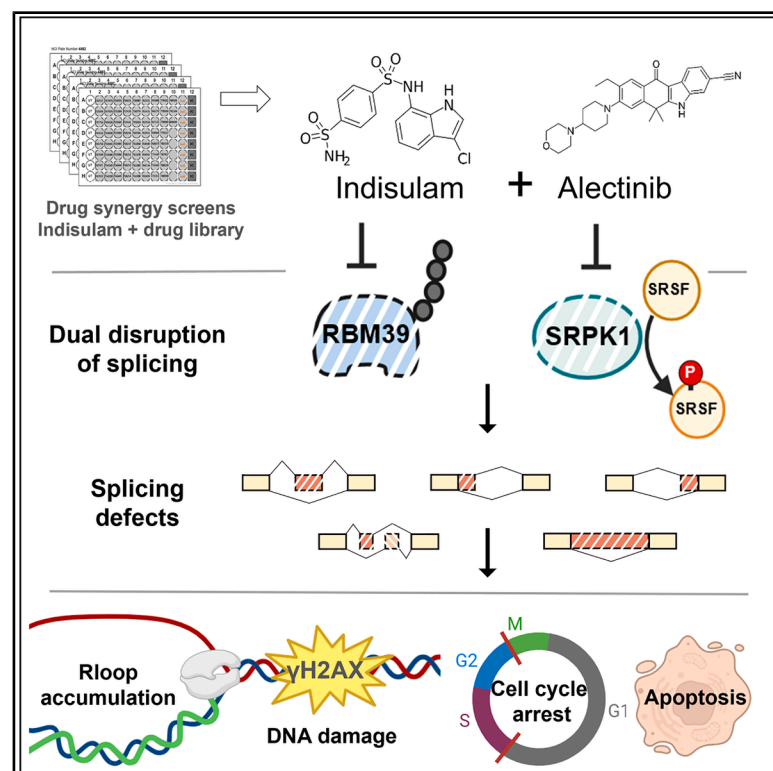


Exploiting the polypharmacology of alectinib for synergistic RNA splicing disruption with RBM39 degraders

Graphical abstract



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In brief

Ma et al. reveal that alectinib functions as a clinically relevant RNA splicing inhibitor through suppression of SRPK1 activity. Co-targeting SRPK1 and RBM39 amplifies splicing disruption, leading to R-loop accumulation, DNA damage, and tumor regression in *MYCN*-driven neuroblastoma, uncovering a therapeutic strategy for hard-to-treat cancers.

Highlights

- Alectinib is a potent and clinically relevant RNA splicing inhibitor via targeting of SRPK1
- Co-targeting SRPK1 and RBM39 enhances splicing disruption in genome stability pathways
- Alectinib synergizes with indisulam to drive strong anti-tumor effects in neuroblastoma
- Combination therapy extends survival in a neuroblastoma mouse model



Article

Exploiting the polypharmacology of alectinib for synergistic RNA splicing disruption with RBM39 degraders

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SUMMARY

Precise control of pre-mRNA splicing is vital for transcriptome integrity, and its disruption is an emerging cancer vulnerability. Here, we use indisulam to degrade RBM39 and show that the clinical ALK inhibitor alectinib can be repurposed to inhibit SRPK1. Co-treatment of indisulam and alectinib inhibited cell proliferation, induced apoptosis, and caused cell-cycle arrest in multiple cancer cell lines, including *MYCN*-amplified high-risk neuroblastoma. RNA sequencing revealed enhanced splicing defects preferentially in DNA repair-related genes following combination treatment, leading to R-loop accumulation and increased DNA damage. In the Th-*MYCN/ALK*^{F1174L} neuroblastoma mouse model, combination therapy induced complete tumor regression and significantly improved survival rates compared with monotherapies. These findings demonstrate that combining indisulam and alectinib is a promising approach to treating aggressive malignancies such as high-risk neuroblastoma, exploiting the untapped polypharmacology of alectinib as an RNA splicing inhibitor and supporting the therapeutic value of co-targeting interdependent splicing factors for synergistic benefit.

INTRODUCTION

The splicing of precursor messenger RNAs (pre-mRNAs) is a critical regulatory step in gene expression, during which non-coding introns are excised to produce functional mRNA.¹ This process is tightly controlled by splicing factors, governing the recognition and selection of splice sites, thereby generating mRNAs with varying exon combinations and consequently diverse protein isoforms.¹ Dysregulated alternative splicing can promote tumorigenesis and chemoresistance. Aryl sulfonamides such as indisulam have emerged as promising anticancer agents by targeting the RNA splicing factor RNA binding motif 39 (RBM39).^{2,3} These molecular glues facilitate the association of RBM39 with the CUL4-DCAF15 ubiquitin ligase, resulting in RBM39 polyubiquitination and subsequent proteasomal degradation.^{2,4} Suppressing RBM39 efficiently inhibits tumor growth and advancement through modulating the transcription of various genes associated with tumorigenesis (reviewed in Xu et al.³).

One cancer type particularly vulnerable to splicing perturbation is neuroblastoma, a common and aggressive extracranial solid tumor in young children, and a major contributor to pediatric cancer mortality.⁵ High-risk neuroblastoma, comprising approximately 40%–50% of all neuroblastoma cases, represents a distinct subtype characterized by aggressive tumor behavior

and an unfavorable prognosis.⁶ One pivotal factor contributing to the progression of high-risk neuroblastoma is the amplification of the *MYC* family transcription factor, *MYCN*, which can block the differentiation of neural progenitors, the key event in neuroblastoma initiation, and plays a crucial role in facilitating the transcriptional repression of tumor suppressor genes.^{6,7} Furthermore, oncogenic gain-of-function mutations in the *ALK* (anaplastic lymphoma kinase) occur in 10%–15% of primary tumors, making *ALK* the most common single-gene alteration in high-risk neuroblastoma for which targeted clinical therapies have been developed.^{8,9} Alectinib, an FDA-approved ATP-competitive ALK tyrosine kinase inhibitor, was initially developed for non-small-cell lung cancer and has shown considerable promise in addressing neuroblastoma in extensive preclinical studies.^{10–12} Besides, lorlatinib, a third-generation ALK/ROS1 tyrosine kinase, is under active investigation in neuroblastoma, where *ALK* alterations are common drivers of high-risk disease.^{13,14} However, tumor heterogeneity and resistance to treatment with ALK inhibitors (and targeted drugs in general) limit their efficacy as monotherapies.¹⁵ Combination therapies, therefore, remain important as a means to reduce the toxicity of any single drug and overcome any potential resistance more efficiently. This approach is particularly advantageous in childhood cancer, where long-term toxicity is a major lifelong issue.¹⁶



Our previous study¹⁷ and others¹⁸ have recognized the exquisite sensitivity of high-risk neuroblastoma to indisulam, with complete responses observed in multiple xenografts, patient-derived xenografts (PDX), and genetically engineered mouse models (GEMMs). Notably, cells with *MYCN* amplification exhibit increased sensitivity to aryl sulfonamides, likely due to their enhanced dependence on RNA processing machinery driven by elevated N-Myc/c-Myc activity, rendering them more susceptible to RNA splicing interference.¹⁹ Through the induction of splicing errors in key oncogenic pathways, indisulam can render cancer cells susceptible to additional disruptions caused by other anticancer drugs. For example, we previously showed that several DNA damage repair (DDR) genes are mis-spliced following RBM39 depletion, increasing sensitivity to PARP inhibitors in ovarian high-grade serous carcinoma.²⁰

In this study, we sought to further define combinatorial vulnerabilities arising from spliceosomal disruption through drug-library screens combining indisulam with FDA-approved anticancer agents across various cancer models. This approach revealed a synergistic interaction between indisulam and the ALK inhibitor alectinib, likely due to activity against a recently discovered secondary target of alectinib, serine-arginine protein kinase 1 (SRPK1).²¹ SRPK1 promotes the nuclear translocation and activation of a family of SR splicing factors (SRSFs). The combination of indisulam and alectinib simultaneously depletes RBM39 and inhibits SRPK1-mediated SRSF phosphorylation, resulting in widespread RNA splicing aberrations. The subsequent failure to resolve accumulated DNA damage and R loops resulted in cell cycle arrest, cell growth inhibition, and apoptosis of neuroblastoma cells *in vitro* and tumor regression *in vivo*. Our study provides important evidence for the co-targeting of splicing factors as a viable strategy to treat aggressive malignancies such as *MYCN*-driven high-risk neuroblastoma.

RESULTS

Combination drug screens revealed synergy between indisulam and alectinib

We previously demonstrated that indisulam-mediated RBM39 degradation causes widespread RNA splicing defects and an enrichment in the aberrant expression of proteins that mediate DDR and cell cycle regulation across various cancer types.^{17,20} This led us to speculate that indisulam likely generates drug-induced vulnerabilities that potentiate the efficacy of existing, clinically approved anticancer compounds, which we have demonstrated with PARP inhibitors.²⁰ RBM39 degraders, such as indisulam and E7820, have been trialed in 47 clinical studies,³ highlighting potential for therapeutic combinations ready for clinical evaluation.

To explore this, we conducted drug-library screens combining indisulam with 147 FDA-approved anticancer drugs (NCI plate no. #4,891 and #4,892) in three different cancer backgrounds with high Myc activity (KURAMOCHI (ovarian high-grade serous carcinoma, c-Myc), KELLY (neuroblastoma, n-Myc), and HCT116 (colorectal cancer, c-Myc). Library compounds were tested at 5 doses (16 nM–10 μ M) and combined with DMSO or

indisulam for 72 h (Table S1). Synergy or antagonism was determined by Bliss independence analysis,²² and compounds were ranked based on the number of dose-pairs with scores > 0.02 and average Bliss scores. Across the three screens, alectinib was a top-ranked candidate (#1 in KELLY and HCT116 and #2 in KURAMOCHI) (Figures 1B and S1A–S1D), indicating a consistent drug-drug interaction. The clinical ALK inhibitor alectinib was therefore selected for further validation in a wider range of drug concentrations.

Depletion of RBM39 by indisulam is dependent on DCAF15 expression, and we previously generated CRISPR-Cas9 knockouts of *DCAF15* to identify RBM39-specific drug-drug interactions.¹⁷ Therefore, we next examined whether this dependency also underlies the observed synergy (Figures 1C and 1D). Co-treatment of indisulam and alectinib demonstrated strong synergistic growth inhibition across a range of doses in KELLY, HCT116, and KURAMOCHI, confirming the synergy observed in our high-throughput screens. Most of the synergistic drug-drug interactions were abrogated in both KELLY and HCT116-*DCAF15*^{KO} cells compared to the wild-type clones (*DCAF15*^{WT}), suggesting that the synergy is dependent on DCAF15-mediated RBM39 degradation. Similar trends were observed by co-treatment of alectinib and E7820, a second aryl sulfonamide RBM39 degrader² (Figures S1E and S1F).

