Deciphering the rules of mRNA structure differentiation in vivo and in vitro with deep neural networks in *Saccharomyces cerevisiae*

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Abstract

In vivo mRNA structure was influenced by versatile factors in the translation process resulting in a significant difference of mRNA structure in vitro. In this study, we elaborated a novel deep neural networks (DNN) model for predicting stable or unstable structural states of mRNA in vivo by fitting six quantifiable features of mRNA structure: ribosome density, minimum free energy, GC content, mRNA abundance, ribosomal initial density and structural relative position. By mutations simulation and well-trained, high-precision models prediction, we discovered the unique effects of these six mRNA structural features on the structural stability of mRNA in vivo. It was worthwhile to point out that we have found a double-sided effect of ribosomal density on the structural stability of mRNA, and it may be speculated that, more stable mRNA structure leaded to higher ribosome density in the region of low ribosomal density and higher ribosomal density resulted in more unstable mRNA structure in the region of high ribosome density.Additionally, the trend and extent of these six features affecting mRNA structural stability in vivo could also be accurately predicted by the DNN model.This is the first time to use DNN method to decipher the difference of mRNA structure in vivo and in vitro, and a good prediction results have been acquired, revealing a "personally" impact of different mRNA structural features on its stability quantitatively. Thus, it was speculated that this frontier analysis method, DNN model, will have great potential in solving the problem of complex regularity in the translation process.

Introduction

mRNA is a key component of the translation system. It is not only carries protein coding information, but also an important vector for translation regulation information by folding into mRNA structures[1], most notably mRNA structures at 5'UTR and RBS region has a significant effect on translation efficiency[2,3]. In recent years studies, in-cell RNA structures showed quite different characteristics in vivo and in vitro using genome-wide high-throughput sequencing analyses (DMS-probing[4,5], DMS-MaPseq[6], icSHAPE[7] and SHAPE-MaP[2]) , some general conclusions have been drawn: the number of in vivo structures was far less than the number of in vitro structures[4], mRNA structures in highly expressed genes, especially long-range base pairing structures, were revealed translation destabilizes[2]. However, it challenges to carry out this difference further and accurately to the specific analysis of each mRNA structure, owing to the dynamic and uniqueness of the mRNA structure itself, that is, each mRNA structure has a ‘personal’ distinctive structure.

In order for the ribosome to proceed translation smoothly, it is necessary to first unwind the mRNA structure, and thus a complex interaction between the ribosome and the mRNA structures occurs during translation, and translating ribosome is one of the most important factors that cause structural differences in vitro and in vivo. Ribosome profiling is a method for determining the exact position of ribosomes in transcriptome with single-nucleotide resolution during translation by deeply sequencing ribosomal protected mRNA fragments[8,9]. This approach can more effectively resolve the interaction between ribosomes and mRNA structure during translation, for

example, the mRNA structure in CDS region influences protein co-translational folding by affecting the efficiency of ribosome translation or is more extreme, with causing ribosomal pauses[10-12]. In addition, factors that affect the mRNA structural stability in vivo have primarily below several parts: the gene features, for instance, gene expression quantity and translation initiation rate; structure features, including structural strength like MFE and structural sequence characteristics; and other factors such as mRNA structure location in gene. Due to the complex influence of these factors, precise predict mRNA structural stability become a great challenge.

Deep learning, or artificial neural networks, is a type of machine learning that solves complex problems by learning “big data”. Deep neural network (DNN), a type of deep learning, has multiple hidden layers and units, which can model complex non-linear relationships, was widely used in natural language processing[13], speech recognition[14] or the sensational alpha Go[15]. At present, deep learning has been applied in biological ‘big data’, such as detection of breast cancer based on histological images[16], analysis of ribosomal stalling sites based on ribosome profiling[17] and prediction of protein-nucleotide binding[18]. We propose a novel deep learning DNN model to fit in vitro mRNA structure features, subsequently, the structure status in vivo was predicted, revealing the impact range of these features on the in vivo mRNA structural stability.

As the simplest eukaryote, *S. cerevisiae* was first and most commonly applied to the research of mRNA structure in vivo and ribosome occupancy. Therefore, in this study, *S. cerevisiae* was selected as the a research species to carry out a series of work on the analysis of mRNA structure in vivo. Among in vivo experiments of detecting mRNA structure, DMS-probing determine the

mRNA structure in *S. cerevisiae* by setting a threshold value of Gini coefficient and Pearson correlation was proved to be effective[4]. Thus, we proposed to mark the in vitro and in vivo mRNA structures by this method and divide them into two classes (0 and 1, structures with stable and unstable trend in vivo), then trained deep learning model using these data. After having a precise model that can predict structural state in vivo, we can mutate the different features of these mRNA structures and decipher the particular influence on structures (Figure 1a).

Results

Constructing a Deep Learning Model with 6 mRNA Structure Features.

We proposed a deep neural network (DNN) model, which fit differences of mRNA structures in vitro and in vivo with the quantifiable features that may influence structures, thereby realizing the ability to predict mRNA structural stability in vivo through the in vitro characteristics (Figure 1a). Unlike the previous mRNA structure prediction models aiming at constructing RNA structures[19], this deep learning model was mainly used to predict whether mRNA structures in vivo featured stable or unstable status. DMS profiling[4] is a chemical probing method, methylated adenine and cytosine bases in single-stranded RNA, especially suggested a global feature of vivo, vitro and denatured mRNA structures by measuring Gini index[20] and Pearson correlation coefficient. A high Gini index reveals a high-structured region, and vice versa a low-structured region. Normally, due to a variety of factors such as translation of body ribosomes, the in vitro structure is stronger than in vivo[4]. Therefore, ‘disappear trend’ (DIS) was designated to measure the

differences of mRNA structure in vivo and in vitro, which is equal to Gini index of mRNA structures in vitro minus the Gini index of mRNA structures in vivo. In this study, the whole mRNA structures of Saccharomyces cerevisiae in vitro were classified into two classes by DIS. Class 1 was structures beyond standard deviations (SD), which represented structures with unstable state. Class 0 was structures below the mean minus standard deviation, which yielded the structure profiles of a stable state. Multiple thresholds of classification were tested and 1 times SD was determined for ensuring the amount of training sets and deep learning model accuracy.

