression of Merlin-e11 that could be associated to a partial rescue of the phenotype. Additionally, we showed an improvement in the cytoskeleton organization after treatment, which could be due to a recovery of the Merlin function as a scaffold protein regulating the linkage between membrane proteins and the cortical cytoskeleton [52,64,65]. Consistent with the role as a tumour suppressor protein, Merlin-deficient cells loose contact inhibition of proliferation [54,66,67] and although much remains to be discovered about the adhesive signalling pathways involved, in this study we showed that the induction of Merlin-e11 by PMO treatment contributed to the recovery of cell-cell contact inhibition of proliferation in fibroblasts derived from NF2 patients harbouring truncating mutations in exon 11.

This work is a proof of concept of the use antisense therapy *in vitro* as a personalized therapy for NF2 patients harbouring truncating variants at exon 11 of the *NF2* gene. Further studies will be needed to elucidate whether there are other mechanisms or designs to apply ASOs as a therapeutic approach for this disease, as in our experience it has not been possible to recover Merlin levels for variants located at in-frame exons 4 or 8, or to correct splicing signals *in vitro* in the four variants located near canonical splice sites tested, although other splicing variants should be tested to confirm these results.

On the other hand, our approach has shown encouraging results when targeting exon 11, although further characterization assays need to be performed to better characterize the function of Merlin-e11 before considering it for further *in vivo* or pre-clinical assays.

MATERIAL AND METHODS

All procedures performed were in accordance with the ethical standards of the IGTP Institutional Review Board, which approved this study and with the 1964 Helsinki declaration and its later amendments.

Samples: samples from 14 NF2 patients from the Spanish Reference Centre were included in the study (Table S1) after providing written informed consent. Samples included in the study were those harbouring a splicing variant or a truncating variant in an in-frame exon of NF2.

Genetic NF2 test: Genetic testing was performed using the customized I2HCP panel [66] in blood or tissue when available. To detect splicing variants, patients were studied both at RNA and DNA levels. Variants were validated by Sanger sequencing and analyzed using CLC-workbench (Qiagen, Hilden, Germany) in all primary cultures established.

Antisense specificity and efficacy: 25-mer specific Phosphorodiamidate Morpholino Oligomers (PMOs) for four patients with splicing variants, and a pair of PMOs to induce exon skipping of exons 4, 8, and 11 of the *NF2* gene were designed, synthesized and purified by Gene Tools (Philomath, OR, USA). Endo-Porter (Gene Tools) was used as vehicle to deliver PMOs into cells, according to the manufacturer's instructions. Patient primary fibroblasts carrying *NF2* germline pathogenic variants were treated with the specific single or paired PMOs. A dose-response and a time course study were performed to set-up treatment conditions and the effect of PMOs was assessed by RT-PCR and Sanger sequencing. Levels of Merlin after PMO treatment were analyzed by Western Blot. For all experiments, the correct effect of the treatment at RNA level was confirmed prior to Western Blot analysis.

Analysis of Merlin-e11 phenotype recovery: an actin immunocytochemistry assay was performed to study the cytoskeletal organization after PMO treatment using Phalloidin (Molecular Probes, Invitrogen). Click-iT™ EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit (Molecular Probes, Invitrogen) was used, according to the manufacturer's protocol, to determine cell proliferation. Cell viability was assessed using RealTime-Glo™ MT Cell Viability Assay (Promega) following the manufacturer's instructions.

***NF2* Cloning and expression:** *NF2* gene was cloned using the Gateway® Gene Cloning system (Invitrogen) after treatment to induce the exon-skipping to a FLAG® Tag Expression Vector. The expression vector was transfected into HeLa cells (Lipofectamine LTX with Plus Reagent, Invitrogen) according to the manufacturer's instructions. Flag expression was assessed by Western Blot (Monoclonal ANTI-FLAG® M2 antibody, Sigma-Aldrich).