Considering the potency of both indisulam and alectinib as monotherapies in high-risk neuroblastoma,^{11,17} we further investigated the combination of indisulam and alectinib in 3 other *MYCN*-amplified neuroblastoma cells (IMR32, SKNBE2C, and IMR5) (Figure 1E). Combination treatment significantly inhibits cell proliferation compared to monotherapies and displays great synergy across a wide range of doses of both compounds on these *MYCN*-amplified cell lines. However, the synergy between indisulam and alectinib was not observed in non-amplified cells (SKNAS, SKNSH, and SHEP) (Figure S1G), which is consistent with our previous finding that indisulam exerts higher toxicity in *MYCN*-amplified neuroblastoma cells compared to non-amplified neuroblastoma cells.

Indisulam and alectinib exhibit synergistic anti-cancer efficacy in high-risk neuroblastoma

In addition to synergistic growth inhibition, alectinib significantly increased the percentage of apoptotic cells when combined with indisulam in KELLY and IMR32, while there was no induction of cell apoptosis by alectinib monotherapy (Figures 2A, 2B, and S1H). Correspondingly, a significant increase in cleaved caspase-3 was detected in the combination treatment group in KELLY cells (Figure 2C). To evaluate cell cycle progression, we used flow cytometry analysis and detected a notable accumulation of cells in the G2/M phases following 48 h treatment with indisulam, which is consistent with a previous study.¹⁸ Co-treatment with indisulam and alectinib increased the percentage of cells in the G1-phase, implying enhanced cell-cycle arrest in both G2/M and G1 phase, with a substantial decrease in the percentage of cells in S-phase (Figures 2D and 2E). These data confirm that indisulam and alectinib act synergistically to suppress the proliferation of neuroblastoma cells, disrupt the cell cycle, and induce cell apoptosis.

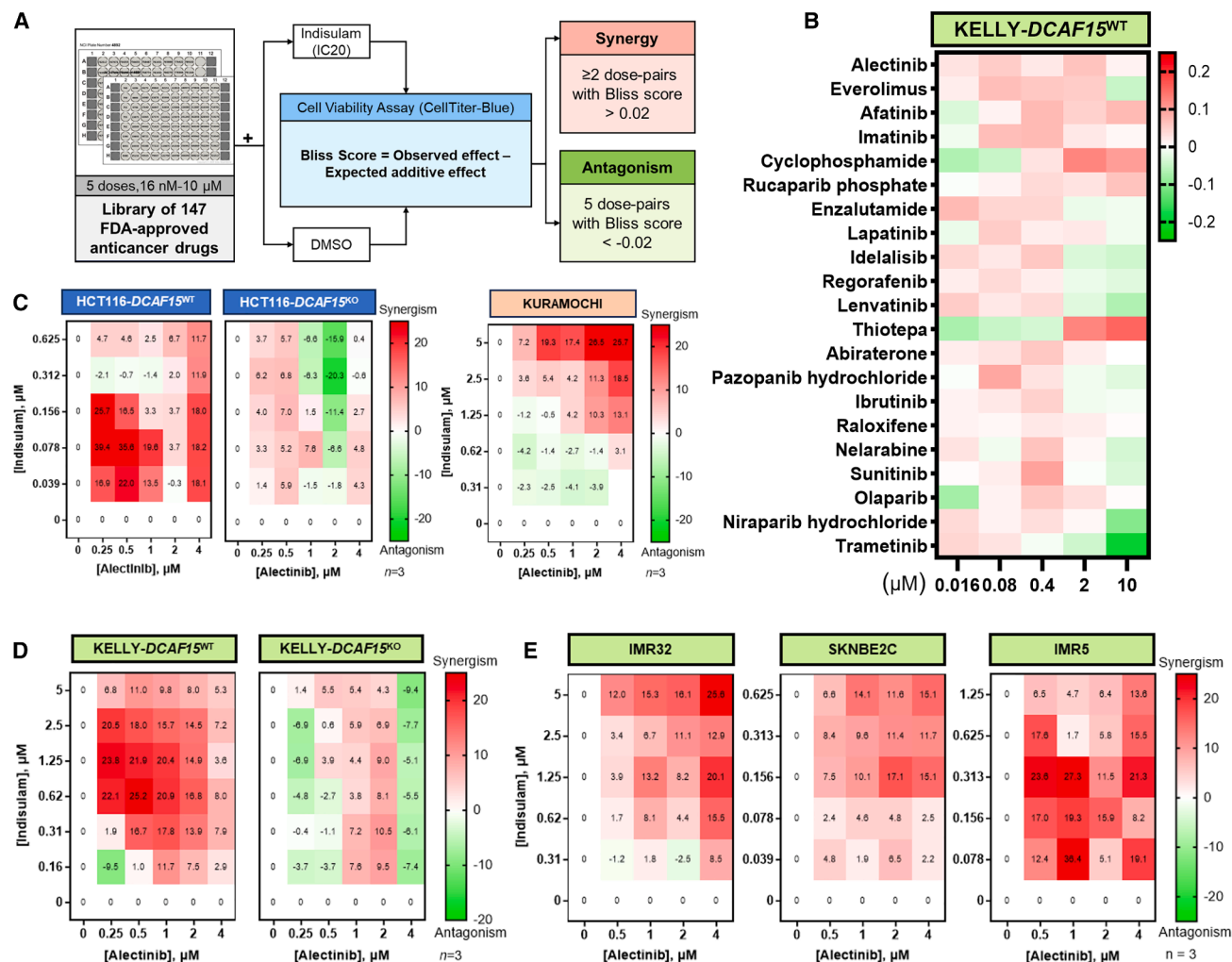


Figure 1. Combination drug screens revealed a synergistic growth inhibition of indisulam and alectinib

(A) Workflow of drug combination screens on neuroblastoma cells. KELLY-DCAF15^{WT} cell clones were treated with 147 FDA-approved anticancer in 5 doses ranging from 16 nM to 10 μ M with or without indisulam. Bliss scores were calculated based on cell viability measured by CellTiter-blue assay. Synergy pairs are defined as more than 2 dose-pairs with indisulam showing Bliss score larger than 0.02. Antagonism is defined as all five doses showing Bliss score lower than -0.02 . $n = 1$.

(B) Heatmap showing Bliss scores of drug combination screens. Drugs were ranked based on the number of dose-pairs (with indisulam) showing Bliss scores > 0.02 and average Bliss scores. Red indicates synergism, and green indicates antagonism.

(C–E) Cell lines used for synergy screen (C and D) and indicated neuroblastoma cell lines (E) were treated with alectinib combined with indisulam for 72 h and analyzed for cell growth by SRB. $n = 3$ biological replicates. Synergy scores were calculated by Synergyfinder (<https://synergyfinder.fimm.fi/>) using the Loewe method.

ALK inhibition by alectinib is not required for the synergistic interaction with indisulam

According to data from the Human Protein Atlas ([proteinatlas.org](https://www.proteinatlas.org/)),²³ ALK and RET, both primary targets of alectinib, are expressed in neuroblastoma cells but not in HCT116 and KURAMOCHI (Figure S2A; Table S2), in which synergy was discovered. To further test whether the synergy is driven by ALK inhibition, we tested a second ALK inhibitor with a distinct chemical scaffold and binding site, lorlatinib, and found that this compound did not enhance sensitivity to indisulam in neuroblastoma cell lines with wild-type ALK or in HCT116 and KURAMOCHI, which lack ALK expression (Figures 3A–3C). High doses of

lorlatinib showed slight synergy with indisulam in KELLY cells, which express a mutant, oncogenic form of ALK (ALK^{F1174L}) (Figure 3C), consistent with other studies showing that lorlatinib exerts higher efficacy in ALK-mutated neuroblastoma,²⁴ implying that the efficacy of lorlatinib is specific to ALK-mutated neuroblastoma. We also transfected KELLY cells with a small interfering RNA (siRNA) targeting ALK (siALK) prior to indisulam dosing (Figures 3D and 3E). We found no significant difference in cell growth inhibition between siALK and non-targeting control (siNTC) transfected cells (Figure 3E), indicating that ALK inhibition alone does not increase sensitivity to indisulam. These findings confirm that ALK inhibition is not a significant

