Alternative pre-mRNA processing by the RNA binding protein Sam68 ensures metabolic reprogramming in the skeletal muscle

Ramona Palombo [1,2*] , Gloria Guizzo [2] , Elisa De Paola [2] , Daniela Caporossi [1] and Maria Paola Paronetto [1,2]

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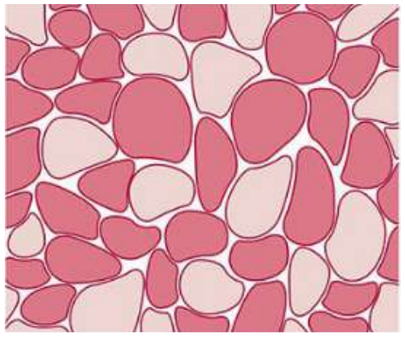
Acknowledgments:

SKELETAL MUSCLE FIBERS

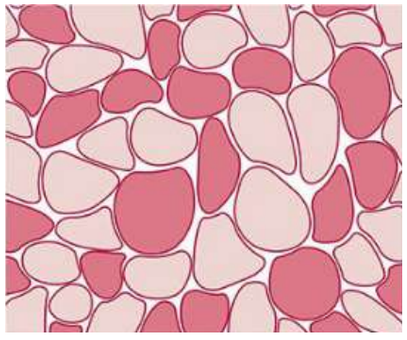
Slow Twitch



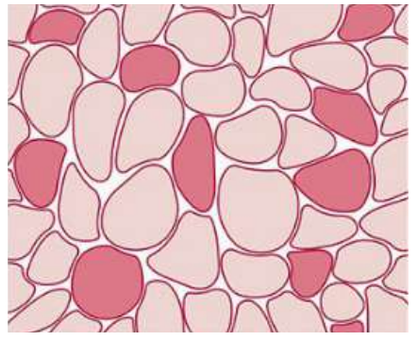
(slow oxidative fibers)

Soleus

Fast Twitch



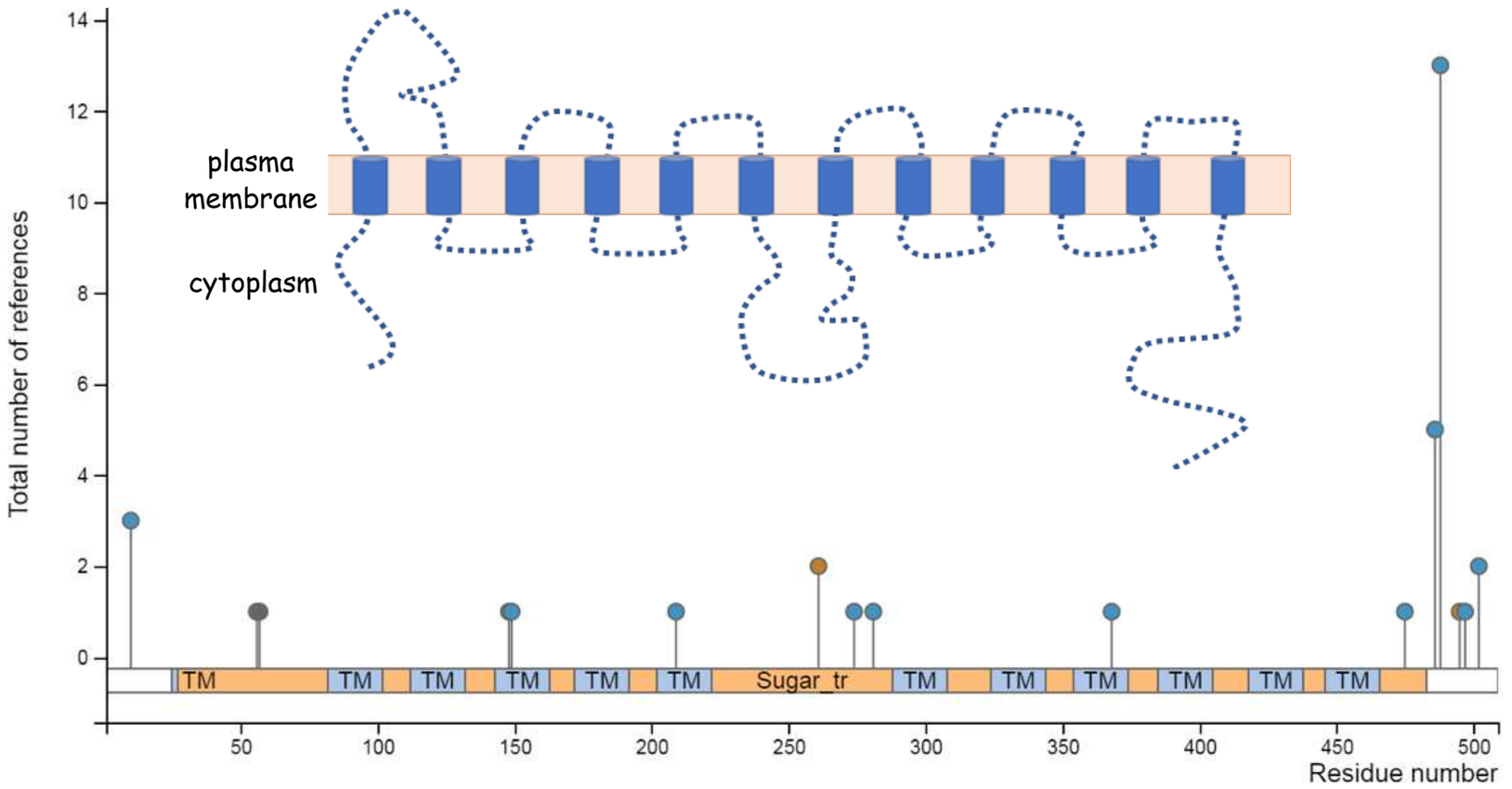
(oxidative fibers)



