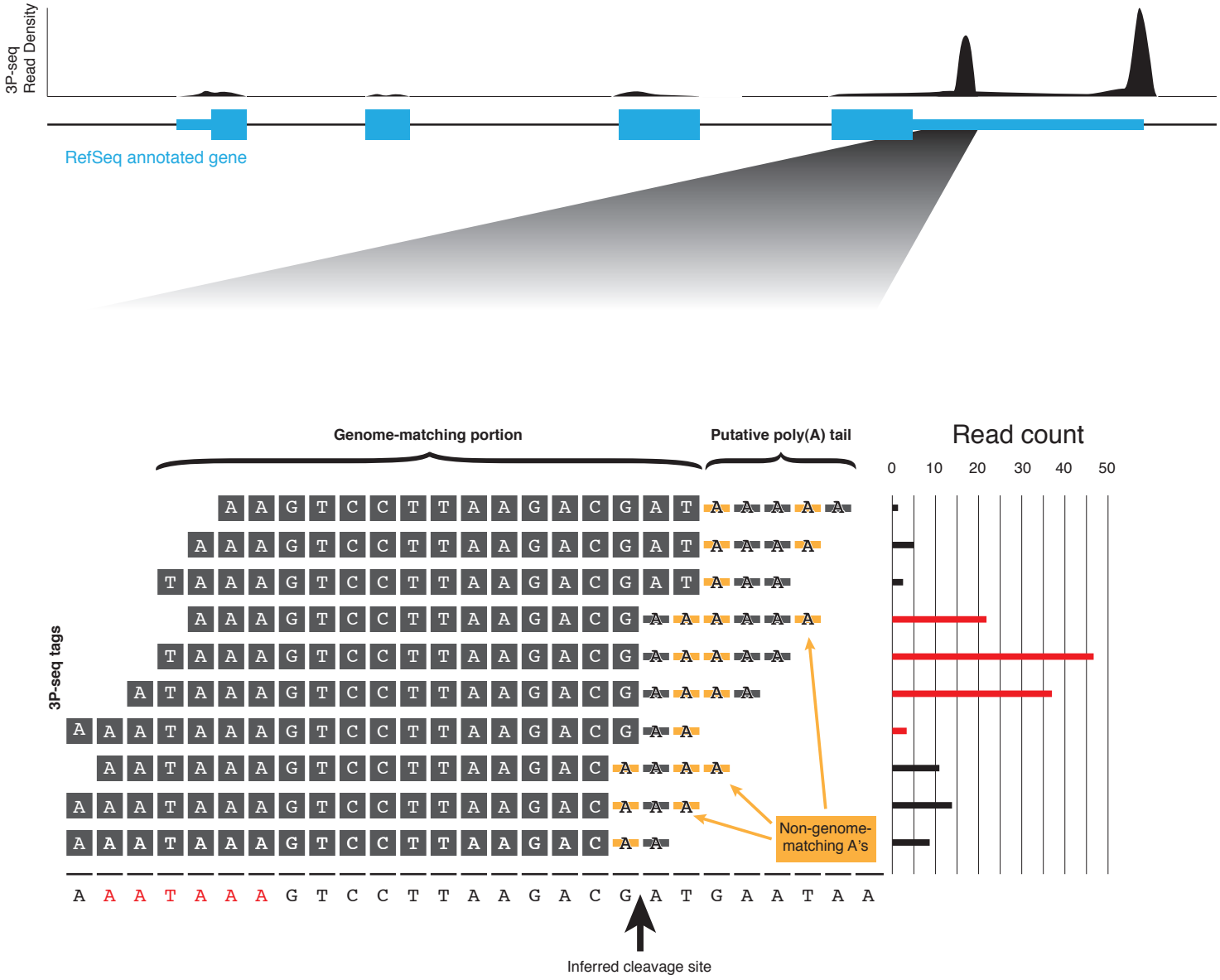


Figure S1. Demonstration of the annotation of an internal cleavage and polyadenylation site from hypothetical 3P-seq data. First, reads are mapped to the genome, and each read is assessed for the presence of a stretch of one or more A's at its 3′ end. Of particular interest are positions in this final A stretch that do not match the genome (highlighted in orange). Starting with the position with the highest count of reads including at least two 3′-end A's (read-count bars highlighted in red), adjacent reads within ±20 nt are gathered. In order to distinguish bona-fide poly(A) sites from the low-level background of non-poly(A)-site derived exonic reads, the clusters were only annotated as poly(A) sites if the following criteria were met:

(1) the cluster contains at least 10% of the reads with non-genome matching A's mapping to the gene
(2) the total number of reads with non-genome-matching A's must be at least 3
(3) at least one read includes four or more 3′-end A's

After a poly(A) site cluster is annotate, all reads within the cluster are set aside, and the previous process is repeated with the remaining reads to annotated additional poly(A) sites. Together, these criteria are designed to prevent occasional reads with sequencing errors from being accidentally annotated as cleavage sites, even in highly expressed genes, while simulltaneously enabling the accurate detection of alternative poly(A) sites in lowly expressed genes.

