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Tandem repeats ubiquitously flank and select translation initiation sites.

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ABSTRACT

Evolutionary divergence in cis-regulatory sequences impacts translation initiation sites (TISs). The implication of tandem repeats (TRs) in TIS selection remains elusive for the most part. Here we employed the TIS homology concept to study a possible link between all categories of TRs and TIS selection. Human and 83 other species were selected, and data was extracted on the entire protein-coding genes (n=1,611,368) and transcripts (n=2,730,515) annotated for those species from Ensembl 102. Two different weighing vectors were employed to assign TIS homology, and the results were assessed in 10-fold validation. On average, every TIS was flanked by 1.19 TRs of various categories within the 120 bp upstream sequence. We detected statistically significant excess of non-homologous TISs co-occurring with human-specific TRs, vice versa. We conclude that TRs are abundant cis elements in the upstream sequences of TISs across species, and there is a link between all categories of TRs and TIS selection.

Keywords: translation initiation site; tandem repeat; genome-scale; TIS selection; homology

Abbreviations
TIS: Translation initiation site
TR: Tandem repeat
STR: Short tandem repeat
ORF: Open reading frame
UTR: untranslated region
HS-TR: Human-specific TR
NHS-TR: Non-human-specific TR
INTRODUCTION

Translational regulation can be global or mRNA specific, and most instances of translational regulation affect the rate-limiting initiation step (1,2). While mechanisms that result in the selection of translation initiation sites (TISs) are largely unknown, conservation of the alternative TIS positions and the associated open reading frames (ORFs) between human and mouse cells (3) implies physiological significance of alternative translation. A vast number of human protein-coding genes consist of alternative TISs, which are selected based on complex and yet not fully understood scanning mechanisms (3-6). The alternative TISs can result in various protein structures and functions (7,8).

Whereas lack of sufficient knowledge has led to the conclusion that TISs are stochastic for the most part, the probability of using a particular TIS differs among mRNA molecules, and can be dynamically regulated over time (9). Selection of TISs and the level of translation and protein synthesis depend on the cis regulatory elements in the mRNA sequence and its secondary structure such as the formation of hair-pins, stem loop, and thermal stability (10-15). In fact, the ribosomal machinery has the potential to scan and use several ORFs at a particular mRNA species (16).

A tandem repeat (TR) is a sequence of one or more DNA base pairs (bp) that is repeated on a DNA stretch. While TRs have profound biological effects in evolutionary, biological, and pathological terms (17-23), the effect of these intriguing elements on protein translation remains largely (if not totally) unknown. There are limited publications indicating that when located at the 5’ or 3’ UTR, short tandem repeats (STRs) (core units of 1-6 bp) can modulate translation, the effect of which has biological and pathological implications (24-28). Abnormal STR expansions impact TIS selection in a number of neurological disorders (29,30).

Based on a TIS homology approach, we recently reported a link between STRs and TIS selection (31). Here, we extended our weighing methods, and developed a comprehensive software package, to study a possible link between all categories of TRs and TIS selection across 84 species.

RESULTS AND DISCUSSION

TRs are ubiquitous cis elements flanking TISs.

A total of 1,611,368 protein-coding genes and 2,730,515 transcripts were investigated across the 84 selected species, which resulted in the extraction of 3,283,771 TRs. 22,791 genes, 93,706 transcripts, and 99,818 TRs belonged to the human species (Supplementary Table 1). On average, there were 1.64 transcripts and 1.97 TRs per gene, and 1.19 TRs per transcript per species (Fig. 1). The highest ratios of transcripts and TRs per gene, at 4.11 and 4.38, respectively, belonged to human. Human ranked 59th among 84 species in respect of the TR/transcript ratio (Supplementary Table 1).
Out of the 93,706 identified protein-coding transcripts in the human genome, there were 50,169 transcripts which had at least one TR in the upstream flanking sequence (53.54% of protein-coding transcripts). At a similarly high rate, from the 22,791 identified protein-coding genes in the human genome, there were 15,256 genes which had at least one transcript flanked by a TR in its upstream flanking region (66.94% of human protein-coding genes). From the 2,850 different types of TRs in the human genome, there were 1,504 different types of TRs that were human-specific (52.77% of the TRs).