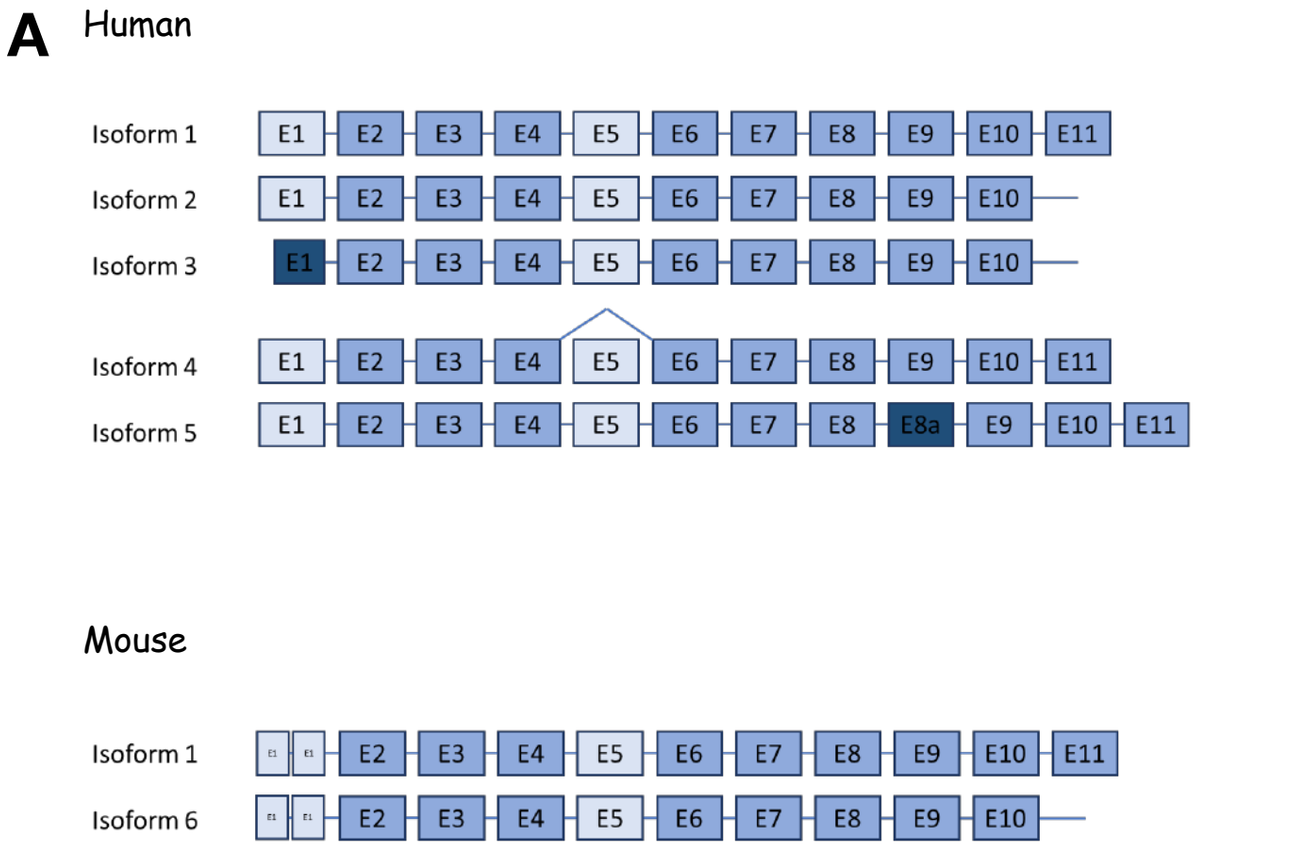
(glycolytic fibers)

