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The long intergenic non-coding RNA CCR492 functions as a let-7 competitive endogenous RNA to regulate c-Myc expression.

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Abstract

In mammals the cell-cycle progression through the G1 phase is a tightly regulated process mediated by the transcriptional activation of early genes in response to mitogenic stimuli, whose dysregulation often leads to tumorigenesis.

We here report the discovery by RNA-seq of cell-cycle regulated (CCR) long intergenic non-coding RNAs (lincRNAs), potentially involved in the control of the cell cycle progression. We identified 10 novel lincRNAs expressed in response to serum treatment in mouse embryonic fibroblasts (MEFs) and in BALB/c fibroblasts, comparably to early genes. By loss-of-function experiments we found that lincRNA CCR492 is required for G1/S progression, localizes in the cell cytoplasm and contains 4 let-7 microRNA recognition elements (MREs). Mechanistically, CCR492 functions as a competing endogenous RNA (ceRNA) to antagonize the function of let-7 microRNAs, leading to the de-repression of c-Myc. Moreover, we show that ectopic expression of CCR492 along with a constitutively active H-Ras promotes cell transformation. Thus, we identified a new lincRNA expressed as an early gene in mammalian cells to regulate the cell cycle progression by upregulating c-Myc expression.

Keywords: non-coding RNA, c-Myc, let-7, ceRNA, cell cycle

1. Introduction

The portion of the genome deputed to the transcription of protein-coding genes in mammals is limited to less than 2%, while the majority of the transcripts is represented by non-coding RNA (ncRNAs). To date, the function and regulation of many short non-coding RNAs such as ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), microRNAs (miRNAs) and small nuclear RNAs (snRNAs) have been well studied. Much less is known about the other group of ncRNAs so far defined as long non-coding RNAs (lncRNAs). LncRNAs have been described as transcripts longer than 200 nucleotides with no evidence of coding for functional proteins. Long intergenic non-coding RNAs (lincRNAs) are lncRNAs which lie within a genomic region devoid of annotated genes. Large-scale sequencing of cDNA libraries estimated that several thousand lncRNAs are transcribed in mammalian cells in a tightly regulated cell and developmental specific way, although only a few of them have been studied [1]. They can exert a variety of different functions, such as epigenetic regulation, transcriptional, post-transcriptional and post-translational processing [2,3].

The cell cycle is the sequence of events that occurs in cells stimulated to grow. A complex network of signalling pathways regulates the entry into the cell cycle and is crucial for the cell homeostasis. Normal cells, like fibroblasts, require growth factors to proliferate, which are provided *in vitro* by the addition of fetal bovine serum (FBS). In serum deprivation conditions, the cells enter in a quiescent state, termed G0, while the addition of serum triggers a proliferative response. This system has been widely used as a model for studying growth control and cell-cycle progression and the identification of the early genes that are induced by mitogens to progress into the in G1 phase and whose genetic and epigenetic alterations led to cell transformation.

miRNAs are short ncRNAs (~22 nucleotides long) that serve as guides for targeting the RNA to imperfectly complementary miRNA recognition elements (MREs) within target mRNAs inducing both translational repression, and mRNA decapping/deadenylation [4]. Several studies have shown that miRNAs can

control the expression levels of genes involved in the cell-cycle regulatory machinery [5,6]. The let-7 miRNA was initially discovered in *Caenorhabditis elegans* [7], and has been shown to be highly evolutionarily conserved [8]. Let-7 is undetectable in embryonic stem cells, but it is expressed at high levels in adult tissues and its expression is deregulated or lost in many cancers [9,10].

In order to characterize the control circuitry underlying cell proliferation, we set up a screening approach to identify novel cell-cycle regulated (CCR) lincRNAs. We found a number of new lincRNAs specifically upregulated by mitogens as early genes. Moreover, by loss-of-function experiments we identified one lincRNA that promotes G1/S progression by upregulating c-Myc expression and whose deregulation contributes to cell transformation.

