



High-precision screen to identify lncRNAs governing specific mitotic stages

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Comment on: Stojic L, Lun ATL, Mascalchi P, *et al.* A high-content RNAi screen reveals multiple roles for long noncoding RNAs in cell division. *Nat Commun* 2020;11:1851.

Received: 09 June 2020; Accepted: 28 June 2020; Published: 30 December 2020.

doi: 10.21037/ncri-2020-005

View this article at: <http://dx.doi.org/10.21037/ncri-2020-005>

The vast majority of human transcripts lack coding potential (1,2). These transcripts are known as noncoding RNAs (ncRNAs) and include many ubiquitous RNAs with housekeeping functions such as ribosomal (r)RNAs and transfer (t)RNAs, and many RNAs with restricted spatiotemporal expression patterns, including long noncoding (lnc)RNAs, micro (mi)RNAs, and piwi-interacting (pi)RNAs, which regulate gene expression programs in a range of cellular processes (3). The study discussed here focuses on lncRNAs, a class of ncRNAs longer than 200 nucleotides that can regulate gene expression on many levels by remodeling chromatin, modulating transcription, affecting posttranslational processes, and altering protein activity (3-5). Through these functions, lncRNAs can affect cell proliferation, differentiation, and apoptosis, thereby contributing to pathologies such as cancer (6,7). Recent reports have highlighted distinct roles of lncRNAs in mitotic cell division (8-11).

Following DNA replication, cells enter a period of division into two daughter cells through mitosis, during which chromosomes condense, orient, and segregate; afterwards, cells undergo cytokinesis, during which the nuclear and cytoplasmic contents divide (12). Chromosome condensation is often tracked by monitoring phosphorylation of histone H3 (PHH3), which marks cells at the G2- to M-phase transition (13). Thus, in a given cell population, the mitotic index (the ratio between the number of mitotic cells and the number of total cells) can

be calculated as PHH3-positive cells relative to total cells. Chromosome segregation requires the precise coordination of spindle assembly and polymerization of microtubules (consisting of α - and β -tubulin dimers) that separate sister chromatids toward opposite poles (14). As soon as mitosis ends, a contractile ring cleaves the cytokinetic bridges connecting the two daughter cells to eventually split them. The microtubules that form cytokinetic bridges are often used to detect cytokinetic progression. If these bridges remain attached, daughter cells do not separate and mitosis is halted (15). In light of growing evidence that lncRNAs govern cell division, Stojic and colleagues recently set out to identify lncRNAs affecting cell division using RNA interference (RNAi) and high-content screening (16).

Stojic *et al.* employed human cervical carcinoma HeLa cells to set up a *tour de force* screen of small interfering (si)RNAs targeting 2,231 lncRNAs (the human Lincode siRNA library), then assessing mitotic traits with state-of-the-art methods. In the first screen the authors focused on defects of chromosome segregation and cytokinesis by visualizing centrosomes stained for the centromeric protein CEP215 and by visualizing the actin cytoskeleton using phalloidin (17). In the second screen, the authors focused on cells entering the M-phase and the maturation of centrosomes by staining for PHH3 and γ -tubulin, respectively (17). In both screens, Hoechst was used to visualize DNA and α -tubulin was used to assess cytoskeletal structures; in the process, the authors obtained highly informative data and strikingly beautiful