Statistics: Statistical analysis was carried out using GraphPad Prism software v7. The Shapiro–Wilk test was used to test normality and for multiple group comparisons, a two-tailed unpaired t test was performed. Statistical significance is indicated by *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

For more information, see Supplementary Materials and Methods.

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AUTHOR CONTRIBUTORS

E.C. conceived the study and wrote the manuscript that was revised, corrected, and improved by all the authors. N.C. performed most of the experimental work, analyzed the data, generated the figures for the paper and contributed to writing the manuscript. I.R, S.B. and A.N. performed experimental work. A.P. and H.S. contributed with sample collection. E.S. and I.B. provided scientific input. All authors approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1. Experimental approach to test antisense therapy for splicing *NF2* variants. In blue, expected outcome after PMO treatment. **(A)** Experimental design for splicing variants that induce exon skipping and result in a truncating protein. **(B)** Experimental design for variants inducing the inclusion of intronic regions as coding sequences, resulting in alterations in the gene reading frame that finally generate a truncating protein. In both approaches, the PMO is expected to mask the aberrant splicing signal and rescue the NF2 phenotype *in vitro*.

Figure 2. (A) Design of the PMO specific for each variant. For the second design (v2), Canonical Splice Sites (CSS), Branch Points (BR), Acceptor and Donor Sites (AS and DS), Exonic Splicing Enhancer sequences (ESE) and Exonic Splicing Silencer (ESS) sequences indicated were taken into account and excluded, when possible, for the sequence design in order to avoid masking the correct splicing signals. The exonic part of the sequence is shown in a gray box and the unlabeled part constitute intronic regions. Tested genetic variants are highlighted in red. **(B) The use of variant-specific PMO did not allow to correct the aberrant splicing signaling.** The effect of the PMOs is shown at cDNA level through dose response and a time course experiments. As, the same effect was observed in both of the PMO designs (v1 and v2), only results from the second design (v2) are shown. Merlins' forms according to the expected molecular weight are indicated next to the bands in the agarose gel. PMO_Ctrl was used as an experiment control. Fib_Ctrl stands for fibroblasts without NF2 mutation, ES for Exon Skipping and UT for Untreated.

Figure 3. Experimental approach to test antisense therapy for truncating *NF2* variants. In blue, expected outcome after PMO treatment**.** PMOs are designed at the donor and acceptor sites of an in-frame exon to induce skipping of exons harboring truncating variants. The generated in-frame deletion is expected to ameliorate the phenotype *in vitro*.

Figure 4. Merlin levels after inducing skipping of in-frame exons harboring truncating variants. Merlin Western Blot after exon-specific PMOs treatment showed a decrease in Merlin total

levels and no presence of exon-less, shorter forms of Merlin-e4 (A) and Merlin-e8 (B). Merlin-e11 was detected and a slight increase was determined in total Merlin levels (C). Increases or decreases in Merlin levels are considered with respect to the untreated sample. Fibroblast control, with no NF2 mutation, are used as experimental control. UT stands for Untreated. NPE stands for Normalized Protein Expression. 0µM are samples treated with Endo-Porter.

Figure 5. E11 PMO treatment of *NF2*$^{+/-}$ fibroblasts induced improvement in actin cytoskeleton organization. Actin immunocytochemistry analysis revealed cytoskeletal abnormalities in patient's derived fibroblasts. Patient_ES11_1 (A) showed a decrease in membrane ruffle formation and Patient_ES11_2 (B) responded to the treatment showing improved culture organization. DAPI was used to stain cell nuclei and Phalloidin is shown in red. Scale bar: 75µm.

Figure 6. Expression of Merlin-e11 could allow a reduction in the proliferation capacity of patient's fibroblasts. Quantification of EdU-positive cells (percentage over DAPI-positive nuclei). A significant decrease was detected in the proliferation rate of treated fibroblasts when compared to Endo-Porter for Patient_ES11_1 (A). Patient_ES11_2 (B) showed the same tendency. (C) Representation of the mean values of both patients. Mean±SD is represented in the bar, representing three independent experiments. *$p<0.05$, **$p<0.01$ denotes statistical significance using unpaired t-test. See also Figure S5.