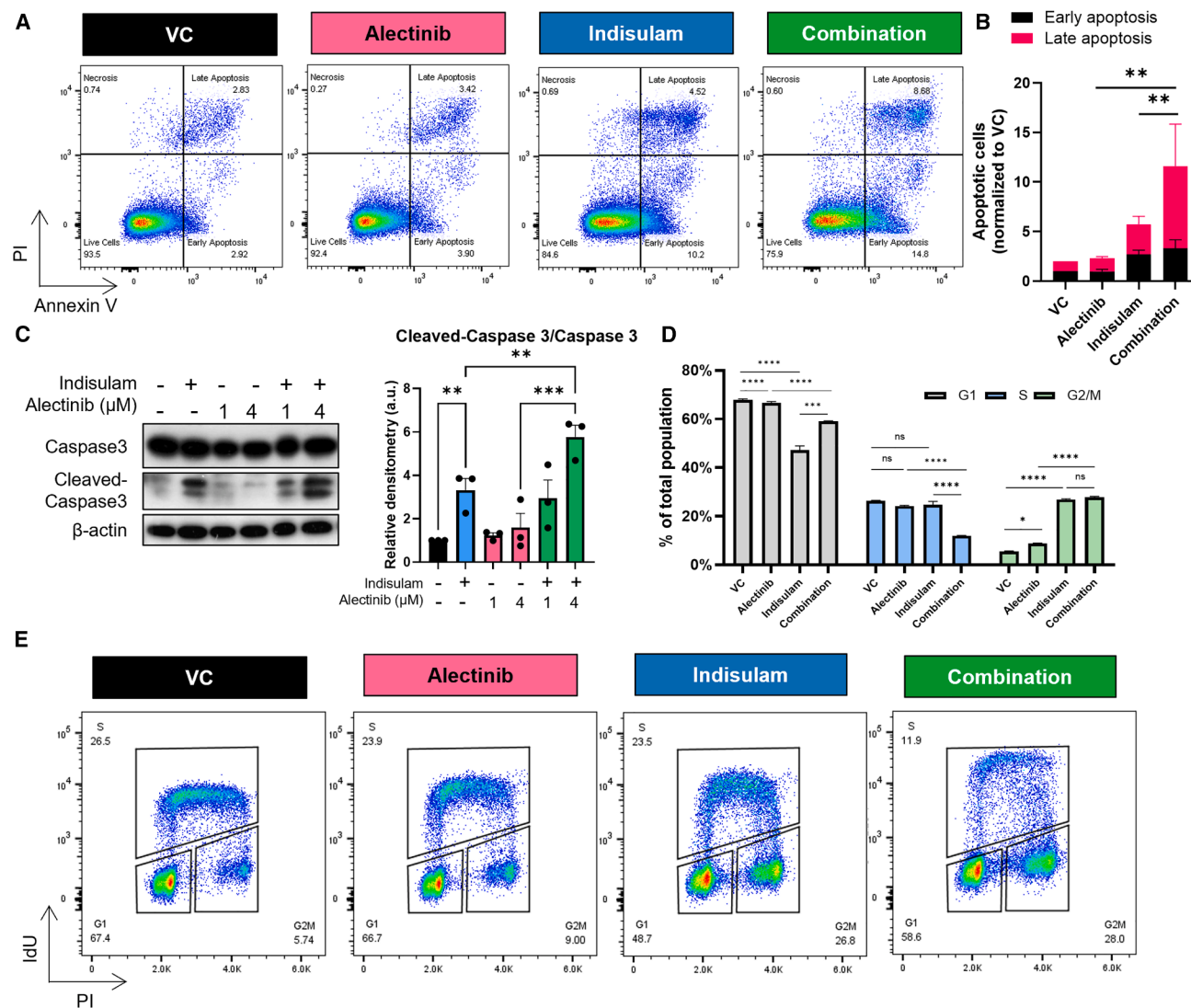


Figure 2. Indisulam and alectinib induce apoptosis and cell cycle disruption in high-risk neuroblastoma

(A and B) Apoptosis analysis via flow cytometry in KELLY cells following exposure to 1.25 μ M indisulam, 4 μ M alectinib, or their combination for 48 h. Cells were labeled with Annexin V and propidium iodide (PI). $n = 3$ biological replicates. Error bars indicate SEM. The significance was calculated by one-way ANOVA.

(C) Western blot of KELLY cells treated with indicated doses of indisulam and alectinib for 48 h; $n = 3$ biological replicates and error bars indicate SEM. Significance was calculated by one-way ANOVA.

(D and E) Profiling of cell cycle distribution in KELLY cells was performed using flow cytometry after 48 h treatment with indisulam, alectinib, or the combination. Cellular DNA content was labeled with PI, while replicating cells were labeled with nucleotide analog, IdU. $n = 3$ biological replicates and error bars indicate SEM. Two-way ANOVA was employed to determine the statistical significance among the groups. n.s., not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

contributor to the synergy we observe between alectinib and the RBM39 degraders indisulam and E7820. This suggests that synergy with RBM39 degradation relates primarily to the inhibition of a secondary target of alectinib.

Alectinib exerts potent inhibition of SRPK1

Comprehensive kinase inhibitor screens indicate that alectinib inhibits an array of additional kinases other than ALK.²¹ The observation that alectinib is a potent inhibitor of SRPK1/2^{21,26} is particularly relevant to our research on aberrant RNA splicing.

Both SRPK1 and 2 play a central role in the regulation of alternative splicing via phosphorylation as well as the activation and nuclear translocation of the SRSFs.^{27,28} In MYCN-amplified neuroblastoma, SRPK1 expression is elevated and associated with reduced survival rates (Figures S2B and S2C). In contrast, lower survival rates are not associated with increased expression of SRPK2 nor SRPK3, indicating an increased dependency on SRPK1 to drive tumor growth in MYCN-amplified tumors. Furthermore, a genome-wide CRISPR study showed that loss of SRPK1 sensitizes various solid tumor cell lines to indisulam

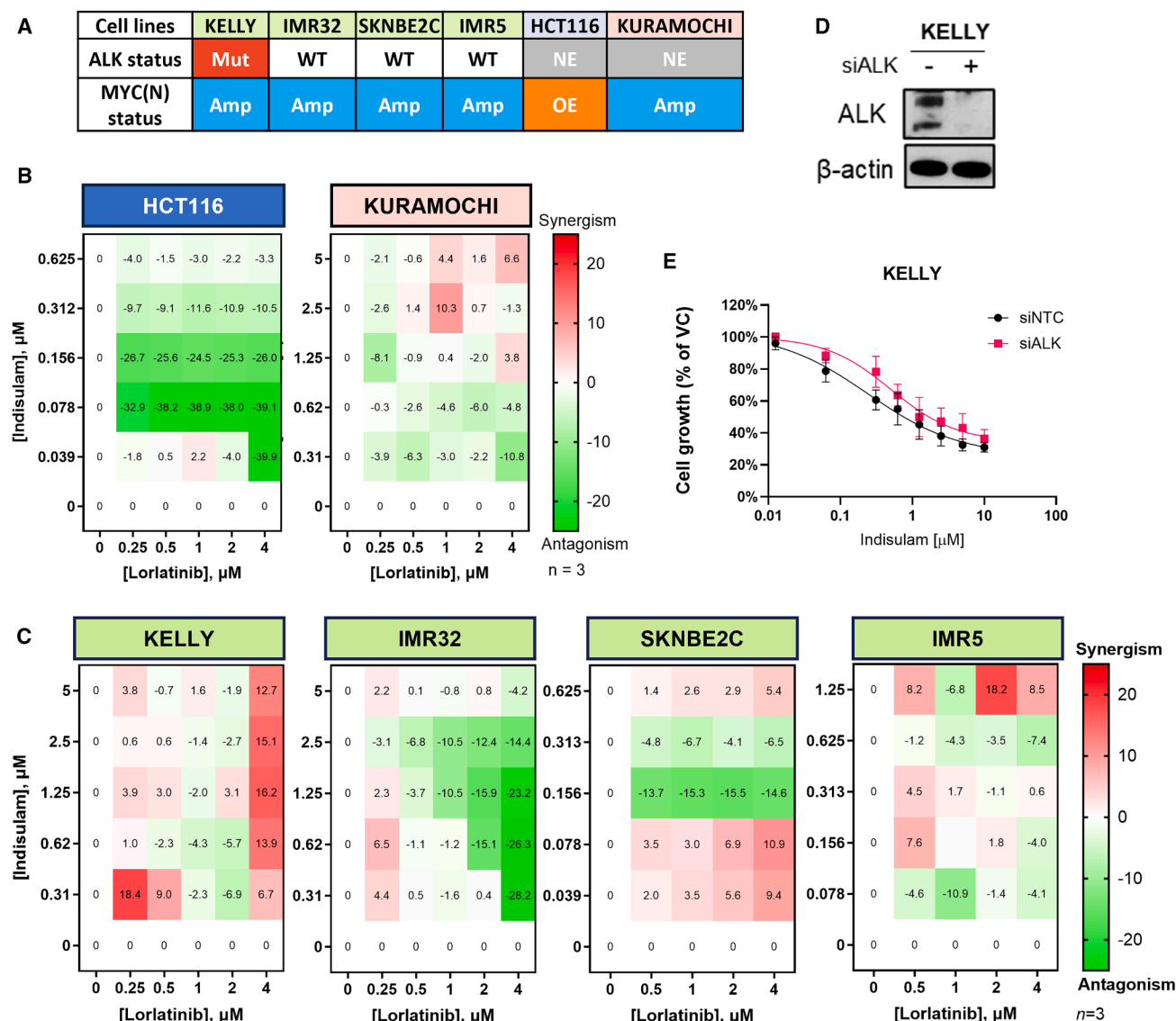


Figure 3. ALK inhibition by alectinib is not critical for driving the synergy with indisulam

(A) Genetic alterations of four neuroblastoma cell lines, HCT116, and KURAMOCHI, Mut, mutation; Amp, amplification; WT, wild type; NE, no expression; and OE, overexpression. Data adapted from Galiger et al.²⁵

(B and C) Indicated cell lines were treated with lorlatinib and indisulam for 72 h. Loewe synergy scores are calculated as described in Figure 1; $n = 3$ biological replicates.

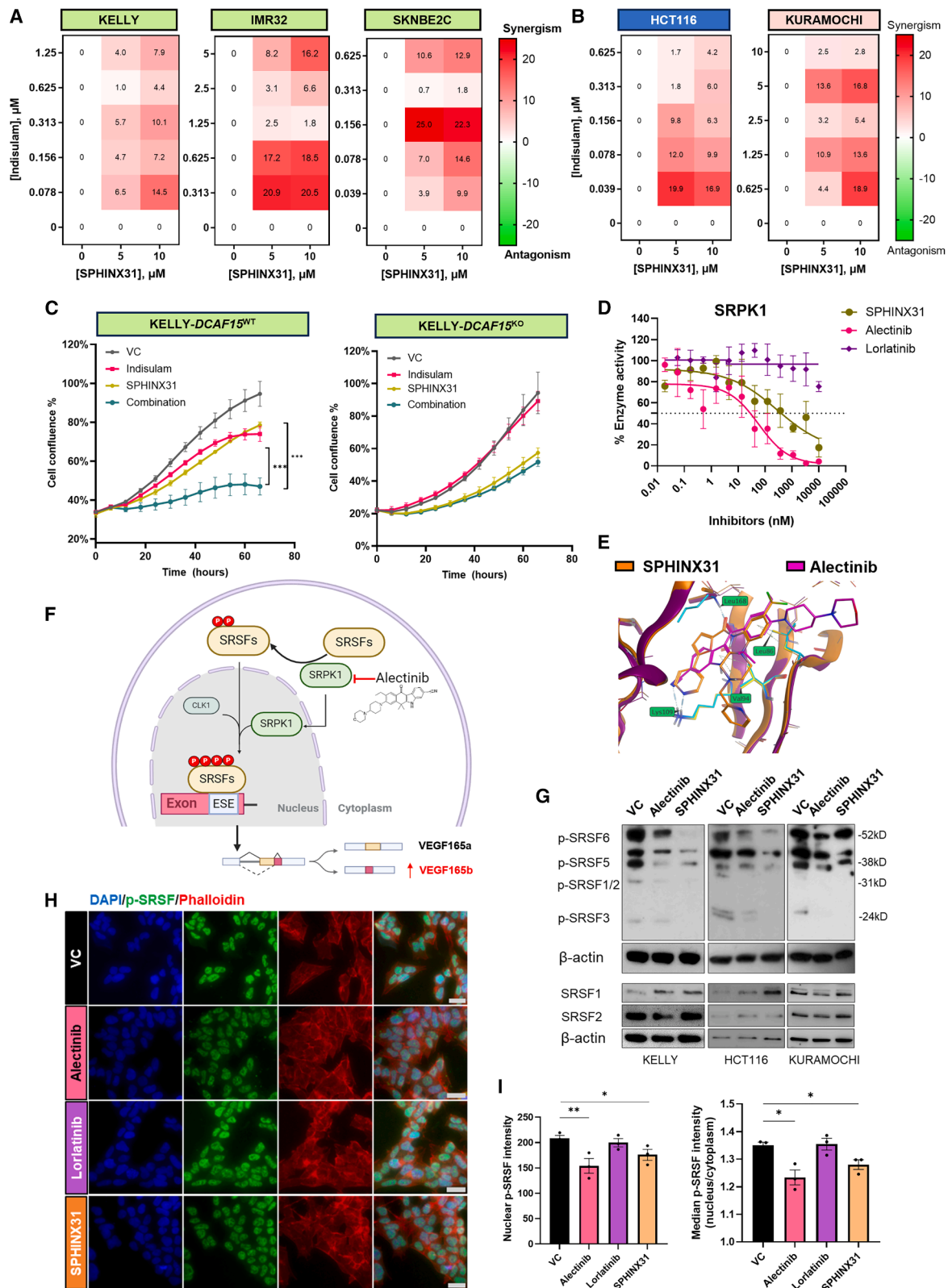
(D) Western blot confirming the knockdown of ALK gene expression in KELLY cells post-transfection with siNTC or siALK. $n = 2$ biological replicates.

