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Original Article

HnRNP A1 tethers KSRP to an exon splicing silencer that inhibits an erythroid-specific splicing event in PU.1-induced erythroleukemia

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Abstract: Exon 16 inclusion is a critical splicing event that triggers the production of a functional protein 4.1R in mature normal erythroblasts, and is obviated in PU.1-induced erythroleukemia cells. Exon 16 contains an exonic splicing silencer (ESS16) that interacts with hnRNP A/B in heterologous cell context. We here show that ESS16 promotes the recruitment of a protein complex containing hnRNP A1 and a 79-kDa protein in nuclear extracts from either proliferative erythroleukemia cells or cells induced to terminal differentiation. By using 2D gel fractionation and mass spectrometry, we unambiguously identified KSRP as the 79-kDa component interacting with ESS16. Furthermore, we show that KSRP slightly decreases in erythroleukemia cells induced to terminal erythroid differentiation. Yet, KSRP inducible knockdown, through stable transfection of small hairpin KSRP RNA, did not alter exon 16 splicing, suggesting that KSRP alone does not modulate the splicing event. Interestingly, absence of hnRNP A1 prevented KSRP from binding to ESS16. Reciprocally, KSRP interaction with ESS16 was recovered when hnRNP A1 expression is restored in hnRNP A1-null cells. Collectively, this study establishes that hnRNPA1 is part of a KSRP-containing RNP complex, and emphasizes that, aside from its function in AU-rich element-mediated mRNA decay and its role in microRNA biogenesis, KSRP associates with hnRNP A1 to bind an ESS. These findings further support the role of members of the KH-domain protein family in organizing large RNA-protein complex formation, rather than primarily in modulating specific splicing events.

Keywords: Alternative splicing, cell differentiation, erythroleukemia, hnRNP, KSRP, PU.1

Introduction

Alternative splicing of messenger RNA precursors (pre-mRNA) is a powerful source of protein diversity. It allows a highly regulated inclusion/removal of exonic or intronic sequences from mature mRNA in a tissue-, sex-, cell context- or developmental stage-specific manners [1-3]. Alternative splicing in healthy cells and aberrant splicing in pathologic conditions can generate protein isoforms that might have no function, distinct functions, or even antagonistic functions [1, 4, 5]. Intron removal and exon splicing by the spliceosome involve a large RNA-protein complex that interacts with the 5' and 3' splice sites, the branch point, and non canonical splicing regulatory sequences that are required to ensure the accuracy of pre-mRNA splicing and exon or intron definition. These sequences are designated, based on their location and their function, as exonic splicing enhancers (ESEs) and silencers (ESSs), and intronic splicing enhancers (ISEs) and silencers (ISSs) [2, 6]. These sequences generally interact with non-spliceosomal factors; ESEs typically bind members of the serine-arginine-rich (SR) protein family [7, 8], whereas ESSs typically recruit the hnRNP factors [9, 10].

During late erythroid development, inclusion of exon 16 in the mature protein 4.1R mRNA is a critical event that is required to endow the protein with a functional 10 kDa internal domain, needed to stabilize the spectrin-actin complex of the membrane skeleton ([11, 12], and references therein). Exon 16 is skipped in early progenitors and massively retained in mature erythroblasts.

Early studies have documented an upregulated expression of PU.1 in mouse erythroleukemia (MEL) cells. These PU.1-induced erythroleukemia cells are blocked in their differentiation ([12], and references therein). PU.1 is an oncoprotein encoded by *spi-1*, a member of the ETS family of transcription factors. Inhibition of exon 16 splicing switch has been correlated with PU.1 upregulation [13]. These PU.1-induced erythroleukemia cells undergo erythroid differentiation, and recover exon 16 splicing switch following cell treatment with either dimethylsulfoxide (DMSO), or through PU.1 forced gene silencing [13, 14]. More recently, we have shown that phosphatidylinositol 3-kinase (PI3K)/AKT-mediated phosphorylation of PU.1 sustains a high level of expression of PU.1 through an autoregulatory loop. The specific inhibition of PI3K/AKT, which is constitutively active in PU.1-induced erythroleukemia cells, triggers PU.1 downregulation in a stepwise manner and induces cell differentiation and the erythroid splicing switch of 4.1R exon 16, in a promoter-dependent manner, and in the absence of DMSO [15]. These data led us to argue that DMSO might act as a chemical inhibitor of the PI3K/AKT signaling pathway [16].

The PU.1-induced erythroleukemia cell model reproduces the endogenous exon 16 splicing patterns from a transfected minigene. It allowed characterizing the *cis* elements involved in the regulation of exon 16 splicing during late erythroid differentiation. Hence, it has been demonstrated that exon 16 is an intrinsically poor splicing substrate because of its weak 5' splice site, and that a complex interplay of splicing enhancers and silencers, present in the exon and the surrounding intronic sequences, controls the developmental stage-specific splicing of exon 16 [17]. Subsequent studies have shown that several splicing factors bind these elements, and modulate exon recognition in erythroid cell context [18-20]. Among these sequence elements, an ESS acts in a constitutive manner. Disruption of this element by targeting specific sequences activated exon inclusion both in proliferative and DMSO-induced cells to late erythroid differentiation [17]. This ESS, called ESS16, can function in a heterologous sequence context and in different cell types [17, 21]. Further investigations have documented that hnRNP A/B bind ESS16 in HeLa nuclear extracts, and that addition of recombinant hnRNP A1 to depleted HeLa nuclear extract restores the silencing effect of ESS16 [21].

To further investigate the regulation of exon 16 erythroid splicing, we here identified hnRNP A1 and the KH-type splicing regulatory protein (KSRP) as components of a ribonucleoprotein (RNP) complex that docks to the exon silencer. Yet, knockdown of KSRP did not alter exon 16 splicing, nor it affected hnRNP A1 expression during induced erythroid terminal differentiation. We further demonstrated that KSRP interaction with ESS16 is conditioned by the presence of hnRNP A1. KSRP is a single-strand RNA binding protein, involved at different levels in RNA metabolism, but mainly known for its ability to promote labile mRNA decay, as well as miRNA precursor maturation. The present study shed lights on a new feature of KSRP, dealing with its recruitment in a multicomponent regulatory splicing complex, that represses exon inclusion.

