

# Functional classification of long non-coding RNAs by *k*-mer content

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**The functions of most long non-coding RNAs (lncRNAs) are unknown. In contrast to proteins, lncRNAs with similar functions often lack linear sequence homology; thus, the identification of function in one lncRNA rarely informs the identification of function in others. We developed a sequence comparison method to deconstruct linear sequence relationships in lncRNAs and evaluate similarity based on the abundance of short motifs called *k*-mers. We found that lncRNAs of related function often had similar *k*-mer profiles despite lacking linear homology, and that *k*-mer profiles correlated with protein binding to lncRNAs and with their subcellular localization. Using a novel assay to quantify *Xist*-like regulatory potential, we directly demonstrated that evolutionarily unrelated lncRNAs can encode similar function through different spatial arrangements of related sequence motifs. *K*-mer-based classification is a powerful approach to detect recurrent relationships between sequence and function in lncRNAs.**

The human genome expresses thousands of lncRNAs, several of which regulate fundamental cellular processes. Still, the overwhelming majority of lncRNAs lack characterized function and it is likely that physiologically important lncRNAs remain to be identified. Moreover, the mechanisms through which most lncRNAs act are not clear, limiting our understanding of the biology that they govern in cells<sup>1–12</sup>.

A major roadblock to progress remains the inability to detect recurrent relationships between lncRNA sequence and function. An understanding of analogous relationships in proteins has enabled the classification of protein families, functional domains, and mechanisms that, in turn, have led to discoveries that have improved the diagnosis and treatment of disease<sup>13,14</sup>. However, with rare exceptions, the functions of lncRNAs are unrecognizable from computational analyses and must be determined empirically<sup>10–12,15–20</sup>. As a result, classification of function in one lncRNA often provides no information about function in others. For example, the *Xist* and *Kcnq1ot1* lncRNAs both repress gene expression in cis (meaning on the same chromosome from which they were transcribed), and both require the Polycomb Repressive Complex to do so<sup>7</sup>. Yet, despite similarities in mechanism, the two lncRNAs share almost no sequence similarity by standard metrics. Using two common sequence alignment algorithms, nhmmer<sup>21</sup> and Stretcher<sup>22</sup>, *Xist* and *Kcnq1ot1* appear just as similar to each other as they do to randomly generated sequences (Supplementary Fig. 1). Thus, comparing the sequence of *Kcnq1ot1* to a known cis-repressive lncRNA (*Xist*) provides no indication that *Kcnq1ot1* is also a cis-repressive lncRNA.

This problem extends to the thousands of lncRNAs that lack characterized functions.

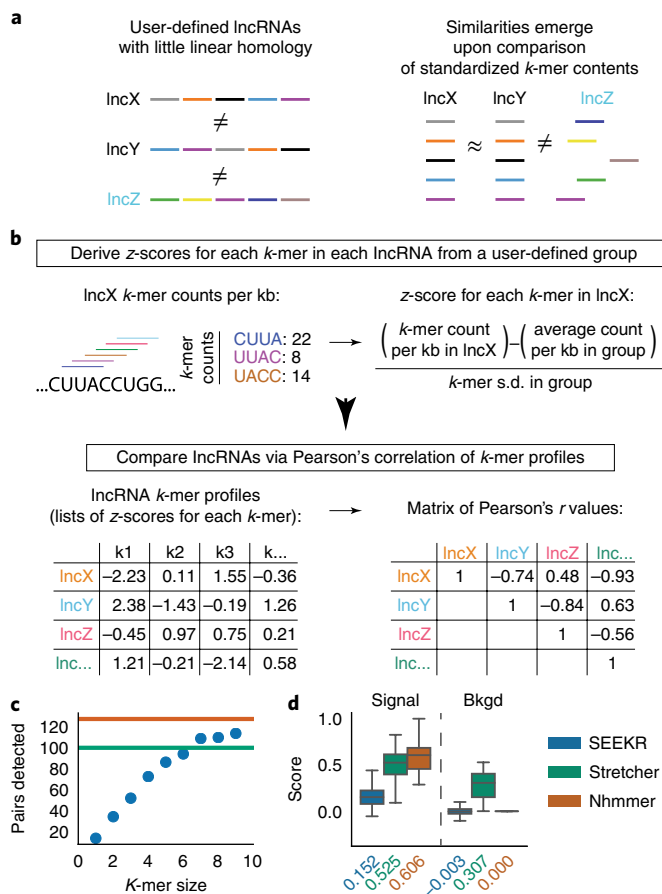
## Results

***K*-mer-based quantitation as a means to compare lncRNA sequence content.** We hypothesized that lncRNAs with shared functions should harbor sequence similarities that confer shared functions, even if conventional alignment algorithms do not detect the similarity. Our rationale follows. First, most lncRNAs probably have no catalytic activity, suggesting that the proteins they bind in cells define their function. Second, proteins often bind RNA through short motifs, or *k*-mers, that are between three and eight bases in length, where '*k*' specifies the length of the motif<sup>23,24</sup>. Third, the mere presence of a set of protein binding-motifs may be more important than their relative positioning within an lncRNA, meaning that functionally related lncRNAs could harbor related motif contents and still lack linear sequence similarity.

To test our hypothesis, we developed a method of sequence comparison, called SEEKR (sequence evaluation from *k*-mer representation). In SEEKR, all *k*-mers of a specified length *k* (that is, *k*=4, 5, or 6, etc.) are counted in one-nucleotide increments across each lncRNA in a user-defined group, such as the GENCODE annotation set<sup>12</sup>. *K*-mer counts for each lncRNA are then normalized by lncRNA length and standardized across the group to derive a matrix of *k*-mer profiles, which consist of *z*-scores for each *k*-mer in each lncRNA. The relative similarity of *k*-mer profiles between any pair of lncRNAs can then be determined via Pearson's correlation (see Fig. 1a,b and Methods).

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**Fig. 1 | Overview and initial test of *k*-mer-based sequence comparison.**

**a**, lncRNAs of related function (names in black) may harbor similar sequence similarity in the form of motif content (colored bars) even if they lack linear homology. **b**, In SEEKR, the abundances of all *k*-mers of length *k* are counted by tiling across each lncRNA in a user-defined group in one-nucleotide increments. *K*-mer counts are normalized for lncRNA length, and standardized across the group to derive z-scores. Similarity is evaluated by comparing lncRNA *k*-mer profiles (lists of z-scores for each *k*-mer in the lncRNAs) with Pearson's correlation. **c**, Number of homologous pairs detected by SEEKR versus *k*-mer length in a test set of conserved lncRNAs. Green and orange lines mark the homolog number detected by Stretcher and nhmmer, respectively. **d**, Signal-to-background ratios for homolog detection via the three methods. Tukey boxplots show the lower, median, and upper quartile of values, and  $\pm 1.5 \times$  interquartile range ( $n = 161$  *r* values for signal,  $n = 12,880$  *r* values for background); outliers are not shown. Bkgd, background.

SEEKR offers advantages relative to existing alignment algorithms. Foremost, SEEKR does not consider positional information in similarity calculations, allowing it to quantify non-linear sequence relationships. For reasons described above, this functionality might suit lncRNAs better than traditional alignment algorithms developed to detect linear sequence homology between evolutionarily related entities<sup>21,22,25,26</sup>. Second, whereas traditional alignment algorithms can only quantify similarity, SEEKR can quantify similarities and differences using Pearson's correlation. Third, SEEKR can quantify relationships in groups of lncRNAs despite differences in overall length, whereas length differences can confound traditional alignment algorithms. For example, conventional alignment of a 20-kb and 4-kb RNA is barely informative (80% of the 20-kb RNA would not align), but their *k*-mer contents can be compared

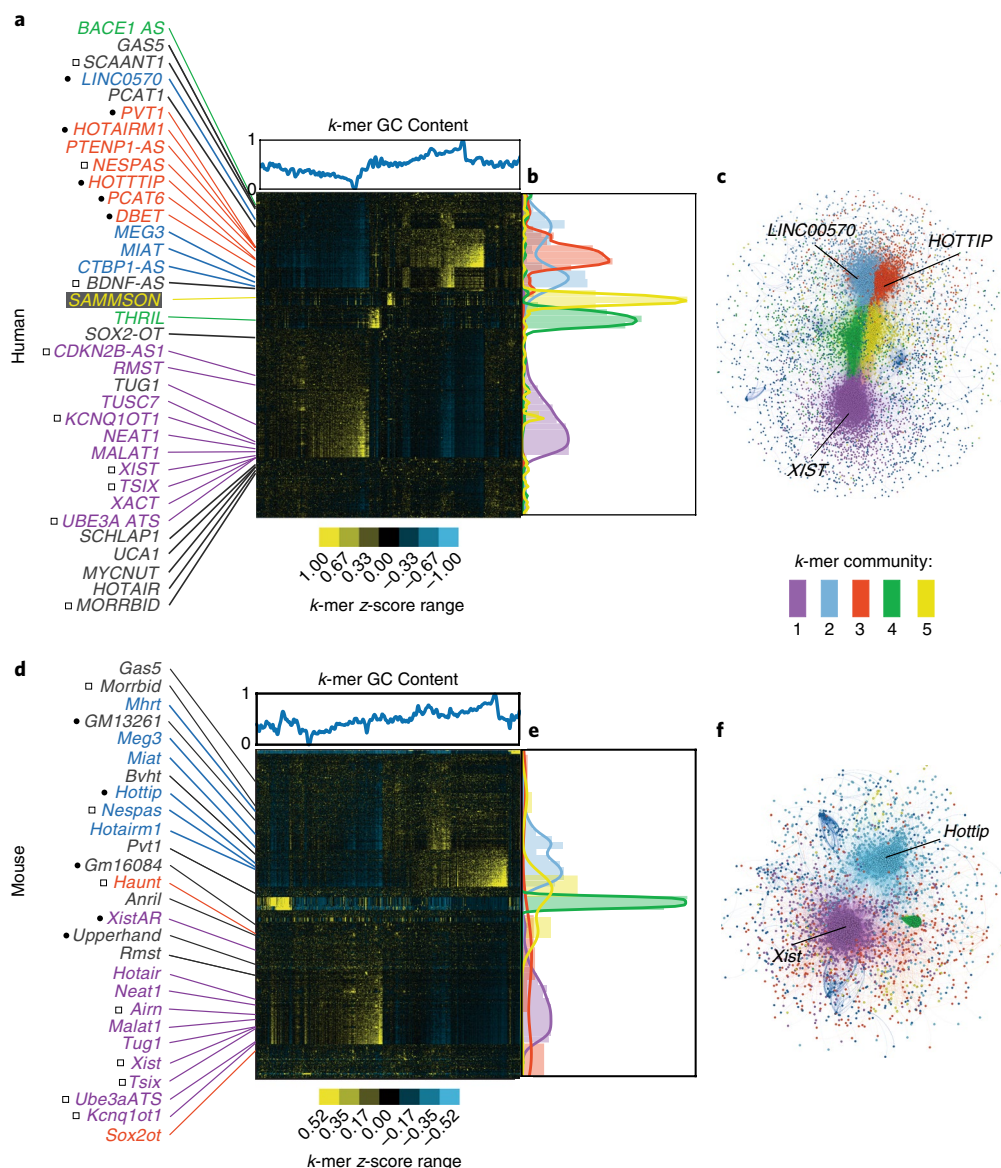
via SEEKR. Lastly, SEEKR is algorithmically efficient; all pair-wise comparisons between human GENCODE lncRNAs can be computed in under 1 min.

