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Journal of Cellular Biochemistry 9999:1–11 (2012)

Journal of Cellular Biochemistry

Involvement of hnRNP A1 in the Matrix Metalloprotease-3-Dependent Regulation of Rac1 Pre-mRNA Splicing

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ABSTRACT

Rac1b is an alternatively spliced isoform of the small GTPase Rac1 that includes the 57-nucleotide exon 3b. Rac1b was originally identified through its over-expression in breast and colorectal cancer cells, and has subsequently been implicated as a key player in a number of different oncogenic signaling pathways, including tumorigenic transformation of mammary epithelial cells exposed to matrix metalloproteinase-3 (MMP-3). Although many of the cellular consequences of Rac1b activity have been recently described, the molecular mechanism by which MMP-3 treatment leads to Rac1b induction has not been defined. Here we use proteomic methods to identify heterogeneous nuclear ribonucleoprotein (hnRNP) A1 as a factor involved in Rac1 splicing regulation. We find that hnRNP A1 binds to Rac1 exon 3b in mouse mammary epithelial cells, repressing its inclusion into mature mRNA. We also find that exposure of cells to MMP-3 leads to release of hnRNP A1 from exon 3b and the consequent generation of Rac1b. Finally, we analyze normal breast tissue and breast cancer biopsies, and identify an inverse correlation between expression of hnRNP A1 and Rac1b, suggesting the existence of this regulatory axis in vivo. These results provide new insights on how extracellular signals regulate alternative splicing, contributing to cellular transformation and development of breast cancer. J. Cell. Biochem. 9999: 1–11, 2012.

KEY WORDS: RAC1; MATRIX METALLOPROTEASE; ALTERNATIVE SPLICING; HNRNP

Pre-messenger RNA (pre-mRNA) alternative splicing can generate multiple protein variants from a single gene. It is a highly controlled process and represents the major source of protein diversity [Nilsen and Graveley, 2010]. One way alternative splicing is regulated is by the interaction of splicing factors such as serine–arginine-rich (SR) proteins and heterogeneous nuclear

Davitte Khauv and Guillermo Risso contributed equally to this work.

Additional supporting information may be found in the online version of this article.

Grant sponsor: Agencia Nacional de Promoción Científica y Tecnológica of Argentina; Grant sponsor: Universidad de Buenos Aires, Argentina; Grant sponsor: Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina; Grant sponsor: European Alternative Splicing Network (EURASNET); Grant sponsor: UICC International Cancer Technology Transfer Fellowship; Grant sponsor: National Cancer Institute; Grant sponsor: National Institutes of Health; Grant number: NO2-CO-41101; Grant sponsor: National Cancer Institute; Grant numbers: CA122086, CA132879; Grant sponsor: Mayo Clinic Breast Cancer Specialized Program of Research Excellence (SPORE) Grant; Grant number: CA116201.

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Received 23 December 2011; Accepted 8 February 2012 • DOI 10.1002/jcb.24103 • © 2012 Wiley Periodicals, Inc.
Published online in Wiley Online Library (wileyonlinelibrary.com).

ribonucleoproteins (hnRNP proteins) with their target sequences in the pre-mRNA [Black, 2003]. Typically, SR proteins recognize intronic or exonic splicing enhancers (ISEs and ESEs, respectively) and hnRNP proteins recognize intronic or exonic splicing silencers (ISSs and ESSs, respectively) [Matlin et al., 2005]. In addition, the regulation of alternative splicing by signaling pathways is an important mechanism required for maintenance of cellular homeostasis and deregulation of alternative splicing is associated with pathological conditions and progression to cancer [Srebrow and Kornblihtt, 2006; Blaustein et al., 2007; David and Manley, 2010; Dutertre et al., 2010]. There has been particular interest in the association between tumor progression and alternative splicing [Venables, 2004; Srebrow and Kornblihtt, 2006; Skotheim and Nees, 2007], though our knowledge about how splicing regulation is controlled by pro-tumorigenic stimuli is limited [Cheng and Sharp, 2006; Cheng et al., 2006; Karni et al., 2007; Valacca et al., 2010].

hnRNPs play important roles in every step of mRNA metabolism. The human hnRNP family consists of at least 24 members, which are among the most abundant nuclear proteins. hnRNP A1, a member of the hnRNP A/B subfamily, has been studied extensively and participates in the regulation of splicing, mRNA export, translation, telomere regulation, and microRNA (miRNA) processing [Izaurralde et al., 1997; LaBranche et al., 1998; Dreyfuss et al., 2002; Chabot et al., 2003; Zhang et al., 2006; Cammas et al., 2007; Guil and Caceres, 2007; Lewis et al., 2007]. Although hnRNP A1 localizes predominantly in the nucleus, it shuttles continuously between the nucleus and the cytoplasm [Pinol-Roma and Dreyfuss, 1992], and this shuttling can be regulated by signaling cascades [van der Houven van Oordt et al., 2000; Guil et al., 2006].

Rac1b, an alternatively spliced isoform of the small GTPase Rac1, was initially identified as expressed in breast and colorectal cancer cells [Jordan et al., 1999; Schnelzer et al., 2000]. Subsequent investigations of purified recombinant Rac1b revealed that it possessed limited GTPase activity, decreased affinity for nucleotides, and differential association with effector proteins [Matos et al., 2003; Orlichenko et al., 2010]. Functional investigation has revealed that Rac1b transforms NIH3T3 cells [Singh et al., 2004], promotes colorectal cancer cell survival [Matos and Jordan, 2008; Matos et al., 2008], and is an essential intermediate in the induction of epithelial–mesenchymal transition (EMT) in mouse mammary epithelial cells exposed to matrix metalloproteinase-3 [Radisky et al., 2005]. EMT is a highly regulated process in which epithelial cells acquire the invasive, motile mesenchymal phenotype [Thiery and Sleeman, 2006]. EMT has been best characterized for its role in development, but recent studies have shown that EMT processes are activated during tumor progression. Matrix metalloproteinases (MMPs) can trigger EMT both in vivo and in cell culture [Egeblad and Werb, 2002; Thiery and Sleeman, 2006; Stallings-Mann and Radisky, 2007]. Treatment of functionally normal mammary epithelial cells with MMP-3 promotes cytoskeletal modifications, loss of cell adhesion, acquisition of an invasive phenotype, down-regulation of epithelial markers and up-regulation of mesenchymal markers [Lochter et al., 1997, 1998; Sternlicht et al., 2000], and was shown to require increased expression of Rac1b [Radisky et al., 2005; Nelson et al., 2008]. However, the processes by which exposure of cells to MMP-3 led to increased expression of Rac1b were unclear.

