mTOR-driven widespread exon skipping renders multifaceted gene regulation and proteome complexity

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Abstract

Mammalian target of rapamycin (mTOR) pathway is crucial in cell proliferation. Many have associated its dysregulation with numerous human pathogenic processes. Previously, we reported 3'-untranslated region (UTR) length dynamics by alternative polyadenylation in the mTOR-activated transcriptome and its impact on the proteome. Here, we further explored the mTOR-activated transcriptome with regard to alternative splicing (AS) events and their functional relevance. We employed cellular models with genetic or pharmacological manipulation of mTOR activity and investigated the changes of transcriptome profile using RNA-Seq experiments and a custom-developed AS-Quant pipeline. Strikingly, we found that hyperactivation of mTOR in cells promotes transcriptome-wide exon skipping/exclusion. These AS events multifariously regulate the proteome: exon-skipping in the coding regions widely affects functional domains in the proteome; exon-skipping in the 5'-UTR can control translation efficiency. Moreover, a number of these exon-skipping events affects potential ubiquitination and phosphorylation sites and produces protein isoforms with varying stabilities. Furthermore, some of these exon-skipping events can switch transcripts from being coding to non-coding or vice versa, suggesting AS as a built-in post-transcriptional molecular switch for gene expression and/or functional regulation. Notably, we found that mTOR-activated widespread exon skipping is in part facilitated by splicing factor Srsf3. Our study reveals previously unappreciated mTOR-coordinated post-transcriptional pathways that regulate cellular proteome. These findings also highlight multifaceted mechanisms of AS in controlling functional proteome.

Introduction

Multilevel pre-mRNA processing, including capping, splicing and polyadenylation, occurs during eukaryotic gene expression[1–3]. Splicing of pre-mRNA is critical as it splices out introns to join exons and produces uninterrupted coding DNA sequences (CDS) for translation[4]. Alternative splicing can occur during pre-mRNA splicing by alternating the inclusions of exons or part of exons to produce mature mRNAs[5–7]. Alternative splicing increases the genome capacity as it potentiates the production of diverse protein isoforms from a gene[8,9]. Previous reports have shown that protein isoforms produced by alternative splicing play distinct roles in various aspects of biology and pathogenesis[10]. Dynamic organization of numerous *trans*-acting RNA-binding proteins (RBPs) and *cis*-acting elements in pre-mRNA upon specific circumstances (*e.g.* aberrant signaling cascade activation in cancers) drives alternative splicing[11,12]. Although alternative splicing is a critical layer of post-transcriptional regulation of gene expression in eukaryotic cells[13,14], there is still much to be explored concerning its regulation and its impact on the resulting proteome.

mTOR is a serine/threonine kinase and is the major component of two multi-protein complexes known as mTOR Complex 1 (mTORC1) and 2 (mTORC2)[15–17]. The heterodimer of tuberous sclerosis complex (TSC1 and TSC2) is a negative regulator of mTORC1. mTOR directly phosphorylates two substrates, p70S6 Kinase 1 (S6K1) and eIF4E Binding Protein (4E-BP), and they in turn promote general protein synthesis. Particularly, studies have shown that mTOR can tightly control the translation of a subset of mRNAs that contain the 5'-terminal oligopyrimidine tract (5'-TOP)[18–21]. Moreover, by activating transcription networks, the mTOR signaling pathway plays a central role in regulating various cellular metabolism pathways such as lipid/mitochondria metabolism and stress response pathways including nutrient/growth factor deprivation, ER stress,

hypoxia, and oxidative stress[15,22]. The dysregulation of mTOR signaling pathway can thus contribute to many pathological conditions. Furthermore, recent studies have also revealed a role of mTOR signaling in post-transcriptional regulations. For example, our previous study showed that hyperactivation of mTOR promotes transcriptome-wide alternative polyadenylation (APA) in the 3'-untranslated regions (3'-UTR) of transcripts, leading to 3'-UTR shortening[23]. This transcriptome-wide changes of molecular event modulate downstream cellular pathways including ubiquitin-mediated proteolysis and ER stress responses.

mTOR kinase has been shown to phosphorylate and activate tumor suppressor p53 (Jung oncogene 2018). However, other regulatory venues, such as post-transcriptional processing by mTOR signaling and its impact on p53, remain largely unexplored. Mdm4 is a key negative regulator of p53[24,25]. Full-length, functional mdm4 protein is generated by the inclusion of exon 6 (exon 7 in humans). The exon 6 skipped mdm4 transcript contains a premature stop codon and degrades rapidly by the nonsense mediated decay pathway (NMD)[26]. Thus, there are numerous ongoing efforts in therapeutically targeting mdm4 exon 6 splicing to regulate p53 activity and the transcriptional control of its target genes in cancers[27].

In this study, we investigate mTOR-driven transcriptome changes in alternative splicing and their impact on the resulting proteome using cellular models with genetic or pharmacologic manipulations of mTOR activity. Strikingly, we discovered that mTOR activation generally prefers exon skipping in pre-mRNA splicing. This transcriptome-wide exon skipping serves as multifaceted post-transcriptional gene regulatory mechanisms in the mTOR-activated proteome. The widespread exon skipping occurrence diversifies the proteome by changing functional domains, post-translational modifications, and protein stability in a cell and tissue specific manner. Additionally, we revealed that a significant portion of these exon skipping events is controlled by

the upregulation of splicing factor srsf3 and its binding on or near the alternatively spliced exons in the mTOR-activated transcriptome. Specifically, the increase of srsf3 expression by mTOR activation suppresses mdm4 exon 6 splicing, which in turn downregulates mdm4 full length transcript expression. This results in p53 activation and an increase expression of apoptotic gene Bax in mTOR-activated cells. Our studies provide an unprecedented insight of a regulatory axis of mTOR and p53 via Srsf3-mediated mdm4 alternative splicing.

Results

Changes in cellular mTOR activity leads to transcriptome-wide exon skipping

To understand the effect of mTOR activation on cellular transcriptome, we first analyzed the differential expression of splicing factors from wild type (WT) and *Tsc1-/-* mouse embryonic fibroblasts (MEFs) (Fig. 1A). Interestingly, we found many splicing factors, including U2AF heterodimer and SRSF proteins, are differentially expressed, suggesting there is transcriptomic alternative splicing regulation by mTOR activation. We then used our custom-developed AS-Quant pipeline[28] to profile alternative splicing events in our previously reported RNA-Seq data from wild type (WT) and *Tsc1*$^{-/-}$ MEFs[23]. Interestingly, in this comparison, we found that 166 cassette type alternative splicing events (66%) favored exon inclusion whereas 86 events (34%) preferred exon exclusion in WT compared to *Tsc1*$^{-/-}$ MEFs (Fig. 1B). These observations suggest that hyperactivation of cellular mTOR leads to widespread exon skipping in cassette type alternative splicing. Other types of alternative splicing events such as alternative 5'-splice site and alternative 3'-splice site also showed partial exon skipping by alternating the splice site (62% and 57%, respectively) (Fig. 1B). To test the idea of general exon skipping of alternative splicing in post-transcriptional processing of pre-mRNA by the activation of mTOR, we inhibited cellular mTOR activity by the treatment of potent mTOR inhibitor, Torin 1, and profiled alternative splicing events using RNA-Seq experiments and AS-Quant analyses. Strikingly, in the case of pharmacological inhibition of mTOR, we found that exon inclusion in cassette type alternative splicing was almost exclusively preferred (497 events; 96%) (Fig. 1C). The exon inclusion by pharmacological inhibition of mTOR was consistent with other types of alternative splicing events with varying degrees (59% in alternative 5'-splice site and 82% in alternative 3'-splice site) (Fig. 1B). To test whether these observations are conserved in humans, we treated two breast cancer cell