Across TR categories 1-4, we detected 660, 101, 339 and 404 different types of human-specific TRs, respectively, the top most abundant of which are represented in Table 1.

**Link between TRs and TIS selection**

We employed two weighing settings for designating homologous vs. non-homologous TISs, one of which was similar to our previous approach (31). In both settings, there was significant co-occurrence of human-specific TRs with non-homologous TISs, and non-human-specific TRs with homologous TISs (Fig. 2). The results were replicated in 10-fold validation (Fig. 3).

In addition to the weighing vectors used, we employed the Needleman Wunsch algorithm (32) to check for the robustness of the proposed link. Human TIS homology was checked for the proteins encoded by the orthologous genes in three species, mouse, macaque, and chimpanzee. A dramatically lower homology was observed for human proteins linked by human-specific TRs (Fig. 4). Similarity calculation between human proteins and the three other species was performed by RESTful API at: https://www.ebi.ac.uk/Tools/psa/emboss_needle (33).

**Evolutionary and biological implications**

Of the 15,256 human genes which had at least one transcript flanked by a TR in their upstream flanking region, there were 2,991 genes which had at least one transcript flanked by a human-specific TR in their upstream flanking region (Supplementary Tables 2 & 3). Text mining of a number of those genes as examples (34,35) yielded predominant expression and functions in the human brain and skeletal muscle (e.g., MYH2, TTN, SLC6A8, CACNA1A, and EIF5AL1) (Table 2). These are examples of expression enrichment in tissues that are frequently subject to species-specific evolutionary processes. Gene ontology and pathway Enrichment analyses were performed on the extracted genes from Table 2, by using the online facility "Enrichr" tool, that is a useful application for the success of any high-throughput gene function analysis (https://maayanlab.cloud/Enrichr/enrich) (36-38). The list of genes in Table 2 was given as input, and “GO Biological Process 2021” and “KEGG 2021 Human” were selected as libraries, respectively (Fig. 5). Interestingly, nervous system development (GO 0007399) was the top enriched ontology term, and the calcium signaling pathway was the top
pathway. Calcium signaling pathways are being increasingly recognized as essential processes in the human brain neurogenesis (39).

Our findings provide prime evidence of a link between all categories of TRs and TIS selection, mechanisms of which are virtually unknown at this time. Our approach was based on homology search, which reliably identifies "homologous" proteins or genes by detecting excess similarity (40). This approach was performed using two weighing vectors and a confirmatory algorithm, which consistently supported the link.

It is possible that asymmetric and stem-loop structures, which are inherent properties of repeat sequences result in genetic marks that enhance TIS selection. Asymmetric TR structures have recently been reported to be linked to various biological functions, such as replication and initiation of transcription start sites (41). It remains to be clarified how this intriguingly abundant reservoir of regulatory elements contributes to TIS selection across species.

Conclusion

We conclude that TRs are abundant cis elements that flank TIS sequences, and contribute to TIS selection at the trans-species level. These findings shed light on an underappreciated aspect of evolutionary biology, which warrants future functional analyses.

MATERIAL AND METHODS

Data collection

All sequences, species, and gene datasets collected in this study were based on Ensembl 102 (https://www.ensembl.org). 84 species were selected, which encompassed orders of vertebrates and non-vertebrates (Fig. 6). Throughout the study, all species were compared with the human sequence, as reference. The list of species was extracted via RESTful API, in Java language. In parallel, a list of available gene datasets of the selected species was collected by using the “biomaRt” package (42,43) in R language.

In the next step, in each selected species, all protein coding transcripts of protein coding genes were extracted. Subsequently, the 120 bp upstream flanking sequence of all annotated protein-coding TISs were retrieved and analyzed. All steps of data collection were performed by querying on the Biomart Ensembl tool via RESTful API, which was implemented in the Java language, except fetching the primary list of available species and gene datasets. For each species, its name, common name and display name were retrieved. For each gene in each species, its gene name, Ensembl ID and the annotated transcript IDs were retrieved, and finally, for each transcript its coding DNA sequence, upstream flanking region and protein sequence were retrieved. All collected data was stored in a
MySQL database which is accessible at
https://figshare.com/search?q=10.6084%2Fm9.figshare.15405267 .