Stable and Unstable mRNA structurescould appear on the same gene, meanwhileseveral structural features differences might be interpreted as the existence of these two states (Figure 2a).Previous studies found that a versatility of factors can affect mRNA structure in vivo, such as ribosome density (RD), gene expression level, tRNA levels and mRNA structure stability itself[2,11,12,21]. Six quantitative features of them were chose for modeling: gene features such as reads per kilobase per million mapped reads (RPKM) of RNA-Seq implied mRNA abundance and initial translation ribosome density (INI) calculated by ribosome profiling data; structure subsequence features such as minimum free energy (MFE) and GC contents of subsequence(GC) represent mRNA structure stability; other features such as structural relative position in gene (POS) and ribosome density of structure region (RD). It’s worth mentioning that RD and INI value in this project were averaged from five different studies ribosome profiling data in wild-type yeast[22-26]. It was revealed that mRNA structure DIS value was remarkably positive

relevant to RD and RPKM, implying that high ribosomal density and high mRNA abundance were not conducive to the formation of the stable mRNA structure (Figure 1b), the results were consisted with observations from prior studies[2,27]. Six features showed significant differences (p value<2.2e-16) of two mRNA structure classes, but only the visual difference was revealed on the RD, RPKM, and GC distributions (Figure 1C). It could be initially concluded that these six features do not affect the mRNA structural stability alone, and it was tough to resolve their contribution to mRNA structural stability from the correlation and distribution. Therefore, we adopted DNN modeling and deciphering the relationship between these complex regulatory factors.

We established the DNN sequential model by Tensorflow deep-learning framework[28]. By adjusting the hyperparameters of the model, the final model has 11 hidden layers (10×64 and 1×1), the activation function adopted ReLU[29] and Sigmoid, the optimization function adopted Adam[30] to accelerate the training process, and batch normalization[31] was added in order to prevent overfitting of the model training (Table S1). The average accuracy of the trained DNN model of 10-fold cross validation reached 99.92%. The DNN model that can accurately predict in vivo mRNA structure status through in vitro structural features were established.

Ribosome density revealed a double-sided effect on mRNA structure.

Translating ribosome is a critical factor that affect mRNA structural stability. Previous studies have shown thata negative correlation between ribosomal translation efficiency and in vivo

mRNA structure stability, that is, high translation efficiency corresponds to a low structural region[2,5,27,32]. Two main hypotheses of this correlation in previous studies were proposed. One is that the mRNA structure forms an energy barrier that reduces the translation efficiency of ribosomes. The other is that the translation of ribosomes causes the in vivo structure to fail to fold into a structure, especially long-paired structures. In order to solve this causal relationship between ribosome and RNA structures, we performed single factor gradient mutations on the ribosome density (RD) of randomly selected 5000 stable structure regions and 5000 unstable structural regions, based on the assumptions that no significant changes in other parameters after RD mutation (Figure 2d). After the RD of 64% unstable structures were weakened to the minimum value, they were transformed into a stable structure. It's also revealed that double-side effect of RD impact mRNA structure stability: Low RD was positively correlated with structural stability and this tendency was more obvious in the original stable structure (Figure 2d, red line). Contrarily, RD in a higher range was negative correlated with structural stability. This double-side effect of ribosome density interpreted that stronger structures hindered ribosomes translation especially high translation rate ribosome (equal to low ribosome density), which would slow translation rate and leaded to increased ribosome density. However, it wasarduous to re-increase its density of high ribosome density region by mRNA structure, but instead unwound the structure and leading to in vivo mRNA structural instability.

We proposed the concept of "feature based structure transition region" of mRNA structure in vivo, which performed by determining the inflection point of DNN model's prediction of various features' gradient mutation. Two "RD based structure transition regions" were revealed from the

single-factor mutation results. It was shown that the positive correlation region was below -2.3656 and the negative correlation region were from -2.36568 to 0.149073 of unstable structures and -2.36568 to 2.26147 of stable structure, representing mRNA structure tended to be stable or unstable as RD increasing (Figure 2d, Table S3). Pronounced effect of higher ribosomal density on the stable mRNA structure was showed compared to the unstable structural transform region. It was worthwhile to mention that the boundary values of these transition regionswere the inflection points of structural transformed curve from five studies'ribosome profiling data with data process method (Corresponding raw datawas showed in Table S4).

Further analysis divided the area into fourgroups (Figure 2d). For the originally stable structures, G1 and G2 groups were closer to the gene 5' end and preferred to locate in lower expression genes (low RPKM) comparing to G3 and G4 groups (Figure 2e). Gene upstream preference and low RPKM was also reflected in the group of remain stable structures as same as group G1 and G2, additionally, the lower initial efficiency translation was obviously observed. The original unstable four-group structure has mild difference, but thegroup of remain unstable structures were more focused on highly expressed genes (Figure 2e). Single-factor gradient mutations were difficult to achieve in "real" experiment, thusthe use of high-precision models to predict the general correlation between in vivo structures and RDs through simulated mutations was exactly the effective way to decipher this rule.

mRNA structure prone to be stable at 5'-end gene.

Our trained model provided accurately prediction of yeast mRNA structure state in vivo throughsix features of the structure. Here, we sought to establish the relationship between mRNA structure and its relative position in the gene CDS region. Previous studies analyzed the in vitro structure of various species to derive a tendency that stable RNA structure lean to form at downstream of the translation initiation codon (AUG), which might favor ribosome formation in ribosome 'Ramp'[33-35]. This feature couldalso be seen from the statistics of the 2 classes (Stable and unstable) mRNA structure in the yeast, with a higher proportion of stable mRNA structure at the 5' and 3' ends of the gene (Figure 3b). We changed the relative position (POS) of the mRNA structure on gene by gradient mutations and found that the state of 86.38% originally unstable structures transformed to stableafter transferring structures to the downstream of AUG (Figure 3a). As the mRNA structure was being transferred to gene upstream, the proportion of stable structures increased. The mRNA structure of the middle part of the gene remained basically unchanged, and the stable state of structure at 3' end (beyond 0.8) revealed the slightly trend of stable-transformed especially to unstable structure.It was indicated that stable mRNA structure tended to form at the 5' and 3' ends of the gene, which wasconsistentwith previous studies[33,35], and stable structure itself was easily formed in vivo at upstream of the gene. Combined with the previous RD results, it can be concluded that the mRNA structure and RD at gene 5' end prone to have a "cooperate effect", in other terms, the structure maintained the ribosome density to a certain level for more efficient translation, and the RD also remained in a certain range to ensure mRNA structure stability. Therefore, we concluded that region below approximately 0.3 was regarded as "POS based positive structural transition region", signifying thatmRNA structure

performed more stable in the gene upstream (approximately <30%). Considering that it's arduous to communicate all the changes of the whole features, mutations in this fraction also carried out using single factor mutation, involving a scheme that mutation only occurred with the feature of relative position (POS).Furtherly, the proposal of co-mutation of the position and RD was designed by changing the POS and RD features to the first structure of the gene initial, similar result was obtained as well (Figure 3c). Small fraction of unstable structures in position mutations revealed unchanged status and obvious RPKM may accounted for this appearance (P value < 0.0001, Figure 3d).

mRNA abundance and initiation RD effect on mRNA structure stability.

