# Functional study of the Ser/Arg-rich splicing factor SRSF5a during zebrafish embryonic development

Marine JORIS[1], Arnaud LARBUISSON[2], Marc MULLER[2], Patrick MOTTE[1]

[1]Functional Genomics and Plant Molecular Imaging, [2]Molecular Biology and Genetic Engineering (GIGA-R), Dpt of Life Sciences, University of Liège, Belgium

**Contact: marine.joris@doct.ulg.ac.be**

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## Introduction

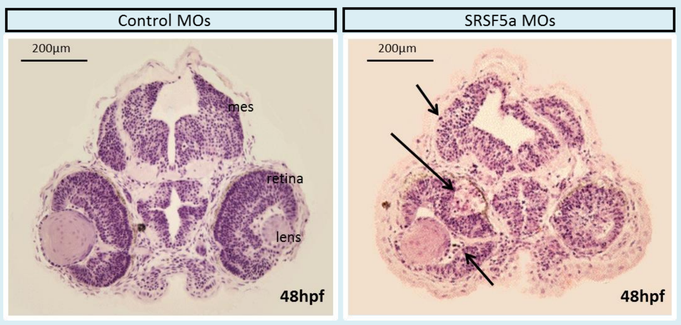
Pre-mRNA splicing is a key process regulating gene expression in eucaryotes. Nuclear pre-mRNA processing involved the removal of introns and the joining of exons by a macromolecular complex called the spliceosome, which consist of five small nuclear ribonucleoproteins (snRNPs) and numerous non snRNPs proteins. Amongst these non snRNPs proteins, the SR proteins family constituted an important group of splicing factors that are involved in constitutive and alternative splicing. SR proteins are structurally related as they are characterized by a C-terminal domain enriched in dipeptide Ser/Arg preceded by one or two RNA-recognition motifs. The structural relationship between these splicing factors allowed us to identify 13 encoding genes for SR proteins in the vertebrate model organism, *Danio rerio* (zebrafish). In this study, we investigate the role of the SR splicing factor, SRSF5a by injecting splice-junction morpholinos in single cell stage embryos, then, we analyse the defects in embryonic development and the impact on the transcriptome using RNA sequencing.

## Functional study

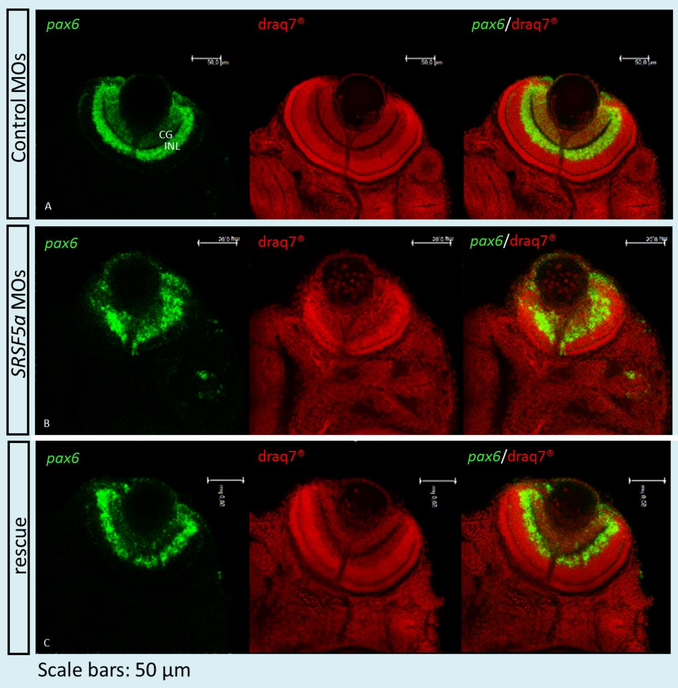
Knock down of *SRSF5a* by splice site blocking morpholinos microinjection reveals its function during zebrafish embryonic development. Indeed, microinjected embryos were analysed and showed developmental delays and defects. Furthermore, to study the role of SRSF5a at the genome wide level we performed high-throughput RNA-sequencing.

### Loss of SRSF5a function causes cell death and an abnormal retinal embryonic development

Hematoxylin/eosin staining showed abnormal organization of cells in the retina but also an increase of cells death in the eye and in the entire brain at 48 and 72 hpf (data not shown).




Fluorescent *in situ* hybridization using *pax6* probe followed by a nucleus staining using draq7® allowed us to visualize the disorganization of the ganglional cells layer and of the inner nuclear layer in the retina at 72hpf. Rescue experiments allowed us to partially restore the wild-type phenotype.