In the present study we identify a key regulator of exon 3b inclusion into Rac1 mRNA. Using a proteomic approach, we find that hnRNP A1 binds to the alternative exon 3b and has a strong repressor activity on this exon inclusion. We show that MMP-3 treatment alleviates exon 3b from splicing inhibition by the disassembly of a repressor complex composed of (at least) hnRNP A1 and A2, leading to an enhanced inclusion of this oncogenic exon. We also compare hnRNP A1 and Rac1b expression in normal breast lobules and in breast cancer tissue, finding that progression to cancer is associated with reduced expression of nuclear hnRNP A1 and increased expression of Rac1b, consistent with the loss of a hnRNP A1 repressive role in breast cancer. These results shed light on how a transforming stimulus such as the disturbed expression of MMPs leads to alternative splicing regulation and ultimately to cellular transformation.

MATERIALS AND METHODS

CELL CULTURE AND MMP-3 TREATMENT

SCp2 and EpH4 stable cell lines bearing a tetracycline-regulated MMP-3 transgene were maintained as described previously [Radisky et al., 2005]. For transgene repression, a 5 mg/ml stock solution of tetracycline in ethanol was diluted 1:1,000 into culture medium and changed daily. To stimulate cells with MMP-3, we used medium that had been conditioned by cells grown in the absence of tetracycline. Conditioned medium from cells grown in the presence of tetracycline was used as control. These conditioned media were analyzed by zymography to verify that MMP-3 was only expressed upon removal of tetracycline [Supplementary Fig. 1; Lochter et al., 1997], and that EMT was induced by extracellular proteolytic activity (not shown). Cells were incubated for 3–5 days in the presence of conditioned media.

CELL TRANSFECTIONS

For experiments requiring higher transfection efficiency in order to analyze endogenous mRNAs, SCp2 cells were used. DNA transfections were carried out in 6-well plates using 6 ml of Lipofectamine 2000 (Invitrogen) and 2 μg of plasmid DNA in 2.5 ml Opti-MEM. RNAi transfections were performed with 5 μl of Lipofectamine 2000 (Invitrogen) and siRNA concentration was 40 nM in a final volume of 2.5 ml Opti-MEM. Cells were plated 24 h before transfection (3.5×10^5 cells for DNA and 1.5×10^5 for RNA). In both cases the medium was changed 4 h after transfection.

CELLULAR FRACTIONATION

Nuclear and cytoplasmic fractions from either SCp2 or EpH4 cells were obtained either by the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, for small scale) according to the manufacturer instructions or by a commonly used laboratory procedure (for large scale). In the latter case, cells were washed and scraped from 10 10-cm plates in ice-cold PBS, spun, and resuspended in 10 mM HEPES–KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF. After incubation for 10 min on ice, cells were dounce-homogenized (15 storkes with

pestle B), and centrifuged at 12,000*g* for 10 min. The supernatant (cytoplasmic fraction) was placed in pre-cooled Eppendorf tubes, adjusted to 10% glycerol and stored at −80°C. The nuclear pellet was resuspended in 25 mM HEPES–KOH pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.6 M AcOK, 0.5 mM DTT, and 0.5 mM PMSF, and kept on ice for 40 min vortexing every 5 min. After a centrifugation step at 12,000*g* for 30 min, the supernatant (nucleoplasmic fraction) was placed in a pre-cooled Eppendorf tube and stored at −80°C. For RNA-binding studies, the nuclear extract was dialyzed against 20 mM HEPES–KOH pH 7.9, 100 mM AcOK, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, and 0.5 mM PMSF for 2 h prior to the binding reaction. Protein concentration in the extracts was measured either by the Bradford assay (BioRad) or with the Quant-iT Protein Assay Kit using the Qubit Fluorometer (Invitrogen).

IN VITRO TRANSCRIPTION

RNAs were synthesized with the Riboprobe in vitro Transcription System (Promega) according to the manufacturer instructions. For radioactively labeled RNAs, [α-^{32}P]-CTP was added to the transcription reactions. Reactions were treated with RQ1-DNase (Promega) and run on a 10% acrylamide/8M urea gel. Bands were cut, eluted overnight, quantified with a scintillation counter and subsequently used for binding reactions.

RNA–BINDING ASSAYS

RNA-affinity purification, UV crosslinking/immunoprecipitation and RNA EMSA assays were performed as described [Rothrock et al., 2005]. Biotinylated RNA oligos were from Dharmacon. The wt exon 3b oligonucleotide sequence was (5′–3′): GTTGGAAGACACATG-TGGTAAAGATAGACCCTCCAGGGGCAAAGACAAGCCGATTGCC.
For the binding reaction, 50 pmol of 5′-biotinylated RNA (Dharmacon) was incubated with 100 μg of SCp2 nuclear extract in a 500 μl binding reaction (3.2 mM MgCl2, 20 mM phosphocreatine, 1 mM ATP, 1.3% polyvinyl alcohol, 25 ng of yeast tRNA, 1 mM DTT, 0.1 μl RNasin (Invitrogen, 40 U/μl), 75 mM KCl, 10 mM Tris, pH 7.5, 0.1 mM EDTA). Binding reactions were incubated with gentle agitation for 30 min at 30°C. Streptavidin agarose beads (Pierce) were pre-blocked in GFB100 (100 mM KCl, 20 mM Tris–Cl, pH 7.5, and 0.2 mM EDTA, pH 8.0), plus 1 μg/μl heparin and washed with GFB100. Pre-blocked streptavidin beads (25 μl) were added to the RNA-binding reactions, with additional heparin, to a final concentration of 0.5 μg/μl, 1.6 mM $MgCl_2$, 100 μl BC400, in a final volume of 1 ml, and incubated for 60 min at 4°C with gentle agitation. RNA–protein-bead complexes were washed with GFB100, resuspended in 2× SDS loading buffer, denatured for 5 min at 95°C, resolved by SDS–PAGE (Acrylamide/Bis 37.5:1, BioRad), and detected by Coomassie staining. The "B mut" oligonucleotide was identical to the wt 3b oligo except for nucleotides at positions 16–25 that were replaced by a linker containing the *Mlu*I restriction enzyme sequence as already described [König et al., 1998].