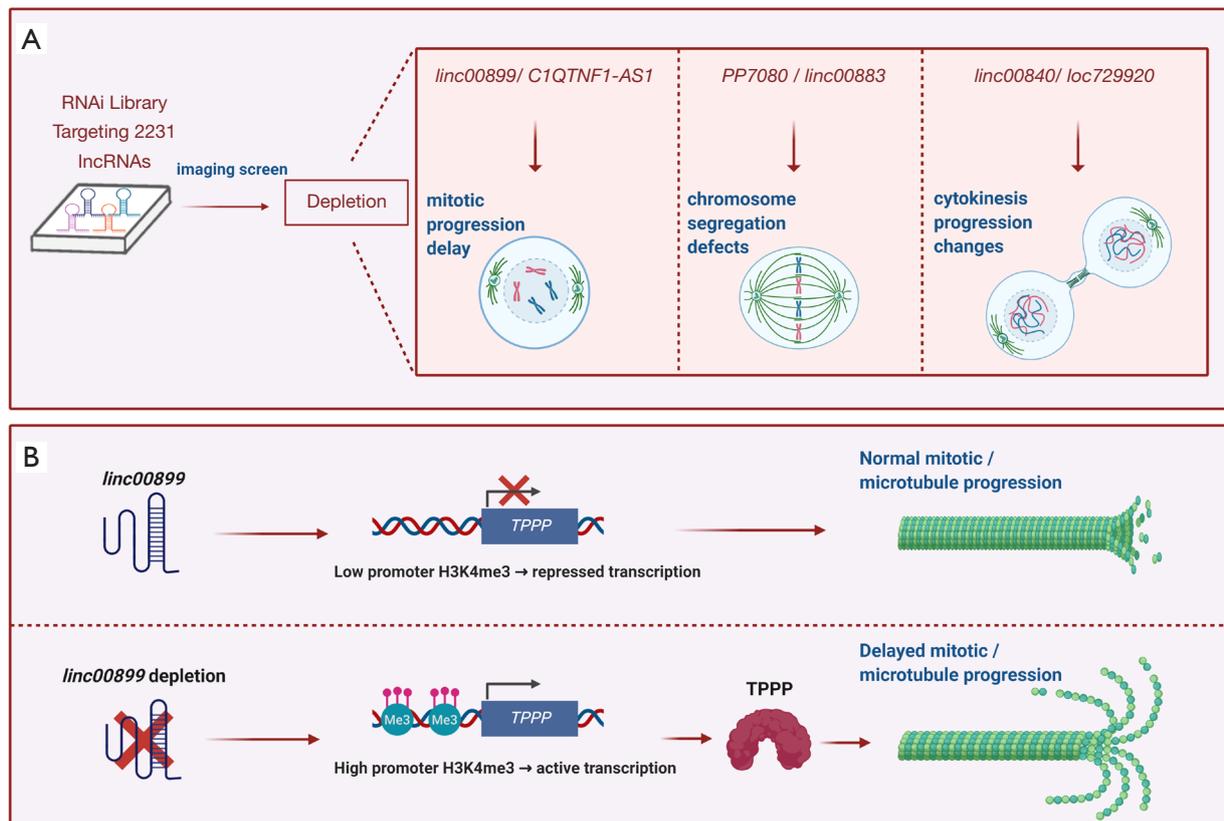


Figure 1 *Linc00899* depletion leads to increased TPPP/p25 production and delayed mitotic progression. (A) Schematic workflow involving high-content imaging RNAi screen to deplete 2231 lncRNAs and identify lncRNAs that have roles in mitosis. *Linc00899* and *C1QTNF1-AS1* depletion induced mitotic progression delay, *PP7080* and *linc00883* depletion induced chromosome segregation defects, *linc00840* depletion induced cytokinesis delay, and *loc729920* depletion shortened cytokinesis; (B) when *linc00899* is expressed, it decreases methylation of the *TPPP/p25* promoter, suppresses transcription of the *TPPP* mRNA, and mitosis progresses normally. When *linc00899* is depleted, increased *TPPP* promoter methylation yields enhanced transcription of *TPPP* mRNA and increased TPPP levels which stabilize microtubules and delay mitotic progression.

images.

Among the most prominent lncRNAs identified as affecting mitosis, silencing *linc00899* and *C1QTNF1-AS1* was found to promote mitotic delay, silencing *linc00883* and *PP7080* increased chromosome segregation errors, silencing *linc00840* doubled the cytokinetic cleavage time, and silencing *loc729970* shortened cytokinesis (summarized in Figure 1A). The authors further analyzed *linc00899* and *C1QTNF1-AS1*, two spliced and polyadenylated lncRNAs spanning ~1.6 and ~1 kb, respectively. *Linc00899* (also known as *loc100271722* or *ENSG00000231711*) lacks a mouse ortholog, but harbors short conserved stretches within exon 1, while *C1QTNF1-AS1* (also known as *ENSG00000265096*) is an antisense RNA transcribed

from a locus that encodes the C1Q and TNF-related 1 (*C1QTNF1/CTRP1*) protein and is conserved between human and mouse.

The authors further confirmed the RNAi-mediated mitotic delay by silencing these lncRNAs using locked-nucleic-acid (LNA) oligomers. Subsequent analysis of differentially expressed genes revealed that depleting *linc00899* changed the expression levels of many transcripts, while depleting *C1QTNF1-AS1* affected mainly *C1QTNF1* mRNA levels. Therefore, the authors focused on *linc00899* and specifically on the differentially regulated *TPPP/p25* mRNA; the encoded product, TPPP (the ubiquitous tubulin polymerization-promoting protein), blocks mitotic spindle formation by promoting tubulin polymerization (18).

