MOLECULAR CARCINOGENESIS 53:883–892 (2014)

Hypoxia-Dependent mRNA Expression Pattern of Splicing Factor YT521 and Its Impact on Oncological Important Target Gene Expression

Marc Hirschfeld,[1,2] Bo Zhang,[1,3,4] Markus Jaeger,[1] Stefan Stamm,[5] Thalia Erbes,[1] Sebastian Mayer,[1] Xiaowen Tong,[3] and Elmar Stickeler[1]*

[1]*Gynecological Hospital, University Medical Center Freiburg, Freiburg, Germany*
[2]*German Cancer Research Center, Heidelberg, Germany*
[3]*Department of Obstetrics and Gynecology, Tongji Hospital, Tongji University, Shanghai, China*
[4]*Klinik für Gynäkologie und Geburtshilfe, St. Elisabeth-Hospital, Bochum, Germany*
[5]*Department of Molecular and Cellular Biochemistry BBSRB, University of Kentucky, Lexington, Kentucky*

The ubiquitously expressed splicing factor YT521 (YTHDC1) is characterized by alternatively spliced isoforms with regulatory impact on cancer-associated gene expression. Our recent findings account for the prognostic significance of YT521 in endometrial cancer. In this study, we investigated the hypoxia-dependency of YT521 expression as well as its differential isoform activities on oncological important target genes. YT521's potential regulatory influence on splicing was investigated by a minigene assay for the specific target gene CD44. Functional splicing analysis was performed by YT521 knock-down or overexpression, respectively. In addition, YT521 expression was determined under hypoxia. The two protein-generating YT521 mRNA isoforms 1 and 2 caused a comparable, specific induction of CD44v alternative splicing ($P < 0.01$). In a number of oncological target genes, YT521 upregulation significantly altered BRCA2 expression pattern, while YT521 knock-down created a significant regulatory impact on PGR expression, respectively. Hypoxia induced a specific switch towards the processing of two non-protein-coding mRNA variants, of which one is described for the first time in this study. The presented study underlines the comparable regulatory potential of both YT521 isoforms 1 and 2, on the investigated target genes in vivo and in vitro. Hypoxia induces a specific switch in YT521 expression pattern towards the two non-protein coding mRNA variants, the already characterized isoform 3 and the newly discovered exon 8-skipping isoform. The altered YT521 alternative splicing is functionally coupled with nonsense-mediated decay and can be interpreted as regulated unproductive splicing and transcription with consecutive impact on the processing of specific cancer-associated genes, such as BRCA2 and PGR. © 2013 Wiley Periodicals, Inc.

Key words: YT521; splicing; hypoxia; BRCA2; cancer

INTRODUCTION

The ubiquitously expressed nuclear splicing factor YT521 (YTH domain containing 1, YTHDC1) was discovered through an interaction of its glutamic-acid/arginine-rich domain with splicing factor htra2-beta1 [1,2]. YT521 expression itself is regulated by alternative splicing processes [3]. The variable inclusion of YT521 exon 6 in mRNA isoforms 1 and 2 results in two corresponding protein products (NCBI Reference Sequences: YTHDC1 transcript variant 1: NM_001031732.2 and YTHDC1 transcript variant 2: NM_133370.2 respectively, Ensemble; Swiss-Prot; Fig. 1A and B). Functional differences of the two YT521 isoforms 1 and 2 have not been reported so far. In addition, four other potential protein coding YT521 mRNA isoforms and one non-coding variant have been characterized [3] (Fig. 1A). One mRNA variant is characterized by simultaneous skipping of the exon cassette 8–9. This skipping creates a frameshift within the downstream nucleotide sequence, resulting in the introduction of a termination codon at the beginning of exon 10 [3]. Since the introduced termination codon is followed by an exon–exon junction more than 55 nucleotides downstream, it might be recognized as a premature termination codon (PTC) [4]. Therefore, the cassette 8–9-skipping YT521 mRNA variant is most likely subjected to nonsense-mediated decay (NMD).

YT521 is involved in splice site selection in a concentration dependent manner [5,6] either by sequestering splicing factors via protein–protein interaction or by binding to nucleic acids via its YTH domain (Fig. 1A) [1,7,8]. The YTH domain

Abbreviations PTC, premature termination codon; NMD, nonsense-mediated decay; RUST, regulated unproductive splicing and translation

None of the authors listed has to declare any competing conflict of interest in relation to the submitted work.

*Correspondence to: Gynecological Hospital, Freiburg University Medical Center, Hugstetterstr. 55, Freiburg 79106, Germany.

Received 5 March 2013; Accepted 19 April 2013

DOI 10.1002/mc.22045

Published online 13 June 2013 in Wiley Online Library (wileyonlinelibrary.com).

© 2013 WILEY PERIODICALS, INC.

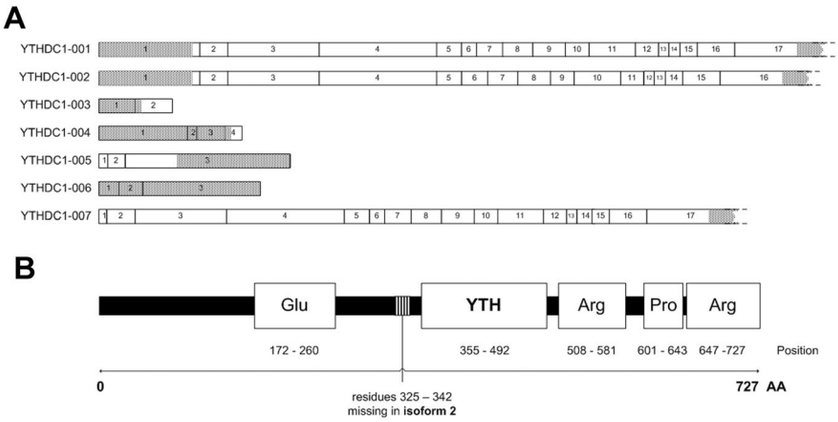

Figure 1. YT521 expression variants due to alternative splicing. (A) YT521 mRNA transcript isoforms (YTHDC1; source: ensemble.org: Gene ID: ENSG00000083896); Isoforms 1 and 2 (YTHDC1-001; -002) code for functional proteins (source: uniprot.org: protein ID's: Q96MU7-1; -2). Isoform 6 (YTHDC1-006) is non-protein coding. The further isoforms YTHDC1-003; -004; -005; -007 are putative protein-coding variants (functional implications unknown; predicted protein; source: uniprot.org: protein ID's: J3KS01; J3KRW0; J3KRG5; J3QR07). *5'- and 3' untranslated regions (UTRs) shaded*. (B) YT521 functional protein domain structure of isoforms 1 and 2. The YTH domain is a novel RNA binding domain and necessary for YT521 to directly influence splice site selection in vivo; Glu, glutamic acid rich region of unknown function; Arg, glutamic acid/arginine-rich regions involved in protein-protein interaction; Pro, proline rich region containing putative SH_3 domains. The skipping of exon 6 due to alternative mRNA splicing results in loss of amino acid (aa) residues 325–342, distinguishing isoform 2 (709 aa) from isoform 1 (727 aa; source: uniprot.org: protein ID's: Q96MU7-1; -2).

represents a novel RNA binding region, which interacts with short, degenerated, single-stranded RNA sequence motifs. The presence of the binding motif in alternative exons is necessary for YT521 to directly influence splice site selection in vivo [6]. Furthermore, YT521 function is regulated by phosphorylation via specific receptor tyrosine kinases [8].