Rac1 MINIGENE CONSTRUCTION

Two fragments of the murine Rac1 gene were PCR-amplified using mouse genomic DNA as template and Platinum HiFi DNA Polymerase (Invitrogen). One fragment contained Rac1 exon 3 and 5′ portion of intron 3. The other fragment contained a 3′ portion of intron 3, exon 3b, the whole intron downstream, and part of exon 4. Fragments were joined by overlapping PCR and cloned into the pcDNA3.1(+) vector (Invitrogen). The minigene was sequenced and confirmed not to have any mutations within the exons, intron/exon boundaries, or intron sequences at a distance of 600 nucleotides from exons. Mutations were performed using standard PCR-mediated mutagenesis using the wild-type minigene as a template.

RNA EXTRACTION, RT, PCR, AND qPCR

RNA was isolated with TRIZOL reagent (Invitrogen) or TRI reagent (Molecular Research Center) and cDNA was synthesized using MMLV-RT (Invitrogen). For quantitative real-time PCR analysis of Rac1 splicing, oligonucleotide primers specific for the exon 3-containing Rac1 splice isoform and for exon 3-excluding Rac1 isoform were used. PCR was carried out using Taq Platinum (Invitrogen) and SYBR green dye. Regular (end point) RT-PCR of Rac1 minigene-derived transcripts was performed by extracting RNA and synthesizing cDNA with a minigene specific primer. PCR was performed with Taq Platinum and minigene specific primers. The ratios of the splicing isoforms were shown not to change between 26 and 32 cycles of PCR, and 28 cycles were routinely carried out. Primer sequences are available upon request.

MASS SPECTROMETRY

Proteomic analysis was performed as described previously [Orlichenko et al., 2010]. Briefly, isolated gel bands were excised, destained, reduced and alkylated with dithiothreitol and iodoacetamide. Proteins were digested for 4 h with 0.6 μg trypsin (Promega) in digestion buffer (20 mM Tris pH 8.1/0.0002% Zwittergent 3–16, at 37°C) followed by peptide extraction with 60 μl of 2% trifluoroacetic acid, then 60 μl of acetonitrile. Pooled extracts were concentrated and then brought up in 0.1% formic acid for protein identification by nanoflow liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) analysis using a ThermoFinnigan LTQ Orbitrap Hybrid Mass Spectrometer (Thermo Electron Bremen) coupled to an Eksigent nanoLC-2D HPLC system (Eksigent, Dublin, CA). The MS/MS raw data were converted to DTA files using ThermoElectron Bioworks 3.2 and correlated to theoretical fragmentation patterns of tryptic peptide sequences from the NCBI nr database using both SEQUEST™ (Thermo Electron, San Jose, CA) and the Mascot™ 3 (Matrix Sciences, London, UK) search algorithms running on a 10 node cluster. The mass spectrometry analysis was done at Mayo Proteomics Research center (Mayo Clinic College of Medicine, Rochester, MN).

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Non-malignant breast (cancer-free prophylactic mastectomy tissue) and breast cancer biopsies were derived from waste surgical material from different deidentified patients, and were formalin-fixed and paraffin-embedded. Sections from four non-malignant and four breast cancer samples were evaluated. For immunohistochemistry, tissue sections were deparaffinized by placing them into three

changes of xylene and rehydrated in a graded ethanol series. The rehydrated tissue samples were rinsed in water and sections were subjected to heat antigen retrieval as described by the manufacturer (Dako, Carpinteria, CA). Slices were incubated with the appropriate primary antibody (Rac1b, Millipore #09-271; hnRNP A1, Abcam #ab5832) for 30 min at room temperature. Sections were then rinsed with Tris-buffered saline/Triton-X100 (TBST) wash buffer, and secondary incubation was with DAKO DUAL+, horseradish peroxidase (HRP) for 15 min. Tissue slices were rinsed with TBST wash buffer and then incubated in 3,3′-diaminobenzidine (DAB+) (K3467, DAKO), and counterstained with modified Schmidt's hematoxylin. Stained slices were scanned and the stain intensity of hnRNP A1 and Rac1b was assessed using the Aperio positive pixel count algorithm in the Imagescope software (Aperio, Vista, CA). The algorithm used is based on spectral differentiation between brown (positive) and blue (counter) staining.

STATISTICAL ANALYSIS

Statistical significance in the splicing assays, both for the minigene and endogenous transcripts, was tested with a two-tailed *t*-test. Whenever a difference is said to be observed between two conditions, a *P*-value <0.05 was obtained except where otherwise indicated.

RESULTS

hnRNP A1 BINDS TO Rac1 ALTERNATIVE EXON 3B

Rac1b, a splicing variant of Rac1, was shown to be necessary and sufficient for mammary epithelial cell transformation [Radisky et al., 2005]. Exposure of functionally normal mouse mammary epithelial cell lines (SCp2 or EpH4) to MMP-3 activates a signaling pathway that leads to the inclusion of exon 3b into Rac1 mRNA, consequently rendering a constitutively active Rac1 protein. In order to gain insight into the mechanisms regulating Rac1 alternative splicing, we investigated which nuclear proteins have the ability to bind the Rac1 mRNA. Comparing the human and murine exon 3b sequence and its flanking introns, there are two conserved regions, one being the exon itself and the other a region in the downstream intron. Taking this into account, we performed RNA-affinity purification using nuclear extracts from SCp2 cells and a 5′-biotinylated murine exon 3b RNA probe. Upon incubation with nuclear extract, proteins bound to the 3b RNA were pulled-down with streptavidin–agarose beads and subjected to SDS-PAGE and Coomassie staining. A doublet of approximately 35 kDa was consistently observed (Fig. 1A) and these bands were isolated, subjected to in-gel trypsin digestion, and analyzed by nanoLC-tandem mass spectrometry, which identified the proteins as hnRNP A1 and hnRNP A2

