Long non-coding RNAs regulate Wnt signaling during feather regeneration

Xiang Lin1*, QingXiang Gao1*, LiYan Zhu1, GuiXuan Zhou1, ShiWei Ni1, Hao Han2 and ZhiCao Yue1‡

ABSTRACT

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts that are involved in a broad range of biological processes. Here, we examine the functional role of lncRNAs in feather regeneration. RNA-seq profiling of the regenerating feather blastema revealed that Wnt signaling is among the most active pathways during feather regeneration, with Wnt ligands and their inhibitors showing distinct expression patterns. Co-expression analysis identified hundreds of lncRNAs with similar expression patterns to either the Wnt ligands (the Lwnt group) or their downstream target genes (the Twnt group). Among these, we randomly picked two lncRNAs in the Lwnt group and three lncRNAs in the Twnt group to validate their expression and function. Members in the Twnt group regulated feather regeneration and axis formation, whereas members in the Lwnt group showed no obvious phenotype. Further analysis confirmed that the three Twnt group members inhibit Wnt signal transduction and, at the same time, are downstream target genes of this pathway. Our results suggest that the feather regeneration model can be utilized to systematically annotate the functions of lncRNAs in the chicken genome.

KEY WORDS: LncRNA, Regeneration, Wnt signaling, Feather follicle, Chicken

INTRODUCTION

Long non-coding RNAs (lncRNAs) are defined as non-protein coding transcripts longer than 200 nucleotides. Recent studies have revealed that lncRNAs are involved in various physiological and pathological processes (Fatica and Bozzoni, 2014; Leisegang et al., 2017; Luo et al., 2016; Michalik et al., 2014; Roeszler et al., 2012; Sun et al., 2013). In development and regeneration, lncRNAs are not only tissue specific, but are also spatially and temporally regulated (Gloss and Dinger, 2016; Xu et al., 2013). It has been shown that lncRNAs are involved in the regulation of major signaling pathways, including Wnt, Notch and TGF-β signaling (Arun et al., 2012; Fan et al., 2014; Li and Kang, 2014; Trimarchi et al., 2014; Wang et al., 2017a, 2015; Xiao et al., 2016; Yang et al., 2016; Yuan et al., 2014; Zhou et al., 2014). However, it remains a challenge to systematically annotate the functional lncRNAs in tissue and organ regeneration.

The avian feather has robust growth and regeneration capability (Lin et al., 2013). Many genes and signaling pathways have been shown to regulate feather regeneration, including Wnt/β-catenin, BMP/TGF-β, Sprouty/FGF, Notch, etc. (Cheng et al., 2018; Chu et al., 2014; Yu et al., 2002; Yue et al., 2012). Of note is the Wnt signaling pathway: we have recently found that the fine-tuning of Wnt signaling is essential for feather regeneration (Chu et al., 2014). Moreover, we have developed methods to manipulate gene expression in the feather follicle by lentiviral-mediated overexpression or RNAi-knockdown (Lin and Yue, 2018; Cheng et al., 2018; Chen et al., 2014; Chu et al., 2014; Xie et al., 2015). Thus the feather follicle offers the opportunity to analyze gene function in vivo.

In recent years, there has been tremendous effort to profile and characterize lncRNAs in the chicken genome in development and diseases (Liu et al., 2018, 2017; Li et al., 2018, 2016, 2012; Ren et al., 2018; Wu et al., 2018; Wang et al., 2017b; Zhang et al., 2017; Lin et al., 2016). The NONCODE database (www.noncode.org) lists a total of 13,085 lncRNA transcripts, corresponding to 9688 lncRNA genes in the chicken genome (Xie et al., 2014; Zhao et al., 2016). This database is curated through a combination of several public resources, including published literature and the latest versions of popular databases such as Ensemble, RefSeq, lncRNAdb and GENCODE. To date, the functions of the majority of these lncRNAs remain unknown.