Reverse transcription (RT) followed by quantitative (q) PCR analysis and western blot analysis verified a rise in *TPPP* mRNA and *TPPP* production when *linc00899* was silenced. Double knockdowns of *linc00899* and *TPPP* confirmed that the mitotic delay by *linc00899* depletion was mediated through the upregulation of *TPPP* in HeLa cells. Depletion of *linc00899* increased α -tubulin acetylation and long-lived microtubules, mimicking the microtubule-stabilizing function of *TPPP* (19), and reduced the levels of end-binding 1 (EB1) protein, which associates with the ends of growing microtubules (20). Accordingly, in *linc00899*-depleted cells, metaphase plates were narrow, with unattached kinetochore pairs predominantly located near the poles. Furthermore, in *linc00899*-depleted cells, the chromosome bi-orientation in metaphase was altered only modestly, while the reduction in interkinetochore distances (inter-KT) of chromosomes suggested that *linc00899* affected the tension to kinetochore pairs in metaphase (21).

To gain insight into how *linc00899* might modulate *TPPP* levels and function, the authors investigated the methylation of the *TPPP* promoter. Strikingly, silencing *linc00899* increased trimethylation of lysine 4 on histone H3 (H3K4me3) at the *TPPP* promoter (Figure 1B), although there were no sequences complementary to the *linc00899* transcript. These findings suggest that an independent ribonucleoprotein complex might enhance the methylation of the *TPPP* promoter. Further study is needed to identify the factors implicated in this transcriptional regulation.

To better understand this regulatory paradigm, the authors overexpressed *linc00899* in HeLa cells and in the retinal pigment epithelial cell line RPE1. While ectopic *linc00899* overexpression did not significantly affect *TPPP* mRNA levels in either cell line, CRISPR system-mediated endogenous *linc00899* overexpression reduced *TPPP* mRNA levels in RPE1 cells. This interesting result suggests that the subnuclear site of *linc00899* production affects function as a repressor of *TPPP* transcription.

Despite an escalation in knowledge of lncRNAs associated with cell proliferation, little is known about the lncRNAs directly implicated in controlling progression through the M-phase. In the comprehensive report by Stojic *et al.*, the authors employed advanced and sophisticated approaches to identify prominent lncRNAs affecting precise segments of mitosis. By choosing informative readout parameters, the authors were able to identify, with high accuracy, the lncRNAs that regulated each step of mitosis. The authors further focused primarily on *linc00899* and the underlying mechanism by which it

delayed mitotic progression. In *linc00899*-depleted cells, *TPPP* was the most prominent differentially expressed gene. Although *TPPP* is a ubiquitous protein, it is most highly expressed in the brain, where *linc00899* is remarkably low. Thus, the regulatory pathway described in the study by Stojic *et al.* might be particularly relevant to brain physiology and neurological diseases. In line with the suggested role for *linc00899*, a recent report uncovered that reducing *linc00899* levels halted cell cycle entry in spinal ependymoma cells, albeit through the retinoblastoma-like protein 2 (RBL2)-dependent forkhead box protein (FoxO) pathway (22). Interestingly, *linc00899* was recently reported to be highly abundant in the serum of acute myeloid leukemia (AML) patients (23); whether the *linc00899*-*TPPP* axis might govern mitosis impairment in AML warrants further investigation. While more complete catalogs of lncRNAs expressed in different cells and growth conditions are needed, the report by Stojic and colleagues charts a successful scheme for the identification of lncRNAs that govern specific stages of cellular division.

Acknowledgments

Funding: This work was funded in its entirety by the National Institute on Aging Intramural Research Program, National Institutes of Health.

Footnote

Provenance and Peer Review: This article was commissioned and reviewed by the Section Editor Xiya Lu, MD (Tongji Hospital of Tongji University, Tongji University School of Medicine, Shanghai, China).

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/ncr-2020-005>). KA serves as an unpaid editorial board member of *Non-coding RNA Investigation* from April 2019 to March 2021. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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doi: 10.21037/ncri-2020-005

Cite this article as: Tsitsipatis D, Amarbat B, Gorospe M, Abdelmohsen K. High-precision screen to identify lncRNAs governing specific mitotic stages. *Non-coding RNA Investig* 2020;4:9.