Whole-genome-scale identification of novel non-protein-coding RNAs controlling cell proliferation and survival through a functional forward genetics strategy

D. P. Tonge1, D. Darling2, F. Farzaneh2 & G. T. Williams1

Identification of cell fate-controlling lncRNAs is essential to our understanding of molecular cell biology. Here we present a human genome-scale forward-genetics approach for the identification of lncRNAs based on gene function. This approach can identify genes that play a causal role, and immediately distinguish them from those that are differentially expressed but do not affect cell function. Our genome-scale library plus next-generation-sequencing and bioinformatic approach, radically upscales the breadth and rate of functional ncRNA discovery. Human gDNA was digested to produce a lentiviral expression library containing inserts in both sense and anti-sense orientation. The library was used to transduce human Jurkat T-leukaemic cells. Cell populations were selected using continuous culture ± anti-FAS IgM, and sequencing used to identify sequences controlling cell proliferation. This strategy resulted in the identification of thousands of new sequences based solely on their function including many ncRNAs previously identified as being able to modulate cell survival or to act as key cancer regulators such as AC084816.1*, AC097103.2, AC087473.1, CASC15*, DLEU1*, ENTPD1-AS1*, HULC*, MIRLET7BHG*, PCAT-1, SChLAP1, and TP53TGF1. Independent validation confirmed 4 out of 5 sequences that were identified by this strategy, conferred a striking resistance to anti-FAS IgM-induced apoptosis.

Non-protein-coding sequences in the genome play crucial functional roles in a range of different cellular processes1–47. Most of the human genome encodes ncRNAs, most of which, at > 200 nucleotides, are classified as lncRNAs. Although the analysis of this vast number of transcripts is still at an early stage, it is evident that many lncRNAs play crucial roles in molecular cell biology, ranging from provision of essential frameworks for RNA processing, through to epigenetic control of gene expression, regulating cell signalling pathways, and more46,48. In the healthy state, lncRNA expression is often tightly restricted to specific tissues at specific times48,49. In particular, Sarropoulos et al.50 have demonstrated that many lncRNAs are dynamically regulated during organogenesis, displaying striking specificity both in tissue and chronological time of expression, emphasising their importance in regulating cell fate50. It is not surprising therefore that deregulation of lncRNAs has been implicated in pathology51,52, including oncogenesis50,53–56. Although demonstrations of the functional importance of individual lncRNAs are impressive and growing, the vast majority—many thousands—of lncRNAs are still entirely uncharacterised; some of these currently uncharacterised lncRNAs are likely to play critical roles in important control processes that have yet to be revealed. This provides strong motivation for the development of high-throughput strategies for the identification of key lncRNAs by targeting their functional activity.

For protein-coding genes, forward genetics approaches, such as unbiased gene modulation/mutation followed by selection for function, have already been widely applied in the identification of critical cell regulators57. In our own studies, functional cDNA expression cloning pinpointed not only several significant regulatory proteins58,59, but also 3 lncRNAs that modulate cell fate: GAS560,61, RBMS-AS1/Je258,59, and NEAT1/Trophoblast Derived Non-Protein Coding RNA60,62. All 3 lncRNAs were identified through the functional activity of short partial sequences, rather than the full-length RNA. In addition, a 24-base oligomer corresponding to part of GAS5 also

1Faculty of Natural Sciences, School of Life Sciences, Keele University, Keele ST5 5BG, UK. 2Molecular Medicine Group, Faculty of Life Sciences & Medicine, School of Cancer & Pharmaceutical Sciences, Kings College London, London, UK. *Email: d.p.tonge@keele.ac.uk
shows strong functional activity. These results are all consistent with the observation that many lncRNAs act through short sequence motifs, although such functional sequences will often be required to act in concert.

Here we describe a genome-scale strategy that builds upon these observations and identifies critical sequences through their functional effects on cell proliferation and survival. Our approach identifies those genes that play a causal role, and immediately distinguishes this group from those that are differentially expressed but do not affect cell function—this represents a key advantage over expression level studies. Furthermore, we use sequences derived from whole genomic DNA, thus overcoming the severe limitations imposed by the highly tissue-restricted expression of lncRNAs. Our application of a powerful sequencing and bioinformatic approach complements an unbiased whole genome-scale screen, resulting in the identification of numerous novel lncRNA regulators with demonstrable function, as well as confirmation of the function of several known lncRNAs. In addition to this new information on genes with clear potential in physiology and pathology, these observations provide proof of principle for the application of this novel strategy to the identification of lncRNAs that play rate-limiting roles in many different cellular activities.

Materials and methods
Library preparation and culture selection methods. All mammalian cells were cultured at 37 °C, 5% CO₂ in IMDM (Iscove’s Modified Dulbecco’s Medium; ThermoFisher #12440053) supplemented with 10% (v/v) Foetal Bovine Serum (FBS; Biosera FB-1001/500). Host cells were the anti-Fas-sensitive Jurkat T-leukaemic cell clone JKM1.

Whole human genomic DNA (Promega G3041) was digested with the restriction enzymes DraI and AanI in two separate reactions. The resulting digests were combined at equimolar concentration to produce our initial “Genomic Digest” (i.e. restriction fragments with blunt termini from the whole human genome). The Genomic Digest was cloned into the Sma1 site of lentiviral expression vector pCDH-CMV4 (a third-generation lentiviral vector with deletion/fusion of the 5’ LTR and truncated (3’) LTR); producing the expression library “CL3c” with inserts in both sense and antisense orientation. pCDH-CMV4 was a gift from Kazuhiro Oka (Addgene plasmid # 72284; http://n2t.net/addgene:72284; RRID: Addgene_72284). The library was introduced into MegaX DH10B electrocompetent cells (ThermoFisher) and amplified. For bulk production of the library in the lentiviral backbone, 8000 cm² of tissue culture plasticware was plated at a density of 1.1 × 10⁴ 293T17 cells/cm² in 1.4 L of DH10B electrocompetent cells (ThermoFisher) and amplified. The library was transduced into Jurkat JKM1 cells at a multiplicity of infection of 3.7, i.e. 3.7 × 10⁴ virus constructs were introduced into each population of 10⁶ cells.

Four replicate 50 mL Jurkat cultures (from 4 independent transductions) were maintained for up to 51 days, subculturing to 2 × 10⁶ cells/mL every 2–3 days. Samples of 5 × 10⁶ cells were taken immediately following transduction (to appraise the range of inserts successfully transduced relative to the starting library) (d0, JCPZ), at day 47 following continuous culture (d47, MFZ), and following 47 days of continuous culture plus additional selection with cytotoxic mouse IgM anti-Fas antibody IPO-4 at a final concentration of 20 ng/mL for 96 h at 37 °C. This d47 + anti-FAS, MF group was prepared by taking 4 × 10⁷ cells from each d47, MFZ replicate and subjecting each population to the additional selection with anti-FAS as described above. Surviving cells were harvested using Ficoll and their DNA isolated (Qiagen #69504). For schematic representation of these studies see Fig. 1.