2. Materials and methods

2.1 Cell culture

Primary mouse embryonic fibroblasts (MEFs) were derived from 13.5d pregnant female mice and BALB/c fibroblasts (ATCC, Manassas, VA, USA) were cultured as previously described [11]. Briefly, the cells were maintained in high-glucose DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sigma), 1 mM sodium pyruvate (Invitrogen), 50 U/ml of penicillin/ml, and 50 ug/ml of streptomycin/ml. Cell synchronization was performed as previously described [12] by starving MEFs in 0.2% serum for 48 hours and BALB/c fibroblasts in 0% serum for 48 hours then released into cell cycle by adding 20% serum for the times indicated (30 minutes, 1 hour and 2 hours).

2.2 RNA extraction and quantitative real-time PCR (RT-qPCR)

RNA extraction and RT-qPCR were performed as previously described [13]. Briefly, RNA was extracted using TRIzol reagent (Invitrogen), following manufacturer instructions. RNA integrity measurements were performed using Fragment Analyzer™ (Advanced Analytical). All samples had RNA Quality Number (RQN) greater than 9.8. RT-qPCR was performed using the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, cat.11732-020) following the manufacturer's instructions. Primers sequences are shown in Supplementary Table S2**.**

2.3 RNA-seq library preparation

For RNA-seq library preparations, 2 μg of total RNA were used as input for the TruSeq RNA Library Prep Kit v2 (Illumina), and libraries were prepared following manufacturer instructions.

2.4 Sequencing and bioinformatics analysis

Libraries were normalized, pooled, and sequenced on the Illumina HiScanSQ Platform. Reads were pre-processed using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), to remove poor-quality reads, and clip adapter sequences. After pre-processing, the obtained high quality paired-end reads were mapped to mouse genome (Mus musculus, assembly mm9) using the TopHat v2.0.0 spliced-mapper (http://tophat.cbcb.umd.edu/), a gapped aligner able to discover new splice-junctions *ab initio*. This resulted in approximately 1.4 billion mappings. Reads mapped from TopHat were then assembled into a parsimonious set of transcripts using Cufflinks v2.0.2. Cufflinks uses TopHat output to build the minimal set of transcripts that can explain the majority of mapped reads. To increase Cufflinks accuracy, we provided it with an annotation of both annotated and predicted transcripts from Ensembl release 68 (July 2012). At this stage, Cufflinks produced approximately 3 x 10^5 transcripts, many of which are single exon transcripts deriving from transcriptional noise, residuals of pre-mRNAs, and exons that Cufflinks was unable to connect due to

the lack of spliced-read information. To clean our annotation from background noise, we designed a filtering pipeline, which allowed us to simultaneously remove low-fidelity transcripts, and to select putative previously unannotated transcripts lacking coding potential. (1) All single exon transcripts, and multi-exon transcripts shorter than 200 bases were excluded; (2) Using Cufflinks read coverage estimation we excluded those transcripts with a maximal coverage below 3 reads per base; (3) We removed all transcripts that have at least an exon overalapping a transcript from RefSeq, UCSC, Ensembl, and Vega annotation sets, and any transcript overlapping known rRNAs, tRNAs, miRNAs and snoRNAs; (4) To estimate the coding potential of novel transcripts, we used two different Support Vector Machines (SVM) trained on different sets of lncRNAs, namely iSeeRNA and Coding Potential Calculator {Kong:2007hx}. Transcripts identified by at least one of these two SVMs as coding, were excluded. The final annotation yielded approximately 750 high-fidelity long non-coding transcripts. To determine which transcripts may have a role in cell proliferation, we then performed differential expression analysis across time-course samples, using Cuffdiff, and selected only those showing significant upregulation upon serum induction with respect to serum-starved cells (point 0 of our time-course).

miRNA target prediction on lincRNA CCR492 was performed using three algorithms: MREdictor [14], RNAhybrid {Kruger:2006ek}, and Pita [15].