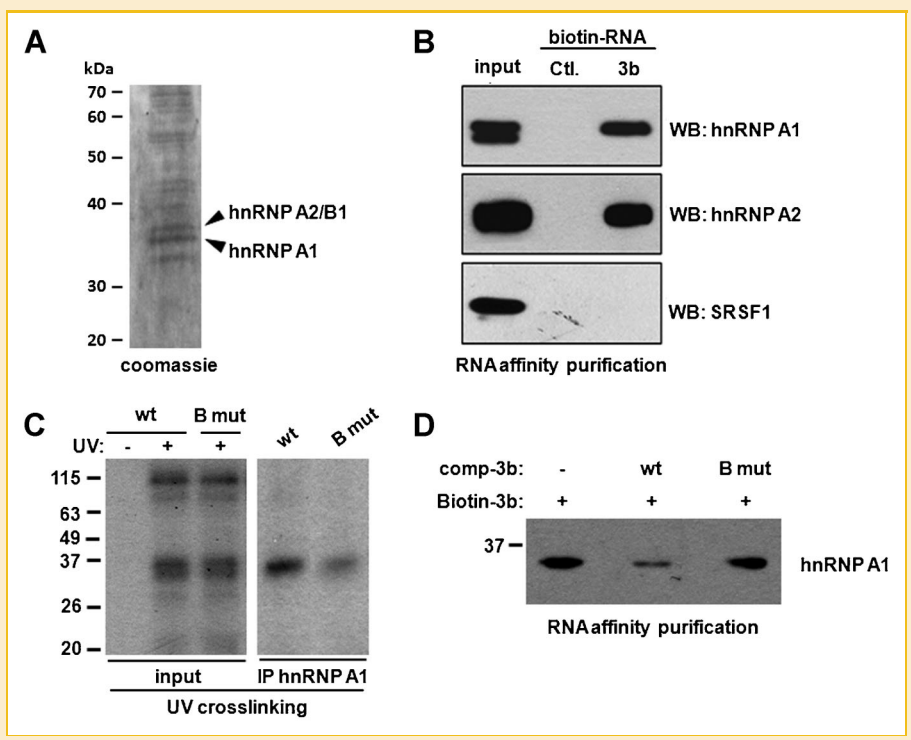

Fig. 1. hnRNP A1 and hnRNP A2 bind to Rac1 exon 3b. A: RNA–affinity purification was performed using a 5′-biotinylated exon 3b RNA oligo and nuclear extract from murine mammary epithelial cells. Proteins bound to the RNA were pulled-down with streptavidin beads and the samples were run on a 12% SDS–PAGE followed by colloidal Coomassie staining. B: RNA–affinity purification was performed as in (A) either with a biotinylated exon 3b RNA (3b) or a control oligo of the same length (Ctl). RNA-bound proteins were analyzed by Western blot with the indicated antibodies. C: Radioactive exon 3b RNA probes (either wild-type "wt" or mutated "B mut," 2–3 $\times$ 10^5 cpm each) were incubated with nuclear extracts from untreated cells (20 mg). Samples were crosslinked by irradiating with 254 nm UV light and treated with RNase. Aliquots of the crosslinking reaction (with and without UV irradiation) were kept as input. Following immunoprecipitation with an anti-hnRNP A1 antibody (4B10, Sigma), samples were loaded on a 12% SDS–PAGE. The gel was dried and exposed to film. D: RNA affinity purification was performed as in (A) but protein binding was competed by an excess of non-biotinylated wt 3b probe (comp-3b: wt) or mutated one (comp-3b: B mut) and Western blot analysis of the bound proteins was performed with an antibody against hnRNP A1.

(Supplementary Fig. 2). To confirm this finding, we performed Western blot analysis of the samples subjected to mass spectrometry analysis with different antibodies: anti-hnRNP A1, anti-hnRNP A2, and anti-SRSF1 (as a control). We found that both hnRNP A1 and hnRNP A2 were pulled down with the exon 3b RNA but not with a control oligo of the same length, and that the splicing factor SRSF1 (previously known as SF2/ASF) did not bind to the Rac1 exon 3b RNA under the same conditions (Fig. 1B). The interaction between these hnRNPs and Rac1 exon 3b was confirmed by RNA electrophoretic mobility shift assay (EMSA)/supershift (data not shown). Similar results were obtained with the EpH4 cell line.

To characterize the hnRNP A1 binding sequence within Rac1 exon 3b RNA, we first searched along the 57-nt sequence for known binding sequences. While no canonical hnRNP A1 consensus sequences were found [Burd and Dreyfuss, 1994], we did identify a 6-nt sequence (UAAAGA) that closely resembles the consensus reported by Chabot and colleagues, matching 5 out of 6 nucleotides [Blanchette and Chabot, 1999] (Supplementary Fig. 3). We found that replacing a 10-nt sequence (hereafter termed B element) containing this putative hnRNP A1 binding site by an unrelated sequence ("B mut") substantially decreased hnRNP A1 binding to exon 3b as measured by UV crosslinking followed by hnRNP A1 immunoprecipitation (Fig. 1C). To confirm this result, we performed RNA affinity purification followed by Western blot as in Figure 1B, but competed the binding reactions with either non-biotinylated wt or non-biotinylated B mut exon 3b RNA oligos. We found that a 30X molar excess of the wt exon 3b RNA greatly diminished hnRNP A1 binding whereas the same molar excess of the B mut exon 3b RNA had no effect (Fig. 1D). These results show that the sequence UAAAGA within exon 3b functions as a bona fide hnRNP A1 binding site.

hnRNP A1 IS A REPRESSOR OF Rac1 EXON 3B INCLUSION

Since hnRNPs are known to have a strong splicing inhibitory activity, we tested whether hnRNP A1 could regulate Rac1 pre-mRNA splicing. As observed in Figure 2A, over-expression of hnRNP A1 diminished exon 3b inclusion into endogenous Rac1 mRNA. In agreement with the identification of hnRNP A2 by our initial RNA affinity-purification experiments, over-expression of hnRNP A2 also decreased exon 3b inclusion levels. We then