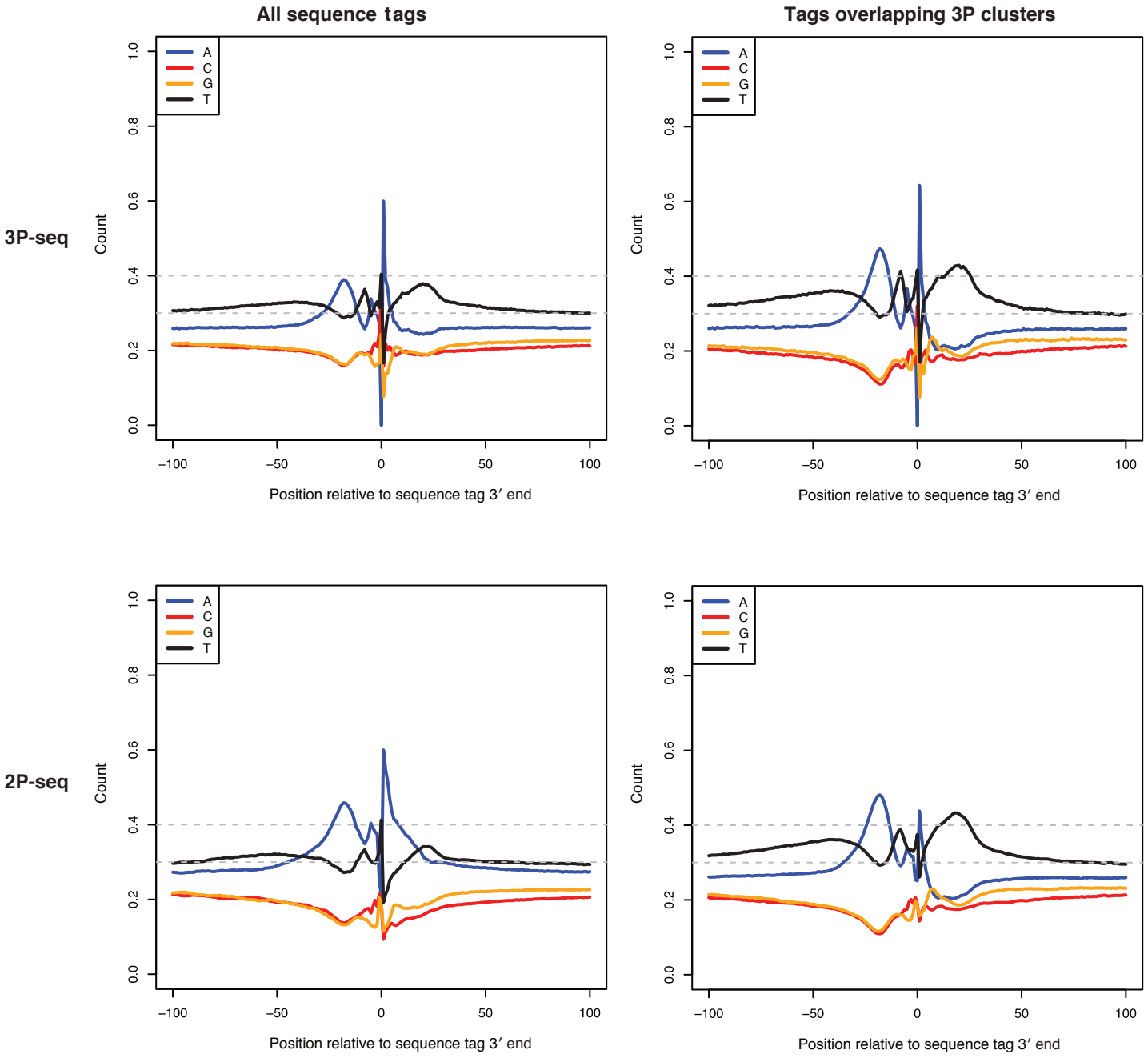


Figure S2. Nucleotide composition relative to 3′ end of all tags (left column) and relative only to reads overlapping 3P tag clusters (right panels).