Materials and methods

Plasmid constructs

A cDNA fragment containing hnRNP A1 coding sequence was obtained by RT-PCR using the following primers: forward primer: 5'-TCGAATTCTCATCATCCTACCGTCAT-3'; reverse primer: 5'-TTGAATTCCTGGCTGTATGTAATTA-3' (underlined sequences were added to include an *Eco* RI restriction site). The PCR product was then digested with *Eco* RI and cloned into *Eco* RI-digested pcDNA3 vector (Invitrogen SARL, Cergy Pontoise). A recombinant plasmid with hnRNP A1 insert in the sense orientation was subsequently used for cell transfection.

ShRNA-mediated KSRP knockdown was achieved using a doxycycline-induced system as previously detailed [14]. Two KSRP silencing targets were tested: 5'...TGAGGTGGTGAGCAGATAA...TTATCTGCTCACCACCTCA...3' and 5'...GTGTGCGCATCCAGTTCAA...TTGAACTGGATGCGCACAC...3'. A scrambled, non-specific shRNA was used as a control [14].

Cell culture, induction and transfection

The PU.1-induced erythroleukemia cell clone 745A was cultured in suspension and induced to terminal differentiation, using either DMSO or hexamethylene bisacetamide (HMBA), as

previously described [14]. CB3 cell line was cultured in the same conditions, but without DMSO or HMBA treatment. This MEL cell line was generated from mice infected by the replication-competent Friend murine leukemia virus (F-MuLV). The virus was integrated within fli-2 locus, and resulted in inactivation of *NF-E2* and *hnRNP A1* genes [22, 23].

Cells were plated onto 6-well plates and transfected with plasmids containing either shRNA targets or cDNAs. Transfection and clone selection procedures were as previously described [14]. For PI3K signaling inhibition, the cells were cultured in the appropriate medium supplemented with 10 or 20 μM of LY294002 inhibitor, as previously described [16].

Total and nuclear protein extracts

Total protein lysate was obtained from cultured cells and treated as previously described [13, 14]. Nuclear extracts were prepared from different cell types according to modified procedures of Dignam's protocol [24, 25]. All steps were performed on ice or in cold room at 4°C, and lysis efficiency was followed up by microscopy. Freshly cultured cells (about 4×10^7) were harvested and pelleted by low speed centrifugation (1500 rpm) at 10°C for 5 min. Cell pellets were washed in 30 volumes of 1X PBS, then resuspended and incubated for 15 min at 4°C, in 1 volume of Buffer A (10 mM HEPES pH 7.9 at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT). Cells were then lysed with 10 strokes in a dounce. Nuclei were spun at 2000 rpm speed for 10 minutes. The supernatants were removed and nuclei were spun one more time at 15000 rpm for few seconds to remove at best the supernatant. Pelleted nuclei were gently resuspended in half the packed nuclei volume Buffer C (20 mM HEPES (pH7.9), 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, 0.5 mM PMSF). Half the packed nuclei volume of nuclear extract Buffer D (same as Buffer C except 1.2 M KCl) was added dropwise, while the nuclei suspension was stirred gently at 4°C. This step results in a final concentration of 0.3 M KCl. The nuclei were then lysed with 10 strokes in a dounce and stirred for 30 min at 4°C. The extracts were spun for 30 min at 15000 rpm and the supernatant was then dialyzed through a 3500 Da cut-off dialysis membrane (ElutaTube Dyalysis kit; Fermentas, St-Rémy les Chevreuse), overnight at 4°C against 50 volumes of Buffer E (20 mM HEPES (pH7.9), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT). The nuclear extracts were further dialyzed for 5 h under the same conditions.

RNA grade HeLa nuclear extracts were obtained from Jena Bioscience (ref. PR-778; Jena, Germany). These extracts are prepared specifically for the purpose of pre-mRNA in vitro splicing and RNA-protein interaction analysis.

Western blot analysis

Immunoblotting experiments were performed as described previously [13, 14]. Fifteen μg of total protein extracts or 30 μg of nuclear extracts were separated by 9% or 12% SDS-PAGE, transferred to nitrocellulose membranes, and probed with specific antibodies: a mouse anti-KSRP monoclonal antibody Ab5 (1:10 diluted serum) [26]; a goat anti-hnRNP A1 antibody Y-15 (1:400) (Santa Cruz Biotechnology, Heidelberg, Germany); a mouse monoclonal anti-hnRNP A1 antibody (4B10; 1:400) (Santa Cruz Biotechnology, the first batch used was generously provided by Dr. Gideon Dreyfuss, University of Pennsylvania, Philadelphia, PA); and a mouse anti-actin monoclonal antibody (1:10000) (Chemicon International, Tmecula, CA, USA). Horseradish peroxidase-conjugated goat anti-mouse IgG (1:10000) (Jackson ImmunoResearch, Suffolk, UK), and horseradish peroxidase-conjugated to protein G (1:5000) (Pierce; Perbio Science France, Brebières, France) were used as secondary antibodies. The immunoreactive proteins were revealed on autoradiography film using ECL+ enhanced chemiluminescence detection kit (Amersham Biosciences AB, Uppsala, Sweden), according to the manufacturer's instructions.

ESS16 in vitro transcription

DNA templates for in vitro transcription were obtained by PCR amplification of ESS16 sequence from recombinant plasmids containing exon 16 minigene [17]. Primers were designed as to contain ESS16 sequence. Forward primer further contained SP6 promoter in its 5' end. Thus, the primer sequences were as follows: forward primer SP6-E16WT-S (ATTTAGGTGACACTATAGAAAGACTAGATGGTGA) and reverse primer HIAS (ACATTAAATTGCTATGTCTG). The PCR product (~50 bp) was purified

through Micro Bio-Spin 30 Columns (cut-off 30 mers; Bio-Rad, Marnes-la-Coquette, France). Uniformly radiolabeled RNA were produced by SP6 RNA polymerase in vitro transcription kit (Riboprobe in vitro Transcription system, Promega, Charbonnières-les-Bains, France) using 500 ng of DNA template, in a 15 µL mixture containing 20 mM rATP, 20 mM rCTP, 20 mM rGTP, 1 mM rUTP and 40 µCi of [α-^{32}P] UTP (800 Ci/mmole), according to the manufacturer's instructions. Unlabeled RNA molecules used as competitors were in vitro transcribed on a large scale using the MEGAscript® SP6 Kit (Ambion, Austin, Texas, USA) and an equimolar amount of each rNTP. The reactions were performed for 3 h at 37°C, followed by degradation of the DNA templates with RNase-free DNase 1. The synthesized RNA was further purified first by phenol-chloroform extraction, then through Micro Bio-Spin 30 exclusion, and finally by ethanol precipitation.