2.5 Proliferation assay and flow cytometry analysis

For cell-growth assay, 5 x 10^4 cells were plated in 35 mm wells and counted at the indicated time point using Scepter™ Automated Cell Counter (Millipore). A growth curve was plotted to examine the effects of the shRNAs on cell proliferation. 72 hours post transfection, the cells were harvested, fixed in 70% ethanol, and stained for flow cytometry analysis. For the One-dimensional cell-cycle analysis, the cells were stained with propidium iodide (PI) solution (0.1% TRITON X-100, 200 mg/ml RNase, 20 mg/ml PI in PBS buffer) for 30 minutes at room temperature. Two-dimensional cell-cycle analysis was performed using

Click-iT EdU-Cell Cycle 633-red assay from Invitrogen according to the manufacturer's protocol with 1 hours of EdU pulse. Acquisition was performed using Becton Dickinson FACS Canto and analysis was done with FACS FlowJo Software.

2.6 Constructs and transfection

shRNAs were constructed using the TRC hairpin design tool (http://www.broadinstitute.org/rnai/public/seq/search), choosing the hairpin sequences provided in Supplementary Table S3. Annealed oligonucleotides were cloned into pLKO.1 vector (Addgene: 10878), and each construct was verified by sequencing. For shRNA transient transfection, 5 ug of PLKO.1 vectors against lincRNAs and control hairpins were incubated with cells for 24 hours. To generate the CCR492 expression plasmid the full length of CCR492 was PCR amplified from MEF cDNA and inserted into pCCLsin.PPT.hPGK.GFPpre using AscI/SalI restriction sites as previously described [16]. The primers are shown below:

CCR492-FW: 5'-TTGGCGCGCCTATCTTTATCCCTGAACTTTCTTTC-3' and CCR492-REV: 5'-GCGTCGACCATCATGTGTCTGTGTACAAG-3'. One deleted mutant of CCR492 was produced by PCR to create pCCL-CCR492-Mutant (Δ162) with a new reverse primer: CCR492 Mut-REV: 5'-GCGTCGACTAACCGCTGATATCTCTCCAG-3'.

A 297-bp fragment of c-Myc 3'UTR containing the let-7 target-miRNA seed region was PCR amplified from c-Myc cells cDNA, and cloned into MluI/SpeI sites of pMIR-Report (Invitrogen, cat. AM5795) to generate pMIR-RLuc-Myc-3'UTR. Primer sequences are as follows: c-Myc-3'UTR-FW: 5'-ACGCGTACTGACCTAACTCGAGGAGGA-3' and c-Myc-3'UTR-REV: 5'-CCCTATTTACATGGGAAAATTGGATACTAGT-3'. Transient transfections of the constructs were performed using Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol.

2.7 RNA/protein extracts and Western blotting

RNA and protein extracts of cytoplasmic and nucleoplasmic fractions were isolated using the PARIS kit (Ambion). For total protein extracts, cells were resuspended in F-buffer (10 mM TRIS-HCl pH 7.0, 50 mM NaCl, 30 mM Na-pyrophosphate, 50 mM NaF, anti-proteases) and sonicated for 3 pulses. Extracts were quantified using bicinchoninic acid (BCA) assay (BCA protein assay kit; catalog no. 23225; Pierce) and were run in SDS-polyacrylamide gels, transferred to nitrocellulose membranes and incubated 16 hours with specific primary antibodies. The antibodies were purchased from Abcam (anti-c-Myc-ab11917), Sigma-Aldrich (anti-b-tubulin-T8328), SantaCruz (anti-Ras-sc520, anti-LaminA-sc20680).

2.8 RNA Fluorescent In Situ Hybridization (RNA-FISH)

5'-biotinylated probes targeting CCR492 were purchased from Eurofins MWG Operon. Probes sequences were as follows:

Probe1:

5'-CTTGCAGGGAGATGGGAAGTCTCCAGTGGCCAAGCTGATGTGAGGA-3';

Probe2:

5'-CATGGGTCTCCATCCAAACATTGGGCTACAGGTTTTCGGCTACAAA-3'.

