

# 2'-Fluoro-modified phosphorothioate oligonucleotide can cause rapid degradation of P54nrb and PSF

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## ABSTRACT

**Synthetic oligonucleotides are used to regulate gene expression through different mechanisms. Chemical modifications of the backbone of the nucleic acid and/or of the 2' moiety of the ribose can increase nuclease stability and/or binding affinity of oligonucleotides to target molecules. Here we report that transfection of 2'-F-modified phosphorothioate oligonucleotides into cells can reduce the levels of P54nrb and PSF proteins through proteasome-mediated degradation. Such deleterious effects of 2'-F-modified oligonucleotides were observed in different cell types from different species, and were independent of oligonucleotide sequence, positions of the 2'-F-modified nucleotides in the oligonucleotides, method of delivery or mechanism of action of the oligonucleotides. Four 2'-F-modified nucleotides were sufficient to cause the protein reduction. P54nrb and PSF belong to *Drosophila* behavior/human splicing (DBHS) family. The third member of the family, PSPC1, was also reduced by the 2'-F-modified oligonucleotides. Preferential association of 2'-F-modified oligonucleotides with P54nrb was observed, which is partially responsible for the protein reduction. Consistent with the role of DBHS proteins in double-strand DNA break (DSB) repair, elevated DSBs were observed in cells treated with 2'-F-modified oligonucleotides, which contributed to severe impairment in cell proliferation. These results suggest that oligonucleotides with 2'-F modifications can cause non-specific loss of cellular protein(s).**

## INTRODUCTION

Short, single- or double-stranded DNA- or RNA-like molecules with different chemical modifications have potential as therapeutics when designed to modulate gene expression through antisense, RNA interference or aptamer-based mechanisms (1). RNase H1-dependent antisense oligonucleotides (ASOs) and siRNAs suppress gene expression

by hybridizing to complementary target RNA to allow RNase H1- or the RNA-induced silencing complex (RISC)-mediated degradation of the targeted RNA, respectively (1,2). Oligonucleotide aptamers can serve as antagonists to inhibit the functions of proteins (3).

Therapeutic oligonucleotides are generally modified on the phosphate backbone and/or ribose sugars to increase nuclease resistance and enhance affinity for target RNAs (1). The phosphorothioate (PS) backbone modification replaces a non-bridging oxygen atom with a sulfur atom and extends half-life of oligonucleotides in plasma from minutes to days (4). Further improvement of nuclease stability and binding affinity to target RNAs of oligonucleotides can be obtained by 2' ribose modifications such as 2'-*O*-methyl, 2'-fluoro (2'-F), 2'-*O*-methoxyethyl (2'-MOE), 2',4'-constrained 2'-*O*-ethyl (cEt) and locked nucleic acid (LNA) (4). The positions of 2' modifications within an oligonucleotide are critical. For example, chimeric RNase H1-dependent ASOs are designed to consist of a central oligo deoxynucleotide region flanked by 2' modified ribonucleotides at both ends (1). Since 2' modifications can block RNase H1 cleavage of RNA strand opposite the modifications, chimeric design of ASOs takes advantage of enhanced pharmacokinetic properties, increased nuclease resistance and increased RNA binding affinity provided by the 2' modifications in the flanking nucleotides, while the central deoxynucleotide region can support RNase H1-mediated cleavage of complementary RNA (1). Studies on therapeutic double-stranded siRNA provide another example of the need for proper positioning of 2' modified nucleotides to achieve nuclease stability, activity and specificity. In contrast to 2'-MOE or 2'-*O*-methyl, the 2'-F-modification of the guide strand of siRNAs is well-tolerated regardless of position, presumably because the 2'-F modified oligonucleotides, when base paired with target RNA, form the type A duplex recognized by the RNAi machinery (5).

Although substantial advantages are conferred by backbone and/or 2' modifications, interactions between modified oligonucleotides and intracellular proteins, as well as the subsequent adverse effects derived from undesirable protein-oligonucleotide interactions have not until recently been systematically evaluated (6,7). Enhanced protein binding has been reported for oligonucleotides with PS-

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modifications compared to those with phosphodiester (PO) linkages (1,8). The increased protein binding facilitates tissue uptake and prevents rapid excretion of oligonucleotides (1,8). In addition to backbone modification, the 2' modifications can further influence protein–oligonucleotide interactions (6,7). For example, oligonucleotides with more hydrophobic 2' modifications bind more proteins (6–8). Our recent studies on identification and characterization of intracellular proteins associated with RNase H1-based PS-ASOs containing different 2' modifications provided insights into potentially desirable and undesirable protein–ASO interactions that can influence activity and intracellular distribution of PS-ASO (6,7). In general, desirable protein–ASO interactions include, but are not limited to, those that facilitate ASO release from endocytic pathways or mediate ASO nucleo-cytoplasmic shuttling, whereas the proteins involved in the undesirable interactions are those that compete with RNase H1 for the ASO/RNA duplex association or that prevent ASO distribution to the correct subcellular sites (9).

We recently showed that P54nrb, PSF and PSPC1, proteins that belong to the *Drosophila* behavior/human splicing (DBHS) family, can bind to phosphorothioate oligonucleotides with different 2' modifications. P54nrb and PSPC1 inhibit the activity of PS-ASOs that act through RNase H1-based mechanism (6). The DBHS proteins contain a conserved arrangement of domains with two N-terminal tandem RNA recognition motifs (RRMs), a NONA/paraspeckle domain and a C-terminal coiled-coil domain (10). DBHS proteins can bind both single- and double-stranded DNA or RNA, interact with each other as homo- or hetero-dimers and are involved in multiple cellular functions such as damage repair, transcription initiation, RNA 3'-end processing, splicing, internal ribosome entry site-mediated translation and nuclear retention of hyper-A-to-I-edited RNAs (11–16). Interactions between P54nrb/PSF and single-stranded PS-ASOs are mainly influenced by the PS modification since a phosphodiester oligonucleotide showed no comparable association with P54nrb/PSF (6). Different 2' moieties can further influence the binding of PS-ASOs to P54nrb to different extents (6). DBHS proteins are enriched in distinct nuclear foci, called paraspeckles (17). As a result of preferential association between DBHS proteins and PS-ASOs, upon transfection or electroporation, PS-ASOs with various 2' modifications were observed in nuclear paraspeckles and related structures (6).

Protein–ASO interactions may affect ASO activity and/or cellular localization, and binding of ASOs may change the fate and/or function(s) of the proteins as well. Here we report that the transfection of PS-oligonucleotides modified with 2'-F, but not with 2'-MOE or cEt, caused sequence-independent reduction of the levels of P54nrb and PSF through proteasome-dependent degradation. Consistent with their important roles in various biological processes, including DNA damage repair, treatment of cells with PS-ASOs modified with 2'-F resulted in significant double-strand DNA damage and cell death. Our results provide an important example of how unexpected adverse effects can be conferred by a commonly used non-natural 2' modification of oligonucleotides.

