

Alternative splicing of the mouse embryonic poly(A) binding protein (*Epab*) mRNA is regulated by an exonic splicing enhancer: a model for post-transcriptional control of gene expression in the oocyte

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Embryonic poly(A) binding protein (EPAB), expressed in oocytes and early embryos, binds and stabilizes maternal mRNAs, and mediates initiation of their translation. We identified an alternatively spliced form of *Epab* lacking exon 10 (c.Ex10del) and investigated the regulation of *Epab* mRNA alternative splicing as a model for alternative splicing in oocytes and early preimplantation embryos. Specifically, we evaluated the following mechanisms: imprinting; RNA editing and exonic splicing enhancers (ESEs). Sequence analysis led to the identification of two single nucleotide polymorphisms (SNPs): one was detected in exon 9 (rs55858A/G), and served as a marker for the parental origin of the alternatively spliced form, and the other was found in exon 10 (rs56574G/C), and co-segregated with the exon 9 SNP. We found that the presence of rs56574G in exon 10 led to the formation of an ESE, leading to efficient exclusion of exon 10. Real-time RT-PCR results revealed a 5-fold increase in the expression of the c.Ex10del alternative splicing variant in animals carrying rs56574G/G in exon 10 compared with rs56574C/C at the same locus. Our findings suggest that SNPs may alter the ratio between alternative splicing variants of oocyte-specific proteins. The role that these subtle differences play in determining individual reproductive outcome remains to be determined.

Keywords: embryonic poly(A) binding protein; oocyte; alternative splicing; exonic splicing enhancer

Introduction

Post-transcriptional modification of mRNAs by alternative splicing is an important mechanism by which genomic complexity is generated from the surprisingly low number of genes currently estimated from the human genome sequence (Black, 2003; Faustino and Cooper, 2003). The fraction of human genes estimated to be subject to alternative splicing has risen from 5% in early predictions to at least 75% in a recent genome-wide exon study (Gardina *et al.*, 2006).

From a single gene, alternative splicing may result in the generation of variable transcripts produced combinatorially through the selection of cassette exons, mutually exclusive exons, alternative 3' or 5' splice sites, alternative promoters or poly(A) sites, which in concert, result in protein diversity (Black, 2003; Faustino and Cooper, 2003). There are examples of hundreds of alternative splicing events from a single gene, which may affect function by adding or deleting functional domains, changing affinities or altering mRNA stability (Babushok *et al.*, 2007). For example, in the case of fibroblast growth factor receptor, a portion of the ligand-binding domain of the receptor is encoded by two alternative exons that are entirely responsible for receptor's interaction with different ligands (Givol and Yayon, 1992).

Regulation of mRNA processing is especially important in oocytes, as oocyte maturation is associated with suppression of transcription (Flach *et al.*, 1982; Newport and Kirschner, 1982a,b; Braude *et al.*, 1988; Schultz, 2002) and new mRNAs are not synthesized until after zygotic genome activation (ZGA) that occurs at the 4- to 8-cell stage in human (Braude *et al.*, 1988) and the 2-cell stage in mouse (Flach *et al.*, 1982). During the period of time that begins with oocyte maturation and lasts until ZGA, gene expression is mainly mediated by activation and suppression of maternal mRNAs accumulated in the oocyte prior to meiotic reactivation (Mendez and Richter, 2001; Schier, 2007).

Gametes express alternative proteins for RNA binding (Gu *et al.*, 1998; Voeltz *et al.*, 2001; Seli *et al.*, 2005), suggesting that RNA metabolism in gametes is tightly and differentially regulated. Although gamete-specific RNA-regulatory proteins have been identified (Voeltz *et al.*, 2001; Seli *et al.*, 2005), and alternative splicing of gamete-specific genes have been described (Gu *et al.*, 1998), mechanisms involved in the regulation of alternative splicing in gametes remain to be elucidated.

