

Intertwined canonical and non-canonical initiation in dual promoters are pervasive and differentially regulate Polymerase II transcription

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Abstract

The diversity and complexity of transcription start site (TSS) selection reflects variation of preinitiation complexes, divergent function of promoter-binding proteins and underlies not only transcriptional dynamics but may also impact on post-transcriptional fates of RNAs. The majority of metazoan genes are transcribed by RNA polymerase II from a canonical initiation motif having an YR dinucleotide at their TSSs. In contrast, translation machinery-associated genes carry promoters with polypyrimidine initiator (known as 5'-TOP or TCT) with cytosine replacing the R nucleotide. The functional significance of start site choice in promoter architectures is little understood. To get insight into the developmental regulation of start site selection we profiled 5' ends of transcripts during zebrafish embryogenesis. We uncovered a novel class of dual-initiation (DI) promoters utilized by thousands of genes. In DI promoters non-canonical YC-initiation representing 5'-TOP/TCT initiators is intertwined with canonical YR-initiation. During maternal to zygotic transition, the two initiation types are divergently used in hundreds of DI promoters, demonstrating that the two initiation systems are distinctly regulated. We show via the example of snoRNA host genes and translation interference experiments that dual-initiation from shared promoters can lead to divergent spatio-temporal expression dynamics generating distinct sets of RNAs with different post-transcriptional fates. Thus utilization of DI promoters in large number of genes suggests two transcription initiation mechanisms targeting these promoters. DI promoters are conserved within human and fruit fly and reflect an evolutionary conserved mechanism for switching transcription initiation to adapt to the changing developmental context. Thus, our findings highlight a novel level of complexity of core promoter regulation in metazoans and broaden the scope for identification and characterization of alternative RNA products generated at shared core promoters.

1 Introduction

2 Transcription is a tightly regulated process initiated by RNA polymerase II (Pol II)
3 in the core promoter region, which is typically -40 to +40 nucleotides with respect to
4 transcription start sites (TSS). There are no universal core promoter elements¹ as they are
5 diverse in their sequence and functions, and the structure-function relationship of core
6 promoters remains poorly understood. Sequencing of capped RNA 5' ends by CAGE (cap-
7 analysis of gene expression) revealed that an overwhelming majority of TSSs are
8 anchored by a purine base at the start site (+1 position) and flanked by pyrimidine in the
9 upstream region (-1 position), thus defining consensus Y₋₁R₊₁ (hereafter called YR-
10 initiation) as canonical initiator in mammals² and in teleosts (zebrafish and tetraodon)³,
11 suggesting generality of conserved initiator among vertebrates. Analysis of core
12 promoters in *Drosophila melanogaster* (invertebrates) revealed a related but more motif-
13 like TC₋₁A₊₁GT initiator sequence^{4,5}. In contrast, transcription initiation of translation-
14 associated genes (ribosomal proteins, snoRNA host genes, translation initiation and
15 elongation factors) is anchored by C₊₁ (cytosine) and flanked by a polypyrimidine stretch⁶⁻
16 ¹¹ (hereafter called YC-initiation). These non-canonical initiators have previously been
17 termed 5'-TOP (terminal oligo-polypyrimidine) in mammalian systems or TCT initiators
18 in *Drosophila*¹² and these YC initiation-dependent genes were shown to be conserved in
19 zebrafish³. *Drosophila* ribosomal protein genes with TCT promoters are recognized by a
20 TFIID-independent transcription initiation mechanism and mediated by the TATA-
21 binding protein (TBP) family member TBP-related factor 2 (TRF2), but not TBP¹³. These
22 results suggest that the non-canonical initiation is specialized for a subset of genes and
23 facilitates a non-canonical initiation complex formation with distinct proteins from that of
24 TBP and TFIID and likely reflecting distinct regulation of transcription initiation¹⁴.
25 However, it is unknown, why such a non-canonical initiation has evolved and has been
26 maintained in evolutionary distant species. Important insight into potential functional
27 significance of the non-canonical initiation is emerging from studies investigating target
28 genes of mTOR pathways that are translationally regulated^{15,16}, and are enriched in 5'-
29 TOP/TCT initiator. The 5'-TOP initiator is defined by a minimum of 4-15 pyrimidine
30 sequences¹⁷. The polypyrimidine stretch proximal to the 5' end of these genes is a target
31 for translation regulation and has been suggested to serve as a target mechanism for
32 oxidative and metabolic stress or cancer-induced differential translational regulation by
33 the mTOR pathway^{15,16,18-20}. The existence of 5'-TOP/TCT promoters raises the questions
34 of how widespread non-canonical initiation is and what is its relationship with canonical
35 initiation.

1 We have generated CAGE datasets³ in zebrafish and profiled all transcription
2 initiators during embryogenesis from the maternal to zygotic transition (MZT) and then
3 through organogenesis. We have extended the detection of YC-initiation in zebrafish to
4 thousands of genes, and made constellation observation of pervasive co-occurrence of YR-
5 initiation and YC-initiation events in shared core promoter. We performed a
6 comprehensive and unbiased analysis of TSSs in promoters and characterized the
7 features and roles of non-canonical initiation by a systematic survey of the base
8 composition within the TSSs in CAGE datasets³. This analysis led us to uncover non-
9 canonical YC-initiation in thousands of genes that are proximal to or intertwined with the
10 canonical YR-initiation in the same core promoter region, thus revealing thousands of
11 what we term dual-initiation (DI) promoter genes. We provide multiple lines of evidence
12 for the functional relevance of dual-initiation, such as sequence composition, differential
13 usage of initiators during development, differential response of initiators during
14 translation inhibition and selective association of snoRNA biogenesis, which is predicted
15 to be processed by splicing from introns of the YC-initiation products of dual-initiation
16 genes. We thus demonstrate that the two initiation types within dual promoters represent
17 composite of promoter architectures and reflect on two regulatory functions, generating
18 distinct sets of RNAs with different post-transcriptional fates. Our findings highlight a
19 novel level of complexity of core promoter regulation during development and broaden
20 the scope for functional dissection of overlaid promoter architectures that act in the
21 complexity of the developing embryo.

1 Results

2 *Non-canonical YC-initiations are pervasively intertwined with canonical YR-* 3 *initiations*

4 To comprehensively map non-canonical initiation events at single nucleotide
5 resolution, we analyzed the start base distribution of (m)RNA 5' ends by pooling CAGE
6 Transcription Start Sites (CTSSs) with at least 1 tag per million (TPM) detectable across
7 12 stages during zebrafish embryo development ³(**Figure 1a**). Majority of CTSSs (71.6%)
8 have canonical ($Y_{-1}R_{+1}$) start sites (**Figure 1a; Supplementary Figure 1a**). The remaining
9 CTSSs have been excluded from further analysis as they include RNA start sites with a
10 well-characterized GG dinucleotide associated with post-transcriptional processing
11 products independent from transcription initiation³ and therefore do not reflect true
12 transcription start sites. Furthermore, we have excluded CAGE signals which represent
13 Drosha-processing sites on pre-miRNAs²¹ and snoRNA 5'-end capping events²².
14 Importantly, a substantial proportion of TSSs possess the non-canonical pyrimidine
15 initiation (labeled $Y_{-1}C_{+1}$ in **Figure 1a**). Majority of YR-initiation (85.97%) and YC-
16 initiation (83.05%) sites mapped within the expected promoter region of ENSEMBL
17 transcripts (500 bases upstream and 300 bases downstream) and thus, support detection
18 of true transcription initiation products. YR-initiation and YC-initiation are highly
19 reproducible across replicates (**Supplementary Figure 1b**). For downstream analysis,
20 we retained only those robustly detected transcripts that are transcribed in at least 2
21 developmental stages and whose promoter expression level is at least 3 TPM. At this
22 filtering threshold, 4201 promoters have YC-initiation and 12056 promoters have YR-
23 initiation (**Supplementary Table 1**). Intersection analysis of gene promoters revealed
24 that 50 (1.19%) genes carry only YC-initiation and 7905 (65.5%) genes have only YR-
25 initiation, thus regulated by a single type of initiator. However, the majority of YC-
26 initiation site-containing promoters (98.81%) also carry YR-initiation sites (**Figure 1a;**
27 **Venn diagram**). This novel class of promoters have collectively called dual-initiation (DI)
28 promoters (**Figure 1b**). The DI promoters identified by CAGE were also confirmed by
29 independent analysis of capped mRNA sequencing at prim 5 stage of development (24h
30 post fertilization), which, though less sensitive than CAGE, has demonstrated hundreds of
31 cases of dual-initiation events and demonstrated statistically significant overlap with
32 CAGE detected dual-initiation promoter genes (**Supplementary Figure 1c**).

33 For all dual-initiation promoter genes, we summed the expression levels of all YR
34 and YC components and genes were classified as either YR-dominant or YC-dominant
35 depending upon the TPM levels of their YR and YC components. The exemplified *sumo2b*

1 gene (**Figure 1b**) has a higher total level of YR-initiations than YC-initiation, thus
2 classified as a YR-dominant gene. We then used the highest expression level of YR and YC
3 CTSSs and determined the position of dominantly used YR and YC TSS. The YR-dominant
4 TSS is located 4 nucleotides downstream to the YC-dominant TSS in the exemplified
5 *sumo2b* gene (**Figure 1b**). The distance between dominant YR-initiation and YC-initiation
6 of all DI promoters at prim 5 stage fall mostly within 30 bases and there is some degree of
7 preference for YC 1 nt upstream of YR (**Figure 1c**). This close proximity between the two
8 types of initiations suggest that the initiation machinery or machineries involved in
9 controlling transcription of these transcripts recognize the same core promoter region.
10 Comparing the expression levels of YR and YC components revealed that the contribution
11 of YC-initiation to the total activity of dual-initiation promoters tends to be relatively
12 small (**Figure 1d; Supplementary Figure 1d**), resulting only in a small portion (8.25%;
13 n=251) of genes as YC-dominant in prim 5 stage (**Figure 1d**). This observation may
14 explain why the non-canonical YC-initiation events largely have been missed in previous
15 studies, which focused on the single dominant TSSs. However, YC-initiation can be
16 dominant over YR-initiation in individual genes even at lowly expressed promoters
17 (**Figure 1d; Supplementary Figure 1d**). In conclusion, we show that non-canonical YC-
18 initiation events are pervasively intertwined with canonical YR-initiation and occur
19 within a small physical distance within the same core promoter regions.

20 *Features of dual-initiation gene promoters*

21 Translational-associated genes such as ribosomal proteins, translation
22 initiation/elongation factors and small nucleolar RNA (snoRNA) host genes are
23 transcribed by 5'-TOP/TCT initiators, thus we asked whether their zebrafish homologs
24 possess single or dual-initiation. The annotation of zebrafish snoRNAs is not
25 comprehensive, therefore we analyzed a size selected RNA library²³ enriched for full-
26 length snoRNA length (18-250 nt) and annotated 176 novel zebrafish snoRNAs
27 (**Supplementary Table 2**). Intersection of the expressed genes from the above listed
28 gene-families revealed that most of these genes carry dual-initiation sites (**Figure 2a**).
29 Gene ontology (GO) analysis of DI promoter genes revealed an enrichment of translation
30 machinery components (translation, translation elongation and translation termination),
31 co-translational proteins targeting to membrane, RNA stability and nonsense mediated
32 decay (**Figure 2b; Supplementary Table 3**). Enrichment of ribosome-related functions is
33 consistent with previous studies describing YC-initiation^{17,24}, associated with such genes
34 while our findings reveal a novel, dual-initiation featuring these promoters (**Figure 2a**).
35 Excluding translation-associated genes from the query list revealed an enrichment of
36 additional unexpected GO terms such as mRNA splicing via spliceosome, telomerase RNA

1 localization, chromosome organization and mitotic cell cycle (**Figure 2b; Supplementary**
2 **Table 3**). In contrast, YR-only initiator genes are enriched for GO terms related to
3 morphogenesis, pattern specification and embryonic development (**Figure 2b**)
4 characteristic of the prim 5 stage of development and highlight the functional distinction
5 of core promoter architectures.

6 Sequence composition around (10 nucleotides) dominant TSSs of both initiation
7 sites revealed higher fraction of pyrimidine sequence adjacent to the YC-initiation (**Figure**
8 **2c**), predominantly with an uninterrupted stretch of at least 4 pyrimidines (**Figure 2d**), a
9 characteristic feature of the 5'-TOP motif (reviewed in¹⁷). We find that the longer an
10 uninterrupted pyrimidine stretch around YC-initiation, the higher is the expression level
11 of dominant YC CTSSs (**Figure 2e**). Translation-associated genes have a longer stretch of
12 pyrimidine sequence (**Supplementary Figure 2a**), which is in agreement with the
13 stringent definition of translationally regulated 5'-TOP mRNAs¹⁵. Dual-initiation promoter
14 genes have shorter 5'-UTR length as compared to single initiation YR promoters (**Figure**
15 **2f**), which may reflect efficient translation as transcripts with longer 5'UTR tend to have
16 lower translational efficiency²⁵.