## MATERIALS AND METHODS

### Cell culture and transfection

HeLa, A431 and mouse hepatocellular SV40 large T-antigen carcinoma (MHT) cells were grown at 37°C, 8% CO<sub>2</sub> in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells at 70% confluency were transfected with oligonucleotides at specified concentration using Lipofectamine 2000 (Life Technologies) at a final concentration of 4 µg/ml, and harvested at specified times after transfection for subsequent analyses. Sequences of oligonucleotides are given in figures.

### RNA preparation and qRT-PCR

Total RNA was isolated using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Real-time quantitative reverse transcription PCR (qRT-PCR) was performed as described previously (6). Primer-probe sets used in this study were: P54nrb-Forward, 5'-GAT TTG GCT TTA TCC GCT TGG-3'; P54nrb-Reverse, 5'-ACA CAT ACT GAG GAA GGT TTC G-3'; P54nrb-Probe, 5'-TTG GCA ATC TCC GCT AGG GTT CG-3'; PSF-Forward, 5'-TGA GCG TCT TCT TCG CT-3'; PSF-Reverse, 5'-AAC CGA TCC CGA GAC ATG TC-3'; PSF-Probe, 5'-TTG CCT CGA CCG CCC CTT GAC-3'; PTEN-Forward, 5'-AAT GGC TAA GTG AAG ATG ACA ATC AT-3'; PTEN-Reverse, 5'-TGC ACA TAT CAT TAC ACC AGT TCG T-3'; and PTEN-Probe, 5'-TTG CAG CAA TTC ACT GTA AAG CTG GAA AGG-3'.

### Western blotting

Cell extracts were prepared in RIPA buffer (Pierce) supplemented with Protease Inhibitor Cocktail (Sigma) and separated on 4–12% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Western blotting was performed as described previously (6). Anti-P54nrb (sc-376865) and anti-PSF (sc-374502) antibodies were purchased from Santa Cruz Biotechnology. Anti-PSPC1 (ab104238), anti-hnRNP K (ab32969), anti-TCP1-β (ab92746) and anti-FUS (ab23439) were purchased from Abcam. Anti-GAPDH (G8795) and anti-γ-Tubulin (T6557) were purchased from Sigma. Anti-phospho-Cyclin D1 (Thr286) (3300) was purchased from Cell Signaling.

### Metabolic protein labeling

HeLa cells were mock-transfected or transfected with 30-nM 2'-F-modified PS-ASO (ISIS404130) in DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin for 18 h. Cells were washed twice with phosphate-buffered saline, incubated for 45 min at 37°C, 8% CO<sub>2</sub> in RPMI1640 media lacking methionine (Gibco), and then pulsed with 35-µCi/ml <sup>35</sup>S-methionine (PerkinElmer) for 20 min. Total protein was extracted using immunoprecipitation (IP) lysis buffer (Pierce) supplemented with Protease Inhibitor Cocktail (Sigma) and subjected to immunoprecipitation using Protein G Magnetic beads (Life Technologies) coated with 10-µg

anti-P54nrb antibody (Millipore 05-950). Beads were boiled and the co-immunoprecipitated proteins were separated on 4–12% gradient SDS-PAGE gels and transferred by iBlot (Life Technologies) to a nitrocellulose membrane. The membrane was subjected to direct autoradiography.

### Isolation of ASO-binding proteins

Neutravidin beads (50  $\mu$ l per reaction) were incubated with 50- $\mu$ l 200- $\mu$ M biotinylated 2'-F-modified PS-ASO (ISIS623496) at 4°C for 2 h in W-100 buffer containing 50-mM Tris, pH 7.5, 100-mM KCl, 5-mM ethylenediaminetetraacetic acid (EDTA), 0.1% NP-40 and 0.05% SDS. After incubation at 4°C for 30 min in blocking buffer (1-mg/ml bovine serum albumin, 0.2-mg/ml glycogen and 0.2-mg/ml tRNA in W-100 buffer), the beads were washed three times with W-100 buffer and incubated at 4°C for 2 h with 300- $\mu$ g HeLa cell extracts prepared in IP lysis buffer (Pierce). After washing three times with 500- $\mu$ l W-150 buffer (50-mM Tris, pH 7.5, 150-mM KCl, 5-mM EDTA, 0.1% NP-40, 0.05% SDS), beads were transferred to a 1-ml column and further washed seven times with W-150 buffer. Proteins were eluted by incubating with 100  $\mu$ l of 50- $\mu$ M competitor ASOs in W-100 at room temperature for 30 min. Eluted proteins were precipitated and separated on 4–12% PAGE for subsequent western analysis.

### Neutral comet assay

HeLa cells were mock-transfected or transfected with 2'-MOE-modified PS-ASOs (ISIS116847) or 2'-F-modified PS-ASOs (ISIS404130) at a final concentration of 30 nM for 16 h. Approximately  $10^3$  HeLa cells were plated per comet slide. The neutral comet assay was performed using CometAssay kit (TREVIGEN) according to the manufacturer's instructions. DNA was stained using SYBR Green (Life Technologies) and visualized using a confocal laser scanning FV1000 Fluoview microscope (Olympus).

### Cell survival assay

HeLa cells were mock-transfected or transfected with oligonucleotides at specified concentrations. The cell survival assay was performed, and percentage of viable cells was calculated using Cell Counting Kit-8 (Sigma) according to the manufacturer's instructions. Cells were incubated with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) at 37°C, 8% CO<sub>2</sub> for 2 h. The percentage of viable cells was determined based on the ratio of absorbance at 450 and 600 nM.

## RESULTS

### Treatment of cells with 2'-F-modified phosphorothioate oligonucleotides reduces levels of P54nrb and PSF

Our recent ASO-binding protein studies suggested that ASO-protein interactions are influenced by the 2' modifications of PS-ASOs (6,7). To study how PS-ASOs with different 2' modifications can affect the proteins to which