Embryonic poly(A) binding protein (EPAB), identified in *Xenopus* oocytes, is the predominant poly(A) binding protein (PABP) during

early *Xenopus* development until ZGA when it is replaced by the ubiquitous somatic cytoplasmic PABP (PABPC1) (Voeltz *et al.*, 2001). EPAB is the only factor identified to date in both known protein complexes (cytoplasmic polyadenylation complex and PUMILIO-2/DAZL/EPAB complex) that bind and stabilize dormant mRNAs in immature oocytes (Padmanabhan and Richter, 2006; Kim and Richter, 2007). EPAB also appears to be the only factor required for both known pathways (cytoplasmic polyadenylation-dependent and independent) that mediate maternal mRNA translational activation upon oocyte maturation (Cao and Richter, 2002; Padmanabhan and Richter, 2006; Vasudevan *et al.*, 2006). Therefore, EPAB seems to play a central role in the translational regulation of maternally derived mRNAs during a critical period of early development when transcription is suppressed. We have recently identified mouse *Epab* expressed in oocytes and early embryos (Seli *et al.*, 2005). Similar to its *Xenopus* ortholog, mouse *Epab* is the predominant PABP until ZGA, which occurs at the 2-cell stage in mouse (Seli *et al.*, 2005).

In the current study, we identified an alternatively spliced form of *Epab* lacking exon 10 (c.Ex10del) and investigated the regulation of *Epab* alternative splicing as a model for alternative splicing in oocytes and early preimplantation embryos. Specifically, we evaluated the following mechanisms: imprinting (expression from maternal or paternal allele only); RNA editing (post-transcriptional single nucleotide substitution) and exonic splicing enhancers (ESEs, sites within an exon that bind splicing proteins).

Materials and Methods

Animals

Adult C3H and C57Bl/6J mice were purchased from Jackson laboratories (Bar Harbor, ME, USA). Mice were bred and maintained according to the Yale University animal research requirements, and all procedures were approved by the Institutional Animal Care and Use Committee (protocol number # 2005-07222). Mice were fed *ad libitum* and housed under a 12 h light cycle.

DNA extraction and single nucleotide polymorphism genotyping

Mouse tail genomic DNA was extracted by standard methods (Laird *et al.*, 1991). Briefly, tail tips were cut and digested overnight with proteinase K (100 µg/ml) in 0.5 ml lysis buffer (100 mM Tris HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) at 55°C with rocking. Following vortexing and centrifugation to pellet hair, 0.5 ml of 100% isopropanol was added to the supernatant and chromosomal DNA was aggregated into a visible clump by rocking manually at room temperature. The DNA was then spooled and dissolved in 0.5 ml TE buffer.

Genotyping for exon 9 single nucleotide polymorphism (SNP), rs55858A/G was performed by polymerase chain reaction (PCR) amplification of DNA using primers exon9F: 5'-TCTACAGCCCAGGCTGTGTAC-3' and IVS9R: 5'-TAGCAATTTAGCCAAAAGAGTTTC-3', followed by HaeIII restriction enzyme digestion (New England Biolabs, Ipswich, MA, USA). Amplifications were carried out using 35 cycles of PCR, in which the initial 5 min for denaturation at 94°C was followed by a touch-down program for 10 cycles of 92°C/30 s, 65°C/20 s (−1°C per cycle) and 72°C/30 s and then 25 cycles of 92°C/30 s, 55°C/20 s and 72°C/30 s, in a 50 µl reaction mix containing 1× PCR buffer (Ambion, Austin, TX, USA), 125 µM dNTP, 0.5 µM primers and 1 unit of SuperTaq polymerase (Ambion). Digested samples were run on a 2% agarose gel and visualized by ethidium bromide staining.

Total RNA isolation, reverse transcription and PCR

Total RNA was isolated after homogenization of adult mouse ovaries in Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and kept at −80°C until use. RNA was treated for genomic DNA contamination using DNase I (Ambion). The quality and concentration of the RNA was determined by measuring the absorbance at 260 and 280 nm. Equal amounts of total RNA (2 µg) were reverse-transcribed to cDNA using oligo

d(T) priming at 37°C for 1 h (Omniscript, Qiagen, Valencia, CA, USA). Then, PCRs were performed on cDNAs using *Taq* DNA polymerase (Qiagen) with specific primer pairs flanking the alternatively spliced exons 10 and 13, respectively, as described in Results. The sequences of primers were: exon8F: 5'-ACGAAACCGCTCTATGTGGCCC-3', exon11R: 5'-TGGGGACTCCAGCAATAA CAGAAGC-3' and exon14R: 5'-CCAGGAACCCA-CAGAGTTCAG-3', PCR conditions were as above.