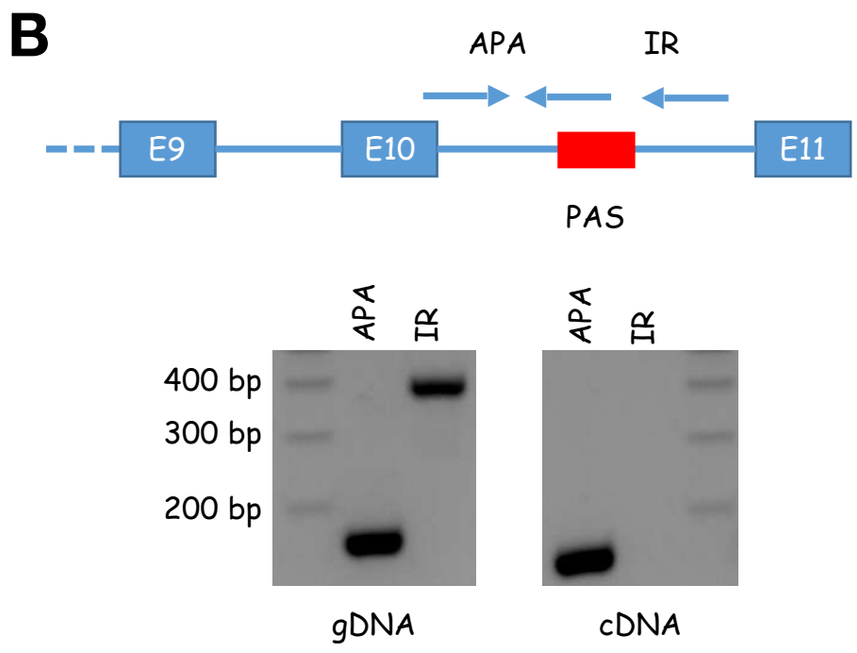
EDL

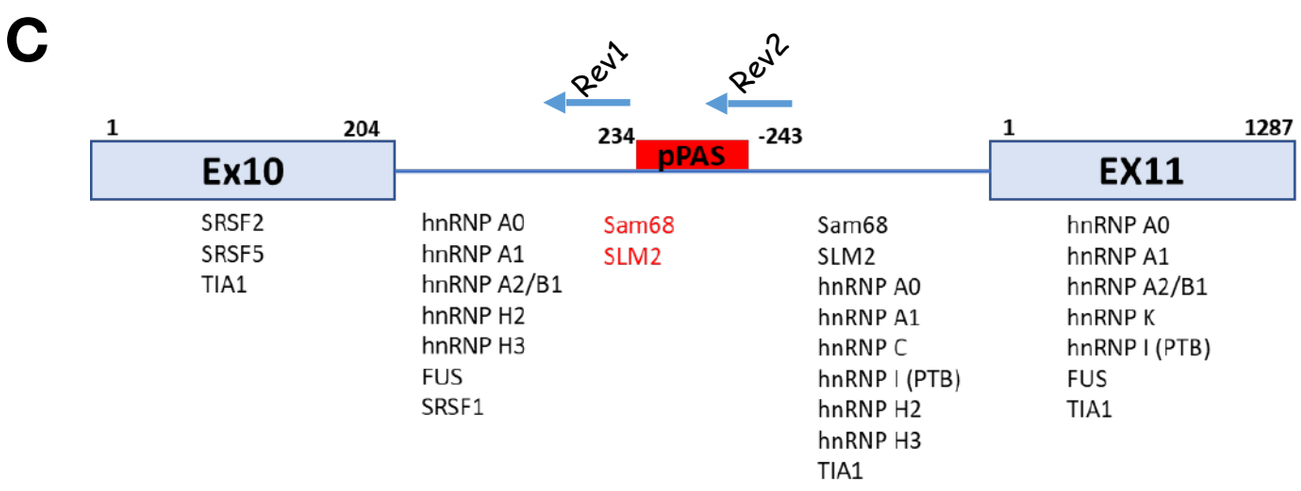
GLUT4

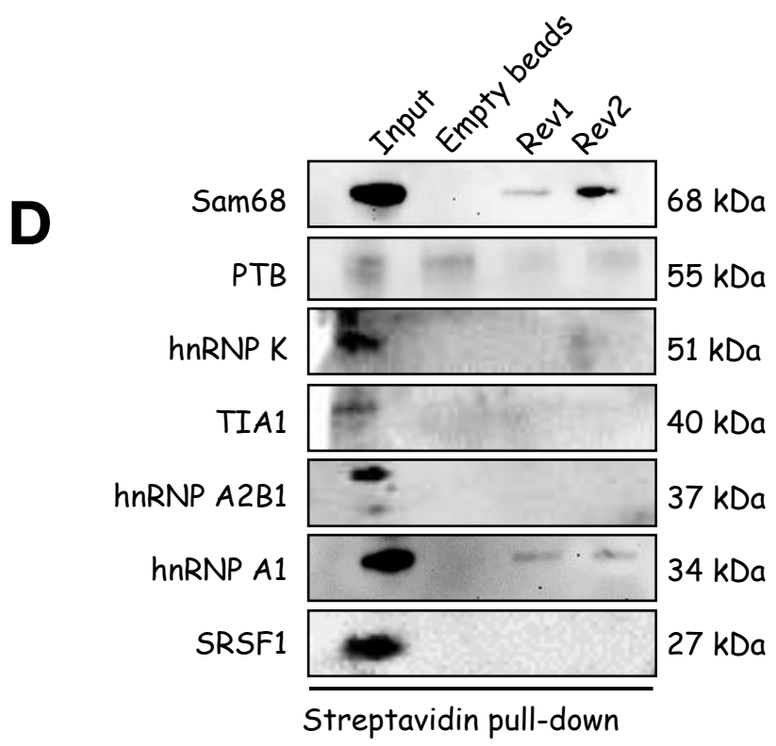


Slc2a4 alternative splicing is regulated by Sam68

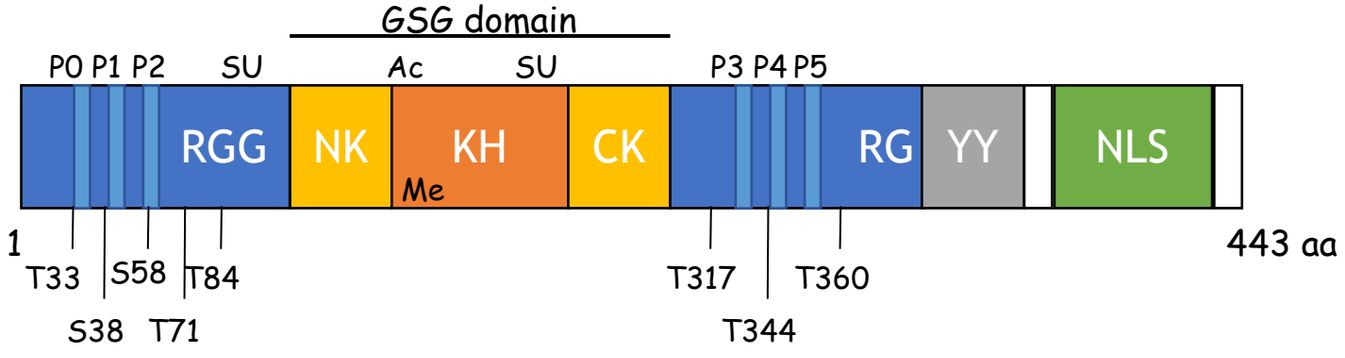




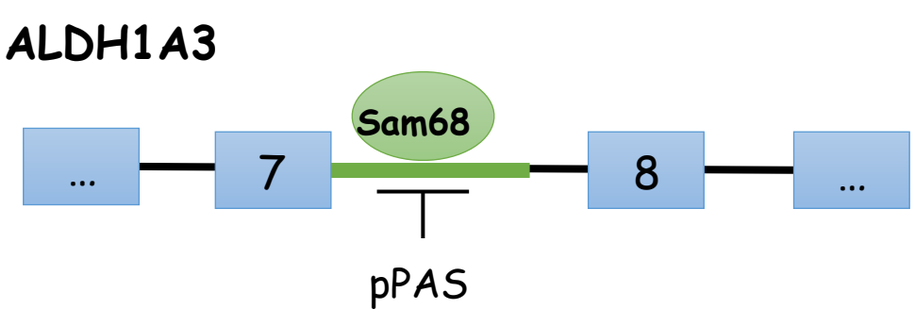




