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Small nucleolar RNA *Snora61* drives self-renewal of intestinal stem cells via initiation of *Lgr5* transcription

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Running title: *Snora61* modulates ISC self-renewal

Abstract

Intestinal epithelium relies on intestinal stem cells (ISCs) for rapid and precise tissue replenishment to maintain gut normal function. The self-renewal maintenance of ISCs is finely regulated by multiple stemness factors and signaling pathways. However, the transcription mechanisms of some key stemness factors remain poorly understood. Here we identify that small nucleolar RNA *Snora61* is highly expressed in ISCs. *Snora61* is mainly distributed in the nucleoplasm. *Snora61* knockout impairs ISC self-renewal and intestinal regeneration. Mechanistically, *Snora61* binds to the promoter region of *Lgr5* gene and engages with RNA-binding protein RBMX to recruit HMGB2 onto *Lgr5* promoter, leading to *Lgr5* transcription and expression. *Snora61* promotes the self-renewal of small intestinal stem cells, which in turn enhances the proliferation of differentiated epithelial cells, thereby contributing to the maintenance of intestinal homeostasis. Conversely, *Snora61* knockout causes reduced LGR5 expression. Deletion of *Lgr5* with *Snora61* displays more severely impaired ISC self-renewal and intestinal regeneration. Our findings reveal a regulatory mechanism of *Lgr5* transcription underlying ISC self-renewal maintenance.

Key words: ISC; *Snora61*; LGR5; RBMX; HMGB2; self-renewal

Introduction

The intestinal epithelium, as a digestive tissue that directly contacts external environment, is subject to continuous mechanical, chemical, and biological damage while absorbing metabolic substances[1]. Once the epithelium is breached, it may lead to wear and tear of the underlying tissue, even systemic infection[2]. Therefore, continuous tissue replacement is crucial for maintaining intestinal homeostasis. The mouse intestinal epithelium renews every 3-5 days[3]. This continuous tissue replacement is provided by the intestinal stem cells (ISCs) located at the base of the crypts. In the crypts, the constantly dividing ISCs proliferate and ultimately differentiate into mature intestinal epithelial cells[4]. Control of ISC stemness is critical for intestinal homeostasis.

Earlier studies have identified LGR5 as a highly specific marker for ISCs[5]. Extensive reports have demonstrated that the self-renewal of ISCs is regulated by various signaling molecules and pathways, such as Wnt, Notch, Hippo, EGF, bone morphogenetic protein, interleukin, and metabolic signals[6]. In the Wnt signaling pathway, LGR5 binds R-spondin to maintain abundance of Frizzled receptors on cell membrane by sequestering the E3 ubiquitin ligases RNF43 and ZNRF3, thereby activating Wnt signaling[7]. LGR5, acting as a critical receptor for ISCs, has been well characterized in terms of its function. However, the precise mechanisms driving expression of LGR5 in ISCs remains elusive.

Small nucleolar RNAs (snoRNAs) are short non-coding RNAs[8]. SnoRNAs are typically classified into two types, C/D box and H/ACA box snoRNAs, which regulate ribosome biogenesis by guiding 2'-O-methylation and pseudouridylation respectively[9]. Recent studies have increasingly revealed that some snoRNAs are also involved in a broader range of transcriptional and post-transcriptional regulatory processes, including modulating mRNA levels[10], participating alternative splicing[11], acetylating rRNA[12], competing ribosomal subunit binding sites[13], and regulating protein secretion and metabolic activities[14]. However, how snoRNAs regulate LGR5 in ISC maintenance is unknown.

HMGB2 belongs to the HMG-box protein family, which is a subgroup of high-mobility group (HMG) superfamily[15]. HMGB2 participates in numerous cellular functions such as transcription[16] and chromatin remodeling[17]. It has been reported that HMGB2 exerts regulatory functions in various types of stem cells. For example, HMGB2 is intricately linked with the activation of human neural stem cells

from a dormant state to an active proliferative phase[18]. The interaction between HMGB2 and OCT4 contributes to pluripotency gene expression in mouse embryonic stem cells[19]. In addition, HMGB2 regulates proliferation and differentiation of human embryonic stem cells (hESCs) and progenitor cells by modulating telomerase activity[20]. Moreover, HMGB2 participates in self-renewal maintenance of hematopoietic stem cells through regulation of latexin transcription[21].

Here we identify *Snora61* as an important noncoding RNA in the regulation of ISC self-renewal maintenance. *Snora61* associates with RBMX to recruit HMGB2 onto *Lgr5* promoter, leading to *Lgr5* transcription and expression.

Results

***Snora61* is highly expressed in ISCs**

To investigate expression profiles of snoRNAs in ISCs, we utilized *Lgr5-eGFP* mice to isolate $LGR5^+$ ISCs and $LGR5^-$ intestinal epithelial cells (IECs) via flow cytometry, followed by snoRNA microarray assay. Through analysis and screening, we identified that H/ACA snoRNA *Snora61* was highly expressed in ISCs (Fig. 1a, b). In addition, we designed shRNAs against the top ten enriched snoRNAs and found that knockdown of *Snora61* most severely impaired organoid formation capacity (Fig. 1c and Supplementary Fig. 1a, b). *Snora61* is located within *Snhg12* gene on mouse chromosome 4 (Supplementary Fig. 1c). The *Snhg* family, which encodes snoRNAs, comprises non-coding genes[22]. Human *SNORA61* shared a similar genomic localization with its mouse counterpart and exhibited 73.68% sequence homology (Supplementary Fig. 1c, d). Knockdown of *SNORA61* in human intestinal organoids exhibited similar inhibitory effect on organoid formation (Supplementary Fig. 1e). Actually, the *Snhg12* gene encodes four snoRNAs: *Snora16a*, *Snora44*, *Snora61*, and *Snord99*. We confirmed that among these four snoRNAs, only *Snora61* was enriched in $LGR5^+$ ISCs (Supplementary Fig. 1f). To further determine tissue-specific expression of *Snora61*, we performed fluorescence *in situ* hybridization (FISH) staining of frozen sections from fetal mice. We observed that *Snora61* was highly expressed in small intestines and spleen (Fig. 1d). Northern blot and RT-qPCR showed similar observations (Fig. 1e and Supplementary Fig. 1g). In addition, *Snora61* was specifically enriched at the tips of budding structures in intestinal organoids, corresponding to the crypt

regions of small intestines[23] (Fig. 1f). This observation was consistent with our staining results from intestinal sections. *Snora61* was predominantly localized in the base of crypts and co-localized with $LGR5^+$ cells (Fig. 1g, h).

To investigate subcellular localization of *Snora61*, we performed subcellular fractionation and FISH staining. We found that *Snora61* was predominantly distributed in the nucleoplasm (Fig. 1i-k and Supplementary Fig. 1h). Collectively, *Snora61* is highly expressed in ISCs and mainly distributed in the nucleoplasm.