(E) KELLY cells were transfected with siALK or siNTC for 16 h, followed by exposure to indisulam for 72 h. Cell growth was measured by the SRB assay. $n = 3$ biological replicates. Error bars indicate SEM.

treatment.²⁹ Thus, we assessed the synergy between indisulam and SPHINX31, a known SRPK1 inhibitor.³⁰ Co-treatment of SPHINX31 and indisulam exhibited synergistic growth inhibition in MYCN-amplified neuroblastoma cells (Figure 4A) and the other two ALK-negative cells, HCT116 and KURAMOCHI (Figure 4B). Time-lapse cell confluency monitoring in KELLY-DCAF15^{WT} and DCAF15^{KO} showed that co-treatment significantly suppressed the growth of KELLY-DCAF15^{WT}, compared to individual drug treatments. Conversely, in the absence of DCAF15, the combination therapy displayed similar potency to

SPHINX31 monotherapy (Figure 4C), suggesting that this interaction is DCAF15-dependent and thus RBM39-dependent.

The inhibition of SRPK1 by alectinib was confirmed through cell-free biochemical assays that reported a half-maximal inhibitory concentration (IC₅₀) of 26 nM. This inhibition is more potent than that of SPHINX31 (IC₅₀ = 375 nM), with lorlatinib showing very little inhibitory action (Figure 4D). Notably, alectinib also inhibited SRPK2 with an IC₅₀ of 700 nM while SPHINX31 showed no inhibitory effect (Figure S2D). Comparison of crystal structures of SRPK1-alectinib and SRPK1-SPHINX31 complexes



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confirmed that alectinib and SPHINX31 interacts with the ATP-binding groove of SRPK1, with reasonable affinity and occupy similar positions within the binding site (Figures 4E and S3A–S3D).

SRPK1 phosphorylates SRSFs, triggering their nuclear translocation and activity in modulating RNA splicing (illustrated in Figure 4F). Both alectinib and SPHINX31 reduced phosphorylated SRSF (p-SRSF) in KELLY, HCT116, and KURAMOCHI cells (Figures 4G and S2E) and decreased the nuclear-to-cytoplasmic ratio of p-SRSF intensity within 4 h in KELLY cells (Figures 4H and 4I), while lorlatinib did not (Figures 4H–4I and S2F). SRSFs are known to promote the selection of the proximal splice site in *VEGF* pre-mRNA, and inhibiting SRPK1 alters *VEGF* splicing to generate the *VEGF165b* isoform.^{21,31} Treatment of both alectinib and SPHINX31 resulted in a switch and increased *VEGF165b* mRNA in high-risk neuroblastoma cells (Figure S2G).

In conclusion, our findings reveal that alectinib is a potent inhibitor of SRPK1, consequently modulating downstream function and RNA splicing through a reduction in the phosphorylation of SRSF splicing factors. Notably, alectinib demonstrates a more potent inhibition of SRPK1 compared to the selective inhibitor SPHINX31, an intriguing observation that could potentially expedite the clinical repurposing of alectinib as an SRPK1 inhibitor.

Co-treatment of alectinib and indisulam exacerbates RNA splicing defects and increases R-loop formation and DNA damage

Proteomic data from our previous study¹⁷ showed that indisulam exposure leads to an increased expression of most SRSF family members (Figure S4A). Here, we observed enhanced nuclear translocation of p-SRSF post-indisulam treatment, which was rescued in *DCAF15*^{KO} cells (Figures S4B and S4C), suggesting that SRPK1/SRSF pathway activation may be a compensatory mechanism following RBM39 degradation. RBM39 has previously been identified as a potential SRPK1 target,³² and we observed a direct interaction between RBM39 and SRPK1 in neuroblastoma cells via proximity ligation assays (Figure S4D). This supports our hypothesis that the synergy between alectinib and indisulam arises from their combined disruption of RNA splicing, by dually targeting interdependent spliceosomal components, limiting potential adaptation.

To evaluate the impact of treatment on alternative splicing, IMR32 cells were treated with alectinib, indisulam, or both for 24 h. RNA sequencing (RNA-seq) and alternative splicing analysis (rMATS) revealed that while indisulam induced widespread splicing changes, particularly skipped exons (SE), and that alectinib alone had a milder effect, combination treatment resulted in

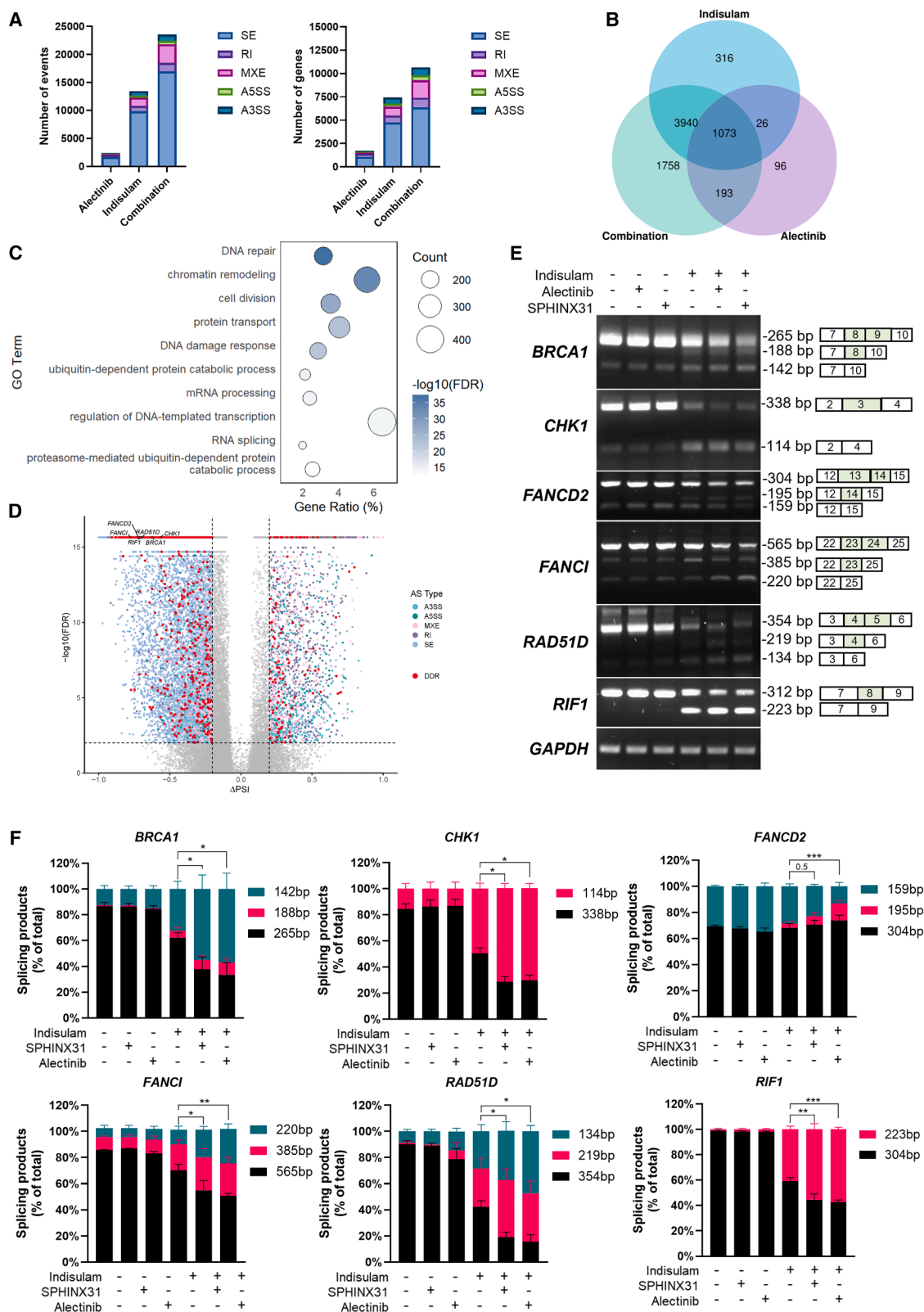
an additional 7,646 altered events (33%) and 1,758 mis-spliced genes (25%) beyond an additive effect (Figure 5A). Notably, genes mis-spliced after alectinib treatment largely overlapped with indisulam targets (Figure 5B), further supporting the likelihood of functional cooperation between RBM39 and SRPK1. Gene ontology (GO) analysis of alternatively spliced genes revealed enrichment in pathways regulating genome stability, including DNA repair (e.g., *FANCD2*, *FANCI*, *RAD51D*, and *RIF1*), chromatin remodeling (e.g., *SMARCA1*, *SMARCD1*, *PRMT2/3/7*, and *INO80*), and DNA damage response (e.g., *BRCA1*, *CHK1*, and *TP53BP1*) (Figures 5C and 5D). Several top-ranked events were validated through custom endpoint PCR of affected exons (Figures 5E and 5F), confirming that splicing disruption was significantly enhanced when combining RBM39 degraders and SRPK1 inhibition in both KELLY and IMR32 cells (Figures 5E, 5F, and S5A); such an effect was not observed with lorlatinib treatment or following ALK knockdown by siRNA (Figures S5B–S5D). These results suggest the synergy observed between alectinib and indisulam may be attributed to the compounded reduction of canonical isoform expression and functional loss in affected pathways, such as DNA repair. For example, expression of the canonical *CHK1* isoform was nearly abolished by the co-treatment of indisulam with either alectinib or SPHINX31. Moreover, *CHK1* protein was depleted following indisulam and alectinib combination treatment (Figure S5E).