Initially, we assessed whether SEEKR could detect previously identified sequence similarities in lncRNAs. We compared *k*-mer profiles via SEEKR for all pair-wise combinations in a set of 161 lncRNAs recently described to be conserved between human and mouse<sup>27</sup>. We also aligned the lncRNAs to each other using two existing alignment algorithms, the hidden Markov model based nhmmer<sup>21</sup>, and Stretcher, an implementation of the global alignment algorithm Needleman–Wunsch<sup>22</sup>. In this test, SEEKR detected known lncRNA homologs nearly as well as, or better than, both algorithms (Fig. 1c). We defined signal-to-background in this assay as the ratio between the median similarity of homologous and non-homologous lncRNAs. By this metric, nhmmer detected homologs the most clearly, as expected (signal-to-background ratio of 0.606: 0.000), followed by SEEKR (signal-to-background of 0.152: -0.003 at *k*-mer length *k* = 6) and Stretcher (signal-to-background of 0.525: 0.307; Fig. 1d). We conclude that *k*-mer-based classification can detect sequence similarity between evolutionarily related lncRNAs.

We next examined whether SEEKR could detect novel forms of similarity between lncRNAs with no known sequence homology. We created *k*-mer profiles for all lncRNAs in the human and mouse GENCODE databases<sup>12</sup>, as well as for select lncRNAs that were not included in GENCODE. Next, we compared *k*-mer profiles between all lncRNAs in each organism using Pearson's correlation and hierarchically clustered the resulting matrices to examine the patterns that emerged. Consistent with our hypothesis, clustering lncRNAs by SEEKR grouped many by known function in human and mouse (Fig. 2). Several known cis-repressive lncRNAs, including *XIST*, *TSIX*, *KCNQ1OT1*, *UBE3A-ATS*, *ANRIL/CDKN2B-AS1*, and *Airn*, clustered together due to high abundance of AU-rich *k*-mers, whereas several cis-activating lncRNAs, including *PCAT6*, *HOTTIP*, *LINC00570*, *DBE-T*, and *HOTAIRM1*, clustered separately due to high abundance of GC-rich *k*-mers (Fig. 2a,d). These patterns were robust over differing *k*-mer lengths (Supplementary Fig. 2). To determine whether this level of clustering was significant, we curated lists of human and mouse cis-activating and cis-repressive lncRNAs from the literature (Supplementary Table 1), and compared average pair-wise *k*-mer similarities between lncRNAs in each list to pair-wise similarities of 10,000 size-matched lists of randomly selected lncRNAs from the respective organism. Human and mouse cis-repressors, and human cis-activators (but not mouse cis-activators), were significantly more similar to each other than expected by random chance (Supplementary Table 2). Concordantly, SEEKR detected significant similarity between the cis-repressive *Kcnq1ot1* and *Xist* lncRNAs where none was found by conventional alignment algorithms (Supplementary Fig. 1). We conclude that lncRNAs of related function can have related *k*-mer profiles even if they lack linear sequence similarity.

Unexpected relationships also emerged in the hierarchical clusters of Fig. 2. Most notably, the lncRNAs *NEAT1* and *MALAT1* showed greater than average similarity to *XIST* in both human and mouse. Among all human lncRNA pair-wise comparisons, their Pearson's *r* values fell in the 99.99th and 99.60th percentile, respectively. Likewise, in mouse the similarities were in the 97.15th and 95.32nd percentiles. The meaning of the similarity between the three lncRNAs is unclear, but we note that all three lncRNAs seed the formation of subnuclear compartments and engage with actively transcribed regions of the genome<sup>28–33</sup>. We speculate that their *k*-mer similarity is related to these shared actions.

**lncRNAs can be partitioned into communities of related *k*-mer content.** We next used a network-based approach to partition lncRNAs into communities of related *k*-mer profiles, reasoning that such communities would provide a framework to understand the



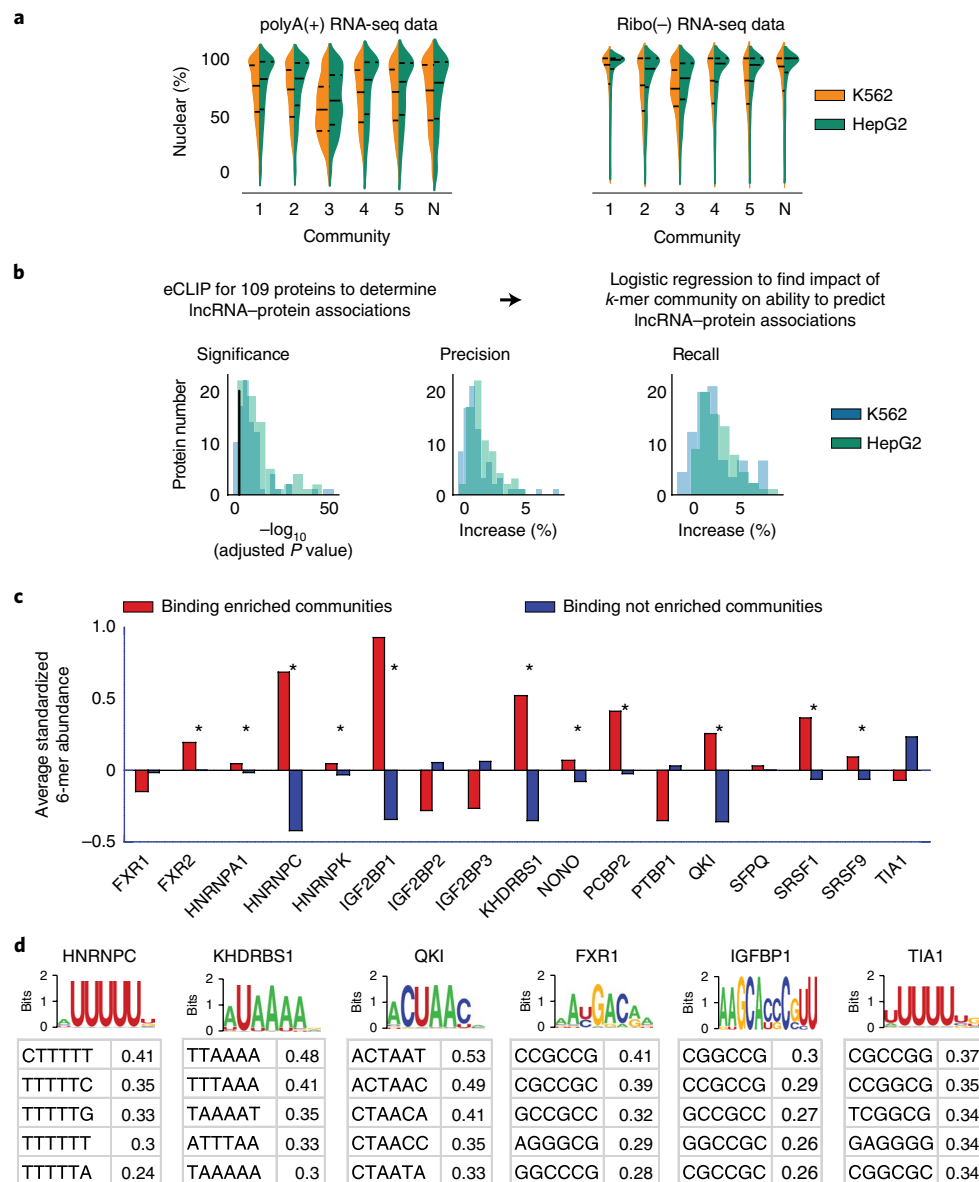
**Fig. 2 | LncRNAs of related function often have related *k*-mer contents.** **a**, Hierarchical cluster of all human GENCODE lncRNAs at *k*-mer length 6, with lncRNAs and *k*-mers on the x and y axes, respectively. *K*-mer z-scores (relative *k*-mer abundance) range from blue (lowest) to yellow (highest). GC content of *k*-mers is shown above the x axis. Locations of select lncRNAs are marked. Left of lncRNA names, black circles indicate cis-activators and squares indicate cis-repressors. **b**, Locations of lncRNAs assigned to communities 1 through 5 via the Louvain/network-based approach. **c**, Network graph of Louvain-assigned lncRNA communities. lncRNA names in **a** are colored by their Louvain community assignment; lncRNAs in gray were assigned to the null. **c,d,e**, Same as **a**, **b**, and **c** but for mouse GENCODE lncRNAs.

predictive value of lncRNA *k*-mer content. We created networks of relationships between all human and mouse lncRNAs in which weighted edges connected lncRNAs in an organism if the Pearson's correlation between their standardized *k*-mer profiles met a threshold for similarity (see Methods). We then used the Louvain method to assign lncRNAs within the largest connected component of the network representations to communities of related *k*-mer profiles<sup>34</sup>. Approximately half of all GENCODE lncRNAs grouped into five major communities in both human and mouse. lncRNAs not assigned to the five most populated communities were assigned to a 'null' community. Our network-based approach and hierarchical clustering grouped lncRNAs in similar ways ( $P < 1 \times 10^{-324}$ , chi-squared; Supplementary Tables 3 and 4), signaling community robustness. lncRNA community assignments and associated summary statistics are provided in Supplementary Tables 5–12

and Supplementary Fig. 3. Differences in human and mouse community structures may be due in part to differences in completeness of lncRNA annotation. In the versions of GENCODE used for this work, there were about twice as many lncRNAs annotated in human (v22,  $n = 15,953$ ) as there were annotated in mouse (vM5,  $n = 8,245$ ; ref. <sup>12</sup>).