Spliced and unspliced forms were gel fractionated in 1.5% agarose and visualized by ethidium bromide staining. PCR fragments were extracted from agarose gel using a gel extraction kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocol, and sequenced at the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University. Sequencing results were compared with mouse *Epab* genomic sequence using Sequencher software version 4.7 (Gene Codes, Ann Arbor, MI, USA).

Quantitative real-time PCR

Gene expression studies by quantitative real-time PCR (qRT-PCR) were carried out on an iCycler (Bio-Rad, Hercules, CA, USA). Tissue cDNA, prepared as described above, was assayed in triplicate. Each experiment was repeated three times and five mutant animals were compared with five litter mate controls. Each 25 µl reaction contained 12.5 µl of 2× SybrGreen supermix (Biorad), 0.4 µM of each primer and 1 µl of template.

In order to quantitate the expression of different splicing variants of *Epab*, the following primers were used: exon9–11F: 5'-GGAACCTCAGCTGTACACAGTG-3', exon 12R: 5'-CTGATGGCCAGGGACATACAC-3', exon10F: 5'-AGCCAGGTGCCT CACACGCAG-3', exon8F: 5'-ACGAAACCGCTCTATGTGGCCC-3' and exon9R: 5'-CCTGGGGTCAGTTGCATTGG-3'. Expression of the target gene was normalized to β-actin levels using primers 5'-GACCTCTATGCAACACAGT-3' and 5'-TTGCTGATCCACATCTGCT-3'. The ΔΔCt (cycle threshold) method was used to calculate relative expression levels. Results were reported as a fold change in gene expression between different genotypes.

Database search of ESE element

The ESE finder program (version 2.0, <http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home>) (Cartegni *et al.*, 2003) (Cold Spring Harbor Laboratory, NY, USA) was used to identify ESE consensus sequences.

Statistical analysis

Since the data from qRT-PCR were normally distributed (as determined by Kolmogorov–Smirnov test), the Student *t*-test was used. Statistical calculations were performed using SigmaStat for Windows, version 3.0 (Jandel Scientific Corp., San Rafael, CA, USA). Statistical significance was defined as $P < 0.05$.

Results

Identification of an additional splicing variant of mouse *Epab* gene

A splicing variant of mouse *Epab* lacking exon 13 has previously been reported (Seli *et al.*, 2005) (Fig. 1A). In the present study, RT-PCR in mouse ovary using primers on exons 8 and 11 revealed two *Epab*-specific bands (Fig. 1B). Sequencing of the lower band revealed an additional splicing variant of the *Epab* transcript lacking exon 10 (c.Ex10del).

Moreover, amplification from exon 8 to exon 14 flanking both alternatively spliced exons, produced four *Epab*-specific bands. Sequencing revealed that these bands corresponded to the full-length *Epab*, alternatively spliced forms lacking exon 10 or exon 13, alternatively spliced form lacking both exons 10 and 13 and a heteroduplex band (Fig. 1C). Domain analysis of the predicted protein revealed that the alternatively spliced form of mouse *Epab* mRNA without exon 13 lacks the PABP domain, whereas the spliced form of *Epab* without exon 10 encodes a full PABP domain (Fig. 2, Table I).

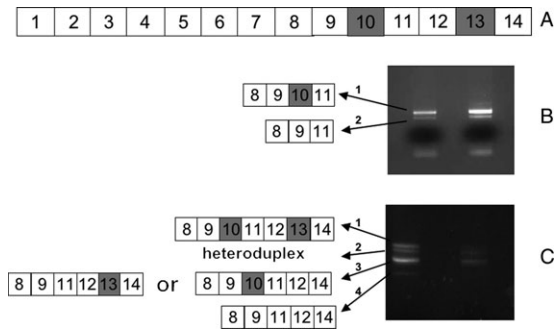


Figure 1: Alternatively spliced forms of *Epab*. (A) The genomic structure of the mouse *Epab* gene consists of 14 exons. Exons 10 and 13 are alternatively spliced. (B) PCR on mouse ovary cDNA with exon8F–exon11R primers detects the expression of alternatively spliced *Epab* mRNA variants with (band 1) and without (band 2) exon 10. The size of the lowest band is consistent with unbound primers. (C) PCR on mouse ovary cDNA with exon8F–exon14R primers detects expression of alternatively spliced *Epab* mRNA variants with and without exon 10 and/or 13. Four *Epab* specific bands were detected by agarose gel fractionation. Sequencing revealed that these bands corresponded to the full-length cDNA (band 1), a heteroduplex band that consists of full-length cDNA paired with the alternatively spliced form lacking exon 10 or 13 (band 2), alternatively spliced form lacking exon 10 or exon 13 (band 3) and alternatively spliced form lacking both exon 10 and exon 13 (band 4).