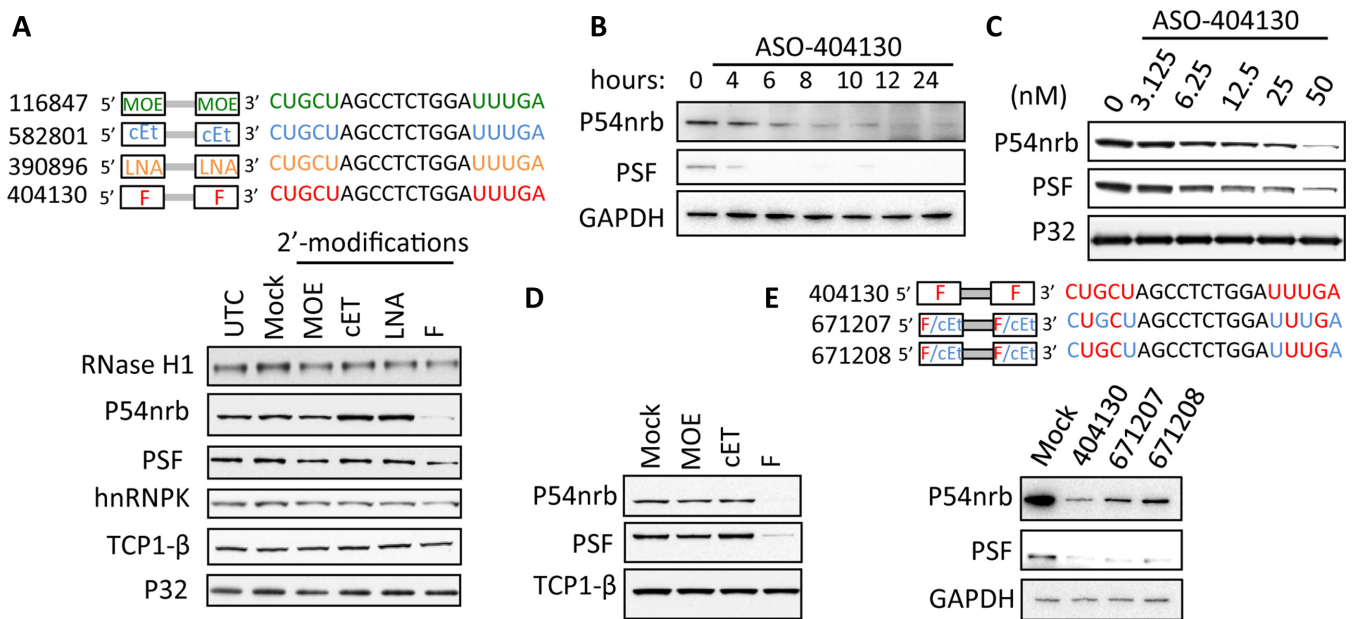
they bind, HeLa cells were transfected at a final concentration of 30 nM for 24 h with fully PS-modified, 5–10–5 gapmer ASOs containing 10 deoxyribonucleotides in the middle flanked at both ends by five nucleotides that are modified with 2'-MOE, cEt, LNA or 2'-F (Figure 1A). The sequences of all ASOs were identical and the sequence is complementary to a region in *PTEN* mRNA. Cell lysates were prepared and protein levels were determined by western analyses. Gapmer PS-ASOs are designed to exert their antisense activity through recruitment of RNase H1 to cleave the target RNAs. The level of RNase H1 protein was not significantly affected by transfection of ASOs as compared with control untreated and mock-transfected cells. However, the previously identified ASO-binding proteins P54nrb and PSF were significantly reduced in HeLa cells transfected with 2'-F-modified PS-ASOs, but not with PS-ASOs modified with 2'-MOE, cEt or LNA.

P54nrb and PSF are known components of nuclear paraspeckles (17). PS-ASOs localize to these foci upon transfection (6). The 2'-F-modified ASOs do not cause a general reduction of proteins in paraspeckles, since no reduction was observed for hnRNPK, a paraspeckle protein that binds PS-ASOs (6). Moreover, levels of TCP1- $\beta$ , another PS-ASO-binding protein that can co-localize with PS-ASOs in the nuclear phosphorothioate bodies (PS bodies), were not reduced by 2'-F-PS-ASO treatment (7), suggesting that preferential association and co-localization will not necessarily alter the levels of the ASO-associated proteins.

The reduction of P54nrb and PSF was rapid. Significant reduction of both proteins was observed as early as 6 h after transfection of cells with 2'-F-ASO (ISIS404130) at a 50-nM final concentration, and greater reduction was observed at later time points (Figure 1B). In addition, reduction of P54nrb and PSF was dependent on the concentration of the 2'-F-modified PS-ASO (Figure 1C). Moderate reduction of both proteins was observed at a relatively low concentration of 2'-F-PS-ASO (6.25 nM), whereas a high concentration of 2'-F-PS-ASO (50 nM) caused an almost complete loss of both proteins. To exclude the possibility that the observed effects were only specific to HeLa cells, we treated mouse MHT cells with the same PS-ASOs (Figure 1D). Substantial reduction of both P54nrb and PSF was observed in cells treated with 2'-F-PS-ASOs but not in cells treated with 2'-MOE- or cEt-modified PS-ASOs, implying that these effects are independent of cell types and species.

The 2'-F-modified PS-ASO (ISIS404130) contains a total of 10 2'-F-modified nucleotides: in the gapmer design there are five 2'-modified residues on each side of a 10-nucleotide deoxy core. To determine the minimal number of 2'-F-modified nucleotides that is required to trigger the loss of P54nrb and PSF, two gapmer PS-ASOs with mixed 2'-cEt and 2'-F modifications were tested (Figure 1E). PS-ASOs ISIS671207 and ISIS671208 contain four and six 2'-F-modified nucleotides, respectively. Each of these ASOs reduced P54nrb and PSF to levels comparable to that in ISIS404130-treated cells. As the four 2'-F-modified nucleotides in ISIS671207 were not placed consecutively, it is not necessary for 2'-F-modified nucleotides to be arranged in tandem in order for ASO treatment to reduce the levels of P54nrb and PSF.





**Figure 1.** 2'-F-modified oligonucleotides cause a reduction in levels of P54nrb and PSF proteins. (A) Gapmer PS-ASOs of the same sequence but modified with 2'-MOE (green), cEt (blue), LNA (orange) or 2'-F (red) on flanking nucleotides were transfected into HeLa cells at a final concentration of 30 nM. After 24 h, levels of indicated proteins were determined by western analysis. P32 served as a loading control. (B) 2'-F-PS-ASO (ISIS404130) was transfected into HeLa cells at a final concentration of 50 nM. P54nrb and PSF levels at the specified time were evaluated by western analysis. GAPDH served as a loading control. (C) 2'-F-PS-ASO (ISIS404130) was transfected into HeLa cells at specified concentration. After 24 h, P54nrb and PSF levels were determined by western analysis. P32 served as a loading control. (D) PS-oligonucleotides modified with 2'-MOE (ISIS116847), cEt (ISIS582801) or 2'-F (ISIS404130) were transfected into mouse MHT cells at a final concentration of 30 nM. Western analysis was performed 24 h after transfection. TCP1- $\beta$  served as a loading control. (E) Levels of P54nrb were determined by western in HeLa cells 24 h after transfection of PS-ASOs with combined cEt and 2'-F modifications at a final concentration of 30 nM. GAPDH served as a loading control.