17 Next, we sought to define the promoter features of YR-components and YC-
18 components of dual-initiation promoters. CAGE defined TSSs have revealed 3 main classes
19 of promoter shapes, namely broad peak, sharp peak and bimodal peaks², and 5'-TOP/TCT
20 promoters were primarily associated with sharp peak promoters of highly expressed
21 genes¹. To explore features of promoter shapes of dual-initiation genes, we first calculated
22 the number of CTSSs and observed that dual-initiation genes have higher number of YR-
23 initiation sites (an average of 6 CTSSs) as compared to their YC constituent (an average of
24 2 CTSSs) or compared to the YR-only genes (an average of 3 CTSSs) (**Figure 2g**).
25 Accordingly, YR component of dual-initiation promoters is typically defined by a broad
26 peak, while YC-initiation events appear mostly sharp (**Figure 2h**). We then asked if
27 positionally constrained motifs characteristic of known promoter architectures can be
28 assigned to either YC and YR-initiation events in DI promoters. We have plotted YR, YY,
29 SS, WW (Y=C/T; R=A/G; S=C/G; W=A/T) dinucleotides and positionally constrained
30 motifs (TATA box, GC box and CCAT motif) with respect to YR and YC-initiation events at
31 fertilized egg and at prim 5 stage. The WW dinucleotide (W-box motif) present in most
32 promoters in zebrafish²⁶ is enriched in both initiators in the fertilized egg but depleted in
33 prim 5 stage (**Supplementary Figure 2b,c**). The finding that YC-initiation is associated
34 with positionally constrained motif previously described for YR-initiation supports YC-
35 initiation detection as indicator of promoter function. Moreover, we have detected similar
36 developmental utilization of sequence determinants of YC transcription start site choice

1 to that previously described for YR-initiation²⁶. However, TATA box, CCAT motif and GC
2 box were not enriched with either initiation events in both stages (**Supplementary**
3 **Figure 2b-c**). Thus, we conclude that YR-initiations peaks of dual-initiation genes are
4 generally broad, while YC-initiations are sharp, however these differences are not
5 reflected in observable differences in the frequency of positionally constrained motifs.
6 Taken together, our results collectively demonstrate the pervasive nature of YC-initiation
7 in the genome which is characteristic not only to translation-associated genes but to
8 previously unappreciated GO categories and often feature TOP promoter-like pyrimidine
9 stretches. These observations suggest that the DI promoter is a novel promoter
10 classification category widely used in the zebrafish genome and which appears to be a
11 composite of canonical and 5'-TOP/TCT promoter features.
12

1 *Differential regulation of YC-initiations and YR-initiations in DI promoters during*
2 *embryogenesis*

3 We have previously shown that two distinct and independently regulated promoter
4 sequence codes such as the W-box and +1 nucleosome positioning signals are often
5 overlaid in individual promoters and used differentially during the maternal to zygotic
6 transition of embryo development²⁶. The existence of such overlapping sequence codes,
7 together with the observation that TCT promoters and canonical initiator may be
8 regulated by different initiation complexes^{12,13} prompted us to hypothesize that
9 intertwined YR-initiation and YC-initiation events may represent differential regulatory
10 principles. Thus divergent regulatory inputs may target dual-initiation promoters, and
11 lead to divergent transcriptional regulation during embryo development. Therefore, we
12 asked about the relationship between the expression dynamics of YR-initiation and YC-
13 initiation during early embryo development. We performed self-organizing map (SOM)
14 clustering between YR and YC expression levels for each gene, and observed the typical
15 zebrafish developmental expression profiles, characterized by two opposing trends. A
16 typical maternal dominant trend includes mRNA expression at early stages originating
17 from the oocyte, which is removed by RNA degradation after zygotic genome activation
18 manifesting as loss of expression typically after 6th to 9th stages (**Supplementary Figure**
19 **3a**, e.g. panels of first column). An opposite zygotic dominant trend features low or no
20 maternal activity followed by the zygotic activation, which also appears as an increase in
21 expression after the 6th to 9th stages of 12 stages analyzed. Additional trends variations in
22 maternal to zygotic activity of YC and YR have also been detected (**Supplementary**
23 **Figure 3a**). Most clusters show similar expression dynamics, while differences may have
24 been masked by the pooling of many genes. Nevertheless, several clusters are
25 characterized by distinct profiles for YR and YC components (**Figure 3a**), where the YR
26 component is expressed both maternally and zygotically, whereas the YC component is
27 either zygotic (top row) or maternal only (bottom row). Correlating the expression levels
28 between YR-initiation and YC-initiation during embryogenesis revealed that a majority of
29 genes (71.4%; n=2947) are positively correlated ($r \geq 0.5$), while a small but distinct
30 proportion (7.5%; n=312) of genes show YC and YR components negatively correlated (r
31 ≤ -0.5) (**Figure 3b; Supplementary Table 4**). To understand the origin of negative
32 correlation in regulation, we plotted the expression profiles of these 312 genes and
33 observed two groups with divergent regulation of YR-initiation and YC-initiation during
34 MZT (**Figure 3c**). YR and YC components show opposite maternal zygotic dominance
35 indicating they are distinctly subjected to maternal mRNA degradation and corresponding
36 zygotic transcription activation²⁶⁻²⁸ (**Figure 3d**). Genes in the top cluster predominantly

1 use YR-initiation during maternal stages, in contrast YC-initiation gets dramatically
2 upregulated at the zygotic genome activation after the mid blastula transition (**Figure**
3 **3c,d**). This trend is demonstrated by translation elongation factor (*eef1g*) gene promoter
4 (**Figure 3e**), the human homolog of which is transcribed by a non-canonical YC-type
5 initiator¹⁷. The other negatively co-regulated cluster (bottom cluster in **Figure 3c**) is
6 primarily driven by YC-initiation in maternal stages and by increased YR-initiation in
7 zygotic stages (**Figure 3d**), as exemplified by the initiation profile of the *psmd6* gene
8 (**Figure 3f**). These results indicate that YR-initiation and YC-initiation are widely used in
9 development and not specific to maternal or zygotic stages. However, they are selectively
10 used for individual genes, which suggests that these genes can respond to differential
11 regulatory inputs. Taken together, the expression dynamics within these 312 dual-
12 initiation promoters indicate independent regulation of YR-initiation and YC-initiation
13 components, which is markedly apparent during the dramatic overhaul of the
14 transcriptome at the MZT.

15 *YC component of dual-initiation promoter genes regulates snoRNA expression*

16 snoRNAs are transcribed by host gene promoters and are spliced out from introns
17 of primary transcripts and subsequently form a riboprotein complex²⁹. Thus snoRNA host
18 genes may carry two functional entities, snoRNA genes and their coding or non-coding
19 host gene. Interestingly, a non-coding host gene (*GAS5*) of snoRNA⁶ has recently shown to
20 have an additional function in maintaining nodal signaling³⁰. In contrast to previous
21 studies in mammals that described snoRNA host genes being transcribed by YC-initiation
22 (5'-TOP/TCT), we showed that zebrafish snoRNA host genes are characterized by dual-
23 initiation (**Figure 2a**). These observations raise the question, whether the dual function of
24 snoRNA host genes is decoupled by YR-initiation and YC-initiation and whether the two
25 initiation events contribute selectively to distinct RNA fates. Indeed, it was previously
26 shown that a 5'-TOP promoter element determines the specific ratio of snoRNA to mRNA
27 production and an artificial canonical YR-initiation containing Pol II promoter is
28 incompatible with the efficient release of snoRNA¹¹. The dramatic transition of maternal
29 and zygotic transcriptomes and the uncovered differential regulation of YC-initiation and
30 YR-initiation at MZT provides an opportunity to address whether YR and YC components
31 of snoRNA host genes are differentially regulated. We thus hypothesized that potentially
32 divergent expression dynamics of YR and YC derived transcripts during MZT could be
33 informative to separate 5' end of the source RNA for embedded snoRNA genes in dual-
34 initiation promoter host genes. To this end, we plotted the expression levels of both YR
35 and YC components of 97 snoRNA host genes (containing 249 snoRNAs) and the

1 expression of snoRNAs²³ at the corresponding developmental stages (**Supplementary**
2 **Figure 4a**). The majority of snoRNA host genes are maternally deposited, and both YR
3 and YC activity as well as snoRNA expression are generally increased after activation of
4 zygotic transcription (**Supplementary Figure 4a**). Correlation of expression levels of
5 snoRNAs with YR and YC components revealed stronger correlation of the YC component
6 with the temporal dynamics of snoRNAs (**Figure 4a**), suggesting YC-initiation to be the
7 likely source for snoRNA host RNA species.

8 To further investigate the observed correlation between snoRNA expression with
9 YC-initiation, we selected two host genes (*kansl2* and *nop53*) whose overall expression
10 levels are comparable but have varying levels of YR and YC components. The snoRNA host
11 gene *kansl2* has a dominant YR-initiation and a minor YC-initiation, while its snoRNA
12 expression levels is low throughout development (**Figure 4b**). On the other hand, the
13 *nop53* host gene predominantly shows the usage of YC-initiation in zygotic stages and
14 corresponding similar dynamics of snoRNA expression levels (**Figure 4b**). We then
15 analyzed snoRNAs expression levels in relation to the expression of YR and YC
16 components of their host genes at the prim 5 stage, by which time post-transcriptional
17 effects of maternal mRNA clearance are eliminated. We classified 97 snoRNA host genes
18 into YR-dominant (N=44) and YC-dominant (N=53) groups and plotted the expression
19 levels of YR-components and YC-components of host promoter and the corresponding
20 snoRNAs (**Supplementary Figure 4b**). The expression levels of snoRNAs from YC-
21 dominant genes is significantly higher (t test; p=0.00037). Since the overall expression
22 levels of YC-dominant genes is significantly higher than YR-dominant genes
23 (**Supplementary Figure 4b**), higher expression levels of snoRNA is expected, and thus it
24 is difficult to distinguish the contribution of two initiators. Thus, we sought to analyze
25 snoRNA expression levels only in those host genes whose overall expression levels are
26 comparable but have significantly varying contribution of YR and YC components
27 between YR-dominant (N=24) and YC-dominant (N=16) genes (**Figure 4c**). Though
28 overall expression levels are comparable, snoRNAs expression levels are significantly
29 higher (t test; p=0.00025) in YC-dominant genes (**Figure 4d**). Taken together, we provide
30 evidence for divergent developmental regulation of two intertwined initiators in snoRNA
31 host genes. Furthermore, the correlation analysis of temporal and expression levels
32 suggests that the YC-initiation better explains snoRNA expression than the YR-initiation.
33 Nevertheless, the localization of snoRNAs in many ribosomal and translation factors
34 suggests that snoRNAs are produced together with the translation and rRNA biogenesis
35 protein machinery encoded by their host genes and hence they are likely also co-
36 regulated.

1 *Differential expression and localization of snoRNA and host RNA in zebrafish*
2 *embryos*

3 The above results suggest that snoRNA host RNAs may be divergently expressed.
4 However, their temporal expression dynamics may not reveal the full extent of
5 differential RNA regulation which emerge from dual-initiation promoter genes. Therefore,
6 we investigated the spatial expression patterns of two newly annotated snoRNAs
7 (**Supplementary Table 2**) embedded in the intron of host gene *nanog* (**Figure 5a**) and
8 *dyskerin* (*dkc1*) respectively (**Figure 5b**). The snoRNA in *nanog* is conserved among
9 teleosts (**Figure 5a**) and is validated by RT-PCR (**Supplementary Figure 5a**). The host
10 gene *nanog* is a transcription factor that regulates genome activation during early
11 zebrafish development^{28,31} with no reported function in rRNA biogenesis. The *nanog* gene
12 carries YR-dominant initiation and low level of YC-initiation (**Supplementary Figure 5b**),
13 with corresponding low level of snoRNA expression. An antisense probe raised against
14 the snoRNA was detected in some but not all nuclei of zebrafish embryos at the sphere
15 stage, whereas an exonic probe detects *nanog* distinctly in the cytoplasm in most cells,
16 indicating the differential transcriptional and/or post-transcriptional fates of the two RNA
17 products generated by the dual-initiation promoter (**Figure 5c-f**).

18 A snoRNA is produced in several copies from introns of the *dyskerin* (*dkc1*) gene
19 and expected to have shared expression pattern with its host gene given their shared role
20 in pseudouridylation of ribosomal RNA. We validated one of the novel snoRNAs by RT-
21 PCR (highlighted in oval shape in **Supplementary Figure 5c**). The *dkc1* gene carries YR-
22 dominant initiation in both maternal and zygotic stages (**Supplementary Figure 5d**),
23 while 3 of 4 minor YC-initiation sites become activated higher in zygotic stages
24 (**Supplementary Figure 5c,e**). Expression of the snoRNA by in situ hybridization in
25 whole mount embryos revealed co-localization with Fibrillarin in highly expressing
26 tissues thus, verifying the expected nucleolar expression profile (**Figure 5g-i**).
27 Furthermore, selective expression of snoRNA in nucleoli were detected as speckles in
28 nuclei of a subset of cells at prim 5 stage notably in the epiphysis, somatic muscle cells,
29 and the ciliary marginal zone of the eye. The host RNA *dkc1* exonic probe was detected
30 ubiquitously in the cytoplasm with elevated activity in overlapping (e.g. epiphysis, ciliary
31 marginal zone of retina) as well as non-overlapping domains (e.g. outer nuclear layer of
32 retina) with snoRNA probe (**Figure 5j-m**). Taken together, these two examples suggest
33 that besides the expected differential subcellular localization of host gene products and
34 embedded snoRNAs they are also activated in partially overlapping domains of the
35 embryo, which is consistent with potential divergence in transcriptional regulation of
36 products from the same core promoter.

1 *Differential fates of YR-initiation and YC-initiation products during translation*
2 *inhibition*

3 SnoRNA host genes are selectively subjected to nonsense mediated decay (NMD),
4 shown by blocking NMD with translation inhibitor cycloheximide, which led to
5 stabilization of several (*UHG* and *GAS5*)^{6,32}, but not all (e.g. *U17HG*⁷, *U87HG*³³, *rpS16*)
6 snoRNA host genes. This result suggests differential stabilization of host RNAs due to
7 differential association of snoRNA host mRNAs with translating ribosomes⁷. We asked
8 whether dual-initiation promoter genes are subjected to differential post-
9 transcriptional/translational regulatory mechanisms involving NMD in zebrafish
10 development. To test post-transcriptional regulation of YR and YC initiated RNAs, we
11 blocked translation/NMD in zebrafish embryos by cycloheximide at 22 somites stage for 2
12 hours until prim 5 stage and performed CAGE analysis (**Figure 6a**). These stages were
13 chosen for the analysis because YC-initiation is broadly active (**Supplementary Figure**
14 **1a; Figure 3b**) by these stages and maternal mRNAs, which could bias monitoring of post-
15 transcriptional control have been cleared from the embryo³⁴. Overall, expression levels of
16 zebrafish *gas5* mildly increased upon cycloheximide treatment with YC-initiation mildly
17 upregulated and YR-initiation downregulated (**Supplementary Figure 6a**), suggesting
18 that *gas5* is regulated by NMD in zebrafish similarly to human yet CAGE-based initiation
19 profile analysis revealed differential response between YR-initiation and YC-initiation. To
20 further demonstrate the response to cycloheximide by individual initiation sites within a
21 single dual promoter, we highlight ribosomal protein (*rps13*) with multiple YR-initiations
22 and YC-initiations (**Figure 6b**). Expression levels of both YC-initiation products are
23 significantly upregulated while YR-initiation products are significantly downregulated
24 (Fisher-exact test; p-value=3.5e-06), suggesting that the intertwined YR-initiation and YC-
25 initiations are independently regulated.