lines (MCF7 and MDA-MB-361) with Torin 1 and conducted alternative splicing profiling experiments. Consistently, a pharmacological inhibition of mTOR significantly affected transcriptome-wide alternative splicing events and increased exon inclusion in cassette type alternative splicing (63% in both MCF7 and MDA-MB-361) (Fig. 1D and 1E). However, unlike the results from MEFs, other types of alternative splicing events did not show significantly exon inclusion-biased alternative splicing upon the inhibition of mTOR (Fig. 1D and 1E). Together, these findings provide evidence that the mTOR activation escalates the frequency of exon skipping during pre-mRNA splicing and reprograms the transcriptome.

mTOR-driven alternative splicing in the 5'-UTR determines translation

To confirm our analyses, we conducted real time PCR analyses on transcript isoforms produced from several genes using total RNAs purified from WT, *Tsc1*$^{-/-}$, *Tsc1*$^{-/-}$ MEFs treated with Torin 1. As shown in Fig 2(A), relative quantitation of exon-included and -excluded transcript isoforms confirmed our AS-Quant analyses. As these transcript isoforms change their coding capacity by deleting part of coding regions and may produce protein isoforms with a different size, we carried out western blot analyses using commercially available antibodies to Trip10. As indicated in the western blot analyses, Trip10 produces two protein isoforms in WT and *Tsc1*$^{-/-}$ MEFs and the stoichiometry of the two protein isoforms alters as suggested in our RNA-Seq data analyses (Fig. 2B). Further examinations of the transcriptome-wide alternative splicing events, we found that the majority of these events occur in the coding regions (coding DNA sequences; CDS) of mRNA (65-68%) and more than 25% of alternative splicing events is found in the non-coding regions predominantly in the 5'-UTR of mRNA. Most of these molecular events alter the length of 5'-UTR without affecting the coding capacity. To examine if changes in the 5'UTR length via

alternative splicing affect translation efficiency, polysome fractionation was conducted using cytoplasmic extracts from mock or torin 1 treated *Tsc1*$^{-/-}$ MEFs and the distribution of two 5'-UTR isoforms was measured by RT-PCR and relative densitometry analysis. In the case of Itch and Gabpb2, transcript isoforms with longer 5'-UTR by exon inclusion formed polysomes more efficiently compared to the transcript isoforms with shorter 5'-UTR in the torin 1 treated cells (Fig. 2D). To a lesser extent, Cenpi transcript isoforms also exhibit differences in translation efficiency in mock treated cells (Fig. 2D). These results suggest that 5'UTR alternatively spliced transcripts driven by mTOR activation promotes different degrees of translation. Interestingly, further investigations on genes undergoing alternative splicing in the 5'-UTR disclosed that most of these genes do not contain 5'-TOP features. Thus, it is likely that 5'-UTR alternative splicing is another process of regulating translation by mTOR.

Alternative splicing reprograms mTOR-activated functional proteome

As indicated in Fig. 1D, the majority of the alternative splicing events controlled by mTOR activation is located within the coding region (CDS) of the mRNA. We asked whether these alternative splicing transcripts affecting the coding region are enriched in certain biological pathways (Fig. 3A). Based on the KEGG pathway enrichment analysis, we found that there are different pathways enriched in alternatively spliced transcripts in WT compared to Tsc1-/- MEFs. More pathways are enriched when comparing the mock treated and the Torin 1 treated MEFs. When compared to alternative splicing in the 5'UTR, those transcripts are enriched profoundly in metabolic, N-Glycan biosynthesis, and pathways relevant to cancer. Interestingly, spliceosome is a major hit for both the coding region and 5'UTR alternative splicing events by mTOR activation. Together, this suggests that alternative splicing changes by mTOR signaling can impact important

biological pathways. Additionally, exon skipping/inclusion by alternative splicing in CDS adds/removes protein domains, we investigated whether widespread exon skipping in the mTOR-activated transcriptome affects functional domains that are connected to biological pathways. Affected exon regions by alternative splicing events were linked to registered Pfam databases and those affected Pfam domains were further clustered by Gene Ontology (GO) term analyses. In our comparison between *Tsc1*$^{-/-}$ mock and Torin 1 treatment, we observed that 114 domains were affected by exon skipping in the mTOR-activated transcriptome (Fig. 3B). These domains, in turn, are interlinked with many GO terms. Interestingly, several Pfam domains like PKinase and PKinase_Tyr are affected in both mock and Torin 1-treated *Tsc1*$^{-/-}$ transcriptome (Fig. 3B), suggesting that signaling cascade pathways upon changes of mTOR activity are not only regulated by mTOR kinase signaling but also modulated by affected kinases through alternative splicing. We next asked whether the deletion/addition of extra domains by alternative splicing affects the stability of protein isoforms. To test the stability of two protein isoforms, we treated cells with cycloheximide to prevent protein synthesis and traced down the amount of protein isoforms by the time course (Fig. 3C). In the case of Sirt2 and mdm2 isoforms, the long protein isoform is more stable than the short form. These results suggest that alternative splicing by mTOR regulates the stability of proteins, affecting the functional proteome. Both Sirt2 and mdm2 protein isoforms are generated by alternatively spliced exon that changes the translational initiation codon, thus affecting the N-terminus of the protein. N-terminal and/or C-terminal of proteins are known to be critical in determining the stability of protein. In many instances, these regions carry potential polyubiquitination sites which can serve as N- or C-degron[29]. Thus, we predicted the unique ubiquitination sites in the protein isoforms generated from mTOR-regulated alternative splicing using the Ubisite database (Fig.4A). We found that out of all the alternative splicing events in the

CDS region, (1130 unique sites gained/6832 of total predicted ubiquitination sites) or 16.5% of ubiquitination sites on protein isoforms are changed by mTOR-activated alternative splicing. These predictions suggest that alternative splicing is a way to regulate the stability of proteins by allowing more or fewer ubiquitination sites for proteasome-mediated degradation. In addition to ubiquitination, other post-translational modification sites can be changed by alternative splicing, which affects a protein isoform's function. Thus, we also predicted potential phosphorylation sites affected by alternative splicing using Scansite4.0 database (Fig. 4B). Similarly, we found (258 unique sites gained/2027 total phosphorylation sites) or 12.7% of phosphorylation sites on protein isoforms are changed by mTOR-activated alternative splicing. These results suggest that mTOR regulates the phosphoproteome via alternative splicing.