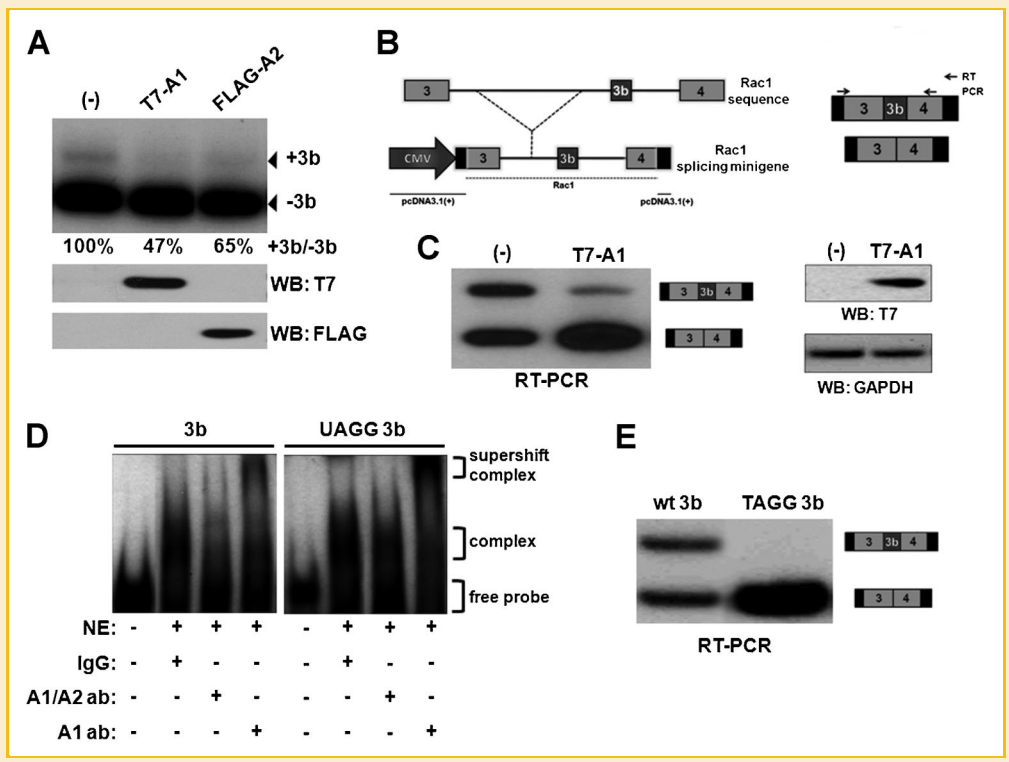

Fig. 2. Rac1 exon 3b splicing is repressed by hnRNP A1. A: SCp2 cells were transfected with expression vectors for hnRNP A1 (T7-A1) or hnRNP A2 (FLAG-A2) and endogenous Rac1 mRNA isoforms containing (3b+) or lacking (3b−) 3b exon were assessed by RT-PCR. The 3b+/3b− ratio relative to the ratio from empty vector (−) transfected cells is indicated under each lane. B: Schematic of the Rac1 splicing reporter minigene (left) and the oligonucleotides used for reverse transcription and PCR amplification of minigene-derived mRNA (right). Transcription is driven by the cytomegalovirus (CMV) promoter. C: Co-transfection of the splicing reporter minigene shown in B with an hnRNP A1-expressing vector leads to exon skipping as assessed by RT-PCR using primers specific for the minigene-derived transcripts (left panel). Over-expression of T7-hnRNPA1 from the transgene was evaluated with a Western blot with an anti-T7 antibody (Novagen) and an anti-GAPDH antibody (Sigma) was used for loading control (right panel). D: RNA–EMSA was performed using in vitro transcribed radioactive exon 3b RNA probes (either wild-type "3b" or mutated "UAGG 3b"; $\sim 1.0 \times 10^7$ cpm/mg, 5×10^4 cpm per reaction) and 0.5 mg of nuclear extract prepared from mammary epithelial cells. For supershifting, 2 mg of each indicated antibody was added to the reaction 5 min before addition of the probe. Migration of the free probe, shifted and supershifted complexes are indicated. E: SCp2 cells were transfected with wild-type or TAGG 3b minigenes and their corresponding transcripts analyzed by RT-PCR as in (C).

constructed a Rac1 splicing reporter minigene consisting of exon 3b, part of its flanking exons (3 and 4), its complete downstream intron and a shortened version of its upstream intron (Fig. 2B, left panel). We found that over-expression of hnRNP A1 decreased Rac1 exon 3b inclusion in transcripts derived from the co-transfected minigene by 10-fold as assessed by radioactive RT-PCR (Fig. 2B, right panel, C). To further define the role of hnRNP A1 in Rac1 pre-mRNA splicing, we created a core hnRNP A1 binding consensus (UAGG, [Burd and Dreyfuss, 1994; Del Gatto-Konczak et al., 1999] within exon 3b. The UAGG-containing exon 3b (termed "UAGG 3b" when referring to RNA and "TAGG 3b" when referring to DNA) binds hnRNP A1 with greater affinity than the wild-type exon as determined by EMSA/supershift (Fig. 2D, compare lanes 4 and 8) and the protein-UAGG 3b complex is more resistant to disruption by an anti-hnRNP A1/A2 antibody that recognizes an epitope within one of the RNA recognition motifs (RRMs) of hnRNP A1 and A2 (Fig. 2D, compare lanes 3 and 7). Consistent with a repressive role for hnRNP A1, creation of this hnRNP A1 binding consensus completely inhibited exon 3b inclusion into Rac1 minigene-derived mRNA (Fig. 2E). Importantly, different mutations in the same region had no effect on Rac1 alternative splicing, ruling out the possibility that the splicing effect of the TAGG motif is due to disruption of a positive regulatory sequence within the exon (Supplementary Fig. 4). These results show that hnRNP A1 functions as a repressor of Rac1 exon 3b splicing, as either its over-expression or its enhanced binding diminish exon 3b inclusion into Rac1 mRNA.

MMP-3 TREATMENT DISASSEMBLES AN hnRNP A1-CONTAINING COMPLEX FROM Rac1 EXON 3B

We then tested whether MMP-3-mediated signaling was acting through an hnRNP A1-dependent pathway to regulate Rac1 pre-mRNA splicing. We reasoned that MMP-3 treatment could be altering the amount of the hnRNPA1-containing complex assembled on Rac1 exon 3b. We assayed for protein complex binding to exon 3b and found that nuclear extracts prepared from MMP-3-treated cells displayed lower exon 3b–protein complex formation than the nuclear extracts prepared from untreated cells (Fig. 3A, wt 3b probe, B). This MMP-3 regulatory effect was lost when using the UAGG 3b as a probe (Fig. 3A, UAGG 3b probe, B). Consistent with these findings, UV crosslinking followed by immunoprecipitation with an anti-hnRNP A1 antibody showed decreased binding to exon 3b upon MMP-3 treatment (Fig. 3C), negatively correlating hnRNP A1 binding with exon 3b inclusion. We also found that inclusion of the 3b exon into minigene-derived mRNA was increased in SCp2 cells treated with MMP-3 (Fig. 3D, wt 3b minigene) as already observed for endogenous Rac1 transcripts [Radisky et al., 2005], but