#### K-mer content correlates with localization and protein binding.

We next examined whether lncRNAs with related *k*-mer profiles shared biological properties. For this analysis, we focused on human lncRNAs, where data from the ENCODE project allowed us to examine lncRNA subcellular localization and protein associations, transcriptome wide. To determine whether *k*-mer content provides information about lncRNA localization, we examined ENCODE subcellular fractionation RNA-sequencing (RNA-seq) experiments



**Fig. 3 | lncRNA localization and protein-binding correlate with *k*-mer content.** **a**, Violin plots of lncRNA localization by *k*-mer community in K562 (orange) and HepG2 (green) cells, as determined from RNA-seq of polyA-selected and ribosome-depleted RNA. N, the null community. Lines show the lower, median, and upper quartile of values (see Supplementary Figs. 13–16 for samples sizes). **b**, From left to right:  $\log_{10}$  significance of increase in likelihood, percentage increase in precision, and percentage increase in recall obtained when lncRNA community information is included in a logistic regression to predict protein association. Black line in the left-hand plot corresponds to a  $\log_{10}$ (adjusted *P* value) of 0.05 ( $n = 3,747$  lncRNAs for HepG2,  $n = 3,278$  lncRNAs for K562). **c**, Eleven of the 17 proteins with experimentally determined PWMs from ref. <sup>23</sup> show significantly increased abundance of motif-matching *k*-mers ( $n = 4,096$ ) in lncRNA communities that are enriched for binding to the protein in question (\* $P < 0.01$ , permutation test). **d**, The most enriched *k*-mers in 300-nucleotide windows surrounding motif matches in CLIP peaks do not always match the motif. PWMs from ref. <sup>23</sup> are shown above average *z*-scores for the top five most enriched *k*-mers in true-positive relative to false-positive binding regions for the protein in question. PWMs and top *k*-mers are shown for all 17 proteins in Supplementary Fig. 5.

performed in HepG2 and K562 cells<sup>35</sup>. For each lncRNA expressed in each cell type, we computed its nuclear ratio and determined whether the distributions of nuclear ratios differed between communities. The majority of communities showed slight but significant differences in their distribution of nuclear ratios, with the largest differences found between communities 1 and 3 (Fig. 3a and Supplementary Tables 13–16). Concordantly, lncRNAs that associate with polysomes in K562 cells<sup>36</sup> were also non-uniformly distributed between communities ( $P = 3.5 \times 10^{-5}$ , chi-squared), and were the most over- and under-represented in the most cytoplasmic and nuclear lncRNA communities, respectively (communities 3 and 1 being the most

cytoplasmic and nuclear, respectively; Supplementary Table 17). Lastly, we used ENCODE data to identify the most cytoplasmic and nuclear lncRNAs in HepG2 and K562 cells and determine which *k*-mers were asymmetrically distributed between lncRNAs in the two compartments. We found that 360 and 27 *k*-mers were significantly enriched in cytoplasmic and nuclear lncRNAs, respectively ( $P$  adjusted  $< 0.05$ ; Kolmogorov–Smirnov test; Supplementary Table 18). Consistent with our RNA-seq and polysome analyses, 58% and 93% of the cytoplasmic- and nuclear-biased *k*-mers were the most enriched in the most cytoplasmic and nuclear lncRNA communities, respectively (communities 3 and 1; see Supplementary



Table 18, last column). We conclude that *k*-mer content provides information about the subcellular localization of an lncRNA.

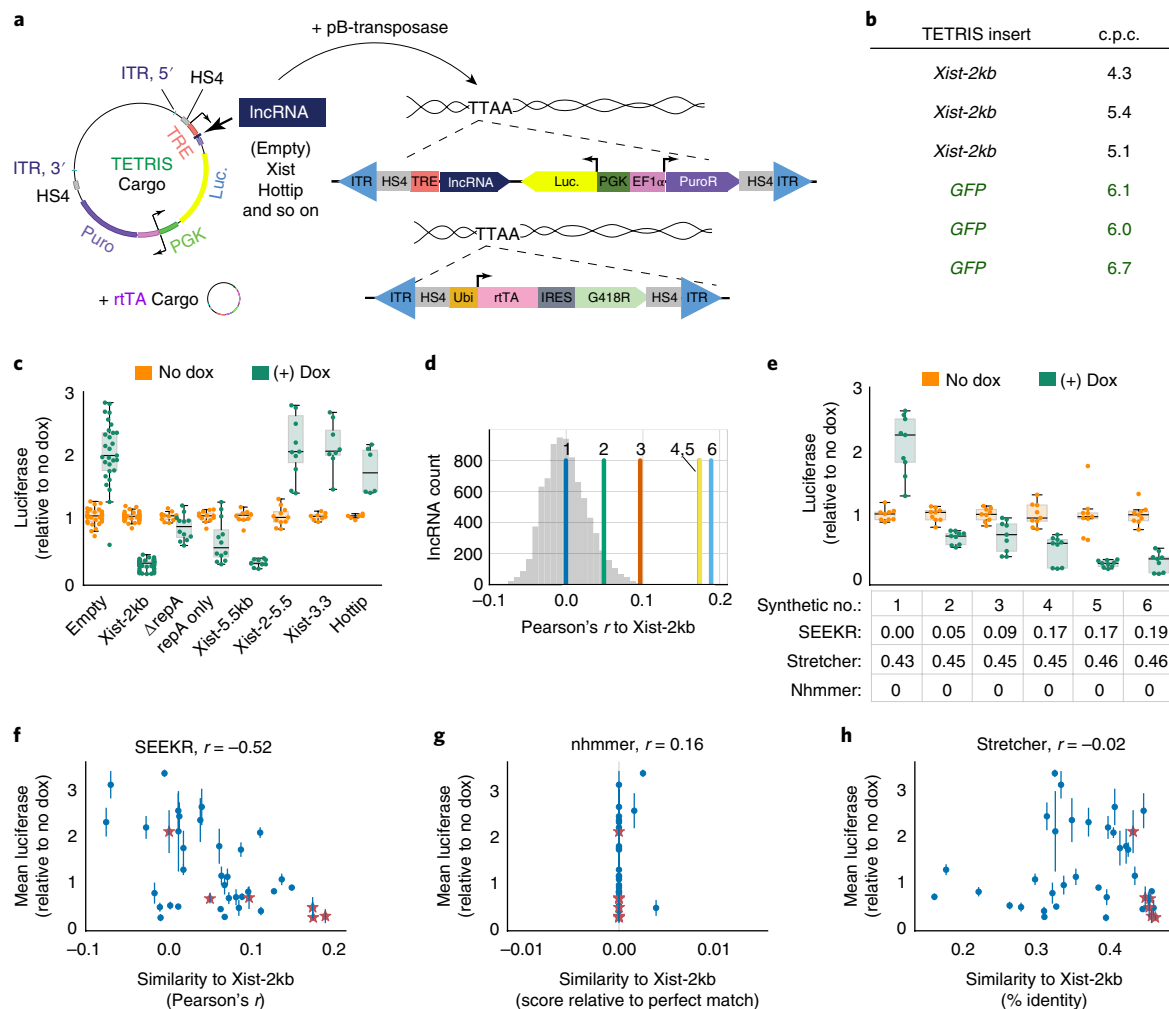
To determine whether *k*-mer content provides information about protein binding in lncRNAs, we examined ENCODE data for 156 enhanced cross-linking immunoprecipitation (eCLIP) experiments performed for 109 proteins in HepG2 and K562 cells<sup>37</sup>. We created binary vectors for each experiment that recorded whether the lncRNAs bound or did not bind a given protein, then built separate logistic regression models for each protein to determine whether *k*-mer community assignments could improve prediction of lncRNA–protein associations over a null model that only included lncRNA length and expression as covariates. LncRNA community assignments significantly increased the log-likelihood of detecting lncRNA–protein associations for the majority of proteins examined (*P* adjusted <0.05; 146 of 156, ~94%; Fig. 3b and Supplementary Table 19). Increases in precision and recall in community-informed models were generally modest but significant (Fig. 3b and Supplementary Table 20). In total, ~17% (25 of 146) of our models had an increase in precision and/or recall of 5% or more. Notably, in all cases in which recall increased, precision also increased, indicating that *k*-mer community information increased the ability to predict true lncRNA–protein associations and simultaneously increased the fidelity of those predictions. When we used individual 6-mers instead of lncRNA communities as predictive features, results were no better than the null model that used only lncRNA length and expression as predictive features. Models with more features than samples are prone to learning noise in their training set, and often lose predictive power due to overfitting<sup>38</sup>. Using individual 6-mers brought the number of features being evaluated to 4,099, more than the number of lncRNAs expressed in HepG2 and K562 cells (3,745). We conclude that *k*-mer content provides information about the protein-binding potential of an lncRNA, but that no single *k*-mer provides an overwhelming portion of that information, and that *k*-mer communities provide a way to collapse high-dimensional *k*-mer matrices down to representative variables for predictive purposes.

Protein binding to RNA is difficult to assess from motif content alone due to the degeneracy of most motifs and the challenge of predicting the effects of RNA structure<sup>24,39–41</sup>. Supporting this notion, we found that the abundance of motif-matching *k*-mers was consistently, but not always, higher in the communities enriched for binding of specific proteins than in the cognate communities not enriched for binding, indicating that factors in addition to motif abundance control protein–lncRNA associations (Fig. 3c). We therefore sought to determine whether *k*-mer content could distinguish between motif matches in lncRNAs that coincide with protein binding events and those that do not. We searched the lncRNAs expressed in HepG2 and K562 cells for matches to binding motifs of the 17 proteins in Fig. 3c, whose position weight matrices (PWMs) were determined from biochemical assays in ref. <sup>23</sup>. We annotated motif matches that fell inside and outside of eCLIP peaks as true and false-positive matches, respectively. As expected, the majority of motif matches fell outside of eCLIP peaks (that is, they were false-positive matches; Supplementary Table 21). We then used SEEKR to compare regional *k*-mer content in 300-nucleotide windows surrounding true and false-positive motif matches. Remarkably, for 13 of 17 proteins examined, *k*-mer profiles of true-positive binding regions were more similar to each other than *k*-mer profiles of randomly selected, size-matched sets of false-positive regions (*P* value <0.005; Supplementary Fig. 4). These data support the notion that binding modules for the same protein in different RNAs often have sequence similarity that extends beyond the protein-binding motif, and that this similarity can be quantified, in part, by local *k*-mer content.