A candidate sequence was considered a TR if it complied with the following four rules: 1 - In the
case of mono nucleotide cores, the number of repeats should be ≥ 6. 2 - In the case of 2-9 bp cores,
the number of repeats should be ≥ 3. 3 - For other core lengths, the number of repetition of cores
should be ≥ 2. 4 - TRs of the same core sequence should not overlap if they were in the same
upstream flanking region.

We categorized the TRs based on the core lengths as follows: Category 1: 1-6 bp, Category 2: 7-
9 bp, Category 3: 10-15 bp, and Category 4: ≥16 bp. This was an arbitrary classification to allow for
possible differential effect of various core length ranges.

Retrieval of data across species

Using the enhanced query (Supplementary Table 4) form on the Biomart Ensembl tool along with the
RESTful API tools, a Java package was developed to retrieve, store, and analyze the data and
information. The source codes and the Java package are available at:
https://github.com/Yasilis/STRsMiner-JavaPackage_PaperSubmission/tree/develop.

Identification of Human-specific TRs

The TIS-flanking upstream 120 bp of all annotated protein-coding transcripts of protein-coding genes
were screened in 84 species for the presence of TRs in four categories based on the core length. The
data obtained on the human TRs was compared to those of other species, and the TRs which were
specific to human were identified. The selected genes were clustered based on their names
(orthologous genes were placed in a cluster). All TRs of each cluster were extracted and categorized
based on the species. In the next step, in each cluster, only TRs that were specific to human (not
detected in other species) were retained and set as reference.

Evaluation of TIS homology

Identifying the degree of homology between two transcripts requires assigning a weight value to each
position of the sequences. Weighted homology scoring was performed in two different weight settings,
as weighing vectors $W_1$ and $W_2$, which can be distinguished by $k = \{1,2\}$. These two weighing vectors
are defined as follow (Eq. 1, 2):

$W_1 = \{0, 25, 25, 25, 12.5, 12.5\}$  \hspace{1cm} (1)

$W_2 = \{0, 25, 25, 25, 12.5, 12.5\}$  \hspace{1cm} (2)
If M is the first methionine amino acid of the two peptide sequences (position of 0 in the two weighing vectors), for all next five successive positions represented by \(i\) in the formula (Eq. 9), we defined five weight coefficients \(w_{k,1}\) to \(w_{k,5}\), observed in the \(W_k\) vector.

Homology of the five amino acids and, therefore, the TIS was inferred based on the value of similarity scoring, in which a similarity of \(\geq 50\%\) was considered "homology". This threshold was achieved following BLASTing three thousand random pair-wise similarity checks of the initial five amino acids of randomly selected proteins as previously described (31).

Scoring human-specific and non-specific TR co-occurrences with TISs.

In both weighing methods, the initial five amino acid sequence (excluding the initial methionine) of the human TISs that were flanked by human-specific TRs and non-human-specific TRs were BLASTed against all the initial five amino acids (excluding the initial methionine) of the orthologous genes in the remaining 83 species. The above was aimed at comparing the number of events in which human-specific and non-specific TRs occurred with homologous and non-homologous (TISs). For computing the number of homologous and non-homologous TISs, we needed to consider a number of assumptions. We defined \(G\) as the set of all human protein coding genes. Therefore, \(g\) denoted a gene that belonged to the \(G\) set (Eq. 3).

\[
G = \{g | g \text{ is a human protein coding gene}\} \quad (3)
\]

We also defined \(T_H(g)\) and \(T_P(g)\) as the set of all annotated transcripts in a gene \(g\), which belonged to human and other species, respectively (Eq. 4, 5).

\[
T_H(g) = \left\{ t | t \text{ was a human protein coding transcript which belonged to the gene, } g \right\} \quad (4)
\]

\[
T_P(g) = \left\{ t | t \text{ was a protein coding transcript which belonged to the gene, } g \text{ but, did not exist in human } \right\} \quad (5)
\]

Moreover, \(T^-\) denoted all filtered transcripts of \(T\) which had at least one human-specific TR at the 120 bp genomic DNA interval upstream of the TIS, while, \(T^+\) denoted all filtered transcripts of \(T\), which had at least one TR at the 120 bp genomic DNA interval upstream of the TIS.

\[
W_2 = \{0, 20, 20, 20, 20, 20\}
\]
The following formula was developed to measure the degree of similarity of two peptides in the two weighing settings (Eq. 6).