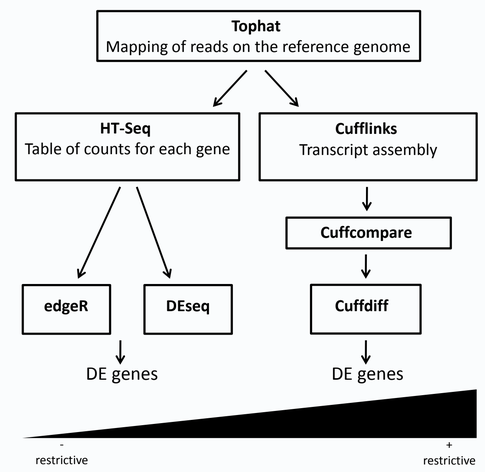

Scale bars: 50 µm

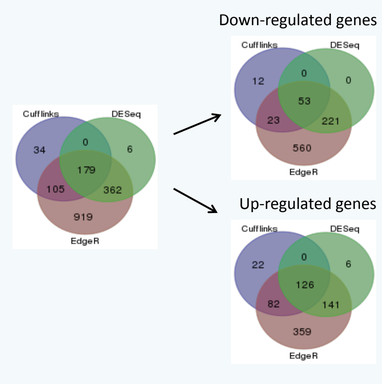
### Transcriptomic analysis

To identify SRSF5a-dependent genes or splicing events during zebrafish embryonic development, total RNA from 48hpf control and morphant embryos was sequenced, yielding a total of $144.10^6$ of 100 bases paired-end reads (22 and $53.10^6$ from control samples, 35 and $34.10^6$ from morphant samples).

#### ➢ Detection of differential gene expression

To determine the genes that are differentially expressed (DE genes) in the 2 conditions (controls vs morphants) we combined the use of 3 methods that allowed us to find 179 DE genes. Out of these genes, 126 were up-regulated and 53 were down-regulated.



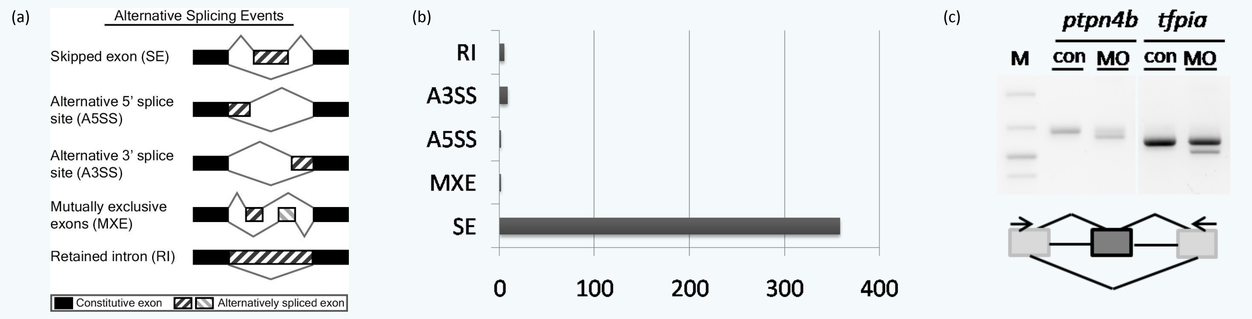




The sequence reads were mapped to the zebrafish genome Zv9 using TopHat[1]. Then we used two different methods to analyse Tophat output; (1) we generated count tables with HT-seq[2] that show how many reads have been mapped in each sample for a given gene or (2) we use Cufflinks[3] that attributes a normalized expression value (Fragment Per Kilobase of exon per million fragments mapped) to each gene. Finally we tested for differential expression by using a statistical test based on a negative binomial distribution (EdgeR[3], DESeq[4] and Cuffdiff[1]).

A **gene ontology (GO)** enrichment analysis[5] of DE genes permitted us to pinpoint biological processes in which SRSF5a is implied. Among these we found eye and nervous system development, which are consistent with morphants phenotypes. Rectangle size is proportional to the number of DE genes implicated in the process.


#### ➢ Detection of differential splicing

To detect differential alternative splicing events from RNA-seq data we used a Bayesian statistical framework called MATS [6]. From the RNA-Seq data, MATS can automatically detect and analyze alternative splicing events corresponding to all major types of alternative splicing patterns. MATS permitted us to identify 377 differential splicing events between control and SRSF5a morphants transcriptome.