26 Next, we expanded the initiation analysis to all ribosomal proteins and
27 subsequently to genome-wide upon cycloheximide treatment. Translation inhibition
28 resulted in an overall upward trend of YC-initiation and downward trend of YR-initiation
29 among ribosomal protein family genes (**Supplementary Figure 6b**). In total, 60% of
30 ribosomal genes have an upregulated YC-initiation while 80% of YR-initiation are
31 downregulated (**Figure 6c; Supplementary Table 5**), corresponding to a significantly
32 different (Kolmogorov-Smirnov test; p=3.5e-06) response between two initiators.
33 However, at the individual gene level only 21 (29.1%) of ribosomal protein genes have
34 significantly different (Fisher's exact test: p<=0.05) dynamics between YR-initiation and
35 YC-initiation (**Figure 6d, Supplementary Table 5**). Subsequently, we have analyzed the
36 response to cycloheximide for all DI promoter genes by classifying them either as YR-

1 dominant (N=1774) or YC-dominant (N=241) based on the YC/YR expression ratio. YR-
2 initiation and YC-initiation products show significantly different response to
3 cycloheximide (Kolmogorov-Smirnov test; p-value=4.44E-05) in YC-dominant genes
4 (**Figure 6e**) and no significant difference in YR-dominant genes (**Figure 6f**). Taken
5 together, these results demonstrate that the two initiation products differentially
6 regulated the YC-dominant subset of DI promoter genes upon cycloheximide treatment.

7 *Dual-initiation promoter genes are conserved across metazoans*

8 Finally, we asked whether DI promoters observed in zebrafish are present among
9 other metazoans. We first re-analyzed transcription initiation of the human snoRNA host
10 gene *GAS5* that is transcribed by a 5'-TOP promoter⁶. Visual inspection of combined CTSSs
11 from FANTOM5²² revealed that *GAS5* utilizes the expected YC-initiation as dominant
12 initiator (indicated by arrow) (**Figure 7a**) but also an unexpected presence of YR-
13 initiation at a comparable expression level. We measured the expression levels of both
14 initiators in individual cell types across FANTOM5 libraries and observed unexpectedly
15 higher levels of YR component of *GAS5* promoter activity than its YC component in
16 multiple cell types (**Figure 7b**). This result demonstrates the presence and differential
17 expression dynamics of two initiations in a dual-initiation promoter in mammals. We then
18 analyzed DI promoters by adapting the pipeline described in Figure 1a to human HepG2
19 cell line²² and *Drosophila melanogaster* S2 cells³⁵. Among expressed genes, 3920 (45%)
20 promoters in HepG2 and 1701 (16%) promoters in S2 cells have intertwined YR-initiation
21 and YC-initiation within the same core promoter (**Figure 7c**). The YC-initiation is
22 dominant in 11.83% and 7.99% of DI promoters in human and *Drosophila* respectively
23 (**Supplementary Table 6**). Furthermore intersection of human and zebrafish
24 orthologous DI promoter genes revealed that 1171 (38.46%) genes share the DI promoter
25 feature indicating high degree of conservation of DI promoters among vertebrates. Gene
26 ontology analysis of DI promoter genes in human has revealed enrichment for translation
27 regulation, mRNA stability, and RNA splicing in human (**Figure 7d**) similar to that in
28 zebrafish (**Figure 2b**) and suggesting that what were previously described as 5'-TOP/TCT
29 promoters, are better described as DI promoters in several cell types both in human and
30 *Drosophila* and argues for redefining non-canonical initiator promoters in these
31 metazoans.

32 We next sought to compare sequence content, analyze expression levels and
33 promoter width of dual-initiation promoters in human and *Drosophila*. In both species, DI
34 promoters have higher C+T content around the TSS as compared to YR-only promoters
35 but lower than YC-only promoters (**Figure 7e**), similar to observations in zebrafish

1 **(Figure 2c)**. Dual-initiation promoters are highly expressed compared to YR-only and YC-
2 only initiation promoters, which appears to be a shared feature among all three species
3 **(Figure 7f; Figure 1d)**. Dual-initiation promoters have higher number of CTSSs, resulting
4 in broad promoter shapes, whereas the YC component shows sharp peaks similar to
5 zebrafish **(Figure 7g compare to Figure 2g)**. The UCSC browser view of the orthologous
6 ribosomal protein genes *RPL38* shows a similar intertwining of YR and YC-initiation
7 events across all three species **(Figure 7h)**. Taken together, the above results
8 demonstrate that DI promoters are pervasive and an evolutionary ancient phenomenon
9 characteristic to distant clade with highly conserved promoter architecture and
10 expression features shared among metazoans and highlight the importance of this novel
11 promoter structure organization in divergent animal systems.

1 Discussion

2 In this study, we demonstrate the pervasive nature of non-canonical transcription
3 initiation intertwined with canonical initiation within the core promoter of thousands of
4 genes in zebrafish development. Thus YC-initiation is utilized by a much larger set of
5 genes than previously reported, which was limited to components of translational
6 machinery^{6,7,12,17}, and characterized as 5'-TOP/TCT initiators. This dual-initiation
7 arrangement represents a novel composite promoter architecture, which encompasses
8 two sets of targets for transcription initiation in individual promoters. By exploiting the
9 dramatic switch of the embryo transcriptome during the maternal zygotic transition, we
10 show that two initiations are uncoupled from each other during this transition,
11 demonstrating the differential use as well as evidence for lack of interdependence
12 between them in many genes. The apparent independent regulation of initiation site
13 selection in dual promoters during the MZT argues for two initiation mechanisms acting
14 both in the oocyte and the early embryo. However, their use is not selective to ontogenic
15 state, instead it appears to alternate among promoters. The remarkable overlap of
16 transcription initiation mechanisms in the same promoter regions suggest that promoters
17 of dual-initiation genes may respond in more than one ways to regulatory inputs acting in
18 different ontogenic contexts, such as the maternal to zygotic transition (**Figure 8a**).

19 We provide evidence that zebrafish snoRNA host genes are transcribed from YC-
20 initiation similar to other model systems^{6,7}. However, we also observe that snoRNA host
21 genes also carry canonical YR-initiation not only in zebrafish but also in mammalian cells.
22 While short read sequencing used either in CAGE and RNA-seq is not suitable to directly
23 trace YR- and YC-specific full length RNAs and thus, unequivocally uncouple the post-
24 transcriptionally generated secondary RNA products from two initiation sites.
25 Nevertheless, we show an association of YC-initiation with snoRNA generation by
26 expression correlation analysis of initiation usage. Our results are in agreement with a
27 previous study, which demonstrate that experimentally replacing YC-initiation (5'-TOP)
28 snoRNA promoter with a YR-initiation site reduce snoRNA production¹¹. Taken together,
29 our observations strongly argue for a combination of transcription initiation mechanisms
30 acting on snoRNA and host genes and raises the question, whether the mixed nature of
31 canonical and non-canonical initiators reflect a shared promoter region being used by two
32 transcription initiation complexes. Thus a regulatory level at transcription initiation can
33 lead to the production of transcripts with distinct post-transcriptional fates, representing
34 two different functional products, such as snoRNAs and host genes products (see
35 examples of spatial expression of *nanog* derived snoRNA and host gene products in

1 **Figure 5)**. This dual role of a promoter in a single ontogenic stage within the same cell
2 expands the transcript repertoire of the cell (see model in **Figure 8b**), and if generally
3 applied by dual promoters, could substantially impact on the a yet unexplored additional
4 layer of diversity of RNAs produced from genes. We hypothesize, that the expansion of
5 utilization of a non-canonical initiation to a wide range of genes could indicate a general
6 transcription regulation paradigm, which represents adaptation to differential regulation
7 of a variety of promoters^{15,18}. Dual-initiation promoter genes are highly expressed
8 compared to other genes (**Figure 1d; Figure 7f**), which is not specific to the contributing
9 YC components, as expression levels of the corresponding YR component alone is also
10 higher than that of YR-only or YC-only initiator genes. This observation either suggest that
11 sharing two alternative initiation mechanisms leads to boost of expression levels or
12 suggest that YC-initiation might be evolutionary co-opted in highly expressed genes. It is
13 interesting to note that the efficiency of transcription correlates positively with
14 translation efficiency and raises the possibility that highly expressed DI promoters
15 contribute to coordination between transcription and translation³⁶. The enrichment of
16 translation and RNA regulation related gene ontology terms in DI promoter genes, along
17 with notable absence of developmental regulator genes, raises the question of why and
18 how this promoter architecture evolved. Important insight into potential functional
19 significance of the non-canonical initiation comes from studies on target genes of the
20 mTOR pathway that are translationally regulated^{15,16}, and are enriched in 5'-TOP/TCT
21 initiator. Polypyrimidine proximal to 5' end of these genes is a target for translation
22 regulation and has been suggested to serve as a targeting mechanism for oxidative and
23 metabolic stress or cancer induced differential translation regulation by the mTOR
24 pathway^{15,16,18-20,37}. Other studies argue for the co-transcriptional regulation of post-
25 transcriptional fates of RNAs, where promoter identity influences cellular localization and
26 translation efficiency of mRNAs under different environmental conditions^{38,39}. Thus, it is
27 plausible that specialization of transcription initiation has co-evolved with post-
28 transcriptional regulation to regulate RNA fates by transcription and the 5'-ends of TOP
29 RNAs reflects such a dual regulatory function. Dual-initiation promoters offer the
30 potential for linking translational regulation to transcriptional regulation in a large range
31 of genes and thus increase the repertoire of genes that may respond to such signals. In
32 this study we have identified many genes, which carry low level of YC-initiation events,
33 which may reflect a non-induced ground state for YC regulation. However there was a
34 notable correlation between the length of polypyrimidine stretch at the 5' end and the
35 expression level of YC (**Figure 2e**). It is not yet possible to distinguish in the CAGE dataset
36 whether this correlation reflects RNA stability or transcriptional differences.

1 Nevertheless, an unanswered question remains, whether the polypyrimidine stretch at
2 the 5'-end is required for selective translation factor binding such as eIF4F complex or
3 also represent distinct transcription regulatory signals acting at the transcription
4 initiation level.

5 The current definition of 5'-TOP mRNA includes a stretch of minimally 4 to 13
6 pyrimidine¹⁷ based on observations restricted to translational-associated genes¹⁷, which
7 have longer pyrimidine stretch also in zebrafish (**Supplementary Figure 2d**). This
8 definition has been suggested to be potentially too stringent, as translationally regulated
9 genes revealed by ribosome profiling are enriched in transcription initiation with "C" and
10 carry only a short pyrimidine stretch^{15,16}. We used a threshold of 1 TPM and identified
11 thousands of YC-initiation sites and thus expanded the pool of genes, which ought to be
12 considered when transcriptomic responses to metabolic stress for example via the mTOR
13 pathway are sought and our results argue for the need for discriminating RNAs produced
14 from the same promoter by using transcriptome analyses with single nucleotide
15 resolution. Many of these genes may respond to post transcriptional signals similarly to
16 5'-TOP promoter genes, however this response is potentially masked in investigations in
17 which the RNAs with distinct initiation profiles are not separately quantified. This
18 possibility is demonstrated by our cycloheximide treatment experiments where YR and
19 YC components of dual-initiation promoters respond differentially to interference with
20 translation/NMD, which implies that C-capped transcripts may be more prone to NMD
21 than their A/G-capped counterparts originating from the same DI promoter. Taken
22 together, our findings provide a framework for future studies to understand coordinated
23 regulation of transcription and translation of thousands of genes.

24 The unexpected widespread presence of YR and YC-initiation intertwined in the
25 same core promoter raises a question as to why this pervasiveness was not seen before.
26 Previous studies analyzing TSSs in a genome-wide level reported multiple TSSs in same
27 core promoter^{2,3,5,22,26}, but downstream analysis is focused on dominant TSSs, majority of
28 which appear as YR, and as a result YC-initiation remained unexplored. Reinvestigation of
29 human and *Drosophila* cell line datasets in this study demonstrated that the dual-
30 initiation is a widespread phenomenon and share similar sequence feature, promoter
31 shapes, expression levels and enriched gene ontology. Dual-initiation promoter genes in
32 three major metazoan model systems spanning a very large evolutionary distance and
33 across many orthologues suggest an evolutionary ancient shared promoter architecture
34 with fundamental functions in multicellular function and development and motivates
35 future investigation into the functional consequences of selective transcription initiation
36 within gene promoters in general. Our studies in zebrafish embryos with dynamic

- 1 spatiotemporal transcriptional patterns underscore the importance of further analysis of
- 2 the dynamics of YR and YC expression profiles across multiple cell types and varying
- 3 physiological states in other model systems.
- 4

1 **Materials and Methods**

2 *Zebrafish CAGE data after cycloheximide treatment*

3 We generated zebrafish CAGE data for translation inhibition experiment. Zebrafish
4 embryos were treated with 100 µg/ml cycloheximide (Sigma-Aldrich) or 0.1 % DMSO as
5 control for 2 hours, starting at 22 hours post fertilization (hpf). Total RNA was extracted
6 from the control and treatment groups at 24 hpf using TRIzol (Invitrogen/ThermoFisher)
7 following the manufacturer's instructions and used for CAGE libraries preparation as
8 described before³, except for the use of oligo-dT primer instead of random primers in the
9 first strand synthesis step. CAGE libraries were sequenced on Illumina MiSeq system.