$$H_k = \sum_{g \in C} \left( \sum_{t_a \in T_g (g)} \sum_{t_b \in T_g (g)} \Theta_k(t_a, t_b) \right)$$  \hspace{1cm} (6)

In this formula, $\Theta$ is a binary function that decides whether the transcripts are homologous or not, and $k = \{1,2\}$ refer to each weight setting. If $S$ function measures the similarity score, $\Theta$ can be defined as follow (Eq. 7):

$$\Theta_k(t_a, t_b) = \begin{cases} 1, & \text{if } S_k(t_a, t_b) \geq 50 \\ 0, & \text{o.w.} \end{cases}$$  \hspace{1cm} (7)

For calculating the similarity score, we used another binary function. We defined $\Phi$ as follows: (Eq. 8):

$$\Phi(x, y) = \begin{cases} 1, & \text{if } x = y \\ 0, & \text{o.w.} \end{cases}$$  \hspace{1cm} (8)

This function takes two amino acids as argument and returns 1 as output if they are the same, and zero if they are not the same. Therefore, $S(t_a, t_b)$ is defined by the following formula (Eq. 9):

$$S_k(t_a, t_b) = \sum_{i=2}^{6} w_{k,i} \Phi(P_i(t_a), P_i(t_b))$$  \hspace{1cm} (9)

In this function, the $i^{th}$ amino acid in the sequence of the transcript $t$, is denoted by $P_i(t)$.

We replicated the comparisons in 10-fold cross-validation. In each-fold, genes in the human non-specific TR group were randomly selected according to the number of genes in the human-specific TRs group. This process was repeated for the two methods (two different weight vectors) and for each of the four categories of TRs. For each category and weighing method, the mean of the result of each round was calculated as a final result. Finally, the Fisher exact test was run for each-fold (Supplementary Table 5).

**DATA AVAILABILITY**

The datasets generated and analyzed during this study are available in the “figshare” repository, with the identifier “10.6084/m9.figshare.15405267”

Also, other source code and software available in the GitHub repository
(https://github.com/Yasilis/STRsMiner-JavaPackage_PaperSubmission/tree/develop)
REFERENCES