So far, biological functions of YT521 are poorly investigated. However, our group could recently demonstrate a significant tumorbiological impact of this nuclear protein. A clear association of low YT521 expression with poor clinical outcome in patients diagnosed with type I endometrial cancer was identified, demonstrating a potential tumor suppressor activity [9]. These findings support the biological importance of splicing regulatory genes in cancer. Recently, a specific sensitivity of YT521 mRNA expression towards hypoxia was reported [2], which represents a key regulatory epiphenomenon in tumor growth inducing a transcriptional cascade that promotes an aggressive cancer phenotype. Experimental studies support its functional implications in cancer progression with regard to angiogenesis, growth factor signaling, immortalization, genetic instability, tissue invasion and apoptosis, respectively [10]. Alterations of YT521 expression might have eminent impact on splicing decision of cancer-associated genes. Recently, we were able to detect the major influence of hypoxia on splicing decisions in another cancer-related factor [11,12]. These findings lead to our hypothesis that hypoxia might also influence development of tumor growth through aberrations in YT521 splicing pattern.

Therefore, we designed the present study to analyze and distinguish the potential functional impact of the different YT521 isoform expression pattern on the splicing regulation of cancer-relevant genes bearing the YTH domain binding motifs (BRCA2, ESR1, PGR, MDM2, and VEGF). Furthermore, we investigated the influence of hypoxia on YT521 mRNA processing in vitro.

We refer in our functional experiments for the YT521 exon 6-inclusion mRNA variant as isoform 1, exon 6-skipping variant as isoform 2 and exons 8 and 9-skipping isoform as isoform 3, respectively.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The established human tumor cell lines HeLa, Ishikawa, SK-OV-3, MCF7, and T47D were cultured in a humidified incubator (37°C, 5%CO_2, 95% air). SK-OV-3, Ishikawa and HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Invitrogen, Darmstadt, Germany), MCF7 cells in Iscove's Modified Dulbecco's Medium (IMDM; Life Technologies, Invitrogen), and T47D cells in Quantum 263 medium (PAA Laboratories GmbH, Pasching, Austria), each supplemented with 10% fetal bovine serum "Gold" (PAA Laboratories GmbH), 1% of 1 mol/L HEPES buffer (Life Technologies), and 100 units/mL penicillin/streptomycin (Sigma–Aldrich Chemie GmbH, Schnelldorf, Germany). For hypoxia experiments, cultures were transferred to hypoxic culture conditions (<1% O_2; mentioned as hypoxia, 18 h) in a hypoxic chamber placed in the same incubator. Cells were cultured in parallel experiments under normal oxygen conditions (95% air, mentioned as normoxia) used as control.

Experiments concluded with the immediate harvesting of treated cultures for RNA extraction or fixation with formaldehyde fixation reagent for immunocytochemistry, respectively.

RNA Extraction and RT-PCR

The GeneMATRIX DNA + RNA + PROTEIN Purification Kit (EURx Ltd., Gdansk, Poland) was used to isolate total RNA from cells according to the manufacturer's protocol. In total, 4 μg RNA, as determined by optical densitometry, were used for complementary DNA (cDNA) synthesis using Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Promega, Mannheim, Germany), RiboLock RNase inhibitor (Fermentas GmbH, St. Leon-Roth, Germany), and random hexamer primers (New England Biolabs GmbH, Frankfurt, Germany) followed by PCR using Taq polymerase (GENAXXON Bioscience GmbH, Ulm, Germany) and specific primers. The sequence of primers and expected amplicon sizes were as follows: RPS18 (305 bp) S: 5′-AACTCACTGAGGATGAGGTG-3′, AS: 5′-CAGACAAGGCCTACAGACTT-3′; YT521 ex8 and ex9 (407, 180, 298 bp) S: 5′-ATCAAACCAGTAAACTCAAATATGTGCT-3′, AS: 5′-CTGTCCATCACGTCCGATCTTTA-3′; YT521 ex6 (178, 124 bp) S: 5′-GAAGTGGAAGCTCTGCATCA-3′, AS: 5′-GAATGTGTCTCTTGCCAAAGC-3′; VEGF ex3/ex7 (381, 297 bp) S: 5′-ACATCTTCAAGCCATCCTGTGTGC-3′, AS: 5′-TTAACTCAAGCTGCCTCGCCTTG-3′; ESTGLv2 Globin (418, 305, 300, 187 bp) S: 5′-AGACACCATGCATGGTGCACCT-3′, AS: 5′-CCATAACAGCATCAGGAGTG-3′; BRCA2 ex2/ex4 (419, 170 bp) S: 5′-CAAGCATTGGAGGAATATCG-3′, AS: 5′-AACATCATCTGCTTGATCC-3′; MDM2 ex4/ex10 (659, 543 bp) S: 5′-CGATTATATGATGAGAAGCAACAAC-3′, AS: 5′-CCTGCCTGATACACAGTAAC-3′; PGR ex2/ex5 (612, 495 bp) S: 5′-GTTTAATCTGTGGGGATGAA-3′, AS: 5′-CGTGTTTGTAGGATCTCCAT-3′; PGR ex5/ex8 (474, 343 bp) S: 5′-ATGGTGTTTGGTCTAGGATG-3′, AS: 5′-TTGTGCAGCAATAACTTCAG-3′; ESR1 ex6/ex8 (320, 136 bp) S: 5′-TGCTGCTGGCTACATCATCT-3′, AS: 5′-CGTTCTTGCACTTCATGCTG-3′. The PCR products were separated by electrophoresis in agarose gel.

Protein Extraction and Western Blot

Total protein of cultured cells was isolated by the GeneMATRIX DNA + RNA + PROTEIN Purification Kit according to the manufacturer's protocol. Protein concentration was determined by BCA Protein Assay Kit (PIERCE, Thermo Scientific, Rockford, IL).

