Widespread Inhibition of Posttranscriptional Splicing Shapes the Cellular Transcriptome following Heat Shock

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SUMMARY

During heat shock and other proteotoxic stresses, cells regulate multiple steps in gene expression in order to globally repress protein synthesis and selectively upregulate stress response proteins. Splicing of several mRNAs is known to be inhibited during heat stress, often meditated by SRp38, but the extent and specificity of this effect have remained unclear. Here, we examined splicing regulation genome-wide during heat shock in mouse fibroblasts. We observed widespread retention of introns in transcripts from ~1,700 genes, which were enriched for tRNA synthetase, nuclear pore, and spliceosome functions. Transcripts with retained introns were largely nuclear and untranslated. However, a group of 580+ genes biased for oxidation reduction and protein folding functions continued to be efficiently spliced. Interestingly, these unaffected transcripts are mostly cotranscriptionally spliced under both normal and stress conditions, whereas splicing-inhibited transcripts are mostly spliced posttranscriptionally. Altogether, our data demonstrate widespread repression of splicing in the mammalian heat stress response, disproportionately affecting posttranscriptionally spliced genes.

INTRODUCTION

During response to stress, eukaryotic cells globally repress protein production, while promoting selective expression of proteins, such as molecular chaperones (Hartl et al., 2011), that are essential for adaptation to stress. This feature of stress response and some specific regulatory programs, including chaperone induction, are evolutionarily conserved from yeast to human (Hartl et al., 2011), whereas others differ between species (Lindquist, 1981). Several steps in gene expression are regulated in response to stress, including well-characterized transcriptional responses (Murray et al., 2004). Regulation of translation also occurs during stress (Lindquist, 1981), espe-

cially in metazoans, via inhibition of translation initiation (Gebauer and Hentze, 2004; Sonenberg and Hinnebusch, 2009) and global pausing of translation elongation (Shalgi et al., 2013). Evidence is also accumulating in support of stress-dependent changes at steps between transcription and translation, including pre-mRNA splicing (Biamonti and Caceres, 2009).

Regulation of RNA splicing during heat shock was first reported in fly, wherein the pre-mRNAs of *Hsp83* and *Adh* accumulate in severe heat shock (Yost and Lindquist, 1986). Similar observations have been made in yeast (Yost and Lindquist, 1991), HeLa cells (Bond, 1988), and other systems, leading to the proposal that splicing is inhibited during heat shock in many organisms (Yost et al., 1990). Later work characterized the role of the splicing factor SRp38 (Fusip1) and its dephosphorylation by PP1 in mediating splicing repression in heat stress (Shi and Manley, 2007; Shin et al., 2004). Additionally, various SR proteins, hnRNPs, and other splicing factors are recruited to nuclear stress bodies (nSBs) during heat stress (Biamonti and Vourc'h, 2010). Interestingly, these nSBs are sites of active transcription directed by HSF1 (Jolly et al., 1999), the major heat-shock transcription factor.

Heat-stress-dependent inhibition of splicing has been observed for only a handful of genes, leaving the generality of these effects unclear. We therefore set out to globally investigate regulation of splicing during the heat-shock response in mammalian cells. We describe here a transcriptome-wide RNA sequencing (RNA-seq) analysis of a time course of mild and severe heat-shock treatments in mammalian 3T3 fibroblast cells. We found evidence for widespread inhibition of splicing, affecting over 1,700 genes, particularly in severe heat shock. The resulting unspliced transcripts are not translated and are instead retained in the nucleus. We also describe a group of mRNAs, many related to oxidation reduction and protein folding, whose splicing is unaffected, and show that these genes are mostly cotranscriptionally spliced.

RESULTS

Extensive Expression and Splicing Changes Occur during Heat Stress

To analyze changes in alternative splicing during heat shock in mammalian cells, we subjected 3T3 mouse fibroblasts to

heat-shock treatments. To explore the kinetic response to acute and chronic heat stress, we treated cells with mild heat stress (42°C) for 2, 4, 8, 12, and 16 hr, or severe heat stress (44°C) for 2 or 8 hr. Transcriptome analysis was conducted using paired-end strand-specific polyA-selected RNA-seq.

Gene expression analysis revealed that ~5,200 genes changed their expression by 2-fold or more in at least one time point relative to control. Clustering samples based on gene expression levels showed that early time points of both treatments clustered together, as well as late time points of mild stress, indicating a partial recovery in the gene expression profile after 12 hr, whereas the 8 hr severe heat-shock sample was an outlier (Figure 1A). The most prominent feature of the gene expression analysis was the downregulation of thousands of genes at 8 hr of severe heat shock (Figure 1B), which were enriched for metabolic processes and transcription (Table S1). Smaller clusters of genes showed increased expression, either early or later in the time course, and were enriched for functions related to stress response and protein folding. Overall, the functional gene groups we observed following heat stress treatments were consistent with previous studies (Murray et al., 2004) and mostly reflect well-understood features of the transcriptional response to stress.

We next analyzed the regulation of pre-mRNA processing. We used a curated collection of the most common types of alternative splicing events in mammals (Wang et al., 2008), including skipped exons, retained introns, and alternative 5′ and 3′ splice sites. For each splicing event, the "percent spliced in" (PSI or Ψ) value was estimated, reflecting the fraction of a gene's mRNAs that include the exon, intron or alternative splice site. Differences in inclusion levels (ΔPSI) between samples, and their significance (using Bayes factors, BFs) were calculated using the MISO algorithm (Katz et al., 2010). Together, just over 4,500 alternative isoform differences were detected (Supplemental Experimental Procedures), and 32% of these changed significantly following heat shock in at least one treatment (1,480 events with BF $\geq$20; Figures 1C–1F).