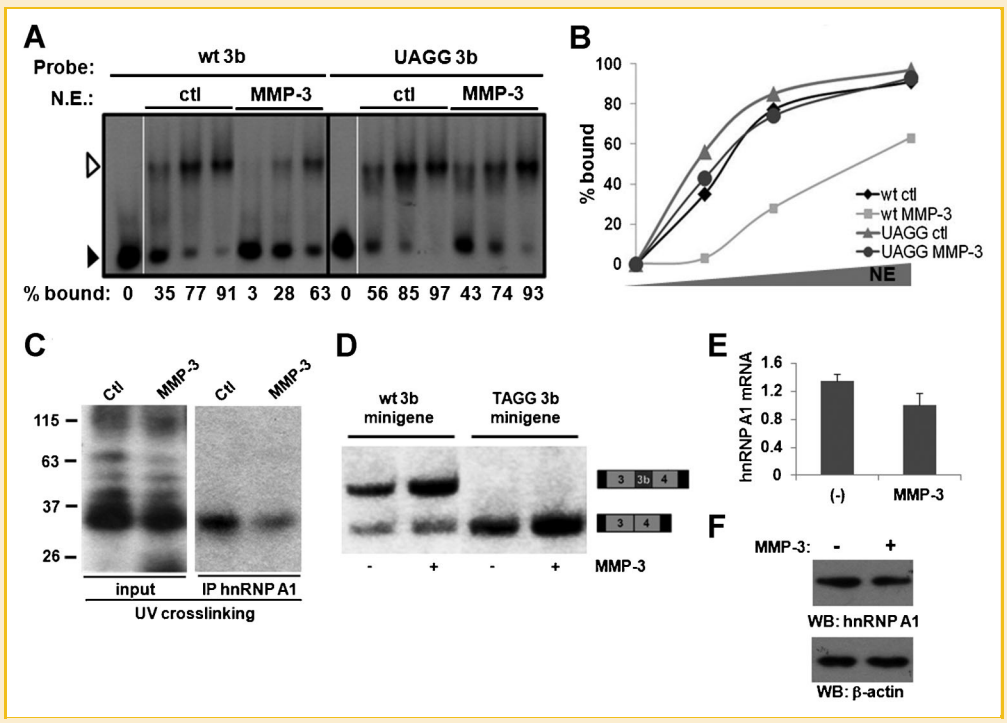

Fig. 3. MMP-3 treatment results in less hnRNP A1 bound to exon 3b. A: RNA-EMSA was performed as in Figure 2D, with increasing amounts (0.2, 0.4, and 0.8 μg) of nuclear extracts prepared from either untreated (Ctl) or MMP-3-treated SCp2 cells (MMP-3). B: Quantification of the results shown in (A), using the NIH image software. C: Radioactive exon 3b RNA probe ($2–3 \times 10^5$ cpm) was incubated with the same nuclear extracts described in (A) (20 mg). Samples were crosslinked by irradiating with 254 nm UV light and treated with RNase. Aliquots of the crosslinking reactions were kept as input control while the rest of the reactions were subjected to immunoprecipitation with an anti-hnRNP A1 antibody (4B10, Sigma). Following immunoprecipitation samples were loaded on a 12% SDS–PAGE. The gel was dried and exposed to a film. D: Cells were transfected with wild-type or TAGG 3b minigene and treated with MMP-3-containing media or left untreated. Minigene-derived transcripts were analyzed by RT-PCR. E: Levels of hnRNP A1 mRNA in untreated and MMP-3 treated cells were analyzed by quantitative real time-PCR. F: hnRNP A1 protein levels in untreated and MMP-3 treated cells were analyzed by Western blot with the indicated antibodies.

that this effect was lost with the TAGG mutant minigene (Fig. 3D, TAGG 3b minigene). Similar results were obtained with EpH4 cells (data not shown). hnRNP A1 mRNA and protein levels showed a slight decrease upon MMP-3 treatment (Fig. 3E,F). These results provide a link between MMP-3-regulated Rac1 exon 3b splicing and hnRNP A1 binding. Treatment with MMP-3 significantly reduces its binding to exon 3b and inclusion of this exon is triggered.

MMP-3-TRIGGERED Rac1 SPLICING REGULATION IS MODULATED BY hnRNP A1

Considering that our results pointed to hnRNP A1 as a repressor of exon 3b splicing, we investigated the effect of its knockdown on exon 3b inclusion levels. To this end, a specific siRNA duplex targeting the murine hnRNP A1 mRNA sequence was designed, which caused a reduction of hnRNP A1 protein levels of approximately 50% (Fig. 4A). Endogenous Rac1 pre-mRNA alternative splicing was measured by quantitative real-time PCR using specific primers for exon 3b-containing (3b+) and exon 3b-lacking (3b−) mRNA isoforms (Fig. 4B), and the results were expressed as 3b+ over 3b− ratio. As observed in Figure 4C, cells transfected with a control siRNA were still responsive to the MMP-3 treatment showing an increase in the 3b+/3b− ratio (2.5-fold effect, siRNA Ctl.). However, when cells were transfected with the hnRNP A1-targeting siRNA, MMP-3 treatment exerted a greater effect (4.5-fold, siRNA hnRNP A1). It is noteworthy that diminishing hnRNP A1 and/or hnRNP A2 protein levels in untreated cells did not relieve exon 3b from repression, suggesting that in their absence other unidentified factor(s) are responsible for maintaining splicing repression (Fig. 4C, black bars and data not shown). Importantly, over-expression of hnRNP A1 decreases exon 3b inclusion into endogenous Rac1 mRNA in a dose dependent manner as assessed by the same quantitative real time-PCR protocol (Fig. 4D). These results suggest that Rac1 exon 3b inclusion is repressed under normal conditions by several factors, including hnRNP A1. While a 50% reduction in hnRNP A1 protein levels does not affect the repressed state of exon 3b splicing, it apparently sensitizes the cells to MMP-3 treatment.

hnRNP A1 AND Rac1B SHOW INVERSE REGULATION IN VIVO

Rac1b was previously identified as overexpressed in breast cancer tissue [Schnelzer et al., 2000]. Since we had identified an inhibitory role for hnRNP A1 as a regulator of Rac1b, we assessed whether progression from non-malignant breast to breast cancer was associated with decreased expression of hnRNP A1. Immunohistochemical analysis of non-malignant breast (obtained from prophylactic mastectomy tissue and determined by pathologic analysis to be cancer-free) and of breast cancer tissue revealed that normal breast tissue showed high nuclear levels of hnRNP A1 and diffuse/punctate cytoplasmic expression of Rac1b in the lobular epithelial cells, while breast cancer tissue showed decreased levels of nuclear hnRNP A1 and increased expression of Rac1b (Fig. 5). These results are consistent with a repressive role for hnRNP A1 on Rac1b expression in vivo.