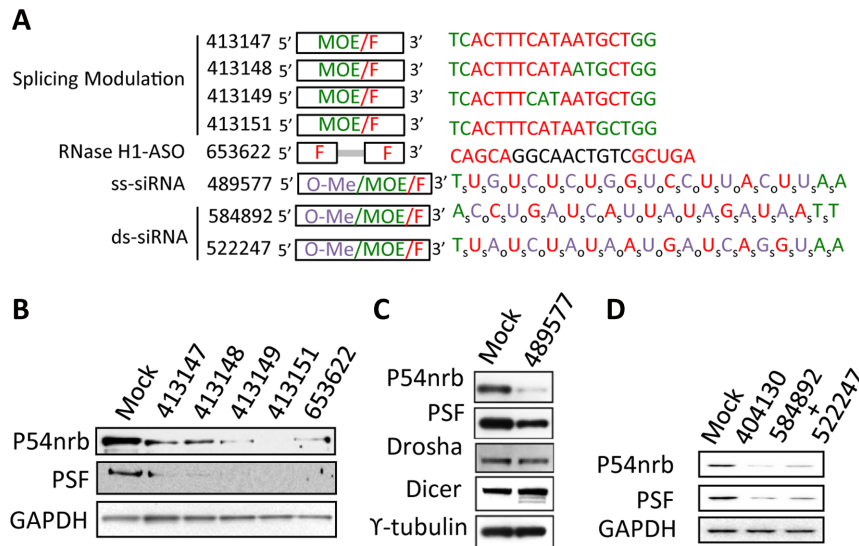
### 2'-F-modified oligonucleotides of different mechanisms of action reduce levels of P54nrb and PSF

The 2'-F-modified gapmer ISIS404130 is designed to exert its antisense activity by recruiting RNase H1 to degrade the target mRNA. 2'-F modifications are used by oligonucleotides that are designed to function through different mechanisms such as splicing modulation or RNA interference. In order to determine whether the reduction in levels of P54nrb and PSF is related to the antisense activity of the 2'-F gapmer, we evaluated 2'-F-modified oligonucleotides designed to modulate splicing and to activate the RNA interference pathway (Figure 2A). The 18-mer splicing-modulating ASOs (ISIS413147, ISIS413148, ISIS413149 and ISIS413151) tested here are fully PS modified with the same sequence but different combinations of 2'-MOE and 2'-F modifications, and were previously validated to modulate splicing (Figure 2A) (18). These splicing ASOs were transfected into HeLa cells at a final concentration of 30 nM. After 24 h, levels of P54nrb and PSF were significantly reduced by each of these oligonucleotides (Figure 2B). An additional gapmer was also tested; the 2'-F-PS-ASO ISIS653622 also significantly reduced levels of the P54nrb and PSF (Figure 2B), indicating that the phenotype we observed is not sequence-specific. We next tested a single-stranded siRNA ISIS489577 modified with 2'-MOE, 2'-O-methyl and 2'-F with a mixed phosphodiester/phosphorothioate backbone that was previously shown to sequence-specifically reduce levels of *PTEN* mRNA through an RISC-mediated mechanism in

animals (19). Significant reduction of both DBHS proteins was observed upon transfection of the single-stranded siRNA into HeLa cells at a final concentration of 30 nM, whereas Droscha and Dicer, key proteins in the RISC pathway, were not affected (Figure 2C). Moreover, reduction of P54nrb and PSF proteins was also observed with a double-stranded siRNA with mixed 2'-O-Methyl, 2'-MOE and 2'-F modifications and alternating PS-PO backbone (Figure 2D). These data suggest that 2'-F-containing oligonucleotides can cause the reduction of P54nrb and PSF regardless the functional properties of the oligonucleotides or whether the oligonucleotides are single-stranded or double-stranded.

### 2'-F-modified oligonucleotides reduce the stability of P54nrb and PSF proteins

The 2'-F-modified gapmer PS-ASO (ISIS404130), at a 30-nM concentration, reduced P54nrb and PSF levels within 6 h (Figure 1B), implying that the 2'-F-ASO acts directly on P54nrb and PSF proteins, rather than on the mRNAs. This is supported by the fact that this PS-ASO has no significant sequence complementarity to either *P54nrb* or *PSF* mRNAs and by our observations in Figure 1A that PS-ASOs with the same sequence but other 2' modifications did not alter levels of P54nrb or PSF proteins, even though all four tested types of 2' modifications (MOE, cEt, LNA and F) were known to support the RNase H1-mediated target RNA reduction. To examine this hypothesis, total RNAs from untreated HeLa cells, mock-transfected cells and cells



**Figure 2.** 2'-F-modified oligonucleotides designed to function through different mechanisms reduce P54nrb and PSF levels. (A) Sequences of the 2'-F-modified oligonucleotides tested are shown. The ASOs designed to modulate splicing and the gapmer ASO are fully PS modified. Both the single-stranded siRNA and double-stranded siRNA oligonucleotides have alternating PS (indicated by lower case 's') and phosphodiester (indicated by lower case 'o') backbone residues. 2'-MOE, 2'-F and 2'-O-methyl are indicated in green, red and purple, respectively. (B–D) HeLa cells were transfected with a final concentration of 30-nM oligonucleotide, and western analysis was performed after 24 h, with  $\gamma$ -tubulin or GAPDH as loading control. (B) Effect of splicing modulators and gapmer on P54nrb and PSF levels compared to mock-transfected cells. (C) Effect of 2'-F-modified ss-siRNA on P54nrb, PSF, Droscha and Dicer levels compared to mock-transfected cells. (D) Effect of 2'-F-modified ds-siRNA on P54nrb and PSF levels compared to mock-transfected cells and cells treated with 2'-F-PS-ASO ISIS404130.

transfected with gapmer PS-ASOs modified with 2'-MOE, cEt, LNA or 2'-F were isolated, and *PTEN*, *P54nrb* and *PSF* mRNA levels were quantified by qRT-PCR. These ASOs were designed to target *PTEN* mRNA and the expected on-target reduction of *PTEN* mRNA was observed (Figure 3A). The 2'-MOE, cEt and LNA PS-ASOs reduced *PTEN* mRNA by ~80% in cells transfected with 30-nM ASO compared to untreated cells; the 2'-F-modified PS-ASO was slightly less active. Importantly, no decreases in *P54nrb* or *PSF* mRNA levels were observed in any of the PS-ASO-treated cells (Figure 3A), although P54nrb and PSF proteins were significantly reduced by the 2'-F-ASOs.