1. Sonenberg, N. and Hinnebusch, A.G. (2009) Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell*, **136**, 731-745.
2. Gebauer, F. and Hentze, M.W. (2004) Molecular mechanisms of translational control. *Nature reviews Molecular cell biology*, **5**, 827-835.
3. Lee, S., Liu, B., Lee, S., Huang, S.-X., Shen, B. and Qian, S.-B. (2012) Global mapping of translation initiation sites in mammalian cells at single-nucleotide resolution. *Proceedings of the National Academy of Sciences*, **109**, E2424-E2432.
4. Na, C.H., Barbhuiya, M.A., Kim, M.-S., Verbruggen, S., Eacker, S.M., Pletnikova, O., Troncoso, J.C., Halushka, M.K., Menschaert, G. and Overall, C.M. (2018) Discovery of noncanonical translation initiation sites through mass spectrometric analysis of protein N termini. *Genome research*, **28**, 25-36.
5. Andreev, D.E., O’Connor, P.B., Loughran, G., Dmitriev, S.E., Baranov, P.V. and Shatsky, I.N. (2017) Insights into the mechanisms of eukaryotic translation gained with ribosome profiling. *Nucleic acids research*, **45**, 513-526.
6. Studtmann, K., Ölschläger-Schütt, J., Buck, F., Richter, D., Sala, C., Bockmann, J., Kindler, S. and Kreienkamp, H.-J. (2014) A non-canonical initiation site is required for efficient translation of the dendritically localized Shank1 mRNA. *PLoS One*, **9**, e88518.
7. Fukushima, M., Tomita, T., Janoshazi, A. and Putney, J.W. (2012) Alternative translation initiation gives rise to two isoforms of Orai1 with distinct plasma membrane mobilities. *J Cell Sci*, **125**, 4354-4361.
8. Bazykin, G.A. and Kochetov, A.V. (2011) Alternative translation start sites are conserved in eukaryotic genomes. *Nucleic acids research*, **39**, 567-577.
9. Boersma, S., Khuperkar, D., Verhagen, B.M.P., Sonneveld, S., Grimm, J.B., Lavis, L.D. and Tanenbaum, M.E. (2019) Multi-Color Single-Molecule Imaging Uncovers Extensive Heterogeneity in mRNA Decoding. *Cell*, **178**, 458-472 e419.
10. Li, J.J., Chew, G.-L. and Biggin, M.D. (2019) Quantitative principles of cis-translational control by general mRNA sequence features in eukaryotes. *Genome biology*, **20**, 1-24.
11. Martinez-Salas, E., Lozano, G., Fernandez-Chamorro, J., Francisco-Velilla, R., Galan, A. and Diaz, R. (2013) RNA-binding proteins impacting on internal initiation of translation. *Int J Mol Sci*, **14**, 21705-21726.
12. Cenik, C., Cenik, E.S., Byeon, G.W., Grubert, F., Candille, S.I., Spacek, D., Alsallakh, B., Tilgner, H., Araya, C.L., Tang, H. et al. (2015) Integrative analysis of RNA, translation, and protein levels reveals distinct regulatory variation across humans. *Genome Res*, **25**, 1610-1621.
13. Babendure, J.R., Babendure, J.L., Ding, J.-H. and Tsien, R.Y. (2006) Control of mammalian translation by mRNA structure near caps. *Rna*, **12**, 851-861.
14. Master, A., Wójcicka, A., Giżewska, K., Poplawski, P., Williams, G.R. and Nauman, A. (2016) A novel method for gene-specific enhancement of protein translation by targeting 5’UTRs of selected tumor suppressors. *PLoS one*, **11**, e0155359.
15. Jagodnik, J., Chiaruttini, C. and Guillier, M. (2017) Stem-loop structures within mRNA coding sequences activate translation initiation and mediate control by small regulatory RNAs. *Molecular cell*, **68**, 158-170. e153.
16. Kochetov, A.V., Allmer, J., Klimenko, A.I., Zuraev, B.S., Matushkin, Y.G. and Lashin, S.A. (2017) AltORFev facilitates the prediction of alternative open reading frames in eukaryotic mRNAs. *Bioinformatics*, **33**, 923-925.
17. Hannan, A.J. (2018) Tandem repeats mediating genetic plasticity in health and disease. *Nat Rev Genet*, **19**, 286-298.
18. Afshar, H., Adelirad, F., Kowsari, A., Kalhor, N., Delbari, A., Najafipour, R., Foroughan, M., Bozorgmehr, A., Khamse, S. and Nazaripanah, N. (2020) Natural selection at the NHLH2 core promoter exceptionally long CA-repeat in human and disease-only genotypes in late-onset neurocognitive disorder. *Gerontology, 66*, 514-522.

19. Press, M.O., McCoy, R.C., Hall, A.N., Akey, J.M. and Queitsch, C. (2018) Massive variation of short tandem repeats with functional consequences across strains of Arabidopsis thaliana. *Genome Res, 28*, 1169-1178.

20. Bagshaw, A.T.M. (2017) Functional Mechanisms of Microsatellite DNA in Eukaryotic Genomes. *Genome Biol Evol, 9*, 2428-2443.

21. Abe, H. and Gemmell, N.J. (2016) Evolutionary Footprints of Short Tandem Repeats in Avian Promoters. *Sci Rep, 6*, 19421.

22. Ohadi, M., Valipour, E., Ghadimi-Haddadan, S., Namdar-Aligoodarzi, P., Bagheri, A., Kowsari, A., Rezazadeh, M., Darvish, H. and Kazeminasab, S. (2015) Core promoter short tandem repeats as evolutionary switch codes for primate speciation. *American journal of primatology, 77*, 34-43.

23. Mohammadparast, S., Bayat, H., Biglarian, A. and Ohadi, M. (2014) Exceptional expansion and conservation of a CT-repeat complex in the core promoter of PAXBP1 in primates. *Am J Primatol, 76*, 747-756.

24. Rovozzo, R., Korza, G., Baker, M.W., Li, M., Bhattacharyya, A., Barbarese, E. and Carson, J.H. (2016) CGG repeats in the 5’UTR of FMR1 RNA regulate translation of other RNAs localized in the same RNA granules. *PLoS One, 11*, e0168204.

25. Todur, S.P. and Ashavaid, T.F. (2012) Association of Sp1 tandem repeat polymorphism of ALOX5 with coronary artery disease in Indian subjects. *Clin Transl Sci, 5*, 408-411.