TABLES

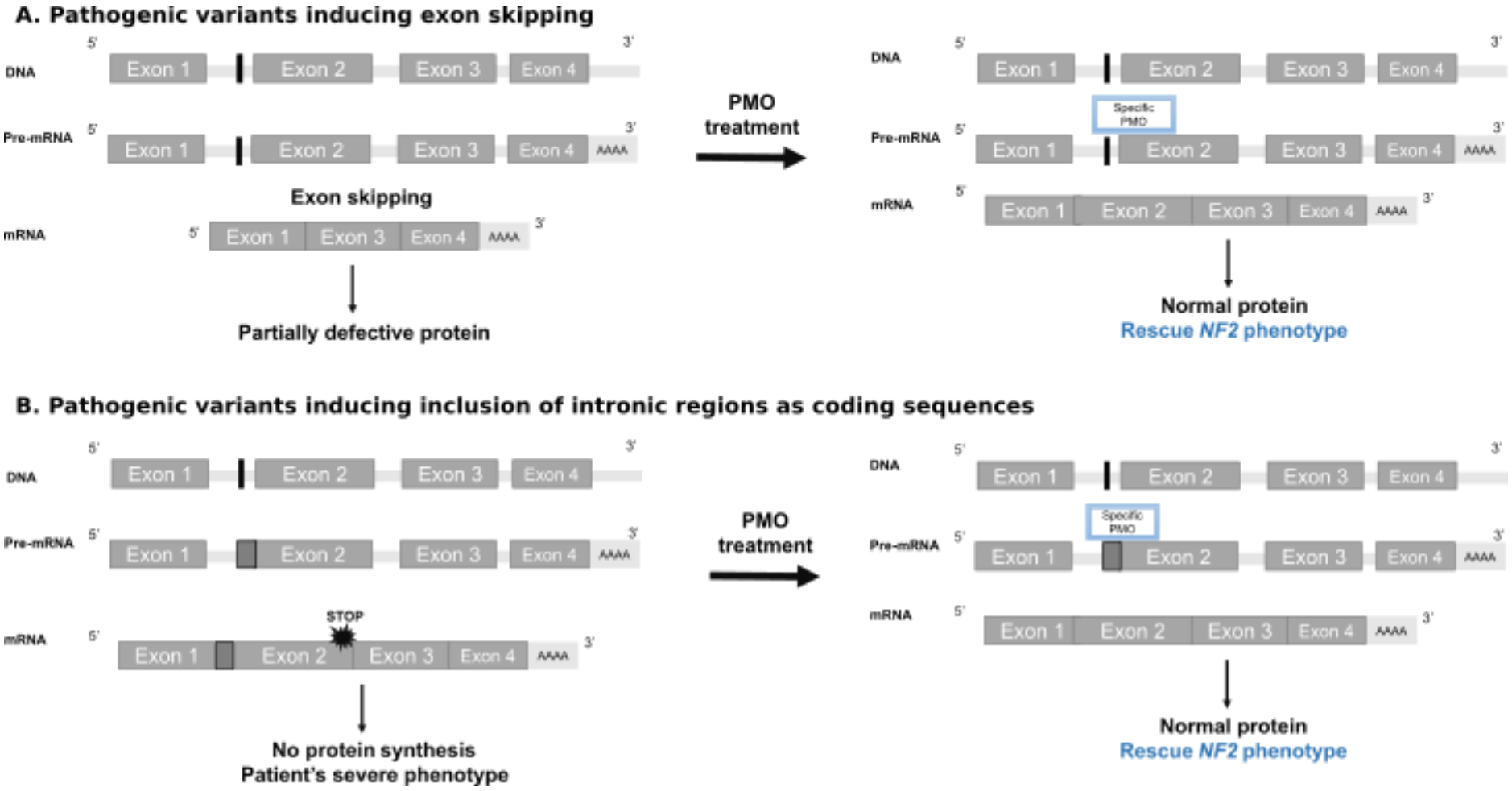
Table 1. NF2 splicing loss-of-function PMO design						
Patient's code	**c.**	**g.**	**p.**	**Variant Effect**	**PMO_Design 1**	**PMO_Design 2**
Patient_Spl_Ctrl (Experimental Control) (PMO_Ctrl)	c.1446_1477ins	g.74409T>A	p.Pro482Profs*39	Insertion of a cryptic exon of 167pb between exons 13 and 14	CATCCCTCAAATCTCTTACCGTTCT*	
Patient_Spl_1	c.1736A>G	g.83045A>G	p.Lys525Asnfs*19	ES of exon 15	**Spl1_PMO_v1**: AGAAAGAGACCCTGGGTACCTTTTT	**Spl1_PMO_v2**: CCTTTTAATGGTATTGTGCTTGCTG
Patient_Spl_2	c.810+1dupG	g.62785dupG	p.Phe271_295del	ES of exon 8	**Spl2_PMO_v1**: GCAGTACACACATGTCCTACCCTCC	**Spl2_PMO_v2:** CCCTCCTTGTCACTGTACGAGATGT
Patient_Spl_3	c.241-9A>G	g.35526A>G	p.Val81Phefs*44	New acceptor site. Inclusion of 8 intronic nucleotides as coding sequence	**Spl3_PMO_v1**: AGTACCTGCAGAACTGCAGAGCAAA	**Spl3_PMO_v2**: GCAGAACTGCAGAGCAAAAGACAAA
Patient_Spl_4	c.241-13T>A	g.35522T>A	p.Val81Phefs*44	New acceptor site. Inclusion of 10 intronic nucleotides as coding sequence	**Spl4_PMO_v1**: AGTACCTGCAGAATTGCTGAGCAAA	**Spl4_PMO_v2**: GCAGAATTGCTGAGCAAAAGACAAA