2 x 10^4 BALB/c fibroblasts were seeded onto 0.1% gelatin-coated glass slide chambers. Then, cells were rinsed once in PBS buffer, fixed in 4% Paraformaldehyde and permeabilized with 0.5% TRITON X-100. Cells were saturated in saturation buffer (2x SSC, 1% BSA) for 2 hours a room temperature. Bioinylated probes were diluted 1:1000 in hybridization buffer (2x SSC, 10% formamide, 100 mg/mL dextran sulfate) and heat denatured and incubated with the cells at 37°C for 3 hours in a humidified chamber protected from light. After the incubation cells were rinsed twice in PBS buffer. DAPI (0.5 mg/ml) was used to visualize cell nuclei and the images were performed with Leica TCS SP5 confocal microscope.

2.9 Monolayer and soft agar colony-formation assays

For monolayer colony-formation assay, 1000 BALB/c fibroblasts were transduced with pCCL-CCR492 and H-RasV12 (Addgene Plasmid 9051: pBABEpuro H-RasV12) expression vectors and plated in 100 mm^2 plates and allowed to grow in appropriate culture medium for 10 days. Fresh media were supplied every 3 days. Colonies were stained with crystal violet dye after formaldehyde fixation. Sft agar colony-formation assays, 3 x 10^4 cells/ml were transduced in the same conditions and seeded on top of a solidified layer in a volume of 2 ml of 0.5% Bacto Agar (Sigma-Aldrich) over 2 ml 0.4% agar base layers in each six-well plate as previously described [17].

2.10 Luciferase assay

For the luciferase assays, 3 x 10^3 BALB/c fibroblasts were seeded per well in a 96-well plate the day before transfection. The cells were transfected with: 25 ng of pMIR-RLuc-Myc-3'UTR vector bearing the tested MRE, 1,25 ng of SV40-Renilla and either 86,9 ng of shControl or 86,9 ng of shCCR492, plus 86,9 ng of empty vector or 86,9 ng of CCR492 Mutant. For complementation assay cells were also transfected with 30 nM miRNA Inhibitor negative control #1 (Ambion) or a 30 nM pool of let-7 miRNA Inhibitors (Ambion). Firefly luciferase activity was assayed after 48 hours using the Dual Luciferase Reporter Assay System (Promega, cat. E1910) and was normalized over the Renilla intensity.

2.11 Statistical analyses for experimental studies

All data are presented as means ± S.E.M. All experimental assays were performed in duplicate or triplicate. Statistical analyses shown in figures represent two-tailed Student t-tests, as indicated.

3. Results

3.1 Cell-cycle re-entry by serum promotes the expression of lincRNAs

To identify lincRNAs expressed as early genes upon mitogen stimulation, we extracted the RNA from cells harvested at different time points after serum treatment (Figure 1A, Figure S1A-B). Short-read gapped alignment yielded approximately 1.4 billion mappings on the mouse genome. Reference annotation based transcript (RABT) assembly of mapped reads generated about 3 x 10^5 potential transcripts. We then designed a filtering pipeline to select for previously unannotated transcripts lacking coding potential. All single exon transcripts, transcripts shorter than 200 bases, transcripts with low coverage (< 3 reads per base on average), transcripts that have been previously annotated and transcripts with putative coding potential were excluded (Figure 1B). Furthermore, we selected only those transcripts whose expression was significantly upregulated at any time point following serum induction. This screening produced a set of 19 novel high-fidelity long intergenic non-coding transcripts upregulated by serum treatment in MEFs (Table S1). We confirmed their serum induction by quantitative PCR (RT-qPCR) (Table S2). The same expression profile was confirmed for 10 of them in BALB/c fibroblasts (Figure 1C, Figure S1D). We named these transcripts cell-cycle regulated (CCR) lincRNAs.