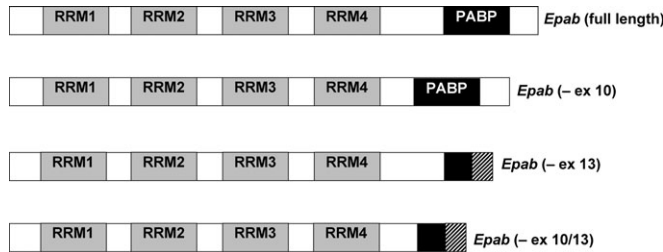


Figure 2: Schematic representation of predicted mouse *Epab* protein encoded by full-length *Epab* mRNA, and alternatively spliced forms excluding exon 10, exon 13 or exon 10/13.

The four RRM (1–4) are indicated by gray boxes, and the PABP domain in the C-terminal region is indicated by a black box. Since the PABP domain is encoded by exons 12 and 13, the (–ex 13) and (–ex 10/13) forms contain only part of the PABP motif. The carboxy termini of the (–ex 13) and (–ex 10/13) forms also differ because of shifting of the exon 14 reading frame, whereas the absence of exon 10 does not result in a frame shift.

Mouse *Epab* exon 10 skipping exhibits monoallelic expression

The mice used for the identification of the c.Ex10del variant were progeny of mated CH3 with C57Bl/6J mice and were heterozygous for an exon 9 SNP (rs55858A/G). SNP genotyping was performed as described in Materials and Methods, using genomic PCR with primers 9F and IVS9R (Fig. 3A and B), followed by HaeIII digestion (Fig. 3C and D). The presence of rs55858G created a new HaeIII site and resulted in a 240 bp fragment, whereas the rs55858A generated for a 307 bp fragment upon digestion (Fig. 3C and D). This SNP allowed us to determine that, consistent with bi-allelic gene expression observed for most genes, *Epab* mRNA containing exon 10 was expressed from both alleles (Fig. 4A). However, c.Ex10del *Epab* was expressed only from the allele containing the rs55858A at the exon 9 SNP (Fig. 4A). This observation could be explained by three possible mechanisms: imprinting, RNA editing or ESEs.

Mouse *Epab* exon 10 skipping is not mediated by imprinting or RNA editing

To determine if monoallelic expression of c.Ex10del *Epab* is mediated by imprinting, we examined heterozygous (rs55858A/G) mice that inherited the rs55858A allele from either the mother or the father (Fig. 4A and B). Regardless of the parental origin of the allele, the shorter *Epab* transcript lacking exon 10, contained the rs55858A SNP, ruling out imprinting as the regulatory mechanism (Fig. 4A and B).

Next, we tested the possibility of RNA editing mechanism, which is a post-transcriptional base substitution (C to U, A to I) catalyzed by enzymes called RNA-editing deaminases (Maydanovych and Beal, 2006). We bred homozygous (rs55858G/G) mice for the exon 9 SNP. In mice homozygous rs55858G/G SNP, we found that the c.Ex10del variant also contained (G). Therefore, while in mice heterozygous for the exon 9 SNP (rs55858A/G), exon 10 skipping preferentially occurred from the rs55858A allele (Fig. 4A and B), alternative splicing still occurred when both alleles contained G (rs55858G/G) at position rs55858 (Fig. 4C). Consequently, RNA editing mechanism was eliminated.

Mouse *Epab* exon 10 skipping is regulated by an ESE

To identify the origin of the monoallelic exon skipping, we performed further sequence analysis and identified an additional SNP in exon 10 (rs56574G/C) that co-segregated with the exon 9 SNP (rs55858A/G). The two SNPs are in ‘linkage disequilibrium’ as rs55858A allele in

Table I. Pattern search and multiple alignment of mouse, human and *Xenopus* PABPs.