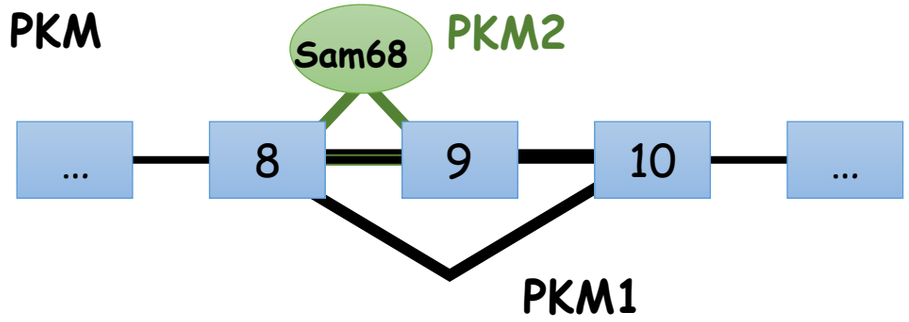
Sam68 promotes glycolytic metabolism

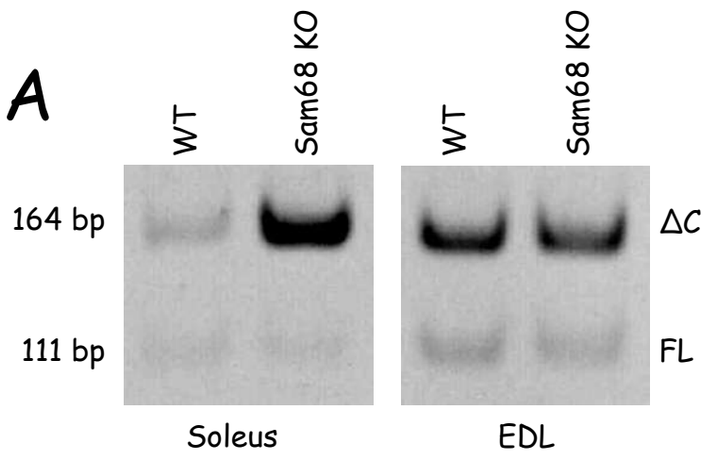


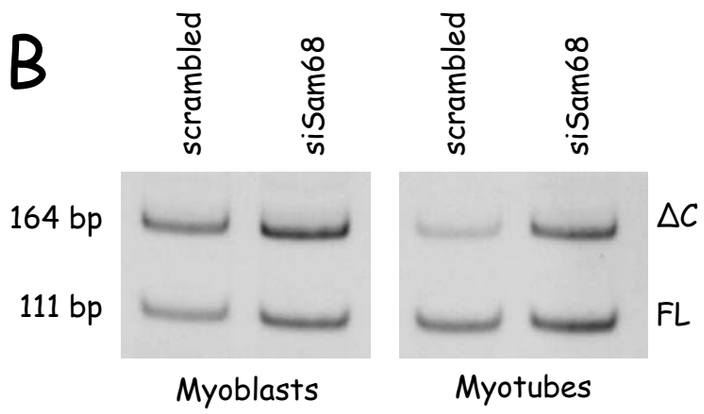
Sam68 modulates *Aldh1a3* pre-mRNA 3'-end processing, binding to an intronic polyadenylation site and preventing its recognition and premature transcript termination (La Rosa P., *et al.,* 2016).

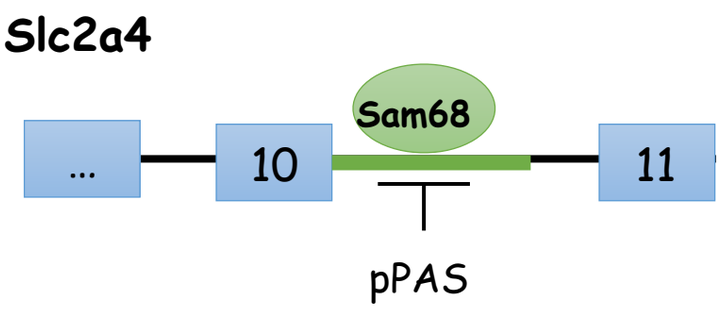


Sam68 is also associated with decreased *PKM1*/*PKM2* ratio, which positively contributed to the glycolysis procedure (Zhao J., *et al.,* 2020).