The strongest effect of heat stress was on the retention of introns, with significant changes in 53% of retained introns detected in at least one heat-shock treatment. Although other types of alternative splicing showed roughly similar proportions of isoforms with increased and decreased inclusion levels, retained introns were substantially skewed toward higher inclusion in heat stress (74%, Figure 1E). These observations, coupled with previous literature documenting splicing repression during heat shock (Shin et al., 2004; Yost and Lindquist, 1986), prompted us to examine intron retention more broadly in our data.

Substantial Amounts of Unspliced mRNAs Are Detected in Severe Heat Shock

We next expanded our analysis to include a comprehensive set of mouse introns, considering all of the ~120,000 introns in genes detected as expressed. We examined the distributions of intron "expression" levels—calculated as RPKM (reads per kilobase of intron per million mapped reads)—and observed that they were significantly skewed toward higher values in all heat-shock samples relative to control (Figures 2A and S1A; fold changes and ΔPSI value analysis shown in Figures S1B

and S1C). These observations suggested that intron retention may be more prevalent in heat shock than initially estimated using our curated collection of retained introns (Figure 1E). Interestingly, we observed that longer introns had larger average increases in retention in heat shock by up to 2- or 3-fold (Figure 2B).

When calculating the expression of introns relative to that of their host genes, we observed a correlation between introns in the same gene (Figures S1D–S1J). Furthermore, normalization of the expression of individual introns to the mean expression of the remaining introns in each host gene yielded similar distributions in heat shock and control conditions (Figure S1K). These analyses indicated that splicing inhibition is largely a gene level rather than an intron level phenomenon, i.e., that splicing inhibition affects some genes across their introns.

It was essential to ensure that technical factors were not confounding our analyses. First, we ruled out genomic DNA contamination as a factor, because the read density in introns exceeded that in intergenic regions by 6- to 8-fold in all samples. Second, we assessed the efficiency of polyA selection and determined that variability in this step could not explain the increase in intronic reads observed in heat shock (Figures S1L–S1O and Supplemental Experimental Procedures). Last, we confirmed that increased intronic expression derives from unspliced transcripts rather than spliced lariats by verifying that the same trends held when we examined reads that span exon-intron junctions (Figures S1P–S1R).

Hundreds of Genes with Significantly Inhibited Splicing in Heat Shock

We next asked which genes have significantly inhibited splicing under heat shock. For each gene, an ANOVA statistical test was used to ask whether the gene's introns, as a group, had significantly different inclusion levels (ΔPSI values) in heat shock than in control. The test is illustrated for the splicing factor *Syf2* (Figures 2C and 2D), splicing of which was significantly inhibited at 2 and 8 hr of severe heat shock (p = $6.8E^{-9}$). Out of 6,274 genes with at least two introns and sufficient expression, the splicing of 1,708 (27%) was significantly inhibited (false discovery rate 0.05), primarily in severe heat-shock conditions (Figure S2C). Intron retention in a representative set of splicing-inhibited genes was validated by quantitative PCR (qPCR) in a biological replicate experiment (Figure S2A). Most genes had significant splicing inhibition in one sample; 40% in two or more time points (Figures 2E and S2D). Splicing-inhibited genes had a median increase in intron inclusion (ΔPSI) of ~18.5% (Figures 2F and S2E), and these genes were somewhat more highly expressed than other genes overall (Figure S2F). This set of genes was enriched for several functions related to gene expression (Table S2), including tRNA synthetases, spliceosome—which might contribute to the observed splicing inhibition—and nuclear pore (see below).