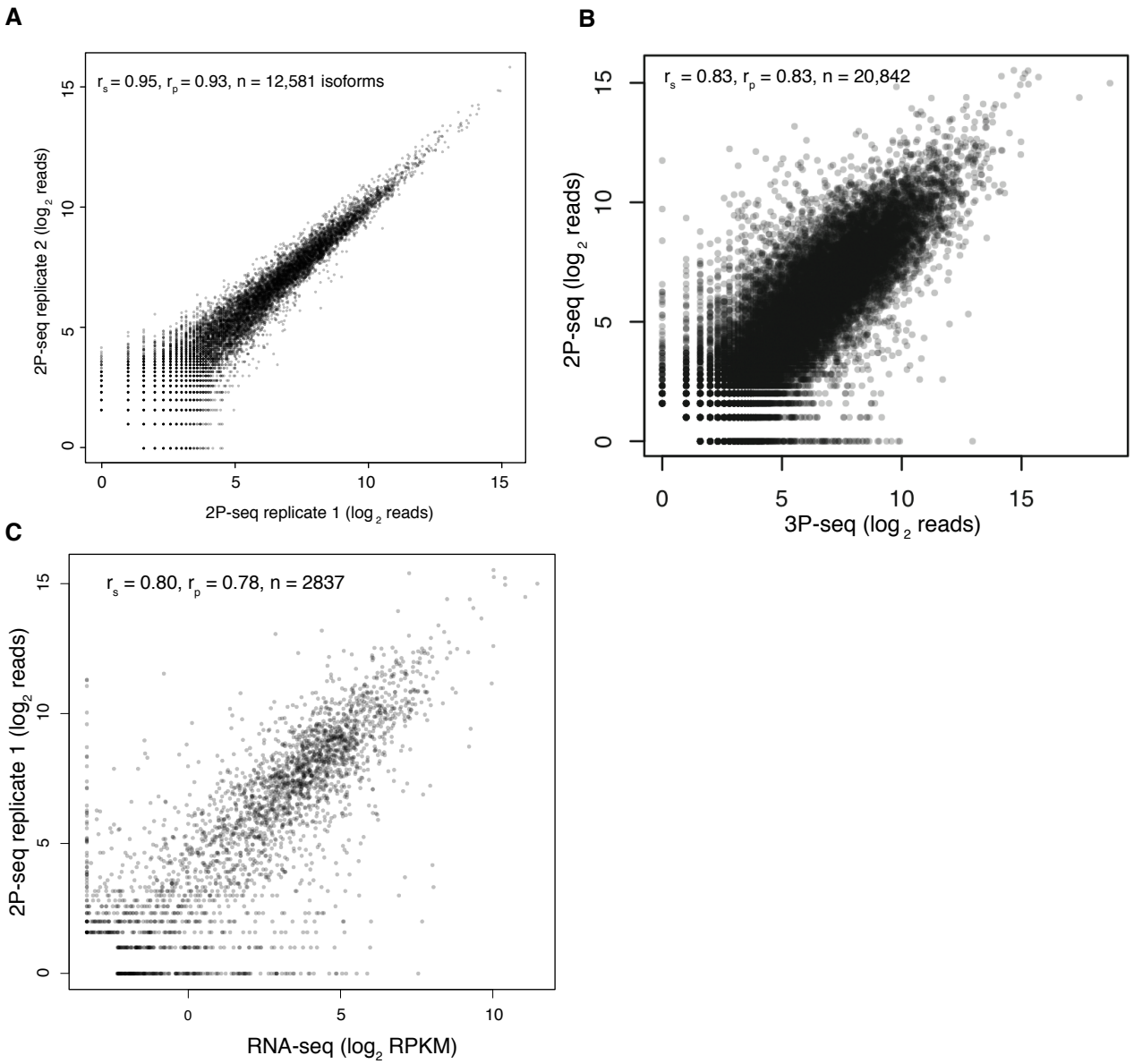
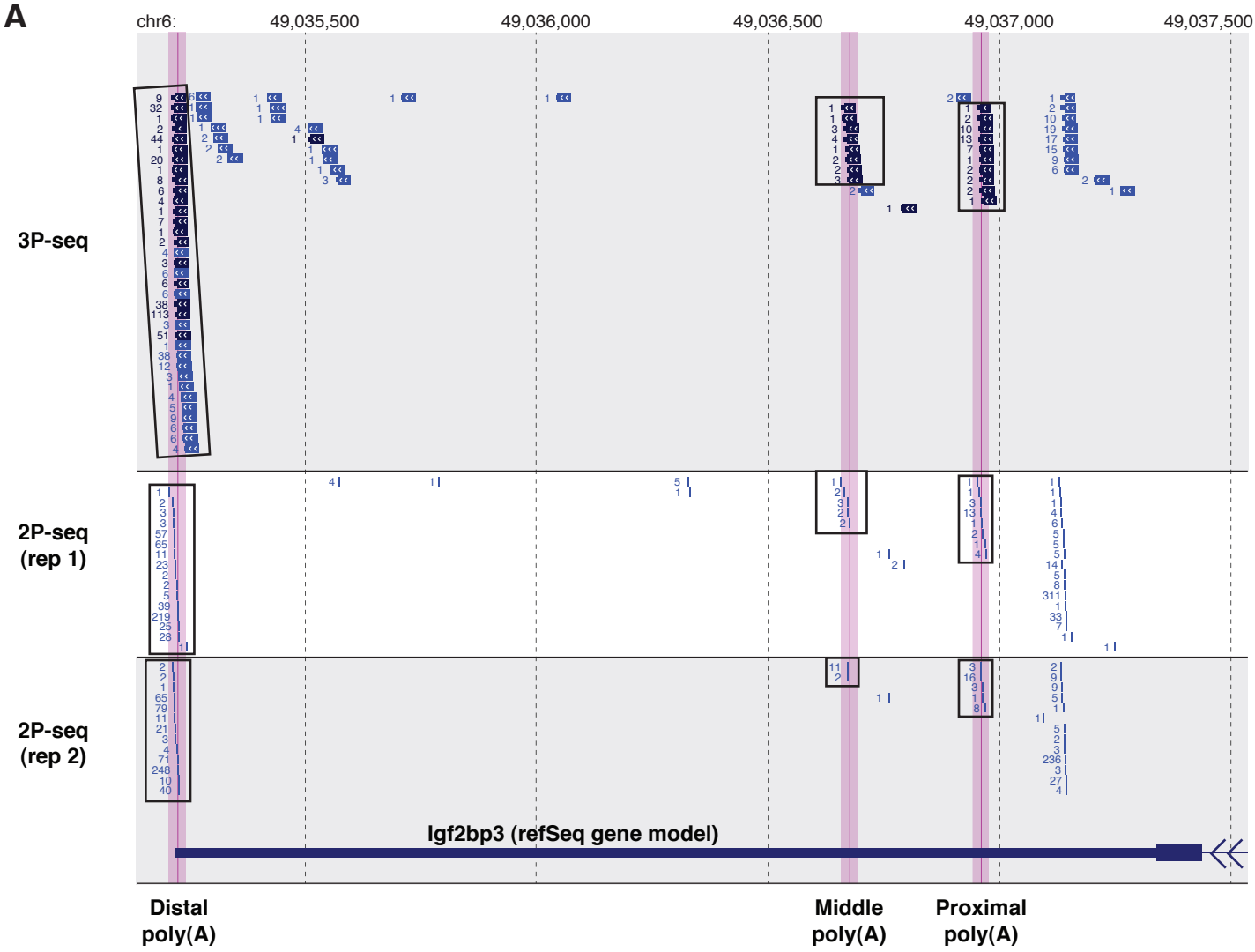