The protein samples were separated by 10% SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (ROTH, Karlsruhe, Germany) by using a Bio-Rad Trans Blot Cell (Bio-Rad Laboratories GmbH, Munich, Germany). Membranes were soaked in a blocking solution (phosphate buffered saline (PBS) with 3% dry nonfat milk, 0.1% Tween 20) for 1 h at room temperature and incubated with the primary polyclonal rabbit antibody anti-YT521 (provided by Stefan Stamm; dilution 1:2000) in blocking buffer overnight at 4°C. After washing, membranes were further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Jackson Immuno Research Europe Ltd., Suffolk, UK; dilution 1:5000) in blocking buffer for 2 h at room temperature. Finally, the immunocomplexes were made visible by an enhanced chemiluminescence Western blot detection kit (PIERCE) and exposed to X-Ray film (FUJIFILM Europe GmbH, Düsseldorf, Germany).

Immunocytochemistry

Following experimental treatment, cell lines Ishikawa, SK-OV-3, and T47D were fixed using ROTI® Histofix formaldehyde fixation reagent (4% formaldehyde in PBS, pH 7, Carl Roth). YT521 protein expression were determined using polyclonal rabbit YT521 antibody (provided by S. Stamm [Stamm´s Lab., Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY] [13]).

For visualization of YT521 expression, antigen retrieval and indirect immunoperoxidase technique were applied. Antigen retrieval was performed by heating the fixed cells on slides for 5 min in a 10 mmol/L sodium citrate buffer (pH 6.0). Inhibition of endogenous peroxidase was performed by 30 min incubation with 3% H_2O_2. Endogenous avidin–biotin was blocked by the use of a commercial biotin blocking system (DAKO, Dako GmbH) for 10 min. After two washes in trissaline buffer, slides were incubated with 1% goat serum for 30 min to block unspecific staining. The sections were exposed to YT521 antibody (1:6000) overnight at room temperature. Slides were incubated with biotinylated anti-rabbit immunoglobulins for 60 min at room temperature and treated with streptavidin–peroxidase (DAKO). Staining was achieved by 3,3-diaminobenzidine (DAB; Vectastain, Vector Laboratories, Inc., Linaris GmbH) and the slides were counterstained with hemalaun.

Cloning, Transfection, and Sequencing

The YT521 isoform 1 expression plasmid was constructed by inserting the whole length of its complementary DNA (cDNA) fragment, which was amplified from HeLa cell cDNA library by PCR into pCMV-Script Vector (Stratagene, Agilent Technologies, Inc., Santa Clara, CA). The YT521 isoform 2 expression plasmid was constructed using a method called PCR-directed motif deletion of plasmid, with isoform 1 expression plasmid as template and a pair of primers flanking exon 6, as recently described [14].

The transfection of minigene ESTGLv2 [15], YT521 expression plasmid or shRNA plasmid of YT521 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) was carried out with Satisfection reagent (Stratagene) according to the manufacturer's protocol. 48 h after

transfection, cells were harvested followed by RNA extraction and RT-PCR. Experiments were performed in triplicate. Sequences of newly created clones or identified PCR products of interest were analyzed and validated by GATC Biotech Company (GATC Biotech AG, Konstanz, Germany).

Nonsense-Mediated Decay (NMD) Assay

It is well established that NMD requires translation for PTC recognition. Drugs or RNA structures inhibiting any step of the pioneer round of translation will inhibit NMD [16]. Puromycin is widely used for NMD target identification since it acts as a protein synthesis inhibitor and has been proven to block the NMD pathway [17]. Cells cultured under hypoxia for 18 h were further treated with puromycin (AppliChem GmbH, Darmstadt, Germany; 300 μg/mL, 8 h) as recently described [18] under normal oxygen conditions (95% air, 5% CO_2). As control cells were cultured in parallel under hypoxia for 18 h and then under normal oxygen conditions for 8 h without puromycin treatment used. Afterwards, total RNA was isolated and semi-quantative RT-PCR was performed to evaluate the YT521 mRNA expression profile.

Equipment for Documentation and Evaluation

Documentation of electrophoresis gels was performed using DIANA II (CCD Camera System, Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany) and data were evaluated by ImageJ software (http://rsb.info.nih.gov/ij/).

Statistical Analyses

The Statistical Package for the Social Sciences software version 15.0.1 (SSPS 15.0.1.) was used for statistical analysis. The data of PCR were analyzed by student's *t*-test and one-way ANOVA. Significance of differences was assumed as $P < 0.05$ at the two-sided test.

RESULTS

YT521 Isoforms 1 and 2 Exhibit Comparable Alternative Splicing Properties

CD44 is an established target gene of YT521 in vivo and was selected to evaluate the splicing properties of the distinct YT521 isoforms [8]. ESTGLv2, a reporter gene containing human CD44 variable exons v4 and v5 (Fig. 2A) [15], was co-transfected with pCMV-YT521-isoform 1 and pCMV-YT521-isoform 2 in MCF7 cells, respectively. Following confirmation of transfection efficacy of YT521 plasmids (Fig. 2B), alternative splicing pattern of the reporter gene were analyzed by RT-PCR. The basic phenotype of ESTGLv2 splicing displayed majorly a single exon inclusion, but no v4 + v5 en bloc skipping. In addition, a small fraction of en bloc inclusion of both exons was visible in YT521 untransfected cells (average v4 + v5 en bloc inclusion proportion: 25.4%, v4 + v5 en bloc inclusion proportion = v4 + v5 en bloc inclusion/all splicing isoforms; Fig. 2C and D). The following co-transfection experiments revealed a specific and significant induction of CD44 v4 + v5 en bloc inclusion in a concentration-dependent manner for both tested YT521 isoforms (pCMV-YT521-isoform 1: from 26.5% to 54.9%, $P < 0.01$; pCMV-YT521-isoform 2: from 24.2% to 53.2%, $P < 0.01$; Fig. 2D). Comparable effects were also detected for genuine CD44 mRNA processing (data not shown).