*Exon Skipping (ES). * Published in Castellanos et al. 2013*

Table 2. Exon-specific PMOs design to target NF2 truncating variants

Exon	Target *NF2* variant	Target region	PMOs designed	PMO_Design 1	PMO_Design 2
4 (n=2)	Any truncating at exon 4	Exon 4_5'	PMO Pair	**PMO_5'ES4**: ACCTGAAAGGAGCAACAAGGGAGAC	
		Exon 4_3'		**PMO_3'ES4**: TTTCTTCTTTGAGCCTACCTTGGCC	
8 (n=5)	Any truncating at exon 8	Exon 8_5'	PMO Pair	**PMO_5'ES8**: TTTATTCTGTGGATCCAATAAGAAC	
		Exon 8_3'		**PMO_3'ES8**: TGCAGTACACACATGTCCTACCTCC	
11 (n=2)	Any truncating at exon 11	Exon 11_5'	PMO Pair	**PMO_5'ES11_v1**: CGCTCCATCTGCGAGGGGTGAAGAA	**PMO_5'ES11_v2**: CGCTCCATCTGCGAGGGGTGAAGAA
		Exon 11_3'		**PMO_3'ES11_v1**: CCTCAGAAATCACCAGTGCTTCGTT	**PMO_3'ES11_v2**: CCTCAGAAATCACCAGTGCTTCGTT