A Group of mRNAs Is Unaffected by Splicing Inhibition

We next asked whether there are genes whose splicing is unaffected by heat shock. We defined "unaffected genes" as genes that had nonsignificant ANOVA p values for intron inclusion in heat shock and mean intron ΔPSI no greater than 10% under
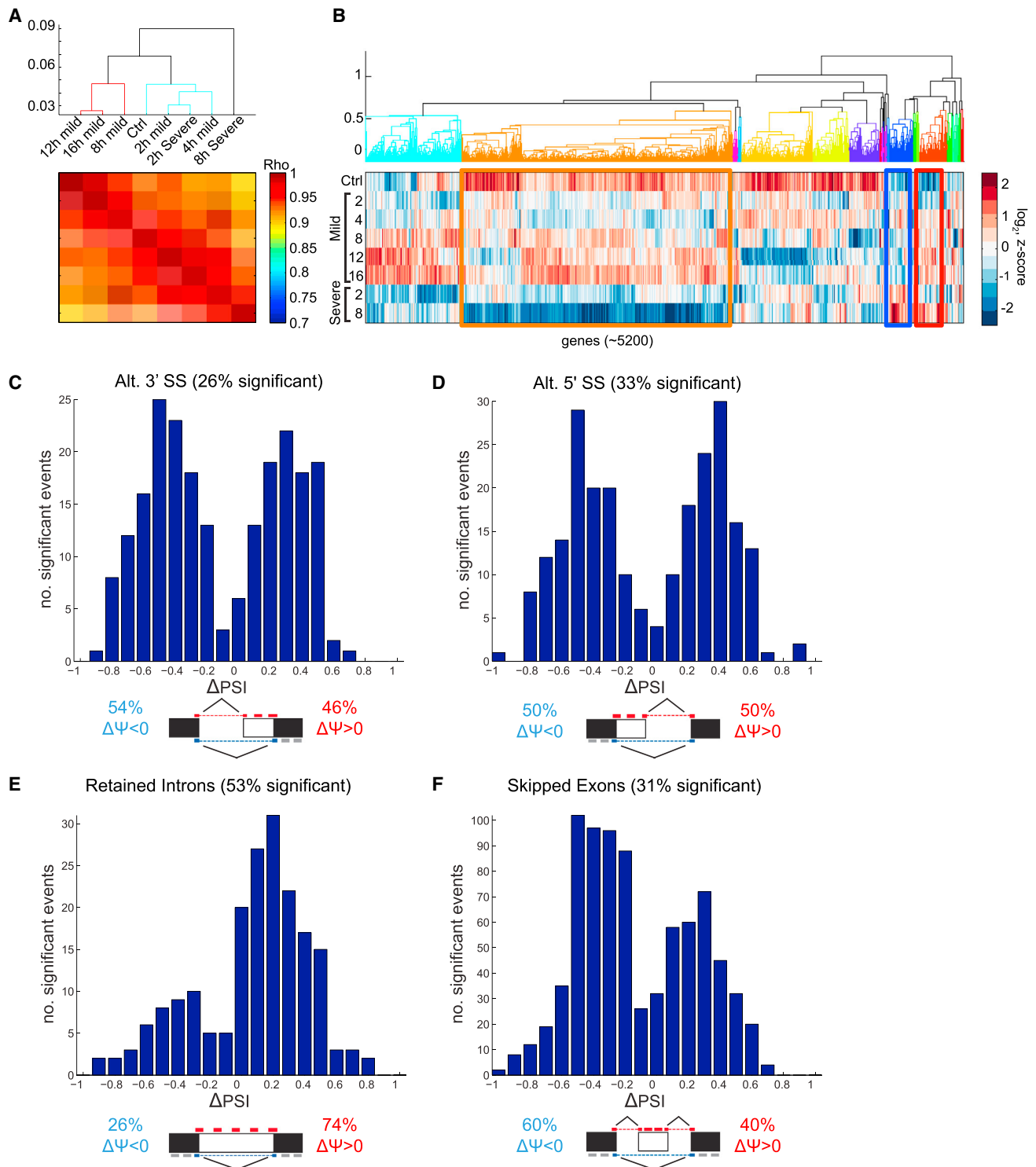
Figure 1. Gene Expression and Alternative Splicing Regulation in Response to Heat Shock

(A) Gene expression RPKM values were grouped using hierarchical clustering; correlations between samples are shown in the bottom panel, similarity tree in top panel. A minimum RPKM of 2 in at least one sample was required, and all values were thresholded to 2.

(B) Gene expression RPKM clustering of ~5,200 genes, which showed an expression change of at least 2-fold in at least one sample compared to control. Examples of clusters are marked and enriched GO terms (using DAVID [Huang et al., 2009]) are listed in Table S1. Clustering was performed on logged expression values, and Z score of log expression is displayed.

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any condition. Furthermore, because intron retention can be harder to detect in lowly expressed genes, we additionally required genes in the unaffected group to be well expressed (above the median of the splicing-inhibited set) in both the 2 and 8 hr severe heat-shock conditions. This yielded a set of 583 unaffected genes. Data for the *Atp1a1* gene are shown as an example (Figure 2G). We verified that introns of a representative set of the unaffected genes were not retained by qPCR in a biological replicate experiment (Figure S2B). Unaffected genes were enriched for functions related to oxidation reduction and protein folding, as well as endoplasmic reticulum and cell surface (Table S2). Although longer introns had higher ΔPSI values overall, the distribution of intron lengths was shifted only slightly toward shorter lengths for the unaffected genes (Figure S2G).

We next asked whether the splicing status of genes was related to their translation during heat stress. Using ribosome footprint profiling data generated previously in this system (Shalgi et al., 2013), we calculated ribosome occupancy (RO) values by dividing the density of ribosome footprint reads in the body of the open reading frame by the gene's RNA-seq expression level. Ribosome footprints capture translated mRNAs in the cytoplasm, whereas RNA-seq should capture total cellular poly(A)+ RNA. Under control conditions, the distribution of RO values for inhibited genes was very similar to all genes (rank sum p = 0.13), whereas the unaffected genes showed a slightly higher level of translation (median $\log_2$ value of 0.65 versus 0.5, rank sum p = $1.8E^{-3}$, Figure S2H). After 2 hr of severe heat shock, the group of unaffected genes still showed a significant shift toward higher RO (median $\log_2$ values of 0.5 compared to 0.3, rank sum p = $1.3E^{-3}$), whereas splicing-inhibited genes showed significantly lower values compared to all genes (median $\log_2$ value 0.19, rank sum p = $6.8E^{-3}$, Figure S2I). Thus, splicing inhibition is associated with reduced mRNA translation during heat shock.