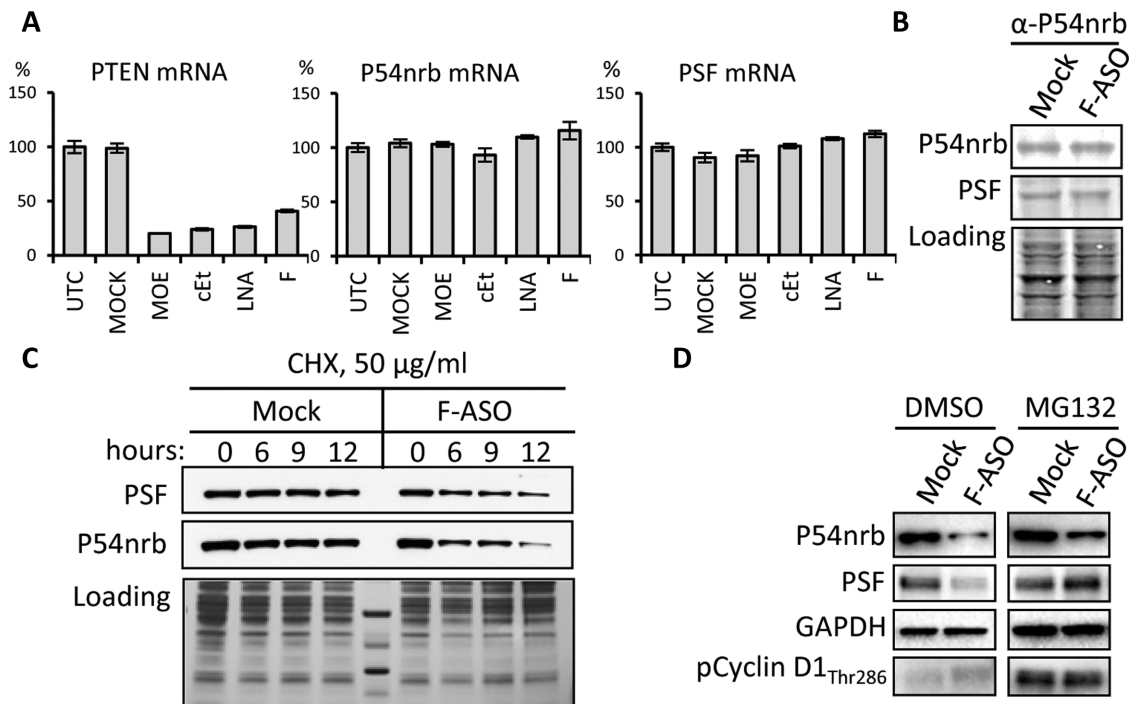
Since treatment of cells with 2'-F-modified oligonucleotides did not alter levels of *P54nrb* or *PSF* mRNAs, P54nrb and PSF protein synthesis was analyzed. HeLa cells were transfected with 2'-F-PS-ASOs (ISIS404130) at 20-nM concentration for 12 h followed by methionine starvation and <sup>35</sup>S-methionine labeling (Figure 3B). Immunoprecipitation was performed using an anti-P54nrb antibody to isolate P54nrb and its heterodimer binding protein PSF. The co-precipitated proteins were separated on a 4–12% PAGE and <sup>35</sup>S-labeled nascent P54nrb and PSF were detected by autoradiography. No significant difference in the levels of nascent P54nrb and PSF proteins was observed between mock-transfected cells and 2'-F-PS-ASO-treated cells (Figure 3B), suggesting that the loss of P54nrb/PSF was not due to the impaired protein production.

Next, the effects of 2'-F-PS-ASOs on the stability of P54nrb and PSF proteins were evaluated. HeLa cells were either mock-transfected or transfected with 2'-F-PS-ASO (ISIS404130) at 20-nM concentration for 12 h, followed by cycloheximide (CHX) treatment. Levels of P54nrb and PSF proteins were examined by western blot over a time course

after inhibition of protein synthesis (Figure 3C). Half-lives of both P54nrb and PSF were greater than 12 h in mock-transfected control cells; however, in the presence of 2'-F-PS-ASOs, the half-lives of P54nrb and PSF were 6 h or less. To determine whether P54nrb and PSF degradation occurred via the proteasome-mediated pathway, HeLa cells were either mock-transfected or transfected with 20-nM 2'-F-PS-ASO for 12 h before the treatment with DMSO (control treatment) or a proteasome inhibitor MG132 for another 4 h (Figure 3D). The effectiveness of the proteasome inhibitor was confirmed by the rescued levels of phosphor-Cyclin D1 (Thy286) protein, which is rapidly degraded by the proteasome pathway under normal conditions (20). We found that the loss of P54nrb and PSF proteins induced by 2'-F-PS-ASO treatment was at least partially rescued in the presence of MG132 (Figure 3D), indicating that 2'-F-modified PS-oligonucleotides can induce proteasome-dependent degradation of these two DBHS proteins.

### Levels of PSPC1, the third mammalian DBHS protein, are reduced by 2'-F-modified oligonucleotide treatment of cells

Both P54nrb and PSF belong to the DBHS family (10). The third annotated member of this family is PSPC1 (10,17). DBHS proteins share ~50% sequence similarity. PSPC1 interacts with both P54nrb and PSF and was found to associate and co-localizes with PS-ASOs in nuclear paraspeckles (6,21). To test whether the 2'-F-modified oligonucleotides also reduced levels of PSPC1, HeLa cells were transfected with the four 2'-F-modified ASOs designed to modulate splicing and two RNase H1 ASOs. We observed a significant reduction of PSPC1 in cells treated with each of the six 2'-F-containing oligonucleotides, indicating that 2'-F-



**Figure 3.** 2'-F-modified oligonucleotides reduce P54nrp and PSF levels by decreasing protein stability. (A) 2'-F-oligonucleotides did not reduce levels of *P54nrp* or *PSF* mRNAs. HeLa cells were transfected with 2'-F-PS-ASO (ISIS404130) at a final concentration of 30 nM. After 24 h, RNA was isolated and indicated mRNAs were quantified by qRT-PCR. On-target reduction of *PTEN* mRNA was evaluated as a positive control for transfection. The error bars represent standard deviations from three experiments. (B) 2'-F oligonucleotides have no effect on translation of P54nrp and PSF. Nascent <sup>35</sup>S-methionine-labeled P54nrp and its heterodimer partner PSF were co-immunoprecipitated from mock-transfected cells or from HeLa cells transfected with 20-nM final concentration of 2'-F-PS-ASO (ISIS404130) and visualized by direct autoradiography. Autoradiography of 5% of the total cell lysate used for immunoprecipitation served as a loading control. (C) 2'-F oligonucleotides reduce P54nrp and PSF stability. Western analysis of P54nrp and PSF from mock-transfected cells or from HeLa cells transfected with 20-nM final concentration of 2'-F-PS-ASO (ISIS404130) treated with CHX (50  $\mu$ g/ml) for 0, 6, 9 and 12 h. Silver-staining of total cell lysate served as a loading control. (D) 2'-F oligonucleotides cause degradation of P54nrp and PSF through the proteasome-mediated pathway. Western analysis of P54nrp and PSF from HeLa cells treated with DMSO or MG132 (10  $\mu$ M) with or without transfection of 2'-F-PS-ASO (ISIS404130). GAPDH served as a loading control. pCyclin D1<sub>Thr286</sub> served as a positive control for proteasome inhibition.

modified oligonucleotides can cause a general reduction of DBHS proteins (Figure 4A). Moreover, dose-dependent reduction of the three DBHS proteins was also observed upon free uptake of ISIS404130 into A431 cells (Figure 4B). Note that the doses that resulted in the reduction of the proteins were within the range that is commonly used to lower target RNAs.