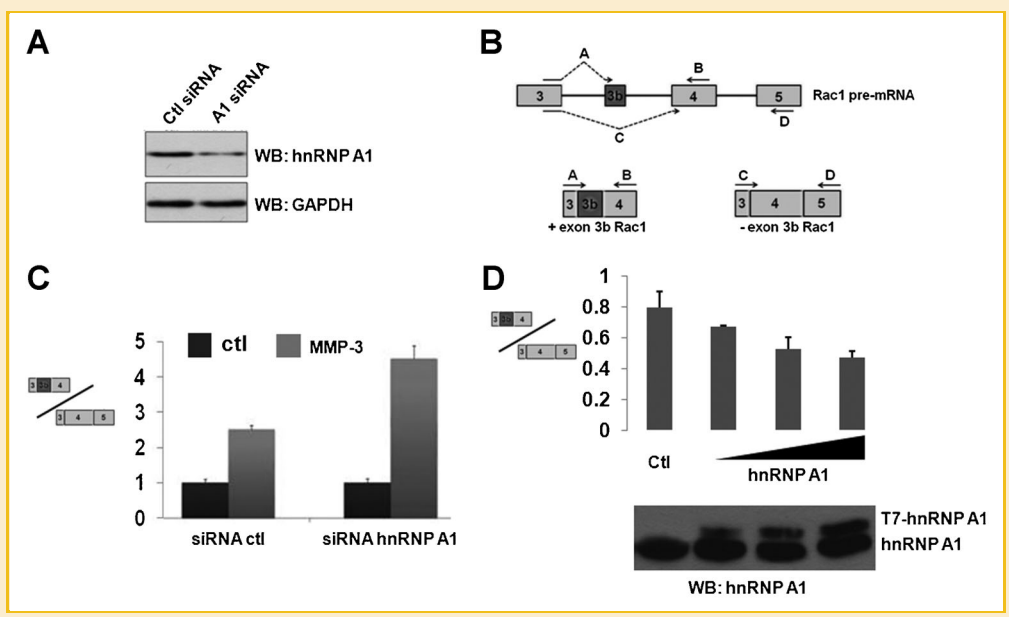

Fig. 4. MMP-3 acts through an hnRNP A1-dependent pathway to control Rac1 pre-mRNA splicing. A: Levels of hnRNP A1 protein after transfecting SCp2 cells with a control siRNA or with a siRNA targeted to hnRNP A1 were assessed by Western blot with the indicated antibodies. B: Schematic of the primers designed to amplify Rac1 mRNAs either lacking (−3b) or containing (+3b) exon 3b by quantitative real time PCR after reverse transcription. C: Endogenous Rac1 transcripts were analyzed in RNA samples prepared from cells transfected with the indicated siRNA, either untreated (Ctl) or treated with MMP-3. Quantitative RT-PCR was performed with the primers shown in (B). Results are shown as the ratio between 3b-containing and 3b-lacking mRNA (3b+/3b−). Mean + standard deviation of a representative experiment run in triplicate is shown. D: SCp2 cells were transfected with empty vector (Ctl) or increasing amounts of hnRNP A1 expression vector (T7-hnRNP A1). Endogenous Rac1 transcripts from transfected cells were analyzed by quantitative RT-PCR as indicated in (B) and (C) (upper panel). Over-expressed and endogenous hnRNP A1 proteins were assessed by Western blot (lower panel).

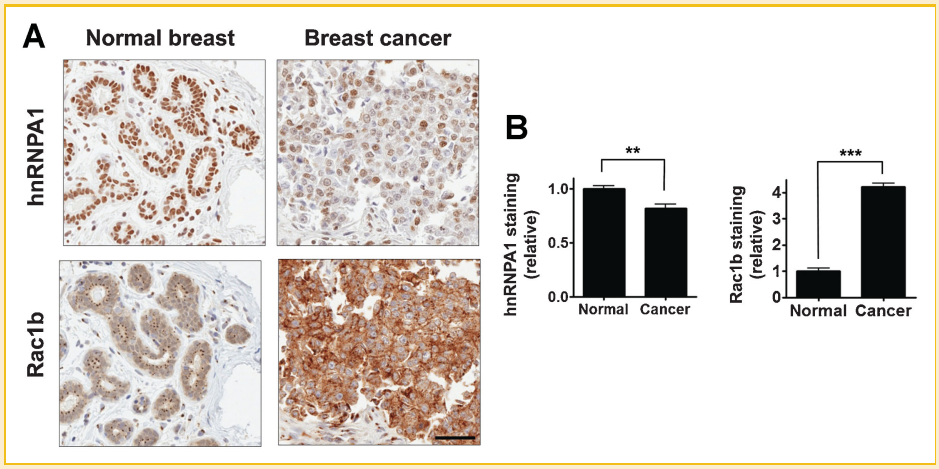

Fig. 5. Decreased hnRNP A1 in breast cancer is associated with increased Rac1b expression levels. A: IHC images of sectioned biopsies of normal (non-malignant) breast (left) or breast cancer (right), stained for hnRNP A1 (top) or Rac 1b (bottom). Scale bar, 50 mm. B: Quantification of IHC staining for hnRNP A1 and Rac1b in normal tissue and in breast cancer. $^{**}P < 0.005$; $^{***}P < 0.001$.

DISCUSSION

We show here that hnRNP A1 is involved in Rac1 alternative splicing regulation in mouse mammary epithelial cells. Moreover, our experiments provide insight into the mechanism by which MMP-3 stimulates exon 3b inclusion into Rac1 mRNA. We find that hnRNP A1 is bound to the alternative exon 3b under normal conditions and is able to repress exon 3b splicing. Upon treatment with MMP-3, hnRNP A1 is displaced from exon 3b, relieving it from the splicing-repressive effect and leading to an enhanced inclusion of the exon. As suggested by RNAi experiments, it is expected that other proteins playing a repressive function cooperate with hnRNP A1 to maintain low inclusion levels of 3b exon under normal conditions. In addition, it is possible to speculate that not only the displacement of hnRNP A1 but also the binding of a positive regulator is required for MMP-3-triggered increase in 3b exon inclusion (Fig. 6). This remains to be elucidated.