Electrophoretic mobility shift assays (EMSA)

Thirty µg of nuclear extracts were mixed with 48 mM potassium glutamate, 2.2 mM $MgCl_2$, 0.4 mM ATP, 50 mM creatine phosphate, 1 mM DTT, and 10 µg of yeast tRNA (used as non-specific competitor). The mix was preincubated at 30°C for 5 min. Then 100000 cpm of ^{32}P-labeled RNA template was added, and incubation was continued for 10 min. When needed, 1.6 µM of unlabeled RNA template (used as specific competitor) was added together with the labeled RNA template. Five µl of each sample was loaded on non-denaturing 4.5% polyacrylamide gel (29:1), containing 2.5% glycerol. RNA-protein complexes were separated by electrophoresis in Tris-glycine buffer (50 mM Tris base, 380 mM glycine, 2.1 mM EDTA) at 18°C for 2 h and 175 V, and visualized on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

UV-crosslinking and RNase protection

RNA-protein mixtures prepared as described above were analyzed by RNase protection of UV-crosslinked complexes. Twenty µl of the mixture was supplemented with 10 µg of heparin, and irradiated with a 3 mm distant UV lamp at 254 nm wavelength, for 10 min. RNAs were then digested with 2 µg of RNase A for 30 min at 30°C. RNase protected complexes were resolved by 9% SDS-PAGE, and visualized by phosphorimaging.

Two-dimensional (2-D) gel electrophoresis and mass spectrometry analysis

RNA-protein complex formation was performed as described above on an analytical scale using 100000 cpm of ^{32}P-labeled RNA, and on a preparative scale using 150 fmoles of unlabeled RNA. The reactions were UV-crosslinked and RNase A-treated as indicated above, then precipitated with 10% trichloroacetic acid (TCA). The protein pellets were resuspended in the iso-electric focusing (IEF) buffer as previously described [27]. The IEF was carried out on dehydrated IEF strips in the 3-10 pI range (GE Healthcare Europe, Orsay, France), for 40.000 Vh at 20°C. Reduction/alkylation of the proteins was achieved with iodoacetamide prior to the second electrophoretic separation on a 10% SDS-PAGE.

The 2-D electrophoresis was performed in parallel on an analytical scale using ^{32}P-labeled RNAs and a preparative scale using unlabeled RNAs. The labeled spots were revealed by autoradiography, and the gel was further stained with Biosafe Coomassie blue to reveal all the fractionated proteins. The preparative gel was stained with SYPRO Ruby. This protocol allowed by superimposition of the autoradiogram and the preparative gel to assign the protein of interest. The spotted areas were cut out of the gel and submitted to mass spectrometry analysis (Dr. J. Garin, Laboratoire de Chimie des Proteines, CEA, Grenoble, France).

Semi-quantitative RT-PCR

Total RNA was isolated from cell lysates using the TriReagent (Molecular Research Center, Cincinnati, OH, USA), according to the supplier's protocol. The semi-quantitative RT-PCR experiments were performed to analyze exon 16 splicing, as previously detailed [17, 28]. Quantification measurements were obtained using ImageQuantMac v1.2 software.

Inducible RNA interference

To knockdown KSRP expression in MEL cells, we used a doxycycline-inducible RNA interference system based on RNA polymerase III H1 promoter activation of KSRP shRNA expres-

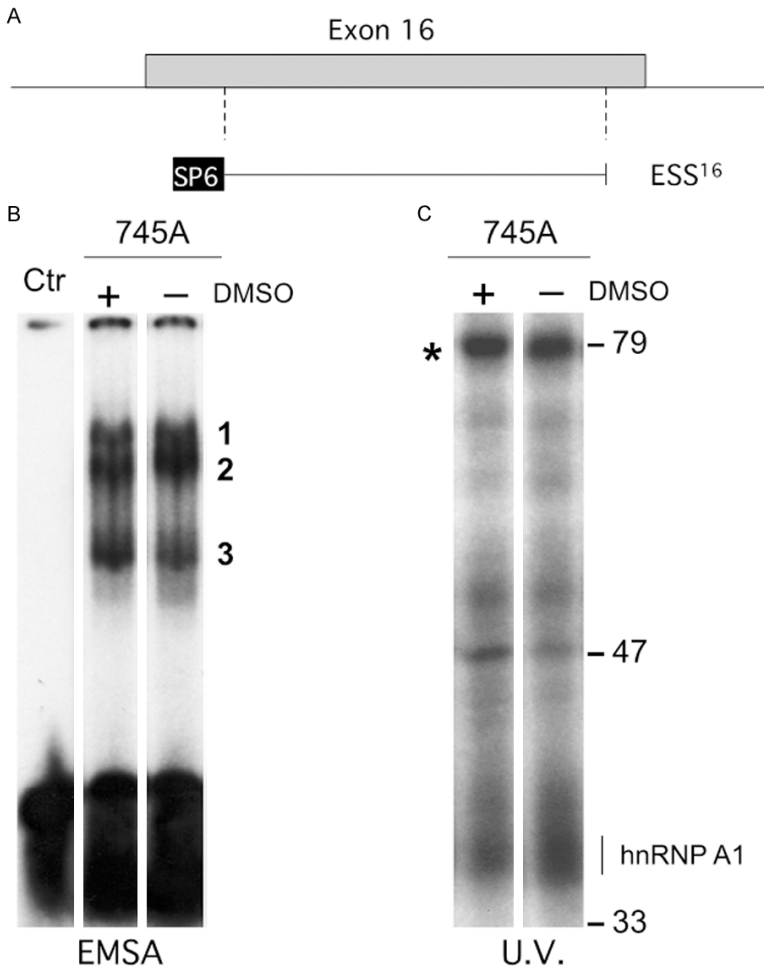

Figure 1. ESS16 binds to a 79 kDa nuclear factor in proliferating and differentiating erythroleukemia cell nuclear extracts. A. ESS16 ^{32}P-labeled RNA probe was in vitro transcribed under the control of SP6 promoter. B. EMSA using labeled ESS16 probe and nuclear extracts from proliferating (-) cells, or cells induced to erythroid differentiation by DMSO (+). 1, 2 and 3 indicate the 3 major complexes revealed. A control sample (Ctr), where the RNA was incubated in the absence of protein extract, shows only free labeled molecules. C. UV crosslinking experiments using ^{32}P-labeled ESS16 RNA and nuclear extracts from proliferating (-) and DMSO-treated (+) 745A cells. The star points to the major crosslinked protein. Size markers are depicted on the right.

sion. The procedure has previously been detailed [14]. The shRNA target sequences and cloning are indicated above.