Intron-Containing Transcripts Are Largely Nuclear and Untranslated

Next, we used the ribosome footprinting data to ask specifically whether the unspliced transcripts that accumulate in heat shock are translated. We were able to detect one or more footprint reads for just ~1,700 introns of the ~47,000 that passed our RNA-seq filters. We defined RO values for each intron, analogously as for mRNAs, and observed that these values were lower in heat shock compared to control (p = $5.4E^{-16}$, rank sum test, Figure S2J). Furthermore, RO values of introns in splicing-inhibited genes were similar in control conditions and lower in heat shock than the RO of introns in other genes (Figures S2K and S2L). Thus, the intron-containing transcripts that accumulate under heat stress are translated to an even lesser extent than the low level that occurs under control conditions.

To determine the subcellular localization of intron-containing transcripts, we fractionated control- and heat-shock-treated cells (2 hr severe) into cytoplasmic, nucleoplasmic, and chromatin fractions (Figure S3A). RNA was extracted from each fraction, and the abundance of representative splicing-inhibited and unaffected genes was assayed by qPCR (Supplemental Experimental Procedures). The retention status of these introns measured by qPCR was largely consistent with the RNA-seq data (Figure S3C). Additionally, introns were nearly absent from cytoplasmic fractions for both unaffected and splicing-inhibited genes (Figure S3B), in line with the very low translation of intron-containing transcripts under both control and heat stress conditions as seen above. Furthermore, we observed substantial accumulation of both intron-containing (Figure S3B) and total mRNA in the nucleoplasm of heat-shocked cells, for splicing-inhibited genes (Figure 3A). High levels of intron retention of two splicing-inhibited genes, *Syf2* (Figures 3B and S3D) and *Map2k3* (Figure S3E), were observed during heat stress using single-molecule RNA FISH. This assay also confirmed the nuclear retention of the unspliced messages. We found no evidence of intron retention for the unaffected gene *Atp1a1* (Figures 3B and S3D). Thus, our data show that during heat stress intron-containing transcripts that derive from splicing-inhibited genes are predominantly retained in the nucleus.

Unaffected Genes Undergo Efficient Splicing

We next explored the properties of unaffected genes to understand how they differ from splicing-inhibited genes. A gene that is transcribed and continues to be efficiently spliced during heat stress should be classified as unaffected. A gene whose transcription was strongly repressed but whose presynthesized spliced messages remained stable during heat stress would also appear as unaffected. To test whether unaffected genes continue to be transcribed under heat-shock conditions, we used 5-ethynyl uridine (EU) metabolic labeling for 2 hr in both control and heat-shocked cells (Supplemental Experimental Procedures). qPCR analysis of labeled RNA showed that the overall transcription of most unaffected genes is only moderately downregulated, to levels between ~40% and 80% of control levels (mean: 64%, Figure 4A). Transcription of splicing-inhibited genes was downregulated to a similar extent, with the exception of *Syf2*, which was upregulated ~1.8-fold (Figure 4A). Notably, we also confirmed the efficient splicing of newly synthesized RNA from the unaffected genes (Figure S4A). Additionally, we assessed the change in levels of chromatin-associated RNA and cytoplasmic mRNA (Figures S4B and S4C) in order to further examine the relative contributions of stabilization and transcription to the overall steady-state levels of unaffected genes. Most unaffected genes showed either no change or a moderate decrease in chromatin-associated RNA accompanied in most cases by a modest increase in cytoplasmic RNA. This pattern further supports a combination of mRNA stabilization and efficient splicing of newly transcribed RNA. Thus, some mRNAs are stabilized in heat stress, but all of the unaffected genes tested continue to be transcribed and efficiently spliced in heat stress.