Figure S3. (A) Reproducibility of 2P-seq measurements of isoform abundance across two biological replicates. As in Figure 1C but considering only isoforms of genes with tandem 3' UTRs. (B) Quantification of 3' UTR isoforms by 3P-seq and 2P-seq. Correlation is 0.83 (by Spearman or Pearson) for n = 20,842 isoforms, removing isoforms that are not expressed in either dataset and adding a pseudocount before taking the log. (C) Comparison between 2P-seq and RNA-seq, as in Figure 1D, but counting only single-isoform genes.



B

	Distal isoform		Middle isoform		Proximal isoform	
	Raw	Normalized	Raw	Normalized	Raw	Normalized
2P-seq rep 1	486	1.0	10	0.021	26	0.054
2P-seq rep 2	557	1.0	13	0.023	31	0.056
2P-seq mean		1.0		0.022		0.055
3p-seq	458	1.0	17	0.037	41	0.090
2P-seq polysomes	44,635	1.0	782	0.018	2806	0.063

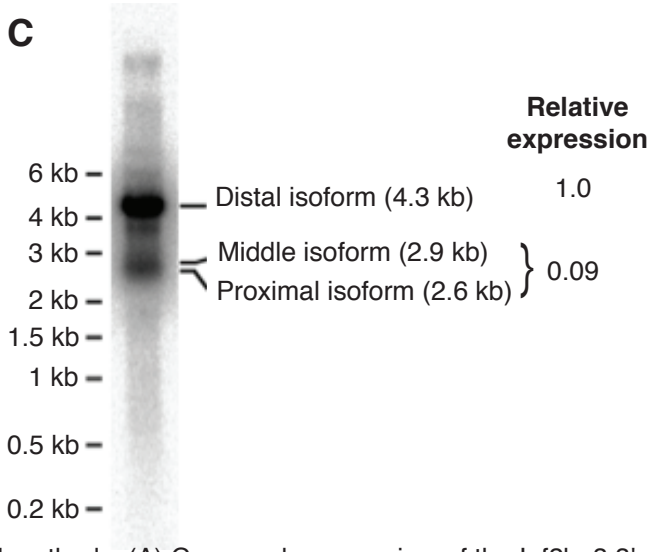


Figure S4. Quantification of Igf2bp3 3'-end isoforms across experimental methods. (A) Genome-browser view of the Igf2bp3 3' UTR showing the 3P tags (top) and 2P tags from two replicates (middle and bottom). Numbers indicate read counts. Three poly(A) sites, called from the 3P tags containing non-genome-matching 3′-end adenosines (dark-blue tags; Figure S1), are shown as vertical magenta bands. The two 2P-seq replicates are from the two 0-hr samples taken before adding actinomycin D. Each tag is shown as a thin line at the implied poly(A) site. Tags used to quantify each 3'-end isoform are boxed. The Igf2bp3 gene is transcribed right to left. A small number of opposite-strand tags, likely derived from a nearby downstream antisense gene, were omitted for clarity. (B) Relative expression of the Igf2bp3 isoforms, as indicated by the read counts from panel A. Isoform expression levels were also measured from the polysome experiment (2P-seq polysomes) by adding the library-normalized expression counts across all six polysome fractions. (C) Relative expression of the Igf2bp3 isoforms, as indicated by an RNA blot of poly(A)-selected RNA probed for Igf2bp3 mRNA. Proximal and middle isoforms could not clearly be distinguished on the blot and hence were quantified together. Their combined expression relative to the distal isoform (0.09) closely matched that determined by 2P-seq (0.08) and 3P-seq (0.12) in panel B.

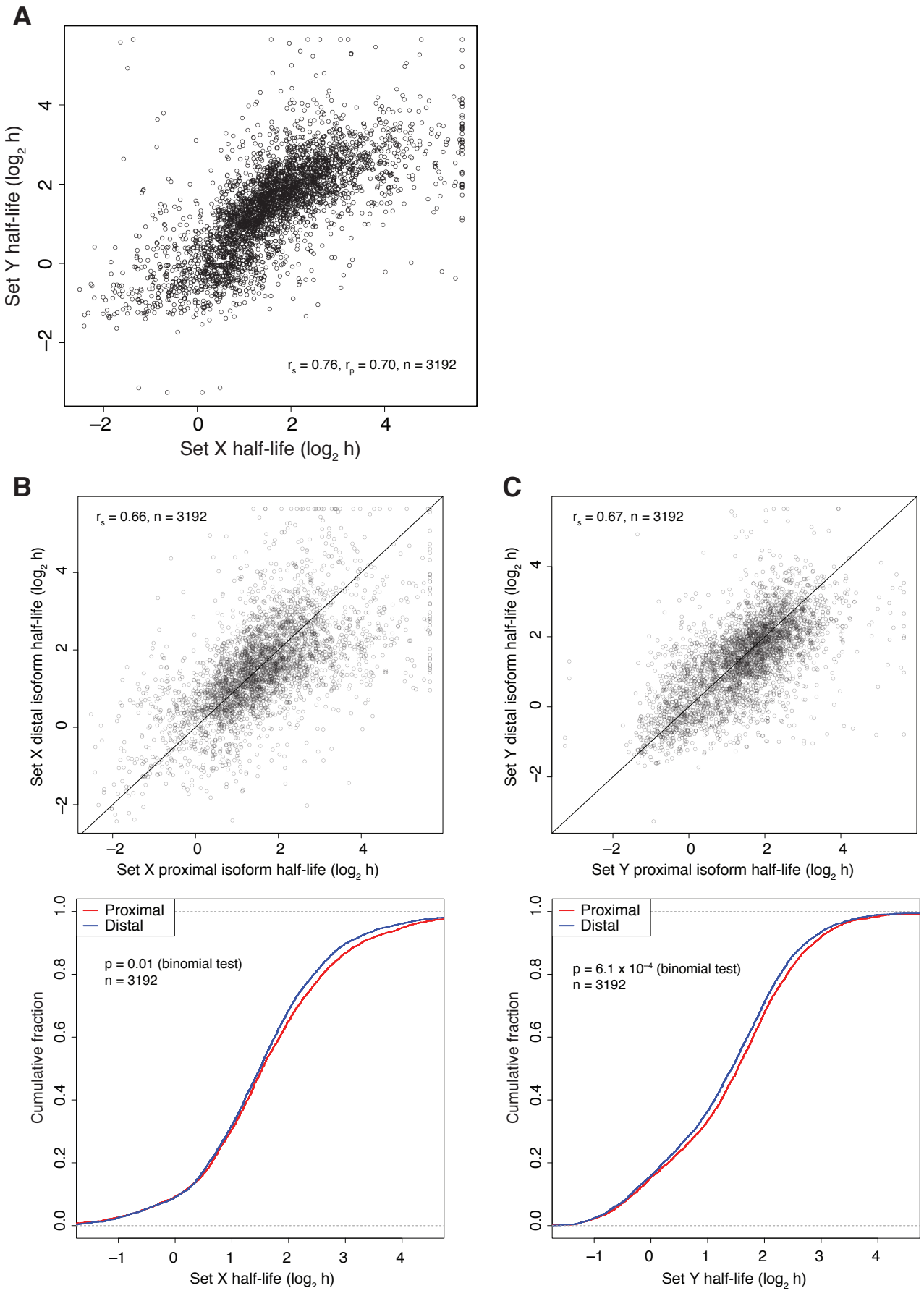
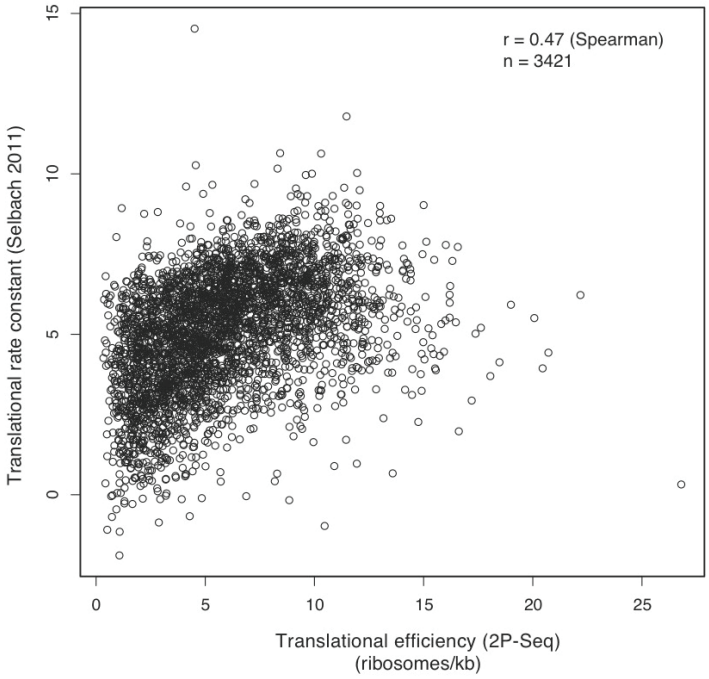