Sequencing and bioinformatic analysis. Extracted DNA was amplified by custom oligonucleotide primers targeting a region of the pCDH-CMV4 genome located adjacent to the Sma1 multiple cloning site, and thus designed to enable the amplification of all insert sequences irrespective of sequence composition (Forward: 5′ catcacaagcttttggc 3′, Reverse: 5′ cgcgagtatcataagg 3′). Amplicoons were size selected using Ampure XP beads at a ratio of 1: 1.8 to remove unincorporated primers but retain the shortest products and sequenced using the Illumina platform with a 300 bp paired-end read metric.

A custom bioinformatic analysis workflow was developed to enable appraisal of the inserts within cells prior to (d0, JCPZ) and (in subsequent experiments) following selection (d47, labelled MFZ, and d47 + anti-FAS, labelled MF—see Fig. 1). This workflow validated inserts as “library-derived” through the presence of flanking vector sequence (recalling that the library was human in origin and transfected into human cells), confirmed the presence of an appropriate restriction site (DraI or AanI), and provided an indication of insert frequency, in addition to reporting the likely direction of insertion (and thus transcription). Sequencing reads were trimmed of low-quality bases and mapped to the latest human genome version (hg38) using BMA-MEM version 0.7.17.2 in simple Illumina mode. Areas of the genome enriched in mapped reads were identified by MACS2 version 2.1.2.20160309.6 and output as a list of genomic intervals in standard bed format. Sequences located within 25 bp of each other were considered to originate from the same overall sequence and thus consecutive intervals within 25 bp of another were combined using bedtools merge (-d 25). The abundance of each insert was determined in each experimental sample using bedtools multicov where the number of reads mapped to each insert was used as a surrogate indicator of the number of cells harbouring that specific insert within a given cell population. We generated lists of overlapping inserts (based upon their genomic coordinates) across the different independently treated replicates using MultiIntersectBed, and these data were used to identify candidate
functional RNAs present in 3 or more independent replicates (Fig. 2). Our initial approach, reported herein, ensured that only very high-confidence hits (functional sequences identified in ≥ 3 independent replicates, plus evidence of vector associated sequence) were considered for further validation. Significant scope exists to appraise those hits present in less than 3 experimental replicates.

**Independent experimental validation of novel cell fate modifying sequences.** Candidate sequences for independent validation (Table 7) were chemically synthesised and inserted into pcDNA3.1 sense expression plasmids (GenScript Limited). Constructs containing candidate sequences and control empty vector (pcDNA3.1 sense) were transfected into Jurkat JKM1 cells. The polyclonal populations were selected in 1 mg/mL geneticin for 14 days, grown to saturation density and challenged with 10–20 ng/mL anti-Fas IgM at 5 × 10⁵ cells/mL, in parallel with vector-only-transfected control cell cultures in the same 24-well plates. Viable (trypan-blue-excluding) cell densities were determined 7–18 days after addition of anti-Fas IgM.

**Results and discussion**

**Development of the genome-scale library (CL3c) and mammalian cell transduction and functional selection.** The observation that some lncRNAs can act through short sequence motifs led us to hypothesise that key functional potential could be revealed by expression of genomic DNA (i.e. in the absence...
of post-transcriptional processing). The first stage in this strategy was the preparation of a lentiviral library containing most of the human genome in both sense and antisense orientations and its transduction into the human T-leukaemic Jurkat clone JKM1, which is highly sensitive to apoptosis induced through ligation of the Fas receptor66 (see “Materials and methods” section). Commercially sourced human DNA was digested by the enzymes DraI and AanI to reduce the genome to a series of fragments that were predominantly in the predicted range of 0.5–4 kb. The resulting fragments were cloned into the pCDH-CMV4 vector in both orientations (the “CL3c library”), ensuring that transcript was generated from the entire genome from both the sense and antisense strands.

Jurkat clone JKM1 cells were transduced with the CL3c lentiviral library and cells harvested 24 h later (d0, JCPZ). These cells were used to evaluate the proportion of our CL3c library that could be successfully transduced into mammalian cells. A series of functional selection experiments followed in which JKM1 cells transduced as above were harvested following 47 days of continuous culture (47d, MFZ). Culture of transduced Jurkat cells over a period of 47 days produced a progressive increase in their rate of proliferation (Fig. 3). This increase was confirmed through directly comparing the proliferation of the 4 replicate cell populations, A–D (doubling time 19.1 h (SD (Standard Deviation) 1.6, n = 4), with standard Jurkat JKM1 control populations that had not received library (doubling time 26.3 h (SD 3.7)), (p = 0.01). To examine the potential role of apoptosis resistance as a mechanism underlying selection during prolonged culture, we induced Jurkat cell death through ligation of the Fas cell surface receptor, the classical experimental system for the investigation of caspase-dependent apoptosis in T-cells58,69–71.

4 × 10⁷ cells from each replicate (d47, MFZ A–D) were treated with a cytotoxic anti-Fas antibody for 96 h (20 ng/mL) (47d + anti-FAS, MF). Wild-type JKM1 cells do not survive such selection with anti-Fas IgM58. Anti-Fas resistance appeared stable in cultures selected with anti-Fas antibody. Notably, a secondary challenge with 20 ng/mL anti-Fas antibody resulted in 62.8% (SD 17.6) survival after 22 h for the replicates A–D (d47 + anti-FAS, MF), compared to 2.5% (SD 1.2) for control cells.

Our custom sequencing and bioinformatic approaches (see “Materials and methods” section) were used to characterise the complexity of inserts present and the extent of genome coverage achieved by the CL3c lentiviral library, evaluate our ability to transduce the entire library into Jurkat mammalian cells, and to determine the effects of functional selection on the sequences remaining within our selected cell populations. Sequencing data were generated at all points throughout the study from the initial Genomic Digest, CL3c library and Jurkat cells following transduction (d0, JCPZ), to the highly selected cell populations following continuous culture (d47, MFZ) and continuous culture plus anti-FAS IgM (d47 + anti-FAS, MF).

**Read-level analysis.** Our amplicon sequencing approach generated an average of ~34 million mapped read pairs per sample (range 24.6–49.7 million read pairs). Our initial approach considered how these mapped
Continuous culture (d47, MFZ) reduced the number of insert sequences by ~77% and continuous culture + anti-FAS and d47 + Fas, MF) were clearly more focused in discrete areas of the genome than in the unselected samples. PCR amplification step as applied to the remaining samples. Sequencing reads in the selected samples (d47, MFZ) were treated with anti-FAS IgM post continuous culture (d47 + anti-FAS, MF_NoD), consistent with marked selection. Strikingly, less than 10% of the total read count was reached when cells were subjected to continuous culture (d47, MF), d0 samples suggesting a broad distribution of reads from across the entire genome. In contrast, just 24.6% of the total read count was reached for those cells treated with anti-FAS IgM post continuous culture (d47 + anti-FAS, MF, NoD), consistent with even more marked sequence selections. These data confirm that our CL3c library is both highly diverse, and that we were able to transduce Jurkat JKM1 cell populations with very many sequences simultaneously (Table 1).

