

Understanding a research article

2024-04-17

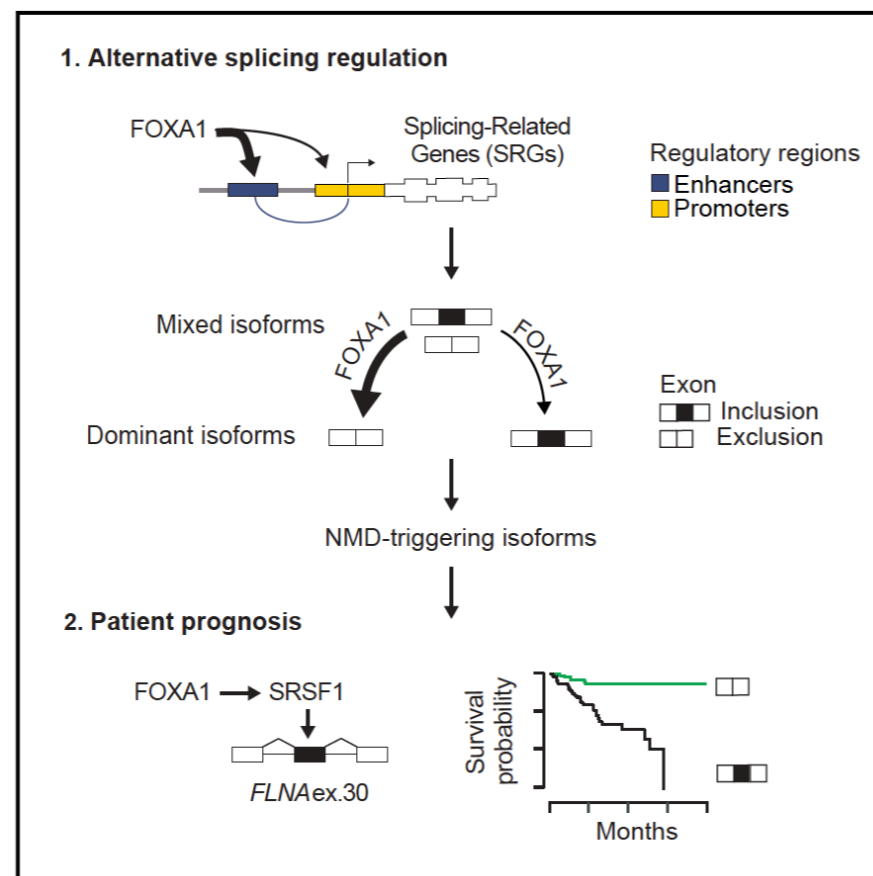
We will analyse...

Article

Cell Reports

FOXA1 regulates alternative splicing in prostate cancer

Graphical abstract



Authors

Marco Del Giudice, John G. Foster, Serena Peirone, ..., Giuseppe Basso, Prabhakar Rajan, Matteo Cereda

Correspondence

p.rajan@qmul.ac.uk (P.R.),
matteo.cereda1@unimi.it (M.C.)

In brief

Del Giudice et al. identify the pioneer transcription factor FOXA1 as a master regulator of alternative splicing in prostate cancer. By controlling splicing factors, FOXA1 buffers the noise of isoform production toward a mRNA dominant product. This regulation impacts on splicing of nonsense-mediated decay-determinant exons influencing patient survival.

Explore the title... what we will read about?

FOXA1 regulates alternative splicing in prostate cancer

Explore the title... what we will read about?

FOXA1

FOXA1 is a **pioneer transcription factor (TF)**, *i.e.* its role consists in opening chromatin to promote gene transcription and in regulating gene expression.

It regulates gene expression by binding on **DNA regulating sequences**. In particular, it is known to bind preferentially on **enhancer** regions (distal regulatory elements).

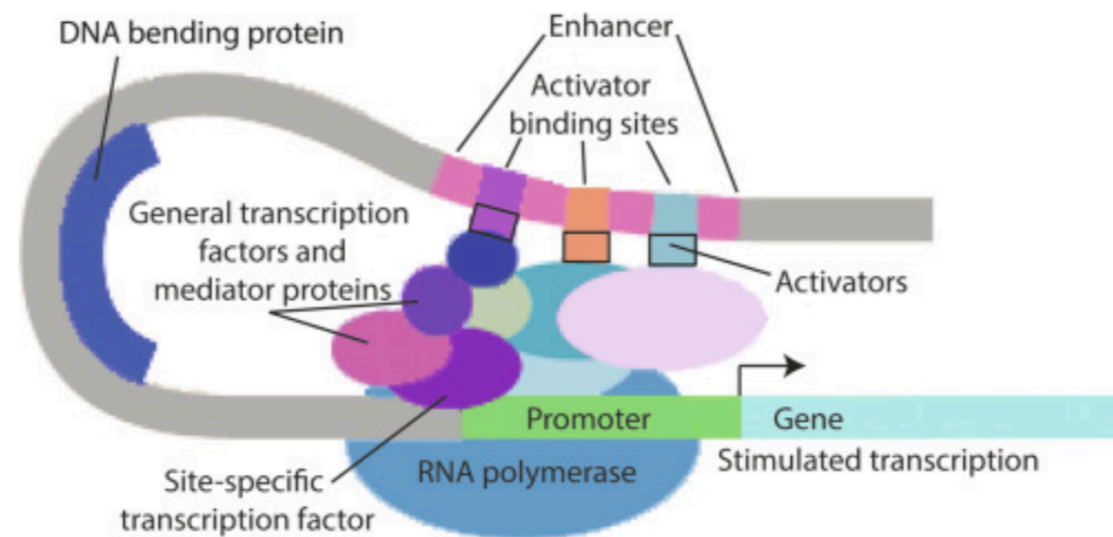
It is involved in the **development** of multiple endoderm-derived organ systems, such as liver, pancreas, lung and prostate.

In prostate cancer, it coordinates its action with the androgen receptor (AR). Nevertheless it also possesses an AR-independent role of regulating **epithelial-to-mesenchymal transition (EMT)**.

In prostate cancer, **mutations** often converge onto the coding sequence and cis-regulatory elements (CREs) of FOXA1, leading to **functional alterations**.

Explore the title... what we will read about?

FOXA1



Explore the title... what we will read about?

alternative splicing

Splicing is a fundamental process by which introns are excluded from a pre-mRNA molecule and exons are joined together.

More than 95% of genes present more than one isoform (mature transcript) . This mechanism is called **alternative splicing**.