n indicates the number of patients derived fibroblasts in which PMOs is tested

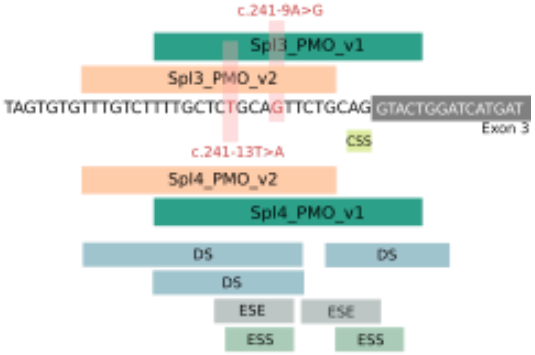

A. Pathogenic variants inducing exon skipping
DNA
Pre-mRNA
mRNA
5'
3'
Exon 1
Exon 2
Exon 3
Exon 4
AAAA
Exon skipping
Partially defective protein
PMO treatment
Specific PMO
Normal protein
Rescue NF2 phenotype
B. Pathogenic variants inducing inclusion of intronic regions as coding sequences
STOP
No protein synthesis
Patient's severe phenotype

A

Patient Spl1

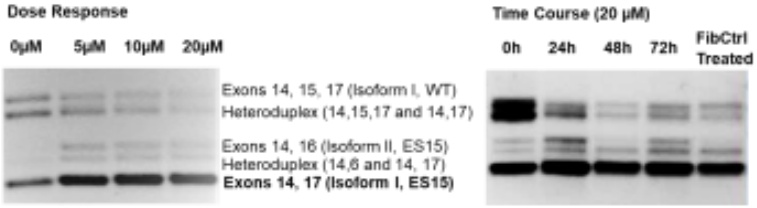
Patient Spl2

Patients Spl3 and Spl4



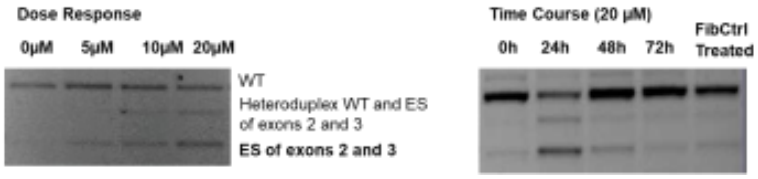
B

B.I. Patient Spl1

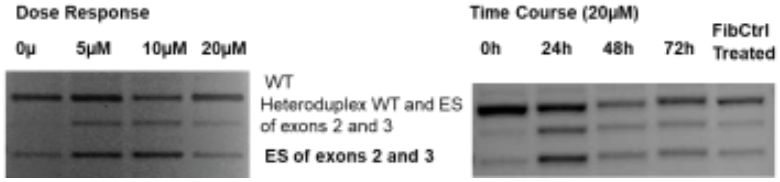