In order to investigate how the sequencing reads mapped across the genome, and in doing so to explore evidence of reduced sequence diversity in our selected samples, a BAMFingerPrint analysis approach was used. In the absence of selection, one expects a broad distribution of reads across the genome, whereas following selection, one expects focused areas of coverage overlaying those areas of the genome with functional capability. Considering reads contained within 97% of all genomic bins (Fig. 4 red dotted line) revealed that >85% (84.5–85.8) and 72.6% (71.4–74.3) of the total read count was reached for the CL3c samples (library before transduction) and JCPZ, d0 samples suggesting a broad distribution of reads from across the entire genome. In contrast, just 24.6% of the total read count was reached for those cells subjected to continuous culture (d47, MFZ) consistent with marked selection. Strikingly, less than 10% of the total read count was reached when cells were treated with anti-FAS IgM post continuous culture (d47 + anti-FAS, MF, NoD), consistent with even more marked sequence selections. These data support our hypothesis that selection reduces the population of sequences to only those with demonstrable function i.e. the ability to evade otherwise lethal stimuli. It is noteworthy that MF (d47 + anti-Fas) replicate D appeared as an outlier in this analysis, and we make provision for this moving forward by including group level data for d47 + anti-FAS, MF samples both with "MF" and without "MF No_D" this replicate for transparency.

**Insert-level analysis.** We next considered our mapped reads as “insert sequences” by identifying regions of increased read coverage on the genome using MACS2 call peak and using these to define the bounds of library inserts. This enabled us to effectively assemble individual reads into “units” with potential function, and was necessary given the sequencing reads generated were shorter than our insert size distribution i.e. they were not full-length. The genomic coordinates corresponding to peaks in sequence coverage across the entire human genome were converted into bed format and the number of reads mapping to these defined locations determined in each sample using bedtools multicov version 2.29.2.

We identified 845,461 different inserts across our experimental sample set, with a size range of between 132 and 8293 bp. In considering reads mapped specifically to inserts, our initial genome digest sample attracted the least reads (7.25 million), as expected, given this comprised input DNA only and was not the result of a targeted PCR amplification step as applied to the remaining samples. Sequencing reads in the selected samples (d47, MFZ and d47 + Fas, MF) were clearly more focused in discrete areas of the genome than in the unselected samples (Fig. 4), even though the total number of reads mapping to inserts was similar (Table 2).

We next considered the diversity of inserts present within our CL3c library, starting transduced host cell population (d0, JCPZ), and highly selected cell populations. The expression of each insert was normalised to the total number of mapped reads in each sample to account for variation in sequence coverage, and the expression of each insert expressed in terms of “reads per million reads mapped”. The chromosome start and stop position of each insert was added and the nominal insert length calculated.

An insert was considered to be present in each sample when 10 or more reads mapped to that location. The CL3c library contained in excess of 485,000 different insert sequences with the required sequence coverage. Approximately 338,000 inserts were successfully transduced into our starting cell population (d0, JCPZ), representing ~70% of all inserts in the CL3c library. In considering the impact of selection upon insert diversity, continuous culture (d47, MFZ) reduced the number of insert sequences by ~77% and continuous culture + anti-FAS.
IgM treatment (d47 + anti-FAS) by ~90%. These data are consistent with marked functional selection (Table 3), revealing only those sequences that enabled cells to survive the selection conditions.

We next considered the number of reads mapping to each insert as a proxy of the number of cells harbouring that same insert within each cell population. In this regard, the more reads mapping to a given insert sequence indicated a greater presence of cells in the cell population with that insert, and potentially the importance and/or functional potency of the insert. Mean insert presence remained stable irrespective of experimental condition at approximately 1.18 reads per million mapped reads per insert, for CL3c, d0, d47, and d47 + anti-FAS, respectively. Median values revealed a higher presence per insert in the starting library (CL3c = 0.69) and d0 samples (d0, JCPZ = 0.40) than the selected sample sets d47, MFZ (0.067) and d47 + anti-FAS, MF (0.122) [d47 + anti-FAS, MF_NoD (0.133)]. The most striking results however related to the range of insert presence, in particular maximal presence, in each context. These data revealed specific inserts in the selected populations that were present in very many cells—indicating that they afforded a clear survival advantage. Maximal presence was increased dramatically in the selected sample sets; CL3c (1466), d0 (433), increasing to d47, MFZ (32,555), d47 + anti-FAS (28,055) [d47 + anti-FAS, MF_NoD (36,988)], suggesting the existence of large cell sub-populations that harbour a specific insert conferring a proliferation/survival advantage. As demonstrated below in Fig. 5, insert presence

Figure 4. BAMFingerPrint analysis—Proportion of the entire human genome covered by sequence data is shown. Considering reads contained within 97% of all genomic bins (inset panel) revealed that > 85% (84.5–85.8) and 72.6% (71.4–74.3) of the total read count was reached for the CL3c samples (library before transduction) and JCPZ, d0 samples suggesting a broad distribution of reads from across the entire genome. In contrast, just 24.6% of the total read count was reached for those cells subjected to continuous culture (d47, MFZ) consistent with marked selection.

| Experimental group                 | Mean mapped reads (Millions) | S.E.M |
|-----------------------------------|-----------------------------|-------|
| Genome digest                     | 7.25                        | 1.87  |
| CL3c library                      | 17.43                       | 1.82  |
| JCPZ (d0)                         | 17.87                       | 1.27  |
| MFZ (47d continuous culture)      | 21.20                       | 3.08  |
| MF (47d continuous culture followed by anti-FAS IgM) | 19.86                       | 2.61  |
| MF (excluding MF_D*)              | 22.02                       | 2.07  |

Table 2. The number of reads mapping to all inserts for each experimental group.

IgM treatment (d47 + anti-FAS) by ~90%. These data are consistent with marked functional selection (Table 3), revealing only those sequences that enabled cells to survive the selection conditions.

We next considered the number of reads mapping to each insert as a proxy of the number of cells harbouring that same insert within each cell population. In this regard, the more reads mapping to a given insert sequence indicated a greater presence of cells in the cell population with that insert, and potentially the importance and/or functional potency of the insert. Mean insert presence remained stable irrespective of experimental condition at approximately 1.18 reads per million mapped reads per insert, for CL3c, d0, d47, and d47 + anti-FAS, respectively. Median values revealed a higher presence per insert in the starting library (CL3c = 0.69) and d0 samples (d0, JCPZ = 0.40) than the selected sample sets d47, MFZ (0.067) and d47 + anti-FAS, MF (0.122) [d47 + anti-FAS, MF_NoD (0.133)]. The most striking results however related to the range of insert presence, in particular maximal presence, in each context. These data revealed specific inserts in the selected populations that were present in very many cells—indicating that they afforded a clear survival advantage. Maximal presence was increased dramatically in the selected sample sets; CL3c (1466), d0 (433), increasing to d47, MFZ (32,555), d47 + anti-FAS (28,055) [d47 + anti-FAS, MF_NoD (36,988)], suggesting the existence of large cell sub-populations that harbour a specific insert conferring a proliferation/survival advantage. As demonstrated below in Fig. 5, insert presence
was far less focused in the library (CL3c) and starting cell population (d0, JCPZ) in comparison with the selected cell populations. CL3c and d0 JCPZ samples present relatively homogenously across the range of presence values with no obvious “peaks” in presence. In contrast the selected cell populations (d47 MFZ and d47 + anti-FAS MF) present with a large increase in focused presence, and a very large increase in the maximal presence values noted. Maximal presence was increased dramatically in the selected sample sets; CL3c (1466), d0 (433), increasing to d47, MFZ (32,555), d47 + anti-FAS (28,055) [d47 + anti-FAS, MF_NoD (36,988)], suggesting the existence of large cell sub-populations that harbour a specific insert conferring a proliferation/survival advantage. The frequency of inserts at each coverage level in the CL3c library is indicated on all panels by way of a blue line to enable comparison between the initial library and the selected samples. The Y axis terminates at a frequency of ≥ 100 for clarity of data presentation—complete data presented in Supplementary Fig. S5.