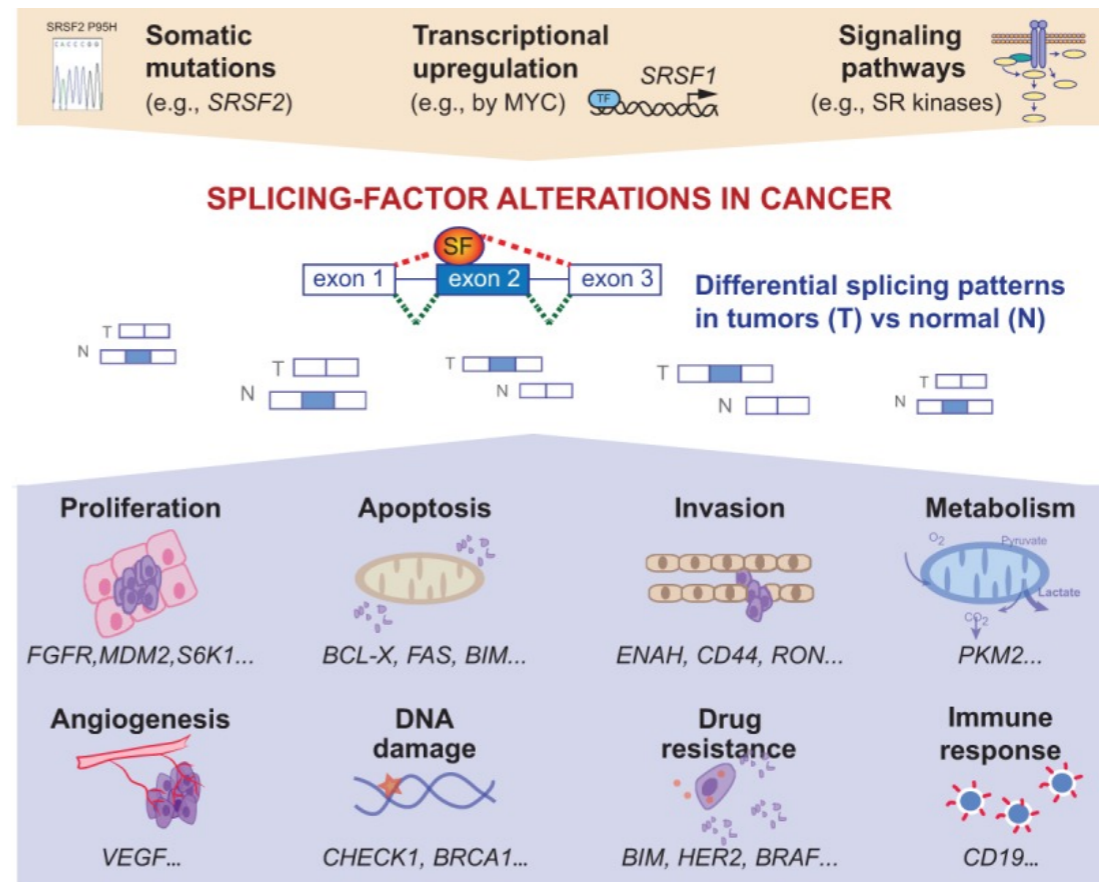
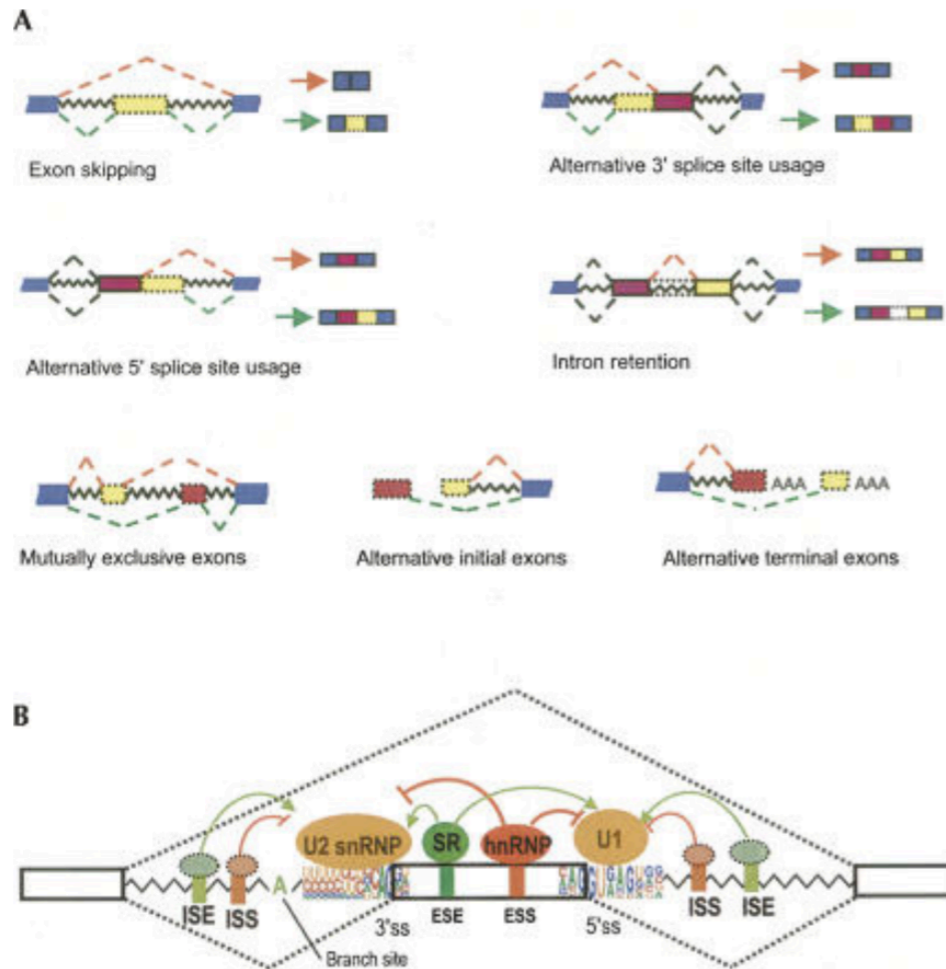
Alternative splicing explains the great quantity of proteins present in the cell respect to the genes that encode for them.

It is mainly regulated by RNA-binding proteins, also called **Splicing-related proteins (SRPs)**, that bind on specific motifs.

Alternative splicing is often **dysregulated in cancer**.

Explore the title... what we will read about?

alternative splicing



Explore the title... what we will read about?

prostate cancer

Prostate cancer is the second most common cancer type worldwide and commonest cause of male-specific cancer death.

Despite advances in diagnosis and treatment, end-stage metastatic castration-resistant prostate cancer is **hardly treatable**.

It presents **high heterogeneity**.

Recurrent activating alterations occur within oncogenic transcription factors like: AR, ERG, FOXA1 and MYC.

Explore the authors... do you know someone?

Marco Del Giudice,^{1,2,10} John G. Foster,^{3,10} Serena Peirone,^{1,4} Alberto Rissone,^{1,2} Livia Caizzi,^{1,2} Federica Gaudino,^{1,2} Caterina Parlato,^{1,2} Francesca Anselmi,^{1,5} Rebecca Arkell,³ Simonetta Guarrera,^{1,2} Salvatore Oliviero,^{1,5} Giuseppe Basso,^{1,2} Prabhakar Rajan,^{3,6,7,8,9,11,*} and Matteo Cereda^{1,4,11,12,*}

¹Italian Institute for Genomic Medicine, c/o IRCCS, Str. Prov. le 142, km 3.95, 10060 Candiolo (TO), Italy

²Candiolo Cancer Institute, FPO—IRCCS, Str. Prov. le 142, km 3.95, 10060 Candiolo (TO), Italy

³Centre for Cancer Cell and Molecular Biology, Barts Cancer Institute, Cancer Research UK Barts Centre, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK

⁴Department of Biosciences, Università degli Studi di Milano, Via Celoria 26, 20133 Milan, Italy

⁵Department of Life Science and System Biology, Università degli Studi di Torino, via Accademia Albertina 13, 10123 Turin, Italy

⁶Division of Surgery and Interventional Science, University College London, Charles Bell House, 3 Road Floor, 43–45 Foley Street, London W1W 7TS, UK

⁷The Alan Turing Institute, British Library, 96 Euston Road, London NW1 2DB, UK

⁸Department of Urology, Barts Health NHS Trust, the Royal London Hospital, Whitechapel Road, London E1 1BB, UK

⁹Department of Uro-oncology, University College London NHS Foundation Trust, 47 Wimpole Street, London W1G 8SE, UK

¹⁰These authors contributed equally

¹¹Senior author

¹²Lead contact

*Correspondence: p.rajan@qmul.ac.uk (P.R.), matteo.cereda1@unimi.it (M.C.)

<https://doi.org/10.1016/j.celrep.2022.111404>

Search the date... is it recent?



Del Giudice et al., 2022, Cell Reports 40, 111404
September 27, 2022 © 2022 The Author(s).
<https://doi.org/10.1016/j.celrep.2022.111404>

Are there any keywords?

No, go on!

Understand the abstract

In this case we do not only have the abstract (called “**summary**”), but also the **graphical abstract**, a **brief description** and the **highlights**. Let’s explore all of them!

In brief

Del Giudice et al. identify the pioneer transcription factor FOXA1 as a master regulator of alternative splicing in prostate cancer. By controlling splicing factors, FOXA1 buffers the noise of isoform production toward a mRNA dominant product. This regulation impacts on splicing of nonsense-mediated decay-determinant exons influencing patient survival.

Title message

Understand the abstract

In this case we do not only have the abstract (called “**summary**”), but also the **graphical abstract**, a **brief description** and the **highlights**. Let’s explore all of them!

Hint: nonsense-mediated decay (NMD)

NMD is a translation-coupled mechanism that eliminates mRNAs containing premature translation-termination codons (PTCs).

PTCs arise from single nucleotide variations or alternative splicing events modifying RNA frame that convert a triplet nucleotide codon into one of three stop codons, i.e. TAG, TGA or TAA.