mTOR-driven exon skipping of mdm4 activates p53

Most alternative splicing events which occurred in CDS in our data affected functional domains of proteins as shown in Fig 3A. However, strikingly, we discovered that numerous cases of alternative splicing in CDS switch the characteristics of a transcript. Specifically, cassette exon inclusion or exclusion transitions the status of the transcript between coding and non-coding (Fig. 5A). Examples of transcripts transition from non-coding to coding and vice versa were validated by RT-PCR (Fig. 5B) Thus, it is likely that hyperactivation of mTOR leads to the skipping of exon in these genes and downregulates gene expression without affecting the amount of mRNA significantly. Interestingly, one of the cases switching from coding to non-coding transition by mTOR activation is mdm4, which is a negative regulator of tumor suppressor p53. The skipping of this alternative exon transforms the Mdm4 transcript from coding to non-coding transcript. Interestingly, this particular exon is preferentially included when mTOR activity is relatively low

(WT and *Tsc1*$^{-/-}$ MEFs plus Torin 1 compared to *Tsc1*$^{-/-}$ MEFs). However, it is skipped to make a non-coding transcript by hyperactivation of mTOR (Fig. 5B). This validates that mTOR activity modulation by knockdown of Tsc1 or by a pharmacological inhibitor Torin1 is able to downregulate mdm4 coding expression. As non-coding transcripts are more prone to be subjected to NMD, we asked whether the coding and noncoding transcripts of mdm4 have differences in mRNA stability. We treated Tsc1-/- MEFs with actinomycin to inhibit transcription and chased down the mRNA level of the two mdm4 mRNA isoforms (Fig. 5C). Consistent with previous studies[26], the non-coding (exon-skipped isoform) of mdm4 displayed a faster degradation compared to coding (exon-included isoform) of mdm4. Together, these results provide evidence that alternative splicing not only alters functional domains in proteins but also serves as a molecular switch to turn on or off the gene expression by making a transition between non-coding and coding transcript. This also suggests that mTOR regulates the stability of select transcripts in the transcriptome. MDM4 exon skipping is also conserved in humans. However, unlike in MEFs, MDM4 exon skipping in humans generates a truncated protein isoform. Similar to MEFs, modulation of mTOR activity by Torin 1 treatment showed a significant change of MDM4 alternative splicing (Fig. 5D). Together, these data suggest a conserved link between mTOR and p53 by MDM4 alternative splicing in humans.

Srsf3 upregulation by mTOR promotes exon skipping

In efforts to explain the widespread exon skipping phenomenon in the mTOR hyperactivated cells, we further analyzed the differential expressed splicing factors between WT and Tsc1-/- MEFs. We found that splicing factor Srsf3 is significantly upregulated in Tsc1-/- MEFs. To identify Srsf3-mediated alternative splicing, we knocked down Srsf3 expression in

Tsc1-/- cells and identified transcriptome-wide exons included events (Fig. 6A). When compared to the alternative splicing dataset between WT and Tsc1-/- MEFs, we found 13.7% (22/166 cases of mTOR-activated exon skipping) is regulated by srsf3. Similarly, 12.9% (64/497 cases) of alternative splicing from pharmacological inhibition of mTOR overlaps with the Srsf3 knockdown alternative splicing dataset. This data suggests that exon skipping by mTOR activation is partly due to the upregulation of srsf3 expression. To confirm Srsf3 binding at or near these alternative splicing exons, we validated srsf3 consensus binding site on mdm4 alternative splicing using RBPMap. We confirmed srsf3 putative binding site(s) near mdm4 alternatively spliced exon 6 (Fig. 6B). Together, our study reveals a new post-transcriptional gene expression regulation by mTOR signaling by widespread exon skipping. This is found to be mediated partly by the upregulation of splicing factor srsf3 (Fig. 6C).

Discussion

In numerous high profiling studies, differential gene expression analysis is by far the most widely used data analysis to understand the changes of transcriptome in a given biological or disease context[30,31]. However, in many cases, the catalog of differential gene expression is not sufficient in providing a thorough understanding of the phenotypic changes in biology as it does not entirely reflect the changes of the functional proteome. Specifically, one of the major drawbacks of differential gene expression analysis is that it does not account for the different mRNA isoforms generated through the post-transcriptional regulatory processing of mRNAs. Previous studies have reported mTOR's involvement in regulating Snrnp 70, a component of the spliceosome, for lipogenesis[32], suggesting mTOR signaling may have a role in splicing. Our study fine-tuned the role of mTOR in post-transcriptional mRNA alternative splicing and investigated individual aspects that influence steady state gene expression and the resulting proteome.

One of the key processes in mTOR-controlled gene expression is the translation activation of 5'-TOP containing mRNAs upon mTOR activation. In fact, 5'-TOP features are well characterized sequence elements that enable translation enhancement upon a specific signaling cascade, making the production of protein independent of mRNA amounts. To build upon mTOR's role in translational control, our findings show that 5'UTR alternative splicing is another way that mTOR affects translation. In addition to protein translation, we also showed that the functional proteome can be globally affected by alternatively spliced protein isoforms due to changes in the protein domains, post-translational modifications such as ubiquitination and phosphorylation as well as protein stability. Mutually exclusive tandem exons, such as pyruvate kinase (PKM1 and PKM2) and splicing factor U2AF1 (U2AF1a and U2AF1b) are known to have different functions in biology[33–35]. Particularly from our post-translational modification prediction analysis, Vcan,

Far1, and Lrcc40 tandem exon splicing protein isoforms have differences in phosphorylation sites. These newly gained residues may be phosphorylated or dephosphorylated under different cellular contexts.

One of the most interesting observations from our study is the transcriptome wide exon skipping by mTOR activation. This suggests that alternative splicing is largely biased towards exon skipping in cells with activated mTOR. Our investigation of alternative splicing in MEFs and human cancer cells showed that the degree of exon skipping in the transcriptome varies among cell types and organisms. This suggests that mTOR signaling diversifies the proteome in a tissue-specific and cell-specific manner with respect to the degrees of exon skipping.

Exon skipping phenomenon can be facilitated by numerous splicing factors and RNA-binding proteins. Our study provided a mechanistic insight of srsf3 involvement in driving a significant portion of the transcriptome-wide exon skipping in hyperactivated mTOR cells. This exon skipping occurrence also has implications in cell growth and apoptosis in highly proliferating cells and cancer cells as we showed that srsf3 expression suppresses mdm4 splicing, resulting in changes in p53 activity. Nonetheless, our study unravels a regulatory axis of mTOR/srsf3/mdm4/p53 in MEFs and human MCF7 cells. In summary, our study recognizes the importance of post-transcriptional regulation on gene expression and the functional proteome by mTOR signaling.

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Declarations

The authors declare no conflict of interest.

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Figure legends

Figure 1. Transcriptome-wide alternatively spliced exon skipping upon mTOR activation. A) Histogram of differential gene expression of splicing factors and RNA-binding proteins (RBPs) comparing between Tsc1-/- and WT MEFs. The x-axis indicates the 300 different RBPs analyzed. The y-axis represents the log2 fold change. B-E) Different categories of alternative spliced exon inclusion events preferentially expressed in B) WT vs. Tsc1-/- MEFs. C) Tsc1-/- mock vs Torin1 inhibitor treatment. D) Human MCF7 cells mock vs. Torin1 treatment. E) Human MDA-MB-361 cells mock vs. Torin1 treatment. Four different types of alternative splicing events are displayed. Alternative spliced exon inclusions are compared between WT and Tsc1-/- MEFs and between mock treated and Torin1 treated samples. Graphs generated to display the total number of exon inclusion events preferred by WT and Tsc1-/- MEFs and between mock treated and Torin1 treated samples.