Snora61 knockout impairs self-renewal of ISCs

To explore the role of *Snora61* in ISC self-renewal maintenance, we generated *Snora61* knockout ($Snora61^{-/-}$) mice through CRISPR/Cas9 technology. *Snora61* deletion was confirmed by genotyping and DNA sequencing (Supplementary Fig. 2a, b). Deletion of *Snora61* did not influence expression levels of *Snora16a*, *Snora44*, or *Snord99* (Supplementary Fig. 2c). We found that $Snora61^{-/-}$ mice exhibited shorted intestine length (Fig. 2a), while $Snora61^{-/-}$ mice displayed normal sizes and morphology of other organs, including brain, heart, liver, spleen, kidneys, and testis (Supplementary Fig. 2d). As expected, *Snora61* knockout caused impaired organoid formation, while *Snora61* overexpression rescued organoid growth and budding (Fig. 2b and Supplementary Fig. 2e). However, knockdown of *Snora16a*, *Snora44*, or *Snord99* did not affect organoid formation (Supplementary Fig. 2f). Moreover, $Snora61^{-/-}$ mice demonstrated reduced crypt numbers across all regions of small intestines (duodenum, jejunum, and ileum) (Fig. 2c). In addition, small intestines of $Snora61^{-/-}$ mice showed decreased cell proliferation (Fig. 2d). Consistently, intestinal organoids derived from $Snora61^{-/-}$ mice also displayed markedly reduced cell proliferation during organoid formation (Fig. 2e and Supplementary Fig. 2g). We next treated $Snora61^{+/+}$ and $Snora61^{-/-}$ mice with 8 Gy X-ray irradiation for radiation-induced damage. We noticed that $Snora61^{-/-}$ mice displayed remarkable inhibition of intestinal regeneration by H&E staining compared with that of littermate $Snora61^{+/+}$ mice (Fig. 2f). In parallel, $Snora61^{-/-}$ mice exhibited more severe body weight loss and a higher disease activity index (DAI) score (Supplementary Fig. 2h, i and Supplementary Table 5).

We also examined the relevant phenotypes in heterozygous mice. Expression levels of *Snora61*

in *Snora61*$^{+/-}$ mice were approximately half of those in *Snora61*$^{+/+}$ mice (Supplementary Fig. 2j). We observed analogous but less severe stemness suppression in *Snora61*$^{+/-}$ mice compared to *Snora61*$^{-/-}$ mice, including shortened small intestine and villus length, reduced crypt number, and impaired organoid formation ability (Supplementary Fig. 2k-m). Taken together, *Snora61* is required for self-renewal maintenance of ISCs.

***Snora61* initiates *Lgr5* transcription in ISCs**

Snora61 canonically functions to mediate pseudouridylation at position 2263 of 28S rRNA (Supplementary Fig. 3a). To determine whether *Snora61* mediated pseudouridylation regulated ISC stemness, we performed N-cyclohexyl-N'-(2-morpholinoethyl) carbodiimide methyl-p-toluene sulfonate (CMC) treatment to assess levels of 28S rRNA U2263 pseudouridylation across different experimental groups (Supplementary Fig. 3b). We found that *Snora61* deletion did not affect abundance of 28S rRNA (Supplementary Fig. 3c). However, *Snora61* deletion caused markedly reduced levels of U2263 pseudouridylation. whereas overexpressing wild-type (WT) *Snora61* could rescue U2263 pseudouridylation but not mutant *Snora61* (Supplementary Fig. 3d, e). Of note, overexpressed WT or mutant *Snora61* in *Snora61*$^{-/-}$ ISCs could restore organoid growth (Supplementary Fig. 3f), suggesting that U2263 pseudouridylation was not involved in the maintenance of ISC self-renewal. We concluded that *Snora61*-mediated ISC self-renewal is independent of its canonical pseudouridylation function.

We next carried out RNA-seq from ISCs of *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice. GO analysis revealed that the downregulated genes were significantly enriched in pathways related to intestinal development, cell proliferation/differentiation, and gene transcription (Fig. 3a). We hypothesized that *Snora61* knockout may impair transcription of key stemness-related genes. Consequently, we analyzed differential expression levels of main stemness-related genes and found that *Lgr5* was most downregulated gene in *Snora61*$^{-/-}$ ISCs (Fig. 3b and Supplementary Fig. 3g). *Lgr5* and its downstream target genes showed various degrees of downregulation, which was particularly evident during post-irradiation repair (Fig. 3c, d). Consequently, the gene expression profile in *Snora61*$^{-/-}$ ISCs showed significantly reduced enrichment in the Wnt signaling pathway post irradiation (Fig. 3e). In addition,

ChIRP-seq analysis showed that *Snora61* was enriched on the *Lgr5* promoter region (Fig. 3f). Sequence alignment indicated complementary base-pairing between *Snora61* and the enriched genomic segment (Fig. 3g). This binding and enrichment were further confirmed by ChIRP-qPCR and dot-blot assays, while mutation of the binding sites of *Snora61* abolished the engagement (Fig. 3h, i). These results indicate that *Snora61* binds to the *Lgr5* promoter via complementary base-pairing sequences.

Dual luciferase reporter assays demonstrated that *Snora61* promotes *Lgr5* transcription, and this effect is dependent on the -2000 ~ -1800 bp binding region (Fig. 3j). Consequently, *Snora61* deletion suppressed chromatin accessibility and transcription activity of *Lgr5* gene (Fig. 3k, l). In parallel, *Snora61* deletion dramatically decreased H3K4me3 levels of the -2000 ~ -1800 bp region of *Lgr5* promoter, whereas increased H3K27me3 levels (Fig. 3m, n). The impaired expression in *Snora61*$^{-/-}$ ISCs was further validated by immunoblotting (Fig. 3o). In addition, we crossed *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice with *Lgr5-eGFP* mice to evaluate proportions of ISCs. We observed that proportions of LGR5^{+} cells were remarkably decreased in *Snora61*$^{-/-}$ mice (Fig. 3p). Consistently, LGR5 expression was lowly expressed in *Snora61*$^{-/-}$ mice crypts and organoids (Supplementary Fig. 3h, i). In addition, LGR5 expression was relatively downregulated in *Snora61*$^{+/-}$ ISCs and in human intestinal organoids with *SNORA61* knockdown (Supplementary Fig. 2j and Supplementary Fig. 1e). Furthermore, we monitored dynamic expression of *Lgr5* and *Snora61* during intestinal repair post irradiation. Time-course analysis revealed that with initial synchronous decline with *Lgr5* due to ISCs loss, *Snora61* expression rebounded ahead of *Lgr5* recovery (Supplementary Fig. 3j). By contrast, mutant *Snora61* could not restore organoid formation ability and LGR5 expression in *Snora61*$^{-/-}$ organoids (Fig. 3q and Supplementary Fig. 3k, l). Collectively, these results indicate that *Snora61* enriches on the *Lgr5* promoter to initiate *Lgr5* transcription in ISCs.

Snora61 associates with RBMX in ISCs

To further explore the molecular mechanism by which *Snora61* promoted *Lgr5* transcription, we performed RNA pull-down assays (Fig. 4a). Through mass spectrometry analysis of the two most prominent differential bands (located around 42 kDa and 30 kDa), we identified a set of binding protein

candidates for *Snora61* (Supplementary Table 6). Based on subcellular localization of these candidates and their regulatory effects on *Lgr5* expression, we ultimately selected RBMX for further validation. RBMX exhibited clear nuclear localization, and its knockdown suppressed *Lgr5* expression (Supplementary Fig. 4a-c). This interaction was further validated by Western blot and RNA immunoprecipitation assays (Fig. 4b, c). RBMX, an RNA-binding protein, contains an RNA recognition motif (RRM) domain. Truncation mapping demonstrated that *Snora61* bound to the RRM domain of RBMX (Fig. 4d). Fluorescence staining revealed that RBMX was co-localized with *Snora61* in ISCs and intestinal organoids (Fig. 4e, f).