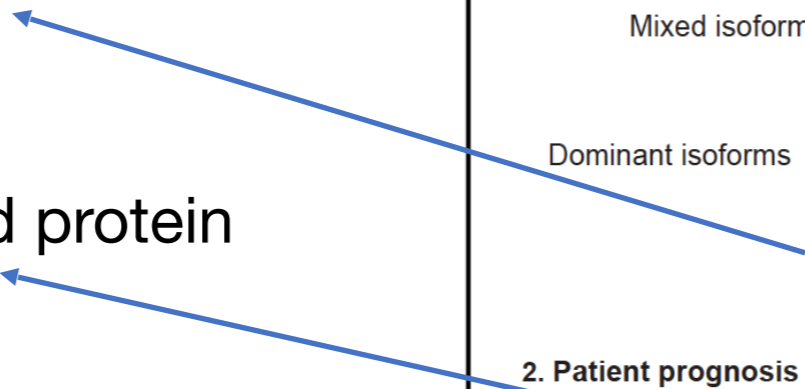
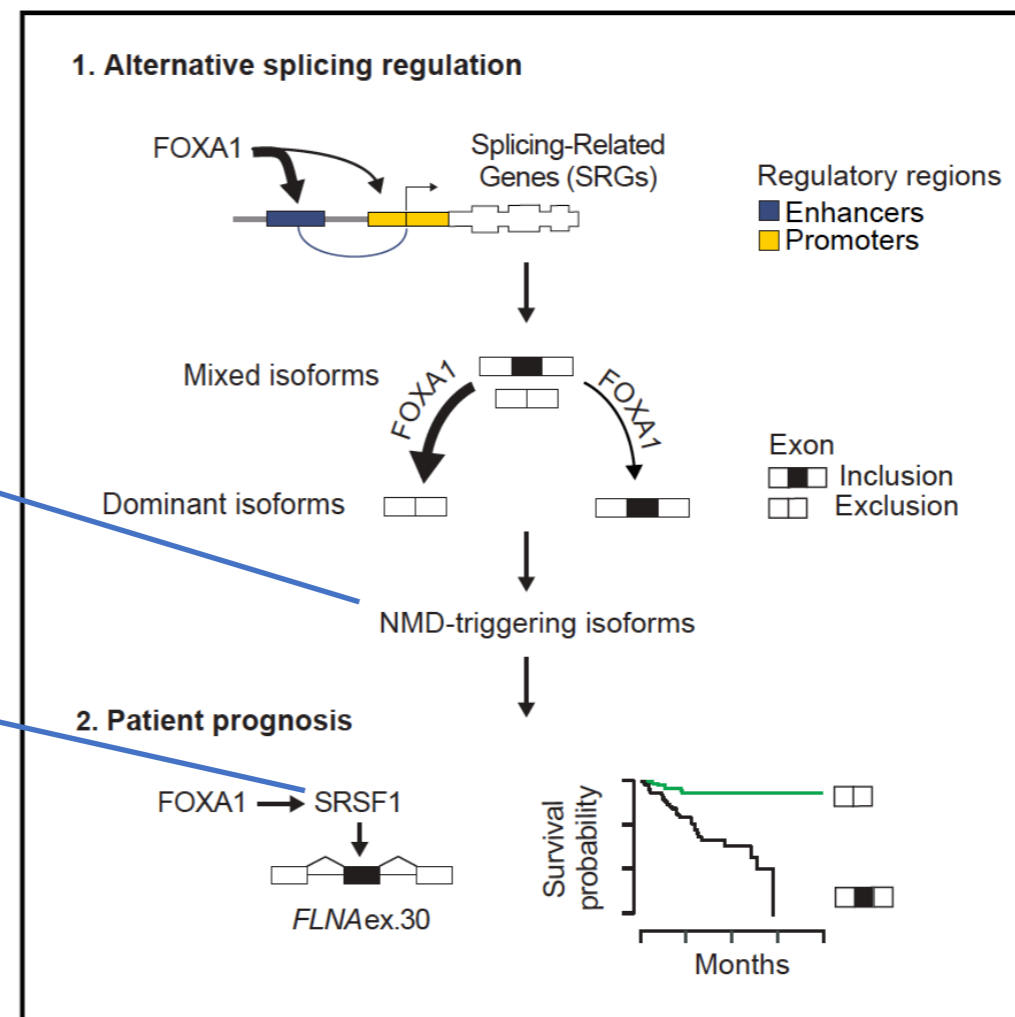
Understand the abstract

In this case we do not only have the abstract (called “**summary**”), but also the **graphical abstract**, a **brief description** and the **highlights**. Let’s explore all of them!

Graphical abstract

Nonsense-mediated decay

Splicing-related protein



Understand the abstract

In this case we do not only have the abstract (called “**summary**”), but also the **graphical abstract**, a **brief description** and the **highlights**. Let’s explore all of them!

Highlights

- FOXA1 is a master transcriptional regulator of splicing factors in prostate cancer
- FOXA1 drives splice isoform production toward an optimal dominant mRNA product
- FOXA1 controls exons triggering NMD, influencing prostate cancer patient prognosis
- FOXA1-controlled SRSF1 enhances inclusion of FLNA exon 30, promoting disease recurrence

Understand the abstract

In this case we do not only have the abstract (called “**summary**”), but also the **graphical abstract**, a **brief description** and the **highlights**. Let’s explore all of them!

SUMMARY

Dysregulation of alternative splicing in prostate cancer is linked to transcriptional programs activated by AR, ERG, FOXA1, and MYC. Here, we show that FOXA1 functions as the primary orchestrator of alternative splicing dysregulation across 500 primary and metastatic prostate cancer transcriptomes. We demonstrate that FOXA1 binds to the regulatory regions of splicing-related genes, including HNRNPK and SRSF1. By controlling trans-acting factor expression, FOXA1 exploits an “exon definition” mechanism calibrating alternative splicing toward dominant isoform production. This regulation especially impacts splicing factors themselves and leads to a reduction of nonsense-mediated decay (NMD)-targeted isoforms. Inclusion of the NMD-determinant FLNA exon 30 by FOXA1-controlled oncogene SRSF1 promotes cell growth *in vitro* and predicts disease recurrence. Overall, we report a role for FOXA1 in rewiring the alternative splicing landscape in prostate cancer through a cascade of events from chromatin access, to splicing factor regulation, and, finally, to alternative splicing of exons influencing patient survival.

Dive into the introduction

1st paragraph

Splicing and its relation with cancer

Pre-mRNA alternative splicing (AS) is a fundamental genetic process underpinning eukaryotic proteome diversity. AS is the selective inclusion of exons or introns into mature transcripts. Catalyzed by the macromolecular spliceosome complex comprising core spliceosomal factors, AS is finely regulated by auxiliary RNA-binding proteins (RBPs), which bind to sequence-specific nucleotide motifs to promote or repress a given splicing event (Cereda et al., 2014; Van Nostrand et al., 2020a). Genomic studies have also shown that somatic cells exploit RBP-mRNA interactions to promote tumor onset and progression (Pereira et al., 2017; Wang et al., 2018).

Dive into the introduction

2nd paragraph

Potential of exploiting splicing for novel cancer therapeutic targets

AS can be affected by somatic alterations leading to dysregulated expression of splicing-related genes (SRGs) (Sebestyén et al., 2016; Seiler et al., 2018). These alterations have uncovered novel cancer therapeutic targets (Lee and Abdel-Wahab, 2016). Small-molecule compounds targeting RBP-mRNA perturbations have entered clinical trials (Bonnal et al., 2020). For instance, pladienolide B derivatives inhibiting the SF3b splicing commitment complex have efficacy for blood and solid cancers (Zhang et al., 2020; Zhou et al., 2020). Similarly, antisense decoy oligonucleotides targeting RBPs have proven effective in preventing the activation of RBP-driven oncogenic programs (Denichenko et al., 2019). Finally, dysregulated AS has the potential to generate neo-epitopes to a greater extent than point mutations, thus potentially expanding the indications for immunotherapies (Frankiw et al., 2019; Kahles et al., 2018).

Dive into the introduction

3rd paragraph

Prostate cancer

The commonest cause of male-specific cancer death is prostate cancer (PC) (Rebello et al., 2021). Despite advances in the diagnosis and treatment of early disease, there are few therapeutic options for end-stage metastatic castration-resistant PC (mCRPC) (Rebello et al., 2021). The disease is difficult to tackle in part due to considerable phenotypic heterogeneity, underpinned by genomic alterations within different oncogenes or tumor suppressors. These impact on transcriptional and translational programs that are fundamental for the cell in complex ways (Rebello et al., 2021).

Dive into the introduction

4th paragraph

Aberrant splicing in prostate cancer

Interestingly, aberrant splicing can contribute to the heterogeneous phenotypes of PC (Paschalis et al., 2018; Rajan et al., 2009). The dysregulation of this mechanism increases with disease aggressiveness toward metastatic disease, with most SRGs being transcriptionally dysregulated throughout PC progression (Zhang et al., 2020). Consequently, the AS landscape fingerprints the spectrum of PC disease states, with many aberrant events associated with oncogenic signals driven by transcription factors (TFs), such as MYC and AR (Phillips et al., 2020; Shah et al., 2020). Consistently, novel therapeutic targeting of highly expressed SRGs (specifically members of the SF3 splicing commitment complex) has been shown to have anti-proliferative effects in PC models (Kawamura et al., 2019; Zhang et al., 2020).