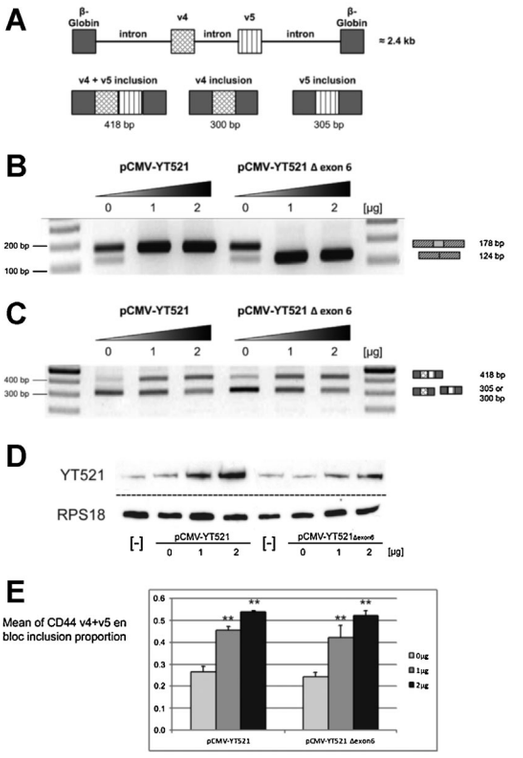

Figure 2. YT521 isoforms 1 and 2 exert comparable impacts on alternative splicing of CD44 exon v4 + v5. (A) Schematic structure of ESTGLv2 minigene [15], with a genomic fragment of human CD44 gene containing variable exons v4 and v5 (*patterned boxes*) into the first intron of a β-Globin (shaded boxes). (B) Expression level of YT521 isoforms 1 and 2 after transfection. Expression plasmids of YT521 isoforms 1 and 2 were transfected in an increasing amount (0, 1, 2 μg). Analysis of YT521 mRNA expression with exon 6 inclusion/skipping by RT-PCR. (C) YT521-regulated splicing pattern of ESTGLv2. Increase of YT521 isoforms 1 and 2 lead to increased proportion of CD44 v4 + v5 en bloc inclusion (*upper band*). Analysis of mRNA expression by RT-PCR. (D) Quantitative YT521 protein expression analysis in T47D cells; comparable results in Ishikawa and SK-OV-3 cells, data not shown. T47D cells were transfected with increasing amounts of expression vectors pCMV-YT521 or pCMV-YT521Δexon6 (0, 1, 2 μg), or "empty" pCMV vector ([−]) used as control; RPS18 protein expression as comparative value and loading control, Western blot. *Dashed line indicates origin from different gels*. (E) Both of YT521 isoforms (1 and 2) cause a significant increase in CD44 v4 + v5 en bloc inclusion proportion (v4 + v5 en bloc inclusion/all splicing isoforms) (**$P < 0.01$, one-way ANOVA: pCMV-YT521-isoform-1 2 μg vs. 1 μg and 1 μg vs. 0 μg; pCMV-YT521-isoform-2 2 μg vs. 1 μg and 1 μg vs. 0 μg).

Impact of YT521 on Potential Target Genes In Vivo

Since YT521 expression was found to correlate with survival of endometrial cancer patients [9], we wanted

to elucidate the role of YT521 in alternative splicing of cancer-related genes bearing YTH domain binding motifs. Therefore splicing pattern of the potential YT521 target genes BRCA2, ESR1, MDM2, VEGF, and PGR were analyzed. To investigate functional implications on target genes, YT521 was either knocked-down by YT521 shRNA or overexpressed by pCMV-YT521-isoform 1 expression plasmid in a set of distinct tumor cell lines (Ishikawa: endometrial cancer; SK-OV-3: ovarian cancer; T47D: breast cancer). Consecutively the endogenous alternative splicing pattern of the potential target genes (BRCA2 exon 3, ESR1 exon 7, MDM2 exons 5 and 6, VEGF exon 4, PGR exons 3 and 6, respectively) were investigated under these different conditions (Fig. 3). Our assays revealed specific alternative splicing changes of BRCA2 (exon 3 inclusion/skipping) by YT521 upregulation (Fig. 3A) and PGR (exon 3 inclusion/skipping) by YT521 knock-down in T47D cells (Fig. 3E), respectively. In detail, YT521 upregulation created a significant induction of BRCA2 exon 3 inclusion ($P = 0.012$; Fig. 3A), while YT521 knock-down lead to a significant decrease of PGR exon 3 inclusion ($P = 0.007$; Fig. 3E), respectively. However, for the other investigated potential target genes or PGR and BRCA2 in other distinct cell lines, only mild and non-significant effects of YT521 were observed (Fig. 3B–D). Efficacy of forced YT521 overexpression or knock-down on protein level was approved by quantitative analysis (Western blot, Fig. 3F).

Hypoxia-Induced Coupling of Alternative Splicing and NMD in YT521 Expression

In the second part of our analyses, we focused on the influence of hypoxia on YT521 expression in human cancer cells. Therefore, five human gynecological tumor cell lines were cultured under hypoxic conditions and the expression levels and pattern of YT521 were examined.

Hypoxia did only cause slight changes in mRNA expression pattern of the YT521 isoforms 1 and 2 in the investigated tumor cell lines on the mRNA level (Fig. 4A). However, different mRNA variants aside from isoforms 1 and 2 were detected as YT521 isoform 3 specific PCR primers were applied. These two hypoxia-induced mRNA isoforms could be identified as the already known YT521 isoform 3, characterized by skipping of the exon cassette 8–9 (amplicon 180 bp), as well as a novel, so far undescribed YT521 mRNA variant solely skipping exon 8 (amplicon 298 bp), respectively (Fig. 4B). Verification of isoform 3 and the novel exon 8-skipping YT521 mRNA isoform was conducted by cloning and sequencing.

In the next experimental step, Ishikawa and SK-OV-3 cells were cultured under hypoxic conditions for 18 h followed by 8 h rescue (under normoxia). The following expression analyses showed a specific disappearance of the YT521 isoform 3 as well as the newly discovered exon 8-skipping mRNA. Even more interestingly, both isoforms were retained in puromycin assay suggesting that these two mRNA variants are definitive targets of NMD (Fig. 4C).

The exclusion of YT521 exon cassettes 8 and 9 in isoform 3 creates a downstream sequence frameshift resulting in the introduction of several PTCs. The frameshift caused by exclusive skipping of exon 8 also leads to the introduction of several PTCs within the downstream sequence of the novel mRNA variant, with the first termination codon located in exon 9 approximately 70 nucleotides downstream of the exons 7–9 junction.

Quantitative analysis (Western blot) identifies oxygen deprivation as inductor of a detectable, though marginal reduction of YT521 protein levels. This effect is reversible, at which comparable levels of YT521 protein to control cells are restored following instauration of normoxia (Fig. 4D). Immunocytochemical detection of YT521 demonstrates the nuclear localization of the splicing factor and confirms the reversible hypoxia-dependent alterations in protein expression levels (Fig. 5).