Results

ESS16 recruits a 79 kDa nuclear factor

Exon 16 was found to contain a splicing silencer [17, 21]. Previous data based on affinity selection assays using HeLa nuclear extracts have suggested that ESS16 recruits hnRNP A/B proteins, among other factors [21]. In an attempt to further characterize the nuclear factors that bind this sequence, we performed in vitro gel shift assays using labeled RNA sequence encompassing the entire ESS sequence (**Figure 1**). Nuclear extracts were obtained from proliferating cells, and from cells induced to terminal erythroid differentiation using either DMSO (**Figure 1B**) or HMBA (Supplementary Figure 1). EMSA consistently revealed three RNP complexes. Their binding specificity was ascertained by competing-out the labeled substrate by adding excess unlabeled ESS16-template (Supplementary Figure 1). These experiments showed similar EMSA patterns obtained with nuclear extracts, either from proliferative cells, or from chemically induced cells, suggesting that, at least under in vitro binding conditions, ESS16 is able to recruit the same splicing factors.

To further characterize the specific RNA-binding proteins that recognize ESS16, UV crosslinking experiments were then performed using the same ^{32}P-labeled RNA template. These experiments revealed, in addition to hnRNP proteins [21], a major nuclear factor of ~79 kDa in size (**Figure 1C**). Again, use of nuclear extracts from DMSO-treated cells revealed a similar UV crosslinking pattern, further supporting the capacity of ESS16 to recruit the same splicing factor in vitro. UV crosslinking experiments were then performed in the presence of various erythroid and non-erythroid nuclear extracts (not shown), and let to an identical pattern, suggesting that the silencer would recruit the 79 kDa component in a cell-specific independent manner. These data support the capacity of ESS16 to recruit the 79 kDa component in vitro, in cell- and differentiation stage-specific independent manners.

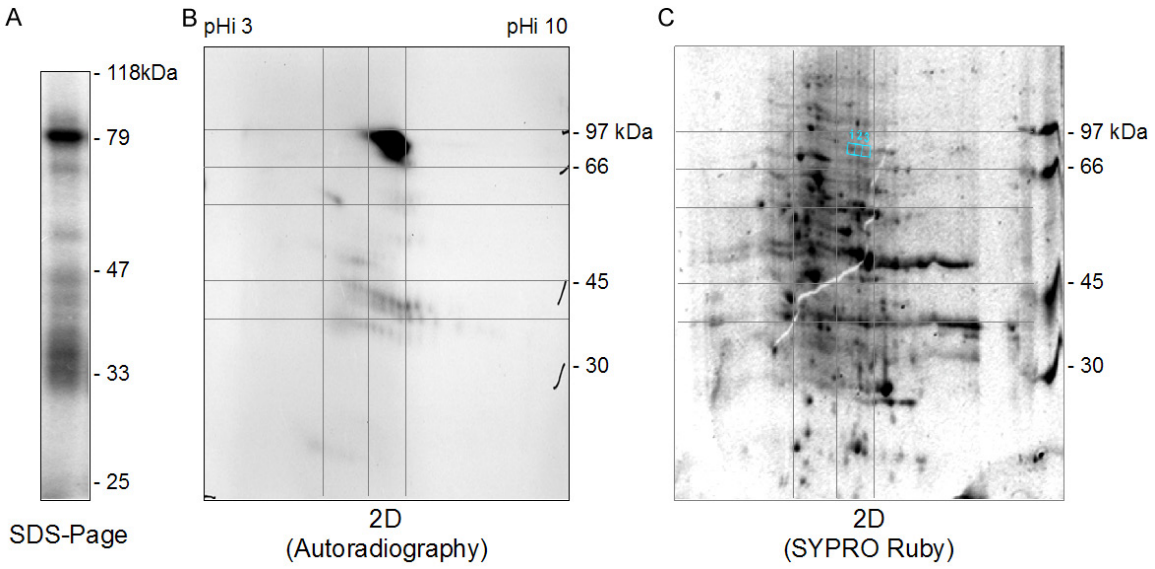

Figure 2. 2-D electrophoretic analysis of ESS16-associated proteins. UV crosslinking assay was performed at an analytical (A, B) and preparative (C) scales, using ^{32}P-labeled (A, B) or unlabeled (C) in vitro transcribed ESS16 RNA probes. A: SDS-PAGE showing the crosslinked proteins including the major 79 kDa component. (B, C) 2-D gel electrophoresis. Proteins were separated by IEF on immobilized pH gradients 3-10 in the first dimension. They were subsequently separated by SDS-PAGE in the second dimension. All three spots fractionated by SDS-PAGE at the 79 kDa position were analyzed by mass spectrometry.