3.2 Characterization of CCR492 a lincRNA that controls cell-cycle progression in fibroblasts

To explore the biological role of the identified lincRNAs we performed loss-of-function experiments by transfecting BALB/c fibroblasts with short hairpin RNAs (shRNAs) against each lincRNA (Table S3). Silencing of 2 lincRNAs (CCR102 and CCR492) showed a significant reduction of cell proliferation (Figure S2B-C). We focused on lincRNA CCR492 which exhibited the highest induction by serum (>4 fold), a comparable expression profile in both MEFs and BALB/c fibroblasts, and the strongest effect on cell proliferation once depleted.

The CCR492 gene maps on chromosome 4 in a 200kb-long intergenic region between the *Bnc1* and *Cntln* protein-coding genes. It is composed of 4 exons and generates a mature transcript of 953 nt (Figure 2A, Figure S2A). The promoter specific H3K4me3 modification and the presence of the H3K36me3 along the gene body indicate its active transcription in fibroblasts (Figure 2A).
CCR492 silencing induced a strong decrease in cell proliferation (Figure 2B-C and D upper panel). Analysis of the cell cycle by fluorescence-activated cell sorting (FACS) revealed an increase of G1-phase cells, together with a reduction of S-phase cells upon CCR492 knockdown (Figure 2D lower panel). To verify that the effect on cell proliferation was not due to off-target effects of the shRNAs, we performed a complementation experiment by expressing a CCR492 deletion mutant lacking the shRNA pairing region. Analysis of cell proliferation showed that ectopic expression of the lincRNA was able to rescue the wild-type proliferation phenotype, supporting the involvement of CCR492 in regulating the cell cycle in fibroblasts (Figure 2E-G).

3.3 CCR492 is a cytosolic lincRNA

Next we examined the subcellular localization of CCR492 in BALB/c fibroblasts by nuclear and cytoplasmic subcellular fractionation (Figure 3A). The RT-qPCR showed that CCR492 RNA was prevalently present in the cell cytoplasm (Figure 3B). We further analysed the cellular distribution of CCR492 by RNA Fluorescent in Situ Hybridization (RNA-FISH). CCR492 signal was visible in the cytoplasm while no signal was evident in the DAPI-stained nuclei (Figure 3C, Figure S3A).

3.4 CCR492 acts as a molecular sponge modulating let-7 activity

Considering that CCR492 is expressed in the cytoplasm in response to growth factor treatment to favour the cell-cycle progression, we hypothesized that it could act as a competing endogenous RNA (ceRNA) for miRNAs targeting genes involved in cell growth. By performing small RNA-seq we identified the top 20 miRNAs expressed in MEFs, and bioinformatically evaluated the presence of the corresponding MREs on CCR492.

Our analysis revealed that CCR492 contains 2 or more putative MREs for 13 of them (Figure S3B). We focused our attention to the let-7 miRNAs, which showed 4 high-affinity MREs to CCR492 (Figure 3D-E, Figure S3C) and are known to play a role in cell growth [10] . To evaluate the relationship between CCR492 and let-7 we performed a cell-growth assay in the presence of let-7 inhibitors {Yang:2014hz}.

The silencing of CCR492 does not affect cell growth in the presence of let-7 miRNA inhibitors indicating that CCR492-dependent phenotype requires the presence of let-7 miRNA (Figure 3F and G). Taken together these experiments show that CCR492 acts by titrating let-7 cellular level to limit its activity.

3.5 CCR492 regulates c-Myc expression

The intersection of the serum-induced genes and those predicted to be let-7 target genes identified 14 putative target RNAs (Figure 4A). Most of these are not related with the cell cycle and are not affected by CCR492 silencing (Figure S4A). Interestingly, c-Myc, which is known to play a central role in cell proliferation, showed a significant decrease of expression in CCR492 silenced cells both at the RNA and protein levels (Figure 4B-C, Figure S4A).