Figure 2. mTOR-mediated 5'UTR alternative splicing transcripts regulate translation. A) RT-PCR validation of mTOR-regulated Camkk2 and Ranbp3 alternative splicing at exon 16 and at exon 3, respectively, in mock treated and 50uM Torin 1 treated Tsc1-/- MEFs. The RNA-seq alignment of the alternatively spliced exon is shown at the bottom. A schematic of the alternative spliced exon is shown on the left. Arrows denote the primer locations. B) Western blot analysis of Trip10 validating the endogenous alternative spliced Trip10 protein isoforms in WT, Tsc1-/- MEFs, control and Trip10 siRNA knockdown in Tsc1-/- MEFs in different exposures. The knockdown of Trip10 in Tsc1-/- MEFs confirms the Trip10 protein isoform bands. HnrnpA1 protein level serves as a loading control. C) Proportion of mTOR-regulated alternative splicing event in different regions of the mRNA. The pie chart on the left compares alternative splicing

events between WT and Tsc1-/- MEFs. The pie chart on the right compares alternative splicing events between mock treated and Torin1 treated Tsc1-/- MEFs. D) RT-PCR and cumulative plots illustrating mTOR-regulated 5'UTR alternative splicing transcripts in fractions obtained from sucrose gradient using cytoplasmic extracts from mock and torin1 treated Tsc1-/- cells. Fraction 5 is the monosome fraction and fraction 7-11 are polysome fractions. A schematic of the alternative splicing event for each transcript is shown on the left of the RT-PCR gel electrophoresis. The cumulative distribution of the alternatively spliced transcripts in each fraction is plotted against the summation of that isoform in all 11 fractions from mock treated and Torin1 treated samples. The x-axis indicates the fraction number and y-axis indicates the cumulative ratio. eef2 and hsp90ab1 are known mTOR-sensitive transcripts containing a 5'TOP motif and serve as a positive control for mTOR translational regulation.

Figure 3. Exon skipping renders mTOR-driven changes in functional proteome A) Affected KEGG pathways by mTOR-activated alternative splicing events using g:Profiler software. KEGG pathway enrichment from alternative splicing in the coding region is shown on the left and 5'UTR specific alternative splicing KEGG analysis is on the right. B) Impacted pfam domains and their associated GO terms by mTOR-activated alternative splicing events. Bioinformatics analysis of the functional pfam domains disrupted by alternatively spliced exons in the coding region (CDS) of the mRNA. Pink: domains gained in hyperactivated mTOR. Light purple: domains lost in hyperactivated mTOR. Green: domains maintained regardless of mTOR activity. Rectangle: Molecular Functions; Diamond: Biological Processes; Triangle: Cellular Pathways. Each of affected pfam domains associated with a GO term is indicated by a linkage to rectangle, diamond, and/or triangle. C) Western blotting analysis of overexpressed flag-tagged Sirt2 protein isoforms

display different stability in HEK293 treated with cycloheximide. Protein isoforms of Sirt2 are generated by alternative splicing of exon 2. Protein isoforms of Mdm2 are generated by alternative splicing of exon 3. HEK293 was transfected with flag-tagged Sirt2/Mdm2 long or short isoform. Cells were treated with 30ug/ml cycloheximide to inhibit translation machinery over a 12 hour period and cells were harvested in increments displayed on the western blot analysis. Cell lysates were run on SDS-PAGE gel for western blot analysis using antibodies against FLAG and Ribosomal protein S6 for loading control. Protein level was quantified using densitometry and normalized to S6 level. Overexpressed cells untreated with cycloheximide were set at 100%. Mean and SEM from two technical repeats were subjected to Two-tailed Student's t-test for statistical analysis. $P<0.05$ as significant (*). n.s. denotes no significance.

Figure 4. mTOR-regulated AS modulates proteome-wide post-translational modifications. A) Bioinformatic analysis of predicted ubiquitination and B) phosphorylation sites uniquely present between protein isoforms generated from mTOR-regulated alternative splicing events between mock treated and Torin1 treated Tsc1-/- cells. The number of unique sites located in the alternative splicing isoforms of the four categories is presented. The percentage of these unique sites is calculated against the total number of sites that are common between two exon-included and -excluded transcripts.

Figure 5. mTOR-driven exon skipping of mdm4 activates p53. A) Schematic diagram illustrating the transition between coding and noncoding transcript caused by alternative splicing of cryptic exons. B) RT-PCR validation of coding-to-noncoding isoform switch in Syce2 and mdm4 alternative splicing in the presence of Torin1 treatment in Tsc1-/- MEFs. C)

Semiquantitative RT-PCR of mdm4 transcripts from Tsc1-/- cells treated with 1ug/ml actinomycin D. Total RNAs were harvested and reverse transcribed into cDNA, which is used as a template for the RT-PCR. Quantitation of the RT-PCR was performed using densitometry and the data represented the data +/- SEM. Statistical analysis was done using two technical replicates with $p<0.05$. D) RT-PCR of human MDM4 alternative splicing transcripts in the presence of mock or Torin 1 treatment in human MCF7 cells. Band intensities were quantified using densitometry and plotted on the right.

Figure 6. Srsf3-mediated exon skipping in mTOR activated cells. A) Venn diagram illustrating the number of exon skipping transcripts overlapped between Mock/Torin1 and control/srsf3 knockdown datasets. On the left is comparing between WT/Tsc1-/- and control/srsf3 knockdown datasets. B) Diagram of putative srsf3 binding sites at or near the mdm4 alternative exon. C) Summary model of mTOR-mediated Srsf3 suppression of mdm4 alternative splicing and the resulting p53 activation.

Methods

Cell Lines

WT, Tsc1-/- MEFs, HEK293, MCF7, and MDA-MB-361 cells were cultured in Dulbecco's Modified Eagle Media (DMEM) (Gibco), in supplements with 10% FBS (Gibco), 100ug/ml streptomycin, and 100U/ml penicillin. All cell lines were cultured at 37˚C with 5% CO2.

Plasmids

cDNAs of mouse Sirt2 long form (NM_022432), Sirt2 short form (NM_001122765), mouse mdm2 long form (NM_010786.4), mdm2 short form (NM_001288586.2) were cloned into p3xFlag-CMV-14 plasmid.