DISCUSSION

The expression of splicing factor YT521 is regulated by alternative splicing, a phenomenon also described for other nuclear proteins involved in the complex nuclear mRNA processing. Out of seven known alternatively spliced mRNA isoforms of YT521, biologically active YT521 protein isoforms solely originate from the wild type (exon 6 inclusion, isoform 1) and exon 6 skipping isoform (isoform 2) according to the Ensembl (http://www.ensembl.org/index.html) and Swiss-Prot (http://expasy.org/sprot/) databases. Our recent study identified YT521 isoform 1 as a potential prognostic factor for endometrial cancer [9]. The functional in vivo experiments of the current study revealed that both YT521 isoforms 1 and 2, irrespectively of their exon 6 inclusion status, exhibited the same functional properties regarding splicing regulation. An explanation for this phenomenon is given by the fact, that exon 6 is not coding for any of the known functional YT521 domains and skipping of this 54 nucleotide exon does not create a downstream sequence frameshift, thereby potentially not altering the splice regulatory function of the truncated protein sequence. The development- and tissue-specific character of alternative splicing events [19–21] may account for the existence of the exon 6 skipping isoform as an evolutionary conserved physiological phenomenon [22,23].

Our previous investigations identified high YT521 expression as a protective factor in endometrial cancer with a potential function as a tumor suppressor by influencing the alternative splicing pattern of specific cancer-associated target genes [9]. YT521 expression-dependent splicing patterns of all known

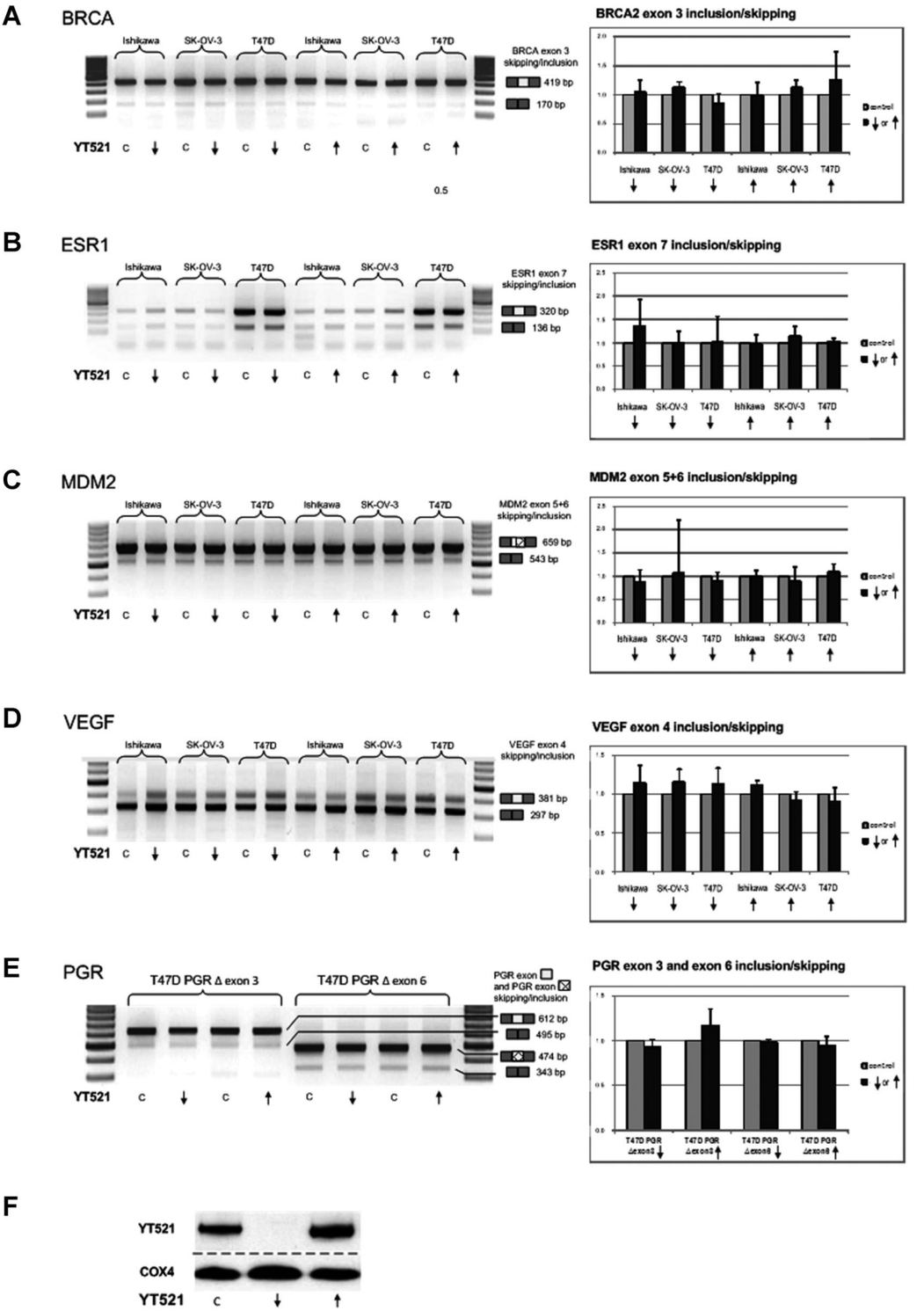
A
BRCA
Ishikawa
SK-OV-3
T47D
Ishikawa
SK-OV-3
T47D
BRCA exon 3 skipping/inclusion
419 bp
170 bp
YT521
c
0.5
BRCA2 exon 3 inclusion/skipping
control
B
ESR1
ESR1 exon 7 skipping/inclusion
320 bp
136 bp
ESR1 exon 7 inclusion/skipping
C
MDM2
MDM2 exon 5+6 skipping/inclusion
659 bp
543 bp
MDM2 exon 5+6 inclusion/skipping
D
VEGF
VEGF exon 4 skipping/inclusion
381 bp
297 bp
VEGF exon 4 inclusion/skipping
E
PGR
T47D PGR Δ exon 3
T47D PGR Δ exon 6
PGR exon and PGR exon skipping/inclusion
612 bp
495 bp
474 bp
343 bp
PGR exon 3 and exon 6 inclusion/skipping
F
YT521
COX4

Figure 3.