Figure S5. Consistency of results when comparing time points 0 h (replicate 1), 1 h, 4 h (Set X) with time points 0 h (replicate 2), 2 h, 8 h (Set Y).
(A) Half-lives for Set X compared with Set Y.
(B) Comparison of proximal and distal isoforms for Set X (compare to Figure 3).
(C) As in (B), comparison of proximal and distal isoform pairs for Set Y.



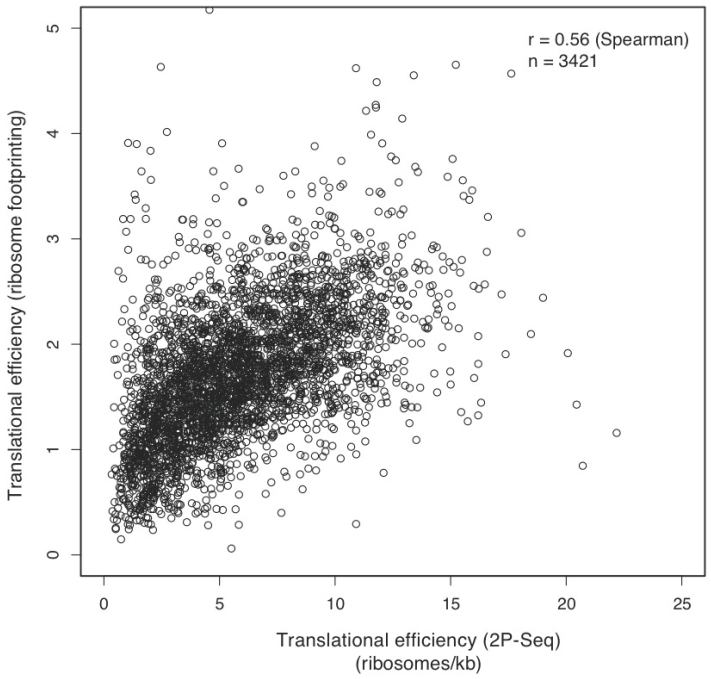


Figure S6. Translational efficiency measurements by polysome profiling followed by 2P-seq (x-axis) compared to translation rates measured by metabolic labeling (left panel, Schwanhäusser 2011) and by ribosome footprint profiling (right panel).