Dive into the introduction

5th paragraph

**Role of TFs,
in particular
AR, ERG,
FOXA1 and
MYC, in
prostate
cancer**

In the heterogeneous genetic landscape of PC, the only recurrent activating alterations occur within key oncogenic TFs: AR, ERG, FOXA1, and MYC (Rebello et al., 2021). Ligand-dependent activation of AR controls a tumorigenic cistrome of androgen-sensitive genes (Pomerantz et al., 2015). FOXA1 is a pioneer TF that reprograms the AR cistrome to drive PC initiation and progression to metastasis (Parolia et al., 2019). In the aggressive neuroendocrine PC (NEPC) subtype, where AR transcription is absent, FOXA1 is essential for proliferation (Baca et al., 2021). Similarly, overexpression of ERG redirects AR and FOXA1 binding to drive invasive PC, illustrating the cooperation between these TFs (Chen et al., 2013; Kron et al., 2017). Finally, aggressive PC is characterized by amplification of MYC, which is the most frequent genomic alteration in NEPCs (Rebello et al., 2021). MYC antagonizes AR transcriptional programs pioneered by FOXA1, underscoring the interdependence of PC on this handful of TFs (Hawksworth et al., 2010; Qiu et al., 2021).

Dive into the introduction

6th paragraph

TFs role in dysregulating splicing in prostate cancer

Of these four TFs, all but FOXA1, have each been implicated in controlling splicing outcomes in PC by modulating SRG expression or influencing inclusion levels of functionally relevant exons (Phillips et al., 2020; Saulnier et al., 2021; Shah et al., 2020). These studies highlight the involvement of distinct TFs in the dysregulation of AS during PC progression. Nevertheless, in the context of PC transcriptional reprogramming cooperatively driven by these TFs, the magnitude of influence exerted by each individual TF to aberrant AS remains to be elucidated. Here, we systematically assess the impact of the four TFs on AS in primary PC and mCRPC patients.

What has been done

What is missing

The contribution of the paper

Figures and tables

Figure 1

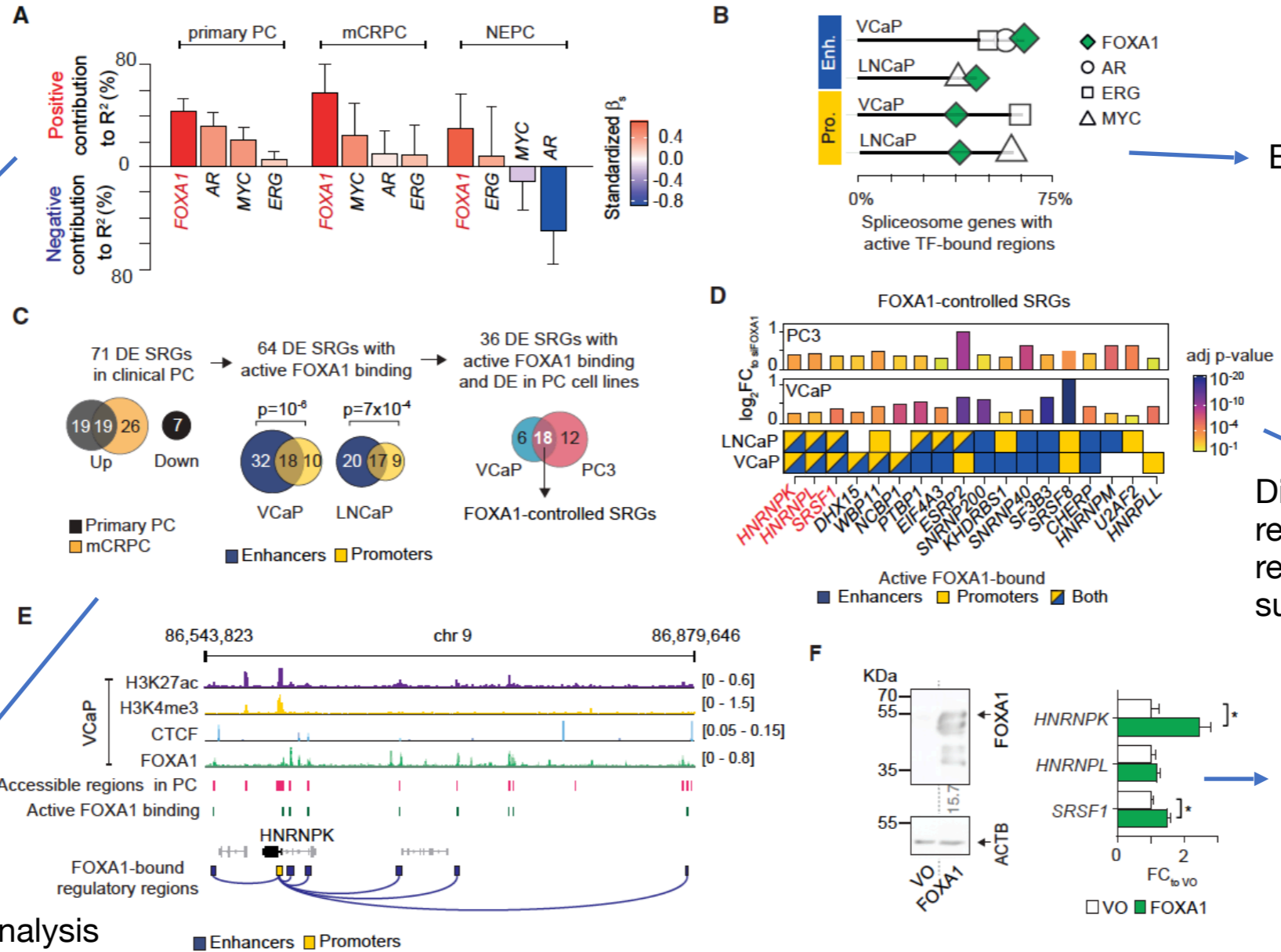


Figure 1. FOXA1 transcriptionally controls splicing-related genes in PC

(A) Results of multivariable covariance analysis between the cumulative expression of SRGs and the expression of TFs in primary PCs, mCRPC, and NEPC. Color key indicates the standardized β coefficients of the model.

(B) Enrichment of spliceosome genes with active TF binding sites within chromatin-accessible promoters (yellow) and enhancers (blue) for the VCaP- and LNCaP-based architectural datasets. The fraction of spliceosome genes with active TF-bound regions for each TF is shown.

(C) Framework used to select FOXA1-controlled SRGs. p values refer to a two-tailed test of equal proportion comparing the proportion of active FOXA1 binding sites on SRG promoters (yellow) and enhancers (blue). DE, differentially expressed.

(D) Bar plots indicate fold change (FC) in expression levels of FOXA1-controlled SRGs upon FOXA1 depletion in VCaP and PC3 cells. Color code indicates DEseq2 adjusted p value. Bottom annotations depict the active FOXA1-bound regulatory regions for each SRG.

(E) ChIP-seq density read tracks of H3K27ac, H3K4me3, CTCF (two overlaid experiments) and FOXA1 (five overlaid experiments) in VCaP cells are shown together with recurrent accessible regions of primary PC from assay for transposase-accessible chromatin using sequencing experiments, active FOXA1 binding sites and RNA PolIII chromatin interaction analysis by paired-end tag sequencing-derived FOXA1-bound regulatory regions.

(F) Representative western blotting images (left panel) of whole-cell lysates from PC3 cells transfected with 2 μ g of plasmid DNA vectors encoding FOXA1 or vector only (VO) control using antibodies to FOXA1 and ACTB. ACTB-normalized mean fold change in protein expression compared with control are shown below the upper blot image. Bar plots (right panel) depict the mean fold change in expression of candidate SRGs measured by qRT-PCR upon FOXA1 overexpression (biological triplicates). Error bars correspond to standard error of the mean. Two-tailed t test was used to compare conditions (* $p \leq 0.05$).

Multivariable covariance analysis gives information about which variable (single TF expression) explain the most the result (cumulative expression of SRPs)