Real-time PCR (RT-PCR) and Real-time quantitative PCR (RT-PCR) analysis

Total RNAs were isolated using the Trizol method recommended by manufacturer's protocol. cDNAs were made by reverse transcription using Oligo-d(T) and superscript III (Thermo Fisher Scientific). SYBR Green fluorescence was used for quantitative real-time PCR reactions. The relative expression between groups was measured using the ΔΔCt method. The list of primers used for RT-PCR and qRT-PCR is:

mouse Camkk2 RT-PCR forward: 5'-GGGAACCCGTTCGAAGGTAG-3'; mouse Camkk2 RT-PCR reverse: 5'-GCAGGGACCACCTTTCACAA-3'; mouse Ranbp3 RT-PCR forward: 5'-AAGCCTGCCGTCGCACCGTCTGTCT; mouse Ranbp3 RT-PCR reverse: 5'-CTTCTCCGGCTTCGGGACTGGAGC-3'; mouse Mdm4 RT-PCR forward: 5'-GCTAAGAAAGAATCTTGTTACATCAGC-3'; mouse Mdm4 RT-PCR reverse: 5'-ATGTCGTGAGGTAGGCAGTGTGTGA-3'; mouse Syce2 RT-PCR forward: 5'-AGCATCGGCAGAGTGAGAAC-3'; mouse Syce2 RT-PCR reverse: 5'-CCGTTTCCACAGTTTGGCAG-3'; mouse Itch RT-PCR forward: 5'-

TTCACAGTGGCCTTCTGGAAACAACG-3'; mouse Itch RT-PCR reverse: 5'-GGAATCAAGCTGTGGTCCACTGTCAGA-3'; mouse Cenpi RT-PCR forward: 5'-AGAGGCTGTGTGCTCGCCGAGGT-3'; mouse Cenpi RT-PCR reverse: 5'-TGAAAGCAGATGACTGGCCTACAGTAG-3'; mouse Gabpb2 forward: 5'-GAGAACCAACTCCTGCGAGTTGTC-3'; mouse Gabpb2 reverse: 5'-TCCCCAAGTCCACCAGAGACATCTTA-3'; human MDM4 forward: 5'-CGTCAGAGCTTCTCCGTGAAAGAC-3'; human MDM4 reverse: 5'-CTCTAGAATGTATGCATTTATGCTC-3'; mouse eef2 RT-PCR forward: 5'-AGTGTCCTGAGCAAGTGGTG-3'; mouse eef2 RT-PCR reverse: 5'-AGATCAGCGGTGAAGCCAAA-3'; mouse hsp90ab1 RT-PCR forward: 5'-CAGAAGGCTGAGGCAGACAA-3'; mouse hsp90ab1 RT-PCR reverse: 5'-AATCATGCGGTAGATGCGGT-3';

siRNA knockdown and antibodies

Cells were transfected with siRNAs synthesized by Integrated DNA Technologies (IDT) using RNAiMax Transfection reagents (Thermo Fisher Scientific) recommended by the manufacturer's protocol. siRNA targeting mouse Trip10: 5'-CCGUGGCUACCGAAGAUU-3'. Antibodies used for western blot analysis include: anti-Trip10 (No. F-10, Santa Cruz Biotechnology), anti-HnrnpA1 (4B10, Sigma Aldrich), anti-Flag (No. F3165, Sigma Aldrich), anti-S6 (No. 2317, Cell Signaling), Secondary antibody against mouse: goat-anti-mouse IgG-HRP (No. sc-2005, Santa Cruz Biotechnology), Secondary antibody against rabbit: goat-anti-rabbit IgG-HRP (No. sc-2004, Santa Cruz Biotechnology).

Polysome isolation and analysis

Polysomes from mock-treated and Torin1-treated Tsc1-/- MEF cell lysates were isolated using a sucrose gradient[36]. Cells were lysed in lysis buffer containing 20mM Tris pH=7.4, 150mM NaCl, 5mM $MgCl_2$, 1mM dithiothreitol, 100mb/ml cycloheximide, 1% TritonX-100. Cytoplasmic extracts were loaded onto the sucrose gradient of 5% to 45% followed by ultracentrifugation of 36,000 rpm for 2 hours at 4°C. Eleven fractions were collected in total. Total RNAs were isolated using the Trizol method stated above. Ten percent (v/v) of the total RNAs from each fraction was used for cDNA synthesis for RT-PCR analysis. The total intensity was determined from the band intensity of the transcript isoform of each fraction combined and set as 100%. The relative ratio of the band intensity from each fraction over the total intensity was calculated and was used to generate the cumulative plots.

mRNA stability

Cells were treated with 1ug/ml actinomycin D to inhibit transcript and were harvested at different time points in the course of 12 hours. Total RNAs were obtained using the Trizol method stated above.

Protein stability

Cells with treated with 30ug/ml cycloheximide to inhibit protein synthesis and were harvested at different time points. Cells were then lysed and lysates were run on SDS PAGE gels for western blot analysis.

RNA-seq Data Analyses

To evaluate the transcriptome features under mTOR-hyperactivation at single-nucleotide resolution, we performed RNA-Seq analyses of poly(A+) RNAs isolated from WT, *Tsc1*$^{-/-}$ MEFs, MCF7 and MDA-MB-361 cells. Paired-end reads were aligned to the mouse mm10 reference genome or human hg38 reference genome using TopHat with up to two mismatches allowed.

AS-Quant (*A*lternative *S*plicing *Quant*itation) pipeline

Alternative splicing analysis was performed using the AS-Quant pipeline[28]. Briefly, the pipeline categorizes the alternative splicing events into cassette exon, mutually exclusive, alternative 5' splice site, and alternative 3' splice site. Reads (n) from these alternatively spliced exons were measured and compared to reads (N) coverage of the rest of the transcripts. A 2x2 Chi-Square test was performed to determine the statistical significance for each alternative splicing event using the ratio of n/N. Events with a p-value <0.1 and a ratio difference <0.1 were considered real alternative splicing exons.

Quantification and Statistical Analysis

Signal intensity from gel electrophoresis or western blots was quantified using the ImageStudio software. Statistical analysis was performed by Two-tailed Student's test unless stated otherwise.

Data Availability

The accession numbers for the RNA-seq data are SRP056624.

Figures

Figure 1.