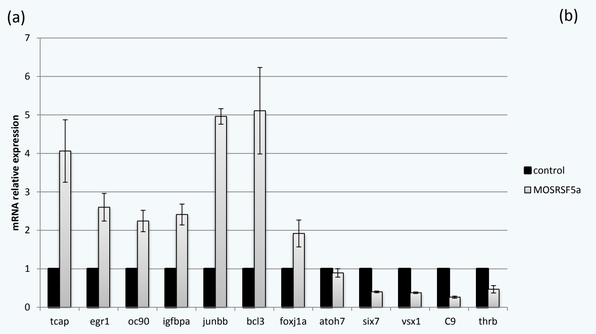

(a) Major alternative splicing patterns. (b) Number of alternative splicing events that differ between the two conditions. Exon skipping is preponderant. (c) Among these differentially spliced genes some will be tested and validated by RT-PCR. Some exon skipping events had already been tested and were in concordance with MATS results. For example the exon 3 (43bp) of *ptpn4b* and the exon 4 (66bp) of *tfpia* were skipped in *SRSF5a* morphants (MO) compared to control embryos (con). A negative control reaction (without reverse transcription) was performed and no amplification was observed (results not shown).

**6.** Shen S, *et al.* (2012) MATS: a Bayesian framework for flexible detection of differential alternative splicing from RNA-Seq data. *Nucleic Acids Res* 40(8):e61.

### Validation of DE genes by quantitative real time PCR analysis

12 interesting genes were chosen to be validated by qRT-PCR. The results were in agreement with RNA-seq. However the fold-change varied among the different approaches.

(a)




(b)

| Gene name | Description | log2FoldChange | | | |
|---|---|---|---|---|---|
| | | EdgeR | DESeq | Cufflinks | qRT-PCR |
| tcap | Titin cap | 2,59438249 | 2,75714014 | 2,80467 | 2,02188 |
| egr1 | Early growth response factor 1 | 0,62045969 | 0,71107524 | 0,806293 | 1,378747 |
| oc90 | otoconin 90 | 2,50735601 | 2,34161925 | 1,96179 | 1,165015 |
| igfbpA | insulin like growth factor binding protein A | 2,16677947 | 2,19189766 | 2,21237 | 1,269788 |
| junbb | junb proto oncogen b | 1,73694681 | 1,84407232 | 2,03194 | 2,310718 |
| bcl3 | b cell lymphoma 3 | 2,2327888 | 2,35697114 | 2,46014 | 2,352740 |
| foxj1a | forkhead box j1a | 1,33370196 | 1,47890577 | 1,40772 | 0,940047 |
| atoh7 | atonal homolog 7 | -1,05118717 | -1,0189777 | -1,03586 | -0,162117 |
| six7 | sine oculis homeobox homolog | -1,14577022 | -0,99895105 | -0,976411 | -1,316877 |
| vsx1 | visual system homeobox 1 | -0,88985455 | -0,7900168 | -0,729386 | -1,405075 |
| C9 | Complement component 9 | -1,67951514 | -1,73124535 | -1,72352 | -1,925773 |
| thrb | Thyroid hormone receptor beta | -1,16502802 | -1,11877313 | -1,1029 | -1,091577 |

(a) qRT-PCR validation of DE genes relative to the expression of these genes in control embryos. Error bar indicates standard error of triplicate qRT-PCR reaction. (b) Table including the 12 genes chosen for validation and the fold change values observed for each approache used.

**1.** Trapnell C, *et al.* (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature protocols* 7(3):562-578. **2.** http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html accessed on august 2013 developped by Simon Anders at EMBL heidelberg ; **3.** Robinson MD, McCarthy DJ, & Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1):139-140.
**4.** Anders S, *et al.* (2013) Count-based differential expression analysis of RNA sequencing data using R and Bioconductor. *Nature protocols* 8(9):1765-1786; **5.** Eden E, Navon R, Steinfeld I, Lipson D, & Yakhini Z (2009) GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics* 10:48.

## Conclusions

Our results suggest an important role of the SRSF5a splicing factor during the embryonic development of *Danio rerio* especially in retina formation and brain development. Rescue experiments provided evidence for the specificity of the splicing morpholino used to perform *SRSF5a* knock-down. RNA-sequencing analysis on control and morphant transcriptomes allowed us to determine SRSF5a dependent genes and the associated biological processes (GO analysis). The defect phenotypes are consistent with the RNA sequencing results as many differentially expressed genes between the two conditions are implicated in eyes and nervous system development. 12 differentially expressed genes were validated by qRT-PCR analysis. MATS program analysis permitted to pinpoint 377 splicing event differences in the two samples. Some exon skipping events had already been validated. Other differentially transcribed genes associated with other splicing events (intron retention, etc) have to be validated in the two samples. Finally, all of these results have to be confirmed with *SRSF5a* mutants generated by TALEN mediated gene targeting.

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