Gene	RRM1	Score	RRM2	Score	RRM3	Score	RRM4	Score	PABP	Score
Mouse EPAB (full length)	13–84 aa	6.6e-27	101–170 aa	4e-23	193–263 aa	2.2e-27	296–365 aa	4.7e-23	524–595 aa	1.3e-20
Mouse EPAB (–exon 13)	13–84 aa	6.6e-27	101–170 aa	4e-23	193–263 aa	2.2e-27	296–365 aa	4.7e-23	Absent	
Mouse EPAB (–exon 10)	13–84 aa	6.6e-27	101–170 aa	4e-23	193–263 aa	2.2e-27	296–365 aa	4.7e-23	524–595 aa	1.3e-20
Mouse EPAB (–exon 10/13)	13–84 aa	6.6e-27	101–170 aa	4e-23	193–263 aa	2.2e-27	296–365 aa	4.7e-23	Absent	
<i>Xenopus</i> EPAB	13–84 aa	9.7e-25	101–170 aa	2.8e-24	193–263 aa	5.7e-26	296–365 aa	8.7e-22	540–611 aa	8.6e-42
Mouse PABPC1	13–84 aa	6.6e-27	101–170 aa	9e-25	193–263 aa	1.2e-30	296–365 aa	1.7e-23	543–614 aa	4.7e-47
<i>Xenopus</i> PABPC1	13–84 aa	5.4e-27	101–170 aa	8.8e-25	193–263 aa	6.1e-27	296–365 aa	4.1e-22	541–612 aa	1.3e-47
Human PABPC1	13–84 aa	3e-27	101–170 aa	9.2e-25	193–263 aa	7.5e-31	296–365 aa	1.8e-23	543–614 aa	9.5e-48

The positions of each structural motif and their similarity to the consensus are shown. The prediction and assignment of the protein structures were performed by using PFAM (<http://pfam.wustl.edu>). Shown are expected values (e values) that describe the number of hits one can expect to see by chance when searching a database of a particular size. An e value of 1 assigned to a hit can be interpreted as meaning that, in a database of the current size, one might expect to see one match with a similar score simply by chance. The lower the e value is the higher the significance of the match.

PABP, poly(A) binding protein; RRM, RNA recognition motifs.

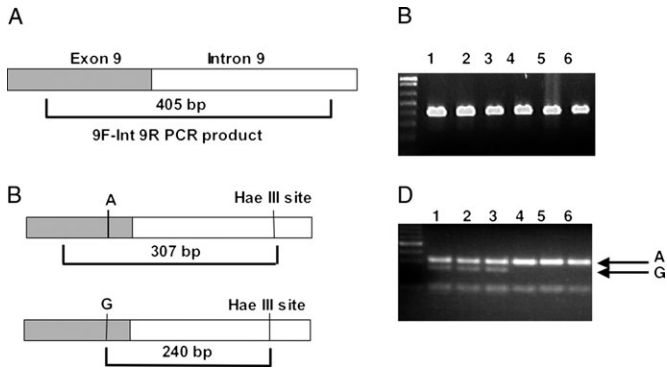


Figure 3: SNP genotyping of exon 9.

(A and B) PCR amplification of genomic DNA was performed using primers exon9F and IVS9R and resulted in a 405 bp product. PCR product was run on a 2% agarose gel and visualized by ethidium bromide staining. (C and D) This was followed by HaeIII restriction enzyme digestion of the PCR product. The presence of rs55858G created a new HaeIII site and resulted in a 240 bp fragment, whereas the rs55858A generated for a 307 bp fragment upon digestion. 1–6 corresponds to samples from the ovaries of six different mice.

exon 9 is always inherited together with rs56574G allele on exon 10, and rs55858G on exon 9 with rs56574C on exon 10.

Preferential splicing can be regulated by the presence of ESEs within the sequence. To identify ESEs that are recognized by individual splicing regulatory proteins (SF2/ASF, SC35, SRp40 and SRp55), we used the ESE finder program version 2.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/ese_finder.cgi?process=home). In this program, consensus motifs obtained with the four SR proteins are detected and the thresholds represent values above which a score for a given sequence is considered to be significant (high score motif). *In silico* analysis using ESE finder program revealed that the presence of rs56574G leads to the formation of a consensus sequence [CCTGAGG] characteristic of an ESE that binds splicing regulatory

protein SRp40, resulting in efficient exclusion of exon 10, as the binding affinity of SRp40 protein for the CCTGAGG sequence is 3.19 whereas it is lower than thresholds value for CCTGAGC sequence.