To determine whether co-treatment leads to increased DNA damage, we assessed γ H2AX levels as a marker for DNA double-stranded breaks.³³ Immunofluorescence and western blot showed significant γ H2AX accumulation following combination treatment compared to monotherapies (Figures 6A, 6B, and S6A). Importantly, this increase was absent when indisulam was combined with lorlatinib (Figures S6B and S6C), indicating the effect is a result of RNA splicing disruption rather than ALK inhibition.

R-loops are DNA:RNA hybrids that are a product of intermediate transcription and must be resolved to permit progression of replication forks.^{34,35} R-loop accumulation results in transcription-replication collisions, which cause replication stress and eventually lead to double-strand DNA breaks.³⁶ Many genes that regulate R-loop maintenance were mis-spliced after combination treatment of indisulam and alectinib. For example, *BRCA1* interacts with the putative RNA helicase senataxin, forming a complex that localizes to R-loops,³⁷ and Fanconi anemia genes recruit RNA processing enzymes to resolve R-loops generated by moderate replication stress,³⁸ while *RIF1* facilitates replication fork protection and efficient restart to maintain genome stability.³⁹ Notably, SR proteins regulated by SRPK1 can also

Figure 4. SRPK1 inhibition synergizes with indisulam in high-risk neuroblastoma

(A and B) Indicated cell lines were treated with indisulam and SPHINX31. Loewe synergy scores were calculated to ascertain synergy. $n = 3$ biological replicates. (C) KELLY-*DCAF15*^{WT} and *DCAF15*^{KO} cells were treated with indisulam and SPHINX31 individually or in combination for 72 h. Cell confluence was monitored every 6 h using the Incucyte live cell imaging system. $n = 3$ biological replicates. Error bars indicate SEM. One-way ANOVA was used to determine significance. (D) SRPK1 enzyme activity measured with ADP-Glo kinase assay with the indicated doses of the compounds. $n = 3$ biological replicates. Error bars indicate SEM. (E) Alectinib (pink) docked into SRPK1 complexed with SPHINX31 (orange). (F) Diagram depicting alectinib inhibition against SRPK1. Made with Biorender. (G) Western blotting of p-SRSF and total SRSF1/2 in KELLY, HCT116, and KURAMOCHI post 48 h treatment: 4 μ M alectinib and 10 μ M SPHINX31 were used. $n = 3$ for KELLY, $n = 2$ for HCT116 and KURAMOCHI. (H and I) Immunofluorescent staining of p-SRSF in KELLY post-treatment. Nuclear intensity and cytoplasmic staining intensity are calculated using DAPI and phalloidin segmentation. Scale bars, 20 μ m. $n = 3$ biological replicates. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.



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impede R-loop formation.³⁴ Dysregulation of these functions leads to accumulation of unresolved R-loops, replication fork breakage, and genome instability.³⁴ We therefore quantified R-loops using the S9.6 antibody.⁴⁰ Treatment with either alectinib or SPHINX31 led to increased nuclear S9.6 staining, indicating the accumulation of R-loops. Combination treatment significantly increased R-loop formation as compared to monotherapies (Figures 6C, 6D, and S6D–S6F). To evaluate the replication stress induced by R-loop accumulation,^{34,41} we quantified the intensity of phosphorylated RPA32 serine 33 (p-RPA), a marker of single-stranded DNA at stalled replication forks. We observed an increase following exposure to the combination treatment compared to monotherapy (Figures 6E and S6E). DNA fiber analysis using bromodeoxyuridine (BrdU) and 5-iodo-2-deoxyuridine (IdU) labeling further revealed a reduction in track length and significantly decreased IdU/BrdU ratio following combination treatment, indicating increased fork stalling and replication stress. Collectively, these findings suggest that dual targeting of SRPK1 and RBM39 leads to the accumulation of unresolved R-loops, promoting replication stress and contributing to subsequent DNA damage in high-risk neuroblastoma.

Combination of indisulam and alectinib improves survival in Th-MYCN/ALK^{F1174L} neuroblastoma model

Gain-of-function mutations in ALK account for 10%–15% of high-risk neuroblastoma.⁹ ALK inhibitors have been shown to be safe and effective in treating ALK-driven neuroblastoma in a phase I trial^{13,14} and are currently being investigated in several phase III trials,^{13,42,43} highlighting their potential for clinical use in ALK-positive neuroblastoma. Considering the clinical relevance and benefit, we used an allograft mouse model implanted with Th-MYCN/ALK^{F1174L} tumor cells derived from GEMMs (Figure 7A).

Tumor-bearing mice were treated (indisulam 10 mg/kg, alectinib 40 mg/kg, or a combination) for 2 weeks with a 45-day follow-up. Both monotherapies significantly enhanced survival; however, all alectinib-treated mice relapsed during treatment, and 5 of the 8 mice in the indisulam monotherapy group relapsed after withdrawal of treatment. We observed complete tumor regression without subsequent relapses in the combination group (Figures 7B and 7C), consistent with the increased efficacy *in vitro*.

Tumor samples were collected for pharmacodynamic analysis after 3 days of treatment. Western blotting showed a clear depletion of RBM39 following indisulam and combination therapy, confirming the on-target effect of indisulam (Figure 7D). Furthermore, treatment with 10 mg/kg indisulam reduced n-Myc expression, as previously reported by Singh et al.,¹⁸ suggesting a

potential link between RBM39 inhibition and suppression of n-Myc (Figure 7D). Additionally, a reduction in p-SRSF and p-ALK confirmed on-target effects of alectinib on SRPK1 and ALK (Figures 7F and S7A). Double-strand DNA breaks measured by accumulation of γ H2AX foci were increased in the combination arm (Figures 7G and 7H), in line with our observations *in vitro*. Indisulam alone did not significantly induce γ H2AX accumulation, possibly due to the transient nature of γ H2AX focus formation. However, clear nuclear fragmentation was observed in mice treated with indisulam and combination therapy (Figures 7G and 7H). Importantly, no significant weight loss and adverse effects were observed during the trial, indicating no significant toxicity (Figure S7B; Table S3), and we observed no significant RBM39 loss in major organs after 3 days of treatment (Figure S7C).

Taken together, these data confirm that in neuroblastoma, the therapeutic benefit of RBM39 degraders can be enhanced through combination therapy with SRPK1 inhibitors, such as alectinib. This study provides proof of concept for the co-targeting of functionally interdependent splicing factors using repurposed clinical agents to more effectively target aggressive childhood cancers such as neuroblastoma. The strategy could be applied more widely to other oncogene-driven cancers that exhibit a high dependency on faithful splicing of mRNA.

DISCUSSION

Indisulam has been recently identified as a molecular glue capable of regulating RNA alternative splicing.² By inducing splicing errors of genes in key oncogenic pathways, indisulam and other spliceosomal inhibitors may render cancer cells susceptible to additional disruptions caused by other drugs. Thus, it holds potential for implementation as a combination therapy. We performed extensive drug screens with FDA-approved anticancer agents that revealed several potential drug-drug interactions with indisulam. Notably, the synergy between indisulam and PARP inhibitors, also observed in our screens, has been corroborated by other studies in high-grade ovarian carcinoma,²⁰ colorectal cancer, and breast cancer,⁴⁴ giving confidence in our screening approach. Our study also highlights the fact that such screens can reveal beneficial interactions based on polypharmacology (engagement of targets beyond the primary focus of clinical development),⁴⁵ presenting rationales for drug repurposing.

High-risk neuroblastoma, which constitutes about 40%–50% of all neuroblastoma cases, is characterized by aggressive behavior and poor prognosis.⁶ Gain-of-function mutations in ALK account for 10%–15% of primary tumors;⁹ hence, a number of trials

Figure 5. AS splicing disruption in neuroblastoma cells following co-treatment of indisulam and alectinib

(A) Number of alternative splicing (AS) events (left) and affected genes (right) detected by rMATS, classified into exon skipping (SE), retained introns (RI), mutually exclusive exons (MXE), alternative 5' splice sites (A5SS), and alternative 3' splice sites (A3SS).

(B) Venn diagram showing overlap of alternatively spliced genes across treatments.

(C) GO enrichment analysis of AS genes in the combination group; dot size reflects gene count, and color indicates $-\log$ FDR (false discovery rate).

(D) Volcano plot of significantly altered SE events in the combination-treated group versus control. For genes with multiple AS events, the one with the largest absolute percent spliced in (PSI) is shown. Events with FDR = 0 (extremely significant, rounded due to numerical limits) were assigned a nominal value of $1e-15$ for display. DDR and genome instability-related genes are highlighted in red, and six of these genes were selected for further experimental validation (labeled).

(E) PCR validation of SE events in DDR-related genes (representative image of $n = 3$ biological replicates). *GAPDH* was included as the control.

(F) Densitometry analysis of AS products. $n = 3$ biological replicates. Error bars indicate SEM, and two-way ANOVA was used to calculate significance.