10 *RNA sequencing of capped RNAs*

11 Total RNA was extracted from 24 hpf embryos using TRIzol reagent
12 (ThermoFisher) and DNase treated using TURBO DNA-free™ Kit (ThermoFisher)
13 according to the manufacturer's instructions. Full length cDNA libraries were prepared
14 using TeloPrime Full-Length cDNA Amplification Kit (Lexogen), designed to capture 5'
15 Capped, polyadenylated transcripts. Two full cDNA libraries were prepared (technical
16 replicates) according to the provided user manual, using 2 µg of total RNA as input, with
17 differing numbers of PCR amplification cycles: 14 and 16 respectively. Sequencing
18 libraries were prepared from both cDNA libraries using the MicroPlex-Library-Prep-Kit-
19 v2 (Diagenode) and sequenced (2x100bp reads) on HiSeq 2500 System (Illumina). For
20 identification of transcription start sites, only reads starting with the 5' TeloPrime
21 adapter were selected, trimmed using cutadapt⁴⁰ and mapped to the zebrafish Zv9
22 zebrafish reference genome and Ensembl version 79 transcript annotations using STAR⁴¹,
23 reporting only uniquely mapped reads. CAGE-like TSS (CITSS) were called using CAGER
24 package⁴². CITSS with at least 0.3 tpm were assigned to Ensembl version 79 promoter
25 regions (500 bp upstream and 250 bp downstream from the annotated transcript start). A
26 given promoter was identified as YR or/and YC-initiation type, if the mean sum (of the
27 two technical replicates) for the corresponding CITSS-initiator signals (YR/YC) was at
28 least 3 tpm, i.e. the same criteria used for CAGE samples.

29 *Publicly available CAGE data on zebrafish, human and fruit fly*

30 CAGE data on zebrafish, human and drosophila were downloaded from previous
31 studies. Mapped zebrafish CAGE data was used from previous study³. Mapped human
32 CAGE data was downloaded from FANTOM5²². Three replicates of HepG2 CAGE data was
33 merged and converted CAGE tags count into tags per million (TPM). Drosophila CAGE raw
34 reads was downloaded from modENCODE³⁵. CAGE libraries were mapped using

1 bowtie2⁴³. We allowed two mismatches and only unique mapping reads were retained.
2 Mapped reads having a “G” mismatch in the first nucleotide was corrected and
3 transcription start site was corrected accordingly.

4 *Downstream analysis of CAGE data*

5 Based on -1 and +1 nucleotides for each CAGE Transcription Start Site (CTSS) we
6 classified Y₋₁R₊₁ (Y: pyrimidine (C/T)) and (R: Purine (A/G)) as canonical initiator^{2,3} and Y-
7 ₁C₊₁ as non-canonical initiator. For all analysis, we selected CTSS with a minimum
8 expression level of 1 tag per million (TPM) in one of the 12 developmental stages. From
9 the above pool of selected CTSSs, we intersected *remaining* CTSSs and included those
10 CTSS with a minimum of 0.5 TPM. Canonical and non-canonical initiators were separately
11 clustered if they overlapped within 20 nucleotides in the same strand resulting a tag
12 clusters (TCs). Expression levels of all CTSS falling within the tag clusters are summed
13 that gives the expression level of tag clusters. CTSS with the highest expression level,
14 within the tag cluster, defines the dominantly used transcription start sites. The width of
15 tag clusters defines promoter shape which is classified as sharp or board. Genes
16 expression levels are calculated by aggregating tag clusters in the assigned promoter
17 region (500 nucleotides upstream and 300 nucleotides downstream of Ensembl
18 annotated TSSs). Canonical and non-canonical expression levels of each gene were
19 calculated by separately aggregating canonical and non-canonical CTSS.

20 *Annotation of zebrafish snoRNAs from size selected small RNA reads*

21 Size selected (18-350 nucleotide) zebrafish small RNA-seq data was downloaded
22 from public dataset²³. Adapters were filtered, and mapped sequence reads to zebrafish
23 genome (zv9) using bowtie2⁴³. Sequence reads were first mapped to ribosomal RNAs
24 (rRNAs) and excluded those mapping to rRNAs. Unmapped reads were then remapped to
25 genome by allowing up to four multi mappings reads. To ensure that snoRNAs are
26 annotated from mapped reads that resemble the expected full-length of snoRNAs, we
27 retained only those mapped reads that longer than 50 nucleotides and potentially
28 represent full-length snoRNAs rather than small RNA fragments. SnoRNAs were
29 annotated by using four different tools, namely Infernal⁴⁴, snoReport⁴⁵, snoGPS⁴⁶ and
30 snoscan⁴⁷. Infernal was used together with covariance model from RFAM⁴⁸. An e-value
31 cutoff of 0.05 for each covariate model provided by RFAM was used. SnoReport, snoscan
32 and snoGPS were used with default parameters for annotation of novel snoRNAs. To
33 retain high confidence snoRNAs, we excluded snoRNAs that have low reads (<5 reads),
34 residing on exons and repeats. Ensembl (version-79) has 312 annotated snoRNAs⁴⁹ and
35 270 of them are supported by at least 5 reads in developmental stages we analyzed. Out of

1 270 snoRNAs from Ensembl, we predicted 264 snoRNAs and annotated 176 novel
2 snoRNAs. We finally quantified snoRNAs expression by counting mapped reads using
3 BEDTools⁵⁰. Total mapped reads were calculated using SAMtools⁵¹ and then converted
4 into reads per million.

5 *Gene Ontology*

6 Gene Ontology analysis was done by using GStats package⁵² from BioConductor⁵³.
7 Over-represented GO terms were corrected for multiple testing with the Benjamini-
8 Hochberg false discovery rate and obtained statistically significant GO terms by applying a
9 p-value cutoff of ≤ 0.05 .

10 *Data visualization*

11 A genome browser view of multiple genes was downloaded from UCSC genome⁵⁴
12 CTSSs and other relevant data were uploaded on UCSC Genome Browser as tracks for
13 visualization. A screenshot of promoter regions with data tracks were downloaded from
14 the UCSC browser. All other figures were made using R.

15 *RNA extraction and RT-PCR amplification*

16 Purification of total RNA was performed using miRNeasy mini kit (Qiagen, Cat.
17 217004) following the manufacturer instructions. cDNA was synthesized using the iScript
18 cDNA synthesis kit (BioRad, Cat. 170-8890) from 200ng of purified RNA and snoRNA
19 sequences were amplified by RT-PCR. Amplified cDNAs were verified by electrophoresis
20 in 4% MetaPhor agarose gel (Lonza, Cat. 50184). We used the following primers for
21 amplification: ***dkc1-snoRNA***: TGATGAACCTTGTTTATCCATTCGC and
22 TGTCAGTCATGTATAATCATCTTGGC; ***nanog-snoRNA***: CGTGTCCATGCTGTTGCTTG and
23 CTTGTATCATCGTGCCTTTAAGACG.

24 *Riboprobes, single and double fluorescent whole-mount in situ hybridization*

25 T3 promoter was linked at the 5' and the 3' end of the full-length cDNA for each
26 amplified snoRNAs for the synthesis of antisense and Sense riboprobes, respectively.
27 Transcription were done by T3 polymerase using digoxigenin (DIG) labelling mix (Roche)
28 or DNP-11-UTP (TSA™ Plus system, Perkin Elmer) according manufacturer's instructions.
29 The probes were subsequently purified on NucAway spin columns (Ambion), and then
30 ethanol-precipitated. Single whole-mount *in situ* hybridizations were performed as
31 described previously⁵⁵. Double fluorescent *in-situ* hybridizations were carried out as
32 described previously⁵⁶.

1 *Whole mount immunofluorescence after ISH hybridization*

2 Embryos were washed in wash buffer (PBS, 0.3% v/v triton), incubated in blocking
3 buffer (PBS 1x, Tween 0.1%, Goat serum 4%, BSA 1%, DMSO 1%) for 3 hours and then
4 incubated with primary antibody over night at 4C (Anti-Fibrillarin, Abcam 38F3, 1:10).
5 Embryos were then washed in wash buffer and blocked 3 hours followed by incubation
6 with the secondary antibody overnight at 4C (Anti-Mouse Alexa 633, 1:500).

7 *Imaging*

8 Microscopy images were obtained with an Olympus DP70 camera fixed on a BX60
9 Olympus microscope. Confocal imaging was performed using a Leica TCS SP5 inverted
10 confocal laser microscope (Leica Microsystems, Germany) Digitized images were acquired
11 using a 63X glycerol-immersion objective at 1024X 1024 pixel resolution. Series of optical
12 sections were carried out to analyse the spatial distribution of fluorescence, and for each
13 embryo, they were recorded with a Z-step ranging between 1 and 2 μm . Image processing,
14 including background subtraction, was performed with Leica software (version 2.5).
15 Captured images were exported as TIFF and further processed using Adobe Photoshop
16 and Illustrator CS2 for figure mounting.

Data availability

CAGE and RNA-seq data tracks can be visualized and downloaded from the DANIO-CODE DCC (<https://danio-code.zfin.org>) and the UCSC genome browser public track hub (Promoterome).

Authors contributions

C.N. and F.M. conceived and coordinated the project. C.N. and Y.H. analyzed data. C.N. and F.M. interpreted results with critical comments from B.L., and J.B.A. E.T.S. R.C. and B.P. designed and performed whole mount in situ hybridization experiments, for which E.T., F.M and B.P. interpreted results. P.C. and A-M. S generated CAGE libraries from cycloheximide-treated embryos. Y.H. performed cycloheximide and RNA seq experiments. C.N. and F.M. wrote the manuscript with contribution from B.L. and J.B.A. All authors read and approved the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Intertwined canonical initiator (YR) and non-canonical initiator YC (alias as TCT/5'TOP) within the same core promoter

(a) A systematic pipeline for identification of canonical (YR) and non-canonical (YC) initiators in the zebrafish developmental promoterome. CTSSs are classified into known YR and YC initiators based on CAGE transcription start sites (CTSSs). (b) UCSC browser views with CAGE data from prim 5 stage to illustrate examples of YR-initiation (*apoba*), YC-initiation (*rps26*) promoters along with a gene promoter with intertwined YR-initiations and YC-initiations (*sumo2b*). YR-initiations and YC-initiations are shown in blue and red colors respectively. Barplot on the right shows the sum of expression levels of YR-initiations and YC-initiations. Highest CTSS represents the dominant transcription start site. The distance between dominant YR and YC in *sumo2b* is four nucleotides. (c) Position of dominant YC-initiation relative to dominant YR-initiation. (d) Contribution of YC-initiation with respect to YR-initiation expression levels in prim 5 stage. The 4151 genes with dual-initiation are sorted according to YC expression levels and grouped into 10 % bins. Abbreviations: TPM, tags per million.

Figure 2. Characteristic features of dual-initiation and single initiation promoter genes

(a) Intersection of translation-associated gene families as indicated with single/dual-initiation promoter genes. (b) Gene ontology (GO) categories of single and dual-initiation promoter genes clustered as indicated in green fields. (c) Sequence composition around dominant YR-initiation and YC-initiation sites of single/dual-initiation promoters. (d,e) Presence of polypyrimidine stretches in DI promoters. X-axis indicates the length of uninterrupted pyrimidine stretch with respect to YC-initiation frequency (d) and expression levels of YC-initiation sorted by increasing frequency of uninterrupted polypyrimidine stretches (e). (f) 5' UTR length of dual-initiation and single initiation YR genes. (g) Frequency of CTSS in single/dual-initiation promoter genes (h) Tag cluster width of single/dual-initiation promoter genes.

Figure 3. Maternal to zygotic transition of YR-initiation and YC-initiation demonstrates selective promoter utilization in early development

(a) Violin plot of expression profiles (tags per million) of YR and YC components of genes during embryo development. X-axis represents developmental stages as indicated. Y-axis indicates the expression levels. Blue and red colours indicate YR and YC components respectively. Numbers indicate genes in the cluster. (b) Correlation of expression levels of

YR-initiation and YC-initiation during maternal and zygotic stages. X-axis indicates genes binned according to their correlation coefficient. Genes with correlation coefficient ($r \geq 0.5$) are positively correlated and genes with correlation coefficient ($r \leq -0.5$) are negatively correlated. (c) Heatmaps show the gene expression profiles of YR-initiations and YC-initiations of 381 negatively correlated genes. Expression values are scaled (row wise) between 0 to 1, separately for YR and YC. Genes are ordered into two groups based on shift from YR to YC (top) and YC to YR (bottom) during maternal and zygotic stages and sorted based on decreasing order of negative correlation in each group. (d) Averaged expression level of YR-initiation and YC-initiation across clustered group of genes. (e,f) UCSC genome browser views of CTSSs for the *eef1g* and *psmd6* gene promoters. YR-initiation and YC-initiation events are shown in blue and red colors respectively. Barplots on the right shows the sum of CTSSs of YR-initiation and YC-initiation events respectively.

Figure 4. Correlation of expression levels of YR and YC components of snoRNA host genes with that of snoRNA expression levels

(a) Correlation of expression levels of YR-initiation and YC-initiation events with snoRNA expression levels across six developmental stages. (b) Stacked bar plot of TPM expression levels of YR (blue) and YC (red) components of *kansl2* and *nop53* genes obtained by CAGE. The expression levels of snoRNA (dark green) calculated from small RNA-seq data are represented in reads per million. Developmental stages are indicated at the bottom. (c) Box plot of TPM expression levels of YR-initiation (blue) and YC-initiation (red), along with combined (black) expression levels of YR-initiation and YC-initiation during prim 5 stage. Based on the dominant expression levels of YR-initiation and YC-initiation, host genes are classified as YR-dominant or YC-dominant genes. (d) Box plot of expression levels of corresponding snoRNAs (green) from YR-dominant and YC-dominant host genes.

Figure 5. Localization of snoRNAs and host mRNA products in the embryo

(a-b) A UCSC browser showing annotated snoRNAs (green) in the introns of *nanog* and dyskerin (*dkc1*). Ensembl annotated genes and snoRNAs are shown as black tracks. Teleost sequence conservation tracks are shown in magenta. Two snoRNAs selected for expression analysis are highlighted in oval. (c-e) in situ hybridization in whole mount zebrafish embryos at the 30% epiboly stage with probes detecting *nanog* coding exon and the snoRNA gene embedded in *nanog*. Probes detected are marked in the panels. (g-j) In situ hybridization with snoRNA probe from the dyskerin gene is detected in the nucleoli of somites (g, overlay in j) as indicated by simultaneous immunohistochemical detection of fibrillarin (h, overlay in m). K, snoRNA gene probe detecting snoRNA expression in the ciliary marginal zone of retina (cmz, arrow), epiphysis (e, black arrowhead) and somites

(s, arrowhead). **(l,n)** Exon probe of *dkc1* indicate cytoplasmic expression in ciliary marginal zone (cmz in **l**) across the retina including the outer nuclear layer (onl, white arrowhead in **k**), epiphysis (e, black arrowhead in **l**) and somites (s, arrowhead in **n**). Inserts in **k** and **l** show head from dorsal view from which magnified view is cropped.