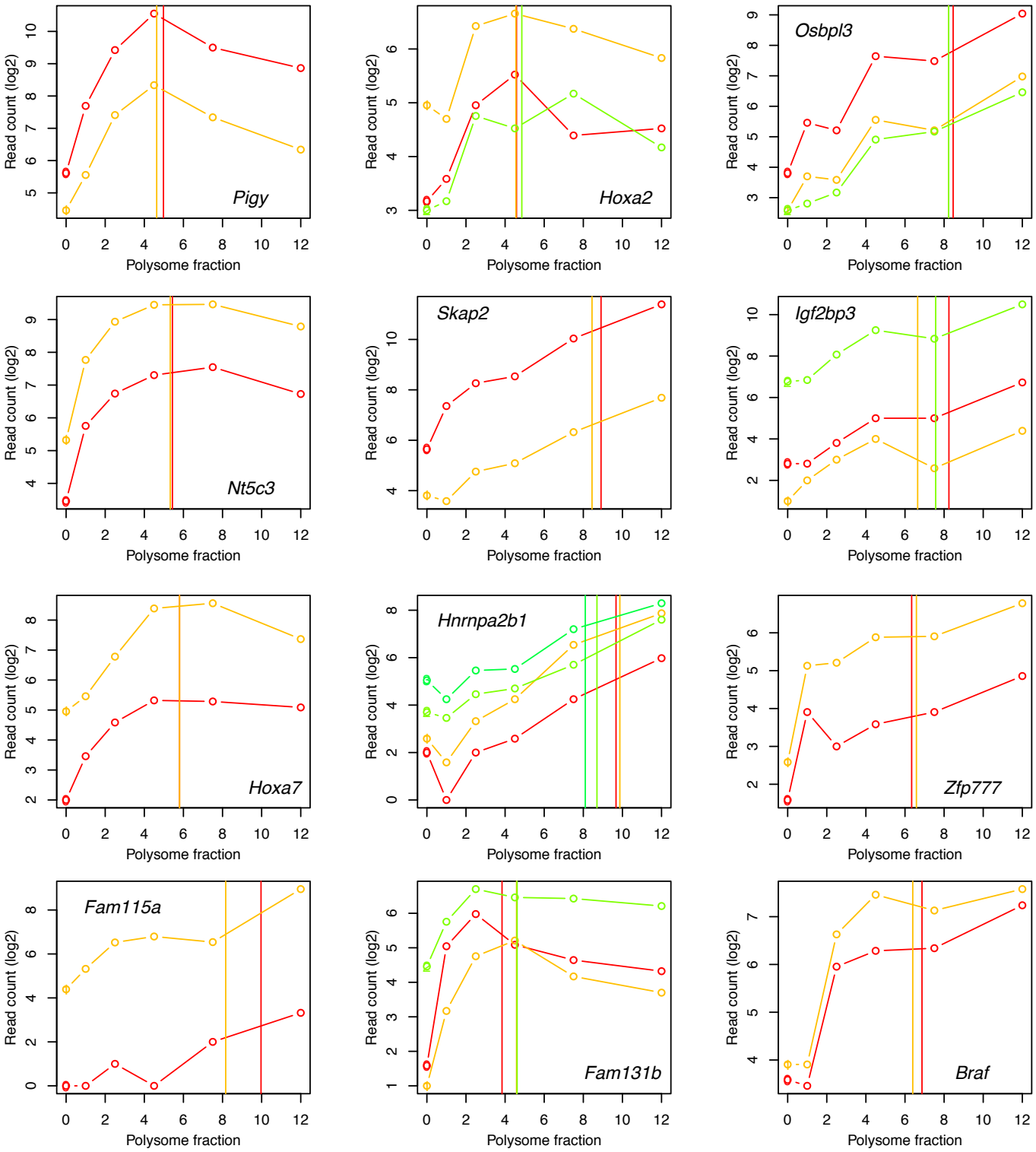


Figure S7. Randomly selected polysome profiles for tandem 3′ UTR-containing genes. From proximal to distal, isoforms are colored red, orange, light green and dark green. See Figure 5C.

Table S1. Comparison of gene-level half-life measurements from our results (2P-seq) and other published datasets, requiring a minimum expression of 10 reads per gene in each control 2P-seq dataset and that the gene be quantified in all other datasets (n = 2149 genes).

Dataset 1	Dataset 2	Pearson Correlation	R^2
2P-seq	Sharova	0.68	0.46
2P-seq	Clark	0.70	0.49
2P-seq	Schwanhäusser	0.62	0.38
2P-seq	Rabani	0.46	0.21
Sharova	Clark	0.69	0.47
Sharova	Schwanhäusser	0.53	0.28
Sharova	Rabani	0.40	0.16
Clark	Schwanhäusser	0.59	0.35
Clark	Rabani	0.40	0.16
Schwanhäusser	Rabani	0.42	0.17

Citations

Clark, M. B. et al. Genome-wide analysis of long noncoding RNA stability. Genome Res 22, 885–898

Rabani, M. et al. Metabolic labeling of RNA uncovers principles of RNA production and degradation dynamics in mammalian cells. Nat. Biotechnol. 29, 436–442 (2011).

Schwanhäusser, B. et al. Global quantification of mammalian gene expression control. Nature 473,

Sharova, L. V. et al. Database for mRNA half-life of 19 977 genes obtained by DNA microarray analysis of pluripotent and differentiating mouse embryonic stem cells. DNA Res 16, 45–58 (2009).