(C–F) MISO was run on a collection of alternative splicing events, and the distribution of ΔPSI values are presented for significantly changing events (BF ≥ 20 in at least one sample compared to control), for (C) alternative 3′ splice sites, 219 events (D) alternative 5′ splice sites, 238 events (D) retained introns, 190 events (E) and skipped exons, 808 events (F). The percentages of significant events out of the total number of events is shown. Percentage of events that show inclusion (red) and exclusion (blue) indicated at the bottom.
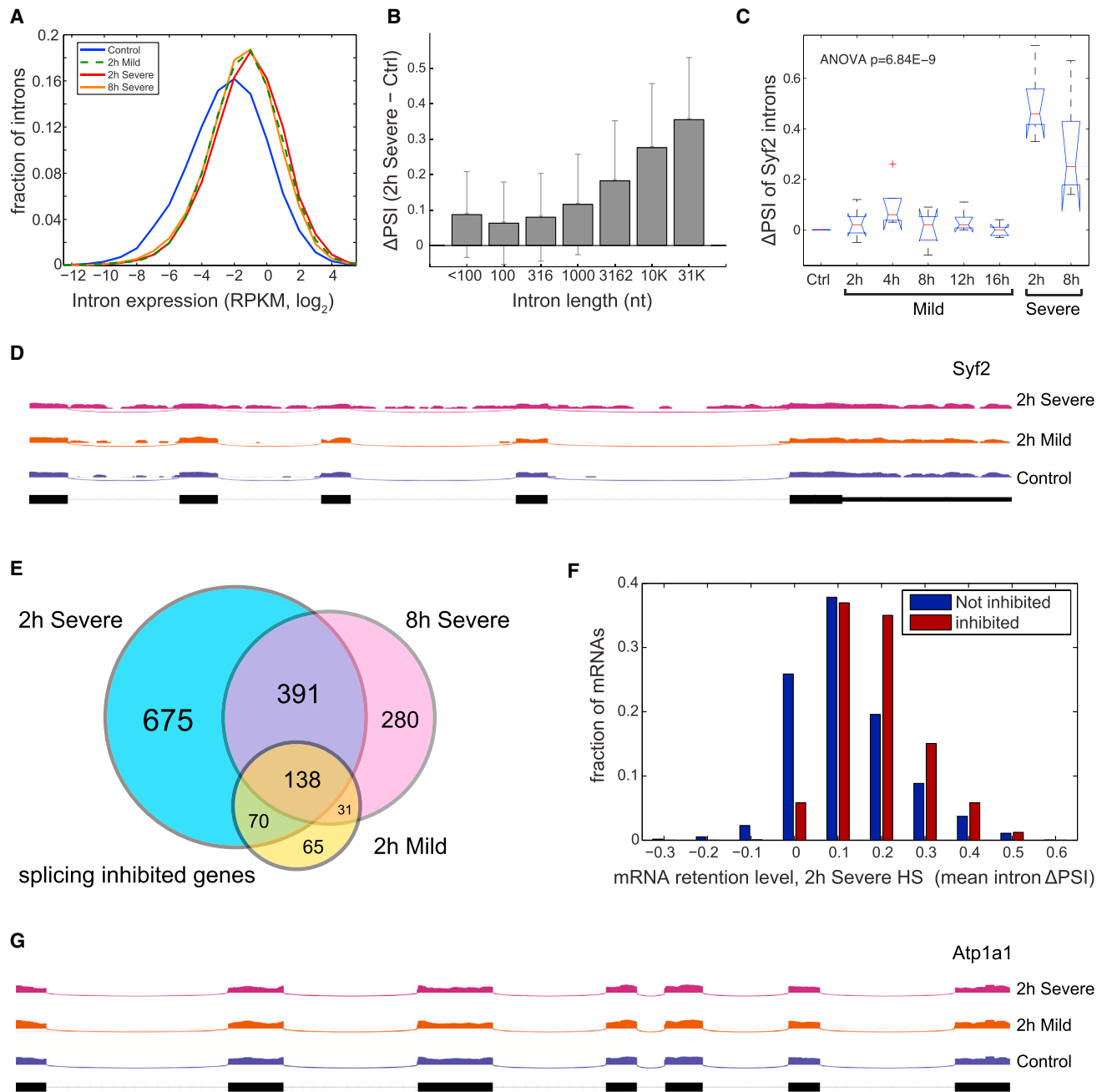
Figure 2. Identification and Characterization of Genes with Significant Splicing Inhibition in Heat Shock

(A) Distributions of RPKM values (in $\log_2$) of all introns in the mouse transcriptome show an increase in heat-shock samples compared to control. Control, 2 hr mild, 2 and 8 hr severe heat-shock conditions are shown; all conditions are shown in Figures S1A–S1C.

(B) Introns were binned by length, and mean and SD of ΔPSI values in each bin in 2 hr severe heat stress are plotted.

(C) Box plot of the ΔPSI values of the six introns of Syf2. The ANOVA test identified significant splicing inhibition ($p = 6.8E^{-9}$) at 2 and 8 hr severe heat shock. For each box, the central mark is the median, the box edges are the 25th and 75th percentiles, the whiskers extend to the most extreme data points not considered outliers, and outliers are plotted individually.

(D) Aligned RNA-seq data ($\log_{10}$) for a portion of the *Syf2* transcript including exons 3–7.

(E) Venn diagram (Venn Diagram Plotter, http://omics.pnl.gov/) depicts the overlap between splicing-inhibited genes in the three samples with the largest extent of splicing inhibition.

(F) mRNA retention levels of splicing-inhibited versus all other genes in 2 hr severe heat shock.

(G) Aligned RNA-seq data ($\log_{10}$) for *Atp1a1*, which is unaffected by the splicing inhibition (exons 4–10 are shown).

See also Figures S1 and S2.

Unaffected Genes Are Mostly Spliced Cotranscriptionally

These findings prompted us to explore how unaffected genes remain efficiently spliced, whereas the splicing of so many other genes is inhibited. Returning to the qPCR data from different cellular fractions, we noted that unspliced RNA decreased dramatically (>10-fold) in the chromatin fraction for unaffected genes in heat stress; however, no significant change was observed for splicing-inhibited genes (Figure 4B). This change suggested that the efficiency of cotranscriptional splicing increases in heat stress, at least for a subset of genes.

Mammalian introns and genes vary in the extent of cotranscriptional versus posttranscriptional splicing (Khodor et al., 2012). To assess the extent of cotranscriptional splicing, we calculated for every gene the ratio between unspliced RNA (exon-intron primers) and the total RNA (exon body primers) in the chromatin fraction. This ratio represents the fraction of chromatin-associated transcripts that remain unspliced, a measure of the extent of posttranscriptional splicing. Interestingly, under control conditions, unaffected genes showed a higher degree of cotranscriptional splicing, with a mean fraction of chromatin-associated unspliced transcripts of just 0.14, compared to 0.42 for the splicing-inhibited RNAs (t test p = 4×10^{-4}, Figure 4C). We confirmed that this difference did not result from previously reported correlates of cotranscriptional splicing (Khodor et al., 2012) (Figures S4D and S4E). These results suggest that a change in the localization or localization-dependent activity of the splicing machinery occurs in heat stress such that posttranscriptional splicing is inhibited, whereas cotranscriptional splicing is enhanced. Such a redistribution of splicing capacity could explain how the unaffected genes continue to be efficiently spliced in heat shock, whereas splicing of more posttranscriptionally spliced genes is inhibited.