**Table 3.** The number of different inserts identified in each experimental group.

| Experimental group                        | Inserts (mean) | S.E.M    | %Reduction |
|------------------------------------------|----------------|----------|------------|
| Genome digest                            | 294,907.00     | 97,030.00|            |
| CL3c library                             | 485,827.00     | 26,833.00|            |
| JCPZ (d0)                                | 337,861.50     | 9140.92  | 30.46      |
| MFZ (47d continuous culture)             | 76,900.75      | 2069.83  | 77.24      |
| MF (47d continuous culture + anti-FAS lgM)| 43,430.75      | 50,235.00| 87.15      |
| MF (excluding MF_D*)                     | 26,838.00      | 5878.10  | 92.06      |

**Figure 5.** Frequency of inserts at defined coverage levels—Initial library (CL3c) and d0 transduced (JCPZ) samples presented relatively homogenously with the vast majority of inserts having a coverage of 20 or less reads per million. In contrast, the selected cell populations (d47 MFZ and d47 + anti-FAS MF) presented with a large increase in focused presence, and a very large increase in the maximal presence values noted. Maximal presence was increased dramatically in the selected sample sets; CL3c (1466), d0 (433), increasing to d47, MFZ (32,555), d47 + anti-FAS (28,055) [d47 + anti-FAS, MF_NoD (36,988)], suggesting the existence of large cell sub-populations that harbour a specific insert conferring a proliferation/survival advantage. The frequency of inserts at each coverage level in the CL3c library is indicated on all panels by way of a blue line to enable comparison between the initial library and the selected samples. The Y axis terminates at a frequency of ≥ 100 for clarity of data presentation—complete data presented in Supplementary Fig. S5.

Annotation and prioritisation of functionally selected insert sequences for further analysis. The data matrix containing insert presence data (normalised as above for sequencing depth), was complemented with a range of additional data to aid interpretation and prioritisation, using a range of custom scripts. The coordinates of restriction enzyme sites (DraI and AanI) were extracted from the human genome and the distance between each insert and a suitable site calculated using the BEDOPS closest-features algorithm. Using the same approach, the distance between each insert sequence and the following features was determined;
information derived from the forward PCR primer and thus reads retaining the ability to orientate a given insert, of independent replicates each insert was identified in, the presence of "directional reads" containing sequence In addition, a range of calculated fields were added to assist in prioritisation, including reporting the number of known features from the GENCODE GRCh38.p13 comprehensive gene annotation superset in GTF format (at
Table 4. Annotation of all library inserts (unselected).

| Feature                        | All inserts (845,461) | High confidence inserts (514,807) |
|-------------------------------|----------------------|----------------------------------|
| Gencode genes                  | 522,106 (62%)        | 328,386 (64%)                    |
| Gencode exons                  | 85,382 (10%)         | 55,777 (11%)                     |
| Gencode introns                | 497,159 (59%)        | 313,598 (61%)                    |
| Intergenic                    | 323,355 (38%)        | 186,421 (36%)                    |
| LNCipedia (V5.2)              | 257,996 (30%)        | 160,962 (31%)                    |
| LNCipedia (V5.2_HC)           | 228,570 (27%)        | 143,279 (28%)                    |
| Cancer IncRNA Census           | 9175 (1.1%)          | 5706 (1.1%)                      |
| Liu et al.73                  | 3029 (0.36%)         | 1864 (0.36%)                     |
| Sarropoulos et al.30          | 34,431 (4%)          | 22,767 (4.4%)                    |

known features from the GENCODE GRCh38.p13 comprehensive gene annotation superset in GTF format (at the gene, intron and exon level) available here:

https://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_human/release_38/gencode.v38.chr_patch_hapl_scaf.annotation.gtf.gz, entries in the LNCipedia database version 5.2\(^{22}\) (standard and high confidence), lncRNAs identified as part of the Cancer IncRNA census dataset\(^{1}\), and lncRNAs identified by Liu\(^{3}\) and Sarropoulos\(^{50}\). In addition, a range of calculated fields were added to assist in prioritisation, including reporting the number of independent replicates each insert was identified in, the presence of “directional reads” containing sequence information derived from the forward PCR primer and thus reads retaining the ability to orientate a given insert, and finally, potentiation scores for 47d and 47d + anti-FAS (calculated as the mean expression of each insert at d47 or 47d + anti-FAS, divided by the mean of each insert at d0). JCZ expressed as a percentage. A separate calculation excluding replicate MF_D was conducted and labelled 47d + anti-FAS_NoD). These operations were performed in RStudio.

Of the 845,461 inserts identified across all samples, 741,912 (87.7%) had a Dra1 or Aan1 site located within 100 bp, 517,732 (61.2%) were supported by directional reads, and 514,807 (60.9% of the total library) had both a suitable restriction enzyme site AND directional read support, herein termed “High Confidence Inserts”. Considering the 845,461 inserts identified across all samples, 522,106 mapped within 100 bp of known genes recorded in the GENCODE GRCh38.p13 comprehensive gene annotation superset. The vast majority of inserts were located within the introns of genes (497,159) with a smaller proportion (85,382) located within exons. By a process of elimination, we identified those inserts that were intergenic on the basis they did not appear within the GENE level annotation file (which naturally encompasses both EXON and INTRON data); intergenic reads accounted for ~ 38% of our total library. Considering reads that were located within 1000 bp of known genes, and therefore within potential gene promoter regions, identified an addition 12,615 inserts (1.5%).

Biotype analysis revealed 361,469 library sequences mapped to protein coding genes, and 142,525 to lncRNAs. Constraining our analysis to exonic regions only revealed 57,459 insert sequences mapped to protein coding genes and 18,960 to lncRNAs, respectively. Comparable results were obtained for both the entire and high confidence datasets. Results of the annotation routine are shown in Table 4.

To identify functional sequences selected in response to continuous cell culture alone (d47, MFZ) and continuous cell culture followed by anti-FAS treatment (d47 + anti-FAS, MF), we prioritised inserts selected in at least 3 independent experimental replicates, and with evidence of directional read support (confirming that the insert sequences were indeed library-derived on the basis that they contained vector sequence prior to trimming). This procedure reduced the many thousands of insert sequences to; 3895, 3778 and 3808 selected inserts for the d47, MF and d47 + anti-FAS, respectively. Biotype analysis was conducted to determine the classification of those sequences that mapped to known genes following comparison with the GENCODE GRCh38.p13 comprehensive gene annotation superset. Of the 3895 d47, MFZ selected sequences, 2458 mapped to known genes; of these, 1653 were protein coding genes and 716 lncRNAs. An additional 64 sequences (2522 in total) were found to be located within 1000 bp of the start of known genes.