Two primarily characteristics of the geneaffecting the structural stability: mRNA abundances and translation initiation ribosome density (could be translated into initiation rate through data processing), which were obtained by RNA-Seq data and ribosome profiling data respectively. mRNA abundance (RPKM value in this project) represented mRNA copy number, which hasa certain correlationbut not equaling to the protein synthesis efficiency collected by ribosome profiling[36-38].From the ratios of different mRNA abundance genes of two classes (class 0 and class 1)structures, it was presented that high mRNA abundance was more likely to cause structural instability while there was mild difference in low mRNA abundance (Figure 3f).The mRNA structure served as a barrier for ribosome translation and it is naturally tough to stay stable for maintain the translation efficiency of highly expressed genes. As there was no strong "translation task" for low-expressed genes, structural instability was not strongly needed, hence,

the structure tended to be preferably stable. According to single factor mutation, the effect of mRNA abundance on mRNA structural stability can be divided into positive and negative correlation regions (Figure 3e).The statistical result of fig.3f was probably a good confirmation of the negative correlation region, reflecting that few stable structures appeared on high expression genes of authentic yeast. However, the positive correlation region couldn't be well explained by fig.3f, assumed that low abundance genes might be affected by other features. mRNA structure and translation efficiency were involved by particular translation initial rate[21]. In our previous correlation analysis, we also found a strong correlation between the ribosomal density in the structural region and the ribosomal translation initiation density of the gene (Figure 1b).mRNA structure was more likely to be stable when the initial ribosome density was either in the lower range (below -3.71493) or in the especially higher extent (from 2.057627 to 3.608730), however, it was apt to be unstable whena subsequent particularly high initial ribosome density reached (beyond 3.608730) (Figure 3g).The lower initial density represented a higher initial rate and higher density was always accompanied with a lower initial rate. It was assumed that lower initial rate was prone to keep the mRNA structural stability while extremely low initial rate was prone to cause structural instability, which could be interpreted as the mRNA structure on low initial rate gene may effectively highlight the effects of its translational barrier function. In this part, structural instability was caused by extremely low initial rate, which was consistent with the conclusion in ribosome density mentioned above that excessively high ribosome density may also lead to structural instability. We have also tried to mutate two features involving with mRNA

abundance and translation initiation rate at the same time, but the structural transformation trend fluctuated greatly and no obvious regularity could be concluded.

Effect of the mRNA Structure Subsequence composition on its stability.

The minimum free energy (MFE) and GC content was used to evaluate the strength of mRNA structure base on its sequence composition[21,33,39,40]. MFE were significantly negative correlated with GC and the Pearson correlation reached -0.45 (Figure 1b). Compared with the mutations on other features observed previously, the result of single-factor mutations presented very special both MFE and GC (Figure 4a,4b). The MFE of unstable structure below -4.14 (corresponding to -47.83 kJ·mol-1) became stable, and the trend of stable structure tendency showed no remarkable change. With mean value 0 (corresponding to -19.18 kJ·mol-1) as the inflection point, the transition of stable and unstable structures had a "V" trend. When the changes of normalized MFE value headed to both sides of the mean 0, the stable and unstable structure would shift toward the opposite state to itself. In general, the reduction of MFE means the enhancement of the mRNA structure in vitro, but it couldn’t fully explain the results of this study. When the MFE was less than mean value 0, the originally unstable structure tended to be stable as the MFE decreased, which was in line with conventional wisdom. Unexpectedly, the instability of the original stable structure was uniquely observed in this condition, which was precisely a confirmation of the conclusion that there existed a double-side effect between the mRNA structure and ribosome density referred to above fig. 2d, supposing that when the original stable mRNA structure continuously to be strengthened its stability by reducing MFE, an increasement

of the ribosome density was appeared until reaching a threshold and the excessive ribosome occupancy would in turn unwind the mRNA structure causing unstable state. Further, in terms of single factor mutation on GC contents, a novel finding was discovered that unstable structures at low GC content were more likely to become stable in particularly. Unlike other influencing factors mentioned above, changes of mRNA structure sequence itself leading to a direct influence on its stability, while the mean value (0 of MFE corresponding to -19.18 kJ·mol-1 and 40.25% of GC contents) was the optimum condition that endowed mRNA structure of keeping its original state.

We proposed that the mRNA structural tolerance could be tested by raising and lowering MFE of the mRNA structural sequence. Randomly selected unstable structures and stable structures were used to carry out gradient-decrease MFE mutations and gradient-rising MFE mutations respectively. As is shown in fig.4c and fig.4d, with the sequential introduction of four gradient mutations, altered status of mRNA structure were divided into four classes of T1-T4, and which were step-up representatives indicating enhancement of the mRNA structure tolerance gradually (Figure 4c and 4d). In conclusion, high gene expression levels and high ribosome densities could lead to more tolerant unstable structures, maintaining their own state of instability, thus make it more difficult to change unstable structures through mutations; High gene expression levels and high ribosome densities could make the stability of the stable structure less tolerant, weakening the state of its own stability, resulting in a state that was more likely to change the stable structure

through mutation; Above all, it was conclusively proved gene expression levels and ribosomal density contributed to structural tolerance.

Phylogenetic conserved mRNA structures prone maintained their in vivo state.

Phylogenetic conserved mRNA structures were supported by DMS-seq and inspected by phylogenetic conservation analysis[4]. Positive and negative mutations in the three features, RD, RPKM and INI, of mRNA structure revealed that conserved mRNA structure was more able to maintain its own stability state compared to random structures when subjected to the same degree of mutation (Figure 4e). Consequently, conserved structure was more tolerant to fluctuations in the intracellular environment than the random structure. It was worthwhile mentioning that the conserved structure not only had a stable state in vivo, but also had an unstable structure measured by DIS and both of them met the tolerant results. It raised the possibility that unstable structure in vivo was also involved a certain function during translation.

Discussion

Considering the invention of large-scale, high-throughput methods for in vivo mRNA structure detection, previous studies have statistically derived the general conclusions of in vivo and in vitro mRNA structural differences, such as, the number of in vitro mRNA structures was more than that in vivo, and the in vivo and in vitro mRNA structural differences were mainly caused by ribosome translation[2,4-7]. Due to multiple factors caused the differences of in vivo and in vitro structures, it was tough to perform a further accurate or "personalized analysis" of mRNA structures in previous studies.