Differential expression analysis results + binding on cis-regulatory elements

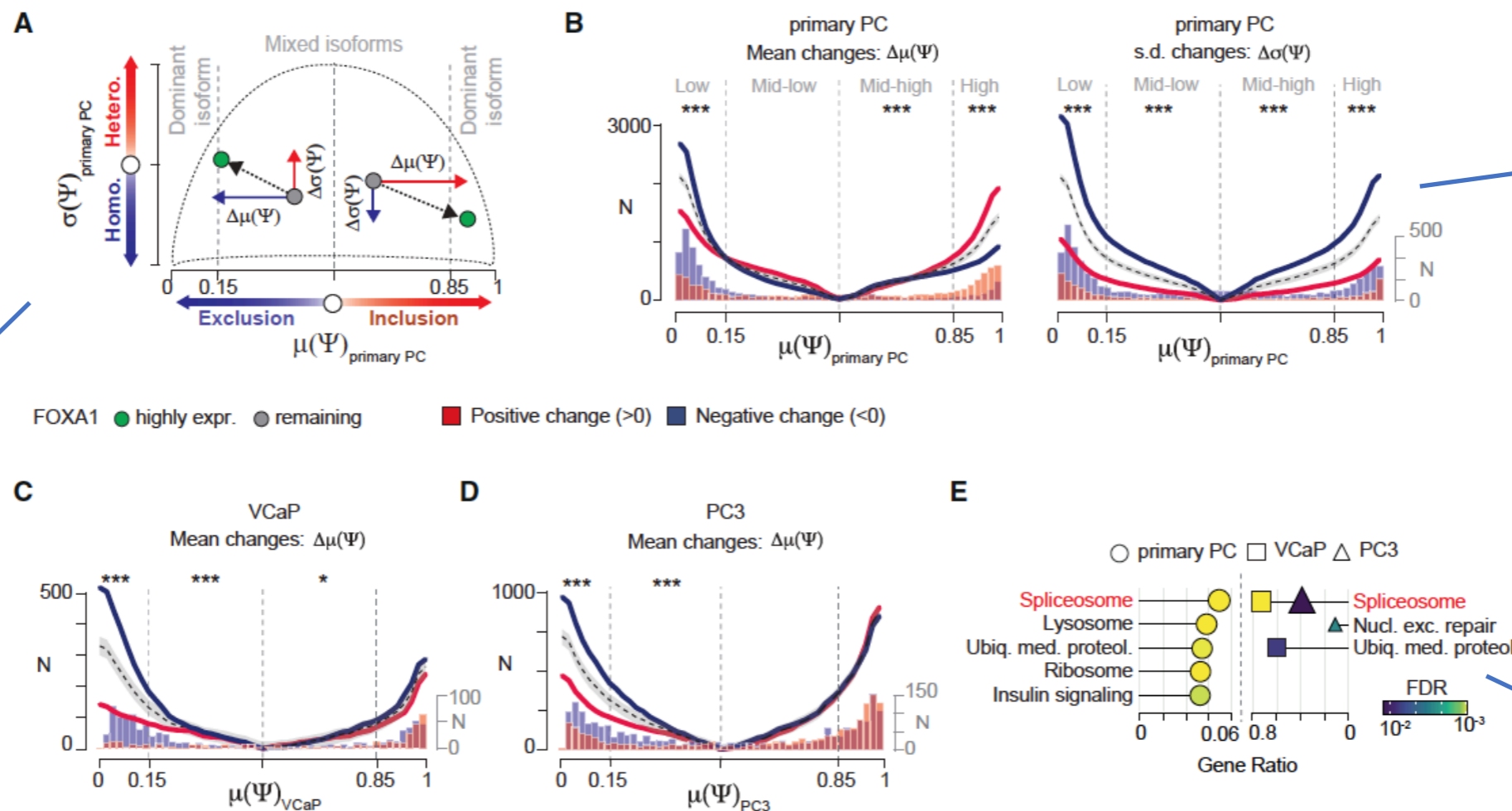
Binding analysis

Differential expression analysis results+ binding on cis-regulatory elements on a subset of SRPs

Validation of SRP differential expression upon high expression of FOXA1 in PC3 cells.

Figures and tables

Figure 2



Variations in event inclusion levels upon FOXA1 high expression

Given a certain mean value or standard deviation of PSI (percent spliced in i.e. percent of reads in which you see the inclusion of an event), how many events are in that window?

Over-representation analysis over differentially included events in primary prostate cancer, VCaP and PC3 cell lines

Figure 2. FOXA1 calibrates the alternative splicing equilibrium of PC by enhancing the production of dominant isoforms

(A) Overview of alternatively spliced exon trajectories in the space defined by mean and standard deviation (SD) of exon inclusion levels (Ψ s). Color codes indicate positive (red) and negative (blue) changes of mean and SD of Ψ s between FOXA1 highly expressing tumors and remaining ones.

(B) Cumulative distribution plots depict the number (N) of exons with either positive (red) or negative (blue) changes ranging from $\mu(\Psi)_{\text{primary PC}}$ of 0.5 (i.e., mixed isoforms) to the boundaries of 0 and 1 (i.e., dominant isoforms). Dashed lines represent the expected mean cumulative distribution of events with inclusion changes generated by 1,000 Monte Carlo simulations. Gray area represents confidence intervals (5%–95%). Histograms of the number of exons with positive and negative changes are superimposed on the x axis. On left panel, a preponderance of blue over red indicates that FOXA1 mostly inhibits exon inclusion, whereas the dominance of red compared with blue indicates a major enhancement of exon inclusion by FOXA1. On right panel, a preponderance of blue over red indicates that exons were more uniformly spliced across tumors by FOXA1, whereas the dominance of red compared with blue indicates more heterogeneous inclusion upon high FOXA1 expression.

(C and D) Cumulative distribution plots depict differentially alternatively spliced events (N) with positive (red) and negative (blue) mean inclusion changes upon FOXA1 depletion in VCaP (C) and PC3 (D) cells ranging from mixed (i.e., $\mu(\Psi) = 0.5$) to dominant (i.e., $\mu(\Psi) = \{0, 1\}$) isoform population. Histograms of the number of exons with positive and negative changes are superimposed on the x axis. A preponderance of blue over red indicates that FOXA1 mostly inhibits exon inclusion.

(E) Over representation analysis performed on genes harboring FOXA1-regulated AS events in primary PCs and cell lines. Shape size and gene ratio indicate the number (from 12 to 59) and the fraction of selected genes in each pathway, respectively. Color key represents the statistical significance (FDR) of the enrichment. Only top 5 enriched pathways (FDR < 0.1), if any, are shown and sorted by statistical significance. For (B–D), stars indicate the significance of two-tailed exact binomial tests comparing the abundances of exons with positive and negative changes against a null hypothesis with probability = 0.5 in four groups of Ψ s. ** $p < 10^{-2}$ and *** $p < 10^{-3}$.

Figures and tables

Figure 3

Nonsense-mediated decay related events. What are they?

Quantifications and differences between FOXA1-regulated and unregulated NMD-related events

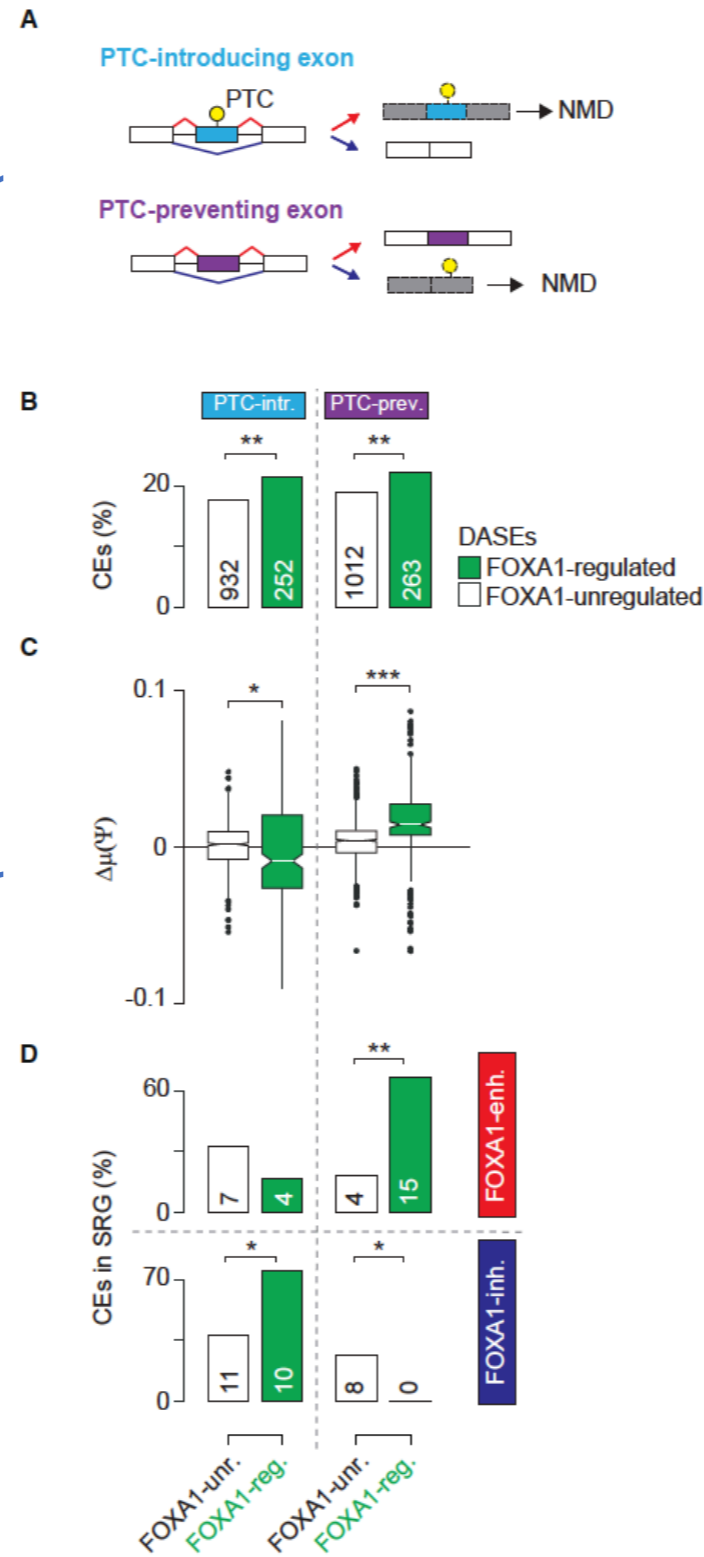


Figure 3. FOXA1 controls nonsense-mediated decay determinant exons

(A) Overview of selective inclusion of premature termination codon (PTC) introducing, or preventing, CEs triggering NMD.