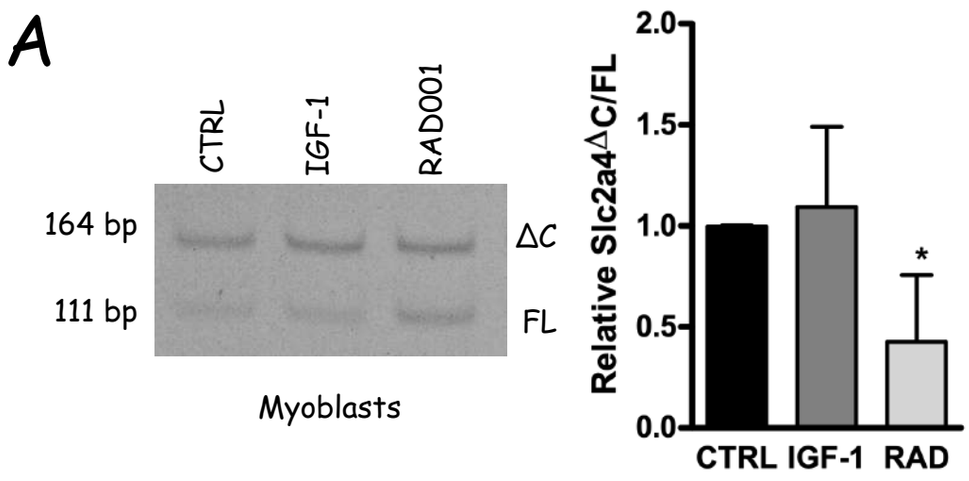








Signaling pathways perturbation affects Slc2a4 splicing.



Conclusions

We identified the RNA binding protein Sam68 as a novel regulator of *Slc2a4* pre-mRNA splicing.

Sam68 is already known to promote glycolytic metabolism in mouse neural progenitor cells by modulating *Aldh1a3* pre-mRNA 3'-end processing and in colorectal cancer by regulating *PKM2* alternative splicing.

The molecular mechanisms driving Sam68 splicing activity on *Slc2a4* transcript may unveil new insights into the role of glucose transport regulation in physiological and pathological conditions, including insulin resistance.

Alternative pre-mRNA processing by the RNA binding protein Sam68 ensures metabolic reprogramming in the skeletal muscle

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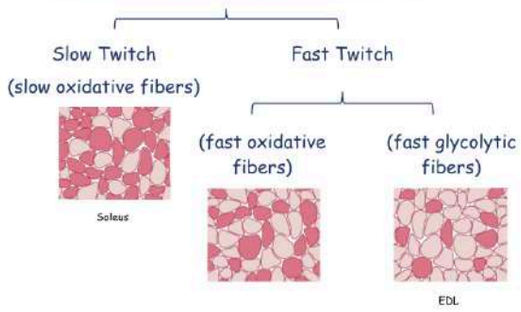
Skeletal muscle is one of the most dynamic tissues in the body, inheriting the ability to fine tune its response to environmental and physiological stimuli, including exercise, diet, disuse, and disease. To achieve skeletal muscle adaptations, transcriptional and post-transcriptional programs are carried out by muscle cells. In addition, a complex network of RNA binding proteins is engaged to achieve alternative splicing programs, thus increasing muscle proteome diversity.
Exercise activates signaling molecules to promote physiological adaptations, such as fiber type transformation, angiogenesis, and mitochondrial biogenesis. The underlying mechanisms involve a complex interplay of signaling pathways and downstream regulators, sensing the energy state and promoting glucose metabolism and fatty acid utilization.
Glucose uptake is a crucial event for energy supply, and GLUT4 protein is the main actor in glucose transport in brain and muscle. GLUT4 pre-mRNA, encoded by *Slc2a4* gene, is affected by alternative splicing: in addition to the main full-length isoform, a shorter transcript is produced, which leads to a truncated protein. We found that the RNA binding protein Sam68 modulates GLUT4 pre-mRNA processing. Sam68 expression promotes the full length GLUT4 isoform, whereas its depletion leads to the truncated GLUT4. Mechanistically, we found that Sam68 binds to and inhibits the recognition of an alternative poly-adenylation signal located in the intron 10 of the pre-mRNA.

Accordingly, *Sam68-/-* muscle biopsies, which are enriched in type I fibers, displayed increased level of the truncated *Slc2a4* isoform. Cross-linking and immunoprecipitation experiments in mouse myoblasts document that stimulation of the IGF1 signaling promotes Sam68 tyrosine phosphorylation and inhibits *Slc2a4* binding.

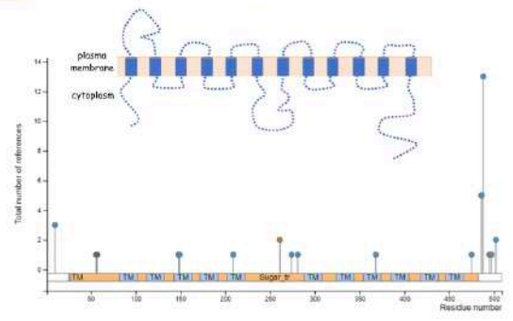
Collectively, our results identify Sam68 as a novel regulator of glucose homeostasis in the skeletal muscle, and highlights an unprecedented link between Sam68, GLUT4 and muscle glycolytic pathway.