In this study, we used a novel deep learning approach to accurately model with labeled mRNA structures in yeast and six quantitative structural features (ribosome density of structure region, minimum free energy and GC content of subsequence, gene mRNA abundance and ribosomal initial density, structural relative position). Laterly, trained deep neural network (DNN) model could accurately predict the stable or unstable status of each mRNA structure in vivo (accuracy reached to 99.92%). Then the rules explaining the effect of these six features on mRNA structural stability were resolved, involving with simulated-mutations of these six features and subsequently predicting by DNN model. We found a double-sided effect between ribosomal density (RD) and mRNA structural stability, i.e., mRNA structural stability was positively correlated with RD in the low ribosome density region and negatively correlated with RD in the high ribosome density region. It might be explained that the mRNA structure was more capable of exerting an energy barrier in the low ribosome density region, consequently, the higher

ribosome density was caused by more stable mRNA structure; and the mRNA structure in vivo was arduous to show stability in the high ribosome density region, owing to the high ribosome occupancy on mRNA structure region. In prior studies, the relationship of RD and mRNA structural stability was not uniform[2,4,27]. In this study, the presumed theory "double-sided effect" on RD and mRNA structural stability was emerged and evidenced by ribosomal density analysis, which was origin from a sparkling thought inspired by the inflection point of RD value at the 'V' shape (Figure 2d). Meanwhile, we found that the mRNA structure at the front of the gene presented more stable status, which was consisted with observations from prior studies[33,35]. What's more, mRNA abundance and initial ribosome density, as members of genetic characteristics, accounted for its various effects on the stability of the mRNA structures to varying degrees. In addition, mutation of mRNA structural subsequence itself could also directly cause changes in the structural state, showing that the mean values of MFE and GC were the optimum condition allowing mRNA structure of keeping its original state. Also, mRNA structural tolerance of MFE adjustment depended more on other factors such as RD or mRNA abundance. Eventually, phylogenetic conserved mRNA structures were prone to be more tolerant in the structural features fluctuations and more capable of keeping their own state when compared with the random mRNA structures. It was worth mentioning that the effect range of these features on the mRNA structural stability could have been provided through the DNN model prediction.

This is the first study to use deep learning approach in deciphering the rules of the in vitro and in vivo differences of mRNA structures. It had been proven that deep neural networks could be used

to solve complex regulatory issues in the translation process in this study. The DNN model accurately predicted the relationship between these six mRNA structural features and its structural states, meanwhile, it also resolved the influence range of these features on structural stability. However, some limitations are worth noting. Previous studies have shown that codon bias and tRNA abundance could affect the ribosome translation rhythm and afterwards influence the mRNA structural stability in vivo[12,21,41]. Codon bias and tRNA abundance were not considered separately in our model, on causality that the ribosome translation information of these two factors were already carried through ribosome density data. Our results are encouraging and should be validated in a larger amount of mutant yeast species. Future works should therefore focus on the following points: codon biases and tRNA abundance substituted for ribosome density, as two respective features, were involved in impact analysis on mRNA structural stability; And more species, like arabidopsis and mouse, as well as more detection methods, like SHAPE-MaP and icSHAPE, should be considered in status prediction of mRNA structure in vivo.

Materials and Methods

Raw Data Preprocessing. *Saccharomyces cerevisiae* RNA structure data was obtained from DMS-profiling experiment (containing in vivo, in vitro and denatured data sets), which was published in the NCBI GEO database, accession number GSE458034. We settled DMS signals of specified genome position in each gene, trimmed G/T sites in gene sequence, then used slide windows of 50nt (full-length slide windows, i.e. after add G and T sited, were about 100nt) to divide trimmed-genes into regions, meanwhile filtered low DMS signal regions (<1 per nt). Gene sequences were retrieved from SGD database assembly R62. Multiple ribosome profiling data (RP) was combined to closer to the true *S. cerevisiae* ribosome occupancy. RP data of wild-type yeast was based on the following studies: Zinshteyn and Gilbert et al. 2013[22], Lareau et al. 2014[23], Albert et al. 2014[24] (BY and RM samples), Young et al. 2016[25] and Nissley et al. 2016[26](Rep1 and Rep2 samples). RNA-Seq data was denoted by Albert et al. 2014 (BY and RM samples)[24]. Then RP and RNA-Seq data were trimmed and mapped to yeast genome (assembly R62) by Bowtie and normalized by RPKM of RNA-Seq data[42]. For the yeast fragments, the off-set 15 nucleotides from 5' end represented the P-site of ribosome's position[36]. Therefore, ribosome occupancy at nucleotides resolution were given by located the genome position of 15th nucleotide of ribosomal fragments. For all these RP data by different studies, ribosome footprint of corresponding position was summed.

Classified RNA Structures from DMS-Probing Data. We defined RNA structure by two metrics: Pearson's correlation coefficient ($r<0.55$) and the Gini coefficient ($Gini<0.14$) as

described method[4]. After removing the G/T sites in the sequence, the 50 nt sliding window was used to divide the CDS region of all genes. Firstly, we determined the structure in vitro by the threshold of r and Gini. In order to more reasonably analyzed the disappearance trend of RNA structure, we proposed a novel measure for structure region, DIS, which was equivalent to subtracting the in vitro and in vivo Gini coefficients of structure region (G). For structure region of length *n*, the DMS signal per sites isS_i, and its DIS and Gini coefficient is:

$$\mathrm{DIS} = G_{vitro} - G_{vivo}$$

$$\mathrm{G} = \frac{\sum_{i=1}^{n}\sum_{j=1}^{n}\left|S_i - S_j\right|}{2n\sum_{i=1}^{n} S_i}$$

Calculation and Standardization Six Features of mRNA Structures. We chose six structural features as a measure of mRNA structure. The feature TE (translation efficiency) was the protein synthesis rate of structure region[43]; MFE was the minimum free energy of a local subsequence, calculated by by Vienna (v.2.1.9) package RNAfold[44] function and visualized by RNAstructure[45]; INI was the translation efficiency at gene initial region (+15nt); RPKM represented the relative number of transcripts of a gene and was calculated by RNA-Seq data; GC represented the GC content of the structural region sequence; POS was the relative position of the structural region on the gene, which was obtained by dividing the sequence length by the central position of the structural region. The distribution of these six features, of which three, TE, INI and RPKM were exponentially distributed, so a logarithmic process was performed. Prior to deep learning training, parameters (except Pos and GC) were normalized ($(x-\mu)/\sigma^2$) to make the model more accurate.

Established Deep Neural Networks Model. We established a deep neural networks model to

predict whether RNA structures in vitro would have stable or unstable tendency in vivo. By labeling known-status structures and predicting a sequence using these six features, a prediction model can be obtained. DIS was used to evaluate the degree of RNA structure destabilization in vivo and its distribution was similar to a normal distribution. To ensure a sufficient number of training set and development set (Train/Dev set), we decided to use ($\mu \pm \sigma$) as the threshold value. Class 1 was defined as DIS greater than $\mu+\sigma$, meaning that such structures had an unstable tendency in vivo; class 0 was tagged as DIS greater than $\mu-\sigma$, representing that such structures had a stable tendency in vivo. Therefore, it became a binary classification problem. In this project, we used the Tensorflow[28], a deep learning framework created by Google Brain team, and Keras API[46] to construct a DNN model, solving the complex problem of RNA structural state prediction in vivo. This framework can greatly simplify the process of building the DNN sequential model by using the built-in functions. Through a large number of hyper-parameter adjustments, the final parameters of the model were determined using ROC curve (Figure S2, Table S1) and 10-fold cross-validation was used to test the accuracy of the model. DNN model of this project, python script of loading model and sample file of stable and unstable structures could be accessed in the github (https://github.com/atlasbioinfo/RNAstructureDisStay/).