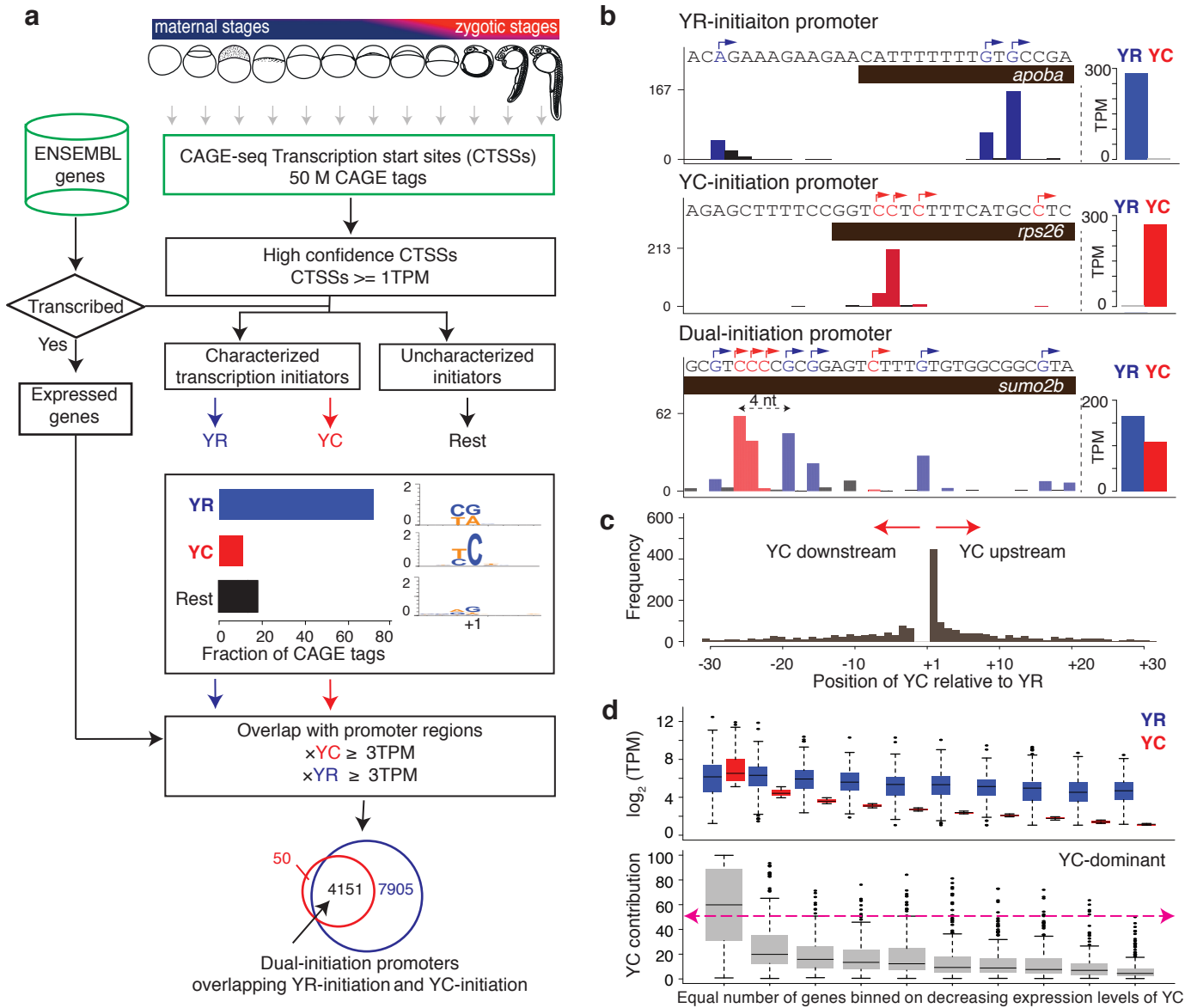
Figure 6. Differential regulation of YR-initiation and YC-initiation during translation inhibition suggest differential translational fates

(a) Experimental design to study response of YC-initiation and YR-initiation products during translation inhibition by cycloheximide. **(b)** A UCSC browser screen shot showing an example of levels of YR and YC components of the dual-initiation promoter gene *rps13*. The bar chart includes sum of all peaks. **(c)** Cumulative frequency of YR-initiation and YC-initiation of all ribosomal protein genes after cycloheximide treatment. X axis indicates the \log_2 fold change of YR-initiation and YC-initiation in cycloheximide and wild type condition. **(d)** Difference of YR-initiation and YC-initiation in individual ribosomal protein genes after cycloheximide treatment. Each bar represents a ribosomal protein gene. Vertical line represents the significant p-value (0.05) determined by Fisher test. **(e)** Behavior of YR-initiation and YC-initiation in YR-dominant (N=1771) and YC-dominant (N=241) genes.

Figure 7. Dual-initiation promoters are conserved in human and *Drosophila*. **(a)** A UCSC browser screenshot of human *GAS5* promoter with FANTOM5 CTSSs summed in hundreds of cell types. CTSSs show transcription of YR-initiation and YC-initiation within same core promoter region. **(b)** Expression levels of YR-initiations and YC-initiations by summing their CTSSs. Promoter are classified as YR-dominant or YC-dominant across individual cell types and their expression is shown in stacked bars. Y-axis shows the expression levels measured in tags per million (TPM). **(c)** Venn diagram with intersection of gene promoters with YR and YC-initiation in human HepG2 and *Drosophila* S2 cells. Dual-initiation (DI) promoters are indicated in the overlap between detected YR-initiation and YC-initiation. **(d)** Enrichment of gene ontology terms of DI promoters in human HepG2 cell line. **(e)** Comparison of C+T sequence content around transcription start sites in DI promoters with YR-only or YC-only initiation promoter in human and *Drosophila*. **(f)** Expression levels of DI promoter genes in human and *Drosophila*. **(g)** Frequency of CTSSs and promoter width of DI promoters in human and *Drosophila*. **(h)** UCSC browser screenshots showing CTSSs in the promoter region of *RPL38* gene in human, *Drosophila* and zebrafish. YR-initiation and YC-initiation peaks are colored as blue and red.

Figure 8. Models for utilization of dual-initiation promoters during development

(a) Dual-initiation promoters are occupied by pre-initiation complexes (PIC) in a cell to generate two different RNA products. PIC forms to generate RNA from YR-initiation site for generating a protein coding mRNA or non-coding RNA gene product from a snoRNA host gene, while the YC-initiation may be utilized by a specialized PIC to produce an RNA which is processed to splice out snoRNAs while the rest of the YC initiated RNA subjected to NMD or other degradation pathways. **(b)** Dual-initiation promoter is utilized divergently by YR and YC associated initiation complexes to adapt to requirements in different cells (for example the oocyte versus zygotically active embryonic lineage cells).



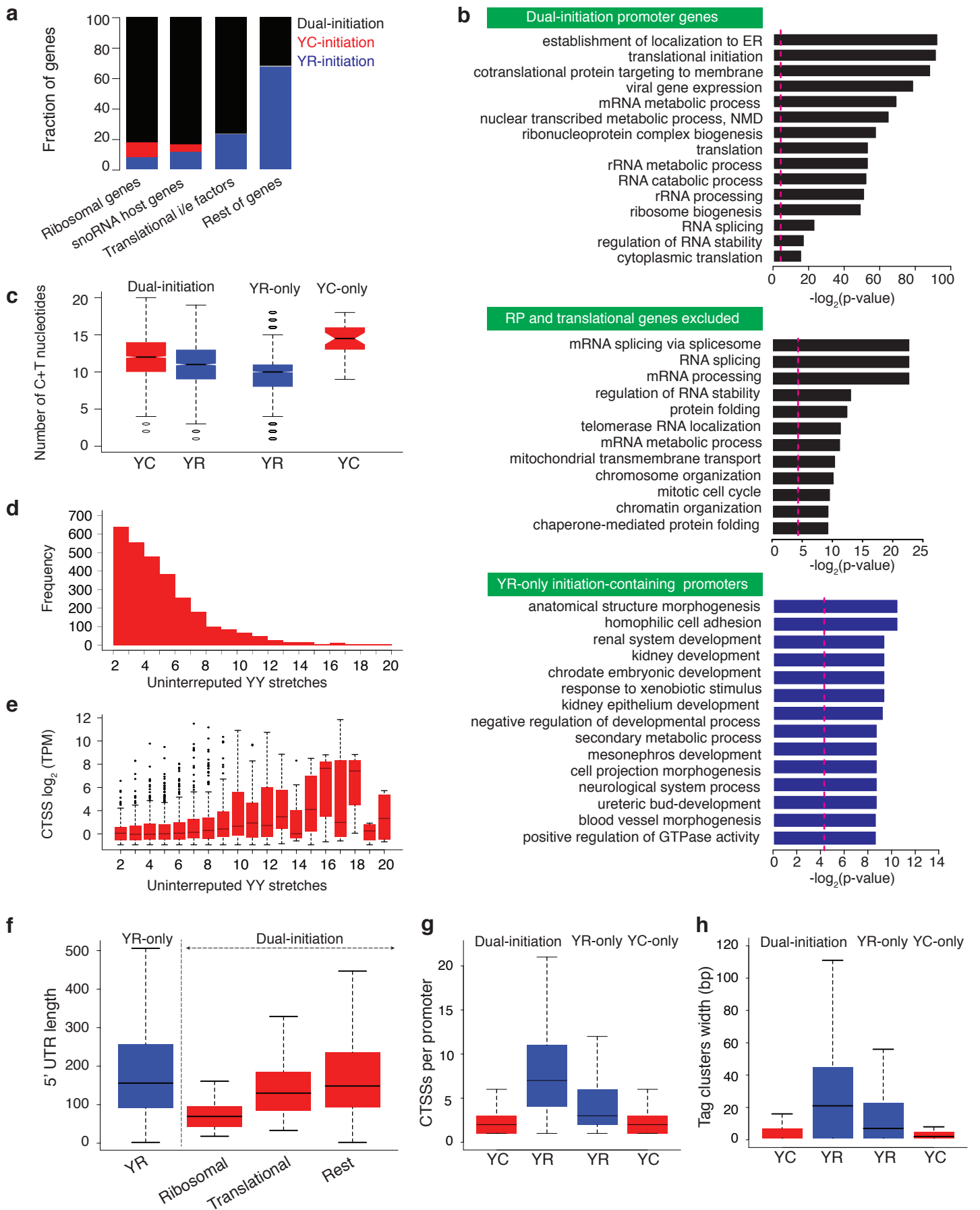


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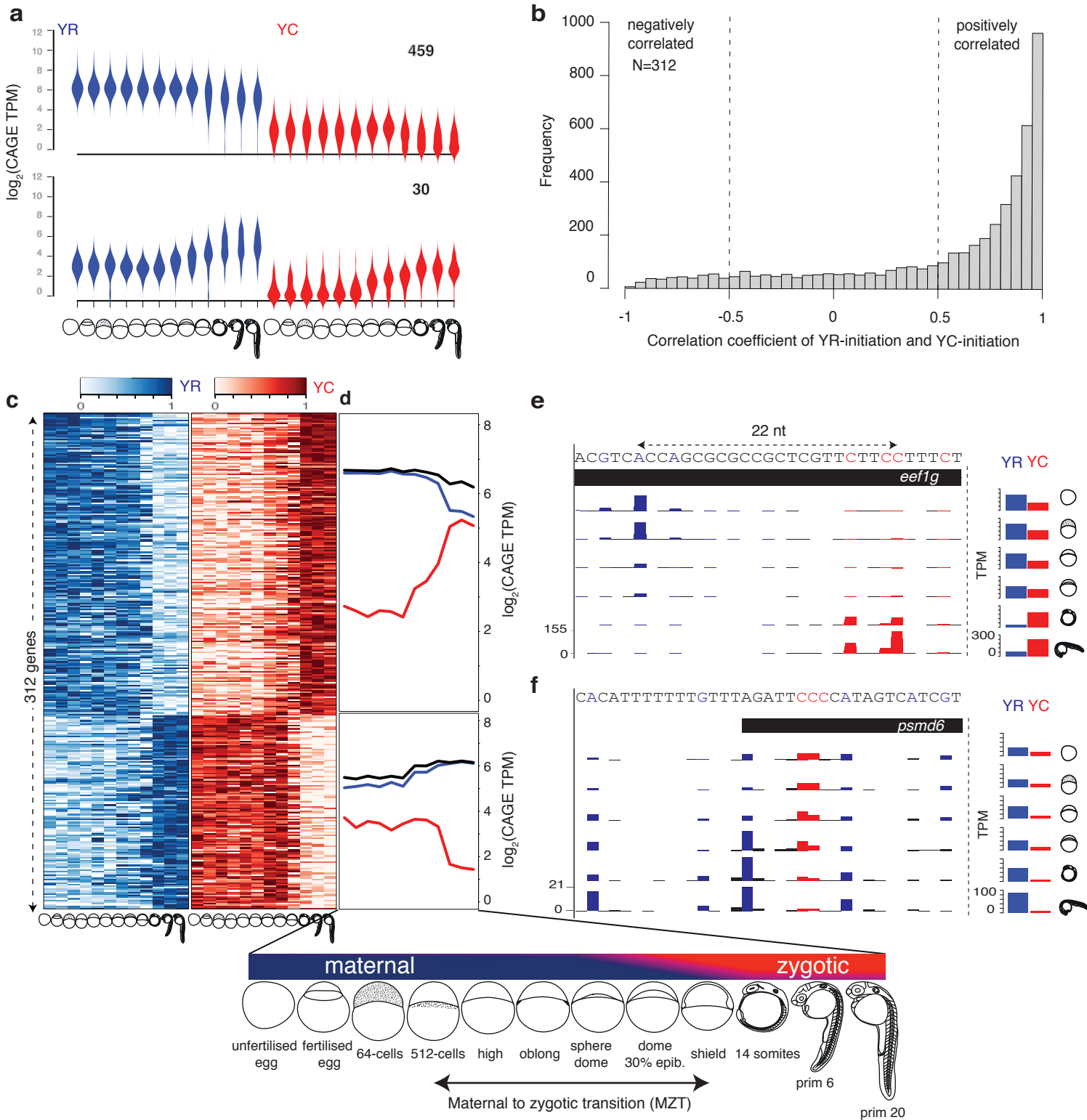