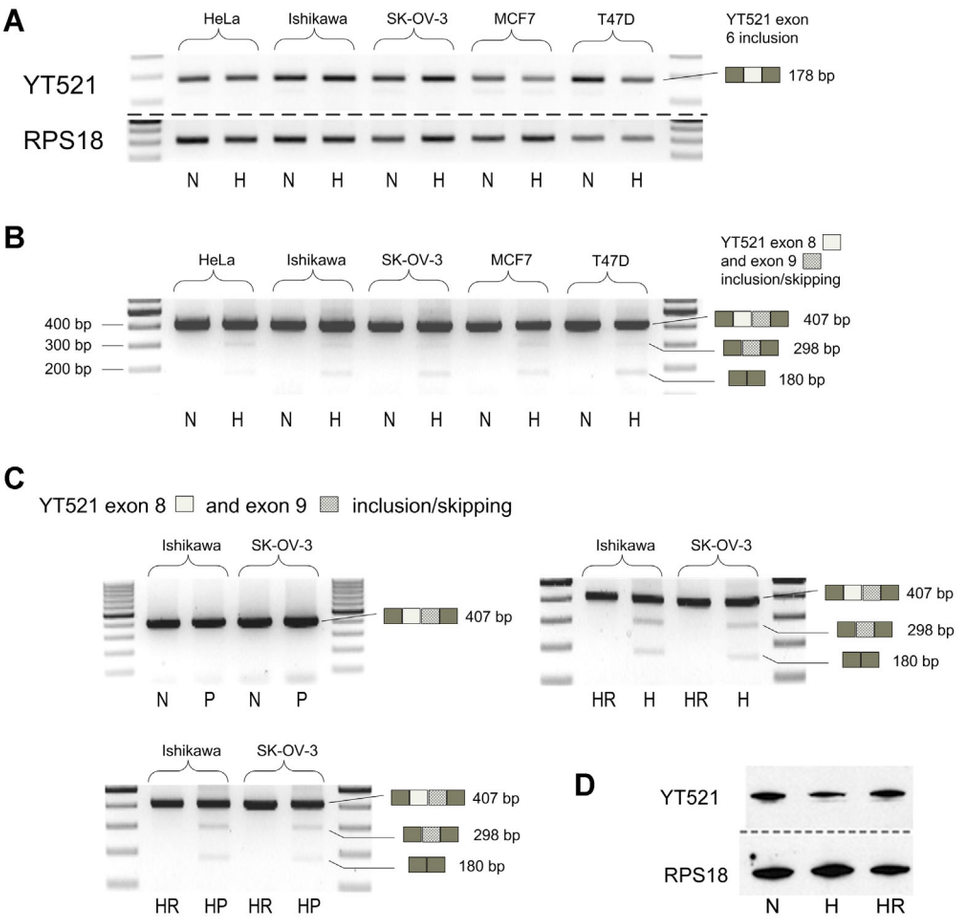

Figure 4. Hypoxia-induced coupling of alternative splicing and NMD in YT521 mRNA expression pattern. (A) Hypoxia exerted no significant impact on the alternative splicing of YT521 exon 6 ($P > 0.05$, hypoxia vs. normoxia), *N: normoxia; H: hypoxia*; RPS18: comparative value as loading control. *Dashed line indicates origin from different gels*. (B) Hypoxia lead to YT521 exon 8-skipping and exons 8 and 9-skipping (isoform 3) in HeLa, Ishikawa, SK-OV-3, MCF-7, and T47D cell lines, N, normoxia; H, hypoxia. (C) Hypoxia-dependent isoform 3 and exon 8-skipping isoform expression and identification as NMD targets. Puromycin treatment alone did not lead to isoform 3 and exon 8-skipping mRNA expression, indicating that these isoforms are not expressed in cells cultured under normoxia; comparison of cells cultured under hypoxia (18 h) versus combined hypoxia (18 h) followed by rescue (normoxia, 8 h) revealed the hypoxia-dependent expression of isoform 3 and exon 8-skipping isoform. In comparison to control group, isoform 3 and exon 8-skipping isoforms were retained after puromycin treatment (300 μg/mL, 8 h), indicating they are definite NMD targets. *N = normoxia, 26 h; H = hypoxia, 18 h; HR = hypoxia, 18 h and rescue under normoxia, 8 h; P = normoxia, 18 h and puromycin treatment, 8 h; HP = hypoxia, 18 h and puromycin treatment under normoxia, 8 h*. (D) YT521 protein expression in T47D cells under hypoxia (H, 18 h); hypoxia and rescue (HR = hypoxia, 18 h and rescue under normoxia, 8 h); control cells (N) cultured under normoxia, RPS18 protein expression as comparative value and loading control, Western blot.

human genes were determined in a recent genome-wide Affymetrix® splice array analysis [6]. Exons of Anub11, Cxc110, Rhot2, Sec24c, and Zfp687 were identified as endogenous targets of YT521. However, the knowledge about the potential role of these genes in tumorigenesis remains limited. Additional data about further targets of YT521 are not described so far. To explore the potential impact of YT521 on cancer,

Figure 3. The influence of YT521 expression on endogenous alternative splicing pattern of potential target genes. YT521 was knocked-down or overexpressed in Ishikawa, SK-OV-3 and T47D cell lines, respectively. The alternative splicing pattern of endogenous potential target genes were quantified and compared with controls. (A) BRCA2 exon 3 inclusion (*upper band*, 419 bp) and skipping (*lower band*, 170 bp). (B) ESR1 exon 7 inclusion (*upper band*, 320 bp) and skipping (*lower band*, 136 bp). (C) MDM2 exons 5 and 6 inclusion (*upper band*, 659 bp) and skipping (*lower band*, 543 bp). (D) VEGF exon 4 inclusion (*upper band*, 381 bp) and skipping (*lower band*, 297 bp). (E) PGR exon 3 inclusion (*upper band*, 612 bp) and skipping (*lower band*, 495 bp); PGR exon 6 inclusion (*upper band*, 474 bp) and skipping (*lower band*, 343 bp). C, control; , YT521 knock-down; ↑, YT521 overexpression; $^*P < 0.05$. (F) Quantitative YT521 protein expression analysis in T47D cells; similar results in Ishikawa and SK-OV-3 cells, data not shown. Knock-down, YT521 knocked down by shRNA of YT521; ↑overexpression, YT521 overexpressed by pCMV-YT521-isoform 1 expression plasmid; control cells (C) cultured under regular conditions, COX4: Cyclooxygenase 4 protein expression as comparative value and loading control, Western blot.

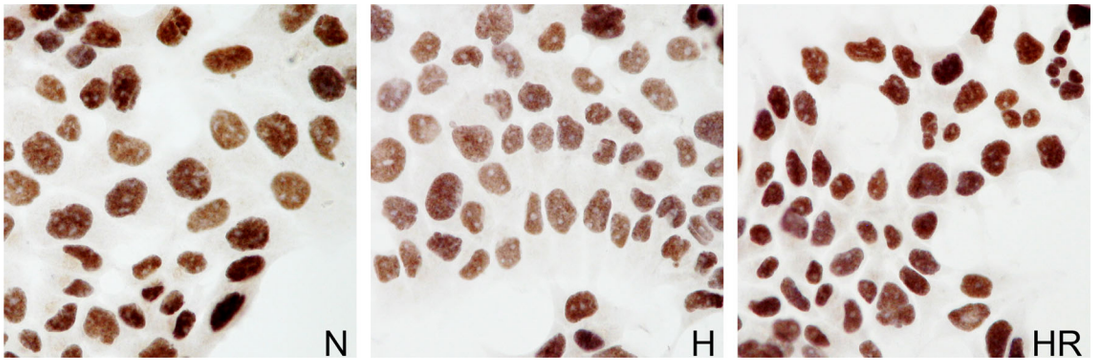

Figure 5. YT521 protein expression in T47D cells. YT521 protein is predominately expressed in nuclear compartments. Hypoxia induces a decrease in YT521 protein expression, which is restored under normal oxygen conditions. *N = normoxia, 26 h; H = hypoxia, 18 h; HR = hypoxia, 18 h; and rescue under normoxia, 8 h*; Immunocytochemistry, YT521 antibody provided by S. Stamm [13]; counterstained with haemalaun, Magnification ×400.