1 SKELETAL MUSCLE FIBERS



Skeletal muscle fibers are classified basing on their speed of contraction and metabolic pathways:
Type 1 (Slow-Twitch) fibers use aerobic metabolism (oxygen fueled) for less-explosive, sustained movements. They do not contact forcefully, so they use less energy.
Type 2a (Fast-Twitch Oxidative) fibers have higher myosin ATPase activity than type 1 fibers, giving them a faster contraction time, and they use both aerobic and anaerobic metabolic pathways.
Type 2b (Fast-Twitch Glycolytic) fibers have a very fast contraction time, using anaerobic metabolism (without oxygen). They have very high force production but fatigue very easily.

2 GLUT4



GLUT4 is the insulin-regulated glucose transporter found primarily in adipose tissues and striated muscle (skeletal and cardiac). Schematic representation of the protein showing the 12 transmembrane domains, the cytosolic C-terminal and N-terminal domains, and the large cytosolic and extracellular loops. On the bottom, a lollipop plot indicates the post-translational modifications of GLUT4, such as phosphorylation (blue), ubiquitylation (yellow), and N-glycosylation (grey). The functional consequences of these post-translational modifications impact cellular localization and function of GLUT4 protein (Bryant, N., *et al.* 2002).

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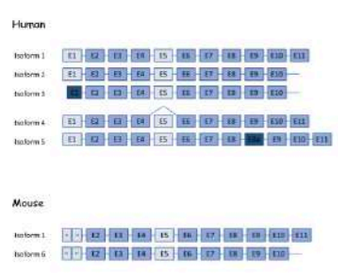
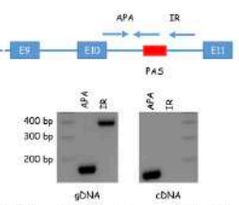


Human and mouse alternative splice isoforms encoding GLUT4. Exons are represented by blue boxes. Human isoform 2 and 3 and mouse isoform 6 display shorter transcripts due to the absence of the terminal last exon 11.

4



Analysis of *Slc2a4* isoforms highlighted the possibility of a cryptic alternative last exon (ALE) residing in the proximal part of intron 10, suggesting the existence of an alternative PAS in this intron and premature termination of the transcript. To verify this hypothesis, we retrotranscribed polyadenylated transcripts using oligo-dT, and we performed RT-PCR analyses using a forward primer in exon 10 and reverse primers located either upstream or downstream of the alternative PAS (APA and IR). A spliced transcript containing intron 10 sequences could be amplified only with the primers situated upstream of the alternative PAS, whereas PCR analysis from genomic DNA could amplify either with primers located upstream or downstream the PAS..

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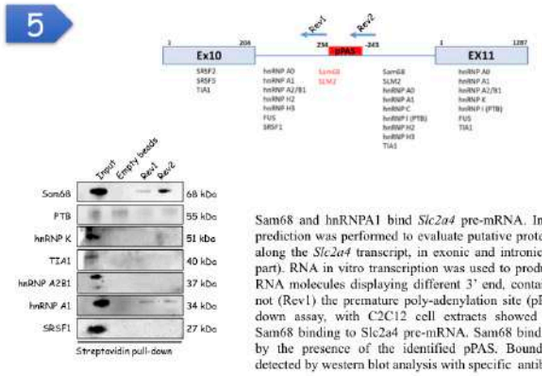
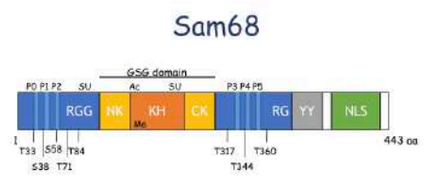


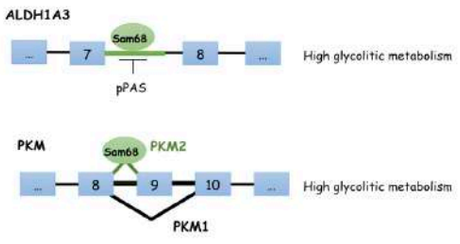
Sam68 and hnRNPA1 bind *Slc2a4* pre-mRNA. In silico analysis prediction was performed to evaluate putative protein binding sites along the *Slc2a4* transcript, in exonic and intronic regions (upper part). RNA in vitro transcription was used to produce biotinylated RNA molecules displaying different 3' end, containing (Rev2) or not (Rev1) the premature poly-adenylation site (pPAS). RNA pull down assay, with C2C12 cell extracts showed hnRNPA1 and Sam68 binding to Slc2a4 pre-mRNA. Sam68 binding is enhanced by the presence of the identified pPAS. Bound proteins were detected by western blot analysis with specific antibodies.