Moreover, SEEKR provides a simple way to visualize the density of specific *k*-mers within eCLIP-enriched regions. We compared the most overrepresented *k*-mers in true-positive binding regions to protein-binding motifs measured *in vitro*<sup>23</sup>, and found that their relationships differed substantially from protein to protein (Fig. 3d and Supplementary Fig. 5). For certain proteins, such as HNRNPC, KHDRBS1, and QKI, the most enriched *k*-mers in true-positive regions matched the PWM for the protein that was determined *in vitro*<sup>23</sup>. We interpret this observation to mean that, for these proteins, motif density plays a dominant role in determining RNA binding *in vivo*, because our *k*-mer data show that motif-matching *k*-mers are more abundant in true-positive regions than they are in false-positive regions. For other proteins, such as FXR1, IGFBP1, and TIA1, the most enriched *k*-mers in true-positive regions did not match the PWM determined *in vitro*<sup>23</sup>. For these proteins, sequence beyond the binding motif may play a dominant role in dictating association with RNA, possibly due to effects from RNA structure. When PWMs were extracted from eCLIP peaks, similar relationships between *k*-mers and *in vitro*-defined motifs were observed (Supplementary Fig. 5). These results show how SEEKR can be used to augment traditional motif-based analyses and provide insights into mechanisms of RNA–protein interaction. SEEKR provides a way to quantify sequence similarities between any number of protein-binding regions, which, in turn, can provide predictive power and identify shared characteristics that are not apparent from PWM-based motif analyses.

**Similarities in lncRNA communities between organisms.** Given (1) that *k*-mer content provides some indication of protein-binding potential in an lncRNA, (2) that sequence specificities of many RNA binding proteins are conserved<sup>23,24</sup>, and (3) that protein binding probably dictates lncRNA function, we hypothesized that *k*-mer contents between communities of functionally related lncRNAs could be conserved even if the lncRNAs themselves lack known evolutionary relationships. In support of this idea, we identified extensive similarity between certain human and mouse lncRNA communities via SEEKR (see Methods and Supplementary Fig. 6). Most notably, lncRNAs in human community 1 (the ‘*XIST*’ community) had *k*-mer profiles that were, as a group, nearly indistinguishable from lncRNAs in mouse community 1 (the ‘*Xist*’ community) and were also similar to lncRNAs in mouse community 4 (*P* <0.0001 for both comparisons). Human community 2 and community 3 (the ‘*HOTTIP*’ community) were both similar to mouse community 2 (the ‘*Hottip*’ community; *P* <0.0001). No other major similarities between mouse and human were apparent. Extending this analysis across greater evolutionary distance, we found *HOTTIP*-like lncRNA communities in ten of ten vertebrates examined as well as in the sea urchin *Strongylocentrotus purpuratus*, and *XIST*-like lncRNA communities in seven of ten vertebrates examined (Supplementary Figs. 7–9; ref. <sup>10</sup>). These analyses demonstrate that, at the level of *k*-mers, subsets of human lncRNAs are more similar to lncRNAs in other genomes than they are similar to lncRNAs in their own genome, supporting the idea that groups of lncRNAs have similar function in different organisms despite lacking obvious linear sequence similarity.

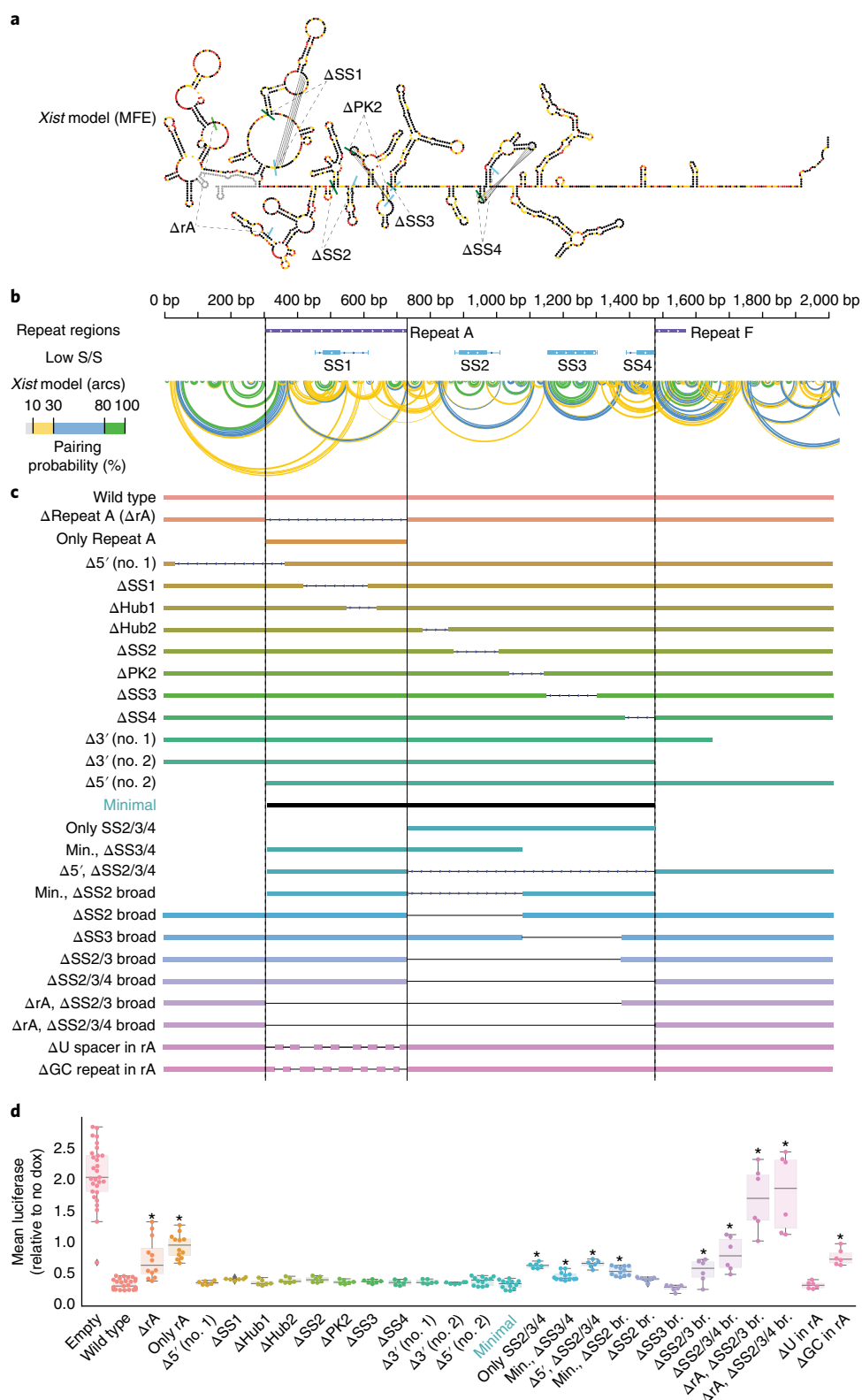
**SEEKR can predict *Xist*-like regulatory potential in lncRNAs.** We next directly tested whether *k*-mer profiles could be used to predict lncRNA regulatory potential. We focused on the ability of certain lncRNAs to repress transcription *in cis*. Cis-repression was one of the earliest characterized functions of lncRNAs, and is essential for normal human health and development. In the most striking example, the *XIST* lncRNA silences nearly all genes across an entire chromosome during X-chromosome inactivation<sup>7</sup>. Cis-repression is also one of most straightforward lncRNA functions to study because, by definition, cis-acting lncRNAs act near their site of transcription.



**Fig. 4 | K-mer content correlates with lncRNA repressive activity.** **a**, Overview of vectors and concept of the TETRIS assay. Luc., luciferase. **b**, Number of TETRIS-IncRNA-cargo insertions per cell (c.p.c.) after 10-d drug selection for two separate cargos, *Xist*-2kb and GFP. Each row represents copy number data from independent replicates. **c**, Luciferase values for different TETRIS-IncRNA-cargos relative to No Dox. Tukey boxplots as in Fig. 1d. Data are from at least six independent luciferase assays from at least two biological replicate derivations of TETRIS cell lines. Exact numbers of assays and replicates performed for each TETRIS-IncRNA-cargo are found in Supplementary Table 22. **d**, Pearson's  $r$  similarity of  $k$ -mer profiles for the six synthetic lncRNAs relative to the first 2 kb of *Xist*. Histogram of similarity of *Xist*-2kb to all other GENCODE M5 lncRNAs is shown in gray. **e**, Effect of synthetic lncRNA expression on luciferase activity. Tukey boxplots as in Fig. 1d. SEEKR, Stretcher, and nhmmer similarities for each synthetic lncRNA relative to the first 2 kb of *Xist* are shown below the graph. **f,g,h**, Pearson's correlation between repressive activity and similarities to *Xist*-2kb as defined by SEEKR, nhmmer, and Stretcher for 33 endogenous lncRNAs/lncRNA fragments (dots) and 6 synthetic lncRNAs (stars) (mean  $\pm$  s.d.). See Supplementary Table 22 for sample sizes in panels **c** and **e-h**.

We developed a reductionist assay to study lncRNA cis-repressive activity in a normalized genomic context, called TETRIS (transposable element to test RNA's effect on transcription in cis). TETRIS enables the sequence of an lncRNA and an adjacent reporter gene to be manipulated in a plasmid, but then rapidly inserted into chromosomes via the piggyBac transposase<sup>42,43</sup>, so that effects of the lncRNA on the reporter can be studied in genomic chromatin (see Fig. 4a and Methods). Under our assay conditions, piggyBac catalyzes 4–7 insertions of each cargo per stably selected cell, and cell density estimates suggest that between 100,000 and 500,000 cells receive insertions and survive selection (Fig. 4b and data not shown). Thus, each TETRIS assay probably surveys 400,000–3,500,000 insertion events. Insertion-site-dependent variations in lncRNA-induced effects are averaged out in the population, bypassing the need to isolate clones of modified cells, and providing the means to quantify lncRNA regulatory potential without influence from genomic position.