To verify whether the effect of CCR492-dependent depletion of let-7 acted directly on the c-Myc RNA, we cloned the 3' UTR of c-Myc (Figure S4B) downstream of a firefly luciferase reporter gene (pRLuc-c-Myc 3'UTR), and analysed the effect of CCR492 knockdown on the c-Myc 3'UTR. The inhibition of CCR492 expression resulted in a significant reduction of the luciferase expression with respect to the control plasmid. Importantly, the inhibitory effect on the luciferase construct by CCR492 silencing was abolished either by the co-expression of the CCR492 mutant or by let-7 miRNA inhibitors (Figure 4D). Taken together these results demonstrate that CCR492, by binding to let-7 family miRNAs, acts as ceRNA reducing the let-7 miRNA repressing activity on c-Myc.

3.6 CCR492 contributes to cell transformation

The above results demonstrate that the expression of CCR492 in response to mitogenic stimuli antagonizes the function of let-7. The finding that CCR492 protects c-Myc mRNA by sequestering let-7 miRNAs prompted us to verify whether CCR492 can cooperate to cell transformation. To this end we transfected BALB/c fibroblasts with a constitutively active mutant of the H-Ras proto-oncogene (H-Ras V12), with and without CCR492. As expected, significantly increased c-Myc expression (Figure 5A). Importantly, the ectopic expression of CCR492 strongly induced the Ras-dependent transformed foci and soft agar colonies compared to either control cells and cells overexpressing H-Ras alone (Figure 5B and C, Figure S5A). Taken together these data show that CCR492 plays a role in the control of the cell cycle and when overexpressed contributes to cell transformation.

4. Discussion

We have here reported the identification of lincRNAs whose expression is induced by mitogens in mouse fibroblasts and the characterization of a novel lincRNA, named CCR492, that plays a role in cell proliferation by upregulating the expression of c-Myc. Mechanistically, CCR492 acts as a ceRNA to compete for let-7 miRNAs binding to c-Myc 3' UTR (Figure 6).

In response to serum treatments quiescent fibroblasts trigger a cellular response that is known to upregulate over one hundred coding genes including the nuclear factors c-Jun, c-Fos, c-Myc, and many genes coding for other cellular components required to allow cells to proceed toward the G1 phase. Only recently, the importance of non-coding RNAs in cell regulatory circuits has become clear. We therefore performed a screening to identify non-coding RNAs involved in the regulation of the cell growth. Since it is impossible to predict RNA function from its primary sequence alone, we performed a screen selection based on their regulation in response to mitogenic signals to identify new lincRNAs, possibly involved in the control of the cell-cycle progression. Thus, we took advantage of the knowledge accumulated from the studies of protein coding genes, which revealed the importance of the early genes in the cell cycle regulatory circuit, to apply the same strategy to screen for lincRNAs.

Our screening identified a number of transcripts induced in starved MEFs by serum, and whose induction could also be reproduced in BALB/c fibroblasts which represent a useful experimental tool to study gene function.

We further characterized the functional role of one of such transcripts that we named CCR492. This is a novel non-coding RNA, which we named CCR492, of 953 base pairs, strongly induced by serum, whose gene, organized in 4 exons, maps in a gene devoid region of chromosome 4 distant about 65 kb from the closest gene. By functional experiments we found that CCR492 contributes to the progression of the cell cycle as its silencing significantly reduces the fibroblasts growth rate affecting the cell entry into the S phase as expected from its induction profile.

We found that CCR492 localizes to the cell cytoplasm. LincRNAs have been shown to function in several contexts to regulate gene expression [18]. In the cytoplasm, lncRNAs have been proposed to function as ceRNAs to modulate the availability of miRNAs, sequestering them from their protein coding target RNAs [19]. In agreement with this model, a number of transcripts that act as potential ceRNAs have been identified in different cellular contexts [20-24]. Inspection of CCR492 sequence revealed the presence of 4 high-affinity recognition elements for the let-7 family of miRNAs, which are known to be involved in the control of the cell cycle [10] suggesting that CCR492 could function as a sponge for let-7 miRNAs. Indeed, in the presence of let-7 inhibitors the effect of CCR492 depletion was abolished demonstrating that CCR492 acts as a ceRNA to the let-7 miRNAs. One important target of let-7 is c-Myc[25] that contains a let-7 MRE on its 3'UTR region and has been recently demonstrated that even modest variations of let-7 levels significantly alter the c-Myc expression [26].