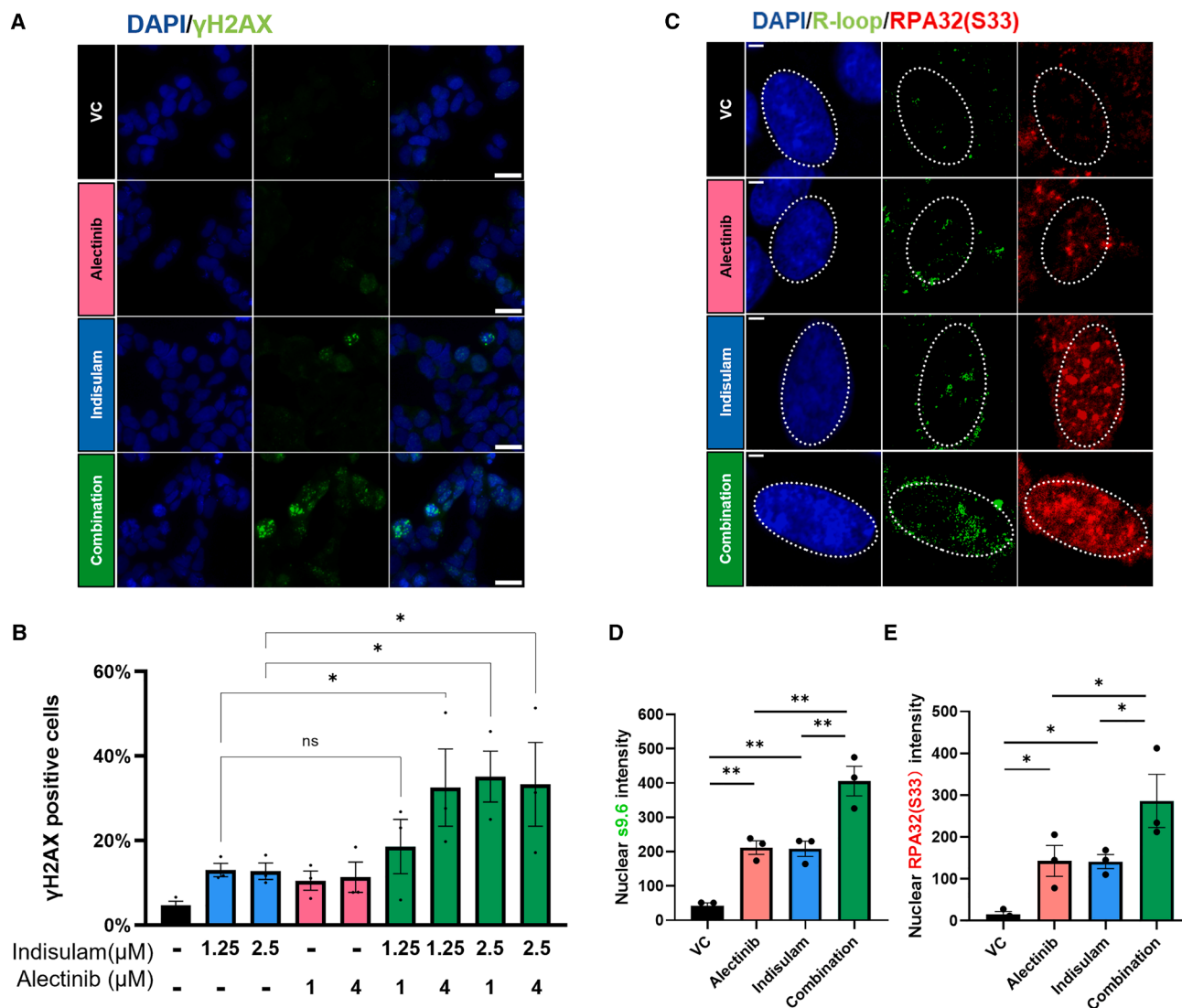


Figure 6. Combination of indisulam and alectinib induces DNA damage, R-loop accumulation, and replication stress

(A and B) Immunofluorescence staining of γH2AX in KELLY cells treated with indisulam in combination with alectinib. Representative images of KELLY treated with 4 μM alectinib and 1.25 μM indisulam are shown. Scale bars, 20 μm. Nuclear foci > 5 indicate positive cells. *n* = 3 biological replicates. Error bars indicate SEM.

(C) Representative images for nuclear S9.6 and RPA32 (S33) foci in KELLY cells. *n* = 3 biological replicates. Scale bars, 2.5 μm.

(D and E) Quantification of nuclear S9.6 intensity (D) and p-RPA32 (E) intensity in KELLY cells. Cells were treated with 4 μM alectinib monotherapy or with 1.25 μM indisulam; nuclear signal intensity was measured by merging with DAPI; *n* > 300 cells from 3 independent experiments were quantified. Significance was analyzed on median values. Error bars indicate SEM.

have investigated the use of alectinib and other ALK inhibitors in this clinical setting. While the more selective ALK inhibitor lorlatinib has shown greater traction for the treatment of mutant ALK-driven neuroblastoma,¹³ treatment resistance remains a significant clinical challenge.⁴⁶ Our study demonstrates that the activity of alectinib against SRPK1 can be exploited therapeutically in combination with other spliceosomal inhibitors. This presents an interesting strategy to treat patients with emerging resistance to ALK inhibitor therapy, including lung cancer where alectinib has demonstrated much greater clinical use⁴² and beyond into other cohorts irrespective of the ALK status. While other inhibitors of

SRPKs and related SRSF regulators, such as cirtuvivint⁴⁷ and TG-003,⁴⁸ are currently in early development, the fact that an established therapeutic tool can be repurposed in this way could accelerate the clinical testing of SRPK1/2 inhibition and co-targeting of splicing factors as an anticancer treatment strategy, particularly in tumors where an ALK inhibitor might normally be indicated.

Limitations of the study

While we have successfully demonstrated the principle of co-targeting functionally interdependent splicing as an anticancer strategy, several resulting questions arise. Our investigation

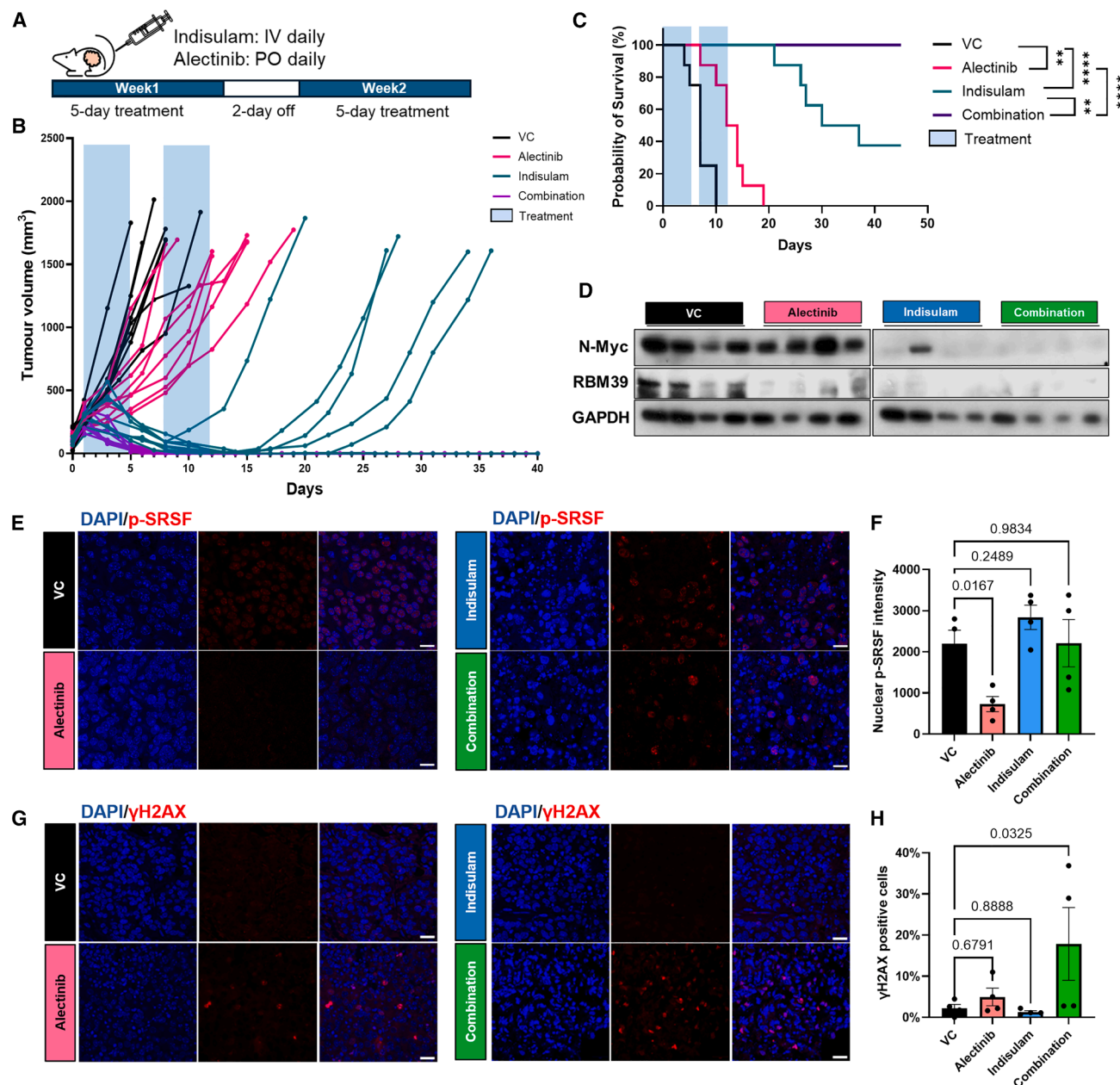


Figure 7. Combination of indisulam and alectinib suppresses neuroblastoma tumor growth

(A) Th-MYC/ALK^{F1174L} GEMM tumor cells were injected into a cohort of mice subcutaneously, with intravenous administration of 10 mg/kg of indisulam, and oral gavage 40 mg/kg of alectinib monotherapy for 2 weeks, with 5 days on and 2 days off.

(B) Mice were randomized into 4 groups: VC (vehicle control), indisulam monotherapy, alectinib monotherapy, and combination therapy. Tumor volume was measured 3 times a week until the humane endpoint was reached. Cyan shade indicates the treatment period. $n = 8$ for each group.

(C) Survival plot of mice bearing Th-MYC/ALK^{F1174L} allografts treated with vehicle or alectinib or indisulam or combination therapy.

(D) Western blotting in tumor tissues after administration of vehicle or alectinib or indisulam or combination therapy for 3 days ($n = 4$ per group).

(E–H) Immunofluorescence staining was performed on tumor sections, with DAPI used for nuclear staining. Nuclear p-SRSF intensity (F) was measured by merging with DAPI. Nuclear γH2AX (H) foci > 5 indicate positive cells. Scale bars, 20 μm. Error bars indicate SEM. n.s., not significant; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

into the functional consequences of combined RBM39 degradation and SRPK1 inhibition underscores their interdependency in regulating alternative splicing, but it is not yet clear if RBM39 is a direct target of SRPK1, as has been suggested by global interac-

tion profiling³² and our co-localization experiments. More broadly, we need further investigation to understand the interactions and co-regulation of different splicing factors that are also cancer targets, such as RBM39 and SRPK1, and to develop

more detailed mechanistic models of how these regulate alternative splicing together.

Interestingly, RNA-seq analysis revealed that dual targeting of SRPK1 and RBM39 synergistically amplifies RNA splicing dysfunction and downstream cellular stress. In addition to DNA repair-related pathways, chromatin remodeling emerged as another key pathway of interest. RBM39 has recently been shown to facilitate epigenomic modification by recruiting MLL1 to promoters and promoting H3K4me3 deposition, beyond its canonical role in splicing.⁴⁹ Consistent with this, we observed that chromatin remodeling genes are a significant group affected by indisulam and alectinib, highlighting a broader role of targeting splicing in epigenetic regulation that warrants further investigation in neuroblastoma cells.

Targeting functionally connected nodes within the splicing machinery may therefore offer an effective strategy to overcome cellular adaptation and enhance therapeutic efficacy in high-risk neuroblastoma. However, as with the targeting of spliceosomal components in general, it is unclear if the sequelae of accumulating R loops, cell-cycle arrest, DNA damage, and eventual cell death that we observe in combined SRPK1/RBM39 interference are lineage-dependent, driven by the mis-splicing and functional loss of a select set of critical genes, or the cumulative consequence of widespread alternative splicing events across multiple functional pathways.