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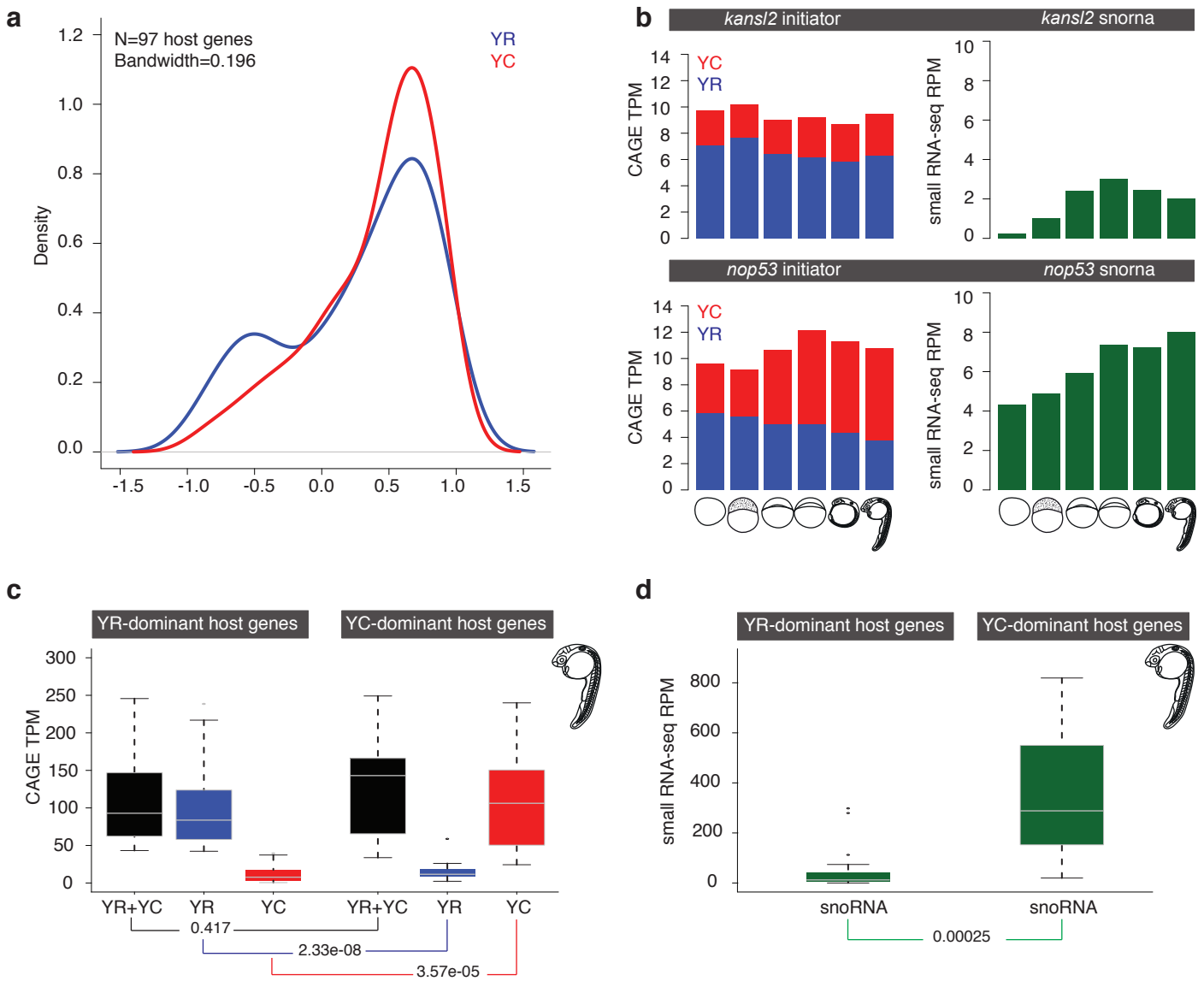


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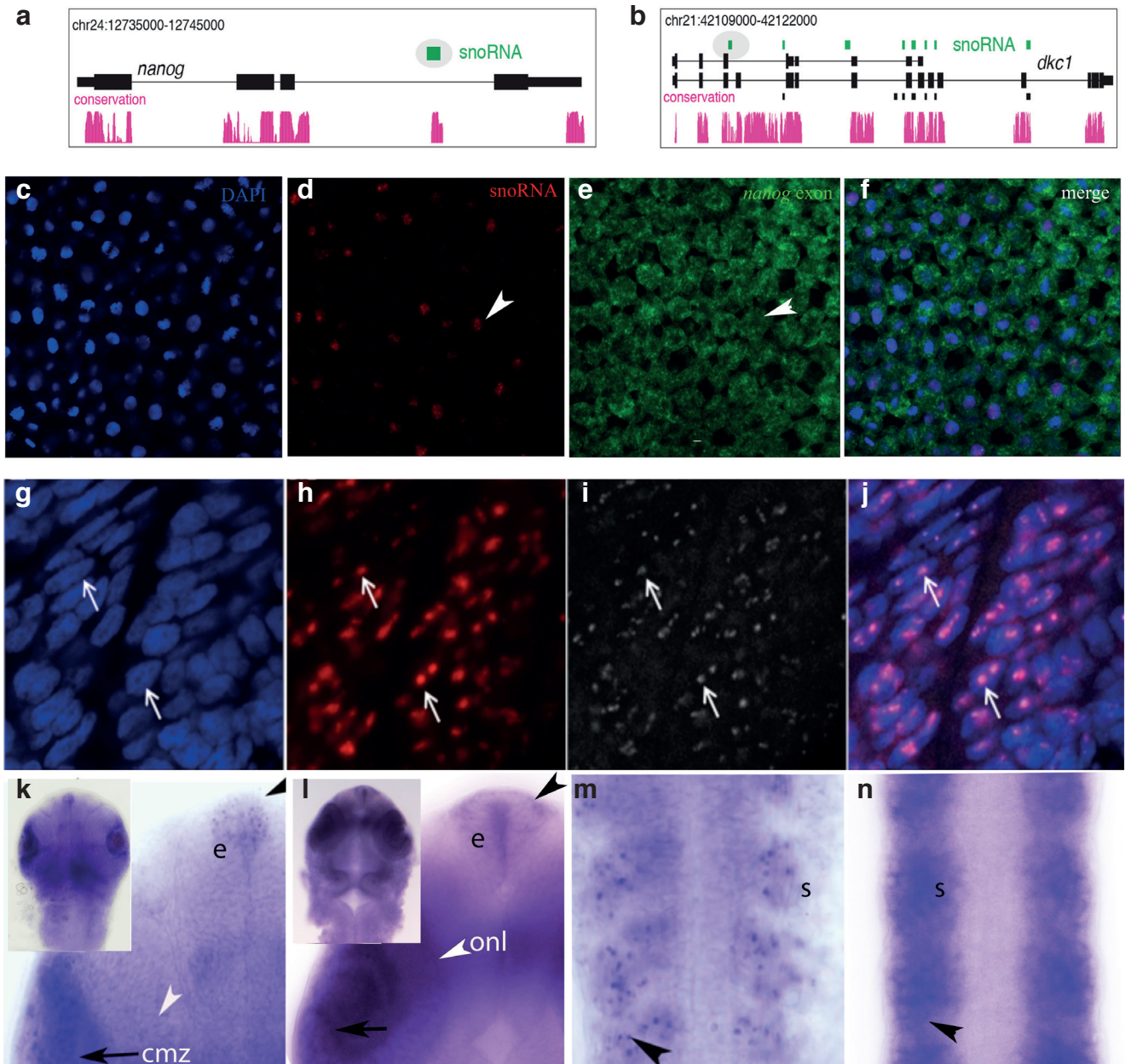


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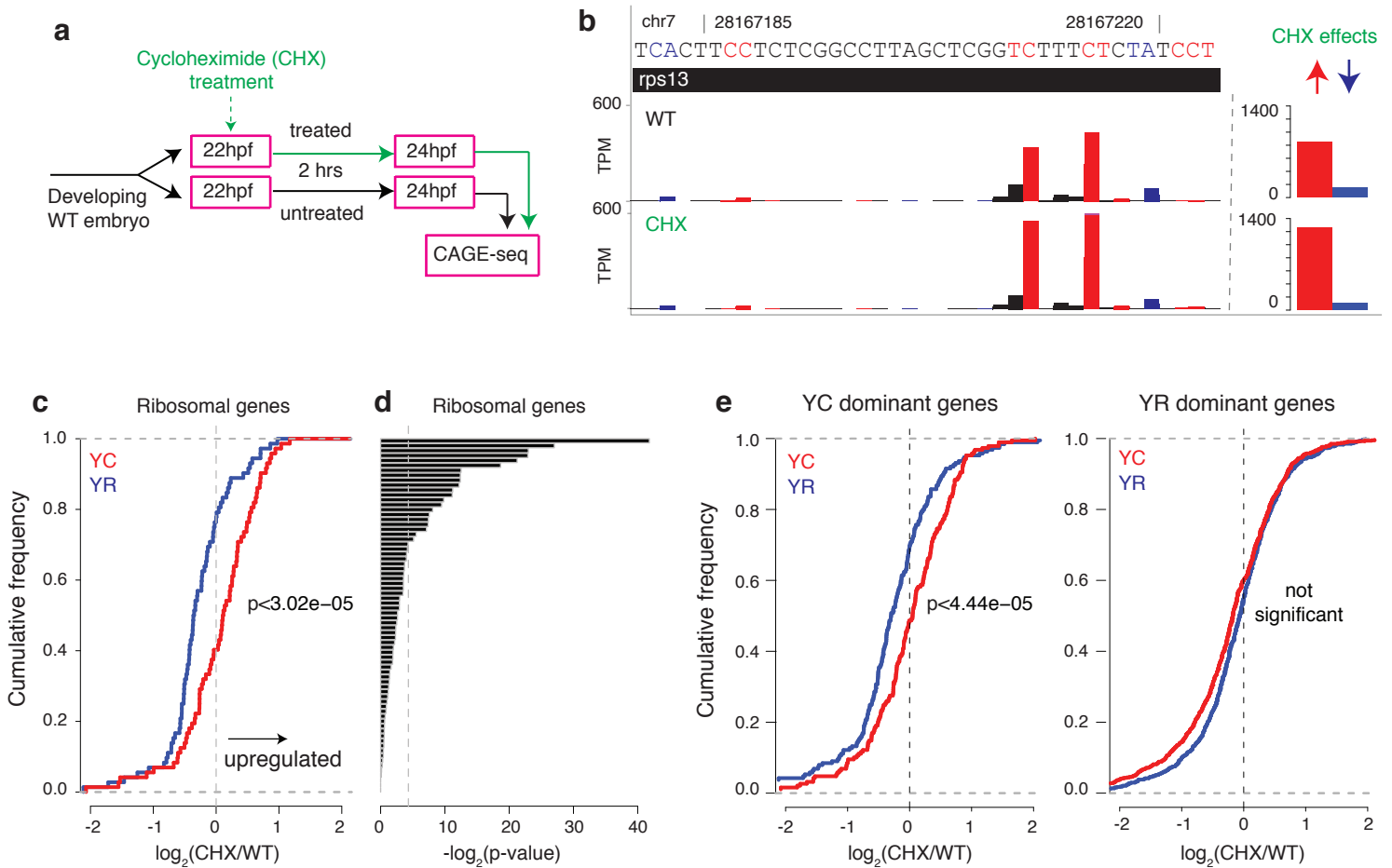


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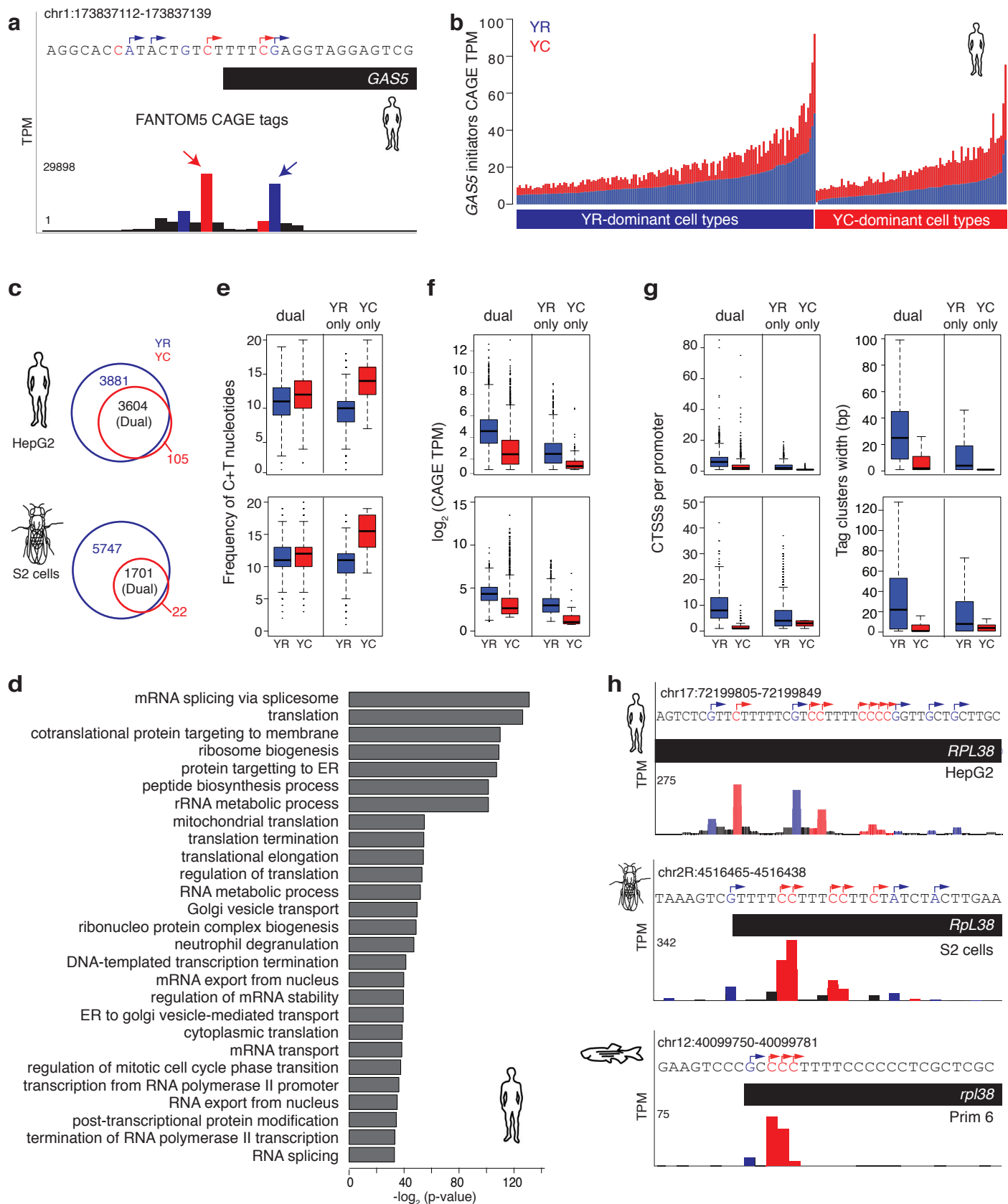