To explore other determinants that might modify the splicing of particular genes, we analyzed the frequency of sequence motifs in introns of unaffected genes relative to introns in all other genes. Despite the nearly identical dinucleotide composition of unaffected introns relative to other introns (Figure S4H), a number of motifs were enriched 1.5- to 2-fold (Table S3; Figures S4F and S4G). These included several GC-rich motifs, some resembling motifs recognized by PPRC1, an RNA binding protein involved in mitochondrial biogenesis (Vercauteren et al., 2009). Many motifs contained G-runs, known binding motifs of the hnRNP F/H family of splicing factors (Caputi and Zahler, 2001; Xiao et al., 2009). G-run abundance correlated with escape from intron retention (Figure S4I), and GGG motifs were depleted in introns of splicing-inhibited genes (Table S3). If G-runs promote efficient splicing in heat stress, this could help to explain our observations that shorter introns are less retained, because G-runs are known to be more abundant in shorter introns in mammals (Xiao et al., 2009). Together, these observations suggest that presence of specific cis-regulatory motifs may facilitate splicing of a subset of introns under heat stress conditions.
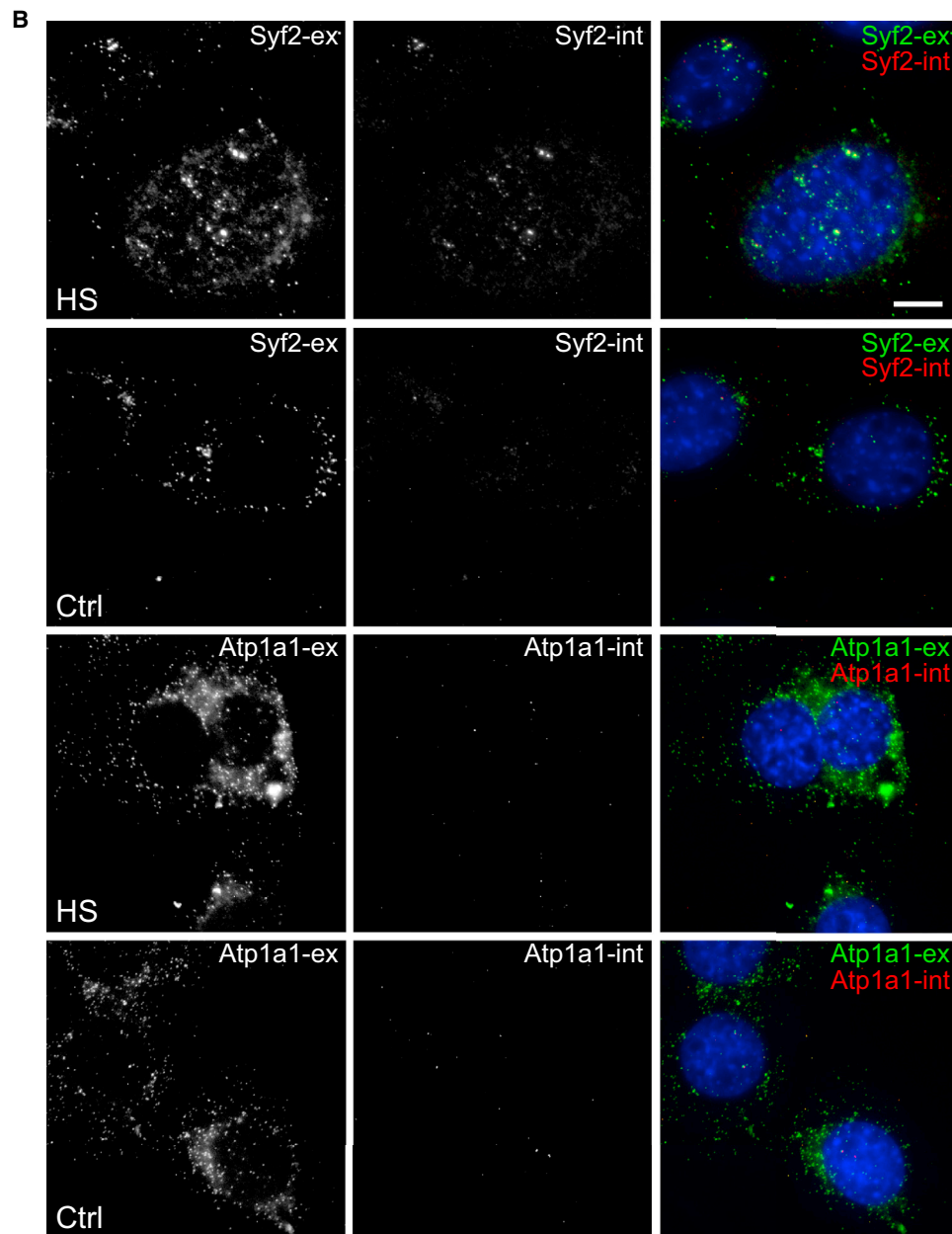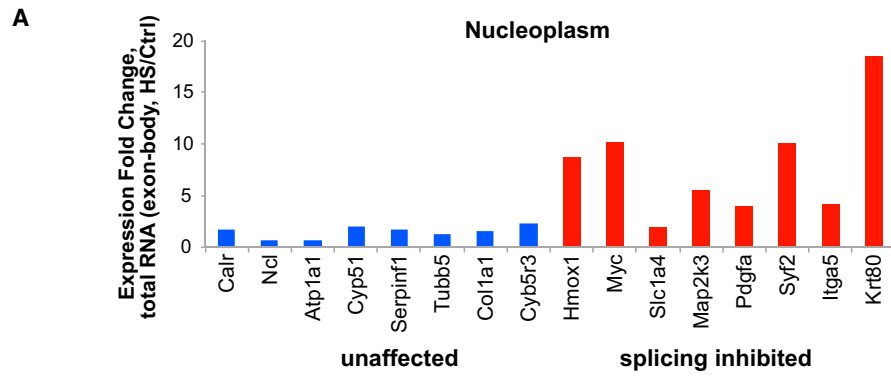
DISCUSSION