we investigated several potential alternatively spliced target exons of cancer-related genes such as BRCA2 [24], ESR1 [25], MDM2 [26], VEGF [27], and PGR [28], respectively. All of these candidate genes exhibit an YT521 binding motif. Interestingly, we were able to identify specific, significant YT521 dependent alternative splicing alterations in BRCA2 (exon 3 inclusion/skipping) in breast ductal carcinoma cell line T47D due to YT521 upregulation and in PGR (exon 3 inclusion/skipping) by a specific YT521 knock-down. For the other genes we could not detect further significant splicing regulatory influence of YT521. However, our data suggest a more specific than an overall impact of YT521 on pertinent tumorbiological genes which might cause vast alterations in cellular behavior. Employing the in vitro systematic evolution of ligands by exponential enrichment (SELEX) technique, we were able to identify the sequence *GAR* as the RNA motif binding to YT521. It is a known as a common cis-acting element for SR protein RNA interactions [6,15].

Hypoxia is a well-known micro-environmental epiphenomenon in solid tumors [29]. Hypoxia-driven changes in alternative splicing pattern were demonstrated in several studies [11,30,31]. Our functional data clearly demonstrate the hypoxia-dependent alterations in YT521 splicing pattern characterized by exon cassette 8–9 or exclusive exon 8 exclusion, respectively, resulting in PTC-exhibiting YT521 mRNA isoforms that are subjected to NMD. This gene expression regulatory process of coupling alternative splicing with NMD is known as regulated unproductive splicing and translation (RUST) [32]. In this study, we could identify hypoxia as a downregulator of YT521 gene expression, which is in accordance with current knowledge and related to a variety of transcriptional and post-translational mechanisms causing global changes in protein synthesis [33,34]. Data obtained on hypoxia-dependency at mRNA level were confirmed by quantitative YT521 protein expression analysis, at which a reversible hypoxia-triggered decrease of YT521 protein could be demonstrated (Fig. 4D, Fig. 5). We postulate that hypoxia-induced YT521 protein downregulation via this RUST pathway exerts decisive biological consequences in tumor progression by alterations in expression profiles of cancer-related genes. RUST is a widespread pathway of gene regulation. One third of the reliably inferred alternatively spliced mRNA isoforms are expected to be targets of degradation through RUST rather than being translated into truncated, but biologically active proteins [35]. Besides transcriptional regulation, RUST provides an additional level of gene expression regulation. Splicing regulation occurs after the decision to transcribe a gene region and RUST is able to provide a rapid way of changing the levels of productive mRNA. This extra regulatory feature accounts for a "fine tuning" in transcriptional regulation.

Although YT521 belongs neither to the SR protein nor hnRNP family, our findings suggest that this gene might also auto-regulate its own expression levels via RUST. Furthermore, the recently described reverse correlation between YT521 protein and mRNA levels in endometrial cancer supports strongly this suggested auto-regulation of YT521 expression [9].

Here, we describe for the first time a hypoxia-induced regulation of gene expression via RUST, by which a striking number of splicing factors and elements of the splicing machinery are auto-regulated, such as PTB [36], SC35 [37], htra2-beta1 [38], and CDC-like kinases (Clks) [39], respectively. Intriguingly, similar unproductive splicing is found in all human SR genes and some hnRNPs [32,40].

In a previously published study, YT521 function of splice site selection was found to be regulated by tyrosine phosphorylation activated by receptor tyrosine kinases [8]. Hypoxia is a known important factor in the activation of receptor tyrosine kinases [41,42]. It is reasonable to postulate that hypoxia activates

signaling pathways, which subsequently activate tyrosine phosphorylation and further exert influences on YT521 mRNA expression pattern, consequently regulating the downstream alternative splicing events that play important roles in tumorigenesis or other pathological processes.

In summary, the two protein isoforms of YT521 exhibit comparable alternative splicing properties in in vivo assay. YT521 expression pattern influence the alternative splicing of cancer-related genes, such as BRCA2 and PGR that play different roles in tumor progression. We identified hypoxia as a key regulator of YT521 mRNA expression pattern and corresponding protein expression profiles via RUST. Further functional investigations on YT521 regulation and its tumorbiological impact in regard to alternative splicing of target genes may focus on hypoxia as well as other micro-environmental alterations involved in signaling pathways in cancer.