Co-expression analysis provides a feasible strategy to predict the functions of lncRNAs (Perron et al., 2017; Xu et al., 2017; Han et al., 2017; Jiang et al., 2015;Guo et al., 2013; Liao et al., 2011). Here, we have systematically analyzed the transcriptional profile during feather regeneration and identified more than 5000 lncRNAs in the process. Using co-expression analysis, we found that hundreds of lncRNAs showed similar expression patterns to either the Wnt ligands or their downstream target genes. The functional significance of a few selected lncRNAs was further validated in vitro and in vivo. Our results reveal that lncRNAs can modulate Wnt signaling and regulate feather regeneration, and suggest that the feather follicle can be used to functionally annotate lncRNAs in the chicken genome.

RESULTS AND DISCUSSION

A feedback loop between Wnt ligands in the epithelium and their inhibitors in the dermal papillae drives feather regeneration

The basic structure of the feather follicle is shown in Fig. S1. After plucking the feather, only the dermal papillae (DP) and the follicle wall were left, which then initiated feather regeneration (Yue et al., 2005). During regeneration, the papilla ectoderm and the follicle sheath epithelium migrated and proliferated to cover the DP and formed the blastema at day 2 (T2). This structure then quickly expanded and re-organized into a new follicle at day 4 (T4; Fig. S1).

To monitor gene expression changes during feather regeneration, we took an unbiased whole-genome profiling approach based on next-generation sequencing technology (RNA-seq data deposited in...
that captures the epithelial-mesenchymal interactions during feather regeneration (Fig. 1D): the Wnt ligands that are expressed in the epithelium drive cell proliferation and maintain the DP markers; the Wnt inhibitors are essential for the activity of the DP and, at the same time, fine tune Wnt signaling to ensure proper regeneration. When feathers are plucked to initiate regeneration, Wnt ligands are removed, therefore the target genes in the DP are downregulated. As regeneration proceeds, Wnt ligands accumulate in the expanding epithelium, leading to the gradual increase in expression of target genes in the DP. The actual expression dynamics of the ligands or the target genes may show different patterns during regeneration (Fig. 1D). Together, our data and analysis suggest that Wnt signaling is a key regulator of feather regeneration, and genes involved in this pathway show distinct dynamic expression patterns in time and space.

Grouping lncRNAs into distinct subsets based on their dynamic expression patterns during feather regeneration

We mapped our RNA-seq data to the NONCODE database, which contains the sequence information of 9688 chicken lncRNAs. We identified more than 5000 lncRNAs in the feather follicle. After removing those lncRNAs that are expressed as zero at one time point (T0/T2/T4), we clustered the remaining 2695 lncRNAs into six subgroups (Fig. 2A). For each subgroup, the expression changes of representative lncRNAs were shown to illustrate its unique dynamic expression patterns during feather regeneration (see Fig. 4). Based on the dynamic expression patterns of these two groups of genes and their interactive behavior, we have built a feedback model that captures the epithelial-mesenchymal interactions during feather regeneration (Fig. 1D): the Wnt ligands that are expressed in the epithelium drive cell proliferation and maintain the DP markers; the Wnt inhibitors are essential for the activity of the DP and, at the same time, fine tune Wnt signaling to ensure proper regeneration. When feathers are plucked to initiate regeneration, Wnt ligands are removed, therefore the target genes in the DP are downregulated. As regeneration proceeds, Wnt ligands accumulate in the expanding epithelium, leading to the gradual increase in expression of target genes in the DP. The actual expression dynamics of the ligands or the target genes may show different patterns during regeneration (Fig. 1D). Together, our data and analysis suggest that Wnt signaling is a key regulator of feather regeneration, and genes involved in this pathway show distinct dynamic expression patterns in time and space.