Of the 3778 d47 + anti-FAS, MF selected sequences, 2405 mapped to known genes; of these, 1647 were protein coding genes and 678 lncRNAs. An additional 55 sequences (2460 in total) were found to be located within 1000 bp of the start of known genes. In considering only those reads that mapped to exons; 401 mapped to protein coding genes and 181 to lncRNAs in the d47 MFZ condition, and 417 and 174 mapped in the d47 + anti FAS MF condition, respectively. Overall, there was an apparent increase in the number of inserts that mapped to the exons of known gene in the selected cell populations (17%) versus the entire unselected dataset (10%). Annotation information is shown in Table 5 and the resulting datasets are available as Supplementary Tables S1–S3.

The d47, MFZ selected dataset (cells subjected to 47d continuous culture) included 43 lncRNAs with known causal roles in cancer including MIR100HG, AZIN1-AS1, HULC, SAMMSON, HOXB-AS3, and SOX9-AS1. Similarly, the d47 + anti-FAS, MF dataset included sequences derived from 40 lncRNAs with known causal roles in cancer including SChLAP1, DDN-AS1, PCAT-1, SOX2OT, DLEU1, and PCA3 (the 6 most abundant in each case)—on occasion, different fragments of the same lncRNA were identified, potentially demonstrating the parts thereof required for function. The presence of established cancer associated lncRNAs within our dataset, identified by a range of unrelated techniques, adds significant support to our more novel approach. After all, one expects our approach to identify known as well as novel sequences.

DDN-AS1
AZIN1-AS1
SOX2OT
HULC
DLEU1
SAMMSON
SOX9-AS1
HOXB-AS3
MIR100HG
SChLAP1
LNCipedia (V5.2_HC)72 228,570 (27%) 143,279 (28%)
Cancer IncRNA Census\(^{1}\) 9175 (1.1%) 5706 (1.1%)
Liu et al.73 3029 (0.36%) 1864 (0.36%)
Sarropoulos et al.30 34,431 (4%) 22,767 (4.4%)

Table 4. Annotation of all library inserts (unselected).
Intersecting the three datasets (Table 5) allowed the identification of sequences present in both the MF and MFZ (536), MF_NoD and MFZ (538), and MF and MF_NoD (3772) conditions. The MF and MF_NoD datasets were very similar (99% similar) in terms of the sequences prioritised, despite replicate D appearing as an outlier during the bin-level analysis (Fig. 3). Furthermore, approximately 13% of sequences originally selected in the d47, MFZ condition were subsequently re-selected following the induction of apoptosis by anti-Fas antibody (d47 + anti-FAS, MF), suggesting that apoptosis resistance may indeed act as the mechanism for selection by continuous culture, for this significant minority of inserts. Annotation information is shown in Table 6 which describes whether the sequences identified in our experiments have been described by others. It is noteworthy that these comparisons are based upon inserts identified on the basis that they were present in at least three independent replicates and supported by evidence of directional read support. When one considers inserts with any evidence of inclusion (i.e. the presence of reads mapping to those loci), then in excess of 99% of inserts present in the MF condition were present in the MFZ condition, as expected.

Four sequences derived from lncRNAs with known causal roles in cancer were selected in both the d47, MFZ and d47 + anti-FAS, MF conditions (AC084816.1*1, AC087473.11, MIRLET7BHG*1, and TP53TG142–45) and as we have observed previously, multiple fragments of certain lncRNAs were independently selected, potentially highlighting the functional elements of these lncRNAs. Considering selection at the gene level, rather than based upon the isolation of identical sequences contained within, we identified 11 lncRNAs with known causal roles in cancer selected in both the d47, MFZ and d47 + anti-FAS, MF conditions including AC084816.1, AC097103.2, AC087473.1, CASC15*3–7, DLEU1*8–13, ENTPD1-AS1*1, HULC*14–22, MIRLET7BH*1, PCAT-123–33, SChLAP134–41, and TP53TG142–45. The presence of these sequences in the anti-Fas selected populations (d47 + anti-Fas, MF) suggests that suppression of apoptosis is one mechanism that contributes, not only to their selection by continuous culture, but also, potentially, to their association with cancer. Several of these genes have indeed been implicated in apoptosis control by independent laboratories. It is striking that multiple functional sequences were identified from within many of these lncRNAs, for example; three functional sequences derived from PCAT-1 were independently selected (2 × selected in 3 MFZ independent replicates and 1 selected in the MF condition). On the other hand, the same specific functional sequence was identified at least 6 times for TP53TG1 (3 × MFZ and 3 × MF). These data emphasise the power of this strategy to identify functional genes and may also help to reveal the specific elements/minimal composition of each lncRNA required for function. Cancer lncRNAs previously implicated in cancers of the blood are denoted with an asterisk.

In order to gain insight into the performance of a given insert in each experimental condition, we calculated the MF to MFZ ratio, indicative of the proportion of cells harbouring each insert following anti-FAS selection (d47 + anti-FAS, MF), relative to their presence in the continuous culture condition (d47, MFZ). Inserts present

| Feature (within 100 bp) | MFZ_Selected (3895) | MF_Selected (3778) | MF_NoD_Selected (3808) |
|-------------------------|--------------------|--------------------|------------------------|
| Gencode genes           | 2458 (63%)         | 2405 (64%)         | 2426 (64%)             |
| Gencode exons           | 643 (17%)          | 651 (17%)          | 662 (17%)              |
| Gencode introns         | 2349 (60%)         | 2297 (61%)         | 2315 (61%)             |
| Intergenic              | 1437 (37%)         | 1373 (36%)         | 1382 (36%)             |
| LNCipedia (V5.2)        | 1296               | 1264               | 1271                   |
| LNCipedia (V5.2_HC)     | 1167               | 1127               | 1133                   |
| Cancer IncRNA Census    | 4                   | 4                  | 4                      |
| Liu et al.              | 26                 | 25                 | 25                     |
| Sarropoulos et al.      | 320                | 308                | 313                    |
| Undescribed*            | 1110 (28.5%)       | 1068 (28.3%)       | 1073 (28.1%)           |

Table 5. Annotation of the selected insert sequences. *Potential novel (undescribed) inserts were identified on the basis that they lacked any annotation in the databases considered herein.

| Feature (within 100 bp) | MF and MF_NoD common (3772) | MF_ and MFZ common (536) | MF_NoD and MFZ common (538) |
|-------------------------|-----------------------------|--------------------------|-----------------------------|
| Known hg38              | 2403                        | 330                      | 334                         |
| LNCipedia (V5.2)        | 1262                        | 161                      | 162                         |
| LNCipedia (V5.2_HC)     | 1125                        | 146                      | 147                         |
| Cancer IncRNA Census    | 40                           | 4 (11)                   | 4 (11)                      |
| Liu et al.              | 25                           | 4                        | 4                           |
| Sarropoulos et al.      | 308                          | 42                       | 43                          |
| Undescribed*            | 1075                        | 165                      | 163                         |

Table 6. Annotation of the selected insert sequences common across the experimental conditions presented below. *When considering inserts contained within the same lncRNA, but not necessarily in the exact same genomic location. *Potential novel (undescribed) inserts were identified on the basis that they lacked any annotation in the databases considered herein.
in the MFZ condition were, on average, present in the MF condition at ratio of 2.66:1 i.e. the inserts were 2.66 times more abundant on re-selection. Considering only those inserts that met the criteria for inclusion in our selected insert datasets (present in at least three independent replicates and with evidence of directional read support) for both the d47, MFZ and d47 + anti-FAS, MF conditions revealed a ratio of > 10.5:1, suggesting that these inserts were more strongly selected.