Presence of rs56574G in exon 10 is associated with a 5-fold increase in the *Epab* c.ex10del variant without a change in total *epab* expression

The effect of the ESE on the efficiency of alternative splicing in different genetic backgrounds was evaluated by qRT-PCR. Mouse strain C57BL/6 is homozygous for rs55858A and rs56574G alleles, and C3H is homozygous for rs55858G and rs56574C alleles.

In order to quantitate the expression of the c.Ex10del variant, we used a forward primer (exon9–11F) that was complementary to exon 9 at its 5' end and to exon 11 at its 3' end, and would not amplify *Epab* mRNA that contains exon 10. Reverse primer (exon12R) was in exon 12. This primer set resulted in a robust and sensitive qRT-PCR reaction with a correlation coefficient of 0.942, and a slope of -3.356 , corresponding to PCR efficiency of 98.6%.

Expression of *Epab* that contains exon 10 was quantified using primers on exons 10 and 12 (exon10F and exon12R). This PCR reaction had a standard curve with a correlation coefficient of 0.999, a slope of -3.395 and a PCR efficiency of 97%. Total *Epab* mRNA expression (including all forms) was quantified using forward and reverse primers at exons 8 and 9, respectively. This resulted in a standard curve with a correlation coefficient of 0.99, a slope of -3.310 and a PCR efficiency of 99.5%.

Quantitative RT-PCR revealed a 5-fold increase in the amount of the c.Ex10del variant in the animals carrying the enhancer (homozygous rs56574G/G) for the exon 10 SNP compared with the mice that did not (homozygous rs56574C/C) at the same locus ($P < 0.001$) (Fig. 5A). This was accompanied by a decrease in the amount of the unspliced *Epab* transcript in the animals that contained the ESE compared with mice that did not have the ESE ($P < 0.01$) (Fig. 5B). There was no significant difference in the

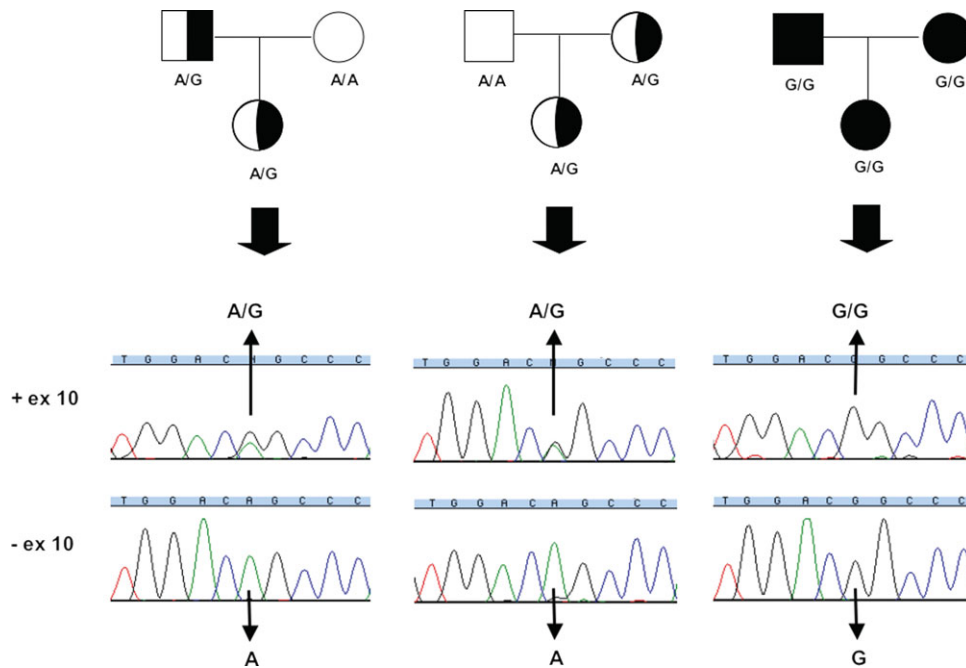


Figure 4: DNA sequences analysis of spliced and unspliced form of *Epab*.