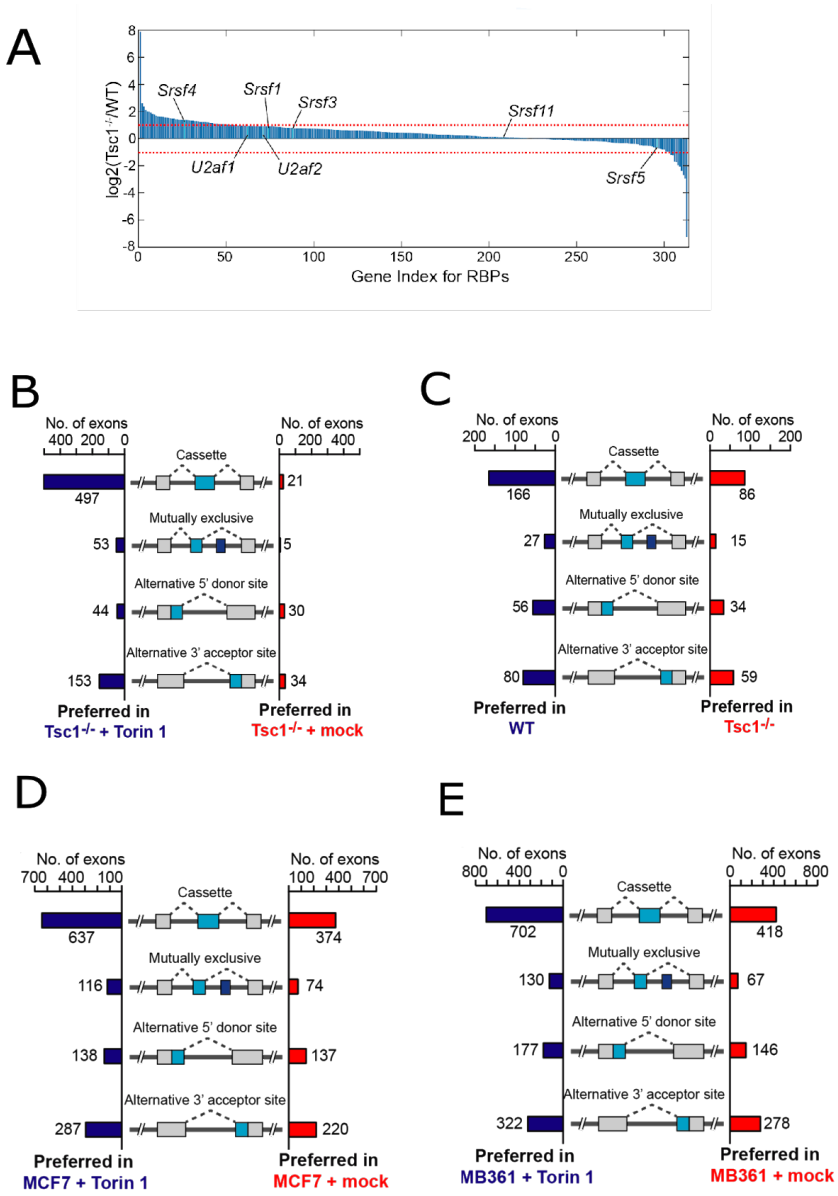


Figure 2.

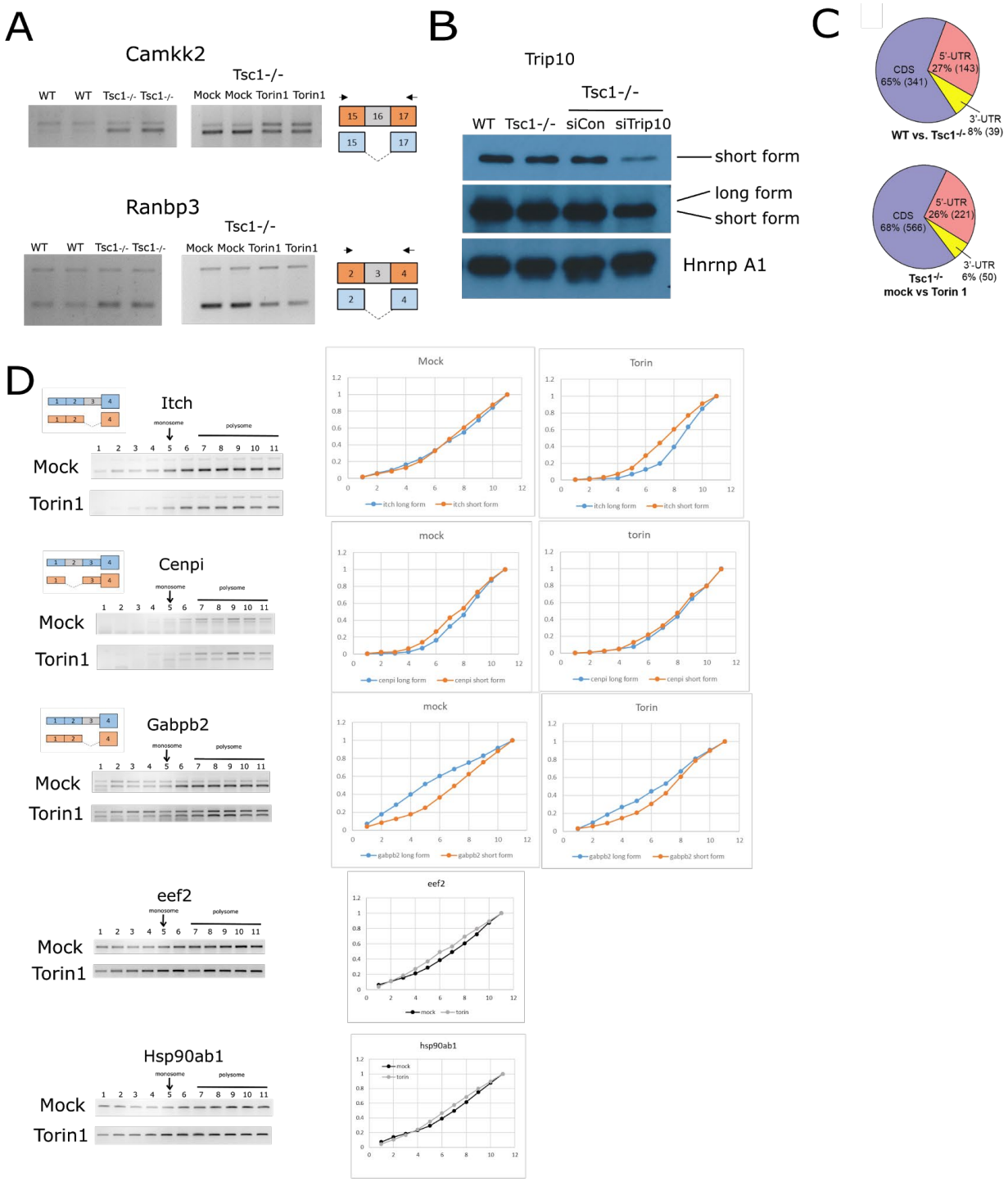

A
Camkk2
Tsc1-/-
WT WT Tsc1-/- Tsc1-/-
Mock Mock Torin1 Torin1
Ranbp3
Tsc1-/-
WT WT Tsc1-/- Tsc1-/-
Mock Mock Torin1 Torin1
B
Trip10
Tsc1-/-
WT Tsc1-/- siCon siTrip10
short form
long form
short form
Hnrnp A1
C
CDS 65% (341)
5'-UTR 27% (143)
3'-UTR 8% (39)
WT vs. Tsc1-/-
CDS 68% (566)
5'-UTR 26% (221)
3'-UTR 6% (50)
Tsc1-/- mock vs Torin 1
D
Itch
Mock
Torin1
Cenpi
Gabpb2
eef2
Hsp90ab1
monosome
polysome
Mock
Torin
mock
torin
itch long form
itch short form
cenpi long form
cenpi short form
gabpb2 long form
gabpb2 short form
hsp90ab1

Figure 3.