6 Sam68



Schematic representation of SAM68 protein domains. SAM68 protein is composed of the GRP33/SAM68/GLD-1 (GSG) domain, formed by a single heterogeneous nuclear ribonucleoprotein particle K (hnRNP K) homology domain (KH) embedded in two flanking regions NK and CK, six consensus proline-rich motifs (P0–P5), arginine/glycine/glycine (RGG) and arginine/glycine (RG) boxes, C-terminal tyrosine-rich domain (YY), and a nuclear localization signal (NLS). Proline residues are responsible for Sam68 interaction with SH3 (Src homology 3) and WW domain containing proteins. Tyrosine phosphorylation on Sam68 by soluble kinases reduces the ability of Sam68 to bind RNA compared to its dephosphorylated state. Methylation (Me) contribute to Sam68 nuclear localization. Other post-transductional modification are reported, such as Sumoylation (SU) and Acetylation (Ac). (Frisone P. *et al.*, 2015)

7 Sam68 SPLICING MODULATION



Sam68 regulates alternative splicing of genes implicated in glucose metabolism. Sam68 modulates *Aldh1a3* pre-mRNA 3'-end processing, binding to an intronic polyadenylation site and preventing its recognition and premature transcript termination (La Rosa P., *et al.*, 2016). Sam68 is also associated with decreased *PKM1/PKM2* ratio, which positively contributed to the glycolysis procedure (Zhao J., *et al.*, 2020).

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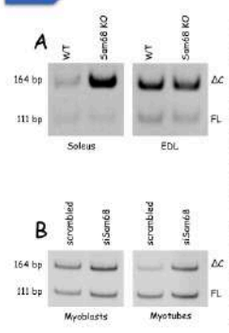

Sam68 regulates *Slc2a4* splicing. *Slc2a4* 3'-end processing splicing event was evaluated in mouse muscle tissue (A) and in C2C12 mouse myoblast (B). PCR products were separated on 2% agarose gel. Primers were designed to produce an amplicon of 111 bp for the full-length isoform (FL), and an amplicon of 164 bp for the shorter *Slc2a4* transcript (ΔC). (A) Sam68 KO soleus muscle shows increased level of *Slc2a4*ΔC isoform. On the contrary, Sam68 depletion does not affect *Slc2a4* isoforms in the fast glycolitic fibers of EDL muscle (extensor digitorum longus). (B) C2C12 myoblast and myotubes (6 days in differentiation medium) were compared for the expression of the splicing isoform of *Slc2a4*. Sam68 silencing enhance *Slc2a4*ΔC isoform in myoblasts and reverted the expression of higher level of Slc2a4FL in myotubes.

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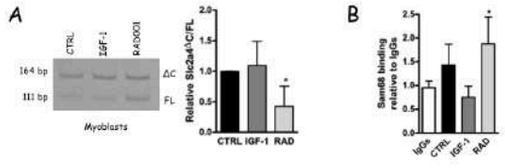

Signaling pathways perturbation affects *Slc2a4* splicing. (A) C2C12 myoblasts treated with IGF-1 and RAD001 showed different *Slc2a4* mRNA processing. IGF-1 and RAD001 promoted *Slc2a4*ΔC and *Slc2a4FL* respectively. Histograms represent the ratio between the two *Slc2a4* isoforms from three independent experiments ± S.D. (B) Histogram bars indicate the binding of Sam68 to *Slc2a4* pre-mRNA normalized to IgGs signal. (* = Student t test p-value <0.05)

Insulin and IGF-1 increase glucose consumption, influencing the glycolytic pathway activation. (Chun Y. Wong, *et al.*, 2020)
RAD001 (everolimus) is an mTOR inhibitor, and reduces the activity of most of the glucose-related metabolic pathways. (Yoshida K., *et al.*, 2017)

Conclusions

GLUT4 is primarily expressed in skeletal muscle to facilitate insulin-stimulated glucose uptake. Insulin and muscle contraction initiate the tyrosine phosphorylation cascade that stimulate GLUT4 translocation from the intracellular GLUT4 storage vesicles (GSVs) to the plasma membrane.

The presence of an alternative isoform lacking the C-terminal portion of the protein (GLUT4ΔC) could modulate the insulin response, as suggested by the differential expression of *Slc2a4*ΔC and *Slc2a4FL* in different skeletal muscles and during myogenic differentiation. Either inhibition of the mTOR signalling pathway or stimulation by IGF-1 affect *Slc2a4* pre-mRNA splicing.

Herein we identified the RNA binding protein Sam68 as a novel regulator of *Slc2a4* pre-mRNA splicing. Sam68 is already known to promote glycolytic metabolism in mouse neural progenitor cells by modulating *Aldh1a3* pre-mRNA 3'-end processing and in colorectal cancer by regulating *PKM2* alternative splicing. The molecular mechanisms driving Sam68 splicing activity on *Slc2a4* transcript may unveil new insights into the role of glucose transport regulation in physiological and pathological conditions, including insulin resistance.