To investigate the role of RBMX, we silenced and overexpressed *Rbmx* in intestinal organoid and examined the effect of RBMX on organoid growth (Supplementary Fig. 4d). We observed that *Rbmx* knockdown inhibited organoid growth in both *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ ISCs, while *Rbmx* overexpression promoted organoid formation (Fig. 4g and Supplementary Fig. 4e). These observations were verified by Ki67 staining (Fig. 4h and Supplementary Fig. 4f). As expected, *Rbmx* knockdown suppressed LGR5 expression, whereas *Rbmx* overexpression enhanced LGR5 expression (Fig. 4i and Supplementary Fig. 4g, h). To assess the functional requirement of the RBMX-*Snora61* complex, we performed rescue assays in sh*Rbmx* organoids. Expression of an RRM-deletion mutant of RBMX failed to rescue impaired organoid formation and low *Lgr5* expression, unlike its full-length protein (Fig. 4j and Supplementary Fig. 4i, j). ChIP analysis confirmed RBMX enrichment at the -2000 ~ -1800 bp region of *Lgr5* promoter, which was abrogated by *Snora61* knockout (Fig. 4k, l). Of note, *Rbmx* knockdown or overexpression did not affect *Snora61* abundance (Supplementary Fig. 4k). Similarly, *Snora61* deletion did not influence expression of RBMX (Supplementary Fig. 4l). Taken together, RBMX associates with *Snora61* in ISCs and cooperatively regulates LGR5 expression.

***Snora61*-RBMX recruits HMGB2 onto *Lgr5* promoter to initiate its transcription**

To further explore how *Snora61*-RBMX engagement regulated *Lgr5* expression, we used anti-RBMX antibody to go through ISC lysates and the most prominently differential band (around 24 kDa) was conducted by mass spectrometry (Fig. 5a and Supplementary Table 7). After evaluating subcellular localization and transcriptional activity of these candidate proteins, we ultimately identified

HMGB2 as an interacting protein of RBMX (Supplementary Fig. 5a). The interaction between RBMX and HMGB2 was further validated by co-IP assay (Fig. 5b, c). Of note, the abundance and interaction of RBMX and HMGB2 in ISCs did not depend on *Snora61* (Fig. 5d). *Snora61*, RBMX, and HMGB2 were co-localized in the nucleus of ISCs and intestinal organoids (Fig. 5e-g). HMGB2, a co-transcription factor, regulates gene transcription by binding to chromatin and recruiting other transcription factors onto gene promoters. We depleted *Hmgb2* in ISCs (Supplementary Fig. 5b) and followed by intestinal organoid formation assay. We observed that *Hmgb2* silencing inhibited organoid growth and LGR5 expression in ISCs (Fig. 5h-k and Supplementary Fig. 5c). In addition, we found that the expression of *Snora61* and HMGB2 is mutually independent (Supplementary Fig. 5d, e). *Hmgb2* knockdown abrogated *Snora61* overexpression increased organoid formation (Supplementary Fig. 5f). However, overexpression of *Hmgb2* failed to restore the impaired organoid formation and *Lgr5* expression in *Snora61*$^{-/-}$ organoids (Supplementary Fig. 5g, h). These data suggest that HMGB2 is involved in *Snora61*-mediated maintenance of ISC stemness.

Through ChIP assay, we observed that HMGB2 was enriched onto the -2000 ~ -1800 bp region of *Lgr5* promoter, while *Snora61* deletion abolished this enrichment (Fig. 5l, m). Moreover, through dual luciferase reporter assay, we noticed that *Hmgb2* overexpression promoted *Lgr5* transcription activity, whereas deletion of -2000 ~ -1800 region abrogated this effect (Fig. 5n). We next generated AAV-mediated *Rbmx* conditional knockout mice (Supplementary Fig. 6a). In this setting, *Hmgb2* overexpression failed to rescue the impaired organoid formation and reduced *Lgr5* expression in *Hmgb2* knockdown ISCs (Supplementary Fig. 6b-d). In addition, *Rbmx* knockout abrogated enrichment of HMGB2 to the *Lgr5* promoter (Supplementary Fig. 6e). Finally, *Rbmx* knockdown inhibited *Lgr5* transcription activity in dual luciferase reporter assay (Supplementary Fig. 6f and Fig. 5n). Altogether, *Snora61* recruits RBMX-HMGB2 complex onto the *Lgr5* promoter to initiate its transcription in ISCs.

LGR5 is required for *Snora61*-mediated ISC self-renewal and intestinal regeneration

To test the physiological role of LGR5 in maintaining ISC self-renewal, we generated *Lgr5* conditional knockout mice using AAV and validated knockout efficiency via Western blot, RT-qPCR

and FACS (Fig. 6a and Supplementary Fig. 7a-c). *Lgr5* knockout dramatically reduced the proportions of $Ki67^+$ cells (Fig. 6b). In addition, *Lgr5* knockout also markedly suppressed expression of OLFM4, another key ISC marker (Supplementary Fig. 7d, e). We noticed that *Lgr5* knockout decreased length of intestinal villi (Fig. 6c). Meanwhile, deletion of *Lgr5* with *Snora61* exhibited much shorter length of intestinal villi (Fig. 6c). Consistently, *Lgr5* knockout impaired formation and budding of intestinal organoids (Fig. 6d) and significantly inhibited cell proliferation in intestinal organoids (Supplementary Fig. 7f). We observed that deletion of *Lgr5* with *Snora61* showed much more severely abolished intestinal organoid formation and cell proliferation (Fig. 6d, and Supplementary Fig. 7f). Moreover, *Lgr5* knockout obviously impaired intestinal regeneration post irradiation exposure (Fig. 6e). Finally, deletion of *Lgr5* with *Snora61* manifested much worse intestinal regeneration post irradiation exposure, along with more severe weight loss and higher DAI scores (Fig. 6e and Supplementary Fig. 7g, h). dFz7-21 is an antagonist of Frizzled receptor[24]. We observed that dFz7-21 treatment abrogated *Snora61* overexpression induced organoid formation capacity in $Snora61^{-/-}$ ISCs (Fig. 6f), suggesting that LGR5-mediated Wnt signaling participates in *Snora61*-induced ISC self-renewal maintenance. Of note, knockdown of *SNORA61* in human intestinal organoids with an *APC* mutation did not affect organoid growth (Supplementary Fig. 7i). Collectively, these results indicate that LGR5 is required for *Snora61*-mediated ISC self-renewal and regeneration.

Discussion

As a digestive organ subjected to continuous long-term damage, the intestinal epithelium relies on ISCs for rapid and precise tissue replenishment to maintain gut normal function. Stemness factors and signaling are involved in the regulation of ISC self-renewal and gut regeneration. However, how *Lgr5* gene expresses remains elusive. In this study, we identified a snoRNA *Snora61* that was highly expressed in ISCs. Knockout of *Snora61* impaired ISC self-renewal and intestinal regeneration. Mechanistically, *Snora61* associated with RBMX to recruit HMGB2 onto *Lgr5* promoter, leading to *Lgr5* transcription. Deletion *Lgr5* with *Snora61* displayed much more severely impaired ISC self-renewal and intestinal regeneration.

Many studies have been reported that noncoding RNAs take part in the regulation of stem cell

maintenance and function. For instance, *Lnc030* maintains breast cancer stem cell stemness by stabilizing SQLE mRNA and increasing cholesterol synthesis[25]. The long non-coding RNA Meg3 mediates imprinted gene expression during mouse embryonic stem cell differentiation[26]. We previously showed that lncRNA *LncBRM* drives self-renewal of liver cancer stem cells by activating YAP1 signaling pathway[27]. *LncKdm2b* regulates self-renewal of embryonic stem cells by promoting transcription of transcription factor ZBTB3[28]. *LncGata6* promotes both maintenance of ISC stemness and intestinal tumorigenesis[29].