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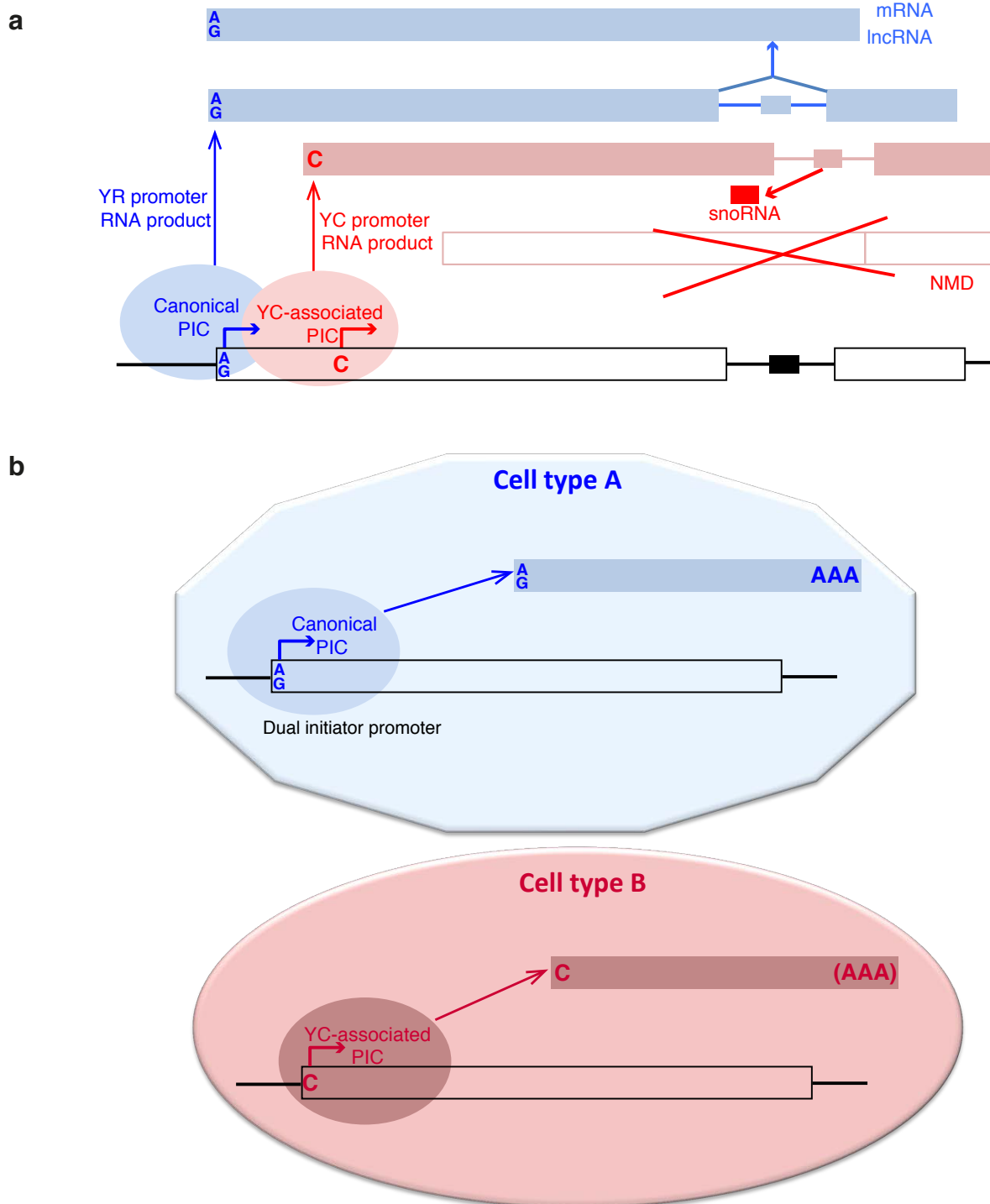
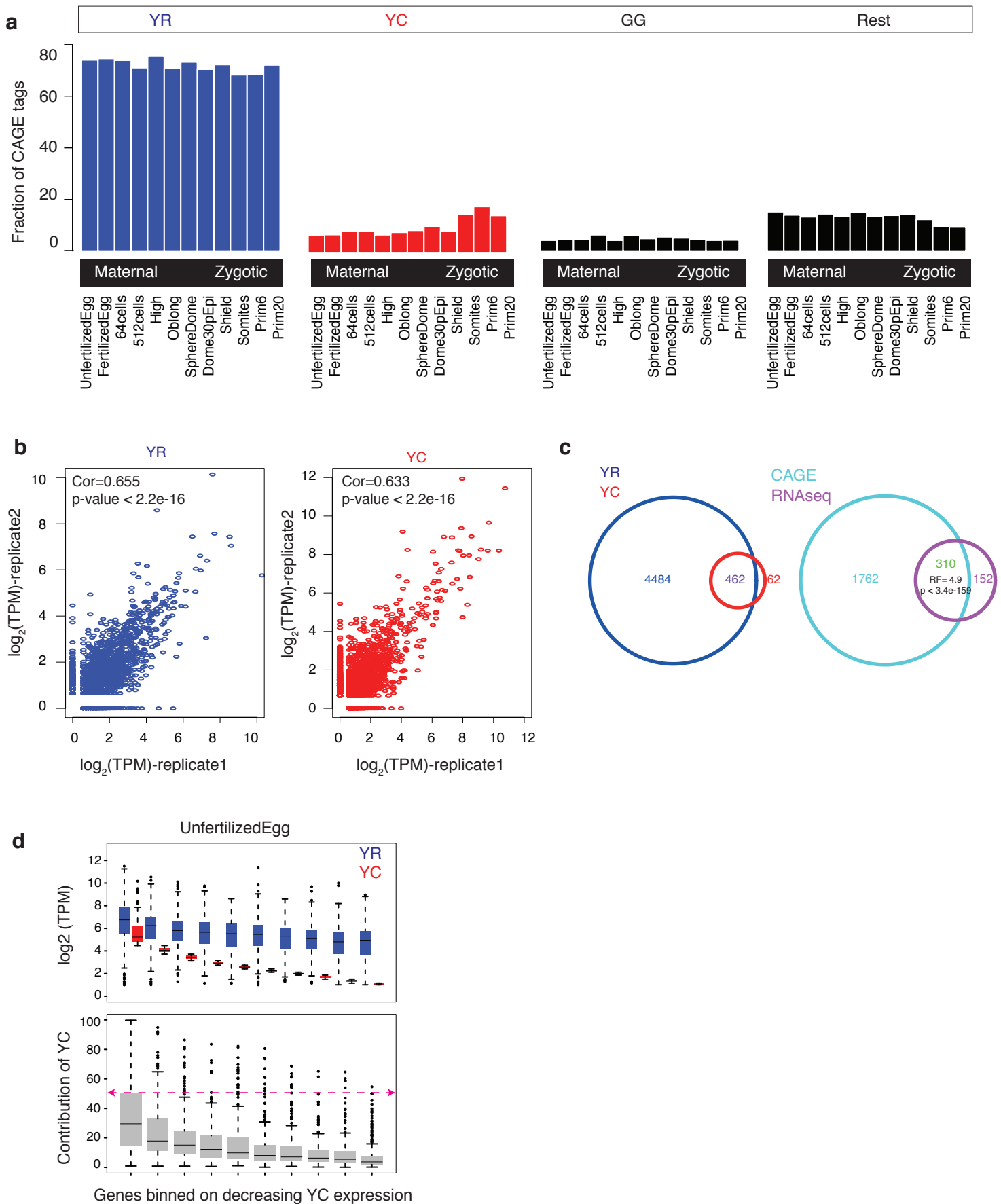


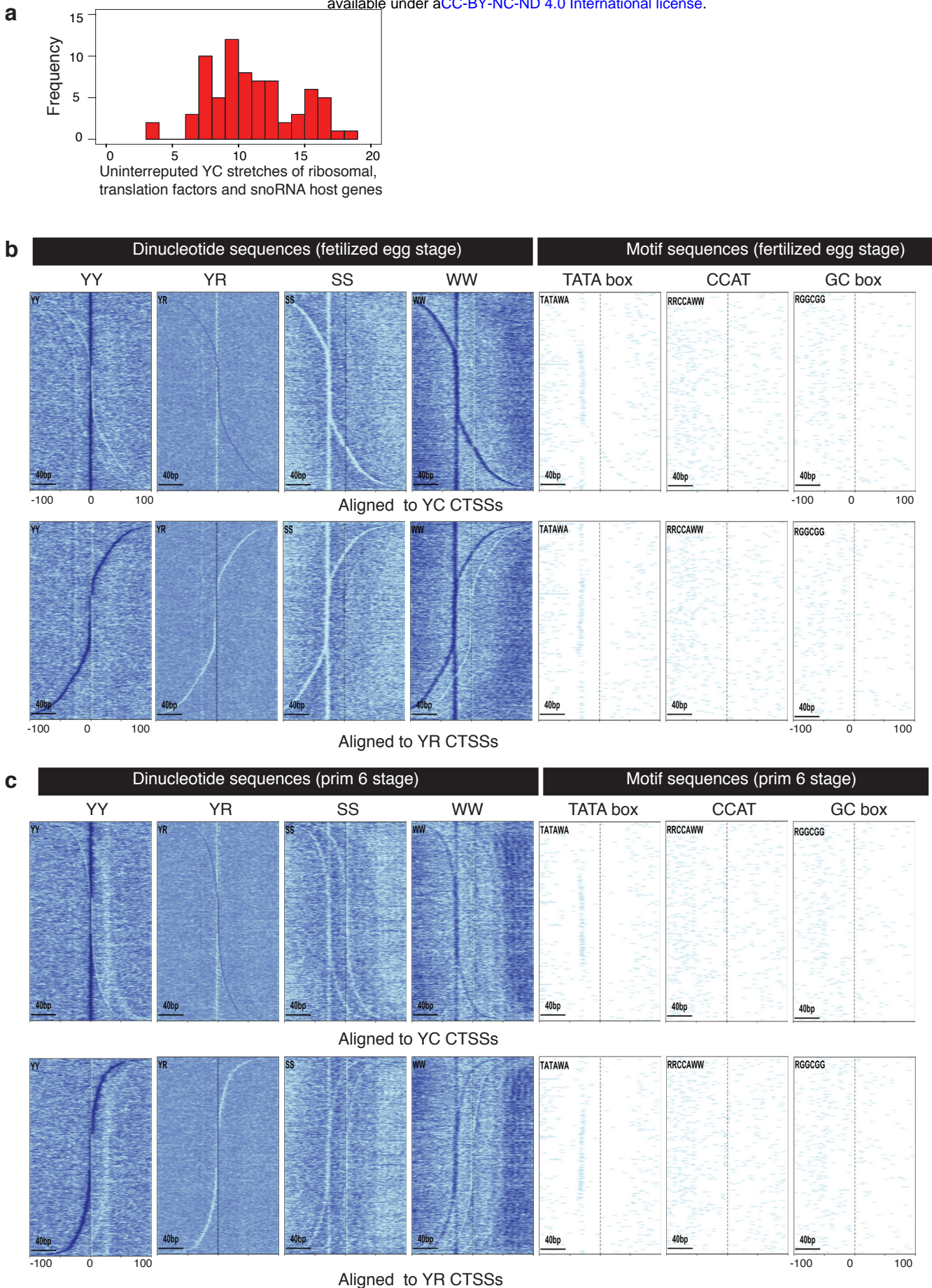
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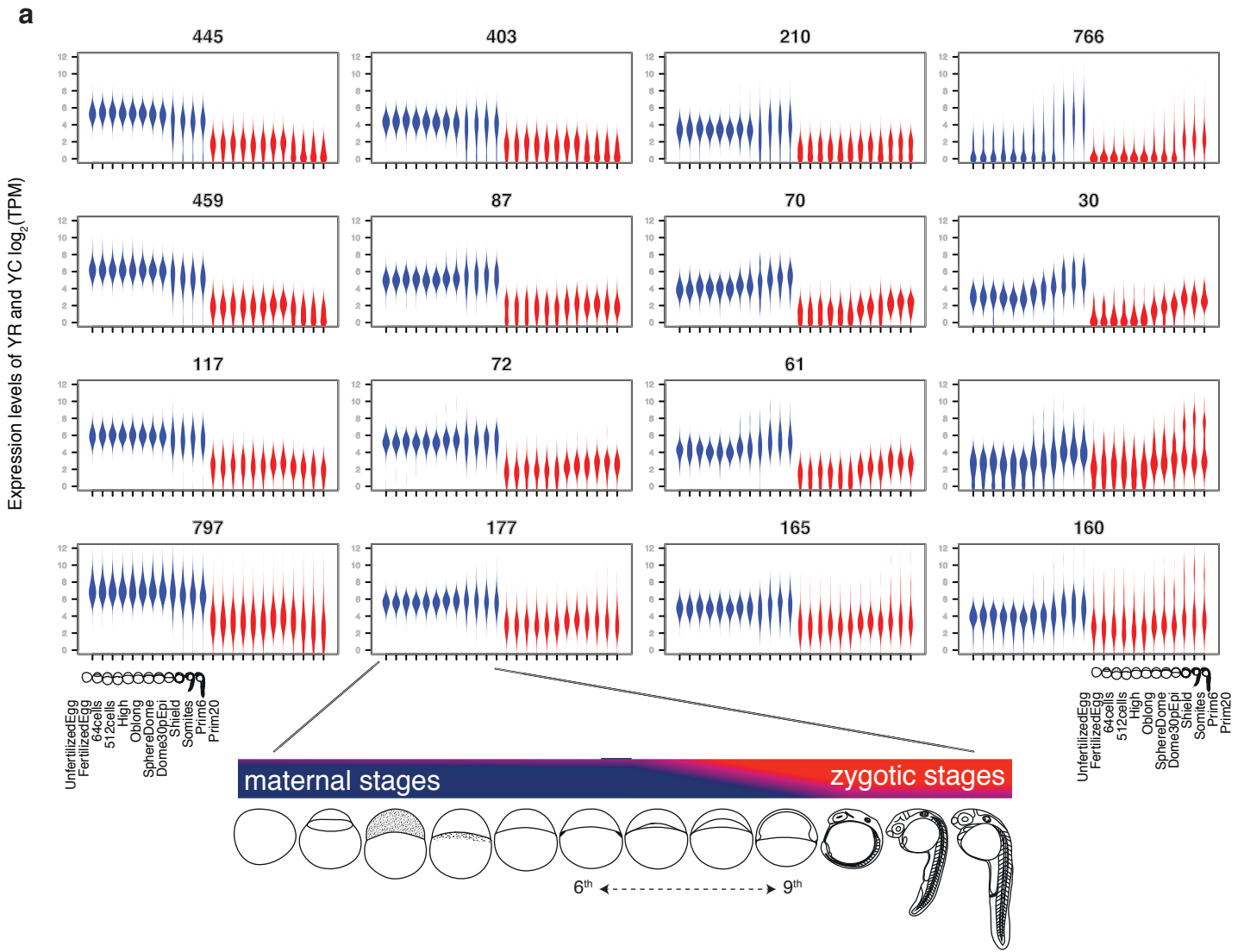
Supplementary Figure 1. Distribution and correlation of YC-initiations and YR-initiations in zebrafish developmental transcriptomes