**Fig. 1.** Wnt signaling is a key regulator of feather regeneration. (A) The number of differentially expressed genes (DEGs) at day 2 (T2) or day 4 (T4) during feather regeneration. (B) The dynamic expression of Wnt ligands (Wnt5a, Wnt4 and Wnt6) and inhibitors (Dkk2, Frzb and Sfrp1). TPM, tag per million (indicates the gene expression levels from RNA-seq). (C) In situ hybridization showing that the Wnt ligands are mainly expressed in the epithelium and are upregulated, whereas Wnt inhibitors are enriched in the mesenchyme and are downregulated. Scale bar: 1 mm. (D) A mathematical model based on the epithelial-mesenchymal feedback interactions in feather regeneration, which is mediated by Wnt ligands (L) and their target genes (T). The simulated phase plane shows that T or L will eventually reach the equilibrium point (black arrow, described as L*, T*) along the trajectory indicated by the brown arrows, regardless of their initial value. The dynamic trends of T or L are also shown. Although L are upregulated in both pattern A and B, T may show different dynamics. a.u., arbitrary unit.
We derived a subset of 687 downregulated lncRNAs as the Twnt group (from cluster 3, which showed similar expression patterns to the Wnt inhibitors Dkk2, Frzb and Sfrp1), and a subset of 355 upregulated lncRNAs as the Lwnt group (from clusters 5 and 6, which showed similar expression patterns to the Wnt ligands Wnt4, Wnt5a and Wnt6; see Table S1).

We then examined the expression patterns of these lncRNAs and tested their function. We randomly chose five lncRNAs: two in the Lwnt group [NONGGAT005351 (lnc5351) and NONGGAT007349 (lnc7349)] and three in the Twnt group [NONGGAT011589 (lnc1589), NONGGAT003500 (lnc3500), and NONGGAT007831 (lnc7831)]. Expression changes during feather regeneration were analyzed using qPCR and in situ hybridization (Fig. 2C and D). The two upregulated lncRNAs were weakly expressed at T0, but increased at T4, mainly in the epithelium. Conversely, the three downregulated lncRNAs were enriched in the DP at T0 and were reduced at T4. These results not only verify the RNA-seq data, but also suggest that the lncRNAs in the Lwnt group were expressed in a pattern similar to the Wnt ligands, whereas the lncRNAs in the Twnt group were expressed in a pattern that was similar to the Wnt inhibitors. Thus we hypothesize that these lncRNAs may function in the Wnt signaling pathway.

**LncRNAs regulate feather regeneration and axis formation**

To investigate the function of lncRNAs, we used lentiviral-mediated RNAi to knockdown the expression of these genes in the feather follicle (Chu et al., 2014; Chen et al., 2014; Xie et al., 2015; Cheng et al., 2018; Lin and Yue, 2018). The knockdown efficiency was examined in the chicken DF1 cell line (Fig. S3) and in vivo (Fig. 3A). The impact of RNAi knockdown was analyzed at two distinct developmental stages during feather regeneration. In the first approach, the feather follicles were harvested at 4 days post infection, when the follicle structures have just formed. Histological analysis by Hematoxylin and Eosin staining, as well as Masson staining, revealed normal regeneration after knockdown of lnc5351 or lnc7349. Knockdown of lnc1589, lnc3500 or lnc7831 retarded the regeneration process: cell proliferation rates were downregulated and pulp formation was reduced, whereas the DP morphology and marker gene expression were maintained (Fig. 3B, Figs S4 and S5). A similar phenotype has been documented after knockdown of Dkk2 or Frzb during feather regeneration (Chu et al., 2014). In the second approach, the feather follicles were allowed to complete their growth cycle and the final feathers were collected to analyze the morphological changes (Yue and Xu, 2017; Fig. 3C). Knockdown of lnc5351 or

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**Fig. 2. Characterization of lncRNA expression in feather regeneration.** (A) A total of 2695 lncRNAs were subject to the Short Time-series Expression Miner software analysis and clustered into six subgroups. Normalized expression levels are represented in shades of red and blue. (B) Expression patterns of 20 representative lncRNAs, with the highest expression levels in the cluster and the total numbers of differentially expressed lncRNAs shown for each cluster. (C,D) qPCR (C) and in situ hybridization (D) showing the expression changes of representative lncRNAs in feather regeneration. *P<0.05; **P<0.001 (Student's t-test). n.s., not significant. Scale bar: 1 mm.