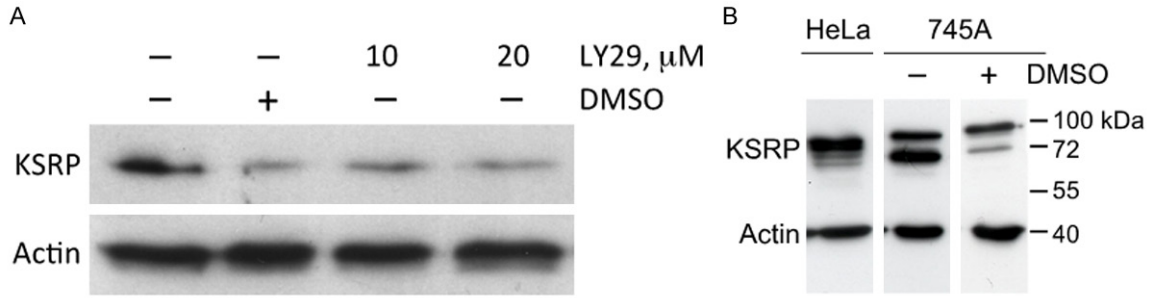

Figure 3. KSRP expression during erythroid differentiation. A. Induction of erythroid differentiation results in decreased total KSRP expression. KSRP expression was analyzed by immunoblotting in untreated (-) cells or cells treated with DMSO (+), 10 μM (10) or 20 μM (20) of LY294002 (LY29) inhibitor. Actin served as a protein quality and sample loading standard. B. KSRP nuclear expression decreases in DMSO-treated cells. Fifteen μg of nuclear protein extracts were loaded on SDS-PAGE and immunoblotted using anti-KSRP antibody. Actin served as a protein quality standard. Nuclear extracts were prepared from HeLa cells, and MEL cells (clone 745 A) before and after DMSO exposure.

Identification of KSRP as ESS16-interacting protein

We sought to identify the 79 kDa protein. UV crosslinked proteins were precipitated at a preparative scale using unlabeled RNA template, and run on 2-D gels in parallel with an analytical ^{32}P-labeled sample (**Figure 2**). This analytical 2-D gel served to visualize the position of the most abundant 79kDa component that interacts with ESS16. Two-D gel analysis of UV-crosslinked factors to ESS16 revealed a string of spots, evocative of a phosphorylated protein (**Figure 2C**). Three areas of interest were cut out on the preparative gel and analyzed by mass spectrometry. From 12 peptide masses determined from the selected spot, we were able to readily predict the protein identity as KH-type Splicing Regulatory Protein (KSRP), a ubiquitous single strand nucleic acid binding protein present in both nuclear and cytoplasmic compartments of the cells [29]. These

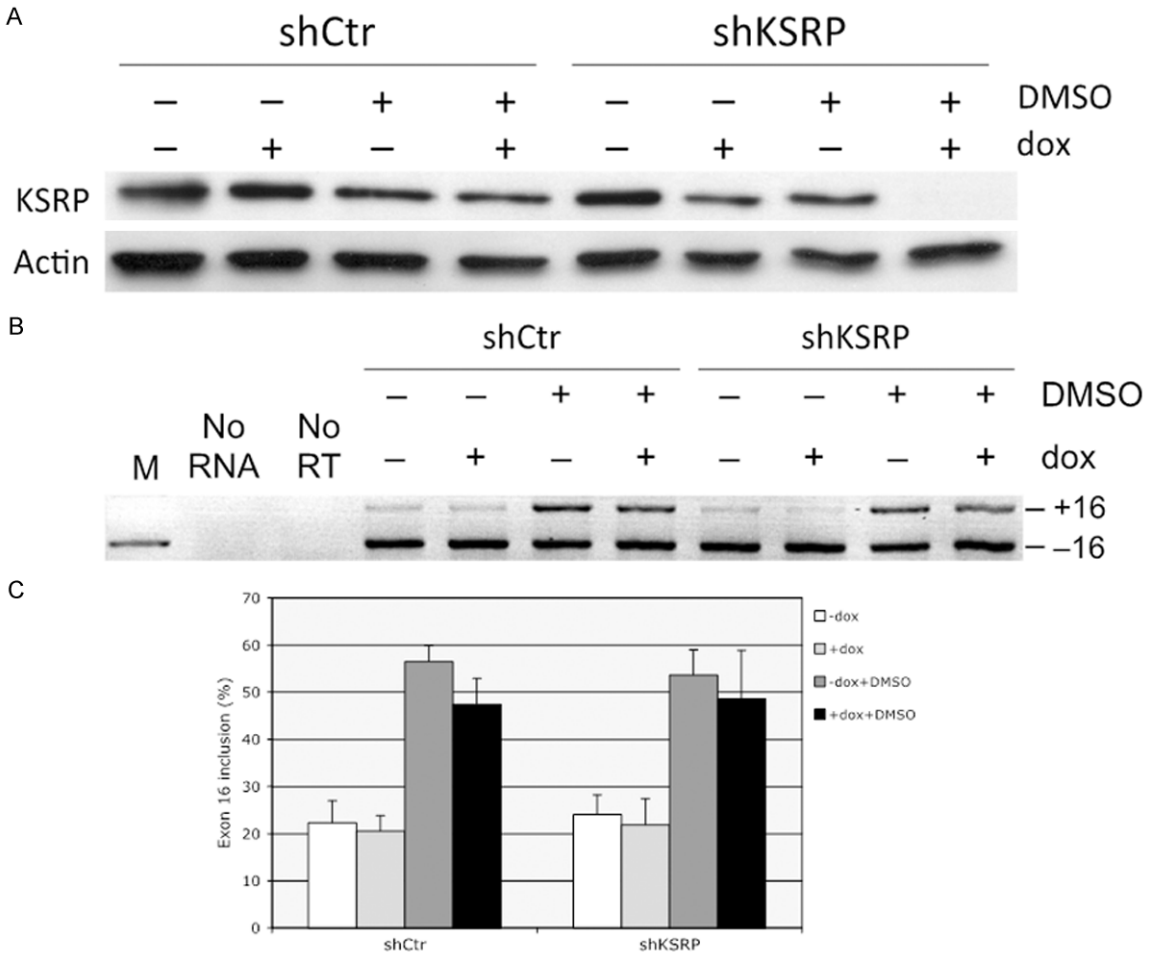

Figure 4. KSRP knockdown does not alter exon 16 erythroid splicing. A. Immunoblotting using anti-KSRP antibody to assess KSRP expression in cells stably transfected with shKSRP, as compared with control cells transfected with the scrambled shRNA (shCtr). Actin immunoblotting served as a loading control. Cells were analyzed in the absence (-) or presence (+) of doxycycline (dox), and before (-) or after (+) DMSO induction to differentiation. B. Exon 16 splicing efficiency was analyzed in shCtr and shKSRP cells in the same culture conditions described in A. Samples without RNA template (No RNA) or without adding reverse transcriptase (No RT) were run as RT-PCR controls. C. Semi-quantitative analysis of exon 16 splicing before and 5 days after DMSO (+DMSO) treatment. Cells were either uninduced (-dox) or induced (+dox) to express shKSRP RNA by adding doxycycline to the culture media. The data correspond to 3 different experiments; they are shown as mean ± SD.

data, together with the above-described UV crosslinking data, established KSRP as a major factor recruited to ESS16.