A

KEGG PATHWAY ENRICHMENT CDS AS

WT vs. Tsc1-/-
Spliceosome
Proteoglycans in cancer
Human papillomavirus infection
Mitophagy - animal

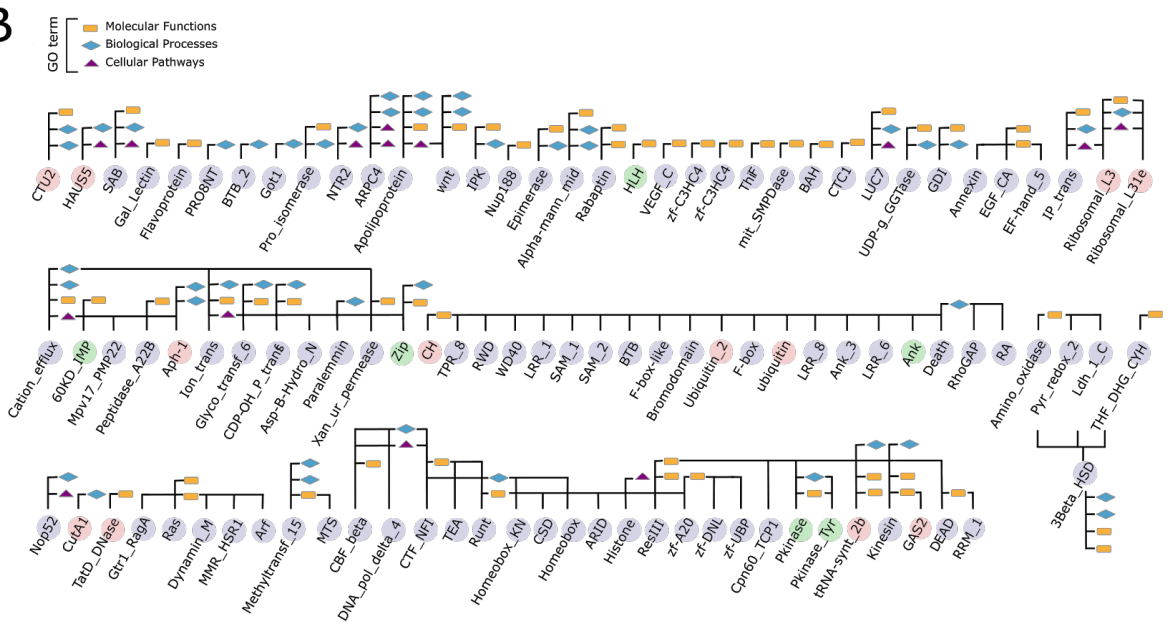
Mock vs. Torin1
Hippo signaling pathway
Human papillomavirus infection
p53 signaling pathway
Oocyte meiosis
Tight junction
Spliceosome
Hippo signaling pathway - multiple species
Cushing syndrome
Cellular senescence
Human T-cell leukemia virus 1 infection
Cell cycle
Autophagy - animal
Proteoglycans in cancer
Ubiquitin mediated proteolysis
Fc gamma R-mediated phagocytosis
PI3K-Akt signaling pathway
Viral carcinogenesis
Wnt signaling pathway
Human cytomegalovirus infection
Endocytosis
Bacterial invasion of epithelial cells
Dopaminergic synapse

KEGG PATHWAY ENRICHMENT 5'UTR AS

WT vs. Tsc1-/-
Metabolic pathways
Spliceosome

Mock vs. Torin1
N-Glycan biosynthesis
Pathways in cancer

B



C

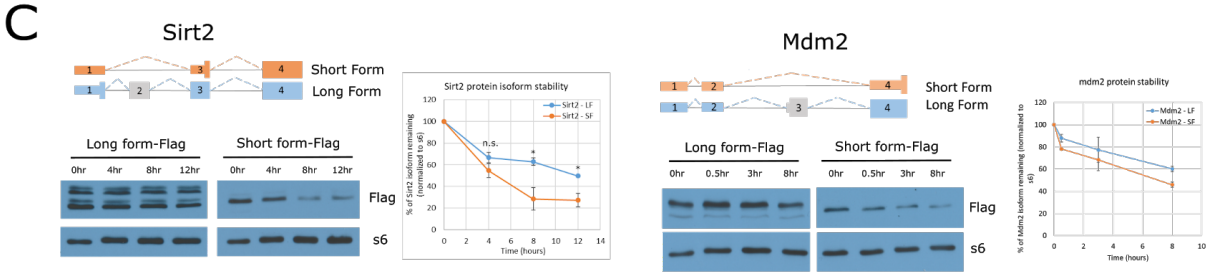


Figure 4.

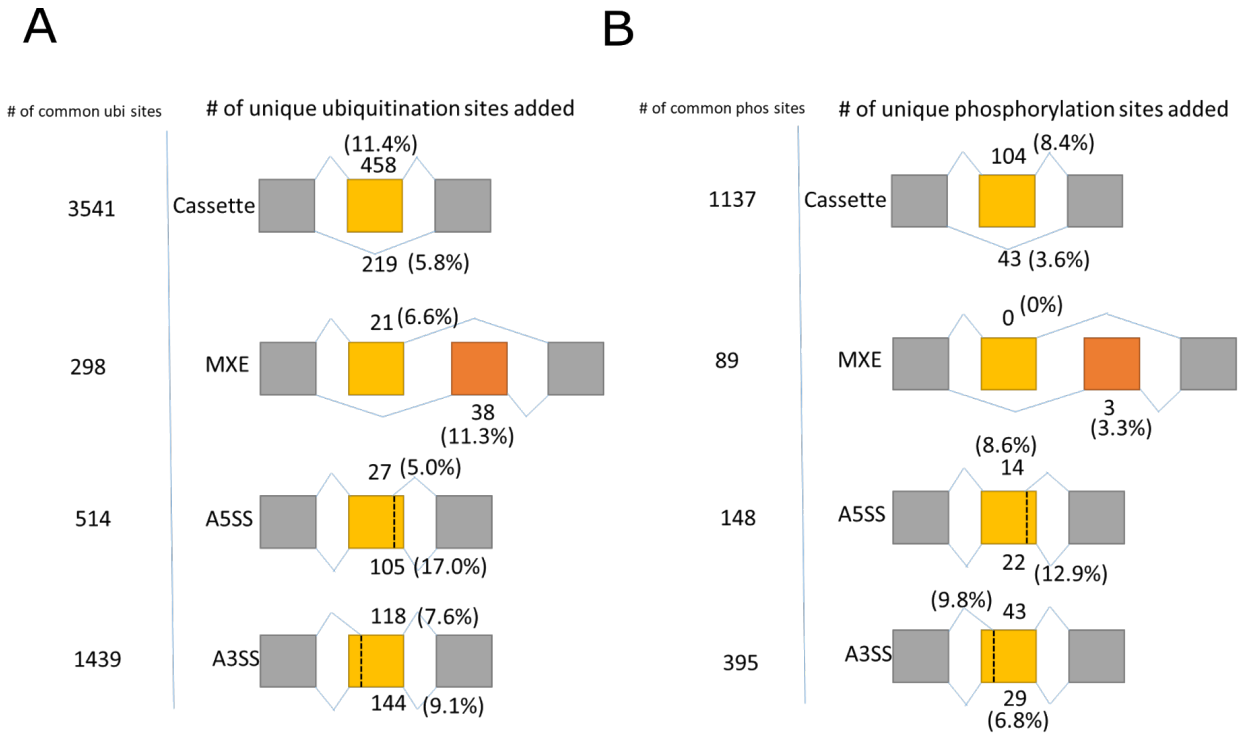

A
# of common ubi sites
# of unique ubiquitination sites added
3541
Cassette
(11.4%)
458
219 (5.8%)
298
MXE
21 (6.6%)
38
(11.3%)
514
A5SS
27 (5.0%)
105 (17.0%)
1439
A3SS
118 (7.6%)
144 (9.1%)
B
# of common phos sites
# of unique phosphorylation sites added
1137
Cassette
104 (8.4%)
43 (3.6%)
89
MXE
0 (0%)
3
(3.3%)
148
A5SS
(8.6%)
14
22 (12.9%)
395
A3SS
(9.8%) 43
29
(6.8%)

Figure 5.