**Independent validation of novel cell fate modifying sequences.** Five selected sequences (Table 7) were independently validated on the basis of their selection in both the MFZ and MF conditions, by producing plasmids (pCDNA3.1 sense backbone) containing each chemically synthesised insert of interest, and used to transduce naive Jurkat JKM1 cells, followed by challenge with anti-Fas IgM (see **Materials and methods** section). Viable cell numbers relative to identically treated control cells receiving empty plasmid were determined. Sequence composition of the validation plasmids is included within Supplementary Table S4.

Four out of the five hits subjected to independent validation conferred significant resistance to anti-FAS IgM mediated apoptosis, evidencing that the sequence alone was sufficient for function (Fig. 6). Whilst the mean number of cells in the Chr5:1373 transfected and challenged populations was numerically larger than controls, this observation was not statistically significant nor was the effect size anywhere near that of the other four validated hits.

**Table 7.** Sequences subjected to independent validation using custom pCDNA3.1 plasmids.

| Validated insert location | MFZ potentiation (%) | MF potentiation (%) | Details |
|--------------------------|----------------------|---------------------|---------|
| Chr13: 24,089,729–24,090,017 | 108.3 | 4619.7 | Within SPATA13 |
| Chr7: 137,120,513–137,121,580 | 695.2 | 7344.3 | Inc-PTN-2-102 |
| Chr5: 102,160,152–102,160,734 | 17,877.4 | 9400.3 | Not Described |
| Chr5: 154,258,210–154,258,883 | 12,371.4 | 6124.2 | Intron GALNT10 |
| Chr5: 137,315,818–137,316,592 | 316.7 | 242.1 | Inc-SPOCK1-1-2 |

**Figure 6.** Independent validation—Jurkat cells (5 x 10⁶ cells/ml) were transfected with our candidate functional sequences (see Table 7) in custom pCDNA3.1 plasmids and challenged with 10–20 ng/ml anti-Fas IgM. Viable (Trypan blue-excluding) viable cell concentrations were determined 7 to 18 days after the addition of antibody (see **Materials and methods** section). The mean viable cells ± standard deviation of 3 replicate cultures are shown. In 4 out of 5 cases, transfected cultures were significantly increased over empty vector transfected controls run in parallel (*p < 0.05).
Concluding remarks

Investigation of long non-protein coding RNAs (lncRNAs) has become a key area in biological and biomedical research. Although the analysis of this vast number of transcripts is still at an early stage, it is evident that many lncRNAs play crucial roles in molecular cell biology. Demonstrations of the functional importance of individual RNAs are impressive and growing, however the vast majority—many thousands—of lncRNAs are still entirely uncharacterised, and some of these currently uncharacterised ncRNAs are likely to play critical roles in the control of cell behaviour that have yet to be revealed. There is thus a need for high-throughput approaches that enable the identification of key lncRNAs from across entire genomes (unrestricted by the physiological expression profiles of specific lncRNAs in specific tissues/biological contexts), based upon demonstrable function. This latter requirement ensures that lncRNAs identified through such studies are actively responsible for the effect seen, for example the evasion of apoptosis, as opposed to being passively modulated as a consequence of changes caused by other genes.

Herein we report a genome-scale screening approach that overcomes the limitations imposed by the highly tissue-specific expression of lncRNAs by interrogating sequences from the whole genome. Combining our established forward genetics approach with next generation sequencing and bioinformatics, we identify many thousands of lncRNAs from across the breadth of the genome, based entirely upon their function. The power of this strategy was confirmed both by multiple hits on established cancer genes, and by confirmation of the resistance conferred by selected identified sequences (Fig. 6), suggesting that, although this screen, like all other screens, produces some false positives, these are not highly represented among the strongest hits.

These data alone represent a significant contribution to the identification of lncRNAs involved in the regulation of apoptosis. Our strategy represents a marked step-change in the rate of functional lncRNA discovery and can be applied to an almost endless range of situations through modification of the selection regime and/or cell types used. Whilst our current investigation utilises a human genomic DNA library applied to human cells, there is further scope to apply this strategy to the functional discovery of other lncRNAs in other species e.g. key model species used in drug discovery, and also in the investigation of conservation of function during evolution through the application of a library to cells from a different species. The identification of lncRNAs that control cell fate is essential to our understanding of molecular cell biology as a whole, and many opportunities in diverse fields are likely to result from our approach and initial data.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. Data are also stored in the NIH Sequence Read Archive under project submission SUB10570351. An annotated script has been deposited on the GitHub website which can be accessed here: https://github.com/dptonge/CL3c_Paper_1/blob/main/CL3c_Paper_1_Script.

Received: 15 September 2021; Accepted: 13 December 2021
Published online: 07 January 2022

References

1. Carlevaro-Fita, J. et al. Cancer LncRNA census reveals evidence for deep functional conservation of long noncoding RNAs in tumorigenesis. Commun. Biol. 3, 56. https://doi.org/10.1038/s42003-019-0741-7 (2020).
2. Bard-Chapeau, E. A. et al. Transposon mutagenesis identifies genes driving hepatocellular carcinoma in a chronic hepatitis B mouse model Europe PMC Funders Group. Nat. Genet. 46, 24–32 (2014).
3. Russell, M. R. et al. Tumor and stem cell biology CASC15-S is a tumor suppressor LncRNA at the 6p22 neuroblastoma susceptibility locus. Cancer Res. 75, 3155. https://doi.org/10.1158/0008-5472.CAN-14-3613 (2015).
4. Yin, Y., Zhao, B., Li, D. & Yin, G. Long non-coding RNA CASC15 promotes melanoma progression by epigenetically regulating PDCD4. Cell Biosci. 8, 42 (2018).
5. Fernando, T. R. et al. The LncRNA CASC15 regulates SOX4 expression in RUNX1-rearranged acute leukemia. Mol. Cancer. https://doi.org/10.1186/s12943-017-0692-x (2017).
6. Yao, X.-M., Tang, J.-H., Zhu, H. & Jing, Y. LncRNA CASC15 in Gastric Cancer. I. Investig. Dermatol. 135, 2464 (2015).
7. Edelmann, J. et al. High-resolution genomic profiling of chronic lymphocytic leukemia reveals new recurrent genomic alterations. I. Investig. Dermatol. https://doi.org/10.1111/bjd.2012.04-423517 (2012).
8. Eleneai, M. O. et al. Delineation of the minimal region of loss at 13q14 in multiple myeloma. Genes Chromosome Cancer 36, 99. https://doi.org/10.1002/gcc.10140 (2003).
9. Wang, L.-L. et al. DLEU1 contributes to ovarian cancer tumourigenesis and development by interacting with miR-490–3p and altering CDK1 expression, I. Cell. Mol. Med. 31, 3055. https://doi.org/10.1111/jcmn.13217 (2017).
10. Lee, S. et al. The effects of DLEU1 gene expression in Burkitt lymphoma (BL): Potential mechanism of chemoinmunotherapy resistance in BL. Oncotarget 8, 27839 (2017).
11. Liu, T. et al. LncRNA DLEU1 contributes to colorectal cancer progression via activation of KPN3. Mol. Cancer. https://doi.org/10.1186/s12943-018-0873-2 (2018).
12. Gao, S. et al. Long noncoding RNA DLEU1 aggravates pancreatic ductal adenocarcinoma carcinogenesis via the miR-381/CXCR4 axis. J. Cell. Physiol. 234, 6746. https://doi.org/10.1002/jcp.27421 (2018).
13. Lu, Y. et al. Long noncoding RNA HULC promotes cell proliferation by regulating PI3K/AKT signaling pathway in chronic myeloid leukemia. Gene 607, 41 (2017).
14. Ghafouri-Fard, S., Esmaeili, M., Taheri, M. & Samsami, M. Highly upregulated in liver cancer (HULC): An update on its role in carcinogenesis, J. Cell. Physiol. 235, 9071 (2020).
15. Ding, Y. et al. The significance of long non-coding RNA HULC in predicting prognosis and metastasis of cancers: A meta-analysis. Pathol. Oncol. Res. 25, 311 (2019).
16. Wang, N. et al. Long non-coding RNA hulc promotes the development of breast cancer through regulating lypd1 expression by sponging Mir-6754–5p. Oncotargets Ther. 12, 10671 (2019).
Wang, C. et al. Long noncoding RNA HULC accelerates the growth of human liver cancer stem cells by upregulating CyclinD1 through miR675-PKM2 pathway via autophagy. Stem Cell Res. Ther. 11, 1–14 (2020).