Inhibition of splicing following heat stress has been observed in multiple systems, suggesting that it may represent an evolutionarily conserved cellular response. Here, we show that this phenomenon is widespread, especially in severe heat stress, where it affects nearly 2,000 genes. But it is not indiscriminate; the splicing of almost 600 genes remained completely unaffected. For genes that were affected, the mean level of intron retention was close to 20% per intron. Because intron-containing transcripts were found to be nuclear retained and untranslated, ~20% of messages from these genes appear not to contribute to protein production in heat stress. However, this proportion could be substantially higher if different introns are retained in different individual transcripts from a gene, and if retention of any intron is sufficient to inhibit nuclear export. Messages from these genes are also less translated under heat stress, perhaps simply because of the increased proportion of incompletely spliced, nuclear-retained transcripts.
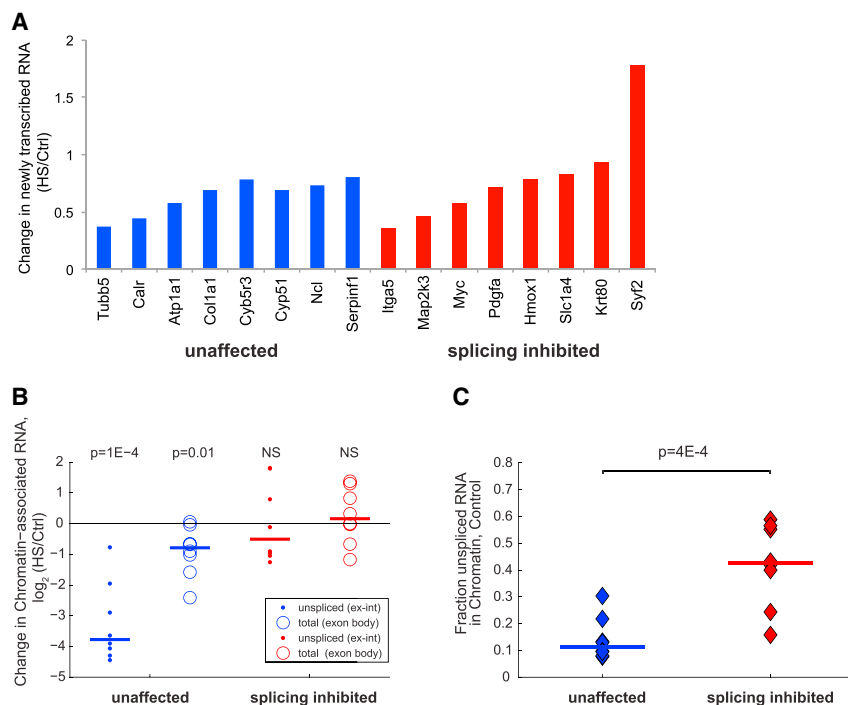
Nuclear retention of some mRNAs has been observed in HeLa cells (Sadis et al., 1988), and export of poly(A)+ mRNAs is inhibited during heat shock in yeast (Saavedra et al., 1996). Here, we observed that genes encoding nuclear pore components were enriched among splicing-inhibited genes, which may contribute to bulk reductions in mRNA export. Certain splicing factors are known to play a role in export of spliced mRNAs in heat shock (Farny et al., 2008), perhaps contributing to the preferential export of spliced versus unspliced transcripts. Nuclear retention of intron-containing messages may prevent production of aberrant proteins, thereby reducing the burden on the chaperone and proteasome machineries. Presence of these transcripts at substantial levels in the cell suggests that they are relatively stable in the nucleus and may have some function, perhaps serving as a reservoir of partially processed transcripts, which can later be spliced and exported to rapidly produce protein when stress conditions abate.

The unaffected genes appear to retain predominant expression of fully spliced mRNAs by two main mechanisms: increased stabilization of pre-existing cytoplasmic mRNAs (DiDomenico et al., 1982; Sadis et al., 1988), and continued generation and efficient splicing of new transcripts. These properties suggest regulation to ensure continued protein production and, consistently, messages from these genes also had increased ribosome occupancy during heat stress. This set of genes was enriched for functions in protein folding, a commonly induced class in proteotoxic stress, and oxidation reduction and energy production, including many genes related to mitochondrial function. Heat stress is known to induce mitochondrial biogenesis in myoblasts and muscle (Liu and Brooks, 2012), so the latter changes might be related to a similar process in fibroblasts.