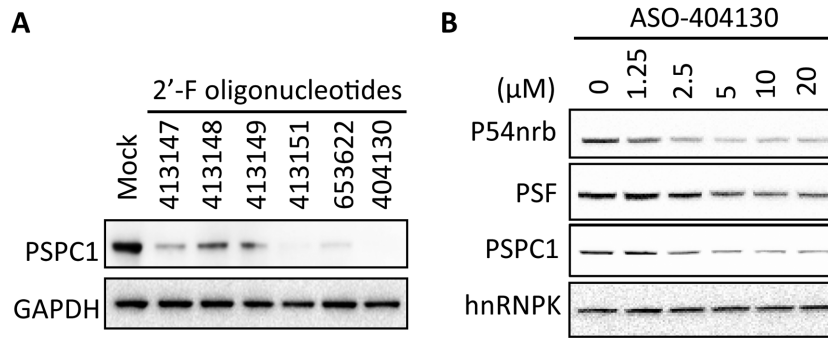
### 2'-F-modified oligonucleotides exhibit higher affinity for P54nrp than 2'-MOE- or cEt-modified oligonucleotides do

We reported previously that association of P54nrp with PS-ASOs was influenced by 2' modifications of the ASOs (6,7). PS-ASOs with cEt, LNA or 2'-F modifications exhibit stronger binding to P54nrp than do those modified with 2'-MOE or 2'-O-methyl (6,7). The fact that oligonucleotides modified with 2'-F, but not with cEt or 2'-MOE, cause the reduction of all three mammalian DBHS proteins prompted us to further investigate these interactions. For this purpose, ASO-binding proteins were isolated from HeLa cell extracts using a biotinylated gapmer PS-ASO with flanking nucleotides 2'-modified with F (ISIS623496) and eluted by competition with non-biotinylated gapmer PS-ASOs of the same sequence but modified on the flanking nucleotides with 2'-MOE, cEt or 2'-F (Figure 5A). The

affinity-selected proteins were analyzed by western blotting (Figure 5B). PSF and P54nrp showed little or no preference for ASO chemistry, but more P54nrp and FUS, another paraspeckle protein, were eluted preferentially by the 2'-F-modified competitor ASOs than cEt- or 2'-MOE-PS-ASOs. Ku70 has been reported to associate equivalently with PS-ASOs of different ribose moieties (6,7) and was detected here to serve as a loading control.

To further evaluate the structure-activity relationship, we designed two gapmer oligonucleotides with the 2'-F-modifications on either the 5' or the 3' flanking nucleotides (referred as 'wings'), and the 2'-MOE-modifications on the other wing (Figure 5A). These two PS-ASOs were used to elute ASO-binding proteins co-precipitated with the biotinylated 2'-F-PS-ASO (ISIS623496). The isolated proteins were then analyzed by western blotting (Figure 5C). P54nrp and FUS had higher affinity for ASOs with 2'-F-modifications on the 3'-wing of the oligonucleotides, whereas PSF and Ku70 showed no preference for the location of the 2'-F-modifications (Figure 5C). Although preferential association of 2'-F-modified ASOs with P54nrp was observed, the loss of DBHS proteins in cells treated with 2'-F-PS-ASO cannot be entirely explained by the binding affinity. Paraspeckle protein FUS can bind preferentially to PS-ASOs with the 2'-F-modification (Figure 5B), but was





**Figure 4.** PSC1 levels are reduced by 2'-F-modified oligonucleotides. (A) Indicated oligonucleotides were transfected into HeLa cells at a final concentration of 30 nM. After 24 h, levels of PSC1 were evaluated by western. GAPDH served as a loading control. (B) A431 cells were incubated with ISIS404130 by free uptake for 40 h at the indicated concentrations. Levels of P54nrb, PSF and PSC1 proteins were evaluated by western. GAPDH served as a loading control.

not reduced in cells treated with 2'-F-PS-ASOs (Figure 5D). These data suggest that affinity may be necessary but not sufficient for 2'-F-modified oligonucleotide to induce the degradation of P54nrb, PSF and PSC1.

As P54nrb, but not PSF or PSC1, binds preferentially to 2'-F-modified oligonucleotides, it is possible that interaction between P54nrb and 2'-F-modified oligonucleotides may induce the degradation of P54nrb, leading to reduced stability of PSF and/or PSC1 which associate with P54nrb. This seems not to be the case since the depletion of P54nrb using an siRNA targeting *P54nrb* mRNA up-regulated levels of both PSF and PSC1 proteins by 2- to 3-fold (Figure 5E). Depletion of PSC1 by siRNA treatment has no effect on the levels of P54nrb or PSF. Moreover, increased level of PSF was observed upon simultaneous knockdown of both P54nrb and PSC1. These results imply that the hetero-dimerization may not be required for the stabilization of DBHS proteins.

#### Treatment of cells with 2'-F-PS-ASOs causes cell death due, at least in part, to unrepaired DNA damage

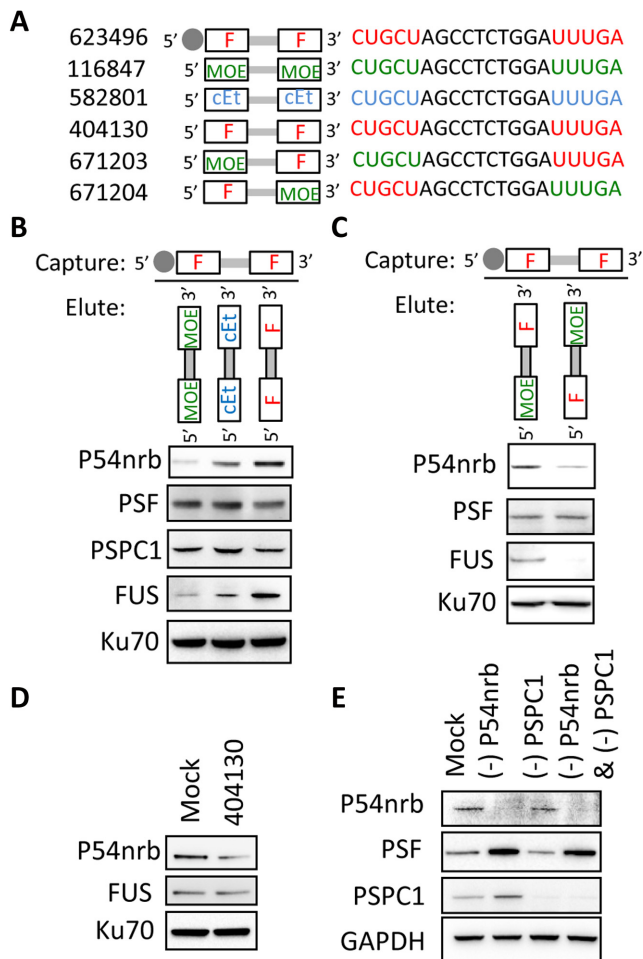
A concentration-dependent decrease in cell viability was observed when HeLa cells were transfected with 2'-F-ASOs (Figure 6A). After 24 h, there was little or no change in cell viability when cells were treated with 2'-MOE-ASOs; but roughly 50% of cells were dead after treatment with 30-nM 2'-F-PS-ASO of the same sequence. Cell viability appears to negatively correlate with the number of 2'-F-modified nucleotides within an oligonucleotide (Figure 6B and C). It has been reported that dual PSC1/P54nrb deficiency results in severe radiosensitivity and delayed repair of DNA double-strand breaks (DSBs). To investigate the effect of 2'-F-modified oligonucleotides on DSBs, a neutral comet assay was performed 16 h after transfection of HeLa cells with 2'-F-PS-ASO (ISIS404130) or 2'-MOE-PS-ASO (ISIS116847) gapmers. Treatment with 2'-F-modified, but not 2'-MOE-modified, PS-ASOs induced a substantial increase in the tail moment of cells (Figure 6D), suggesting that DSBs have accumulated in 2'-F-PS-ASO-treated cells. These results suggest that by depleting cells of DBHS proteins, 2'-F-PS-ASOs induce DSB accumulation that con-

tributes to the observed toxicity of 2'-F-modified oligonucleotides.