Liu, T. et al. LncRNA HULC promotes the progression of gastric cancer by regulating miR-9–5p/MYH9 axis. Biomed. Pharmacother. 121, 109607 (2020).

Dong, Y. et al. Long non-coding RNA HULC interacts with miR-613 to regulate colon cancer growth and metastasis through targeting RTKN. Biomed. Pharmacother. 109, 2035 (2019).

Lu, W. et al. Long non-coding RNA HULC promotes cervical cancer cell proliferation, migration and invasion via miR-218/TDP52 axis. Oncog. Targets. Ther. 13, 1109 (2020).

Qiao, L. et al. Knockdown of long non-coding RNA prostate cancer-associated ncRNA transcript 1 inhibits multidrug resistance and c-Myc-dependent aggressiveness in colorectal cancer Caco-2 and HT-29 cells. Mol. Cell. Biochem. 441, 99 (2018).

Bi, M., Yu, H., Huang, R. & Tang, C. Long non-coding RNA PCAT-1 over-expression promotes proliferation and metastasis in gastric cancer cells through regulating CDKN1A. Gene 626, 337 (2017).

Zhao, B., Hou, X. & Zhan, H. Long non-coding RNA PCAT-1 over-expression promotes proliferation and metastasis in non-small cell lung cancer cells. Int. J. Clin. Exp. Med. 8, 18482 (2015).

Qiao, L. et al. Down regulation of the long non-coding RNA PCAT-1 induced growth arrest and apoptosis of colorectal cancer cells. Life Sci. 188, 37 (2017).

Ren, Y. et al. The long noncoding RNA PCAT-1 links the microRNA miR-215 to oncogene CRKL-mediated signaling in hepatocellular carcinoma. J. Biol. Chem. 292, 17949 (2017).

Yu, W., Chang, J., Du, X. & Hou, J. Long non-coding RNA PCAT-1 contributes to tumorigenesis by regulating FSCN1 via miR-145–5p in prostate cancer. Biomed. Pharmacother. 95, 1112 (2017).

Zhang, D. et al. Long noncoding RNA PCAT-1 promotes invasion and metastasis via the miR-129–5p–HMGB1 signaling pathway in hepatocellular carcinoma. Biomed. Pharmacother. 95, 1187 (2017).

Huang, J., Deng, G., Liu, T., Chen, W. & Zhou, Y. Long non-coding RNA PCAT-1 acts as an oncogene in osteosarcoma by reducing p21 levels. Biochem. Biophys. Res. Commun. 495, 2622 (2018).

Zhen, Q. et al. LncRNA PCAT-1 promotes tumour growth and chemoresistance of oesophageal cancer to cisplatin. Cell Biochem. Funct. 36, 27. https://doi.org/10.1002/cbf.3314 (2018).

Ge, X. et al. Overexpression of long noncoding RNA PCAT-1 is a novel biomarker of poor prognosis in patients with colorectal cancer. Med. Oncol. https://doi.org/10.1007/s12032-013-0588-6 (2013).

Liu, L. et al. Inducing cell growth arrest and apoptosis by silencing long non-coding RNA PCAT-1 in human bladder cancer. Tumor Biol. 36, 7685. https://doi.org/10.1007/s13277-015-3490-3 (2015).

Bai, X. et al. Long non-coding RNA SChLAP1 regulates the proliferation of triple negative breast cancer cells via the miR-524–5p–HMGA2 axis. Mol. Med. Rep. https://doi.org/10.3892/mmr.2021.12085 (2021).

Du, Z. et al. SChLAP1 promotes to non-small cell lung cancer cell progression and immune evasion through regulating the AUF1/PD-1 axis. Autoimmunity 54, 225 (2021).

Li, et al. Long noncoding RNA SChLAP1 forms a growth-promoting complex with hnRNPI in human glioblastoma through stabilization of ACTN4 and activation of NF-κB signaling. Clin. Cancer Res. 25, 6869 (2019).

Huang, K. & Tang, Y. SChLAP1 promotes prostate cancer development through interacting with EZH2 to mediate promoter methylation modification of multiple miRNAs of chromosome 5 with a DNMT3a-feeding loop. Cell Death Dis. 12, 188. https://doi.org/10.1038/s41419-021-03455-8 (2021).

Mehra, R. et al. Overexpression of the long non-coding RNA SChLAP1 independently predicts lethal prostate cancer. Eur. Urol. 70, 549 (2016).

Li, Y. et al. Long noncoding RNA SChLAP1 accelerates the proliferation and metastasis of prostate cancer via targeting miR-198 and promoting the MAPK1 pathway. Oncol. Res. 26, 131 (2018).

Zhang, J., Shi, Z., Nan, Y. & Li, M. Inhibiting malignant phenotypes of the bladder cancer cells by silencing long noncoding RNA SChLAP1. Int. Urol. Nephrol. 3, 711–716 (2016).

Preissner, J. R. et al. The long noncoding RNA SChLAP1 promotes aggressive prostate cancer and antagonizes the SWI/SNF complex. Nat. Publ. Gr. https://doi.org/10.1038/ng.2771 (2013).

Xiao, H. et al. TP53TG1 enhances cisplatin sensitivity of non-small cell lung cancer cells through regulating miR-18a/PTEN axis. Cell Biochem. 8, 23 (2018).