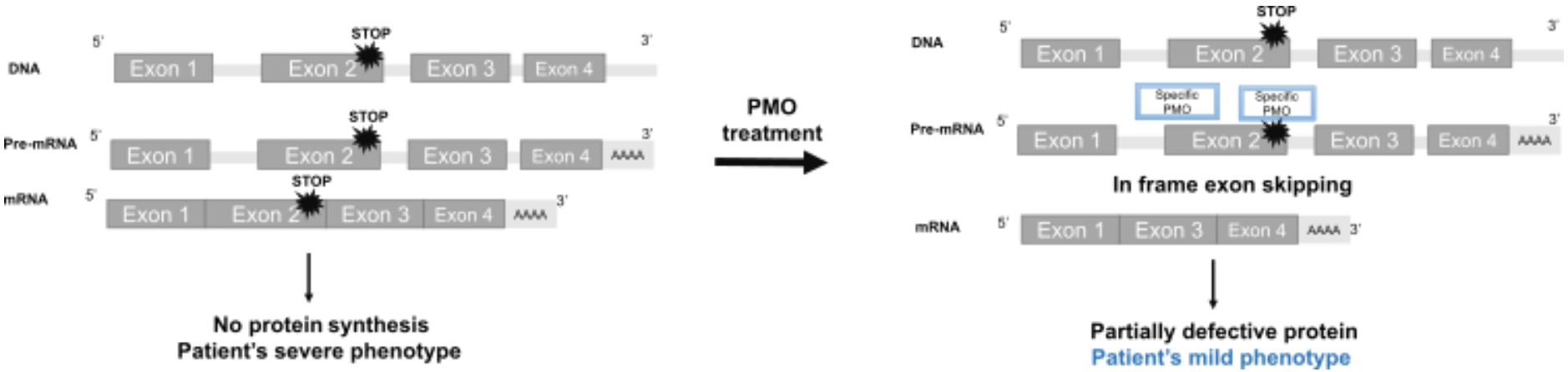
B.II. Patient Spl2

B.III. Patient Spl3

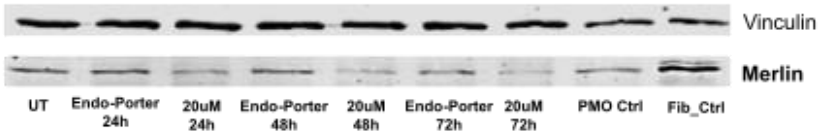


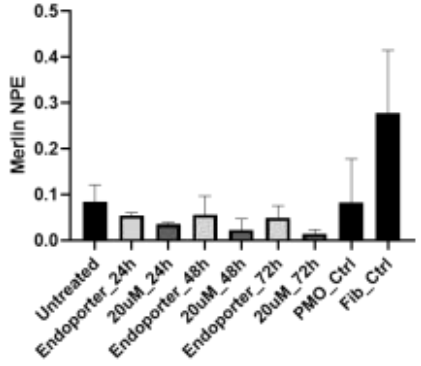
B.IV. Patient Spl4



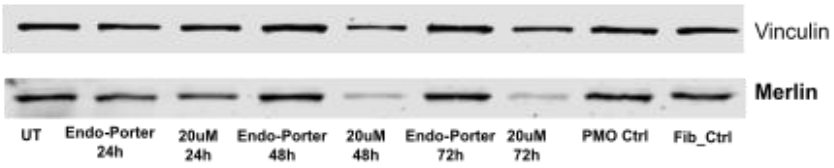

DNA
5'
3'
Exon 1
Exon 2
Exon 3
Exon 4
STOP
Pre-mRNA
AAAA
mRNA
No protein synthesis
Patient's severe phenotype
PMO
treatment
Specific PMO
In frame exon skipping
Partially defective protein
Patient's mild phenotype

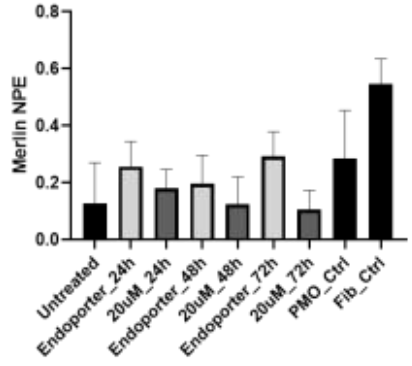
A. Truncating mutation in exon 4: inducing skipping of exon 4 (ES4)



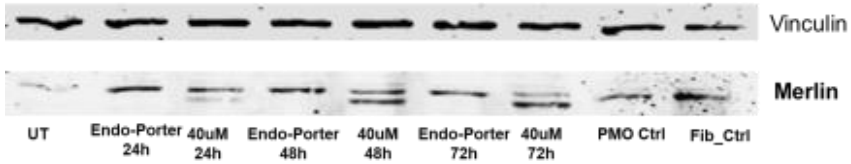


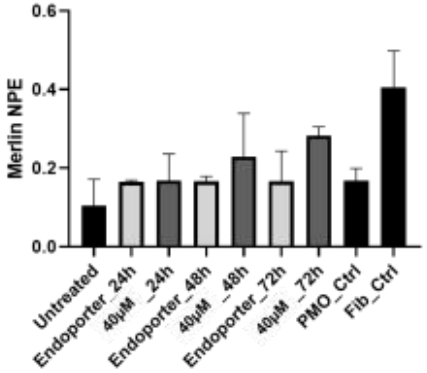
B. Truncating mutation in exon 8: inducing skipping of exon 8 (ES8)