When exploring the link between cancer and splicing, two kinds of cancer-associated alterations in splicing patterns can be distinguished. One group is composed by alterations that are attributable to mutations that create or disrupt splice sites or splicing enhancers and silencers. However, in only few cases has a causal relationship been determined [Venables, 2004; Srebrow and Kornblihtt, 2006]. In the second group, no mutations have been observed in *cis*-acting splicing elements within the involved genes, suggesting that the alterations could be due to changes in the activity of *trans*-acting splicing regulators [Ghigna et al., 1998; Stickeler et al., 1999; Venables, 2006]. Rac1 belongs to this second group, together with Ron, CD44, prolactin receptor, fibroblast growth factor receptor, and MDM2 [Srebrow and Kornblihtt, 2006;

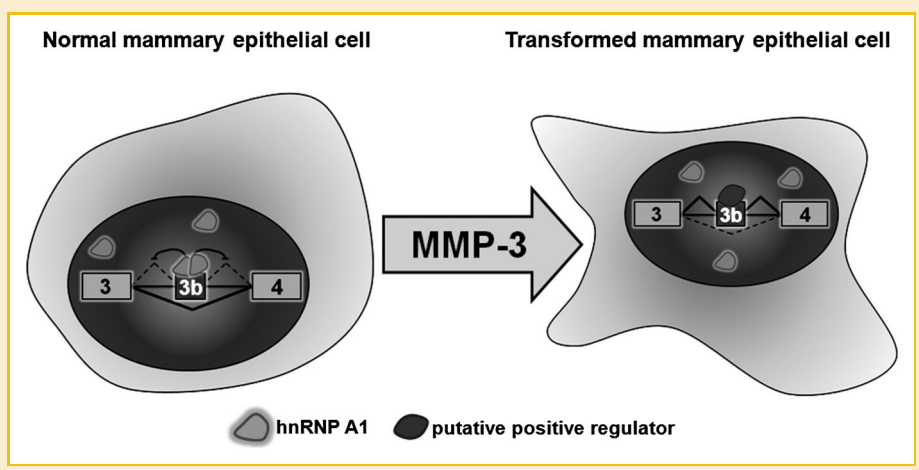

Fig. 6. Schematic model summarizing the presented results.

Ghigna et al., 2008]. In this respect, a strong connection between expression levels of a splicing factor, alternative splicing and changes in cellular behavior related to tumorigenesis has been described for Ron pre-mRNA. Over-expression of SRSF1 has been postulated to regulate malignant transformation by inducing production of ΔRon, a constitutively active isoform of the Ron receptor tyrosine kinase, in turn leading to the loss of epithelial phenotype and acquisition of migratory capacity [Ghigna et al., 2005]. SRSF1 is up-regulated in many human tumors, partially due to an amplification of its gene *SFRS1*. By ectopic expression of SRSF1 in cultured cell lines, this factor has been defined as an oncoprotein that functions in establishment and maintenance of cellular transformation by altering the splicing pattern of several cancer-related genes [Karni et al., 2007]. Recently, SRSF1 and SRSF3 (previously known as SRp20) levels have been shown to regulate Rac1 splicing in human colorectal tumor cells in a PI3K- and catenin/TCF4-dependent manner. It is noteworthy that although the authors of this latter study did not assess hnRNP A1 involvement in Rac1 splicing in these cells, a mutation that disrupts the hnRNP A1 consensus binding site we report in the present work was found to greatly increase exon 3b inclusion [Goncalves et al., 2009].

Culture and animal models in which de-regulation of MMPs expression is associated with tumorigenesis have been well described [Sympson et al., 1994; Lochter et al., 1997; Sternlicht et al., 1999]. In particular, expression of an MMP-3 transgene targeted to mouse mammary epithelial cells was shown to induce spontaneous neoplastic progression in the mammary gland [Sternlicht et al., 1999]; and aberrant MMP expression has been linked to activation of EMT in human breast cancer progression as well [Radisky and Radisky, 2010]. Here, we show an inverse pattern of expression for hnRNP A1 and Rac1b in non-malignant breast tissue and in breast cancer; further studies will be necessary to define a potential role for MMPs in this process.

During the last few years, the involvement of alternative splicing regulation in EMT has been the focus of gene-specific as well as high throughput studies. Not only the expression of epithelial specific splicing factors but also alternative splicing switches have been proposed as crucial regulatory points for this cellular process and for early steps of metastatic progression [Ghigna et al., 2005; Warzecha et al., 2009; Valacca et al., 2010; Brown et al., 2011]. Recently, an EMT-associated splicing signature controlled by different splicing factors including members of the hnRNP family has been proposed [Shapiro et al., 2011].

A vast body of work links hnRNP A1 activity with different aspects of tumorigenesis. This protein functions as an auxiliary factor for the processing of mir18-a, a microRNA that belongs to a miRNA cluster with oncogenic potential [Guil and Caceres, 2007; Michlewski et al., 2011]. hnRNP A1 is highly expressed in proliferating and transformed cells [Biamonti et al., 1993] as well as in breast, colon, and lung cancers, among others [Zerbe et al., 2004]. Silencing of hnRNP A1 and A2 promotes apoptosis in a variety of human and mouse cancer cell lines, while has no effect on normal epithelial and fibroblastic cell lines [Patry et al., 2003], and hnRNP A2 controls invasive cell migration of ovarian carcinoma cells through alternative splicing of TP53INP2 [Moran-Jones et al., 2009]. Our work provides another physio-pathological context in which hnRNP A1 activity affects the malignant phenotype; identification of the pathways that regulate hnRNP A1 function may provide new insight toward early indicators of cancer development as well as novel approaches to anticancer therapies.

ACKNOWLEDGMENTS

We thank Javier Cáceres (Edinburgh, UK), Emanuelle Buratti (Trieste, Italy), Benoit Chabot (Sherbrook, Canada), and Adrian Krainer (Cold Spring Harbor, US) for kindly providing hnRNP A1 expression vector, hnRNP A2 expression vector, antibody against hnRNP A1/A2, and antibody against SRSF1, respectively. We thank the Kornblihtt laboratory for helpful discussion. This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica of Argentina; Universidad de Buenos Aires, Argentina; Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina and European Alternative Splicing Network (EURASNET) (to AS); a UICC International Cancer Technology Transfer Fellowship; Federal funds from the National Cancer Institute, National Institutes of Health under Contract NO2-CO-41101 (to FP); funds from the National Cancer Institute (CA122086 and CA132879) (to DCR); and by the Mayo Clinic Breast Cancer Specialized Program of Research Excellence (SPORE) grant CA116201 (PI James Ingle MD).

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