Gao, W., Qiao, M. & Luo, K. Long noncoding RNA TP53TG1 contributes to radioresistance of glioma cells via miR-524–5p/RAB5A axis. Cancer Biother. Radiopharm. https://doi.org/10.1089/cbr.2020.3567 (2020).

Shao, M., Ma, H., Wan, X. & Liu, Y. Survival analysis for long noncoding RNAs identifies TP53TG1 as an antioncogenic target for the breast cancer. J. Cell. Physiol. 235, 6574 (2020).

Zhang, Y. et al. Long noncoding RNA TP53TG1 promotes pancreatic ductal adenocarcinoma development by acting as a molecular sponge of microRNA-96. Cancer Sci. 110, 2760 (2019).

Morris, K. V. & Mattick, J. S. The rise of regulatory RNA. Nat. Rev. Genet. 15, 432–437 (2014).

Statello, L., Guo, C. J., Chen, L. L. & Huarte, M. Gene regulation by long non-coding RNAs and its biological functions. Nat. Rev. Mol. Cell Biol. https://doi.org/10.1038/s41580-020-00315-9 (2021).

Mattick, J. S. The state of long non-coding RNA biology. Non-Coding RNA 4, 17 (2018).

Kopp, E. & Mendell, J. T. Functional classification and experimental dissection of long noncoding RNAs. Cell 172, 393–407 (2018).

Sarzopoulos, L., Marin, R., Cardoso-Moreira, M. & Kaessmann, H. Developmental dynamics of IncRNAs across mammalian organs and species. Nature 571, 510–514 (2019).

Wapinski, O. & Chang, H. Y. Long noncoding RNAs and human disease. Trends Cell Biol. 21, 354–361 (2011).

Iyer, M. K. et al. The landscape of long noncoding RNAs in the human transcriptome. Nat. Genet. 47, 199–208 (2015).

Schmitt, A. M. & Chang, H. Y. Long noncoding RNAs in cancer pathways. Cancer Cell 29, 452–463 (2016).

Dong, Y. & Chinnaiyan, A. M. The role of non-coding RNAs in Oncology. Cell 179, 1033–1055 (2019).

Unfried, J. P. et al. Identification of coding and long noncoding RNAs differentially expressed in tumors and preferentially expressed in healthy tissues. Cancer Res. 79, 5167–5180 (2019).

Taniue, K. & Akimitsu, N. The functions and unique features of Incris in cancer development and tumorigenesis. Int. J. Mol. Sci. https://doi.org/10.3390/ijms19020463 (2021).

Stark, G. R. & Gudkov, A. V. Forward genetics in mammalian cells: Functional approaches to gene discovery. Hum. Mol. Genet. 8, 1925 (1999).

Sutherland, L. C. et al. LUCA-15-encoded sequence variants regulate CD95-mediated apoptosis. Oncogene 19, 3774–3781 (2000).

Mourtada-Maarabouni, M. et al. Functional expression cloning reveals proapoptotic role for protein phosphatase 4. Cell Death Differ. 10, 1016–1024 (2003).

Williams, G. T. et al. Isolation of genes controlling apoptosis through their effects on cell survival. Gene Ther. Mol. Biol. 10, 255 (2006).

Pickard, M. R. & Williams, G. T. Molecular and cellular mechanisms of action of tumour suppressor GASS LncRNA. Genes. https://doi.org/10.3390/genes6030484 (2015).
62. Klee, C., Prinz, F. & Pichler, M. Involvement of the long noncoding RNA NEAT1 in carcinogenesis. Mol. Oncol. https://doi.org/10.1002/1878-0261.12404 (2019).
63. Pickard, M. R. & Williams, G. T. The hormone response element mimic sequence of GAS5 lncRNA is sufficient to induce apoptosis in breast cancer cells. Oncotarget. https://doi.org/10.18632/oncotarget.7173 (2016).
64. Frank, E. et al. The IncRNA growth arrest specific 5 regulates cell survival via distinct structural modules with independent functions. Cell Rep. https://doi.org/10.1016/j.celrep.2020.107933 (2020).
65. Chilón, I. & Marcia, M. The molecular structure of long non-coding RNAs: Emerging patterns and functional implications. Crit. Rev. Biochem. Mol. Biol. 55, 662 (2020).
66. Williams, G. T., Critchlow, M. R., Hedge, V. L. & O’Hare, K. B. Molecular failure of apoptosis: Inappropriate cell survival and mutagenesis? Toxicol. Lett. 102–103, 485–489 (1998).
67. Zhang, Y. et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 9, R137 (2008).
68. Quinlan, A. R. & Hall, I. M. BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics 26, 841–842 (2010).
69. Fujino, M. et al. Distinct pathways of apoptosis triggered by FTY720, etoposide, and anti-Fas antibody in human T-lymphoma cell line (Jurkat cells). J. Pharmacol. Exp. Ther. 300, 939–945 (2002).
70. Gottlieb, R. A., Nordberg, J., Skowroński, E. & Babior, B. M. Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification. Proc. Natl. Acad. Sci. U.S.A. 93, 654 (1996).
71. Longthorne, V. L. & Williams, G. T. Caspase activity is required for commitment to Fas-mediated apoptosis. EMBO J. 16, 3805–3812 (1997).
72. Volders, P.-J. et al. LNCipedia: A database for annotated human lncRNA transcript sequences and structures. Nucleic Acids Res. 41, D246. https://doi.org/10.1093/nar/gks915 (2013).
73. Liu, S. Y. et al. CRISPRi-based genome-scale identification of functional long noncoding RNA loci in human cells. Science 355, 7111. https://doi.org/10.1126/science.aab7111 (2017).
74. Zhou, L. et al. Long non-coding RNA PCAT-1 regulates apoptosis of chondrocytes in osteoarthritis by sponging miR-27b-3p. J. Bone Miner. Metab. 39, 139–147 (2020).

Acknowledgements
Work in the Molecular Medicine Group at King’s is supported by the Wellcome Trust, Medical Research Council (MRC), LifeArc and Biochemical Biomedical Research Council (BBSRC), as well as the Experimental Cancer Medicine Centre at King’s College London, the Cancer Research UK Centre at King’s Health Partners and by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy’s and St Thomas’ NHS Foundation Trust and King’s College London.

Author contributions
D.P.T. developed and implemented the next generation sequencing and bioinformatics approach reported herein, performed the library preparations and bioinformatic analyses, designed the validation plasmids, analysed and interpreted the resulting data, and prepared the manuscript. D.D. prepared the CL3c library at scale, performed the library preparations and bioinformatic analyses, designed the validation plasmids, analysed and interpreted the resulting data, and prepared the manuscript. G.T.W. conceived and developed the CL3c library, performed all cell culture and selection experiments reported herein, interpreted the resulting data, and refined the manuscript. F.F. provided expert advice and access to state-of-the-art lentiviral processing facilities, analysed and interpreted the resulting data, and refined the manuscript. G.T.W. conceived and developed the CL3c library, performed all cell culture and selection experiments reported herein, interpreted and refined the manuscript, and prepared the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-03983-5.
Correspondence and requests for materials should be addressed to D.P.T.
Reprints and permissions information is available at www.nature.com/reprints.
Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.
© The Author(s) 2022