26. Shirokikh, N.E. and Spirin, A.S. (2008) Poly(A) leader of eukaryotic mRNA bypasses the dependence of translation on initiation factors. *Proc Natl Acad Sci U S A, 105*, 10738-10743.

27. Usdin, K. (2008) The biological effects of simple tandem repeats: lessons from the repeat expansion diseases. *Genome Res, 18*, 1011-1019.

28. Kumari, S., Bugaut, A., Huppert, J.L. and Balasubramanian, S. (2007) An RNA G-quadruplex in the 5′ UTR of the NRAS proto-oncogene modulates translation. *Nature chemical biology, 3*, 218-221.

29. Krauß, S., Griesche, N., Jastrzebska, E., Chen, C., Rutschow, D., Achmüller, C., Dorn, S., Boesch, S.M., Lalowski, M. and Wanker, E. (2013) Translation of HTT mRNA with expanded CAG repeats is regulated by the MID1–PP2A protein complex. *Nature communications, 4*, 1-9.

30. Glineburg, M.R., Todd, P.K., Charlet-Berguerand, N. and Sellier, C. (2018) Repeat-associated non-AUG (RAN) translation and other molecular mechanisms in Fragile X Tremor Ataxia Syndrome. *Brain research, 1693*, 43-54.

31. Arabfard, M., Kavousi, K., Delbari, A. and Ohadi, M. (2018) Link between short tandem repeats and translation initiation site selection. *Human genomics, 12*, 1-11.

32. Needleman, S.B. and Wunsch, C.D. (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. *Journal of molecular biology, 48*, 443-453.

33. Madeira, F., Park, Y.M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., Basutkar, P., Tivey, A.R., Potter, S.C. and Finn, R.D. (2019) The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic acids research, 47*, W636-W641.

34. Thierry-Mieg, D. and Thierry-Mieg, J. (2006) AceView: a comprehensive cDNA-supported gene and transcripts annotation. *Genome biology, 7*, 1-14.

35. Szklarczyk, D., Gable, A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M., Doncheva, N.T., Morris, J.H. and Bork, P. (2019) STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic acids research, 47*, D607-D613.
36. Chen, E.Y., Tan, C.M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G.V., Clark, N.R. and Ma’ayan, A. (2013) Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC bioinformatics, 14, 1-14.

37. Kuleshov, M.V., Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M. and Lachmann, A. (2016) Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic acids research, 44, W90-W97.

38. Xie, Z., Bailey, A., Kuleshov, M.V., Clarke, D.J., Evangelista, J.E., Jenkins, S.L., Lachmann, A., Wojciechowicz, M.L., Kropiwnicki, E. and Jagodnik, K.M. (2021) Gene set knowledge discovery with Enrichr. Current protocols, 1, e90.

39. Suzuki, I.K., Gacquer, D., Van Heurck, R., Kumar, D., Wojno, M., Bilheu, A., Herpoel, A., Lambert, N., Cheron, J. and Polleux, F. (2018) Human-specific NOTCH2NL genes expand cortical neurogenesis through Delta/Notch regulation. Cell, 173, 1370-1384. e1316.

40. Pearson, W.R. (2013) An introduction to sequence similarity (“homology”) searching. Curr Protoc Bioinformatics, Chapter 3, Unit3 1.

41. Georgakopoulos-Soares, I., Mouratidis, I., Parada, G.E., Matharu, N., Hemberg, M. and Ahituv, N. (2021) Asymmetron: a toolkit for the identification of strand asymmetry patterns in biological sequences. Nucleic Acids Research, 49, e4-e4.

42. Durinck, S., Spellman, P.T., Birney, E. and Huber, W. (2009) Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. Nature protocols, 4, 1184.

43. Durinck, S., Moreau, Y., Kasprzyk, A., Davis, S., De Moor, B., Brazma, A. and Huber, W. (2005) BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. Bioinformatics, 21, 3439-3440.

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Authors' contributions
A.M.A.M performed and analyzed the experimental data. M.A. and H.O. contributed to data collection. K.K. and M.O. conceived, designed, supervised the project, and wrote the manuscript with input from all authors.