Single factor mutation and prediction. We performed single-factor mutation experiments on the six features of mRNA structure to test the effect of these features on the structural stability. From the development set, 5,000 unstable structures and 5000 stable structures were randomly selected. Each of the other parameters was subject to 100 equal parts gradient mutation ranging

from its minimum value to its maximum. After that, the model of the mutated structure was predicted again using the DNN model. Transformed mRNA structure was defined as the change of the mRNA structure status (i.e., be classified to another group) after the mutation. The ratio of transformed mRNA structure was equal to the number of transformed structures divided by the total structures. In the results of gradient mutation, with the increase of each one of the six features, the increase of mRNA structural stability was named as “positive correlated transition region”, and the decline of mRNA structural stability was regarded as “negative correlated transition region”.

Ribosome density feature analysis. mRNA structures were divided into 5 groups, G1 to G4 and “stable” or “unstable” structures. G1 to G4 groups were according to the midpoints of “ribosome density based positive and negative correlated transition region”. mRNA structure remained stable during the mutation of the ribosome density gradient was named “stable”, and the structure that remained unstable was called “unstable”.

mRNA structural tolerance analysis of MFE. Randomly selected 5000 high MFE mRNA structures and 5000 low MFE mRNA structures (MFE threshold: $\mu \pm \sigma$) were performed four times gradient mutations (decrease 1 SD of MFE per mutation for high MFE structures and increase 1 SD of MFE for low MFE structures). The status of mRNA structure after each mutation were recorded as the basis for measuring structural tolerance.

The original mRNA structure state was changed at the first mutation was called “T1”, that is, the least tolerate mRNA structure and the state transformed until the 4th mutation was named “T4”,

that is, the most tolerant mRNA structure. mRNA structures with constant state during mutation process were not considered and minimal back-mutation structures was trimmed either.

Tolerance analysis of conserved structures. Conservative structural data from Rouskin's study[4]. Random structure was randomly extracted from the total mRNA structure and excluded the list of conserved structures. Three parameters, RD, RPKM and INI, that may fluctuate within the cell, a one-fold standard deviation mutation was performed (Up was +SD and down was -SD).