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DEVELOPMENT
lnc7349 produced normal feathers. In contrast, knockdown of lnc1589 (11.8%, n=17), lnc3500 (41.2%, n=29) or lnc7831 (23.5%, n=17) produced feathers with reduced rachis, a phenotype that suggests perturbed Wnt signaling (Chu et al., 2014; Yue et al., 2006). By combining the different viruses, a stronger phenotype of splitting vanes and/or axis can be obtained, which suggests that the effects of lncRNAs are additive (Fig. 3C).

Together, these results suggest that lncRNAs can regulate feather regeneration in vivo. LncRNAs regulate Wnt signaling

To further investigate whether the lncRNAs indeed regulate Wnt signaling, two independent tests were performed in DF1 cells. In the first test, we used a luciferase reporter pGL3-7TCF, which contains the 7×TCF promoter sequence to monitor canonical Wnt/β-catenin signaling. When the expression of lnc1589, lnc3500 or lnc7831 was knocked down, the reporter activity was increased, which suggests activation of Wnt signaling (Fig. 4A). In the second test, we quantified the expression of known Wnt target genes including Dkk2, Frzb, NCAM (also known as NCAM1), SMA (ACTA2) and Des using qPCR analysis. Knockdown of lnc1589, lnc3500 or lnc7831 increased the expression of Wnt target genes (Fig. 4A). These data suggested that the three lncRNAs in the Twnt group are bona fide inhibitors for Wnt signaling. Manipulating the two lncRNAs in the Lwnt group showed no significant change, possibly because of functional redundancy.

The relevance of these results was further examined in vivo. Gene expression profiling revealed that after Wnt5a overexpression or lnc3500/lnc7831 knockdown, the expression of Wnt target genes was increased in the regenerating feather follicle (Fig. 4B). Pathway analysis further demonstrated that the Wnt signaling is top of the list of perturbed pathways (Fig. 4C). These in vivo data confirm that the lncRNAs regulate Wnt signaling during feather regeneration.

Fig. 3. Knockdown of lncRNAs disrupt feather regeneration and axis formation. (A) RNAi knockdown efficiency in the feather follicles in vivo. *P<0.05; **P<0.01; ***P<0.001 (Student’s t-test). (B) Representative histology of regenerated feather follicles after knockdown of lncRNAs. Knockdown of lnc1589, lnc3500 or lnc7831 resulted in atrophic pulp, but the DP characteristics were retained, as is shown by SMA and NCAM staining. The numbers indicate the occasions with the desired phenotype. (C) Representative morphology of regenerated feathers. Knockdown of lnc1589 (11.8%, n=17), lnc3500 (41.2%, n=29) or lnc7831 (23.5%, n=17) diminished the feather axis. Knockdown of lnc5351+7349 or lnc1589+3500+7831 produced a stronger phenotype of split axis/vanes. The control virus was produced by the empty viral vector. Scale bars: 1 mm in B; 2 mm in C.
Conclusions

Once considered the noise in transcription, lncRNAs have been shown to play important roles in a wide range of biological processes (reviewed in Batista and Chang, 2013; Fatica and Bozzoni, 2014). Although there has been tremendous effort to identify lncRNAs in chicken development and diseases (Liu et al., 2018, 2017; Li et al., 2018, 2016, 2012; Ren et al., 2018; Wu et al., 2018; Wang et al., 2017b; Zhang et al., 2017; Lin et al., 2016), there are very few reports to functionally annotate them (Roeszler et al., 2012; Arriaga-Canon et al., 2014; Cai et al., 2017; Fan et al., 2017). The nomenclature of the chicken lncRNAs has not reached a consensus and the current databases are still under constant improvement. Nonetheless, the available databases such as NONCODE do offer an entry point for further experimental testing.