#### DISCUSSION

In this study, we showed that 2'-F-modified phosphorothioate oligonucleotides cause reduction of levels of all mammalian DBHS proteins through the proteasome-mediated pathway. Treatment of cells with the 2'-F-modified oligonucleotides resulted in elevated levels of DSBs and cell death compared to cells treated with 2'-MOE-modified oligonucleotides. The DSB repair pathway is likely impaired in 2'-F-modified oligonucleotide-treated cells due to the simultaneous loss of P54nrb, PSF and PSC1, proteins that are functionally redundant in DSB repair (22). The loss of DBHS proteins occurred when cells were treated with oligonucleotides with as few as four sporadically placed 2'-F-modified nucleotides independent of sequence, position or mechanism of action of these oligonucleotides.

To cause the reduction of DBHS proteins, 2'-F-modified PS-oligonucleotides should interact with these proteins, individually or in complexes, differently than PS-oligonucleotides modified with 2'-MOE or cEt do. The difference may come from different binding affinity or different binding sites or both. Interaction between DBHS proteins and PS-oligonucleotides with different ribose moieties was identified by the previously reported ASO-protein complex pull-down experiments (6). These interactions have been further validated by showing the accumulation of PS-modified oligonucleotides with various ribose moieties, delivered into cells by transfection or electroporation, in the DBHS protein-enriched nuclear paraspeckle foci (6). Relatively strong association of DBHS proteins with PS-modified oligonucleotides was suggested by our previous observations that (i) PS-modified oligonucleotides can compete out the long non-coding RNA *NEAT1*, which is naturally paraspeckle-localized and DBHS protein-bound, out of paraspeckle foci (6); (ii) PS-modified oligonucleotides not only can join pre-existing paraspeckle foci but also can recruit diffuse DBHS proteins from the nucleoplasm to trigger *de novo* assembly of paraspeckle-like foci independent of *NEAT1* RNA (6); and (iii) PS-modified oligonucleotides can co-migrate with DBHS proteins from paraspeckles to



**Figure 5.** P54nrB preferentially associates with 2'-F-modified oligonucleotides. (A) Oligonucleotides used in this experiment. (B) ASO-binding proteins were isolated from HeLa cell extracts using a biotinylated gapper 2'-F-PS-ASO (ISIS623496). Proteins were eluted from beads by competition with indicated gappers. The affinity-selected proteins were analyzed by western blot. Ku70 served as a loading control. (C) Western analysis following ASO-pull down as described in panel (A) indicated that P54nrB has higher affinity for oligonucleotides with the 2'-F modification on the 3' side. Ku70 served as a loading control. (D) Transfection of 2'-F-oligonucleotides reduced P54nrB but not FUS protein levels. HeLa cells were transfected with 2'-F-PS-ASO (ISIS404130) at a final concentration of 30 nM, and protein levels were determined by western analysis. (E) Western analysis of proteins isolated from HeLa cells following treatment of cells with siRNAs targeting *P54nrB* or *PSPC1* mRNA. Cells were transfected with siRNA at 3-nM final concentration and harvested after 36 h.

perinucleolar caps upon inhibition of RNA pol II (6). The phosphorothioate linkage contributes to the affinity for P54nrB and for other cellular proteins in general, as phosphodiester oligonucleotides bind far less protein than do PS oligonucleotides (6–8) and binding is correlated to the number of PS linkages (6). In addition to the backbone modifications, various chemical modifications to the ribose influence the association of oligonucleotides with proteins (6,7). Our previous studies showed that the hydrophobicity of 2' moiety is important for ASO–protein interaction, since, in general, oligonucleotides with more hydrophobic modifications, such as 2'-F, cEt or LNA, bind more proteins than the ASOs with more hydrophilic modifica-

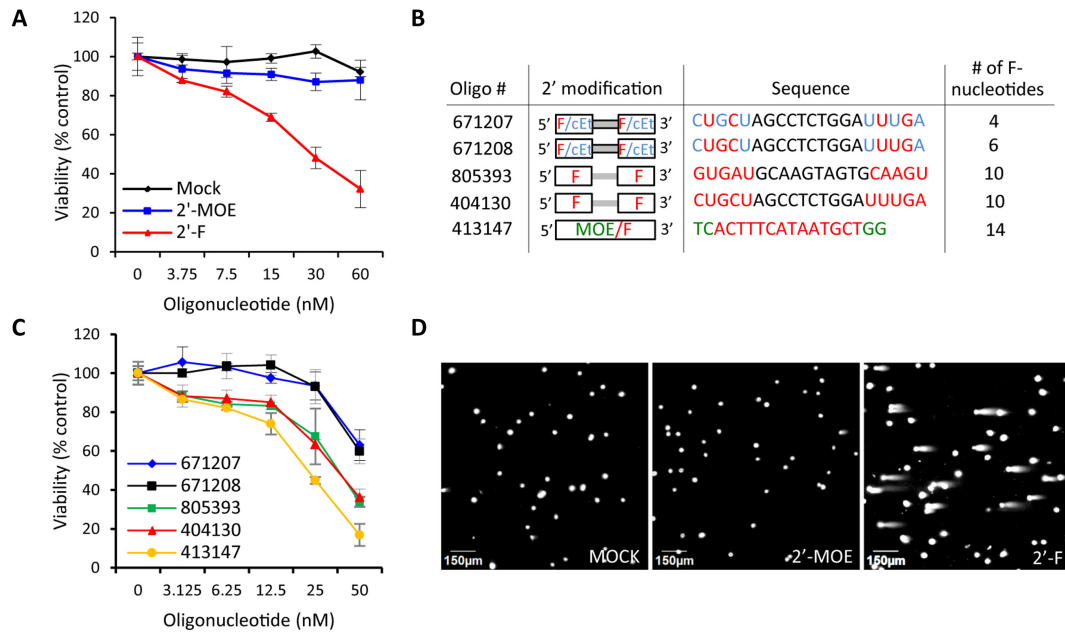
tions such as 2'-O-methyl or 2'-MOE (6,7). We showed here that this is the case for the association of PS-ASOs with P54nrB but not with PSF or PSPC1 (Figure 5B), and the results suggest that the loss of DBHS proteins may not simply be a consequence of high affinity binding of 2'-F-modified oligonucleotides with individual DBHS proteins; however, it is possible that P54nrB may attract 2'-F-modified oligonucleotides to the P54nrB-PSF or P54nrB-PSPC1 heterodimers, rendering conformational change of the entire protein complex and causing the reduction of all proteins in a complex.