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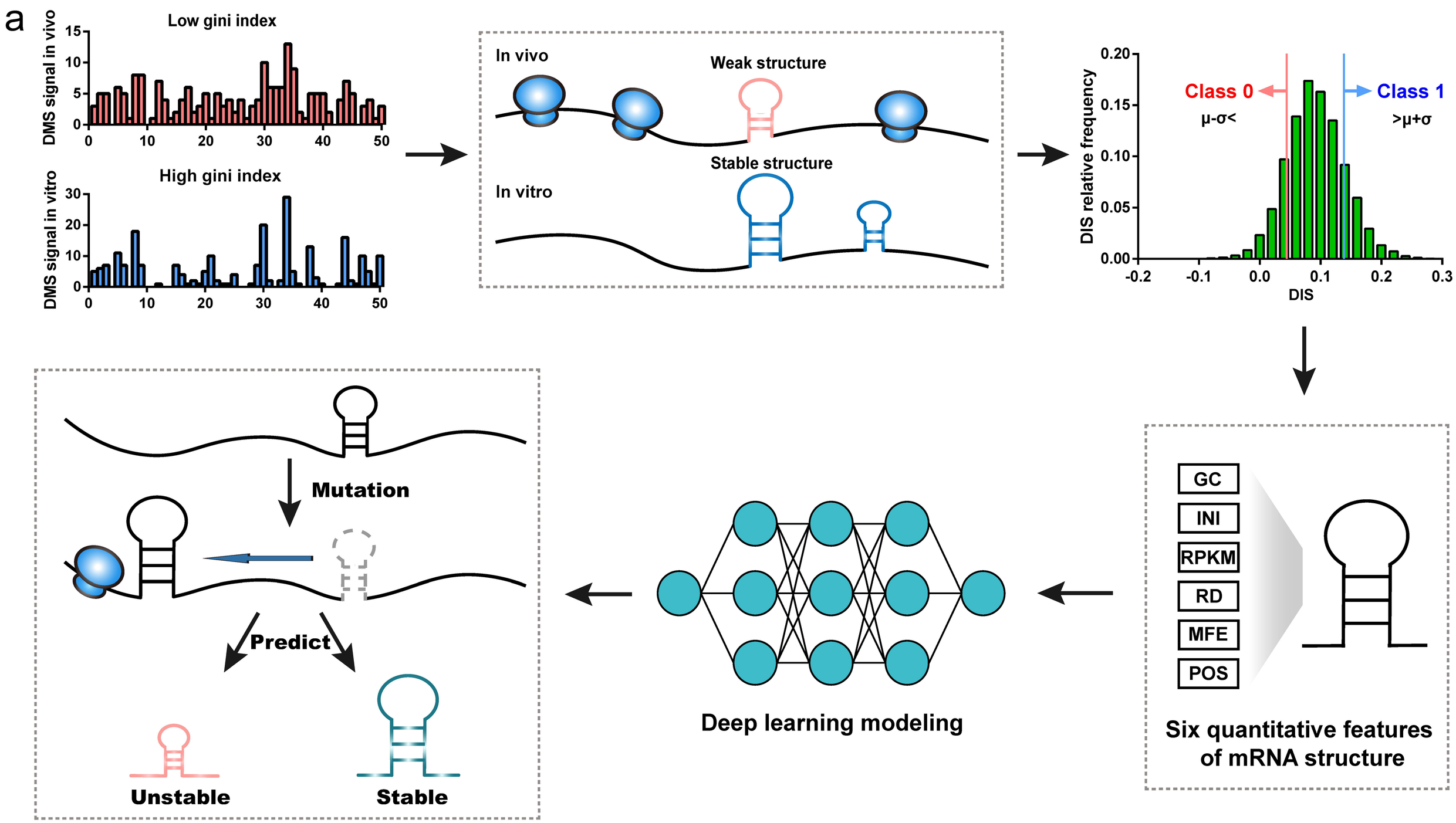
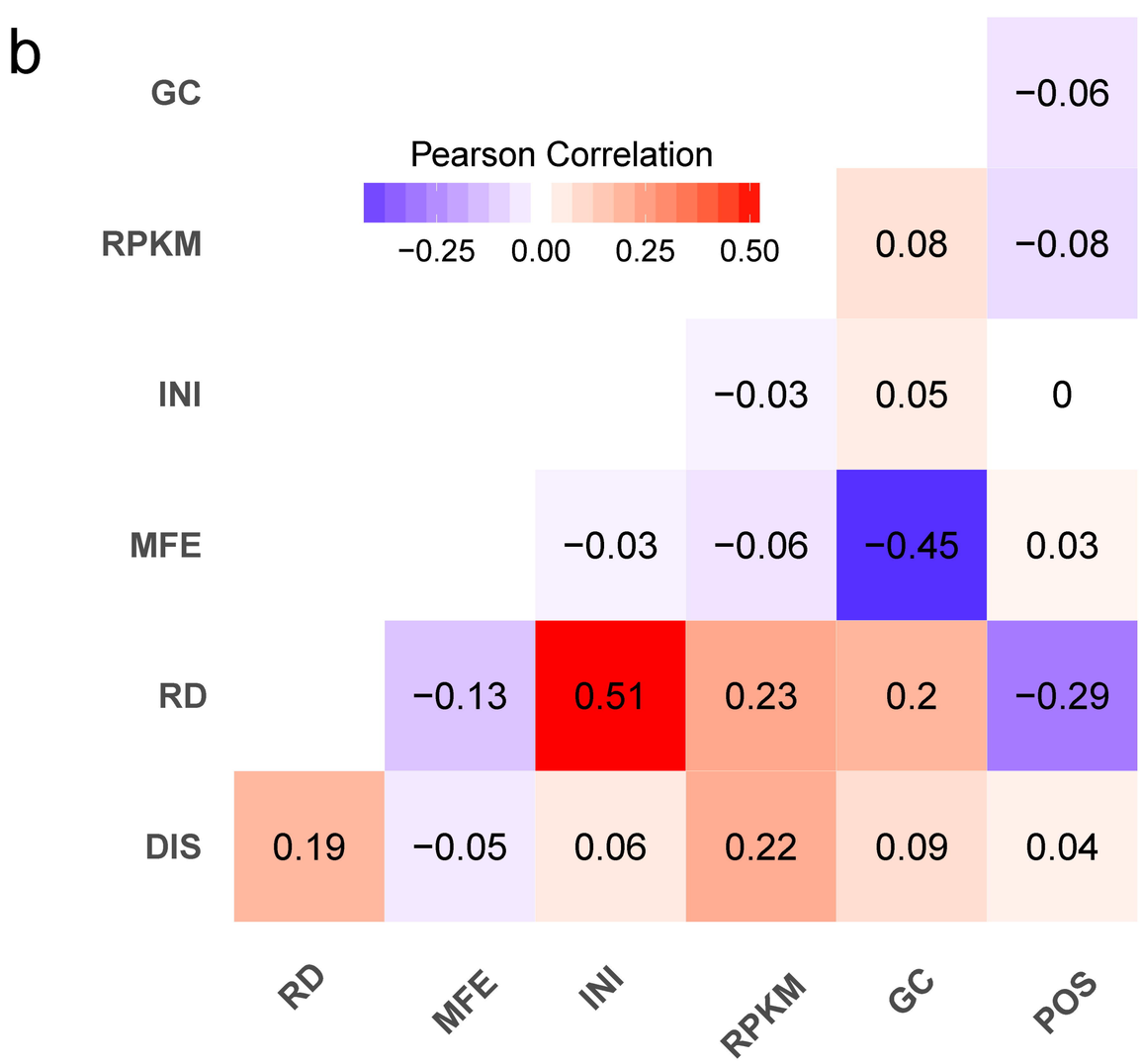