Further work to resolve such mechanistic questions will be important to understand what the most relevant pharmacodynamic readouts will be for future discovery experiments and which predictive biomarkers can best identify patients most likely to benefit from such combinations (i.e., for stratification).

In summary, the present study supports a model in which co-treatment with indisulam and alectinib synergistically impacts RNA splicing through simultaneous RBM39 degradation and SRPK1 inhibition. This dual RNA splicing interference exacerbates splicing errors in key genes regulating genome stability, leading to R-loop accumulation and increased DNA damage. These events eventually result in cellular apoptosis and growth inhibition in high-risk neuroblastoma *in vitro*, as well as suppression of tumor growth *in vivo*. These insights may drive the clinical application of splicing inhibitors, ultimately aiming to improve the prognosis for patients with high-risk neuroblastoma.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Anke Nijhuis (a.nijhuis@imperial.ac.uk).

Materials availability

All materials generated in this study are available upon request.

Data and code availability

- The RNA-seq data generated in this study have been deposited in NCBI's Gene Expression Omnibus (GEO),⁵⁰ under the accession number GEO: GSE299818 and are publicly available.
- The paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.M., A.N., and H.C.K.; methodology, Y.M., E.P., Y.X., S.Q., and A.N.; investigation, Y.M., E.P., B.M.d.C., Y.X., C.J., S.Q., N.Z., C.W., L.C., H.C.K., and A.N.; visualization, Y.M., Y.X., C.J., S.Q., and A.N.; funding acquisition, L.C., H.C.K., and A.N.; project administration, H.C.K. and A.N.; supervision, A.N., H.C.K., and L.C.; writing – original draft, Y.X., L.C., H.C.K., and A.N.; and writing – review & editing, all authors contributed to the review and editing of the paper.

DECLARATION OF INTERESTS

The authors declare that they have no conflicts of interest.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-RBM39	Sigma-Aldrich	Cat# HPA001591; RRID:AB_1079749
Mouse anti- β -actin	Abcam	Cat# ab6276; RRID:AB_2223210
Mouse anti-phospho-Histone H2A.X (ser139) γ H2AX	Sigma-Aldrich	Cat# 05-636; RRID:AB_309864
Mouse anti-phosphoepitope SR proteins	Millipore	Cat# MABE50; RRID: AB_10807429
Mouse anti-SRSF1	Invitrogen	Cat# 324600; RRID: AB_86976
Rabbit anti-SRSF2	Abcam	Cat#ab204916; RRID: AB_2909393
Rabbit anti-pChk1-S345	Cell Signaling Technology	Cat# 2348; RRID:AB_331212
Mouse anti-Chk1	Cell Signaling Technology	Cat# 2360; RRID:AB_2080320
Mouse-anti-BrdU	BD Biosciences	Cat# BD347580; RRID:AB_10015219
Rat anti-BrdU	Abcam	Cat# ab6326; RRID:AB_305426
HRP-conjugated goat anti-rabbit polyclonal	Invitrogen	Cat# 31460; RRID:AB_228341
HRP-conjugated goat anti-mouse polyclonal	Invitrogen	Cat# 31340; RRID:AB_228339
AF488-conjugated goat anti-mouse	Invitrogen	Cat# A11001; RRID:AB_2534069
AF647-conjugated goat anti-rat	Invitrogen	Cat# A21247; RRID:AB_141778
S9.6	Millipore	Cat# MABE1095; RRID:AB_2861387
Rat anti-phospho-RPA32-S33	Novus	Cat# NB100-544; RRID:AB_10002227
Chemicals, peptides, and recombinant proteins		
Indisulam	Sigma-Aldrich	Cat# SML1225
E7820	Sigma-Aldrich	Cat# SML2950
Alectinib (<i>in vitro</i>)	Selleckchem	Cat# S2762
Alectinib Hydrochloride (<i>in vivo</i>)	MedChemExpress	Cat# HY-13011A
Lorlatinib	MedChemExpress	Cat# HY-12215
SPHINX31	Cambridge bioscience	Cat# CAY21582
IdU	Sigma-Aldrich	Cat# I7125
BrdU	Sigma-Aldrich	Cat# B5002
Agarose	Sigma-Aldrich	Cat# A9539
HALT protease and phosphatase inhibitor cocktail	Thermo Scientific	Cat# 78442
Sulforhodamine B	Sigma-Aldrich	Cat# S1402
Cy5.5-conjugated Annexin V	BD Biosciences	Cat# 559925
Propidium Iodide staining solution	Invitrogen	Cat# 00-6990-50
Formaldehyde, Methanol-free	Thermo Scientific	Cat# 11586711
Triton X-100	Sigma-Aldrich	Cat# T8787
DAPI	Sigma-Aldrich	Cat# D9542
Phalloidin-647	Cell Signaling Technology	Cat# 8940S
Critical commercial assays		
CellTiter-Blue® Cell Viability Assay	Promega	Cat# G8081
RNeasy Mini Kit	Qiagen	Cat# 74106
High-Capacity RNA-to-cDNA kit	Applied Biosystems	Cat# 4387406
Q5 High-Fidelity PCR kit	New England Biolabs	Cat# E0555
PowerTrack™SYBR Green Master Mix	Applied Biosystems	Cat# A46012
Duolink® Proximity Ligation Assay	Sigma-Aldrich	Cat# DUO92008

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
ADP-Glo™ Kinase Assay	Promega	Cat#V6930,
Deposited data		
RNA sequencing data	N/A	Data available through the NCBI GEO Series accession number GSE299818
Experimental models: Cell lines		
KELLY, IMR32, SKNBE2C, IMR5, SKNAS, SKNSH, SHEP	Gift from Prof Louis Chesler (Institute of Cancer Research)	N/A
HCT116	Gift from Professor Iain McNeish lab (Imperial College London)	N/A
KURAMOCHI	Gift from Dr Haonan Lu (Imperial College London)	N/A
Experimental models: Organisms/strains		
Mouse	Breed in Prof Louis Chesler's lab (Institute of Cancer Research)	129 × 1/SvJ mice
Oligonucleotides		
ON-TARGETplus Non-targeting Control Pool	Horizon Discovery	D-001810-10-05
ON-TARGETplus siALK	Horizon Discovery	L-003103-00-0005
Software and algorithms		
SynergyFinder	N/A	RRID:SCR_026127
rMATS	(Shen et al.) ⁴⁵	https://maseq-mats.sourceforge.io/
The Database for Annotation, Visualization and Integrated Discovery (DAVID)	Laboratory of Human Retrovirology and Immunoinformatics	https://davidbioinformatics.nih.gov
GRCh38 human reference genome	Ensembl	https://useast.ensembl.org/Homo_sapiens/Info/Index
Annealing temperature calculator	New England Biolabs	https://tmcalculator.neb.com/#!/main
ImageJ Fiji V2.0.0	Schindelin et al. ⁵¹	https://imagej.net/software/fiji/
Prism 10	GraphPad	https://www.graphpad.com/features
CellProfiler (V4.2.6)	Broad Institute	RRID:SCR_007358
Other		
Lipofectamine®2000	Invitrogen	Cat# 11668-019
SYBR™ Safe DNA Gel Stain	Invitrogen	Cat# S33102
Mini-PROTEAN precast gels	Bio-Rad	Cat# 4561033
Supersignal West Pico PLUS chemiluminescent substrate	Thermo Scientific	Cat# 34580

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Cell lines and cell culture

Human neuroblastoma cell lines KELLY and IMR32 were cultured in MEM (Gibco) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), 1 mM sodium pyruvate, and 2 mM L-glutamine. SKNBE2C, SKNAS, SKNSH, SHEP were cultured in DMEM (Gibco, with GlutaMAX Supplement) with 10% FBS and 1% P/S. KURAMOCHI was maintained in DMEM (Gibco) with 10% FBS, 2 mM L-glutamine, 5.6 mM glucose, and 1% P/S. HCT116 was cultured in RPMI 1640 with 10% FBS, 2 mM L-glutamine, and 1% P/S. All cells were authenticated and routinely tested for mycoplasma. Cells were maintained at 37°C with 5% CO₂.

KELLY drug synergy screen

A library of FDA-approved drugs (approved oncology drugs set IX, plates 4891–4892) was acquired from the United States National Cancer Institute Developmental Therapeutics Program (<https://wiki.nci.nih.gov/display/NCIDTPdata/Compound+Sets>). All drugs ($n = 147$) were combined with either vehicle control (DMSO) or a sublethal dose of indisulam (IC₂₀ = 0.1 μM) in a pairwise approach in KELLY cells. 5 doses of the selected drugs were combined with indisulam, ranging from 16 nM to 10 μM. All drug combinations were tested in parallel, and cell viability was measured using the CellTiter-Blue assay. Each combination had 3 technical replicates.

Synergy was evaluated using the Bliss independence model. The Bliss score was defined as the difference between the observed inhibition in the combination treatment and the expected additive inhibition, where the expected value was calculated as:

$$E_{additive} = I_{indisulam} + I_{drug} - (I_{indisulam} \times I_{drug})$$

Here, we represent the fractional cell viability inhibition relative to vehicle control. Thus:

$$Bliss\ score = Observed\ inhibition_{combination} - E_{additive}$$

Positive Bliss scores (>0) indicate synergy, zero indicates additivity, and negative values (<0) indicate antagonism. Drugs were categorized as showing synergism with indisulam if at least two dose-pairs had Bliss scores >0.02; and drugs were categorized as showing antagonism with indisulam if five dose-pairs had Bliss scores < -0.02. Drugs were ranked based on the number of dose-pairs having Bliss scores >0.02 and the average bliss score of all dose-pairs.

In vivo allograft model of neuroblastoma

Syngeneic tumors derived from TH-MYCN/ALK^{F1174L} genetically engineered mouse models (GEMMs), were implanted into immune competent 129 × 1/SvJ mice. Mice with both sexes were randomly enrolled into 4 treatment groups (*n* = 8 each group) and treatment began when the implanted tumors reached a volume of over 200 mm³. The dosing schedule continued for 2 weeks, with daily administration for 5 days followed by 2 days off. Alectinib was dissolved in saline containing 40% PEG300, 10% DMSO, and 5% Tween, and was administered orally at a dose of 10 μL/kg. Indisulam was dissolved in saline with 3.5% DMSO and 6.5% Tween80 and was administered intravenously at a dose of 5 μL/kg. Tumor tissues were excised two hours after the final dose of each compound. Half of the tumor was fixed in 4% PFA, while the other half was snap-frozen for subsequent analysis. Investigators were blind for the analysis of the study.