Despite being discovered in the 1960s, snoRNAs has predominantly been focused on their traditional roles in the nucleolus, especially modifying ribosomal RNA (rRNA) and small nuclear RNA (snRNA). For instance, long noncoding RNA ZFAS1 promotes *SNORD12C/78*-mediated 2'-O-methylation via NOP58 recruitment in colorectal cancer[30]. *SNORD113-114* clusters maintain hematopoietic stem cell self-renewal via modulating translation machinery including rRNA 2'-O-Me modifications, pre-rRNA processing, and 60S ribosome assembly[31]. G-patch protein GPATCH4 activates DHX15 to regulate snoRNA-guided 2'-O-methylation of rRNA[32]. Over the past decades, thousands of snoRNAs have been identified in the genome, yet the targets, mechanisms, and functions of most snoRNAs remain unresolved issues in the field of nucleic acid biology. Tissue-specific expression patterns and subcellular localization of snoRNAs, which extend beyond the nucleolus, suggest that certain snoRNAs may play non-canonical roles in various cell types. In this study, *Snora61* was distributed throughout the nucleus, not limited to the nucleolus, suggesting it may exert its functions beyond modifying 28S rRNA. Here we showed that *Snora61* regulated ISC stemness in a non-canonical manner. Of note, *Snora61* originates from non-coding regions of *Snhg12* gene, which encodes four snoRNAs. However, only *Snora61* was highly expressed in ISCs and regulated ISC stemness. On the other hand, in the mouse irradiation model, we observed a transient increase in the relative level of *Snora61*, which facilitated the initiation of *Lgr5* expression in response to intestinal damage and repair. However, the molecular mechanism underlying the selective and dynamic generation of specific snoRNAs under homeostatic and pathological conditions need further investigation.

Since snoRNAs lack catalytic domains and functional motifs, they typically exert their functions by interacting with specific RNA-binding proteins (RBPs)[11]. RBPs constitute a critical regulatory network that regulates stem cell pluripotency[33]. In previous studies, researchers mainly focused on the roles of RBPs in post-transcriptional regulation processes, including alternative splicing, RNA processing and modification, RNA nuclear export, transcript stability regulation, and translation. For instance, SON regulates splicing of transcripts encoding pluripotency regulatory factors that is an essential factor for maintaining hESCs[34]. RBM15-mediated m^6A modification on mRNA regulates differentiation of hematopoietic stem and progenitor cells during embryonic development[35]. THOC5, a component of the THO complex, interacts with pluripotency gene transcripts and controls self-renewal and differentiation of ESCs by regulating export of pluripotency gene mRNA[36]. IGF2BP maintains MYC protein levels by stabilizing *Myc* mRNA, thereby regulating growth and division of individual neural stem cells[37]. RBPMS is recruited to active ribosomes of hESCs and modulates translation of key stemness factors through mRNA translational specialization[38]. Here we demonstrated an aspect: RBPs interact with ncRNAs and function as intermediary molecules to modulate transcriptional regulation of key stemness genes.

Here we showed that *Snora61* enrichen onto *Lgr5* promoter region. Given that *Snora61* itself lacked transcription factor activity and could not directly promote transcription, we identified an RNA-binding protein RBMX as a specific binding partner of *Snora61*. RBMX recruited co-transcription factor HMGB2 onto *Lgr5* promoter, thereby initiating *Lgr5* transcription and expression. Although we established a regulatory axis of *Snora61*-RBMX-HMGB2-*Lgr5* in ISCs, RBMX might play diverse potential roles in both pre- and post-transcriptional regulation in the regulation of ISC stemness. For instance, RBMX participates in alternative splicing of pre-mRNA[39]. RBMX promotes degradation of telomere-derived noncoding RNA TERRA to influence telomere stability[40]. Moreover, RBMX also maintains proper sister chromatid cohesion[41]. Whether the interaction between *Snora61* and RBMX might affect other potential mechanisms still remains further investigation.

In this study, we compared the transcriptomes of ISCs from *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice to identify key genes or pathways regulated by *Snora61*. We noticed that *Snora61* knockout altered the

expression of a series of stemness-related genes (including *Lgr5, Msi1, Tert, Clca4a, Olfm4, Ascl2, Cdca7, Axin2, Smoc2, Lrig1,* and *Sox9*). *Lgr5* exhibited the most pronounced change among these stemness-related genes. Based on ChIRP-seq assay, *Lgr5* was a bona fide target of *Snora61*. Through this study, we established the *Snora61*-RBMX-HMGB2-*Lgr5* axis playing a critical role in the regulation of ISC stemness. Nonetheless we could not exclude *Snora61* might be involved in other stemness-related genes or pathways for the ISC stemness regulation. In addition, GO analysis indicated that genes downregulated upon *Snora61* knockout were also enriched in pathways involving nutrient absorption, energy metabolism, and protein phosphorylation. Therefore, *Snora61* may participate in the regulation of intestinal stem cell stemness and maintenance with other pathways.

Methods

Ethics

All experiments in this study were conducted in compliance with relevant ethical regulations and approved by the Institutional Committee of the Institute of Biophysics, Chinese Academy of Sciences. Both male and female mice were housed under specific pathogen-free conditions with a 12 h light/dark cycle, temperature of 25±2°C, and 50% relative humidity. Mice were randomly assigned to experimental groups, and no sex-dependent differences were observed in any of the analyses. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Biophysics, Chinese Academy of Sciences. No biases were introduced with respect to patient sex or gender.

Antibodies and reagents

Anti-Ki67 (Cat# 11-5698-82, Cat# 14-5698-80), 488 Donkey anti-Rabbit, Secondary Antibody (Cat# A21206), 594 Donkey anti-Rabbit, Secondary Antibody (Cat# A21207) and 488 Donkey anti-Mouse, Secondary Antibody (Cat# R37114) were purchased from Invitrogen. Anti-EpCAM (CD326) (Cat# 118211) was purchased from Biolegend. Anti-Olfm4 (Cat# 14369), Anti-H3K4me3 (Cat# 9751T), anti-H3K27me3 (Cat# 9733), Anti-RBMX (Cat# 14794S) and Anti-Histone H3 (Cat# 4499) were purchased from Cell Signaling Technology (Danvers, USA). Anti-Nucleolin (Cat# ab129200), Anti-

RBMX (Cat# ab190352) and Anti-HMGB2 (Cat# ab124670) were purchased from Abcam. Anti-HMGB2 (Cat# 14597-1-AP), Anti-Nucleolin (Cat# 10556-1-AP) and Anti-LGR5 (Cat# 30007-1-AP) were purchased from Proteintech. Anti-HMGB2 (Cat# K108316P) and Anti-RBMX (Cat# K007062P) were purchased from Solarbio. Anti-HMGB2 (Cat# WH0003148M5) was purchased from Sigma. Anti-RBMX (Cat# AF5263) was purchased from Affinity. Anti-EEA1 (Cat# SC-6415) was purchased from Santa Cruz. Anti-Lamin A/C (Cat# HY-P80204) and Protein A/G Magnetic Beads (Cat# HY-K0202) were purchased from MCE. Anti-β-actin (Cat# RM2001), Anti-Flag-tag (Cat# RM1002), Goat anti-mouse IgG (Cat# RM3001) and Goat anti-rabbit IgG (Cat# RM3002) were purchased from Ray Antibody. All antibodies were used at the manufacturer's recommended concentrations. F-actin staining kit (Cat# 23122) was purchased from AAT Bioquest. The Dual Luciferase Reporter Gene Assay Kit (Cat# RG027) and Streptavidin Magnetic Beads (Cat# 23122) were purchased from Beyotime. The Biotin RNA Labeling Mix (Cat# 11685597910), T7 RNA polymerase (Cat# 10881767001) and Blocking Reagent (Cat# 11096176001) were purchased from Roche. The Chemiluminescent Nucleic Acid Detection Module (Cat# 89880), NE-PER Nuclear and cytoplasmic extraction (Cat# 78833) and Supersignal West pico plus (Cat# 34577) were purchased from Thermo. The Organoid Growth Medium was purchased from Stem cell (Cat# 06005) and BioGenous (Cat# K2003-HC, Cat# K-2605). The Matrigel was purchased from Corning (Cat# 356237).