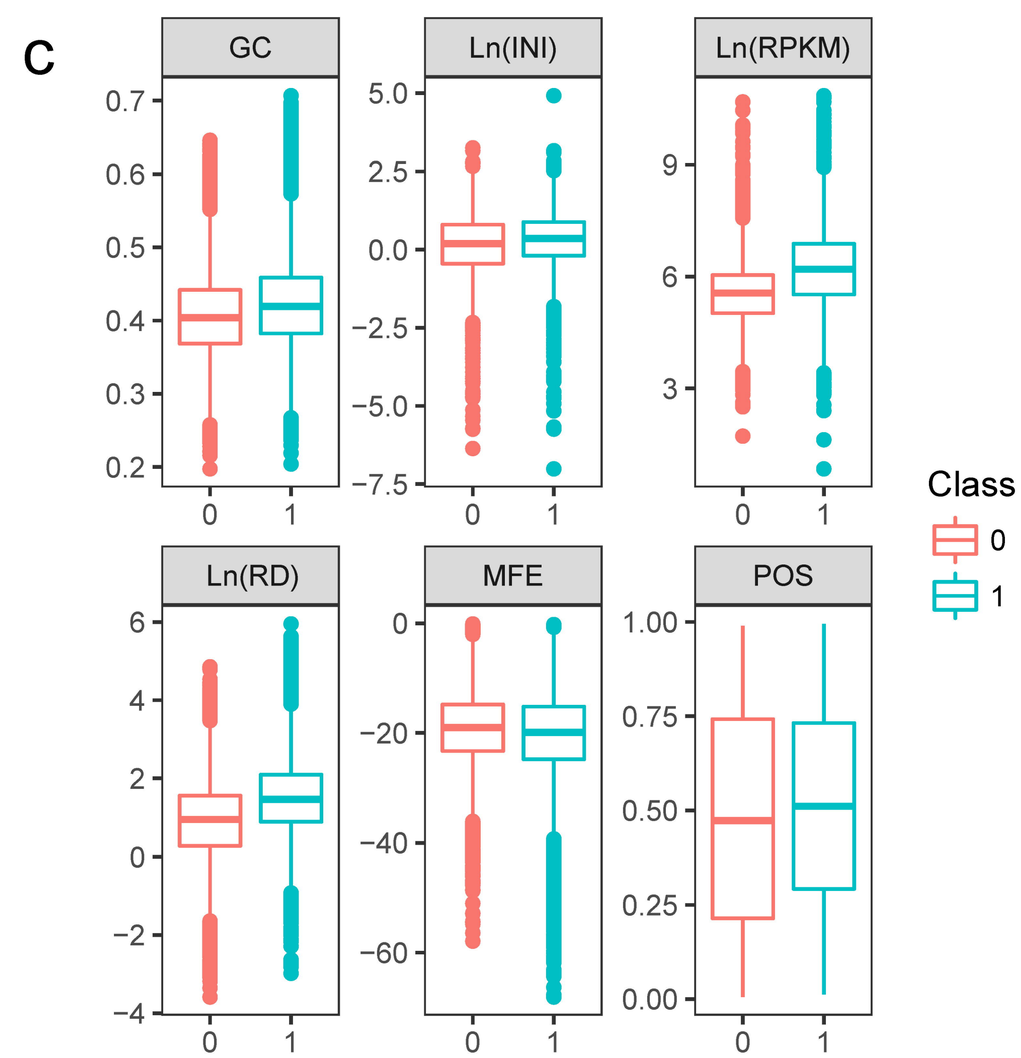
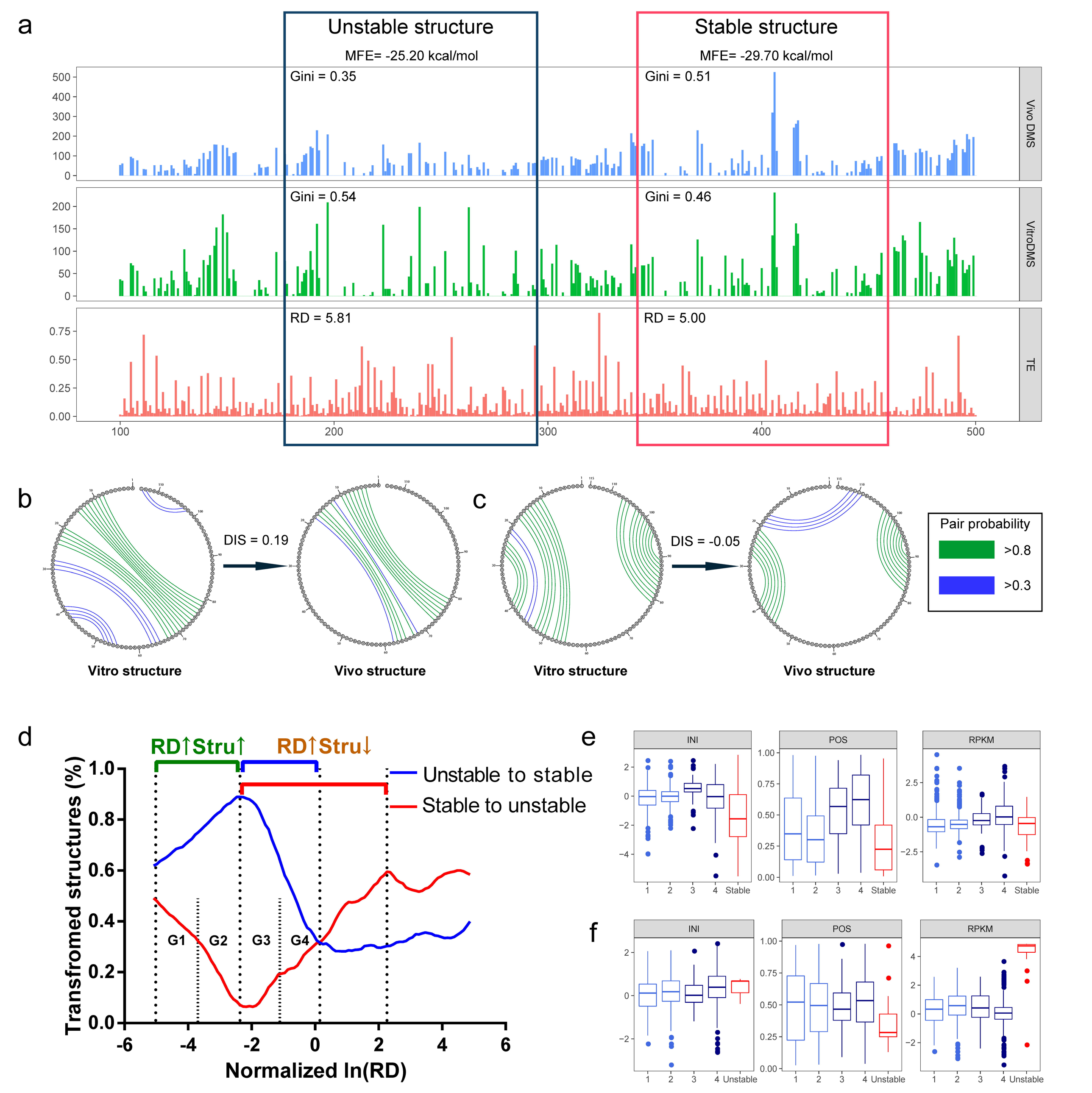
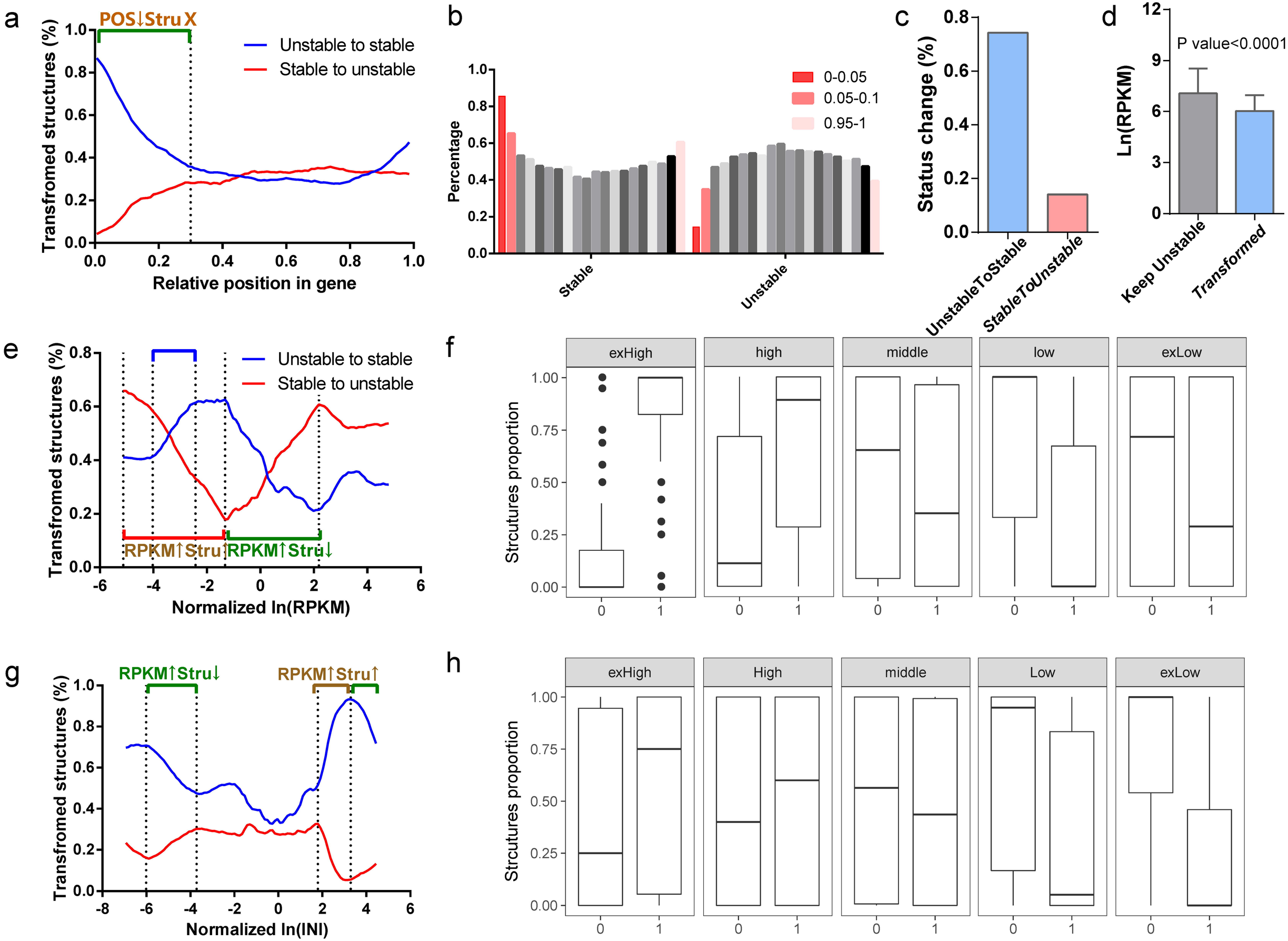
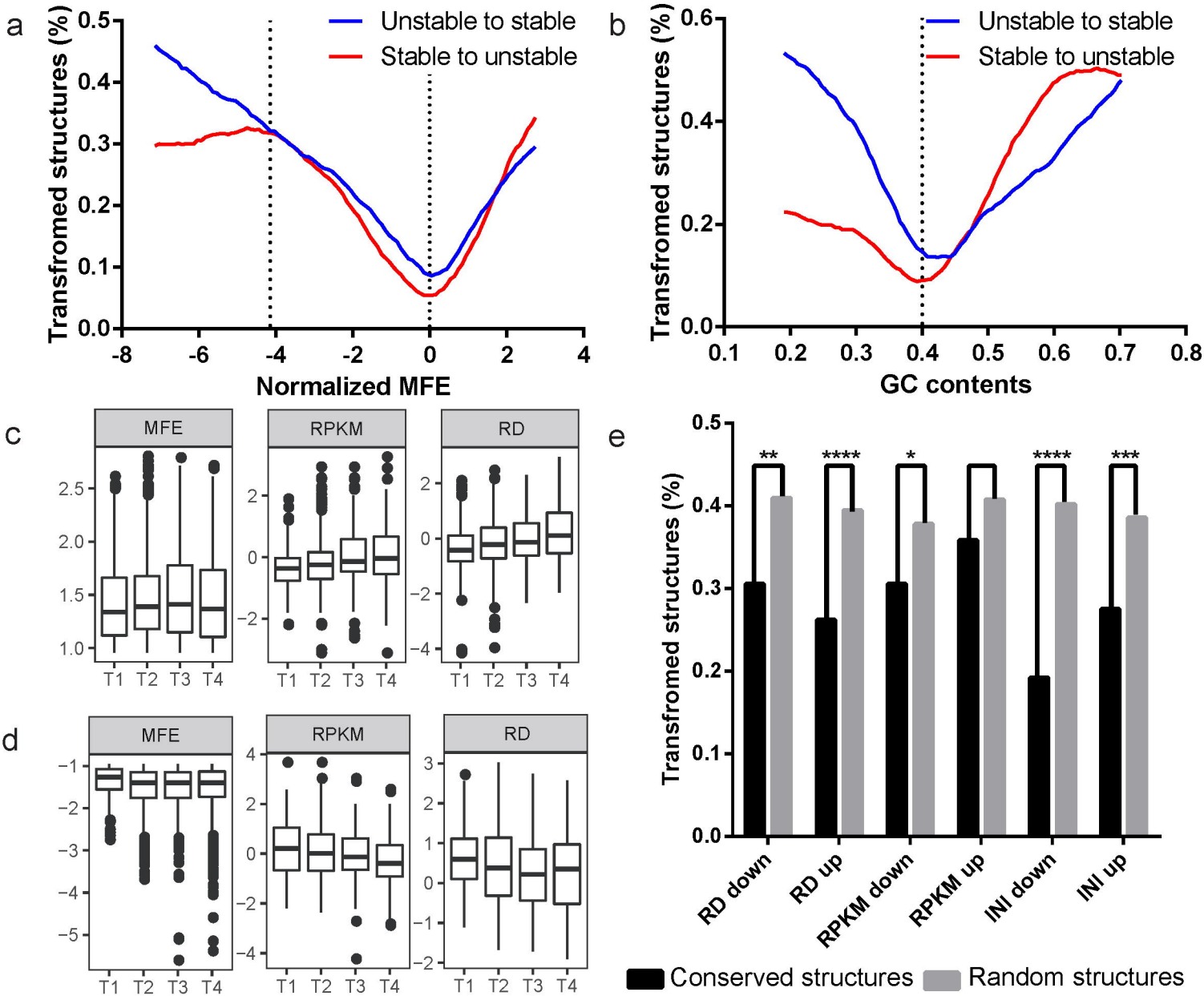