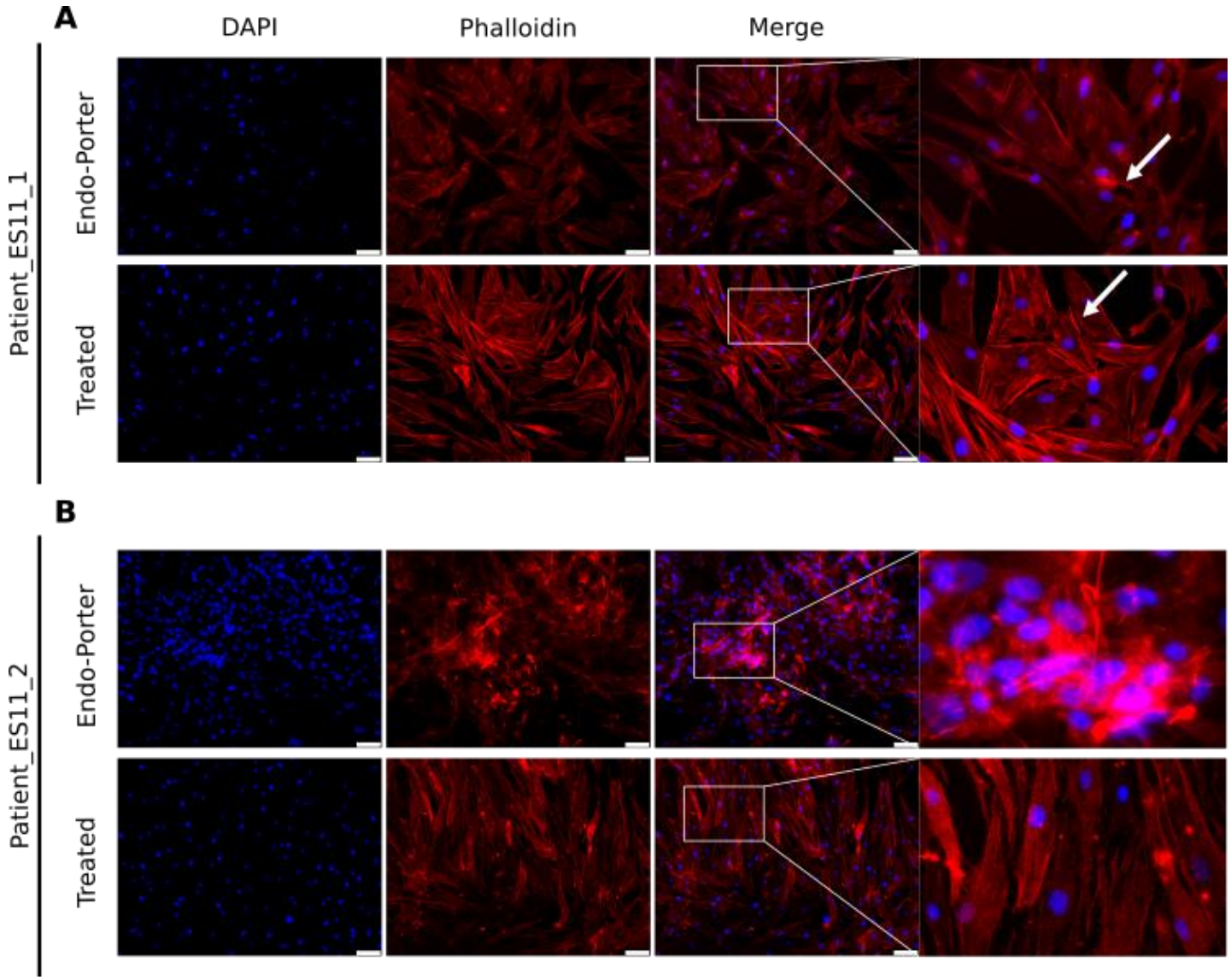


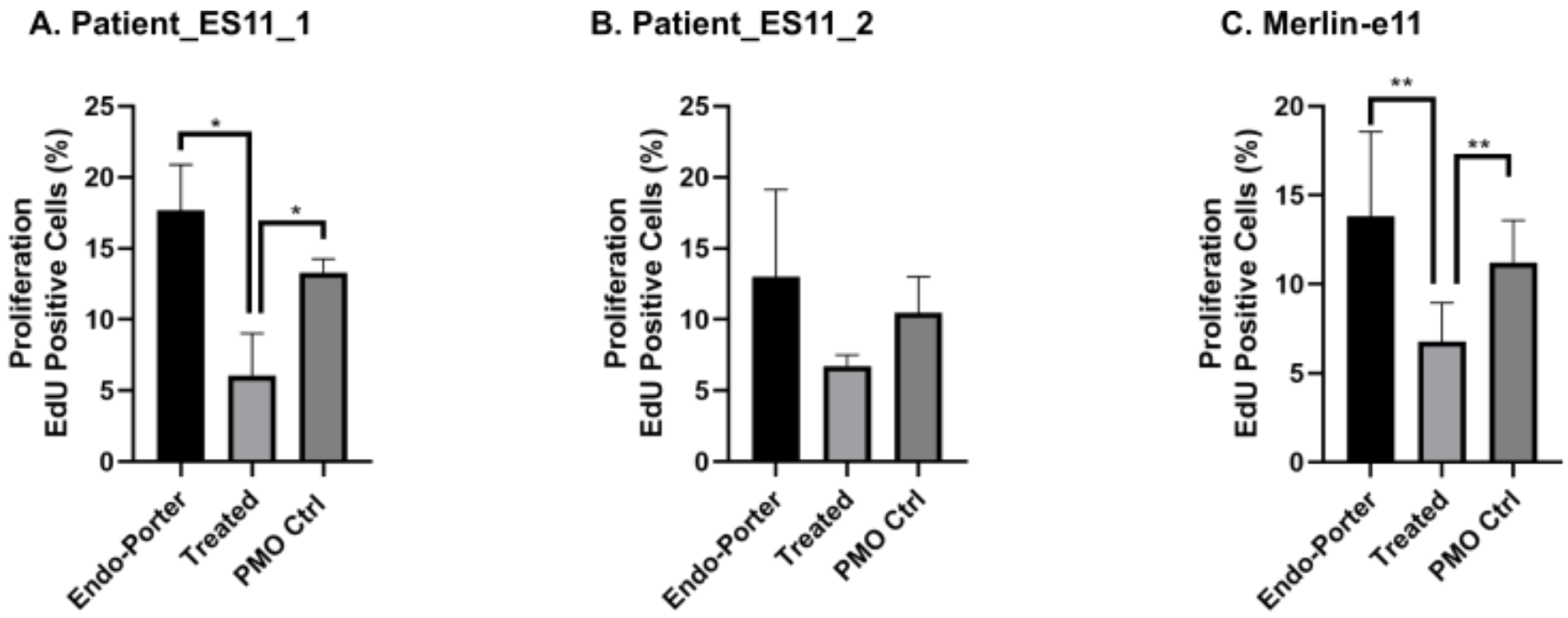


C. Truncating mutation in exon 11: inducing skipping of exon 11 (ES11)






A
DAPI
Phalloidin
Merge
Patient_ES11_1
Endo-Porter
Treated
B
Patient_ES11_2
Endo-Porter
Treated


A. Patient_ES11_1
B. Patient_ES11_2
C. Merlin-e11
Proliferation
EdU Positive Cells (%)
0
5
10
15
20
25
*
**
Endo-Porter
Treated
PMO Ctrl