REFERENCES

1. Hartmann AM, Nayler O, Schwaiger FW, Obermeier A, Stamm S. The interaction and colocalization of Sam68 with the splicing-associated factor YT521-B in nuclear dots is regulated by the Src family kinase p59(fyn). Mol Biol Cell 1999;10:3909–3926.
2. Imai Y, Matsuo N, Ogawa S, Tohyama M, Takagi T. Cloning of a gene, YT521, for a novel RNA splicing-related protein induced by hypoxia/reoxygenation. Brain Res 1998;53:33–40.
3. Stamm S, Riethoven JJ, Le Texier V, et al. ASD: A bioinformatics resource on alternative splicing. Nucleic Acids Res 2006;34:(Database issue):D46–D55.
4. Gardner LB. Nonsense-mediated RNA decay regulation by cellular stress: Implications for tumorigenesis. Mol Cancer Res 2010;8:295–308.
5. Nayler O, Hartmann AM, Stamm S. The ER repeat protein YT521-B localizes to a novel subnuclear compartment. J Cell Biol 2000;150:949–962.
6. Zhang Z, Theler D, Kaminska KH, et al. The YTH domain is a novel RNA binding domain. J Biol Chem 2010;285:14701–14710.
7. Stoilov P, Rafalska I, Stamm S. YTH: A new domain in nuclear proteins. Trends Biochem Sci 2002;27:495–497.
8. Rafalska I, Zhang Z, Benderska N, et al. The intranuclear localization and function of YT521-B is regulated by tyrosine phosphorylation. Hum Mol Genet 2004;13:1535–1549.
9. Zhang B, zur Hausen A, Orlowska-Volk M, et al. Alternative splicing-related factor Y T521: An independent prognostic factor in endometrial cancer. Int J Gynecol Cancer 2010;20:492–499.
10. Harris AL. Hypoxia—A key regulatory factor in tumour growth. Nature Rev 2002;2:38–47.
11. Hirschfeld M, zur Hausen A, Bettendorf H, Jager M, Stickeler E. Alternative splicing of Cyr61 is regulated by hypoxia and significantly changed in breast cancer. Cancer Res 2009;69:2082–2090.
12. Hirschfeld M, Jaeger M, Buratti E, et al. Expression of tumor-promoting Cyr61 is regulated by hTRA2-beta1 and acidosis. Hum Mol Genet 2011;20:2356–2365.
13. Daoud R, Mies G, Smialowska A, Olah L, Hossmann KA, Stamm S. Ischemia induces a translocation of the splicing factor tra2-beta 1 and changes alternative splicing patterns in the brain. J Neurosci 2002;22:5889–5899.
14. Hansson MD, Rzeznicka K, Rosenback M, Hansson M, Sirijovski N. PCR-mediated deletion of plasmid DNA. Anal Biochem 2008;375:373–375.
15. Stickeler E, Fraser SD, Honig A, Chen AL, Berget SM, Cooper TA. The RNA binding protein YB-1 binds A/C-rich exon enhancers and stimulates splicing of the CD44 alternative exon v4. EMBO J 2001;20:3821–3830.
16. Maquat LE. Nonsense-mediated mRNA decay: Splicing, translation and mRNP dynamics. Nat Rev Mol Cell Biol 2004;5:89–99.
17. Carter MS, Doskow J, Morris P, et al. A regulatory mechanism that detects premature nonsense codons in T-cell receptor transcripts in vivo is reversed by protein synthesis inhibitors in vitro. J Biol Chem 1995;270:28995–29003.
18. Asselta R, Rimoldi V, Guella I, et al. Molecular characterization of in-frame and out-of-frame alternative splicings in coagulation factor XI pre-mRNA. Blood 2010;115:2065–2072.
19. Yeo G, Holste D, Kreiman G, Burge CB. Variation in alternative splicing across human tissues. Genome Biol 2004;5:R74.
20. Ule J, Ule A, Spencer J, et al. Nova regulates brain-specific splicing to shape the synapse. Nat Genet 2005;37:844–852.
21. Sugnet CW, Srinivasan K, Clark TA, et al. Unusual intron conservation near tissue-regulated exons found by splicing microarrays. PLoS Comput Biol 2006;2:e4.
22. Lareau LF, Green RE, Bhatnagar RS, Brenner SE. The evolving roles of alternative splicing. Curr Opin Struct Biol 2004;14:273–282.
23. Keren H, Lev-Maor G, Ast G. Alternative splicing and evolution: Diversification, exon definition and function. Nat Rev Genet 2010;11:345–355.
24. Farrugia DJ, Agarwal MK, Pankratz VS, et al. Functional assays for classification of BRCA2 variants of uncertain significance. Cancer Res 2008;68:3523–3531.
25. Garcia Pedrero JM, Zuazua P, Martinez-Campa C, Lazo PS, Ramos S. The naturally occurring variant of estrogen receptor (ER) ERDeltaE7 suppresses estrogen-dependent transcriptional activation by both wild-type ERalpha and ERbeta. Endocrinology 2003;144:2967–2976.
26. Jeyaraj S, O'Brien DM, Chandler DS. MDM2 and MDM4 splicing: An integral part of the cancer spliceome. Front Biosci 2009;14:2647–2656.
27. Matsuyama M, Chijiwa T, Inoue Y, et al. Alternative splicing variant of vascular endothelial growth factor-A is a critical prognostic factor in non-small cell lung cancer. Oncol Rep 2009;22:1407–1413.
28. Cork DM, Lennard TW, Tyson-Capper AJ. Alternative splicing and the progesterone receptor in breast cancer. Breast Cancer Res 2008;10:207.
29. Noman MZ, Messai Y, Carre T, et al. Microenvironmental hypoxia orchestrating the cell stroma cross talk, tumor progression and antitumor response. Crit Rev Immunol 2011;31:357–377.
30. Fukumura D, Xu L, Chen Y, Gohongi T, Seed B, Jain RK. Hypoxia and acidosis independently up-regulate vascular endothelial growth factor transcription in brain tumors in vivo. Cancer Res 2001;61:6020–6024.
31. Kikuchi Y, Kakeya T, Nakajima O, et al. Hypoxia induces expression of a GPI-anchorless splice variant of the prion protein. FEBS J 2008;275:2965–2976.
32. Lareau LF, Brooks AN, Soergel DA, Meng Q, Brenner SE. The coupling of alternative splicing and nonsense-mediated mRNA decay. Adv Exp Med Biol 2007;623:190–211.
33. Liu L, Simon MC. Regulation of transcription and translation by hypoxia. Cancer Biol Ther 2004;3:492–497.
34. Papandreou I, Powell A, Lim AL, Denko N. Cellular reaction to hypoxia: Sensing and responding to an adverse environment. Mutat Res 2005;569:87–100.
35. Green RE, Lewis BP, Hillman RT, et al. Widespread predicted nonsense-mediated mRNA decay of alternatively-spliced transcripts of human normal and disease genes. Bioinformatics (Oxford, England) 2003;19:i118–i121.
36. Wollerton MC, Gooding C, Wagner EJ, Garcia-Blanco MA, Smith CW. Autoregulation of polypyrimidine tract binding

protein by alternative splicing leading to nonsense-mediated decay. Mol Cell 2004;13:91–100.
37. Sureau A, Gattoni R, Dooghe Y, Stevenin J, Soret J. SC35 autoregulates its expression by promoting splicing events that destabilize its mRNAs. EMBO J 2001;20:1785–1796.
38. Stoilov P, Daoud R, Nayler O, Stamm S. Human tra2-beta1 autoregulates its protein concentration by influencing alternative splicing of its pre-mRNA. Hum Mol Genet 2004;13:509–524.
39. Hillman RT, Green RE, Brenner SE. An unappreciated role for RNA surveillance. Genome Biol 2004;5:R8.
40. Ni JZ, Grate L, Donohue JP, et al. Ultraconserved elements are associated with homeostatic control of splicing regulators by alternative splicing and nonsense-mediated decay. Genes Dev 2007;21:708–718.
41. Mishra OP, Ashraf QM, Delivoria-Papadopoulos M. Tyrosine phosphorylation of neuronal nitric oxide synthase (nNOS) during hypoxia in the cerebral cortex of newborn piglets: The role of nitric oxide. Neurosci Lett 2009;462:64–67.
42. Toby IT, Chicoine LG, Cui H, Chen B, Nelin LD. Hypoxia-induced proliferation of human pulmonary microvascular endothelial cells depends on epidermal growth factor receptor tyrosine kinase activation. Am J Physiol 2010;298:L600–L606.