We found that the silencing of CCR492 results in a significant decrease of Myc expression in fibroblasts. Importantly, we could demonstrate that this regulation is via the c-Myc 3'UTR and it is abolished by let-7 inhibitors. Thus, our results are compatible with a model (Figure 5D) in which CCR492, binding to the let-7 miRNAs, releases c-Myc from let-7 dependent inhibition of expression.

In agreement with this result we found that the constitutive expression of CCR492 in fibroblasts cooperates with mutated H-Ras to promote cell transformation.

Let-7 family of microRNAs must be tightly regulated in the cells as their deregulation leads to developmental alterations and cancer [9,10]. However, their regulation is quite complex as in mammals the family of let-7 microRNAs is present in multiple copies in the genome. Let-7 expression is regulated at the transcriptional and post-transcriptional level. In spite of their different genome organization let-7 precursors share conserved structural elements and the sequence of the mature microRNA is highly conserved. One way to regulate let-7 expression is by Lin28 proteins which inhibit the mature form by their specific interaction with let-7 precursors [27-29]. Another simple and effective way to

regulate let-7 function is to inhibit their activity by direct interaction with the mature microRNAs as all let-7 genes share the same seed to bind to their targets. Indeed this is accomplished by the expression of non-coding RNAs containing multiple MREs. In humans, the lncRNA H19 functions as a sponge to antagonize let-7 to regulate muscle differentiation [24]. We here reported a different lincRNA that is not conserved hence performs the same function of inhibiting let-7 activity in mouse in a different cellular context. Thus demonstrating the parallel evolution of different molecules to achieve the same regulatory mechanism establishing the importance of this mechanism for the control of let-7 microRNAs.

5. Conclusions

We here report, for the first time, the identification of a lincRNA expressed as an early gene in mammalian cells whose expression is required for the fine tuning of the cell cycle progression. LincRNA CCR492 regulates c-Myc expression in the G1 phase of the cell cycle acting as a sponge to let7 microRNAs.

Authorship and disclosures

MM, FN, AK, SR, FA, DD, CP, and GB performed the experiments and discussed the data; DI and RC made the analyses; MM, DI, and SO were involved in study design analyzed the data and wrote the paper. The authors declare that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

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FIGURE LEGENDS

Fig.1. Discovery of novel serum-induced lincRNAs through RNA-Seq in MEFs. (A) Experiment layout to identify serum-dependent transcription. Starved MEFs were induced with serum for the times indicated. (B) A schematic overview of the lincRNAs discovery pipeline employed. (1) Paired-end reads from Illumina sequencing are mapped to the mouse genome; (2) A parsimonious set of transcripts justifying the observed read is built using Cufflinks reference annotation based transcript (RABT) assembly; (3) Transcripts are filtered by length, number of exons, and coding potential; (4) Transcripts induced by serum are selected at each time point. (C) RT-qPCR validation of representative lincRNAs in BALB/c fibroblasts with specific oligonucleotides. The gene expression values were normalized to β-actin. Data are presented as mean values $\pm$ SD of 3 independent experiments; $^{**}P < 0.01$, $^{*}P < 0.05$ by the Student's *t*-test.