Animals

Villin-Cre, *Cas9*-KI and *Lgr5-eGFP* mice were purchased from the Jackson Laboratory. The *Snora61*$^{-/-}$ mice were generated by the gene editing facility of Institute of Biophysics using the CRISPR/Cas9 system. The specific procedure is as follows: the sgRNAs targeting *Snora61* were designed by online CRISPR design tool (https://crispor.gi.ucsc.edu/). The sgRNA sequences are listed in Supplementary Table 1. Approximately 250 zygotes in the C57BL/6 background were injected with corresponding single guide RNA (sgRNA) donors and subsequently transferred to the uterus of pseudo-pregnant ICR females, from which viable founder mice were obtained. After extracting genomic DNA from the offspring mice, mice with successful knockout were identified by PCR and next-generation sequencing. Through multiple generations of breeding, stably inherited homozygous

mice were ultimately established. All the mouse strains were C57BL/6 background and maintained under specific pathogen-free conditions. Male and female mice aged 6 to 10 weeks were used. This study is compliant with all relevant ethical regulations regarding animal research.

Cell lines

HEK293T and MC38 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). HEK293T and MC38 cells were cultured in DMEM medium supplemented with 10% FBS, 100 μg/ml penicillin G and 100 U/ml streptomycin.

Human intestinal tissues and CRC samples

Primary human CRC samples were collected from patients pathologically diagnosed with colorectal cancer from the Department of General Surgery, Peking University Third Hospital with informed consent and the approval from the Ethics Committee of Peking University Third Hospital. The participants were selected randomly, without potential self-selection bias or other biases. Patient information is presented in Supplementary Table 8. Fresh human tissues/samples were minced into 1 mm^3 pieces and then digested with digestion buffer (0.1% IV collagenase, 0.01% DNase, 0.05% protease dissolved in serum-free DMEM) at 37°C for 30 min. The supernatant was then filtered through a 100 μm filter and centrifuged at 150 g for 5 min. Organoids were cultured using the obtained cells with the Human Colonic Organoid Kit (Cat# K2003-HC) / Human Colorectal Cancer Organoid Kit (Cat# K-2605).

SnoRNAs sequencing and microarray assay

For snoRNAs sequencing, 5×10^5 $LGR5^+$ ISCs and $LGR5^-$ IECs were collected from small intestine crypts from *Lgr5-eGFP* mice aged 8 weeks by FACS sorting and were detected by confocal microscopy (purity over 95%). Total RNA was extracted using a standard RNA-extraction protocol and then snoRNA microarray assay analysis was performed by Yingbio Tech Company.

Small intestine organoid formation assay

Small intestines from indicated mice aged 8 weeks were isolated, cut open longitudinally and washed using phosphate buffer saline (PBS) several times. The intestinal villus was removed. Then, intestines were cut into 5 mm pieces, resuspended with Collagenase digestion buffer (1mg/mL

collagenase I in PBS) and incubated for 20 min at 37°C. Tissue pieces were transferred into 10 ml ice cold PBS in 15 ml tube. Shake vigorously by hand for one minute and collect first elution components. Repeat for total three times and check crypts under microscope. Usually, crypts were enriched in the second elution component. Crypts were further filtered through 70 μm cell strainer. Crypts were suspended with IntestiCult™ Organoid Growth Medium at density of 200 crypts/25 μl, mixed with Matrigel at 1:1 ratio, seeded into 6-well plate and cultured for 2-3 days. Primary organoids usually undergo 2-3 serial passages to remove contaminating cells. Organoid imaging or collection is conducted 48 to 72 hours subsequent to passaging, based on their growth progression.

shRNA knockdown and gene overexpression system

Silencing of indicated genes was performed by short hairpin RNA. shRNAs of indicated genes were designed on online RNAi designer (https://rnaidesigner.thermofisher.com/rnaiexpress/). 2–3 shRNAs of each target gene were selected and cloned into pSicoR Puro lentivirus vector, and the shRNAs were listed in Supplementary Table 2. Genes for overexpression were constructed into pLVX-IRES-Puro vector. For rescue experiments used in this study, synonymous mutations (i.e., codon substitutions) were introduced into shRNA-targeted region during design of overexpression plasmids, which ensured that the original shRNAs did not interfere with rescue effect. Lentiviral vectors were co-transfected with packaging plasmids psPAX2 and pMD2G into HEK293T cells. Lentiviruses in supernatants were harvested at 48 and 72 h after transfection and filtered through 0.45 μm filters. Recombinant lentiviruses were concentrated by ultracentrifugation (25000 rpm, 2 h, 4°C) and dissolved with Organoid Growth Medium. For gene silencing or overexpression in organoid cells, organoids from indicated mice intestine were cultured for 3 days. Organoids were broken and collected by centrifugation. Then broken organoids were resuspended with 50 μl complete organoid media and 1:1 mixed with lentivirus solution containing 6 mg/ml polybrene (Sigma-Aldrich). Mixed with Matrigel at 1:1 ratio and seeded into 6-well plate, organoids were cultured at 37°C for 3 days. Organoids were passaged and maintained and gene silencing or overexpression efficiency was analyzed by real-time qPCR.

CRISPR/Cas9 knockout system

Lgr5/Rbmx-deletion mice were established using CRISPR/Cas9 approaches provided by Zhang's lab. The sgRNAs were designed by online CRISPR design tool (https://crispor.gi.ucsc.edu/). The sgRNA sequences are listed in Supplementary Table 1. In brief, sgRNAs were cloned into pAAV-U6-gRNA-CBh-mCherry (Addgene, catalog 91947). AAV vectors were co-transfected with packaging plasmids pHelper and pAnc80 into HEK293T cells. AAV were harvested at 72 h after transfection by repeated freezing and thawing and filtered through 0.22 μm filters. AAV was injected into *Villin*-Cre^{+}*Cas9*-KI mice aged 6 weeks via the tail vein. The gene knockout efficiency was analyzed by RT-qPCR and Western blot.

Northern blot

Total RNA was extracted from different organs and tissues from WT mice aged 8 weeks with standard TRIzol methods, and then subjected to electrophoresis on Urea page gel for 1 h in 0.5×TBE. Samples were transferred to positively charged NY membranes for 1 h in TBE buffer. After UV cross-linking (265 nm ultraviolet with energy of 200,000 μJ/cm^2) and prehybridization, membranes were incubated with biotin-labeled probes at 65°C for 16–20 h. After washing with washing buffer, biotin signals were detected with Chemiluminescent Nucleic Acid Detection Module (Cat# 89880) according to the manufacturer's instructions. For detecting snoRNAs only, inverse complementary sequences were used for probes.

Immunofluorescence staining

Organoids or frozen sections were fixed by 4% paraformaldehyde (PFA) for 30 min, and then permeated by 0.5% triton X-100 for 30 min. After blocking with 10% donkey serum for 1 h, primary antibodies were added and incubated overnight at 4°C. After washing with PBS, fluorescence-conjugated secondary antibodies were incubated at RT for 2 h or overnight at 4°C. The DAPI was incubated at RT for 5 min. After sealing, the samples were observed by confocal microscopy (Nikon A1R+).

Fluorescence *in situ* hybridization

Organoids or frozen sections were fixed with 4% PFA for 20 min at room temperature, permeabilized with PBS containing 0.5% TritonX-100 for 20 min, prehybridized with hybridization

buffer (50% formamide, 5× SSC, 500 ng/μl yeast tRNA, 1× Dehardt's solution, 500 ng/μl sperm DNA, 50 ng/μl Heparin, 2.5 mM EDTA, 0.1% Tween-20, 0.25% CHAPS) for 1h at 62°C, incubated with biotinylated or cy5-labeled probes at 62 °C for 2 h, and then washed three times with SSC washing buffer. After blocking with 10% donkey serum, samples were subjected to IF staining and visualized with Nikon A1R+confocal microscopy.