(B) Bar plots show the proportion of PTC-introducing and PTC-preventing exons among FOXA1-regulated and FOXA1-unregulated exons. Numbers of exons in each category are indicated.

(C) Distribution of mean inclusion changes of NMD-determinant FOXA1-regulated and FOXA1-unregulated exons.

(D) Bar plots show the proportion of PTC-introducing and PTC-preventing exons among FOXA1-regulated and FOXA1-unregulated exons. Exons are stratified according to their positive (red) and negative (blue) mean inclusion change upon high expression of FOXA1. The number of exons in each category is indicated. Stars indicate statistical significance of two-tailed Fisher's exact test (B and D) and Wilcoxon rank-sum test (C). * $p < 0.05$, ** $p < 10^{-2}$, *** $p < 10^{-3}$.

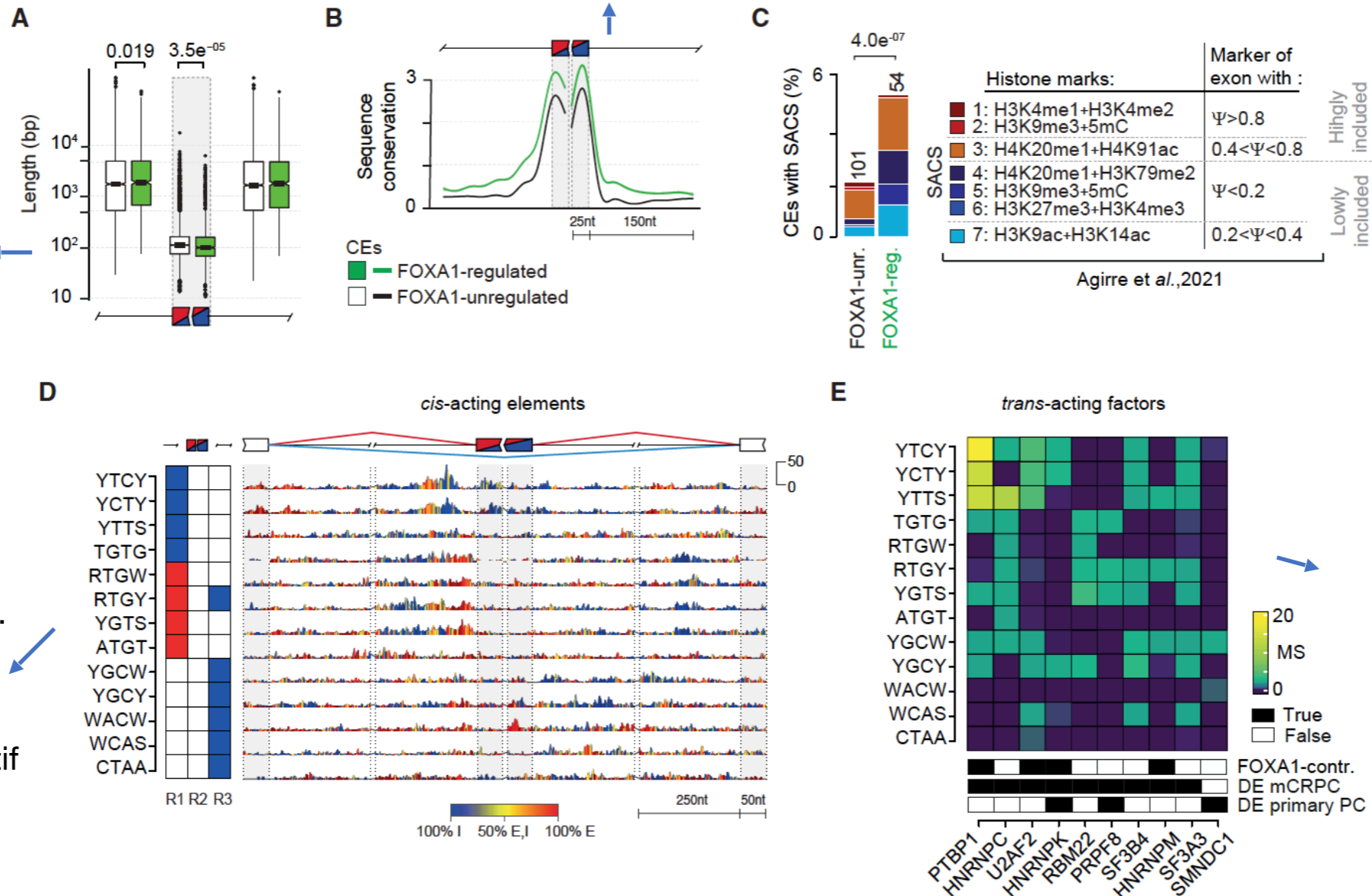
Figures and tables

Figure 4

Length distribution of exons and flanking introns

Enriched motifs with their splicing map (i.e. in how many events, divided by enhanced (red), silenced (blue), both (yellow), the motif is found in each relative position

PhyloP data (conservation across 100 species) of FOXA1-regulated and unregulated exons



SACS stands for Splicing-Associated Chromatin Signatures. Quantity and description of chromatin signatures.

Matching Score (MS) between enriched motifs and SRPs

Figure 4. FOXA1 mediates exon silencing by controlling trans-acting factors within an exon definition mechanism

(A) Length distributions of exon and flanking introns for FOXA1-regulated and -unregulated cassette exons. p values of two-tailed Wilcoxon rank-sum test are reported if significant.

(B) Distribution of smoothed conservation scores (PhyloP, 100 vertebrates) of FOXA1-regulated and -unregulated exons in exonic and flanking intronic regions.

(C) Bar plots show the fraction of SACS marked exons in FOXA1-regulated and FOXA1-unregulated exons (left panel). Color indicates SACS type. Corresponding histone modifications and categories of marked exons are reported as described in Agirre et al. (2021).

(D) RNA splicing map of multivalent RNA motifs enriched at FOXA1-regulated exons. Left color-coded panel indicates the regions at exon/intron junctions where motifs were enriched at inhibited (blue) or enhanced (red) exons. The right panel depicts the nucleotide-resolution RNA splicing map of each motif at the FOXA1-regulated exons, and their flanking exons. The color key indicates whether the position-specific contribution originates from enhanced (E) (red), inhibited (I) (blue), or both (yellow) sets. Maximum RNA motifs enrichment score of the top tetramer, which is used for all tetramers, is reported on the right. nt, nucleotides.

(E) Heatmap shows the association between enriched multivalent RNA motifs and cognate SRGs that were differentially expressed in primary PCs or mCRPCs in terms of matching score (MS).

Figures and tables

Figure 5

A. Survival analysis results on NMD-related differentially included events

B. Summary of the survival analysis... For how many events the inclusion is positive for survival (HR<1)? or negative for survival (HR>1)?

C. Six most prognostic events. Red ones are more included by FOXA1, while blue ones are more excluded.

D. Multivariable covariance analysis that indicates the direction and the magnitude of differential inclusion correlation with FOXA1 expression