a
Low gini index
DMS signal in vivo
High gini index
DMS signal in vitro
In vivo
Weak structure
Stable structure
In vitro
DIS relative frequency
Class 0
μ-σ<
Class 1
>μ+σ
DIS
Mutation
Predict
Unstable
Stable
Deep learning modeling
GC
INI
RPKM
RD
MFE
POS
Six quantitative features of mRNA structure


b
Pearson Correlation
−0.25 0.00 0.25 0.50
GC −0.06
RPKM 0.08 −0.08
INI −0.03 0.05 0
MFE −0.03 −0.06 −0.45 0.03
RD −0.13 0.51 0.23 0.2 −0.29
DIS 0.19 −0.05 0.06 0.22 0.09 0.04
RD MFE INI RPKM GC POS


c
GC
Ln(INI)
Ln(RPKM)
Ln(RD)
MFE
POS
Class
0
1


a
Unstable structure
MFE= -25.20 kcal/mol
Stable structure
MFE= -29.70 kcal/mol
Gini = 0.35
Gini = 0.51
Vivo DMS
Gini = 0.54
Gini = 0.46
VitroDMS
RD = 5.81
RD = 5.00
TE
b
DIS = 0.19
Vitro structure
Vivo structure
c
DIS = -0.05
Vitro structure
Vivo structure
Pair probability
>0.8
>0.3
d
RD↑Stru↑
RD↑Stru↓
Unstable to stable
Stable to unstable
Transfromed structures (%)
G1
G2
G3
G4
Normalized ln(RD)
e
INI
POS
RPKM
Stable
f
Unstable


a
POS↓Stru X
Unstable to stable
Stable to unstable
Transfromed structures (%)
Relative position in gene
b
0-0.05
0.05-0.1
0.95-1
Percentage
Stable
Unstable
c
Status change (%)
UnstableToStable
StableToUnstable
d
P value<0.0001
Ln(RPKM)
Keep Unstable
Transformed
e
Unstable to stable
Stable to unstable
RPKM↑Stru↑
RPKM↑Stru↓
Transfromed structures (%)
Normalized ln(RPKM)
f
exHigh
high
middle
low
exLow
Strcutures proportion
g
RPKM↑Stru↓
RPKM↑Stru↑
Transfromed structures (%)
Normalized ln(INI)
h
exHigh
High
middle
Low
exLow
Strcutures proportion


a
Transfromed structures (%)
Unstable to stable
Stable to unstable
Normalized MFE
b
Transfromed structures (%)
Unstable to stable
Stable to unstable
GC contents
c
MFE
RPKM
RD
T1
T2
T3
T4
d
MFE
RPKM
RD
T1
T2
T3
T4
e
Transfromed structures (%)
RD down
RD up
RPKM down
RPKM up
INI down
INI up
Conserved structures
Random structures