METHOD DETAILS

Synergy analysis by SynergyFinder

To evaluate drug interaction, cells were treated with varying concentrations of two compounds alone or in combination. The concentrations used were primarily 2-fold serial dilutions around drug IC₅₀ or matched those from the control assays. Following 72 h of treatment, SRB assay was performed as described above. Raw OD values were normalized to vehicle-treated controls, and synergy was evaluated using the SynergyFinder web application (RRID:SCR_026127).

The Loewe additivity model was employed to determine drug interaction. Synergy scores were calculated using dose-response matrices, and interaction profiles were visualized with heatmaps. A synergy score greater than 10 indicates synergy, -10 to 10 indicates additivity, and less than -10 indicates antagonism.

Sulforhodamine B (SRB) assay

Cells were fixed with 10% cold trichloroacetic acid, stained with 0.4% SRB for 30 min, and washed with 1% acetic acid. Bound dye was solubilized in 10 mM Tris-base, and OD measured at 565 nm. Results were normalized to vehicle control.

siRNA transfection

Cells were seeded in 96-well plates and cultured overnight before transfection. ON-TARGETplus Non-targeting Control Pool (Horizon Discovery, D-001810-10-05) and ON-TARGETplus siALK (Horizon Discovery, L-003103-00-0005) (30 nM final concentration) were delivered using Lipofectamine 2000 (Invitrogen, 11668-019) in Opti-MEM (Gibco), following the manufacturer's instructions. Opti-MEM only and Lipofectamine-only conditions were included as controls. Cells were incubated for 16 h prior to further treatment.

Western blotting

Whole-cell lysates were prepared using RIPA buffer (Sigma Aldrich) with 1% HALT protease and phosphatase inhibitor cocktail (Thermo Scientific). Equal amounts of protein from each sample were separated on a 4–20% Bis-Tris SurePAGE gel (GenScript) and then transferred onto PVDF membranes. The membranes were blocked with 5% milk for 1 h at room temperature, followed by an overnight incubation at 4°C with CHK1 (Cell Signaling Technology Cat# 2360, RRID:AB_2080320), p-CHK1 (Cell Signaling Technology Cat# 2348, RRID:AB_331212), γH2AX (Sigma-Aldrich Cat# 05–636, RRID:AB_309864). HRP-conjugated secondaries and chemiluminescent substrate were used for detection.

Endpoint and quantification PCR reactions

1500ng RNA was utilized for reverse transcription. The resulting cDNA templates were subsequently employed for qPCR using the PowerTrack SYBR Green Master Mix (Applied Biosystems) or Endpoint PCR utilizing the Q5 High-Fidelity PCR Kit (New England Biolab). PCR products were resolved on 2% agarose gels and quantified with Fiji V2.0.0.

Target genes	Primer (5' to 3')	Tm
<i>BRCA1</i>	AACCTCTGAGGACAAAGCAGC CTTCTCAGTGGTGTCAAATC	62
<i>CHK1</i>	GTGGAAGACTGGGACTTGGT ACCCCTGCCATGAGTTGATG	68
<i>FANCD2</i>	CGGTTACAGGCTTCCCAAGT GCTGTTCTTGAATGCAGCCT	67
<i>FANCI</i>	GAGTCAGGCCGAGAAGGTTT CAACGGCAGCAGGTTTCTC	68
<i>RAD51D</i>	TGGCTCAGTTCTCGGCTTT AGATGTCAAATGCATGCACCA	66
<i>TP53BP1</i>	GCTGTTGCTGAGTCTGTTGC TGCCTACTTCCCGATTGTT	68
<i>RIF1</i>	AGGTGGGCAAACTGGTCAT TGGGTGCTCCACTACGAAAT	67
<i>VEGF165b</i>	AAGAAATCCCTGTGGGCCTT TGGTGAGAGATCTGCAAGTACGTT	60
<i>VEGF165</i>	AGAGCAAGACAAGAAAATCC TACAAACAAATGCTTTCTCC	53
<i>GAPDH</i>	GGCTGCTTTTAACTCTGG GGAGGGATCTCGCTCC	62

Immunofluorescence staining

KELLY cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and blocked in 5% BSA. Subsequently, they were incubated overnight at 4°C with anti-phosphoepitope SR proteins antibody (1:100, Millipore Cat# MABE50, RRID:AB_10807429) or γ H2AX (1:500, Sigma-Aldrich Cat# 05-636, RRID:AB_309864) in blocking buffer, and then stained with a mix of Alexa Fluor 488-conjugated goat anti-mouse antibody (1:500, AF488, Thermo Scientific), DAPI (1 μ g/ml, Biotium, Cat#40043), and Phalloidin-647 (1:50, Cell Signaling Technology Cat# #8940S) for 1 h at room temperature. The cells were washed again three times with PBS-T before imaging with an INCell Analyzer 1000 microscope (GE Healthcare) at 20x magnification.

S9.6 (1:250, Millipore Cat# MABE1095, RRID:AB_2861387) and phospho RPA32-S33 (1:250, Novus Cat# NB100-544, RRID:AB_10002227) were detected in KELLY cells fixed with cold methanol at -20°C for 10 min and slides were imaged using the Aurox laser-free confocal microscope. To confirm the specificity of the S9.6 antibody, RNase H (Ribonuclease H, 1:100, NEB Cat#M0297S) was applied to the fixed cells before antibody incubation.

Image analysis was performed using Cell Profiler V4.2.6, with nuclear regions masked by DAPI staining and cytoplasmic regions masked by Phalloidin staining, excluding areas overlapping with DAPI.

SRPK1/2 kinase activity assay

The SRPK1 Kinase Enzyme System (#VA7558, Promega) and SRPK2 Kinase Enzyme System (#VA7561, Promega) were utilized with the ADP-Glo Kinase Assay (#V6930, Promega), following the manufacturer's protocol. Briefly, 1 μ L of inhibitor, diluted in 1X kinase reaction buffer, was added to the wells of a 384-well low-volume plate and combined with 8 ng of SRPK1 enzyme. Following this, 2 μ L of a substrate/ATP mixture (100 μ M ATP, 0.1 μ g/ μ L MBP) was added. The reaction was incubated at room temperature for 1 h. After incubation, 5 μ L of ADP-Glo Reagent was added to each well, and the plate was incubated at room temperature for 40 min. Subsequently, 10 μ L of Kinase Detection Reagent was added, and the plate was incubated for an additional 30 min. Luminescence was recorded.

Proximity Ligation Assay (PLA)

The Duolink Proximity Ligation Assay (PLA) was used to detect protein-protein interactions in KELLY cells. Samples were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with the Duolink blocking solution. Primary antibodies against RBM39 (1:500, Proteintech Cat# 67420-1-Ig, RRID:AB_2882660) and SRPK1 (1:250, Abcam Cat# ab90527, RRID:AB_2050339) were applied overnight at 4°C. Species-specific PLA probes (Sigma-Aldrich) were added, followed by ligation and rolling-circle amplification according to the manufacturer's instructions.

DNA fiber analysis

Cells were sequentially pulse-labeled with 10 μ M BrdU and 200 mM IdU for 30 min each. KELLY cells were spotted on SuperFrost slides, air-dried for 5 min, and lysed with a buffer (200 mM Tris-HCl, pH 7.5, 50 mM EDTA, 0.5% SDS) to release DNA and fixed in 75% methanol/25% acetic acid, and then treated with 2.5M HCl for 1 h to denature the DNA. After blocking with 1% BSA in PBS-T for 1 h.

BrdU was detected by anti-BrdU antibody (1:50, Abcam Cat# ab6326, RRID:AB_305426) followed by AF647-conjugated secondary antibody (1:100, A21247, Invitrogen). IdU was labeled with mouse anti-BrdU antibody (1:50, BD Biosciences Cat# 347580, RRID:AB_10015219) and AF488-conjugated secondary antibody (1:100, Thermo Fisher Scientific Cat# A-11001, RRID:AB_2534069). Slides were mounted and imaged using a laser-free confocal microscope, with DNA track lengths measured in Fiji V2.0 (RRID:SCR_002285).

Immunoassays on tissue samples

Snap frozen tumors were lysed in 5% CHAPS buffer and western blotting was carried out for RBM39 (Proteintech Cat# 67420-1-Ig, RRID:AB_2882660), ERK1/2 (Cell Signaling Technology Cat# 4695, RRID:AB_390779), pERK1/2 (Cell Signaling Technology Cat# 9101, RRID:AB_331646), phosphopeptide SR proteins (Millipore Cat# MABE50, RRID:AB_10807429) and GAPDH (Cell Signaling Technology Cat# 2118, RRID:AB_561053). ALK Meso Scale Discovery immunoassays were performed as previously described⁵²

Immunofluorescence on tissue sections

Tumors sections were deparaffinized and rehydrated using Xylene and a graded series of alcohol. They were then boiled in 1% citric-acid buffer for 20 min and allowed to cool to room temperature. The sections were blocked for 1 h in TBS with 0.01% Triton (TBST) and 5% BSA. Afterward, γ H2AX antibody (Cell Signaling Technology Cat# 9718, RRID:AB_2118009) was applied at a 1:100 dilution overnight at 4°C. Alexa Fluor 568 goat anti-rabbit antibody (1:1000) was incubated at room temperature for 1 h. Images were captured using a confocal microscope (LSM700, LSM T-PMT) and processed with ZEN2009 software (Black version, Zeiss).

RNA sequencing and alternative splicing analysis

Total RNA was extracted and mRNA enriched using poly-T oligo-attached magnetic beads. Strand-specific libraries were constructed and quantified by Qubit and Bioanalyzer and sequenced on an Illumina NovaSeq 6000 platform using an S4 flow cell with 150 bp paired-end reads (Novogene, Cambridge, UK). Sequencing reads were quality-filtered and clean reads were aligned to the human reference genome (hg38) using HISAT2. Gene-level counts were generated using featureCounts, and differential expression analysis was performed using DESeq2. Resulting *p*-values were adjusted using the Benjamini-Hochberg method to control the false discovery rate (FDR), with significance defined as adjusted *p*-value ≤ 0.05 .

Alternative splicing analysis was conducted using rMATS (v4.3.0), which detects five splice event types: skipped exon (SE), mutually exclusive exon (MXE), alternative 5' splice site (A5SS), alternative 3' splice site (A3SS), and retained intron (RI). Differential splicing events were identified based on junction-spanning reads, with significance set at FDR < 0.05 .

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 10. For normally distributed data, a Student's *t* test was applied to two-group comparisons; one-way or two-way ANOVA for three or more groups. Specific tests are detailed in figure legends.