Real-time quantitative PCR

Total RNA was extracted with standard TRIzol methods. cDNA was synthesized using 5× All-In-one RT Mastermix (Abm, Vancouver, Canada) and analyzed on QuantStudio1 qPCR system using specific primer pairs listed in Supplementary Table 3. The relative expression level was calculated and normalized to endogenous *Gapdh*.

Transcriptome analysis

For transcriptome RNA-seq analysis, crypts were isolated from *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice aged 8 weeks. The isolated crypts were seeded to generate organoids using the aforementioned method. Approximately 12 hours later, the cultures were harvested and centrifuged to remove contaminating cells, yielding primary organoids. Then total RNA was extracted with standard TRIzol methods and subjected to RNA sequencing by Beijing Genomics Institute. Subsequent data analysis was conducted by R studio.

Flow cytometry

For flow cytometric analysis, crypts were isolated from *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice aged 8 weeks and digested into single cells by TrypLE Express (gibco, Cat# 12605). EpCAM^{+}LGR5^{+} cells were analyzed with FACS Aria IIIu instrument (BD Biosciences). Data was collected with FACS Aria IIIu instrument and analyzed by FlowJo Version 10.8.1 software.

Dot blot

DNA fragments corresponding to different regions of the *Lgr5* promoter were amplified by PCR and purified via gel extraction (primers are listed in Supplementary Table 4). 1μg DNA sample was mixed with 20×SSC at a 2:1 volume ratio, followed by denaturation at 95°C for 10 minutes and immediately placed on ice. The annealed products were spotted onto nylon membranes at consistent

intervals and subjected to UV cross-linking. Unbound DNA was removed by washing the membranes with 2×SSC. Prehybridization of the membranes was performed using hybridization buffer at 65°C for 2 hours. Subsequently, the *Snora61* probe was denatured and added to the hybridization solution, followed by overnight incubation at 65°C. After stringent washing, the membranes were blocked, incubated with secondary antibodies, and chemiluminescent signals were detected.

ChIRP-seq and ChIRP-qPCR

Crypts isolated from WT mice aged 8 weeks were digested into single cells. ISCs were isolated by FACS and cross-linked with 1% formaldehyde at 37°C for 10 min and quenched by 0.1 M glycine for 5 min. Then cells were washed twice with PBS, lysed with Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, supplemented with PMSF, proteinase inhibitor cocktails and M5 RNase inhibitor) and sonicated to produce 200~500 bp DNA fragments. Lysates were incubated with biotinylated antisense probes against *Snora61* or scramble probe for 4 hours at 37°C. Streptavidin magnetic beads were then added to isolate probe-binding complexes. After washing, DNAs were extracted from beads. Subsequent DNA library construction, ChIRP-seq, and data analysis were performed by Novogene Co., Ltd. (Beijing). The analysis results were further validated by ChIRP-qPCR using primer pairs listed in Supplementary Table 3.

ChIP assay

Crypts isolated from WT mice aged 8 weeks were digested into single cells and cross-linked with 1% formaldehyde at 37°C for 10 min and quenched by 0.1 M glycine for 5 min. Then cells were washed twice with PBS, lysed with Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, supplemented with PMSF, proteinase inhibitor cocktails) and sonicated to produce 200~500 bp DNA fragments. Lysates were pre-cleared with Protein A/G magnetic beads (MCE, Cat# HY-K0202) and then incubated with 4 μg indicated antibodies overnight at 4°C. Protein A/G magnetic beads were then added to isolate antibody-binding complex. After washing steps, DNA were extracted from beads and analyzed by qPCR using primer pairs listed in Supplementary Table 3.

Dual luciferase reporter assay

For luciferase reporter assay, the full-length promoter of *Lgr5* (from −2000 to 0 upstream from

transcriptional start site) or truncated *Lgr5* promoter was cloned into pGL3 vector. *Snora61* sequence was constructed into pLVX-IRES-Puro vector for overexpression. MC38 cells were seeded in 24-well plate one day before transfection. For transfection of each well, 100 ng pGL3 plasmid, 1 ng pRL-TK and 500 ng pLVX-IRES-Puro plasmid were used. Luminescence signals were analyzed using Dual Luciferase Reporter Gene Assay Kit (Beyotime, Cat# RG027) according to the manufacturer's protocol.

Nuclear run-on assay

Crypts were isolated from *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice aged 8 weeks and digested into single cells by TrypLE Express. Cells were suspended in nuclear extraction buffer (10 mM Tris-HCl, 150 mM KCl, 4mM MgOAc, pH 7.4), followed by centrifugation to collect cell nuclei pellets. Pellets were lysed by lysis buffer (nuclear extraction buffer supplemented with 0.5% NP-40), followed by sucrose density gradient centrifugation to prepare transcriptional active crude nuclei components. Crude nuclei were incubated with biotin labeling mix and RNase inhibitor at 28°C for 5 min. RNAs were extracted using Trizol reagent according to the manufacturer's instructions. DNA was digested by DNase I for 15 min at room temperature. RNA transcripts were enriched with streptavidin affinity beads, followed by reverse transcription and RT-PCR analysis.

Chromatin accessibility assay

For chromatin accessibility assay, crypts were isolated from *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice aged 8 weeks and digested into single cells by TrypLE Express. Cells were suspended with Lysis buffer (60 mM KCl, 10 mM HEPES, 1 mM EDTA, 1 mM DTT, 0.075% NP-40 and 1 mM PMSF, pH 7.6) to remove cytoplasmic components. Nuclei pellets were resuspended with DNase reaction buffer (1 mM EDTA, 0.1 mM EGTA, 1 mM MgCl2, 0.5 mM $CaCl_2$ and 5% Sucrose), added 0 U or 2 U DNase I and incubated at 37°C for 5 min, followed by genomic DNA extraction and qPCR analysis of indicated chromatin regions.

Nuclear and cytoplasmic fraction isolation

Cytoplasmic and nuclear fraction were extracted using NE-PER Nuclear and cytoplasmic extraction according to the manufacturer's protocol. Briefly, the cells were suspended and lysed with cytoplasmic extraction buffer and then centrifuged to separate the nuclear fraction from the

cytoplasmic fraction. Subsequently, the nuclear fraction was lysed with nuclear extraction buffer.

Nucleoplasm and nucleolus fraction isolation

Break nuclei by sonicating the nuclear fraction on ice using several bursts of 10 sec at 400W at 10s intervals for 10 times. After each burst, verify the appearance of the suspension by examining a drop of the suspension by phase contrast microscopy. Stop the sonication when intact nuclei cannot be detected in the suspension. Very gently add 880 mM sucrose under the sonicated nuclei with a micropipettor and then centrifuge 20 min at 2000 g, 4°C. After centrifugation, collect the supernatant, which contains the nucleoplasmic fraction. Resuspend the purified nucleolar fraction in a minimal volume of 340 mM sucrose buffer and check the quality of purification by visualizing under a phase contrast microscope.

RNA pull-down assay

For RNA pull-down assay, 1×10^5 crypts were isolated from WT mice aged 8 weeks. Crypts cell nuclear fraction was collected using NE-PER Nuclear and cytoplasmic extraction as previously described and lysed with RIPA lysis buffer (1% Deoxycholate sodium, 150 mM NaCl, 50 mM Tris-HCl, 0.1% SDS and 1% Triton X-100, pH 7.2). Supernatants were incubated with 2 μg biotin-labeled *Snora61* or antisense control probe overnight at 4°C and incubated with 60 μl Streptavidin Magnetic Beads (Beyotime) for 4 h. Then precipitated components were separated with SDS-PAGE and silver staining. For pull-down assay shown in Figure 4a, the two most prominent differential bands (located at approximately 42 kDa and 30 kDa) were cut for mass spectrometry analysis. Candidate proteins identified by mass spectrometry for these two bands are presented in Supplementary Table 6.