(A and B) Female mice heterozygous (rs55858A/G) for the exon 9 SNP that inherited the A allele maternally (A) or paternally (B) expressed the spliced variant (lower frames) from the A allele. (C) In mice homozygous (rs55858G/G) for the exon 9 SNP, mRNA of the spliced variant contained G.

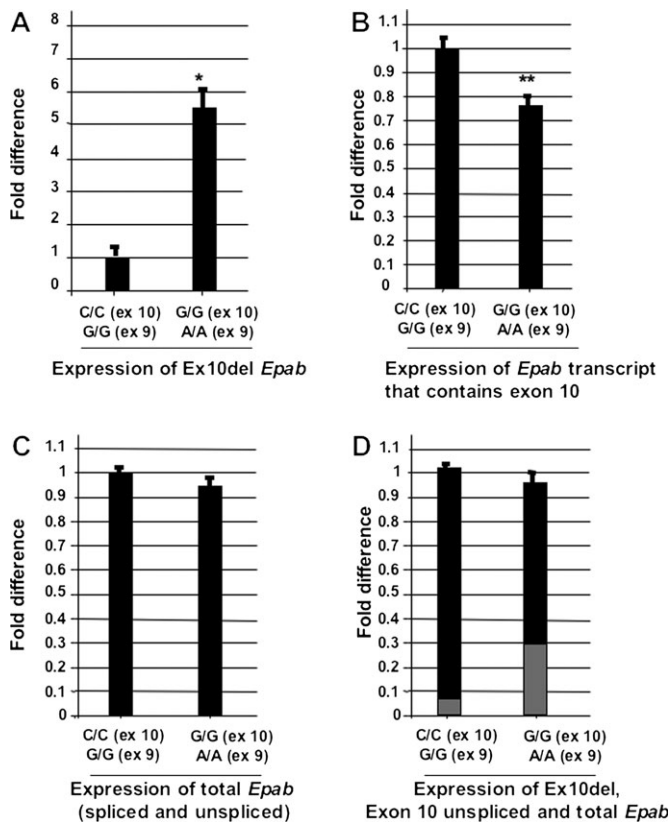


Figure 5: Expression of *Epab* splice variants in mice homozygous rs56574G/G or rs56574C/C for the exon 10 SNP was quantified using real-time PCR (qRT-PCR).

(A) qRT-PCR revealed a 5-fold increase in the expression of the c.Ex10del variant in the animals carrying the enhancer (homozygous rs56574G/G) for the exon 10 SNP compared with the mice that did not (homozygous rs56574C/C) at the same locus ($*P < 0.001$). (B) The animals that contained the ESE showed a decrease in the amount of the *Epab* transcript that contains exon 10 compared with mice that did not have the ESE ($**P < 0.01$). (C) There was no significant difference in the total *Epab* mRNA content of the two different genotypes, suggesting that the rs56574G SNP resulted in an increase in alternative splicing without changing overall of *Epab* expression. (D) Relative ratios of exon 10 unspliced (black) and c.Ex10del (grey) within total *Epab* mRNA is shown for animals carrying the enhancer (homozygous rs56574G/G) for the exon 10 SNP compared with the mice that do not (homozygous rs56574C/C) at the same locus.

total *Epab* mRNA content of these two different genotypes (Fig. 5C and D), suggesting that the rs56574G SNP resulted in an increase in alternative splicing without changing overall of *Epab* expression.

Discussion

Following their inception within the nucleus, pre-mRNAs undergo capping at the 5' end and cleavage and polyadenylation at the 3' end, to increase their stability and facilitate their transport into the cytoplasm (Izaurralde *et al.*, 1994; Flaherty *et al.*, 1997; Zhao *et al.*, 1999; Proudfoot, 2004). In addition, pre-mRNAs are further processed by splicing, which consists of the removal of non-essential transcript sequences termed introns. Splicing requires a 5' splice site that contains a conserved recognition consensus sequence, as well as a discrete 3' splice site that delimits the exon–intron boundary. In between these two regions, an intronic consensus branch point sequence must be present to facilitate the reaction (Staley and Guthrie, 1998; Jurica and Moore, 2003). In addition to these consensus sequences, there are a number of *cis* elements located in exons and introns known as

exonic or intronic splicing enhancers or silencers, due to their stimulating or repressing effects, respectively (Liu *et al.*, 1998; Schaal and Maniatis, 1999; Ladd and Cooper, 2002).