E. Survival analysis on the inclusion of *FLNA* exon 30 of patients stratified by optimal value of inclusion

F. Number of patients with prognostic *FLNA* exon 30 inclusion upon FOXA1 high expression or not.

G. PSI values of *FLNA* exon 30 in PC3 measured by ddPCR in wild type conditions and upon FOXA1 silencing

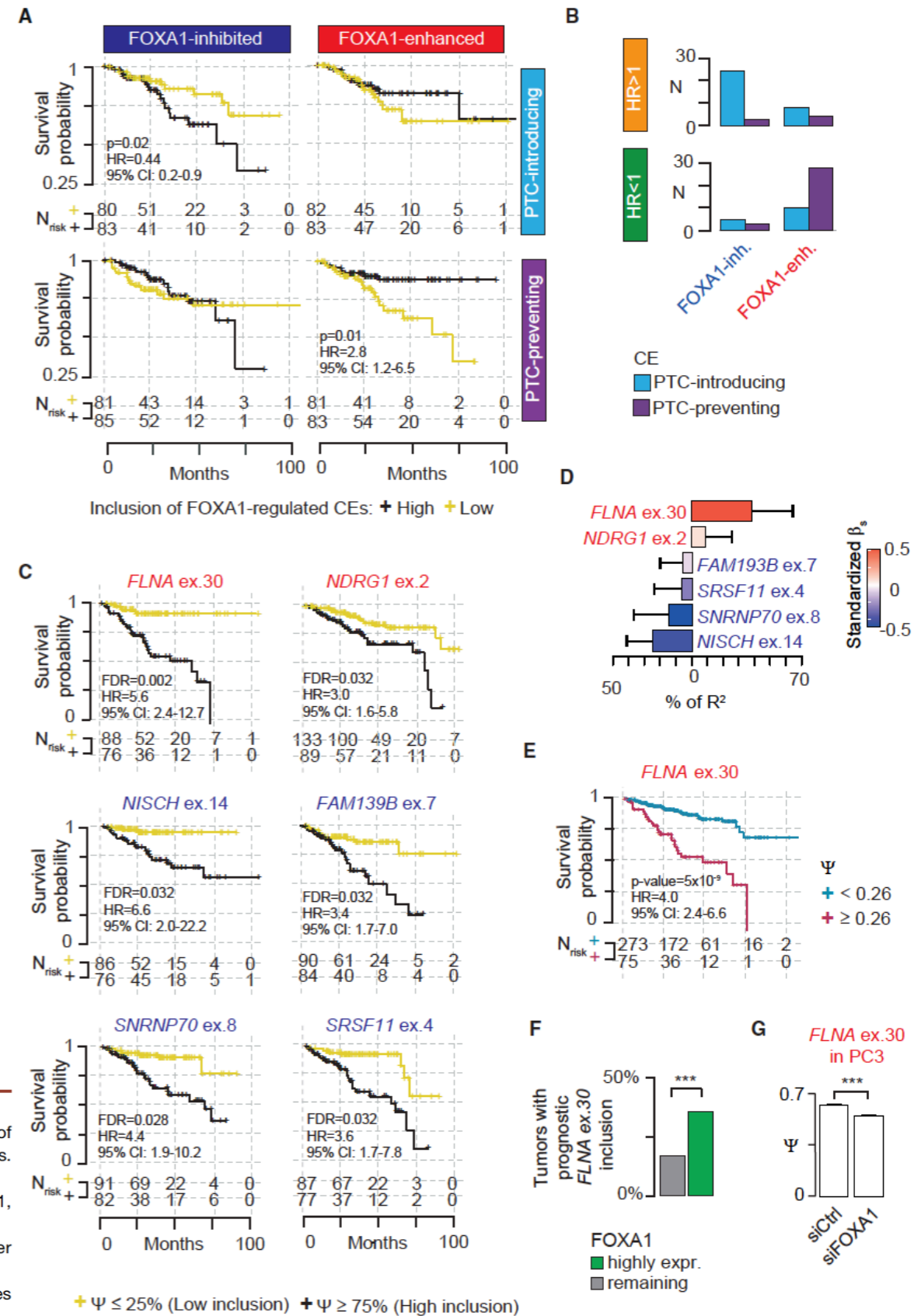


Figure 5. FOXA1-regulated NMD-determinant exons predict PC patient prognosis

(A) Kaplan-Meier plots of disease-free survival for primary PC patients stratified according to the 25th and 75th percentile of the cumulative inclusion levels of NMD-determinant exons that are inhibited or enhanced by high FOXA1 expression. Numbers of patients at risk (N_{risk}) are reported at each time point on the x axis. Univariate HRs with 95% confidence intervals (CI) and two-tailed log rank test p values are shown where statistically significant.

(B) Bar plots show the number of FOXA1-inhibited or -enhanced NMD-determinant exons with a significant harmful (HR > 1, top panel) or favorable (HR < 1, bottom panel) impact on patient disease-free survival (two-tailed log rank test p < 0.05).

(C) Kaplan-Meier plots of disease-free survival for primary PC patients with low and high inclusion of the six most prognostic harmful exons (FDR < 0.05). Number of patients at risk (N_{risk}) are reported at each time point on the x axis. Univariate HRs with 95% CI and two-tailed log rank test FDR are shown.

(D) Results of multivariable covariance analysis between FOXA1 expression and the inclusion levels of the six most prognostic harmful exons. Color key indicates the standardized β coefficients of the model.

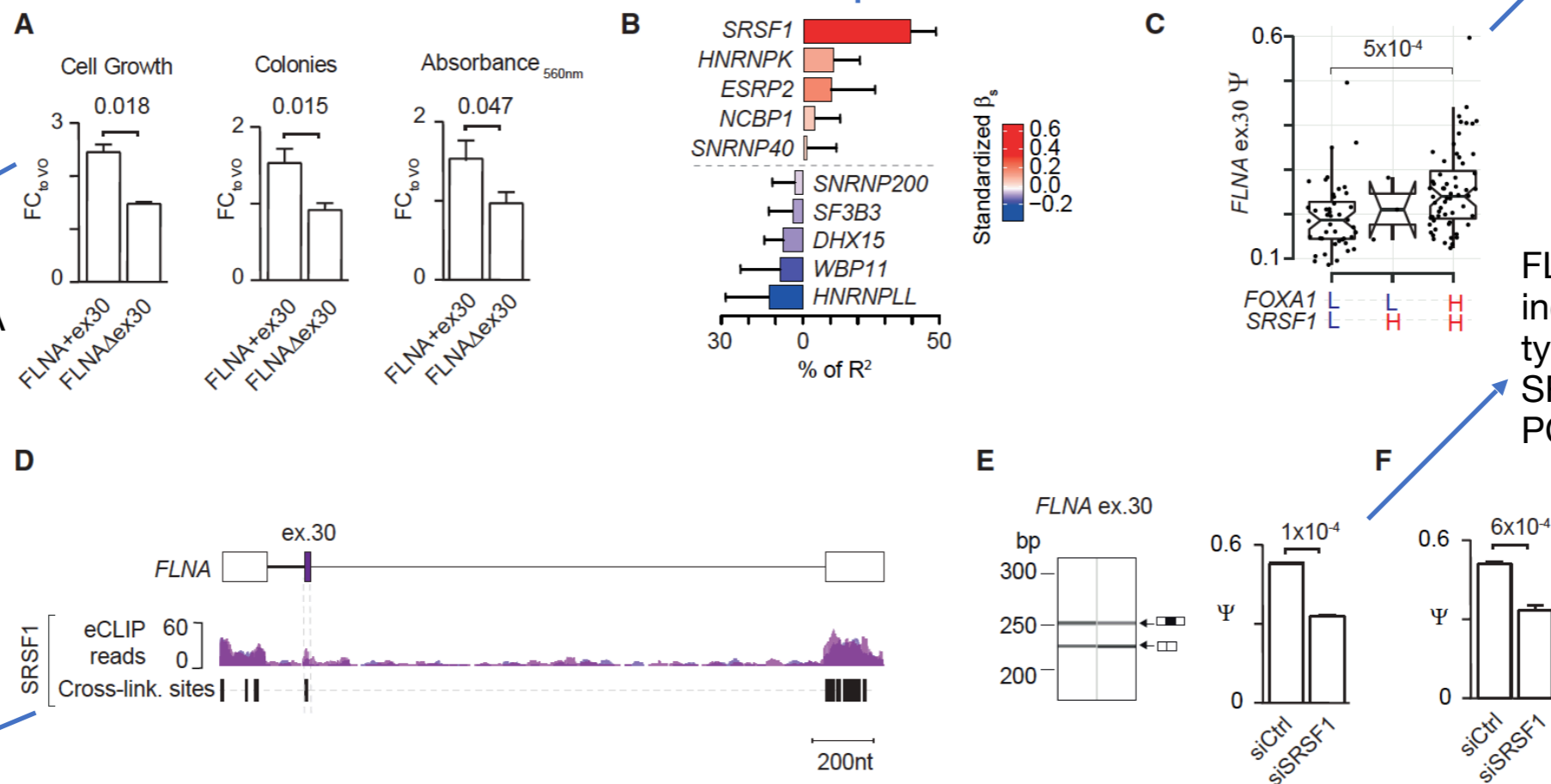
(E) Kaplan-Meier plots of disease-free survival for primary PC patients stratified on the optimal *FLNA* exon 30 inclusion level (i.e., $\Psi \geq 0.258$, maximally selected rank statistics = 5.35). Number of patients at risk (N_{risk}) are reported at each time point on the x axis. Univariate HRs with 95% CI and two-tailed log rank test FDR are shown.

(F) Bar plots show the proportions of high FOXA1 expressing and remaining tumors with *FLNA* exon 30 $\Psi \geq 0.258$.

(G) Bar plots show Ψ s of *FLNA* exon 30 in PC3 cells measured by ddPCR upon FOXA1 depletion with one siRNA duplex (si1, 40 nM for 72 h). For (F) and (G), two-tailed t test was used to compare conditions: ***p < 0.001.