We validated TETRIS by comparing effects that expression of different lncRNAs had on luciferase activity. A cell line created from a vector that lacked an lncRNA insert (TETRIS-Empty) showed an approximately twofold increase in luciferase activity upon addition of doxycycline, representing our baseline for the assay (Fig. 4c). We attribute this mild activation to the close proximity of the dox-inducible and luciferase promoters, and to the fact that both promoters are contained within the same insulated domain<sup>44</sup>. By contrast, expression of the first 2 kb of *Xist* repressed luciferase fivefold relative to uninduced control (Fig. 4c). The twofold activation and fivefold repression were stable across 9 and 16 independent derivations of TETRIS-Empty and TETRIS-*Xist*-2kb cell lines, respectively (mean  $\pm$  s.d. of  $2.03 \pm .50$  and  $0.23 \pm .08$ ), demonstrating that TETRIS assays result in reproducible effects on luciferase activity. For its repressive effect, *Xist* requires 'Repeat A', a 425-nucleotide-long element contained within its first 2 kb<sup>45</sup>. In the context of TETRIS, deletion of Repeat A resulted in a significant, but not



**Fig. 5 | Mapping of elements required for repression by *Xist*-2kb in TETRIS. **a,b**, Minimum free energy (MFE) (**a**) and arc-based structural models (**b**) of the first 2 kb of *Xist* from ref. <sup>41</sup>; green and blue bars mark starts and stops of indicated regions; locations of *Xist* repeats<sup>7</sup> and predicted stable structures; low S/S, regions of low SHAPE reactivity and Shannon entropy from ref. <sup>41</sup> are also shown. **c**, Deleted regions. **d**, Effects on luciferase after dox addition. \*Bonferroni-corrected  $P < 0.001$  relative to Wild-type/*Xist*-2kb via Student's  $t$  test. Tukey boxplots show the lower, median, and upper quartile of values, and  $\pm 1.5 \times$  interquartile range (see Supplementary Table 22 for sample sizes and exact  $P$  values). Min., Minimal; br., broad.**

complete, de-repression of luciferase, whereas expression of Repeat A alone resulted in repression relative to control, but at reduced levels compared to *Xist*-2kb ('*ΔrepA*' and '*repA only*'; Fig. 4c). Similarly, expression of the first 5.5 kb of *Xist* caused a fivefold repression of luciferase, whereas deletion of the first 2 kb from the 5.5-kb construct caused complete loss of repressive activity ('*Xist*-5.5 kb' and '*Xist*-2-5.5'; Fig. 4c). Expression of either the final 3.3 kb of *Xist* or the *Hottip* lncRNA had no repressive effect (Fig. 4c). These experiments demonstrate (1) that TETRIS is a suitable assay to measure repression by cis-acting lncRNAs in a normalized genomic context, and (2) in the assay, sequence elements in addition to Repeat A cooperate to encode repressive function in the 5' end of *Xist*.

We next used TETRIS and SEEKR to test our hypothesis that *k*-mer content can predict lncRNA regulatory potential. We reasoned that we could design entirely synthetic lncRNAs that lacked linear sequence similarity to any known lncRNA but nonetheless had robust *Xist*-like repressive activity. We generated six synthetic lncRNA sequences in silico with varying levels of *k*-mer similarity to the first 2 kb of *Xist*, and cloned them into TETRIS to measure their effects on luciferase activity. As measured by SEEKR, the lncRNAs had Pearson's similarities to *Xist* that ranged from average (a Pearson's *r* of ~0) to 3 s.d. above the mean similarity for all mouse lncRNAs (a Pearson's *r* of 0.19, more similar to *Xist*-2kb than all other lncRNAs in the mouse genome; see Fig. 4d). Using nhmmer or Stretcher to align the synthetic lncRNAs to the first 2 kb of *Xist* produced either no alignments (nhmmer) or alignments that differed by only 3% across all six synthetic lncRNAs (Stretcher; see Fig. 4e, grid below graph). Via BLAST, the lncRNAs had no significant similarity to the mouse genome or to each other (not shown). The lack of informative alignments was expected because the synthetic lncRNAs have no evolutionary relationship with *Xist*, any region in the genome, or each other. Nevertheless, as envisioned, the synthetic fragments that SEEKR classified to be most similar to *Xist* had the highest repressive activity (Fig. 4e). These data directly demonstrate that evolutionarily unrelated lncRNAs can encode similar function through different spatial arrangements of related sequence motifs. Thus, *k*-mer content can be used to predict lncRNA regulatory potential.

We next examined whether SEEKR could predict *Xist*-like repressive activity in endogenous lncRNAs. We cloned into TETRIS 33 lncRNAs or lncRNA fragments that had a range of *k*-mer similarities to the first 2 kb of *Xist*. Included in our final set of fragments were several conserved lncRNAs and/or shorter fragments contained within them (*Airn*, *Hottip*, *Kcnq1ot1*, *Malat1*, *Neat1*, and *Pvt1*), as well as many lncRNAs with uncharacterized functions (Supplementary Table 22). Again, the more *Xist*-like an lncRNA fragment was at the level of *k*-mers, the more likely it was to repress in TETRIS; the Pearson's *r* value between *Xist*-likeness at a *k*-mer length of 6 and luciferase activity upon dox addition was -0.41 ( $P=0.02$ ). Including the 6 synthetic lncRNAs in the correlation brought the Pearson's *r* value to -0.52 ( $P=0.0007$ ; Fig. 4f). Nhmmmer and Stretcher had no ability to predict repressive activity, demonstrating that these algorithms cannot detect sequence signatures correlated with repressive activity in this setting ( $P=0.32$  and  $0.91$ , respectively; Fig. 4g,h). lncRNA fragment length also had no ability to predict repressive activity ( $r=0.03$ ,  $P=0.84$ ).

Lastly, we examined whether *k*-mer profiles associated with sequence elements required for repression by *Xist*-2kb might increase our ability to predict repressive activity in other lncRNAs. To determine the elements in *Xist*-2kb required for repression, we made a series of 26 deletions (Fig. 5). Surprisingly, 15 of the deletions, including ones that removed predicted stable structures, pseudoknots, and ~40% of Repeat A ('*ΔSS1*', '*ΔSS2*', '*ΔPK2*', '*ΔSS3*', and '*ΔSS4*'; see bottom panel in Fig. 5; ref. <sup>41</sup>), had no significant effect on repression. However, removal of all 8 GC-rich portions

of Repeat A, but not its U-rich linkers, caused an approximately threefold reduction in repression ('*ΔGC repeat in rA*' versus '*ΔU spacer in rA*'), as did removal of 3 predicted stable structures and their intervening sequences in the 742 nucleotides immediately downstream of Repeat A ('*ΔSS2/3/4 broad*'; ref. <sup>41</sup>). Co-deletion of Repeat A and the stable structures had an additive effect, causing a near complete loss of repression (the '*ΔrAΔSS234 br*' mutant), whereas expression of Repeat A or the stable structures alone had half the repressive potency of *Xist*-2kb ('*Only rA*' and '*Only SS234*'). Expression of both regions together had the same repressive potency as *Xist*-2kb ('*Minimal*'). Thus, in TETRIS, the major elements required for repression are contained between nucleotides 308 and 1,476 of *Xist*. Based on prior structural models<sup>41,46</sup>, we infer that the elements are comprised of protein binding sites, spacer sequences, and stable structures.

Having mapped the elements responsible for repression in *Xist*-2kb, we attempted to extract subsets of 6-mers from them that increased our ability to predict *Xist*-like repression. We also examined whether *k*-mer variance across lncRNA communities or *k*-mer nucleotide composition could be used to extract subsets of outperforming 6-mers, and whether different *k*-mer lengths had better predictive power than  $k=6$ . No rationally designed subset of 6-mers could predict repression better than the full 6-mer profile of *Xist*-2kb, nor could any other *k*-mer length (Supplementary Fig. 10). These results support the ideas that different lncRNAs can encode similar function through related, but not necessarily identical, sequence solutions, and that the full complement of 6-mers may be a broadly effective search tool to identify such similarities (not too relaxed, not too stringent).

## Discussion

Collectively, our data support the notion that many lncRNAs function through recruitment of proteins that harbor degenerate RNA-binding motifs, and that spatial relationships between protein-binding motifs in these lncRNAs are often of secondary importance to the concentration and effectiveness of the motifs themselves. By this logic, an lncRNA may merely need to present the appropriate motifs embedded within the appropriate structural contexts to achieve a specific function. Thus, different lncRNAs probably encode similar function through vastly different sequence solutions, and non-linear sequence comparisons can be used to discover similarities between them. By extension, because the RNA-binding motifs of many proteins are conserved<sup>23,24</sup>, it is plausible that groups of lncRNAs rely on similar motifs to encode related function in different organisms even though they lack direct evolutionary relationships. This concept is supported by our observation that lncRNA communities with related *k*-mer contents exist in human, mouse, and other organisms. We propose that non-linear sequence homology—in which the relative abundance of a set of protein-binding motifs is conserved, but the sequential relationships between them are not—is prevalent in lncRNAs. To quantify non-linear homology, we introduce SEEKR, a method to compare sequence content between any group of lncRNAs, regardless of the size of the group, the evolutionary relationships between the lncRNAs being analyzed, or the differences in their lengths. Each lncRNA (and each functional domain within each lncRNA) has its own *k*-mer signature, which can encode information about protein binding and RNA structure. SEEKR provides a simple way to tie this information to a biological property.

**URLs.** SEEKR, <https://github.com/CalabreseLab/seekr>; nhmmer, <http://hmmer.org/download.html>; Biopython, <http://biopython.org/>; CHAMP, <https://github.com/wweir827/champ>; Gephi, <https://gephi.org/>; ENCODE RNA-seq, <http://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeCshlLongRnaSeq/>; MEME, <http://meme-suite.org/doc/fimo.html>.



## Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at <https://doi.org/10.1038/s41588-018-0207-8>.