Fig. 2. CCR492 depletion suppresses the G1/S phase progression. (A) Schematic representation of the chromosomal location of the CCR492 lincRNA. The arrowhead indicates the transcription direction. Chromatin structure is shown from fibroblasts ChIP-seq data for histone modifications [30]. Positions of shRNAs directed against exon 4 are indicated. (B) Expression of CCR492 in BALB/c fibroblasts transfected with either a non-targeting control (shControl) or shRNAs targeting lincRNA CCR492 (shCCR492 #1, #2). (C) Cell-growth curve assay in control and CCR492 silenced fibroblasts. (D) Upper panel: bright field of control and CCR492 silenced fibroblasts. Lower panel: EdU-CellCycle 633-red flow cytometry analysis. Cells were pulsed with EdU for 1 hours following 72 hours transfected with shControl or shCCR492 #1, #2. Scatter plot histograms of EdU-labeled cells were stained for DNA content (X-axis) and EdU (Y-axis). The following populations are shown: G0/G1, S, and G2/M. (E) Schematic representation of the CCR492 wild type and mutant (Δ 162) used for the rescue experiment. (F) Expression levels of the endogenous CCR492 were determined

by RT-qPCR. Data are presented as mean values ± SD of 3 independent experiments; **P < 0.01, *P < 0.05 by the Student's *t*-test. (G) Cell-growth curve assay of CCR492 silenced fibroblasts in the absence or presence of the mutant CCR492.

Fig. 3. CCR492 acts as a natural decoy for let-7. (A) Western blot of subcellular fractionation in BALB/c fibroblasts. Controls of subcellular fractionation are β-Tubulin for cytoplasm, and Lamin A/C for nucleus. (B) RT-qPCR of subcellular fractionation in BALB/c fibroblasts. Controls of subcellular fractionation are β-actin for cytoplasm, and U1 for nucleus. (C) Single-molecule visualization of CCR492. Top left shows RNA-FISH performed with 5'-biotinylated probe #1 in BALB/c fibroblasts. Bottom left shows the negative control. Middle panels show staining with DAPI, and the right panels show merged images. (D) Analysis of potential MREs in CCR492. (E) Schematic representation of the 4 bioinformatics predicted let-7 binding sites in CCR492. (F) Expression level of CCR492 in control and CCR492 silenced fibroblasts, in the absence or presence of let-7 inhibitors.
(G) Cell growth assay with let-7 Inhibitors in CCR492 depleted BALB/c fibroblasts. Expression levels of the endogenous CCR492 were determined by RT-qPCR. Data are presented as mean values ±SD of 3 independent experiments; **P < 0.01, *P < 0.05 by the Student's *t*-test.

Fig. 4. CCR492 is a post-transcriptional regulator of c-Myc**.** (A) Venn diagram of serum-induced genes versus let-7 target genes in BALB/c fibroblasts. (B) RT-qPCR of c-Myc mRNA expression in control or CCR492 silenced BALB/c fibroblasts. (C) Western blot of c-Myc protein expression in control or CCR492 silenced BALB/c fibroblasts (D) Dual luciferase assay. Upper panel: schematic representation of the c-Myc 3'UTR cloned downstream to the Firefly luciferase open reading frame (pRLuc-Myc 3'UTR). Lower panel: luciferase level obtained by RLuc-c-Myc 3'UTR transfected in control or CCR492 silenced BALB/c fibroblasts. In addition, complementation assay using the CCR492 mutant

insensitive to the silencing or with let-7 Inhibitors is shown. Data are presented as mean values ± SD of 3 independent experiments; **P < 0.01, *P < 0.05 by the Student's *t*-test.

Fig. 5. CCR492 ectopic expression cooperates with H-RasV12 for cell transformation. (A) WB analysis of c-Myc and H-Ras expression levels in BALB/c transfected either with a construct expressing H-RAsV12 alone or together with CCR492. (B) Soft agar quantification colony assay of BALB/c fibroblasts transfected as in A. The data represent the means ±SD of two independent experiments (C) A representative bright field image of soft agar colonies.

Fig. 6. Model of the interactions between CCR492, let-7, and c-Myc.
Left panel shows cells expressing CCR492. Right panel shows the effect of the let-7 microRNA on the c-Myc transcript.