(a) Classification of CTSSs based on dinucleotide frequencies around CTSSs Y-axis indicates fraction of CTSSs. (b) Correlation of canonical YR-initiation and non-canonical YC-initiation between two replicates of prim 5 stage. (c) Intersection of genes with YR-initiation and YC-initiation from 5' end capped RNA-seq (left). Intersection of dual-initiation genes from RNA-seq and CAGE-seq (right). (d) Contribution of YC-initiation with respect to YR-initiation expression levels in unfertilized egg stage. Genes are sorted according to YC expression levels and grouped into 10% bins.



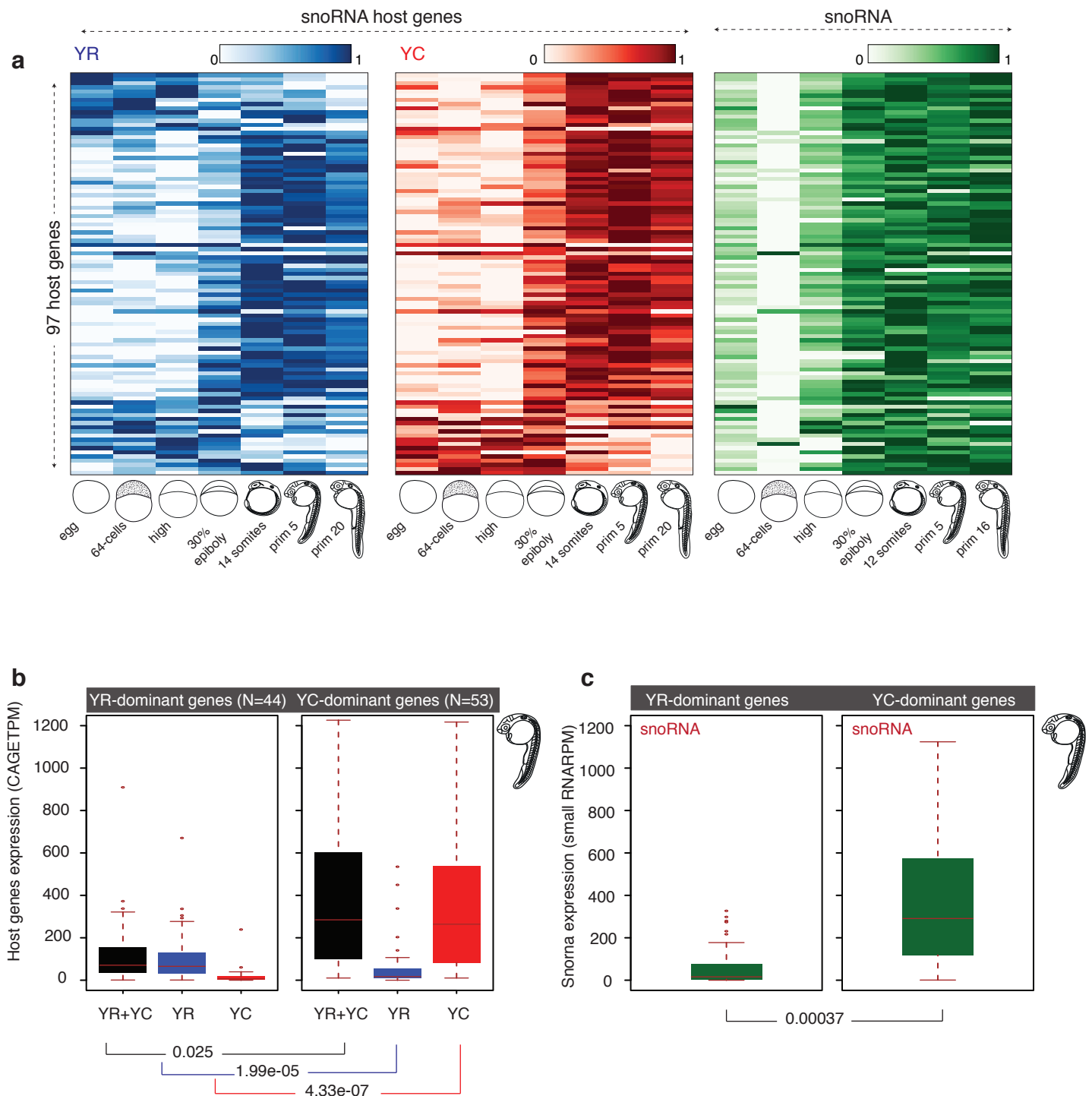
Supplementary Figure 2. Features of dual-initiation promoter genes

(a) Frequency of uninterrupted polypyrimidine stretches around YC-initiation sites of translational-associated genes (ribosomal proteins, translation initiation/elongation factors and snoRNA host genes). X axis indicate the maximum length of uninterrupted stretches of pyrimidine sequence. (b-c) Distribution of dinucleotide (YY/YR/SS/WW; Y=C/T; R=A/G; S=C/G; W=A/T) sequence content and (TATA, CCAT and GC box) motifs with respect to YR-initiation and YC-initiation of dual-initiation promoters in (b) fertilized egg and (c) prim 5 stage. Genes are aligned based on distance between YR and YC and aggregated to the +1 position of YR and YC dominant CTSS respectively.



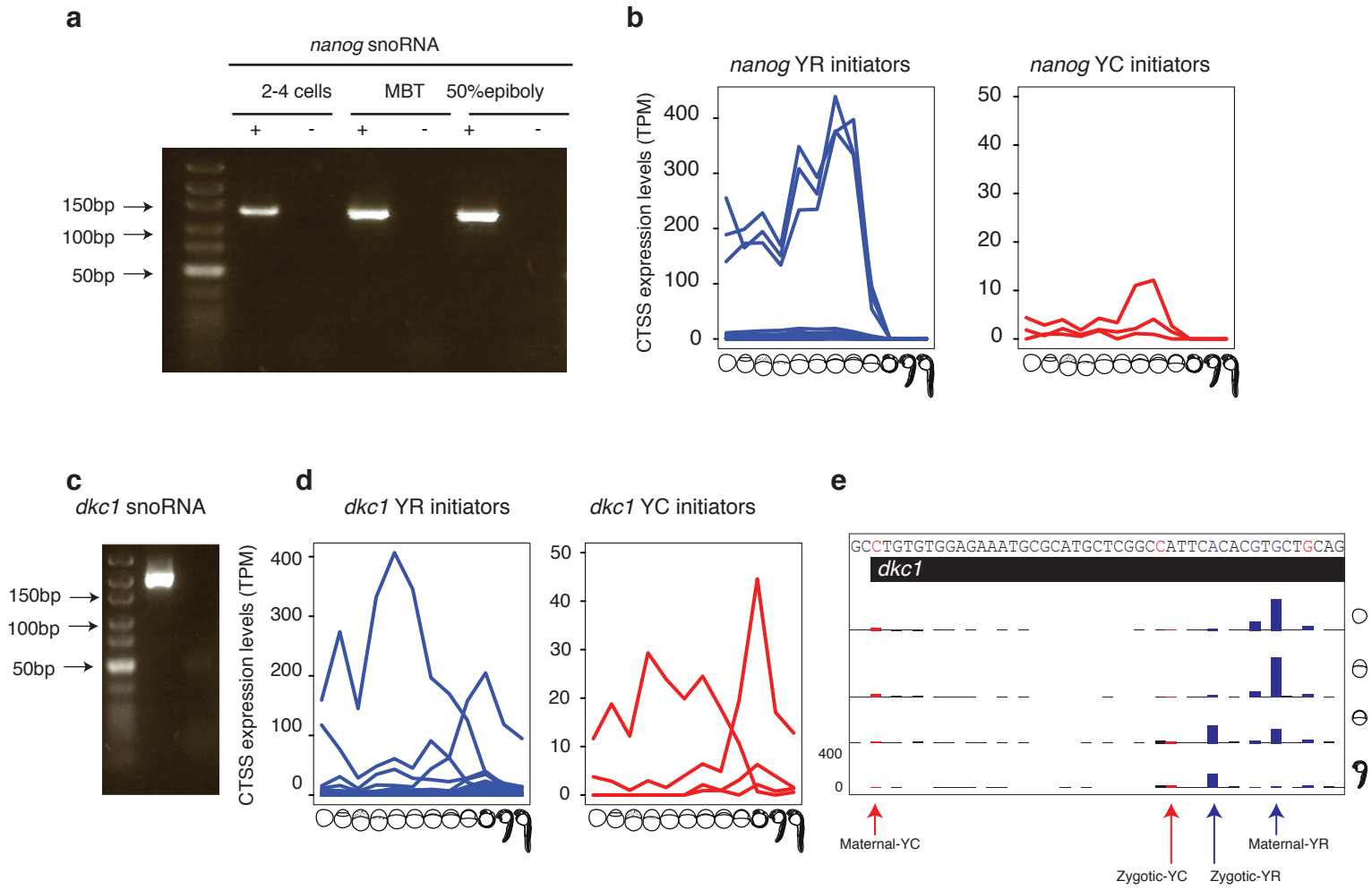
Supplementary Figure 3. Expression dynamics of YR-initiation and YC-initiation during zebrafish embryo development

(a) Self organizing map clusters of the TPM expression profiles of YR and YC components of genes during maternal and zygotic stages. Developmental stages along the x-axis are shown at the bottom. Y-axis indicates the expression levels. Blue and red colors indicate YR and YC components, respectively. Numbers above panels indicate the number of genes in each cluster.



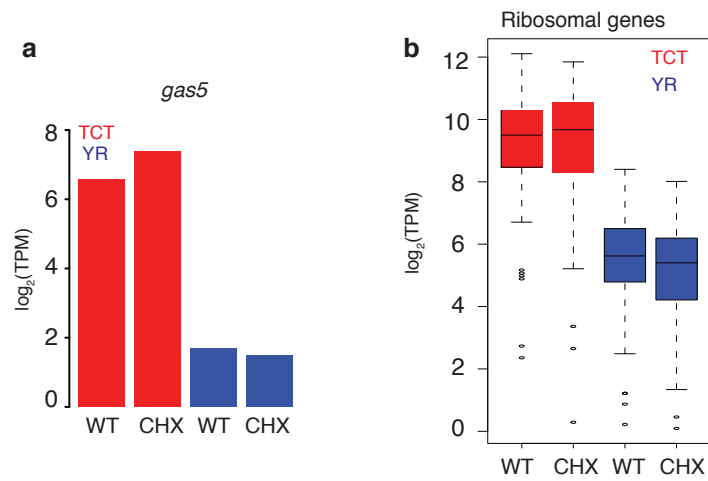
Supplementary Figure 4. Correlation of YR and YC components of snoRNA host genes with snoRNA expression levels

(a) Heat maps showing the expression levels of YR and YC components of snoRNA host genes along with snoRNA expression levels. Genes are sorted based spearman's correlation values between YR-initiation and YC-initiation. Expression levels are scaled between 0-1 for each row separately for YR, YC and snoRNA. (b) Expression levels of YR (blue) and YC (red) initiators, along with combined (black) expression levels of YR and YC. Host genes are divided into two groups (YR-dominant or YC-dominant) based on dominant expression of initiators. Y-axis indicate tags per million. (c) Expression levels of snoRNAs transcribed from YR and YC-dominant genes. Y-axis indicate reads per million.



Supplementary Figure 5. Quantitation and dynamics of snoRNAs and YC-initiations and YR-initiations of their host genes

(a) Validation of *nanog* snoRNA expression by RT-PCR in three developmental stages. Predicted size of PCR fragment is 131 bp (b) Expression level and developmental dynamics of individual YR-initiation and YC-initiation in the *nanog* promoter region. X-axis indicate the developmental stages. (c) Validation of *dkc1* snoRNA expression by RT-PCR in prim 5 stage. (d) Expression level and developmental dynamics of individual YR-initiation and YC-initiation in the *dkc1* promoter region. X-axis indicate the developmental stages. (e) A UCSC browser screen shot of *dkc1* gene with CTSSs. YR-initiation and YC-initiation are colored blue and red respectively.



Supplementary Figure 6. Effect of translation inhibition on YR-initiation and YC-initiation products of dual-initiation promoters

(a) Expression levels of YR-initiation and YC-initiation products of *gas5* after cycloheximide treatment. Blue and red color indicates YR-initiation and YC-initiation respectively. (b) Expression dynamics of YR-initiation and YC-initiation of all ribosomal protein genes.