Here, we show that lncRNAs are involved in the regulation of feather regeneration. In our genome-wide profiling, more than 5000 lncRNAs were detected in the feather follicle. Clustering analysis revealed differential expression dynamics of these lncRNAs, suggesting that they may have distinct roles in feather regeneration. Indeed, we showed that a few selected lncRNAs can regulate Wnt signaling and disrupt feather regeneration.
Wnt signaling is a well-known modulator of organ regeneration. In mammalian hair follicles, Wnt signaling is important for maintaining the DP (Kishimoto et al., 2000) and is required for hair follicle formation (Alonso and Fuchs, 2003; Tsai et al., 2014). When Wnt signaling is attenuated, feather regeneration is disrupted (Chu et al., 2014). Here, we show that the Wnt signaling is among the most active pathways during feather regeneration. Components of this pathway showed distinct expression patterns: Wnt ligands are mainly expressed in the epithelium and are upregulated, whereas the inhibitors are enriched in the DP and are downregulated. These results are in line with the concept that epithelial-mesenchymal interactions control feather regeneration: that a feedback loop exists between Wnt ligands in the epithelium and Wnt inhibitors (at the same time as being target genes of Wnt signaling) in the mesenchyme. According to our mathematical model, the concentration of ligands will increase, whereas that of the target genes will decrease as regeneration proceeds. The levels of Wnt ligands and downstream target genes will finally reach an equilibrium.

Aided by this model and the dynamic co-expression patterns of lncRNAs, we were able to predict the function of a subset of lncRNAs in feather regeneration. Indeed, through individual verification, we confirmed the expression pattern and function of a few selected lncRNAs. Both in vitro (in DF1 cells) and in vivo (in the feather follicle), these lncRNAs can modulate Wnt signaling. Although we have to acknowledge that, because of the large amount of lncRNAs in each of the Lwnt (355) and Twnt (687) groups, these selected lncRNAs cannot be claimed to be ‘representative’, they do offer clues for future tests of their function. More systematic approaches, such as RNAi screening in DF1 cells or in vivo, may help to address this issue further. Moreover, Wnt signaling may not be the only pathway that is crucial for feather regeneration. Nonetheless, our results represent the first effort towards systematically annotating functional lncRNAs in feather regeneration. Genome localization analysis revealed that the five chosen lncRNAs are not in close proximity to any known Wnt ligands or inhibitor genes, making it unlikely that these lncRNAs are co-regulated by known genes in proximity (Fig. S6). Further investigation will be required to clarify the details of how these lncRNAs regulate Wnt signaling, and at the same time how they are regulated during feather regeneration.

MATERIALS AND METHODS

Experimental animals

Three- to six-month-old chickens (Gallus gallus domesticus) were purchased from local farms and housed in the Animal Facility Center, Institute of Life Sciences, Fuzhou University. All experimental protocols were approved by Fuzhou University Experimental Animal Ethics Board, Institute of Life Sciences, Fuzhou University. Three- to six-month-old chickens (Gallus gallus domesticus) were approved by Fuzhou University Experimental Animal Ethics Board, Institute of Life Sciences, Fuzhou University. All experimental protocols purchased from local farms and housed in the Animal Facility Center.

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Whole-genome expression profiling and data analysis

Following Wnt5a overexpression or Inc3500/Inc7831 knockdown, the regenerating feather follicles were collected at 4 days post infection (Chu et al., 2014). Total RNAs were extracted using RNAiso Plus reagent (Takara). Total RNAs were sequenced at Novogene and mapped to a current version of the NCBI database. Data from the GEO deposit GSE42017 were further mapped to the NONCODE database using the Bowtie 2 program (bowtie-bio.sourceforge.net/bowtie2). Differentially expressed genes were identified by at least a twofold change, with a false discovery rate of P<0.001. The differentially expressed genes were further analyzed using the PANTHER program (www.pantherdb.org) to identify the perturbed pathways. For clustering of lncRNAs, the STEM program was used (Ernst and Bar-Joseph, 2006; www.cs.cmu.edu/~jemst/). For each cluster, the 20 lncRNAs with the highest expression levels averaged from T0, T2 and T4 were chosen to show the dynamic expression pattern. A more stringent filter of fourfold expression change (T2 versus T0) was applied to reach a final set of lncRNAs as the Twnt/Lwnt group from Cluster 3 and Clusters 5 and 6, respectively. The specific lncRNAs with their expression levels are listed in Table S1.