KSRP slightly decreases during erythroid differentiation

ESS16 predominant impact in proliferative erythroleukemia cells is overridden by the activation of splicing enhancers through their interaction with positive splicing factors [18-20, 30]. We therefore asked whether ESS16 weakening during erythroid differentiation is accompanied by KSRP quantitative change in DMSO-treated cells. Total and nuclear proteins were prepared from cells before and after DMSO exposure. Immunoblotting analysis using an anti-KSRP specific antibody revealed a slight downregulation of KSRP expression in total, as well as in nuclear cell extracts after DMSO treatment (**Figure 3**). KSRP appeared either as a single band or generally as a doublet, as has been previously observed [26, 31], but the biochemical or molecular basis underlying this feature remains to be elucidated.

It has been reported that PI3K/AKT signaling activation regulates KSRP distinct functions, sometimes in opposite ways (see [29], for review). PI3K is constitutively activated in eryth-

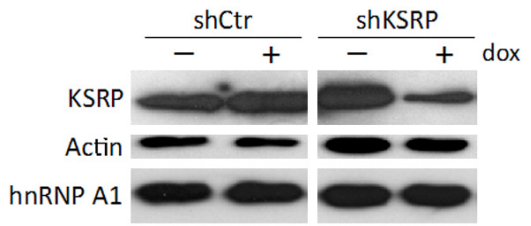

Figure 5. KSRP knockdown does not alter hnRNP A1 expression in erythroleukemia cells. HnRNP A1 and KSRP expression was assessed by western blot. Cells were stably transfected with shKSRP or with the scrambled shRNA (shCtr), the expression of which is directed upon addition of doxycycline (dox). (+) and (-) denote the presence or absence of doxycycline, respectively. Actin immunoblot served as quality and loading control.

roleukemia cells, which activates a PU.1 autoregulation loop, and inhibition of the PI3K/AKT signaling results in cell erythroid differentiation [15]. We tested the implication of specific inhibition of PI3K-AKT phosphorylation cascade on the expression of KSRP. As shown in **Figure 3A**, inhibition of PI3K-AKT signaling pathway by LY294002 resulted in a decrease of KSRP. These data revealed that KSRP expression slightly declines in cells induced to terminal differentiation, either by DMSO or inhibition of PI3K/AKT signaling.

KSRP knockdown does not alter exon 16 splicing

To address the physiological significance of KSRP interaction with ESS16, we examined exon 16 splicing in cells stably transfected with inducible shRNA KSRP. KSRP expression was assessed to ascertain the specificity of the transfected shRNA (**Figure 4A**). KSRP silencing was constantly more efficient in DMSO-treated cells, consistent with a decreased expression in cells induced to terminal erythroid differentiation as compared to proliferating cells (see above). KSRP expression was unchanged in cells transfected with the scrambled shRNA (shCtr cells), and its downregulation remained again strictly associated with DMSO treatment of these cells (**Figure 4A**).

Exon 16 inclusion was determined by analytical (**Figure 4B**) and semi-quantitative (**Figure 4C**) RT-PCR in shCtr- and shKSRP-transfected cells, before and after DMSO exposure. These experiments showed that KSRP knockdown did not alter exon 16 splicing pattern. Instead, exon 16 inclusion remained associated with induction of cell differentiation. Altogether, these data suggest that KSRP expression level alone does not affect the erythroid regulation of exon 16 splicing.

KSRP knockdown does not alter hnRNP A1 expression

It has been shown that activation of PI3K-AKT signaling impairs KSRP function in the degradation of ARE-containing transcripts, and hence causes increased accumulation of a set of KSRP targets. Among these transcripts, three encode members of the hnRNP family, mainly implicated in pre-mRNA splicing: hnRNPA1, hnRNPF, and hnRNPA/B [32]. We and others have shown that PI3K is constitutively activated in PU.1-induced erythroleukemia cells [15], which exhibit high level of expression of hnRNP A1.

In keeping with these findings, we tested whether KSRP is implicated in modulating the level of hnRNP A1 in erythroid cells. The endogenous KSRP was silenced using a doxycycline inducible shRNA system, and immunoblot analysis of hnRNP A1 was performed. As shown in **Figure 5**, KSRP knockdown did not change the endogenous hnRNP A1 expression, when compared with cells transfected with the control shRNA, suggesting that modulation of the expression of hnRNP A1 does not correlate with KSRP expression in these erythroid cells.

KSRP needs hnRNP A1 to interact with ESS16

The recruitment of KSRP on ESS16 on one hand, and the unperturbed exon 16 splicing in KSRP knockdown cells on the other hand, prompted us to test whether KSRP interaction with ESS16 is independent of hnRNP A1 binding. To address this issue, we first performed an EMSA on labeled ESS16 in the presence of nuclear extracts, obtained from three different cell clones: in addition to PU.1-induced erythroleukemia cells used in most experiments described above and previous studies (745A clone), we purified nuclear extracts from CB3 and CB3-A1 cells. CB3 cells are erythroleukemia cells missing *hnRNP A1* locus, and therefore unable to produce hnRNP A1 splicing factor [22]. CB3-A1 cells correspond to the same CB3 clone where we restored hnRNP A1 expres-