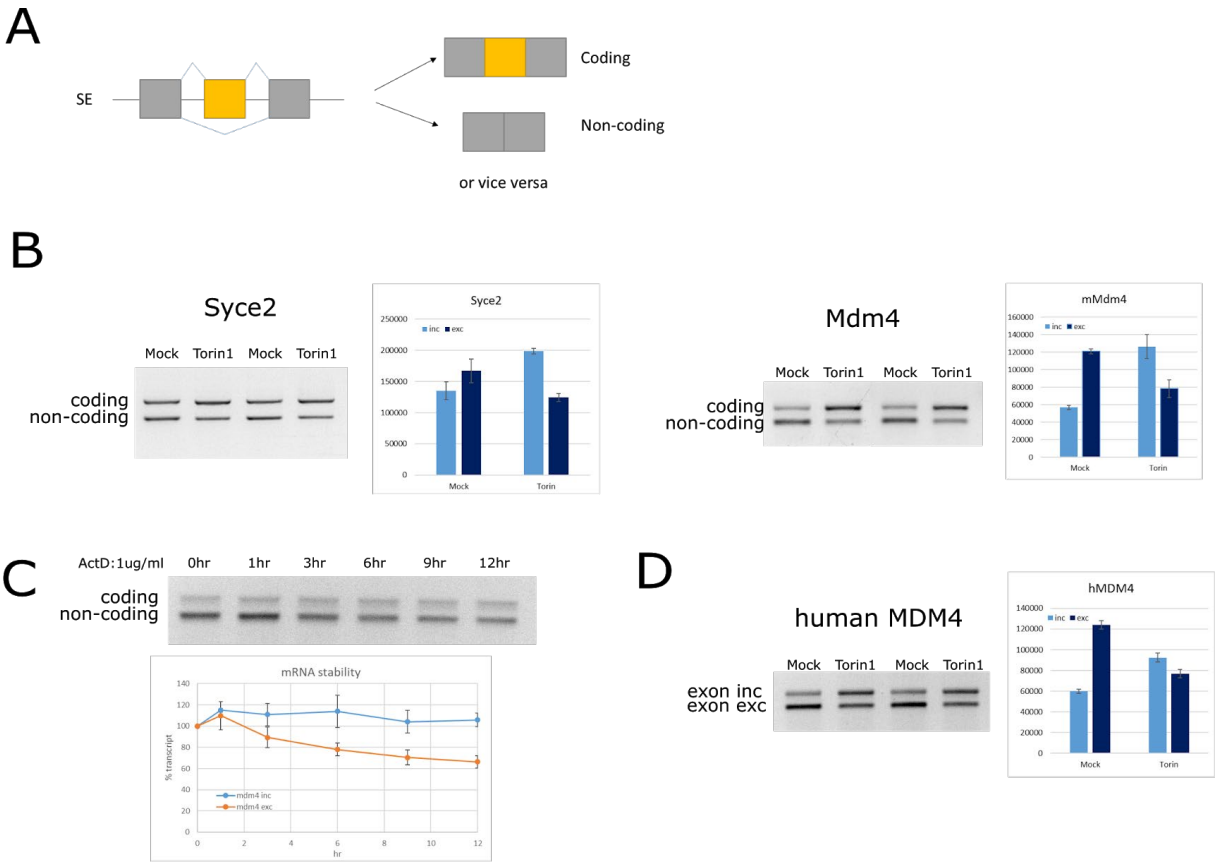
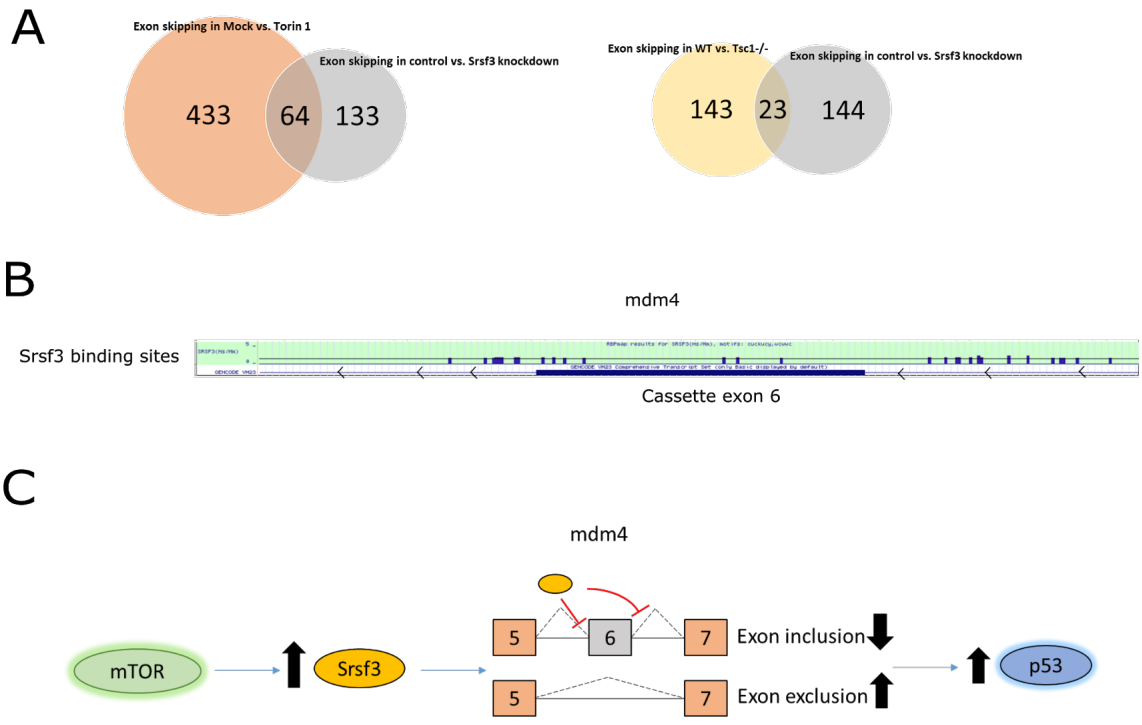

A
SE
Coding
Non-coding
or vice versa
B
Syce2
Mock
Torin1
Mock
Torin1
coding
non-coding
Syce2
inc
exc
Mock
Torin
Mdm4
Mock
Torin1
Mock
Torin1
coding
non-coding
mMdm4
inc
exc
Mock
Torin
C
ActD:1ug/ml
0hr
1hr
3hr
6hr
9hr
12hr
coding
non-coding
mRNA stability
% transcript
hr
mdm4 inc
mdm4 exc
D
human MDM4
Mock
Torin1
Mock
Torin1
exon inc
exon exc
hMDM4
inc
exc
Mock
Torin

Figure 6.


A
Exon skipping in Mock vs. Torin 1
Exon skipping in control vs. Srsf3 knockdown
433
64
133
Exon skipping in WT vs. Tsc1-/-
Exon skipping in control vs. Srsf3 knockdown
143
23
144
B
mdm4
Srsf3 binding sites
Cassette exon 6
C
mdm4
mTOR
Srsf3
5
6
7
Exon inclusion
5
7
Exon exclusion
p53