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## Author contributions

J.M.K., P.J.M., and J.M.C. conceived the study. J.M.K., D.S., and J.M.C. performed the computational analysis. S.O.K., K.L., D.M.L., M.D.S., J.S.W., A.R.B., K.M.W., and J.M.C. designed and performed the TETRIS assays. D.W.C., C.R.H., S.W., Q.C., and J.M.K. built the website. J.M.K. and J.M.C. wrote the paper.

## Competing interests

The authors declare no competing interests.

## Additional information

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## Methods

**Kcnq1ot1 versus Xist comparison.** *Kcnq1ot1* was aligned to *Xist* using nhmmer and Stretcher with default parameters. To assess significance of the alignments, we generated 1,000 pseudo-*Kcnq1ot1*s that were the same length as real *Kcnq1ot1* but composed of nucleotides randomly selected from a distribution of the mononucleotide content of *Kcnq1ot1* (0.335A: 0.205G: 0.202C: 0.258T). We then aligned the pseudo-lncRNAs to *Xist* with nhmmer and Stretcher as well as compared their *k*-mer contents relative to all other mouse lncRNAs at *k*-mer length *k* = 6 via SEEKR.

**SEEKR.** In SEEKR, a matrix of *k*-mer counts for a user-defined set of lncRNAs is created by counting all occurrences of each *k*-mer in each lncRNA in one-nucleotide increments, and then dividing those counts by the length of the corresponding lncRNA. Z-scores are then derived for each *k*-mer in each lncRNA by subtracting the mean length-normalized abundance of each *k*-mer in the group of lncRNAs being analyzed from the length-normalized abundance of the *k*-mer in the lncRNA in question, and then dividing that difference by the standard deviation in abundance of that *k*-mer in the group of lncRNAs being analyzed. We refer to the array of z-scores for each *k*-mer in a given lncRNA as its *k*-mer profile. Similarity between any two lncRNAs can be calculated by comparing their *k*-mer profiles with Pearson's correlation.

Our rationale for length normalization in SEEKR follows. Without length normalization, *k*-mer profiles become difficult to interpret for lncRNAs of different lengths. For example, an RNA that is ten times longer than another RNA will have ten times the number of *k*-mers. Without normalization, these lncRNAs would be considered dissimilar by SEEKR, regardless of the similarity in their relative concentrations of *k*-mers. By length normalizing, SEEKR creates a list of relative *k*-mer concentrations in a given lncRNA that is robust to differences in length. The idea that length normalization is important is supported by studies of known cis-repressive lncRNAs. At 18 kb, the *Xist* lncRNA is the most potent cis-repressive lncRNA known. At least three other known cis-repressive lncRNAs are longer than *Xist*, but less potent: *Airm*, *Kcnq1ot1*, and *Ube3a-ATS* are 90 kb, 85 kb, and 1.1 Mb, respectively<sup>7</sup>. Of these, the longest lncRNA, *Ube3a-ATS*, is the least potent, arguing that length alone does not account for lncRNA potency. In certain biological contexts, lncRNA length may be relevant, or it may have varying influence on lncRNA function. However, what these contexts might be and to what extent length does or does not affect lncRNA function in them are not known and difficult to predict. We also note that Pearson's correlation inherently normalizes for length. Thus, comparisons of *k*-mer content that use Pearson's correlation will eliminate length as a variable.

**GENCODE lncRNA annotations.** All GENCODE annotations used in this work were from human build v22 and mouse build vM5<sup>12</sup>. For each lncRNA, only the major splice annotation was considered (the –001 isoform). In total, there were 15,953 human and 8,245 mouse transcripts. The heat maps in Fig. 2 were generated with GENCODE annotations plus the additional lncRNA sequences downloaded from the UCSC genome browser<sup>17</sup>: *SAMMSON*, *XACT*, *UBE3A-ATS*, *MORRIBD*, and *NESPAS* (human); and unspliced *Airm*, *Anril*, *Bvht*, *Haunt*, *Morrbid*, unspliced *Tsix*, *Ube3a-ATS*, *XistAR*, and *Upperhand* (mouse).

**Conservation analysis.** Ninety-three pairs of human and mouse GENCODE lncRNAs were recently identified as putative homologs due to their high conservation at the DNA level<sup>27</sup>. These 93 lncRNAs, plus an additional 68 lncRNA pairs that had equivalent names in mouse and human GENCODE annotations, formed the final set of 161 homologs that were used for the conservation analysis of Fig. 1c. For the Fig. 1c experiment, 'signal' values were computed as the mean of the 161 homolog-to-homolog measurements in each of the three algorithms; likewise, background values were computed as the mean of the remaining 12,880 non-homologous comparisons. Homologous pairs were defined as being 'detected' if the signal value/average similarity (as determined via SEEKR, nhmmer, or Stretcher) was higher for homolog-to-homolog measurements than it was for all other lncRNA-to-non-homolog comparisons. For this analysis, nhmmer was downloaded as part of the HMMER package (see URLs) and was run with --nonnull2, --nobias, --noali, and -o flags set. Stretcher was used as part of Biopython (see URLs) and was run with --gapopen = 16, and --gapextend = 4.

**Hierarchical clustering and labeling.** Hierarchical clustering was performed with the R package 'amap' using Pearson's as a distance metric and average linkage<sup>48</sup>, and was visualized with Java Treeview<sup>49</sup>. We used *k*-mer length *k* = 6 for our main analyses because it performed well in evolutionary comparisons (Fig. 1c), and it provided a feature number ( $4^6 = 4,096$  features) that was only marginally larger than the average length of a GENCODE lncRNA (1,152 and 1,471 nucleotides for human and mouse lncRNAs, respectively).

**Clustering of known cis-activating and cis-repressive lncRNAs.** We performed a literature review to curate lists of experimentally verified cis-repressive and cis-activating lncRNAs in mouse and human (Supplementary Table 1). We calculated the mean pair-wise similarity between all lncRNAs in each of these groups, and compared those means with the distribution of mean similarities calculated

from pair-wise comparisons of 10,000 randomly selected, size-matched groups of lncRNAs in their respective organism to generate *P* values that describe the likelihood that the similarity observed between the functionally related cis-acting lncRNAs was greater than would have been expected from random chance (Supplementary Table 2).

**Network analysis and lncRNA community definition.** Networks of lncRNAs were formed from a weighted adjacency matrix in which edges between any two lncRNAs were kept only if their Pearson's *r* value was at least 0.13. We selected the lncRNAs within the largest connected component of this network representation and used the Louvain algorithm<sup>51</sup> at default resolution parameter to assign lncRNAs to communities of related *k*-mer profiles (using the Python package 'louvain-igraph'). This decision was supported through use of the recently developed CHAMP algorithm<sup>50</sup> (see URLs), which found a wide domain of optimality around the default resolution parameter. We retained assignments for the lncRNAs present in the top five most populated communities, and assigned the remaining lncRNAs, including those not found in the largest connected component of the network representation, to the null community, which served as an important outgroup for our comparisons of *k*-mer content and biological properties in Fig. 3. Multiple Pearson's *r* value thresholds between 0.12 and 0.21 were tested for human lncRNAs and we found little to no difference in community definition, correlation with lncRNA localization, or ability to predict protein-binding patterns (not shown). Gephi was used for network visualization (see URLs). Community colors were automatically assigned by Gephi according to the size of each community.

We also compared communities generated with 5-mers and 7-mers to those generated with 6-mers. We created contingency tables that compared the distribution of lncRNAs in each of the five major 6-mer communities plus the null to the distribution of lncRNAs in each of the five major 5-mer and 7-mer communities plus their respective nulls. *P* values comparing communities between the *k*-mer lengths were all  $< 1 \times 10^{-324}$  (chi-squared), indicating that community definitions are largely stable when 5-mers, 6-mers, or 7-mers are used (Supplementary Tables 9 and 10). This stability, the quality of our TETRIS predictions when using 6-mers (Supplementary Fig. 10), and the computational inefficiency of performing operations on matrices of *k*-mers with length *k* = 7 or greater provided additional support for our decision to use 6-mers for the bulk of our analyses.

We applied the same *r* value threshold and community assignment logic that we used for human lncRNAs to define lncRNA communities using *k*-mer length *k* = 6 in all other organisms.

**Comparing lncRNA groups in hierarchical clusters to lncRNA communities found by Louvain.** Clusters of lncRNAs with similar *k*-mer content in human and mouse (from Fig. 2) were created by manually making cuts in the dendrogram of the hierarchical clusters that maximized the visual similarity of *k*-mer profiles between lncRNAs in each cluster. Five cuts were made in the hierarchical cluster from each organism to approximate the five major communities found by the Louvain algorithm. We measured the similarity of the manually made clusters to the five major Louvain-defined communities by creating a contingency table that compared lncRNA distributions between the two methods. We then tested whether the distributions of lncRNAs across the two sets of communities were significantly similar via a chi-squared test. In both human and mouse, the *P* value was  $< 1 \times 10^{-324}$  (Supplementary Tables 3 and 4).

**lncRNA localization analysis.** Localization data were downloaded from ENCODE (see URLs) as fastq files and aligned to GRCh38 with STAR using default parameters<sup>47,51</sup>. FeatureCounts was used to tabulate the number of reads aligning to our set of lncRNAs<sup>52</sup>. We then filtered out all lncRNAs with  $< 0.1$  reads per kilobase of transcript per million aligned reads (RPKM) from each community, and calculated the number of reads in the nuclear fraction over the total number of reads from both the nuclear and cytosolic fractions for each lncRNA.

To determine whether specific *k*-mers were enriched in cytosolic or nuclear lncRNAs, we selected cytosolic- and nuclear-enriched subgroups of lncRNAs that were expressed in HepG2 or K562 cells. Because the subcellular distribution values for HepG2 or K562 expressed lncRNAs were not normally distributed (Fig. 3a), we needed to employ different thresholds to define cytosolic and nuclear so that the two groups would include similar numbers of lncRNAs. 'Cytosolic' lncRNAs were defined as any lncRNA that was more than 50% cytosolic, which resulted in 2,801 transcripts, and 'nuclear' lncRNAs were defined as any lncRNA that was more than 95% nuclear, which resulted in 4,576 transcripts. To determine the average difference in *k*-mer abundance between lncRNAs in the two compartments, we calculated the mean value of the z-scores for each *k*-mer in each group, and then used the difference between the means as the metric to calculate the nuclear-enrichment score (Supplementary Table 18). To test for significant differences between the distributions of z-scores between lncRNAs in the two compartments, we used a Kolmogorov-Smirnov (KS)-test and calculated an adjusted *P* value using a Bonferroni correction. This analysis yielded 387 *k*-mers whose distributions differed significantly between cytosolic and nuclear lncRNAs (*P* value  $< 0.05$ ; Supplementary Table 18).