Table S2. Gene ontology categories significantly associated with high mRNA stability or low mRNA stability. Asterisk indicates significance after Bonferroni correction at the p = 0.05 cutoff level. Background gene sets were selected to closely match the gene expression levels of the foreground (test) sets.

GO category associated with high mRNA stability	p
cytoplasmic part	1.40E-25 *
oxidoreductase activity	1.60E-11 *
extracellular space	1.50E-10 *
mitochondrial part	9.00E-10 *
mitochondrial membrane	1.10E-09 *
organelle inner membrane	6.90E-09 *
structural constituent of ribosome	1.50E-07 *
organelle membrane	2.90E-07 *
mitochondrial respiratory chain	3.50E-07 *
respiratory chain	3.50E-07 *
oxidoreductase activity, acting on NADH or NADPH	1.40E-06 *
ribosome	2.50E-06 *
carbohydrate metabolic process	3.80E-06 *
proton-transporting two-sector ATPase complex	5.30E-06 *
ion transmembrane transport	1.00E-05 *
mitochondrial membrane part	1.00E-05 *
small molecule metabolic process	1.20E-05 *
hydrogen transport	1.70E-05 *
cellular carbohydrate metabolic process	3.30E-05 *
small molecule catabolic process	3.70E-05 *
carbohydrate catabolic process	8.60E-05 *
intracellular part	0.00018
generation of precursor metabolites and energy	0.00022
learning or memory	0.00048
transmembrane transport	0.00051

GO category associated with low mRNA stability	p
regulation of cellular process	7.00E-26 *
regulation of biological process	8.20E-24 *
regulation of metabolic process	1.30E-23 *
intracellular	1.10E-21 *
nucleic acid binding	2.70E-16 *
sequence-specific DNA binding transcription factor activity	6.10E-14 *
DNA binding	1.80E-09 *
cation binding	8.90E-09 *
ion binding	8.90E-09 *
positive regulation of biological process	1.20E-06 *
membrane-bounded organelle	1.00E-05 *
intracellular membrane-bounded organelle	1.00E-05 *
organ morphogenesis	5.00E-05 *
cell differentiation	6.40E-05 *
signal transduction	9.00E-05 *
intracellular organelle	0.00024
negative regulation of biological process	0.00027
signaling receptor activity	0.00034
tube development	0.00048
multicellular organismal development	0.00057
anatomical structure development	0.00064
signal transducer activity	0.00072
transmembrane signaling receptor activity	0.00094
organ development	0.0014
system development	0.0017

Table S3. Gene ontology categories enriched among tandem-UTR-containing genes with significantly stabilized (top) or destabilized (bottom) distal 3' UTR isoforms. Shown are raw values used for hypergeometric test, as well as percentages of significant genes. False discovery rate is indicated by q.

Distal isoform more stable than proximal isoform

GO category	p	q	Up-regulated %	Control %	Up-regulated in category	Total up-regulated	Total in category	Total
integral to membrane	1.25E-04	0.21	18.3%	14.1%	135	737	389	2750
intrinsic to membrane	2.16E-04	0.21	18.3%	14.3%	135	737	393	2750
proteinaceous extracellular matrix	2.18E-04	0.21	1.5%	0.5%	11	737	15	2750
extracellular matrix	2.18E-04	0.21	1.5%	0.5%	11	737	15	2750
membrane part	2.32E-04	0.21	21.3%	17.0%	157	737	468	2750
cell adhesion	3.61E-04	0.24	2.2%	1.0%	16	737	27	2750
biological adhesion	3.61E-04	0.24	2.2%	1.0%	16	737	27	2750
cytoplasmic part	1.13E-03	0.60	21.0%	17.3%	155	737	475	2750
extracellular region part	1.18E-03	0.60	7.5%	5.2%	55	737	143	2750
developmental process	1.68E-03	0.78	10.2%	7.6%	75	737	209	2750

Proximal isoform more stable than distal isoform

GO category	p	q	Down-regulated %	Control %	Down-regulated in category	Total down-regulated	Total in category	Total
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	8.75E-03	1	1.3%	0.4%	6	469	12	2750
ubiquitin ligase complex	1.26E-02	1	1.7%	0.7%	8	469	20	2750
sequence-specific DNA binding transcription factor activity	1.26E-02	1	4.9%	3.1%	23	469	85	2750
nucleic acid binding transcription factor activity	1.26E-02	1	4.9%	3.1%	23	469	85	2750
regulation of biological process	1.42E-02	1	24.7%	20.8%	116	469	573	2750
neuron projection	1.68E-02	1	1.1%	0.4%	5	469	10	2750
biological regulation	1.72E-02	1	25.4%	21.6%	119	469	593	2750
regulation of cellular process	2.10E-02	1	23.5%	19.9%	110	469	547	2750
system development	2.23E-02	1	2.1%	1.1%	10	469	30	2750
cellular component biogenesis at cellular level	3.94E-02	1	1.1%	0.4%	5	469	12	2750