Our observations connecting splicing inhibition with posttranscriptional splicing and escape from inhibition with cotranscriptional splicing raise intriguing mechanistic possibilities. The evidence that cotranscriptional splicing may actually increase in efficiency in heat stress may relate to nuclear stress bodies (nSBs) (Biamonti and Vourc'h, 2010). nSBs are known to contain various splicing factors and are sites of active transcription by HSF-1 (Biamonti and Caceres, 2009; Biamonti and Vourc'h, 2010). Therefore, it is possible that a function of nSBs in heat stress is to recruit splicing machinery to sites of HSF-1-directed active transcription and protect it locally from splicing-inhibitory

A

B

(legend on next page)

Figure 4. Transcription and Cotranscriptional Splicing of Splicing-Inhibited and Unaffected Genes

(A) EU metabolic labeling, performed for 2 hr in either control or heat-shocked cells (during 2 hr of severe heat shock), demonstrates a modest change in levels of newly transcribed RNA for a representative set of unaffected (blue) and splicing-inhibited (red) genes.

(B and C) Unaffected genes are mostly cotranscriptionally spliced under both normal and heat-shock conditions. (B) Fold change ($\log_2$ heat shock/control) of the levels of unspliced (filled circles) and total (open circles) chromatin-associated RNA, for unaffected (blue) and splicing-inhibited (red) genes. Medians are shown as bars. p value for a t test for difference from 0 is denoted. (C) Fraction of cotranscriptionally unspliced RNA for unaffected (blue) and splicing-inhibited (red) genes, represented as the ratio between unspliced message (measured by exon-intron qPCR) and the total message level (measured by exon body qPCR), serves as a proxy for the extent of cotranscriptional splicing. Medians are shown as bars.

See also Figures S3 and S4.

functions, to enable highly efficient splicing to occur when it is cotranscriptional. Conversely, pre-mRNA released from genes that undergo transcription outside of nSBs or undergo posttranscriptional splicing might encounter a less conducive splicing environment, as a result of U1 snRNP inhibition by dephosphorylated SRp38 or other mechanisms. For example, the fly homolog of the mammalian SRSF1 splicing factor has been observed to change its localization near active genes dependent on the level of transcriptional induction in heat stress (Champlin and Lis, 1994). Thus, the subnuclear location of splicing might be a critical component of stress-dependent regulation. Although cotranscriptional splicing has been the subject of a number of recent studies (Khodor et al., 2012), this work provides the first indications that the extent of cotranscriptional splicing may play a major role in shaping the cellular transcriptome under stress conditions.

EXPERIMENTAL PROCEDURES

Intron Retention Analysis with MISO

All introns in expressed genes (total of ~120,000) were collected from Ensembl annotation files to generate a gff3 file, which was pickled using MISO. MISO was run with default settings (single end mode, fast MISO) to calculate ΔPSI values for each heat-shock sample compared to control. Introns were first filtered to exclude those that overlapped an annotated Ensembl exon. Further-

more, introns were filtered to contain at least 20 reads supporting both inclusion and exclusion isoforms in the control and at least two other samples, and ten exclusion reads in at least one sample. These filters resulted in a set of ~47,000 introns that participated in the ANOVA analyses for identification of splicing-inhibited genes, and in the motif analysis. See Supplemental Experimental Procedures for additional controls.

qPCR Measurements of ΔPSI

RNA samples were Turbo DNase treated and absence of contaminating DNA was confirmed using a no-RT control in each experiment (data not shown). For each gene, a pair of exon-body primers was designed as well as a pair of exon-intron spanning primers for an adjacent intron (primers listed in Table S4). Genomic DNA (3 μg), prepared from 3T3 cells using QIAGEN DNeasy kit, was used for standardization in all qPCR experiments. qPCR values for exon-intron primer pairs and exon-body primer pairs were each standardized to genomic DNA (using ΔCt) in order to compare between unspliced (exon-intron primers) and total RNA (exon- body primers) within the same gene. PSI values for intron retention were calculated as standardized unspliced divided by standardized total RNA values.

Other detailed experimental procedures can be found in the Supplemental Experimental Procedures. Complete lists of splicing-inhibited and unaffected genes and related expression and splicing data are provided in Tables S5 and S6, respectively.

ACCESSION NUMBERS

RNA-seq data have been deposited to the NCBI Sequence Read Archive under accession number SRP035393.

Figure 3. Localization of Splicing-Inhibited and Unaffected mRNAs in Heat Shock

(A) Splicing-inhibited mRNAs are retained in the nucleus. Total RNA amounts (measured by qPCR with exon-body primers) were assayed in the nucleoplasmic fraction in control and heat-shock (2 hr severe) samples. Presented are fold changes for a group of unaffected (blue) and inhibited (red) genes.

(B) RNA FISH demonstrated the intron retention and nuclear retention in heat shock. Shown are the splicing-inhibited *Syf2* gene and the unaffected *Atp1a1* gene in control and heat-shock cells (2 hr severe). Exon probes, covering the mature mRNA, are shown in the left panel, intron probes (intron 2 for *Syf2* and intron 1 of *Atp1a1*) in the middle panel, and a merge (introns, red; exons, green; DAPI, blue) in the right panel. Scale bar, 10 μm.

See also Figures S2 and S3.

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