Co-immunoprecipitation (Co-IP) assay

For Co-IP assay, 1×10^5 crypts were isolated from WT mice aged 8 weeks. Crypts cells nuclear fraction was collected using NE-PER Nuclear and cytoplasmic extraction and lysed with RIPA lysis buffer. Supernatants were incubated with anti-RBMX antibody (1:50) or IgG isotype. Then precipitated components were separated with 12% SDS-PAGE electrophoresis, followed by silver staining. For co-IP assay shown in Figure 5a, the band of RBMX at approximately 42 kDa and the most prominently differential band around 24 kDa was cut for mass spectrometry analysis. Candidate

proteins identified by mass spectrometry for these bands are presented in Supplementary Table 7.

Statistical and reproducibility

For statistical evaluation, an unpaired Student's *t*-test was applied for calculating statistical probabilities in this study. For all panels, at least three independent experiments were performed and representative experiments are shown. Data were analyzed by using Microsoft Excel or GraphPad version 9. *P*-values smaller than 0.05 were regarded as statistical significance.

Data Availability

The raw RNA sequence data reported in this paper have been deposited in the Genome Sequence Archive in National Genomics Data Center, China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences under accession codes CRA026331 (https://ngdc.cncb.ac.cn/gsa/browse/CRA026331), CRA033390 (https://ngdc.cncb.ac.cn/gsa/browse/CRA033390), CRA033391 (https://ngdc.cncb.ac.cn/gsa/browse/CRA033391), and CRA033396 (https://ngdc.cncb.ac.cn/gsa/browse/CRA033396). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the iProX partner repository with the dataset identifier PXD070722 (https://proteomecentral.proteomexchange.org/?pxid=PXD070722). The raw ChIRP-seq data have been deposited in the GEO database (GSE315692) that have been publicly accessible at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE315692. The remaining data are available within the Article, Supplementary Information, or Source Data file. All full uncropped blots for every associated figure are available in the source data file, with light dotted lines indicating the areas presented in the figures. Source data are provided in this paper.

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Author contribution

J.H., Y.L. and Y.X. performed experiments; J.H. designed the project, analyzed the data and wrote the paper. Y.L., Y.X., Z.X., Z.Y., H.G, J.Z., Z.Z. Y.D., F.P. provided technical assistance and analyzed data; Z.F. initiated the study, organized, designed, and wrote the paper.

Competing Interests

The authors declare no conflict of interest exists.

Figure legends

Figure 1. *Snora61* is highly expressed in ISCs. (a) Scatter plot of H/ACA snoRNAs microarray analysis of LGR5^{+} ISCs and LGR5^{-} IECs. (b) RT-qPCR analysis of Top10 enriched H/ACA snoRNAs in (a) in LGR5^{+} ISCs and LGR5^{-} IECs. n=3 biological independent experiments. Exact *P*-values from left to right: $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, 0.0002, 0.0429, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$. (c) Intestinal organoid formation assay upon snoRNAs knockdown and scramble treated control. n=4 biological independent experiments. Exact *P*-value: $P < 0.0001$. (d) Fluorescence *in situ* hybridization of *Snora61* in frozen sections from fetal mice. Scale bar, 500 μm. (e) Northern blot of *Snora61* in indicated organs and tissues. 18S rRNA served as a loading control. (f-h) Fluorescence *in situ* hybridization of *Snora61* in organoids (f) and small intestines (g, h). Scale bar, 50 μm (f), 100 μm (g) and 10 μm (h). (i) Distribution analysis of *Snora61* in cytoplasmic and nuclear fraction by RT-qPCR. n=3 biological independent experiments. (j) Distribution analysis of *Snora61* in nucleolar and nucleoplasmic fraction by RT-qPCR. n=3 biological independent experiments. (k) Fluorescence *in situ* hybridization of *Snora61* in ISCs. Scale bar, 5 μm. * $P < 0.05$, *** $P < 0.001$. Data were analyzed by an unpaired two-side Student's *t*-test and shown as means ± SD. Data are representative of at least three independent experiments.

Figure 2. Deletion of *Snora61* impairs self-renewal of ISCs and intestinal regeneration. (a) Anatomical examination of small intestine length of *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice. n=5 mice for each group. Exact *P*-value: $P < 0.0001$. (b) Intestine organoid formation assay of *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice under *Snora61* overexpression and control. Scale bar, 100 μm. n=5 mice for each group. Exact *P*-values from left to right: $P < 0.0001$, $P < 0.0001$. (c) H&E staining of indicated intestinal regions (duodenum, jejunum, and ileum) from *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice. Scale bar, 100 μm. The number of crypts (per field) was calculated. n=5 fields for each group. Exact *P*-values from left to right: $P < 0.0001$, $P < 0.0001$, 0.0007. (d) Representative images of Ki67 staining of small intestinal tissues from *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice. Scale bar, 50 μm. The number of Ki67^{+} cells (per crypt) was calculated. n=30 crypts for each group. Exact *P*-value: $P < 0.0001$. (e) Representative images of Ki67

staining of small intestinal organoid from *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice. Scale bar, 50 μm. The proportion of Ki67^{+} cells was calculated. n=5 mice for each group. Exact *P*-value: *P* <0.0001. (f) H&E staining of small intestine tissues from indicated mice at different time points (D0–D7) after 8 Gy radiation. Scale bar, 100 μm. The number of crypts (per field) was calculated. n=3 mice for each time point. Exact *P*-values from left to right: 0.0213, 0.1012, 0.2302, 0.0132, 0.0161. * *P* < 0.05, *** *P* < 0.001 and ns (not significant) *P* > 0.05. Data were analyzed by an unpaired two-side Student's *t*-test and shown as means ± SD. Data are representative of at least three independent experiments.

Figure 3. *Snora61* knockout impairs expression of LGR5 in ISCs. (a) Gene Ontology (GO) analysis of genes with downregulated expression in *Snora61*$^{-/-}$ organoids compared to *Snora61*$^{+/+}$ organoids. (b) Transcriptome analysis of indicated genes expression from *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ organoids. (c) RT-qPCR analysis of indicated genes from *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ crypts. n=3 biological independent experiments. Exact *P*-values from left to right: 0.0005, 0.0068, 0.0023, 0.0190, 0.0072, 0.0003. (d) RT-qPCR analysis of indicated genes from *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ crypts post irradiation. n=3 biological independent experiments. Exact *P*-values from left to right: *P* < 0.0001, 0.0001, 0.0011, *P* < 0.0001, *P* < 0.0001, *P* < 0.0001. (e) Gene Set Enrichment Analysis (GSEA) of expression of Wnt signaling pathway related genes from *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ crypts post irradiation. (f) Enrichment analysis of *Snora61* on *Lgr5* gene promoter by ChIRP-seq. Enrichment peak plots were generated and visualized using IGV software. (g) Sequence alignment of the complementary region between *Lgr5* promoter and *Snora61*/mut*Snora61*-2. (h) Enrichment of *Snora61* on *Lgr5* gene promoter by ChIRP-qPCR assay. n=4 biological independent experiments. Exact *P*-value: *P* <0.0001. (i) Dot blot of enrichment of *Snora61* or mut*Snora61* on *Lgr5* gene promoter. (j) Dual luciferase reporter assay of *Snora61* function on *Lgr5* transcription activation. FL, full-length (represent −2000~0 region upstream *Lgr5* transcriptional start site). n=3 biological independent experiments. Exact *P*-value: *P* <0.0001. (k) DNase I assay of chromatin accessibility in *Lgr5* promoters of *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ ISCs by RT-qPCR. n=3 mice for each group. Exact *P*-value: 0.0002. (l) Nuclear run-on assay of transcription activities of *Lgr5* in *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ ISCs. n=3 mice