An alternative possibility is that the binding site(s) on DBHS proteins or DBHS dimers for 2'-F-modified oligonucleotides is different from that for oligonucleotides modified with 2'-MOE or cEt. RRM1 of DBHS proteins is a canonical RRM and is responsible for nucleic acid binding, whereas RRM2 may not bind nucleic acid since three of four conserved residues essential for its RNA binding activity are substituted (10). DBHS proteins may recognize PS-oligonucleotides through RRM1. However, phosphorothioate modification and/or ribose moiety of oligonucleotides may permit the association of oligonucleotides with other motifs of DBHS proteins with no expected nucleic acid binding activity. The 2'-F-modified PS-oligonucleotides may bind to a unique region of DBHS proteins, causing protein conformational change(s) and degradation. Identification of the exact binding sites on protein surface of DBHS members for PS-oligonucleotides with different 2' modifications requires future investigation.

It is also possible that the 2'-F-modified PS-oligonucleotides may disrupt protein–protein interactions among DBHS proteins. DBHS proteins have extensive hydrophobic surface areas that are buried upon dimerization (10). Approximately half of the residues of each core conserved DBHS region (~300 amino acids) are directly involved in the dimer interaction. Since hydrophobic residues on a protein surface could reduce its stability (23–25), prevention of DBHS dimerization may destabilize these proteins upon exposing hydrophobic surface. In addition, conformational change(s) of DBHS proteins may occur to minimize surface hydrophobicity, exposing proteolytic sites. It has been suggested by the P54nrB/PSPC1 heterodimer crystal that P54nrB interacts PSPC1 transiently (10), raising the possibility that dynamic exchanges of dimer partners among DBHS proteins provide oligonucleotides with more hydrophobic 2'-F modification an opportunity to bind to the dimer interface and prevent dimerization. However, hetero-dimerization was found not to be required for the stabilization of DBHS proteins, since the level of PSF protein was not reduced with double knockdown of its heterodimer partners, P54nrB and PSPC1 (Figure 5E). Alternatively to disrupting dimerization among DBHS proteins, binding of 2'-F-ASOs to the DBHS proteins may recruit other proteins to the complex(s) to trigger further conformational change and protein degradation.

DBHS proteins have been implicated in DNA double-stranded break recognition and repair (22). P54nrB and PSF serve as stimulatory factors in a reconstituted non-homologous end joining system (26). P54nrB and PSF were observed at sites of DNA damage shortly after laser mi-





**Figure 6.** 2'-F-modified oligonucleotides cause severe cell death via induction of DNA damage. (A) HeLa cell viability was analyzed using a WST-8 assay 24 h after mock transfection or transfection of cells with the indicated concentrations of 2'-F-PS-ASO (ISIS404130) or 2'-MOE-PS-ASO (ISIS116847). The error bars represent standard deviations from four independent experiments. (B) The chemical modifications, sequences and numbers of the 2'-F-modified nucleotides of the tested oligonucleotides are shown. (C) HeLa cell viability was analyzed using a WST-8 assay 18 h after transfection of cells with the indicated concentrations of different oligonucleotides. The error bars represent standard deviations from four independent experiments. (D) HeLa cells were mock transfected or were transfected with 2'-F-PS-ASO (ISIS404130) or 2'-MOE-PS-ASO (ISIS116847). Cells were analyzed after 16 h using a neutral comet assay. Representative images are shown. Severity of DNA damage was reflected by the intensity of comet tail relative to the head. Scale bars, 150  $\mu$ M.

croirradiation (27). P54nrb<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) are mildly radio-sensitive; however, deficiency in both P54nrb and PSPC1 almost completely blocks recovery from double-stranded DNA damage (22). A compensatory up-regulation of PSPC1 was reported in P54nrb<sup>-/-</sup> MEFs (22), which may explain the modest phenotype observed in P54nrb<sup>-/-</sup> MEFs and suggests that P54nrb and PSPC1 are at least partially functionally redundant in DSB repair. We found that both PSPC1 and PSF were substantially up-regulated in HeLa cells depleted of P54nrb (Figure 5E), suggesting that (i) there is functional redundancy among DBHS proteins and (ii) roles of DBHS proteins, at least in certain cellular processes, are indispensable. DSBs accumulated in HeLa cells transfected with 2'-F-PS-ASO (Figure 6D). No DSBs were observed in the cells transfected with 2'-MOE-modified PS-oligonucleotides with the same sequence. These data imply that the DNA damage observed in 2'-F oligonucleotide-treated cells was not due to the PS linkages or to off-target reduction of DNA repair pathway proteins by oligonucleotides. In addition, it has been reported that homopyrimidine or pyrimidine-rich oligonucleotides may invade dsDNA by displacing homologous pyrimidine-rich strand and forming a (PNA)<sub>2</sub>-DNA triplex with the complementary strand (28–30), leading to inhibition of replication or site-specific DNA damage as was suggested previously (31,32). However, the F-ASO-induced toxicity appears not to be unique to the pyrimidine-rich ASOs. ASOs ISIS404130 and ISIS805393 are both fully PS-modified, with 10 deoxyribonucleotides flanked at both ends by five

2'-F-modified nucleotides, but are different in pyrimidine-richness. ISIS404130 contains a pyrimidine-rich region of five consecutive pyrimidines and two 5'-pyrimidines, whereas ISIS805393 contains a 5'-purine but no consecutive pyrimidines (Figure 6B). Survival curves for cells treated with ISIS404130 or ISIS805393 overlapped each other (Figure 6C), suggesting that a pyrimidine-rich motif is not required for the observed cell death. Although we cannot exclude the possibility that 2'-F oligonucleotides induce DSBs through other pathways, simultaneous loss of all DBHS proteins likely contributes to the observed phenotype. Other impaired cellular processes, related to the loss of multi-functional DBHS proteins, may also contribute to the toxicity of 2'-F phosphorothioate oligonucleotides.

2'-F modification of therapeutic oligonucleotides confers stability toward nucleases, prolonged half-life in human plasma, increased nucleic acid binding affinity and reduced immune stimulation (1,33). In addition, the 2'-F modification supports both RNase H1 and RISC mechanisms (1,5). Moreover, splicing modulation oligonucleotides with 2'-F modifications can recruit ILF2/3 to pre-mRNA to trigger exon skipping, a unique property not found with other 2'-modified oligonucleotides (18). These advantages qualified 2'-F as one of the most commonly used 2' substituents in oligonucleotide-based drug discovery programs. Our data indicate that 2'-F oligonucleotide treatment of cells reduces levels of multi-functional DBHS proteins. Although it is possible that position and/or combination of the 2'-F-modified nucleotides in an oligonucleotide or the structure of an oligonucleotide may influence the toxic effect to dif-

ferent extents, caution should be taken when interpreting results obtained using 2'-F-modified oligonucleotides.

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