Using only the lncRNAs from community 3, we repeated the process of applying the Louvain algorithm to define communities and measure cellular localization in order to rule out the possibility that potential subcommunities were responsible for the cytosolic nature of community 3. The Louvain algorithm found four main subcommunities and all smaller subcommunities were grouped into a fifth community. The results of analysis of variance tests indicated that there were no significant differences between any of the communities for either the polyA-selected or ribosome-depleted RNA-seq data. We performed this analysis again for community 1, but no subcommunities were found to be significantly different (Supplementary Fig. 11). This uniformity of cellular localization among possible subcommunities provides biological support for our original community definitions.

**lncRNA polysome association.** A recent study found 229 lncRNAs in GENCODE v22 that were polysome-associated in K562 cells<sup>36</sup>. A chi-squared test showed that these 229 lncRNAs were non-randomly distributed between the communities ( $P$  value =  $3.5 \times 10^{-5}$ ; Supplementary Table 17). The expected values for the chi-squared test were calculated by filtering all communities for lncRNAs expressed in K562 cells, dividing the number of lncRNAs in each community by the total number of expressed lncRNAs (3,277), and multiplying by the number of polysomal lncRNAs (229).

**lncRNA–protein association data.** eCLIP data were downloaded from ENCODE<sup>35,37</sup>. For each of the 156 eCLIP experiments ‘bed narrowPeak’ data (representing sites of protein binding that passed an ENCODE-defined threshold for enrichment over background; refs. <sup>35,37</sup>) were pooled from available biological duplicates. Genomic coordinates were overlapped with lncRNA exon coordinates annotated by GENCODE. Any lncRNA that overlapped with one or more eCLIP peak was considered as having a true binding interaction with the given protein. lncRNA expression data were collected from ENCODE RNA-seq experiments in the same cell type as that of the eCLIP experiment (HepG2 or K562).

For each protein, a vector was built for each lncRNA that encoded whether the protein–lncRNA pair did or did not interact. Next, two feature matrices (null and full) were constructed. The null matrix included the log normalized values for length and expression of each of the lncRNAs. The full matrix included log normalized length and expression, as well as an additional five columns that corresponded to each of the five lncRNA communities. Each lncRNA was assigned a value of 1 in the column representing its community.

**Models of protein associations.** To address whether lncRNA communities contained information about lncRNA–protein associations, we used a machine learning model<sup>33</sup>. We tested whether providing the model with the community data allowed it to predict interactions better than a corresponding null model that was not given the community data but still included lncRNA length and expression values as covariates. Logistic regression models were implemented with scikit-learn, using default parameters<sup>33</sup>. The significance of the additional community information was measured with a likelihood ratio test (LRT), where the LRT statistic,  $D$ , was defined as:

$$D = 2 \times (\log(\text{full model likelihood}) - \log(\text{null model likelihood}))$$

A chi-squared distribution was used to determine the corresponding  $P$  value for the LRT statistic.  $P$  values were adjusted with a Bonferroni correction for the 156 comparisons.

To quantify the extent of the effect that community inclusion had on prediction of lncRNA–protein interactions, we used a leave-one-out-cross-validation approach to measure precision and recall metrics<sup>33</sup>, defined as:

$$\text{Precision} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Positives}}$$

$$\text{Recall} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}}$$

In our model, precision is the number of lncRNAs correctly predicted to bind a protein, divided by the total number of lncRNAs the model predicted to bind a protein. Recall is the number of lncRNAs the model correctly predicted to bind a protein, divided by the total number of lncRNAs found to bind a protein according to the eCLIP data. For each lncRNA, the logistic regression models were allowed to train on all other lncRNAs except the single ‘left out’ lncRNA. After training, both models were asked to predict whether the left out lncRNA did or did not bind the protein. This procedure was repeated for all lncRNAs in each eCLIP dataset to calculate precision and recall.

The methodology for training and testing the raw  $k$ -mer models was exactly the same as described above except that the 5 community features were replaced by the 4,096 relative  $k$ -mer abundance features.

### Calculating the abundance of motif-matching $k$ -mers in lncRNA communities.

The data for the bar graph in Fig. 3c were generated by the following approach. Of the 109 proteins on which eCLIP was performed in ref. <sup>37</sup>, 79 showed significant association with at least one  $k$ -mer community over the null (Supplementary Table 19). Of these 79 proteins, binding motifs for 17 were determined via an *in vitro* binding assay in ref. <sup>23</sup>. The PWMs for each of these 17 proteins contained relative weights for each motif matching 6-mer, representing the likelihood that the  $k$ -mer in question would bind the protein in question. We multiplied the weight of each motif-matching 6-mer by its average standardized abundance in each of the six communities, including the null, to obtain  $k$ -mer abundances that were scaled by the likelihood that the  $k$ -mer in question matched the binding motif in question. For each of the 17 proteins, sums of the weighted abundance for all motif-matching  $k$ -mers were created for the communities in which protein binding was enriched and not enriched over the null, respectively, then divided by the number of communities in each group to obtain the average weighted abundance of motif-matching  $k$ -mers in the binding-enriched and binding-not-enriched groups. These abundances are plotted in Fig. 3c. For proteins that had more than one PWM reported in ref. <sup>23</sup>, the average abundance shown in Fig. 3c is comprised of the weighted abundance averaged over all reported PWMs. To calculate significance, we shuffled the communities in the binding-enriched and binding-not-enriched groups 10,000 times and determined how often the difference in  $k$ -mer abundance between the randomly shuffled binding-enriched and binding-not-enriched groups was greater than the difference between the real binding-enriched and binding-not-enriched groups.

### Measuring $k$ -mer similarity surrounding motif matches in lncRNAs.

The lncRNAs expressed in HepG2 and K562 cells were examined for motif matches to the 17 proteins for which eCLIP data was reported in ref. <sup>37</sup> and whose PWMs were determined via a high-throughput *in vitro* assay in ref. <sup>23</sup> by using FIMO at a threshold of  $P < 0.01$  (from the MEME suite, see URLs; ref. <sup>54</sup>; Supplementary Table 21). Each motif match was then labeled as a true positive if it overlapped an eCLIP peak, or a false positive if it did not. For each protein, the sequences surrounding the center of each true- and false-positive motif match (up to 150 bp on either side of the center, or up to the end of the gene, whichever came first) were collected and their  $k$ -mer contents were analyzed with SEEKR. Significance of the similarity between true-positive regions was measured by a permutation test against randomly selected sets of false-positive regions controlling for both the size of the set and the number of overlapping regions in the set (Supplementary Fig. 4).

**Identifying motifs from eCLIP peaks.** To find motifs in eCLIP peaks for the 17 proteins listed in Fig. 3c, we extracted the subset of sequences from eCLIP peaks whose CLIPper-defined  $P$  value was  $< 0.001$  (peaks with the highest read densities relative to control; ref. <sup>37</sup>). We searched these sequences for motifs using DREME at default parameters as a part of the MEME-ChIP package<sup>55</sup>.

### Human-to-mouse and human-to-other community similarity calculations.

To evaluate the similarity between human and mouse lncRNA communities, we calculated the distribution of similarities between all pair-wise combinations of lncRNAs within each human  $k$ -mer community (‘human-to-self’), and compared this distribution to: (1) a distribution of pair-wise comparisons made between all other human lncRNAs excepting lncRNAs from the community in question (‘human-to-other-human’), (2) distributions of all pair-wise comparisons made between all lncRNAs in each of the five mouse lncRNA communities (‘human-to-mouse’), and (3) distributions of all pair-wise comparisons made between all human and mouse lncRNAs that did not fall into one of the five major communities (‘human-to-null’). We then performed a permutation test to determine whether a given human community was similar enough to a mouse community to overcome its intrinsic similarity to other lncRNAs in the human genome. The expectation was that, for related communities, the human-to-mouse distribution would be more similar to the human-to-self distribution than it would be to the human-to-other-human and human-to-null distributions. Bonferroni-adjusted  $P$  values were calculated by permutation tests where we iteratively subsampled 0.1–1% of each distribution, re-measured the mean pair-wise similarities, counted the number of trials in which the human-to-mouse mean subsample was closer to the human-to-other-human mean than it was to the human-to-self mean, and, finally, divided by the total number of trials performed (36,000). This bootstrapping procedure provided a statistical framework to determine whether the similarities uncovered between human and mouse communities were greater than would have been expected from random chance. For example, in each of 36,000 tests, the distribution of similarities between a randomly selected subset of lncRNAs from human community 1 and size-matched subsets of lncRNAs from mouse community 1 was always more similar to the distribution of similarities between all pair-wise comparisons of the human community 1 subset than it was similar to the distribution of similarities between the human community 1 subset and size-matched subsets of non-community 1 human lncRNAs (see upper left panel in Supplementary Fig. 6, ‘H-1 versus M-1’ plot—the H-1-versus-H-1 distribution in red is nearly indistinguishable from the H-1-versus-M-1 distribution in purple).



To generate the plots in Supplementary Figs. 8 and 9, identical analyses were performed that compared human lncRNA communities to lncRNA communities from rabbit, dog, opossum, chicken, lizard, coelacanth, zebrafish, stickleback, Nile tilapia, elephant shark, and sea urchin<sup>10</sup>. In these latter cases, the human *XIST* and *HOTTIP* lncRNAs were doped into the lncRNA annotation set from the organism in question to find the homologous communities that were the most *XIST*- and *HOTTIP*-like (Supplementary Fig. 7).