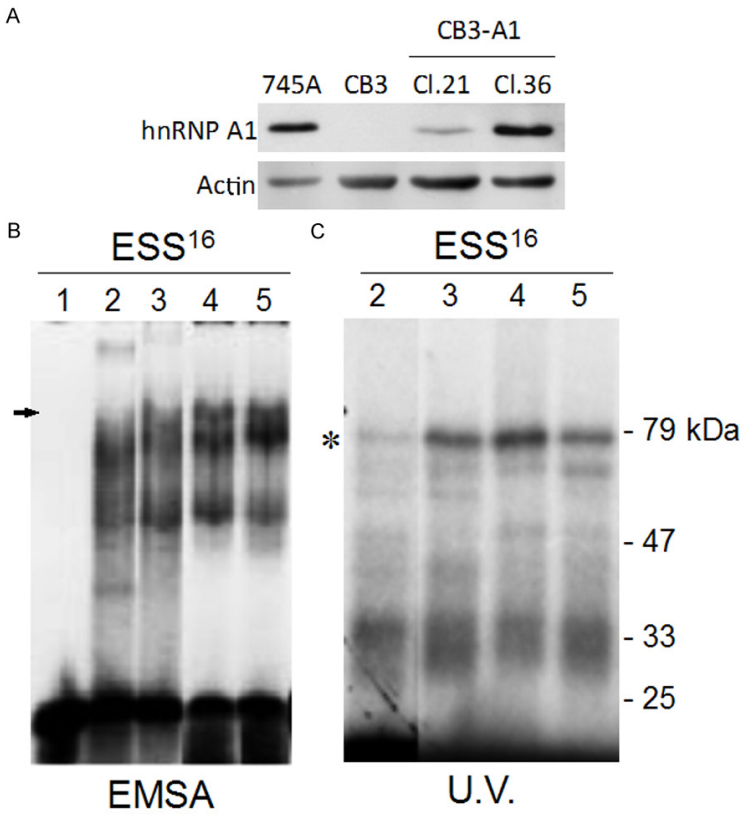

Figure 6. KSRP recruitment to ESS16 requires hnRNP A1. A. Immunoblot analysis of erythroleukemia cell clones using anti-hnRNP A1 specific antibody. The 745A clone serves as a control. It corresponds to the clone used in most experiments described in this work. CB3 is an uninducible clone that does not express hnRNP A1, due to the insertion of F-MuLV at Fli-2 site. CB3-A1 are CB3-derived clones, where hnRNP A1 cDNA was stably transfected. Several clones were analyzed. Two of them are shown: Cl.21 and Cl.36. This latter was used in subsequent experiments. Anti-actin antibody was used as quality and loading control of SDS-PAGE fractionated proteins. B. EMSA using ESS16 substrate and nuclear extracts obtained from CB3 cells (lane 2), CB3-A1 cells (lane 3), proliferating 745A cells (lane 4) or DMSO-treated 745A cells (lane 5). Lane 1: labeled RNA without nuclear extracts. The arrowhead points to the disruption of complex 1. C. UV crosslinking using ESS16 probe and nuclear extracts obtained from CB3 cells (lane 2), CB3-A1 cells, Cl36 (lane 3), proliferative (lane 4), or differentiating (lane 5) 745A cells. Size markers are indicated on the right. The star points to KSRP position.

sion by stable transfection of hnRNP A1 cDNA (**Figure 6A**).

Use of nuclear extracts from 745A cells promoted the formation of the 3 complexes as seen before (see above). Interestingly, complex 1 was disrupted when the probe was incubated with nuclear extracts from CB3 cells (**Figure 6B**, Lane 2). Most remarkably, gel shift assays using nuclear extracts from CB3-A1 cells revealed the formation of complex 1 in addition to complexes 2 and 3 (**Figure 6B**, lane 3). The recovery of complex 1 indicated that hnRNP A1 contributes to this RNP complex.

RNA-protein interactions were then analyzed by UV crosslinking assays, using total nuclear extracts from CB3 cells. As shown in **Figure 6C**, KSRP failed to assemble efficiently to ESS16 in these conditions (Lane 2). Use of CB3-A1 nuclear extracts re-established KSRP binding to ESS16. Altogether, these experiments demonstrated that the recruitment of KSRP to ESS16 requires the presence of hnRNP A1.

Discussion

KSRP is a multifunctional post-transcriptional regulator. It was originally described as a nuclear factor regulating transcription, the c-src pre-mRNA splicing, or the apolipoprotein B editing. Afterwards, KSRP was extensively studied for its ability to promote mRNA decay and the biogenesis of distinct sets of microRNAs (miRNAs) in distinct cell lines (see [29] for review). In fact, along with mRNA decay, KSRP may have a relevant role in virtually all steps of mRNA metabolism, including pre-mRNA splicing, pre-mRNA 3' end processing, mRNA export and localization, and may even modulate translation of specific mRNAs (see [29], for review).

Few data have described KSRP as splicing factor: it was implicated as an activator in c-*src* exon N1 splicing in neuronal cells, through its interaction with a splicing enhancer called downstream control sequence (DCS) [31]. Its homolog in Drosophila PSI acts as a splicing inhibitor by binding to a repressor element for the P-element third intron [33]. We suspected KSRP to be involved in 4.1R exon 16 splicing. Data gathered here show that KSRP binds ESS16 both in proliferating and differentiating cells. However, despite its recruitment to the exon, KSRP does not seem to modulate exon

splicing during erythroid development. In fact, KSRP expression and function in alternative splicing regulation appear to have different features depending on cell types: exon N1 inclusion in c-*src* mRNA increases, yet KSRP level does not change during neuronal differentiation upon DMSO treatment [26], whereas KSRP expression slightly decreases upon DMSO induction of erythroleukemia cells to terminal erythroid differentiation without affecting exon 16 splicing (this work). These observations suggest that both splicing events are not dependent on KSRP expression change *per se*.

A recent study has documented that KSRP function is required to maintain P19 cells in a multipotent undifferentiated state and that its inactivation can orient cells towards neural differentiation [34]. In the erythroid cell model, KSRP knockdown did not seem to affect erythroid differentiation, as evidenced by the regulated switch of 4.1R exon 16 following cell treatment with DMSO.

KSRP is able to recruit the exosome, the deadenylating enzyme PARN, and the decapping complex, and promote the decay of ARE-containing mRNAs. Moreover, tethering KSRP to a non-ARE-containing mRNA elicits mRNA decay ([35]; see [29] for review). It has been shown that KSRP triggers mRNA degradation in a dephosphorylated form; its phosphorylation impairs its interaction with ARE-containing mRNA and, therefore, attenuates its function in destabilizing mRNA molecules, such as β-catenin mRNA [36]. On the other hand, PI3K/AKT activation inhibits KSRP ability to promote decay of myogenin mRNA and activates its ability to favor maturation of myogenic miRNAs [37]. Involvement of KSRP as an ARE-binding protein in hematological diseases has not been established, although it seems to play an important role in hematopoietic cell lineage development through the regulation of selected miRNA maturation (see [38] for references).