for each group. Exact *P*-value: *P* <0.0001. (m, n) Enrichment analysis of indicated histone modifications on *Lgr5* promoter by ChIP assay. n=3 mice for each group. Exact *P*-values *P* < 0.0001 (m) and *P* < 0.0001 (n). (o) Western blot of LGR5 expression of indicated mice crypts. The relative intensity of LGR5 was quantified. n=3 biological independent experiments. Exact *P*-value: 0.0009. (p) FACS analysis of the proportion of LGR5^{+} cells in *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ crypts. n=5 mice for each group. Exact *P*-value: *P* <0.0001. (q) Organoid formation assay of indicated small intestinal organoids. Scale bar, 100 μm. * *P* < 0.05, ** *P* < 0.01, and *** *P* < 0.001. Data were analyzed by an unpaired two-side Student's *t*-test and shown as means ± SD. Data are representative of at least three independent experiments.

Figure 4. *Snora61* associates with RBMX in ISCs. (a) Silver staining image of pull-down assay of *Snora61*-binding proteins. (b) Immunoblotting analysis of RBMX in RNA pull-down samples by beads, *Snora61* sense and scramble probes in mice ISCs. (c) RNA immunoprecipitation (RIP) assay of enrichment of *Snora61* with RBMX by RT-qPCR. n=3 biological independent experiments. Exact *P*-values from left to right: 0.0062, 0.0600, *P* < 0.0001. (d) Validation of binding ability of *Snora61* with indicated RBMX truncations by RNA pull-down assay, followed by Western blot. (e, f) Immunofluorescence staining of RBMX in ISCs (e) and organoids (f). Scale bar, 10 μm (e), 50 μm (f). (g) Small intestinal organoid formation assay of *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice under *Rbmx* knockdown, overexpression and control. Scale bar, 100 μm. n=3 biological independent experiments. (h) Immunofluorescence staining of Ki67^{+} cells of indicated small intestinal organoid in (g). Scale bar, 50 μm. (i) Western blot of LGR5 and RBMX expression of indicated small intestinal organoids in (g). (j) Organoid formation assay of indicated small intestinal organoids. Scale bar, 100 μm. n=5 biological independent experiments. Exact *P*-values from left to right: *P* < 0.0001, *P* < 0.0001, *P* < 0.0001. (k, l) ChIP assay of enrichment of RBMX on *Lgr5* promoter in *Snora61*$^{+/+}$ (k) and *Snora61*$^{-/-}$ (l) ISCs. n=3 biological independent experiments. Exact *P*-values: *P* < 0.0001 (k) and 0.4353 (l). ** *P* < 0.01, *** *P* < 0.001 and ns (not significant) *P* > 0.05. Data were analyzed by an unpaired two-side Student's *t*-test and shown as means ± SD. Data are representative of at least three independent experiments.

Figure 5. *Snora61*-RBMX recruits HMGB2 onto *Lgr5* promoter to initiate its transcription and expression. (a) Silver staining image of co-immunoprecipitation (co-IP) assay of RBMX-binding proteins. (b) Immunoblotting analysis of HMGB2 in co-IP samples by IgG and anti-RBMX in ISCs. (c) Validation of binding ability of HMGB2 with indicated RBMX truncations by co-IP assay, followed by Western blot. (d) Immunoblotting analysis of HMGB2 in co-IP samples by anti-RBMX in *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ ISCs. (e-g) Immunofluorescence staining analysis of HMGB2 in small intestines (e), organoids (f) and ISCs (g). Scale bar, 10 μm (e), 50 μm (f) and 10 μm (g). (h) Intestinal organoid formation assay of *Snora61*$^{+/+}$ and *Snora61*$^{-/-}$ mice under *Hmgb2* knockdown and control. Scale bar, 100 μm. n=3 biological independent experiments. Exact *P*-values from left to right: $P < 0.0001$, 0.0020. (i) Immunofluorescence staining of Ki67^{+} cells of indicated small intestinal organoids in (h). Scale bar, 50 μm. The proportion of Ki67^{+} cells was calculated. n=5 organoids for each group. Exact *P*-values from left to right: $P < 0.0001$, $P < 0.0001$. (j) Western blot of LGR5 and HMGB2 expression of indicated small intestinal organoids in (h). (k) RT-qPCR analysis of *Lgr5* expression of indicated small intestinal organoids in (h). n=3 biological independent experiments. Exact *P*-values from left to right: $P < 0.0001$, $P < 0.0001$. (l, m) ChIP assay of enrichment of HMGB2 on *Lgr5* gene promoter in *Snora61*$^{+/+}$ (l) and *Snora61*$^{-/-}$ (m) mice ISCs. n=3 biological independent experiments. Exact *P*-values: $P < 0.0001$ (l) and 0.1050 (m). (n) Dual luciferase reporter assay of HMGB2 function on *Lgr5* transcription activation. FL, full-length (−2000~0 region upstream *Lgr5* transcriptional start site). n=3 biological independent experiments. Exact *P*-values from left to right: $P < 0.0001$, $P < 0.0001$. ** $P < 0.01$, *** $P < 0.001$ and ns (not significant) $P > 0.05$. Data were analyzed by an unpaired two-side Student's *t*-test and shown as means ± SD. Data are representative of at least three independent experiments.

Figure 6. LGR5 is required for *Snora61*-mediated ISC self-renewal and intestinal regeneration. (a) Western blot of LGR5 expression of indicated mice crypts. (b) Representative images of Ki67 staining of small intestinal tissues from sgCtrl and sg*Lgr5* mice. Scale bar, 100 μm. The number of Ki67^{+} cells (per crypt) was calculated. n=30 crypts for each group. Exact *P*-value: $P < 0.0001$. (c) H&E

staining of small intestine tissues from indicated mice. Scale bar, 100 μm. Length of villus was calculated. n=5 mice for each group. Exact *P*-values from left to right: $P < 0.0001$, $P < 0.0001$, $P < 0.0001$. (d) Intestinal organoid formation assay of indicated mice. Scale bar, 100 μm. n=5 mice for each group. Exact *P*-values from left to right: $P < 0.0001$, $P < 0.0001$, $P < 0.0001$. (e) H&E staining of small intestine tissues from indicated mice at different time points (D0, D3, D5) after 8 Gy radiation. Number of crypts (per field) were calculated. Scale bar, 100μm. n=5 fields for each time point. Exact *P*-values from left to right: $P < 0.0001$, $P < 0.0001$, 0.0462, 0.5447, 0.5447, 0.5447, 0.0034, 0.0005, 0.0294. (f) Intestinal organoid formation assay of indicated mice. The used concentration of dFz7-21, 100 μM. Scale bar, 100 μm. n=5 for each group. Exact *P*-values from left to right: $P < 0.0001$, $P < 0.0001$, $P < 0.0001$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and ns (not significant) $P > 0.05$. Data were analyzed by an unpaired two-side Student's *t*-test and shown as means ± SD. Data are representative of at least three independent experiments.

Editor's Summary:
This study identifies Snora61, a snoRNA enriched in intestinal stem cells (ISCs). Snora61 knockout impairs ISC function. Snora61 binds the Lgr5 promoter, recruiting RBMX and HMGB2 to activate Lgr5 transcription.

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