In the current study, we investigated the alternatively spliced form of *Epab* lacking exon 10 (c.Ex10del) as a model for alternative splicing in oocytes and early preimplantation embryos. Owing to its gamete- and early embryo-specific expression (Seli *et al.*, 2005), *Epab* provided a suitable model. To determine the mechanism resulting in the observed monoallelic expression of the spliced form of *Epab* lacking exon 10, we evaluated imprinting, RNA editing and ESEs. The SNP detected in exon 9 (rs55858A/G) served as a marker for the parental origin of the alternatively spliced form. Neither genomic imprinting nor RNA editing was responsible for the alternatively spliced forms of the *Epab* mRNA (Fig. 4). Another SNP in exon 10, rs56574G/C, co-segregated with the exon 9 SNP of *Epab*. We found that the presence of rs56574G led to the formation of an ESE characteristic for splicing regulatory protein SRp40, resulting in the efficient exclusion of exon 10. Consequently, in mice heterozygous for the rs56574G/C SNP, exclusion of exon 10 occurred preferentially from the allele containing rs56574G, whereas in mice homozygous for rs56574C, exclusion of exon 10 still occurred, although to a significantly lesser extent (Fig. 5A).

ESEs are exonic *cis*-elements that work with classical splicing signals (5' splicing site, 3' splicing site and branch site) by binding-specific serine/arginine-rich splicing regulatory proteins (SR proteins) to promote accurate splicing (Birney *et al.*, 1993; Smith and Valcarcel, 2000). SR proteins are characterized by the presence of RNA recognition motifs (RRM) and by a distinctive carboxy terminal domain that is highly enriched in Arg/Ser dipeptides (the RS domain) (Robberston *et al.*, 1990). SR proteins binding to ESEs is thought to promote exon definition by directly recruiting the splicing machinery through their RS domain and/or by antagonizing the action of nearby silencer elements (Cartegni *et al.*, 2002).

We performed sequence analysis of *Epab* for SR protein-binding sites using the ESE finder program (Cartegni *et al.*, 2003). Putative ESE motifs that agree with the consensus sequences for specific SR proteins have high values in the ESE finder program, and a decrease in the score relative to a threshold value is indicative of a decrease or loss of enhancement of transcript splicing (Cartegni *et al.*, 2003). Although our *in silico* findings were highly suggestive of regulation by ESEs, confirmation was performed using qRT-PCR, which showed that the presence of rs56574G resulted in a 5-fold increase in alternative splicing of *Epab* exon 10 (Fig. 5A).

EPAB plays an important role in the regulation of maternal mRNA translational activation upon oocyte maturation through both polyadenylation-dependent and independent mechanisms (Cao and Richter, 2002; Padmanabhan and Richter, 2006; Vasudevan *et al.*, 2006). In addition, EPAB seems to play a critical role by promoting protection of the maternal transcripts from deadenylation (Voeltz *et al.*, 2001; Kim and Richter, 2007). *Epab* exon 10 encodes for the region of the EPAB protein that is between the 4th RRM and the C-terminal PABP domain. In PABPC1, which shows high homology to EPAB, this region mediates interactions with other proteins. Therefore, the two *Epab* isoforms may play different roles in the regulation of maternal mRNA stability and translation. Additional studies will be necessary to determine whether full length and alternatively spliced forms of *Epab* affect translational activation of maternal genes differentially.

ESEs have been shown to regulate alternative splicing in somatic cells, and exon skipping due to ESEs has been associated with pathologies such as spinal muscular dystrophy (Lorson *et al.*, 1999). In the current study, we showed that ESEs are functional in mammalian oocytes, as they regulate the alternative splicing of *Epab*. We also demonstrated that an SNP may result in the formation of an ESE and

consequently alter the expression of full length and alternatively spliced forms of an oocyte-specific gene. Further studies will be necessary to determine whether alterations in gene expression mediated by similar mechanisms may underlie individual differences in reproductive function.

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