A study has identified a set of transcripts, including hnRNP A1, that are targets of KSRP and whose expression is increased by PI3K-AKT activation [32]. Both KSRP knockdown and PI3K-AKT activation were found to increase the stability and the steady-state levels of these target mRNAs [32, 39]. With this in mind, we focused on PI3K-AKT signaling, and showed that specific inactivation of PI3K-AKT signaling cascade has no effect on hnRNP A1 expression in erythroleukemia cells. We further showed that specific knockdown of KSRP has no repercussion on hnRNP A1 expression in cells stably transfected with inducible shKSRP RNA.

RNA binding of KSRP is mediated by four KH domains that occupy the middle region of the protein. However, the broad range of targets recognized by KSRP has been emphasized [40]. One would therefore anticipate that this flexibility in the recognition of the RNA targets might parallel the wide range of KSRP functions in RNA metabolism. Computational analysis of 4.1R mRNA using the AREsite database (http://rna.tbi .univie.ac.at/AREsite), led to identify 3 ARE consensus within the 3'UTR, but not the pentamers found in exon 16. However, the presence of an ARE consensus motif alone is not sufficient to qualify a gene as a true in vivo target of ARE-binding proteins. Indeed, the ARE consensus sites found in exon 16 are not located in A and U rich region, which is a favorable context for ARE-binding proteins, as previously discussed [41, 42].

Co-immunoprecipitation studies have shown that KSRP associates with different components of pre-mRNA splicing, mRNA degradation, miRNA processing, and transcriptional regulation machineries (see [43], for review). For instance, KSRP was found as a component of a complex involved in c-src pre-mRNA splicing, and as a component of ApoB editing complex. Here, we found KSRP as a component of a RNP complex including hnRNP A1, and acting through an ESS that have been shown to govern exon exclusion in proliferative erythroid cells and in erythroleukemia cells, which are blocked in their differentiation.

Interestingly, KSRP interacts with hnRNPA1, and it also controls the decay of hnRNPA1 mRNA in defined non erythroid cells [32]. In some cases, this interaction reflects competing functions between these two factors [44]. In ARE-containing transcript targeting, AUF1p45 and hnRNPA1 were found as part of the KSRP-containing RNP complex but do not directly interact with KSRP targets [32]. An earlier study has shown that KSRP induces the assembly of 5 proteins, including hnRNP F and hnRNP H, onto the intronic splicing enhancer of the c-*src* exon N1 [31]. In a different cell context, we here found KSRP as a component of a complex

including hnRNP A1 and recruited to a splicing silencer that had been shown to govern exon exclusion in proliferating erythroid cells. Moreover, addition of purified hnRNP A1 in vitro (not shown), or restored expression of hnRNP A1 in total nuclear extracts (**Figure 6B** & **6C**), dramatically enhance KSRP binding to ESS16, indicating that KSRP recruitment depends necessarily on the presence of hnRNP A1. Consistently with our findings, hrp48 and PSI, the hnRNP A1 and KSRP homologs in *Drosophila*, respectively, were found to regulate many of the same splicing events. In fact, every identified target of PSI was similarly affected by hrp48 knockdown [45]. These data led the authors to hypothesize that hrp48 might be an obligate partner of PSI; however, hrp48 does not appear to require PSI to regulate splicing.

Other members of the KH-domain protein family, namely hnRNP K, hnRNP E1 and hnRNP E2, function as regulators of erythroid cytoplasmic mRNAs, such as reticulocyte 15-lipoxygenase (LOX) and α-globin. These hnRNP proteins are endowed with a broad variety of functional roles, suggesting that they may act as bridging molecules that facilitate the assembling of regulatory multiprotein complexes [46]. Consistent with these observations, our work further support that KSRP, and possibly other members of the KH-domain protein family, associate with other proteins in multicomponent regulatory systems, without directly altering a particular splicing event.

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Disclosure of conflict of interest

None to disclose.

Abbreviations

ARE, AU-rich element; EMSA, electrophoretic mobility shift assay; ESS, exon splicing silencer; DMSO, dimethylsulfoxide; HMBA, hexamethylene bisacetamide; KSRP, KH-type splicing regulatory protein; MEL, mouse erythroleukemia; PI3K, phosphatidylinositol 3-kinase; RNP, ribonucleoprotein.

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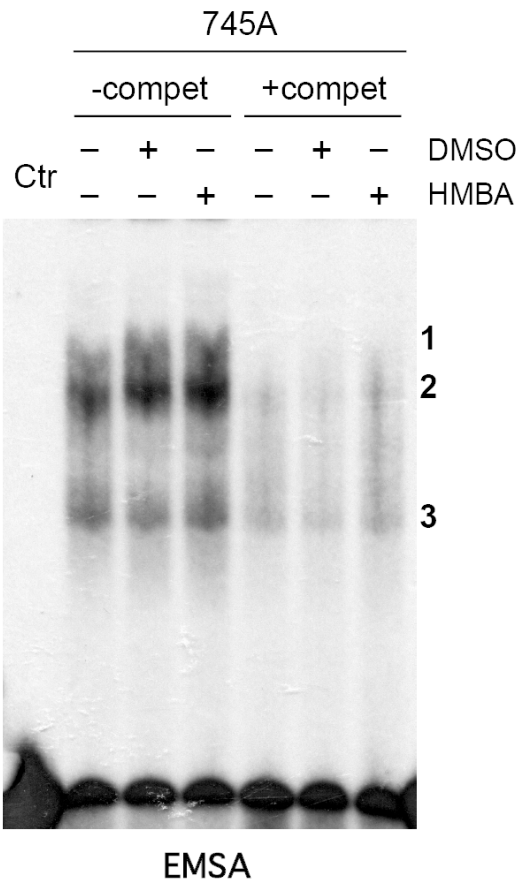

Supplementary Figure 1. ESS16 binds specifically to MEL nuclear factors in a stage-specific independent manner ESS16 ^{32}P-RNA probe was in vitro transcribed under the control of SP6 promoter. EMSA using labeled ESS16 substrate and nuclear extracts from proliferating uninduced (–) MEL cells, or cells induced either by DMSO (+DMSO) or HMBA (+HMBA). The experiments were carried out in the absence (-compet) or the presence (+compet) of excess unlabeled template. 1, 2 and 3 indicate the 3 major complexes revealed. A control sample (Ctr), where the RNA was incubated in the absence of protein extract, shows only free labeled molecules.