CONFLICT OF INTEREST
The authors declare no competing interests.
TABLE AND FIGURES LEGENDS

**Table 1:** The top most abundant human-specific TRs flanking TISs.

**Table 2:** Example of genes enriched in the brain and skeletal muscle, based on STRING (https://string-db.org/) (35).

**Fig. 1:** Ratios of genes, transcripts, and TR counts for each species. The horizontal axis shows the percentage of each entity, and the vertical axis shows each species. Species can be cross-referenced in Supplementary Table 1.

**Fig. 2:** Abundance interval of the genes, transcripts, and TRs to each other.

**Fig. 3:** Average of 10 experiments performed to evaluate the link between TRs and TISs in each of the four TR categories. Each histogram shows the number of homologous TISs against non-homologous TISs based on two different weighing methods.

**Fig. 4:** 10-fold cross-validation for evaluating the link between TRs and TISs in Categories 1-4. Each histogram shows the number of homologous TISs vs. nonhomologous TISs, based on two different weighing methods, as follows: category 1 (a), category 2 (b), category 3 (c), and category 4 (d).

**Fig. 5:** Protein homology check of TISs flanked by human-specific and non-specific TRs. Every chart shows the distribution of similarity abundance between human proteins and three species: mouse, macaque, and chimpanzee in the same gene. For each panel, the first row shows the distribution that was constructed by BLASTing human proteins, which were produced by human-specific TR (HS-TR) genes. Similarly, the second row of each panel shows the distribution that was constructed by BLASTing human proteins which were produced by non-human-specific TR (NHS-TR) genes. The Needleman Wunsch algorithm (upper panel) was used as a confirmatory measure to our two weighing methods (middle and lower panels). In each method, we detected a significant difference in the distribution of HS-TR genes vs. NHS-TR genes.

**Fig. 6:** Enrichment analysis of sample genes in the human-specific TR (HS-TR) compartment. Development of the central nervous system and calcium signaling pathways received the lowest p-values for enrichment significance. a) Gene ontology enrichment analysis. b) Pathway enrichment analysis.

**Supplementary Table 1:** The number of genes, transcripts and extracted TRs for each species in our study. The rows of the table are sorted from large to small, based on the ratio of the number of TRs to the number of genes and transcripts in each species.

**Supplementary Table 2:** The list of all human genes and their Ensembl gene ID, which contained human-specific TRs in their TIS-flanking sequence for categories 1, 2, 3, and 4.

**Supplementary Table 3:** The list of all human specific TRs and their abundance.
Supplementary Table 4: The list of queries that were used to communicate with the Ensembl data repositories.

Supplementary Table 5: The number of homologous and non-homologous events in the two groups of human specific TRs and non-human specific TRs and their P-values, calculated by Fisher exact test in each method across categories 1, 2, 3 and 4.
Table 1: The top most abundant human-specific TRs flanking TISs.

| TR                | Core Length |
|-------------------|-------------|
| (CT)3             | 2           |
| (TC)3             | 2           |
| (GC)3             | 2           |
| (T)6              | 1           |
| (CG)3             | 2           |
| (GGC)3            | 3           |
| (CTG)3            | 3           |
| (CGCC)3           | 4           |
| (GGGCC)3          | 5           |
| (TGTGTTT)3        | 6           |
| (CGCGCC)3         | 6           |
| (GGGGCGC)3        | 7           |
| (CCCGCCG)4        | 7           |
| (GCTGCGGG)3       | 8           |
| (AGGGCGGGG)4      | 9           |
| (CCTCCCG)4        | 7           |
| (CCGGGGG)3        | 7           |
| (TTTTTGT)3        | 7           |
| (AGCCCAGC)3       | 8           |
| (CCCCCGC)3        | 7           |
| (ACCCTCC)3        | 8           |
| (AGCCCAGC)3       | 9           |
| (GTG GTG TT)2     | 10          |
| (ATT TAAA A TT)2   | 11          |
| (AAAATAAAATA A2)  | 11          |
| (TGCGGGCGGG)2     | 12          |
| (CCCCGCCCCA)2     | 10          |
| (CCCCGCCCGG)2     | 11          |
| (CGGAAGTGAGAG)2   | 12          |
| (AAGTGGGAAACTG)2  | 14          |
| (TTCATAGATTT)2    | 13          |
| (ATA TGT T)2      | 10          |
| (CCCCGCCCT)2      | 10          |
| (CCCCGAGGCTCCCGG)2| 16          |
| (CCGGCGTGTACCGAGACTGGCGT)2 | 25 |
| (ACCTGGAGGGCTGGG)2| 16          |
| (CCCTGCCCTGTCTGTCCCTGCC)2 | 25 |
| (ACCATCCCACCTCCCT)3| 18          |
| (CCCTGCCCTGTCTGTCCCT)2 | 20          |
| (ACCATCCCACCTCCCT)3| 18          |
| (CCCCACCTCCCTACCCAT)4 | 18          |
| (ACACGCGAGTCCGCAAGCGCAGCGAGCTCGCCAGCCGCG)2 | 38 |
| (TGAGTGCGCACAGGAGAGACTGACGTGCGCCGCCG)2 | 36 |
| (ACTCTCTCTTCTCGGCGGTGATTGAGTCCGACCAGGCGGTCC)2 | 41 |
Table 2. Example of genes enriched in the brain and skeletal muscle, based on STRING (https://string-db.org/)(35).

| Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol | Gene Symbol |
|-------------|-------------|-------------|-------------|-------------|-------------|
| ACSL6       | DMPK        | KRT23       | PHF8        |
| ADAM22      | DOK6        | KRT73       | PLEC        |
| ADSS1L      | EFHC1       | KRT8        | PPP1CC      |
| AKAP7       | EIF3K       | L3MBTL1     | PPP1R14A    |
| ARHGAP42    | EIF5A1      | LCAT        | PRIMA1      |
| ASIC1       | ELMO1       | LMNA        | PTBP1       |
| ASRGL1      | ENSG00000258947 | MBNL1 | REG1B      |
| ATXN10      | EPB41L4B    | MPRIP       | RYR1        |
| C11orf63    | EXTL3       | MTDH        | RYR3        |
| C19orf12    | FAM101B     | MYH2        | SCIN        |
| CACNA1A     | FMNL3       | NEK3        | SERHL2      |
| CACNA1F     | FOXK1       | NOL3        | SERPINB6    |
| CACNA1G     | FOXP1       | OBSCN       | SIPA1L3     |
| CAPNS2      | GABRB2      | OLIG1       | SLC25A27    |
| CDK16       | GDF11       | PAMR1       | SLC4A1      |
| CELF4       | GSK3A       | PANK2       | SLC6A8      |
| CELF6       | GSTM2       | PCDH7       | SLIT2       |
| CEP55       | HCN2        | PCDAH10     | SPEG        |
| CERCAM      | HDAC4       | PCDAH12     | SYN1        |
| CKB         | HDAC8       | PCDAH13     | SYN1GAP1    |
| CLIP2       | HRC         | PCDAH7      | TCF3        |
| COL3A1      | INPPSK      | PCDH14      | TMEM132A    |
| COPRS       | ITSN1       | PCDH5       | TMEM59L     |
| CRIPT       | KCNA2       | PCDH6       | TRNP1       |
| CROCC       | KCNC1       | PCDH9       | TTN         |
| DAO         | KIAA1191    | PCDHGC4     | ZFHX3       |
| DCTN2       | KRT10       | PDLIM4      |             |
Fig. 1: Abundance interval of the genes, transcripts, and TRs to each other.
Fig. 2: Average of 10 experiments performed to evaluate the link between TRs and TISs in each of the four TR categories. Each histogram shows the number of homologous TISs against non-homologous TISs based on two different weighing methods.
Fig. 3: 10-fold cross-validation for evaluating the link between TRs and TISs in Categories 1-4. Each histogram shows the number of homologous TISs vs. nonhomologous TISs, based on two different weighing methods, as follows: category 1 (a), category 2 (b), category 3 (c), and category 4 (d).
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Fig. 5: Enrichment analysis of sample genes in the human-specific TR (HS-TR) compartment. Development of the central nervous system and calcium signaling pathways received the lowest p-values for enrichment significance. a) Gene ontology enrichment analysis. b) Pathway enrichment analysis.
Fig. 6: Ratios of genes, transcripts, and TR counts for each species. The horizontal axis shows the percentage of each entity, and the vertical axis shows each species. Species can be cross-referenced in Supplementary Table 3.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.xlsx
- SupplementaryTable2.xlsx
- SupplementaryTable3.xlsx
- SupplementaryTable4.docx
- SupplementaryTable5.docx