Figures and tables

Figure 6



Proliferation indexes upon or without FLNA exon 30 inclusion

Multivariable covariance analysis between FLNA exon 30 inclusion levels and SRG expression

FLNA exon 30 levels of inclusion upon high/low expression levels of FOXA1 and SRSF1

FLNA exon 30 schematic event. SRSF1 binding signal and significant crosslink sites

FLNA exon 30 inclusion levels in wild type condition or upon SRSF1 silencing by PCR splicing assay

FLNA exon 30 inclusion levels in wild type condition or upon SRSF1 silencing by ddPCR

Figure 6. FLNA exon 30 inclusion promotes PC cell growth and is controlled by SRSF1

(A) Bar plot shows mean fold change in PC3 cell growth (left panel) measured by MTT assay following transfection with 100 ng of plasmid DNA vector encoding *FLNA* with or without exon 30 (i.e., *FLNA*+ex30 or *FLNA*Δex30, respectively, or VO control, biological triplicates). Bar plot shows mean fold change in PC3 clonogenic potential (middle and right panels) measured by crystal violet assays following transfection with 2 μg of plasmid DNA vector encoding *FLNA* with or without exon 30 (i.e., *FLNA*+ex30 or *FLNA*Δex30, respectively, or VO control). Both colony number (middle panel) and staining intensity (right panel) are shown (five biological replicates). Two-tailed t test was used to compare conditions.

(B) Results of multivariable covariance analysis between *FLNA* exon 30 inclusion levels and SRG expression levels. Color key indicates the standardized β coefficients of the model.

(C) Distribution of *FLNA* exon 30 inclusion levels in primary PC patients stratified by high or low expression ($\geq 75^{\text{th}}$ and $\leq 25^{\text{th}}$ percentile, respectively) of *FOXA1* and *SRSF1*. Two-tailed Wilcoxon rank-sum test was used to compare conditions. Only significant results are reported.

(D) SRSF1 eCLIP density read distribution in HepG2 cells in the alternatively spliced region of *FLNA* exon 30. Significant crosslinked sites detected by iCounts for SRSF1 are shown in black.

(E and F) Bar plots show Ψ 's of *FLNA* exon 30 in PC3 cells upon depletion of *SRSF1* with one siRNA duplex (40 nM for 72 h) in PC3 cells quantified by (E) endpoint PCR splicing assays using the QIAxcel capillary electrophoresis device and (F) by ddPCR. Representative capillary gel electrophoretogram (QIAxcel) shows two bands representing *FLNA* transcripts including or excluding exon 30 which were quantified to determine Ψ (E) (left panel). Two-tailed t test was used to compare biological triplicates of the different conditions.

Extract messages from discussion

1st paragraph

Overview of the paper

In this study, by analysis of transcriptomics, protein-mRNA interactions, epigenomics, and chromosome conformation, we reveal that the pioneer TF FOXA1 orchestrates AS regulation in PC impacting on patient survival.

Extract messages from discussion

2nd paragraph

Why FOXA1 is the predominant hallmark of dysregulation of SRGs?

Collectively, our results indicate that FOXA1 expression is a predominant hallmark of the transcriptional dysregulation of SRGs. As a pioneer factor, FOXA1 opens up nucleosomal domains for DNA binding by distinct TFs (Fei et al., 2019; Lupien et al., 2008). This pliant mechanism (Ramanand et al., 2020) may explain why FOXA1 hallmarks the global SRG dysregulation to a greater extent than the non-pioneer TFs, of which AR and MYC are documented to impact splicing regulation in PC (Phillips et al., 2020; Shah et al., 2020). Therefore, FOXA1 may open multiple channels to transmit transcriptional signals to SRG loci as exemplified by a common pioneer function for AR- and MYC-driven PC transcriptional programs (Barfeld et al., 2017).

Extract messages from discussion

3rd paragraph

Mechanisms of FOXA1 regulation over alternative splicing events

By assessing AS changes in primary PC and cell lines, we demonstrate that FOXA1 calibrates the landscape of exon utilization toward an equilibrium that solidifies the production of dominant isoforms. This phenomenon is largely achieved by silencing lowly included exons in a consistent manner across tumors, but crucially also by enhancing highly included ones. Therefore, FOXA1 ultimately limits protein diversity toward isoforms that are functional for cells. We show that exons responding to FOXA1 are alternatively spliced by an “exon definition” mechanism, being shorter with longer flanking introns, strongly conserved across species, and, for a small fraction, marked by chromatin modifications (Agirre et al., 2021; Keren et al., 2010). A smaller exon size and higher intronic sequence conservation have been associated with a greater exon silencing, under evolutionary constraints, to control relative isoform frequencies (Baek and Green, 2005). By integrating analyses of cis-acting elements and trans-acting factors, we demonstrate that FOXA1 calibrates AS by enlisting splicing factors under its transcriptional control, including binding of PTBP1, U2AF2, and HNRNPC at 3' ss (König et al., 2010; Sutandy et al., 2018; Xue et al., 2009), and HNRNPK at upstream intron-exon boundary and within downstream introns, respectively (Van Nostrand et al., 2020a, 2020b). It is fascinating that FOXA1 increases the inclusion of exons that are already highly included while reducing lowly included exons. This latter group indicates that FOXA1 is a genuine regulator of AS and not just an enhancer of splicing efficiency per se.

Main message

Extract messages from discussion

4th paragraph

FOXA1- orchestrated auto- regulation of SRGs

It is likely significant that FOXA1-mediated AS preferentially impacts on SRGs themselves, suggesting that FOXA1 may be involved in a known regulatory feedback loop exploited by splicing factors to modulate their own protein expression levels (Lareau et al., 2007). Interestingly, our results indicate that high FOXA1 expression in PC mostly inhibits the inclusion of NMD-determinant PTC-introducing “poison” exons. We hypothesize, therefore, that FOXA1-mediated AS restricts proteome diversity by influencing isoform degradation, particularly in SRGs. Recently, MYC has been implicated as a regulator of AS-coupled NMD in PC (Nasif et al., 2018; Pervouchine et al., 2019; Phillips et al., 2020). It is tempting to speculate that FOXA1, as a pliant regulator, may pioneer MYC to control transcription of specific SRGs and fine-tune AS in PC. Further functional studies are necessary to determine whether FOXA1 cooperates with specific TFs, chromatin modifiers, and RNA polymerase II, to rewire the AS landscape of PC.

| Possible
mechanism
| What is
needed?

Extract messages from discussion

5th paragraph

Clinical effect

Clearly the systems-wide impact on AS mediated by FOXA1 is likely to have a profound effect on cancer severity. From a clinical perspective, we found that FOXA1 enhanced the inclusion of two NMD-determinant exons that are strong biomarkers of disease recurrence. Of these, we established a role for the FOXA1-enhanced PTC-preventing exon 30 in the cancer gene *FLNA* as a promoter of PC cell growth. We demonstrate that the inclusion of *FLNA* exon 30 is controlled primarily by SRSF1, which was the first proto-oncogenic splicing factor enacting some of the oncogenic functions of MYC (Das et al., 2012).

Extract messages from discussion

6th paragraph

Summary of results

In summary, we reveal a novel role for the pioneer TF FOXA1 in orchestrating AS regulation in PC at different stages of gene expression. By transcriptionally regulating *trans*-acting factors, FOXA1 exploits an exon definition model to control relative isoform expression thereby fine-tuning proteome diversity. This splicing equilibrium favors the production of dominant isoforms, especially including those that escape NMD. FOXA1-mediated splicing regulation affects clinically relevant coding regions of the genome underlying PC patient survival.

1

2

3

4

5

Limitations of the study

1st paragraph

Our characterization of AS regulation in PC is limited to the contribution of four key oncogenic TFs with recurrent activating alterations across PC patients. In light of a long tail of oncogenic drivers underpinning a heterogeneous disease, we cannot exclude the influence of other transcriptional regulators. The analysis of FOXA1-mediated AS regulation was limited to primary PCs as splicing data for mCRPCs were not available. Although we recapitulated our results on metastatic PC cells, the generalizability of our findings to other clinical PC disease states remains to be elucidated.

1

2

Limitations of the study

2nd paragraph

Our work is based on novel computational analyses that provide unique insights into AS regulation by FOXA1, including the involvement of candidate SRGs and, to a minor extent, chromatin regulators. However, the mechanistic details as to how FOXA1 modulates SRG expression, cooperates with epi-transcriptional regulators, and affects AS decisions remain questions to address in future studies. Although we highlighted candidate prognostic AS events that could be exploited as biomarkers and therapeutic targets, further studies are required to determine their value in the context of FOXA1. Furthermore, a lack of pre-clinical phenotyping in our study limits the immediate clinical translation of our findings.

3

4

5

Limitations of the study

3rd paragraph

A potential confounder in the analysis of PC transcriptomes from bulk sequencing experiments is the contamination in low purity samples arising from benign prostatic epithelial, stromal, or immune cells. However, we performed computational validations showing that FOXA1 orchestrates AS regulation regardless of purity constraints ([Figure S6](#); [STAR Methods](#)).

6