**Generation of plasmids for TETRIS assays.** The pTETRIS-cargo vector was created from components of a cumate-inducible piggyBAC transposon vector (System Biosciences), pGI4.10-Luciferase (Promega), and pTRE-Tight (Clontech). Briefly, a 567-bp fragment containing a minimal mouse PGK promoter was cloned into a SacI site in pGI4.10-Luciferase to generate pGI4-PGK-Luc-pA. The reverse complement of PGK-Luc-pA was cloned into a vector containing the bovine growth hormone polyA site. The entire bGHPa-[reversePGK-Luc-pA] was cloned into NotI and SalI sites of the piggyBAC vector (System Biosciences). The cumate-inducible promoter in the piggyBAC vector was then replaced with the Tetracycline Responsive Element (TRE) from pTRE-Tight (Clontech) via Gibson assembly to generate pTETRIS-cargo in Fig. 4a, in which the lncRNA, the luciferase gene, and a gene encoding puromycin resistance are all flanked by chicken HS4 insulator elements, and inverted terminal repeats recognized by the piggyBAC transposase. The rtTA-cargo vector from Fig. 4a was generated by cloning the hUbiC-rtTA3-IRES-Neo cassette from pSLIK-Neo (Addgene Plasmid no. 25735) into SfiI and SalI sites in a piggyBAC transposon vector (System Biosciences). The piggyBAC transposase from System Biosciences was cloned into SmaI and HindIII sites in pUC19 (NEB) to allow propagation of the transposase on ampicillin plates.

**Generation of TETRIS-lncRNA Cargo vectors.** lncRNA fragments were PCR-amplified from genomic DNA or bacterial artificial chromosomes using Phusion DNA Polymerase (NEB), or commercially synthesized (Genewiz, IDT), and cloned via Gibson assembly into the SmaI site of pTETRIS-Cargo. Insert size was verified by restriction digestion, and the 5' and 3' end of each insert was verified by Sanger sequencing. To generate mutant *Xist*-2kb constructs, the 2-kb fragment of *Xist* was subcloned into pGEM-T-Easy, and the regions in question were deleted using site-directed mutagenesis, or by synthesis of a mutated fragment and re-cloning back into compatible sites in pGEM-*Xist*-2kb (Genewiz). Deletions were verified by Sanger sequencing and then assembled into the SmaI site of pTETRIS-Cargo. The sequences of all inserted fragments, including *Xist*-2kb mutations, are listed in Supplementary Table 22.

**Estimation of TETRIS copy number per cell.** Genomic DNA was prepared from biological triplicate derivations of TETRIS-*GFP* and TETRIS-*Xist*-2kb cell lines. qPCR signal (SoFast, Biorad) from the genomic DNA was compared to signal from a molar standard amplified from increasing amounts of the corresponding TETRIS plasmid (Supplementary Table 23).

**TETRIS assays.** To generate stable TETRIS-lncRNA cell lines,  $8 \times 10^5$  E14 embryonic stem cells (ATCC CRL-1821) were seeded in a single well of a 6-well plate, and the next day transfected with 0.5  $\mu$ g TETRIS cargo, 0.5  $\mu$ g rtTA-cargo, and 1  $\mu$ g of pUC19-piggyBAC transposase. Cells were subsequently selected on puromycin (2  $\mu$ g ml<sup>-1</sup>) and G418 (200  $\mu$ g ml<sup>-1</sup>) for 6–12 d. Due to the efficiency of piggyBAC cargo integration and the rapidity of puromycin selection, all observable death from drug selection occurred within ~3 d after addition of puromycin and G418 (that is, cells with puromycin resistance were invariably resistant to G418). For luciferase assays,  $1 \times 10^5$  cells per well of a 24-well plate were seeded in triplicate from each biological replicate preparation of a stable TETRIS-lncRNA cell line. At 24 h post seeding, medium was changed to include doxycycline at a final concentration of 1  $\mu$ g ml<sup>-1</sup>. After 2 d growth in dox-containing media, cells were lysed with 100  $\mu$ l passive lysis buffer (Promega), and luciferase activity was measured using Bright-Glo Luciferase Assay reagents (Promega) on a PHERAstar FS plate reader (BMG Labtech). Luciferase activity was normalized to protein concentration in the lysates via Bradford assay (Biorad). Each lncRNA fragment was assayed at least in triplicate from at least two independent biological replicate preparations of stable TETRIS-lncRNA cell lines.

**Synthetic lncRNA design.** Synthetic lncRNAs were designed by generating 10,000,000, 1,650-nucleotide-long lncRNAs in silico that were composed of nucleotides randomly selected based on a given input ratio. To generate synthetic lncRNAs 2 through 6, the input ratio was the mononucleotide content of the 2,016-nucleotide-long fragment of *Xist* inserted into TETRIS (0.203A: 0.262G: 0.204C: 0.331T). To generate synthetic lncRNA 1, the input ratio was an equal proportion of mononucleotides (0.250A: 0.250G: 0.250C: 0.250T). Synthetic

lncRNAs with the specified *k*-mer similarity to the 2-kb fragment of *Xist* were then selected and synthesized as geneBlocks (Integrated DNA Technologies) and Gibson assembled into the SmaI site in TETRIS. Similarities in *k*-mer content to the 2-kb fragment of *Xist* are relative to all other mouse GENCODE lncRNAs.

**Visualization of *Xist* structural models.** Minimum free energy and probability-arc structural models of *Xist*-2kb were generated using SHAPE-MaP data from ref.<sup>41</sup>, the visualization package VARNA<sup>56</sup>, and a modified version of the IGV browser<sup>57</sup>. Predicted pseudoknots and regions of low SHAPE reactivity and low Shannon entropy in *Xist*-2kb are from ref.<sup>41</sup>.

**TETRIS predictions for *k*-mer sizes and subsets.** We measured SEEKR's ability to capture the relationship between an lncRNA's *Xist*-likeness and its repressive ability in the TETRIS assay using *k*-mers from size one to eight. In each case, the correlation is measured using the means of all biological and technical replicates of each real and synthetic lncRNA, and by normalizing *k*-mer counts of *Xist*-2kb and the lncRNA in question in context with all mouse GENCODE lncRNAs. This process was repeated for select subsets of *k*-mers that had the potential to increase our ability to predict repressive activity in TETRIS. Individual subsets were created by counting and normalizing *k*-mers as normal with SEEKR then removing columns of the resulting count matrix that were not included in a given subset. Additionally, we randomly generated 100,000 *k*-mer subsets each containing between 2 and 4,095 *k*-mers, and measured each of the subsets' Pearson's *r* values relative to our TETRIS data (Supplementary Fig. 10).

**Statistical analyses.** All statistics were performed in Python or R. Details of statistical analyses are described in the corresponding sections. All multiple comparison tests were adjusted using a Bonferroni correction. *P* values are reported as exact values except in cases where the *P* value was calculated using a permutation test, and no random samples were found to be more extreme than the observed value. In these cases, *P* values are reported as ( $P \leq 1/n$ ), where *n* is the number of permutations performed.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Code availability.** SEEKR is open source and available on GitHub (see URLs). A web instance of SEEKR is hosted at <http://seekr.org/>. A library for counting small *k*-mer frequencies in nucleotide sequences is available as Supplementary Software.

## Data availability

The datasets generated during and/or analyzed during the current study are available within the article and its supplementary information files.

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## Life Sciences Reporting Summary

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### ► Experimental design

#### 1. Sample size

Describe how sample size was determined.

For each lncRNA that was assayed via TETRIS, we required that stable TETRIS-lncRNA cell lines were derived at least twice from transfections that occurred on different days, and that the effect of lncRNA expression for each stable cell line was measured at least in triplicate. Data collected for multiple (>3) derivations of TETRIS-Xist, TETRIS-Empty, TETRIS-synthetic, and TETRIS-natural-lncRNA cell lines over the course of ~18 months by several different researchers indicated that this was an appropriate sample size to capture the variability of the assay.

#### 2. Data exclusions

Describe any data exclusions.

No data were excluded.

#### 3. Replication

Describe whether the experimental findings were reliably reproduced.

TETRIS assays were highly reproducible across multiple researchers in my lab.

#### 4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

lncRNAs were grouped by their relative kmer contents, and this grouping was defined using the network-based approach described in the methods.

#### 5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Researchers that performed the TETRIS assays did not have prior knowledge of the levels of kmer similarity to the 5' fragment of Xist; also, the assays were performed in small groups of lncRNAs over the course of 18 months. This blinded expectations for any overall trend that may or may not have emerged.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

## 6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

n/a Confirmed

- ☐ ☒ The exact sample size ( $n$ ) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- ☐ ☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
- ☐ ☒ A statement indicating how many times each experiment was replicated
- ☐ ☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- ☐ ☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- ☐ ☒ The test results (e.g.  $p$  values) given as exact values whenever possible and with confidence intervals noted
- ☐ ☒ A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- ☐ ☒ Clearly defined error bars

See the web collection on [statistics for biologists](#) for further resources and guidance.

## ► Software

Policy information about [availability of computer code](#)

### 7. Software

Describe the software used to analyze the data in this study.

Code to implement SEEKR is provided as a supplementary file, and will be deposited in GitHub upon acceptance of the manuscript. Pre-existing software used for the analysis of data are listed below:

- \* Python (2.7, 3.6)
- \* nhmmer (3.1b2)
- \* Biopython (1.69)
- \* Java Treeview (1.1.6r4)
- \* R (3.2.2)
- \* amap (0.8-14)
- \* CHAMP (1.0.4)
- \* Gephi (0.9.1)
- \* louvain-igraph (0.6.1)
- \* scikit-learn (0.19.1)
- \* MEME Suite (4.12.0)
- \* STAR aligner (2.5.3a)
- \* Subread analysis package (1.5.2)
- \* matplotlib (2.0.2)
- \* seaborn (0.7.1)

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The *Nature Methods* [guidance for providing algorithms and software for publication](#) may be useful for any submission.

## ► Materials and reagents

Policy information about [availability of materials](#)

### 8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No restrictions. All plasmids that do not get deposited directly in Addgene will be available without restriction upon request. Code to implement SEEKR is included as a supplementary file and will be deposited on Github.

### 9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

We did not generate any data with antibodies for this work.

## 10. Eukaryotic cell lines

- State the source of each eukaryotic cell line used.
- Describe the method of cell line authentication used.
- Report whether the cell lines were tested for mycoplasma contamination.
- If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by [ICLAC](#), provide a scientific rationale for their use.

We used E14 mouse embryonic stem cells for all TETRIS assays.

E14 cells were received from ATCC and were verified to be diploid by a third party.

The cells were not tested for mycoplasma directly, but routine imaging with DAPI as part of another study indirectly confirmed they were mycoplasma negative.

No commonly misidentified cell lines were used.

## ► Animals and human research participants

Policy information about [studies involving animals](#); when reporting animal research, follow the [ARRIVE guidelines](#)

### 11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

No animals were used.

Policy information about [studies involving human research participants](#)

### 12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.