Mathematical modeling of the feedback interactions during feather regeneration

We have built a two-compartment interactive model to monitor the dynamic expression changes of the Wnt ligands in the epithelium and the inhibitors and/or the target genes in the DP. [L] represents the concentration of Wnt ligands and [T] represents the concentration of the inhibitors/target genes. Then we have:

\[
\frac{d[L]}{dt} = mL \cdot \frac{L^{m}}{1 + [L]^{m}} - L \cdot \alpha \cdot \n
\frac{d[T]}{dt} = mL \cdot \frac{L^{m}}{1 + [L]^{m}} - L \cdot \alpha \cdot \n
\]

Here, \( m \) represents the maximum synthesize rate, \( n \) represents the Hill coefficient, \( \alpha \) represents the background synthesize rate, \( L \) represents the degradation rate, and \( t \) represents time. Results were simulated by the MATLAB software (uk.mathworks.com/products/matlab.html).

Plasmids

The vector used for RNA knockdown was pLL3.7 (Addgene plasmid #11795, deposited by Luk Parijs). Sequences targeted in RNAi were: GC-ACGCGTAGTTCGCCGTT for Inc1589, GGAGGTAGTTGCTCATAA for Inc3500, GCATTGAGCCGGTAA for Inc7831, GGCCAAATGG-CTAACAAACC for Inc5351 and GGACACCTATTCCGCCGAT for Inc7349. These targets were designed using the Thermo Fisher Scientific BLOCK-it RNAi Designer (maidesigner.thermofisher.com/mairexpress) and blasted in Genbank and NONCODE to ensure the specificity. The RNAi knockdown efficiency was monitored both in DF1 cells and in vivo, as previously described (Lin and Yue, 2018). For pGL3-7TCF, the 7×TF promoter sequence (from the T7Gp plasmid; Addgene plasmid #24305, donated by Roel Nusse) was PCR amplified using the following primers: forward 5′-TGCAGGGTACCGAGCTCTTA-3′, reverse 5′-ACCATGGTG-GCTTTAACC-3′. The PCR fragment was cut with SacI and HindIII, and ligated to the luciferase reporter plasmid pGL3-basic (Promega).

Histology, immunostaining and in situ hybridization

Histology; immunostaining and in situ hybridizations were processed as previously described (Yue et al., 2006). Tissue samples were harvested at designated time points. Paraffin sections or cryosections were collected at 8 μm thickness. Antibodies were obtained from the Developmental Study Hybridoma Bank and used at a 1:50 dilution: anti-NCAM (SE), anti-SMA (1E12) and anti-Brdu (G34). For Brdu staining, animals were given an intraperitoneal injection of 50/μg Brdu (Sigma-Aldrich) and samples were collected 1 h later. For Massion staining, a commercial kit from ZSGB-Bio was used, following the manufacturer’s instructions. Probes for in situ hybridization were: Inc1589 [nucleotides (nt) 231-606], Inc3500 (nt 1468-1710), Inc7831 (nt 425-842), Inc5351 (nt 80-460), Inc7349 (nt 68-561), Wnt4 (nt 831-1180; NCBI NM_204783.1), Wnt5a (nt 382-1227; NCBI NM_204887.1), Wnt6 (nt 191-507; NCBI NM_001007594.2), Dkk2 (nt 961-1260; NCBI XM_420494.2), Fzrb (nt 521-771; NCBI NM_204772.2), Sfrp1 (nt 755-1212; NCBI NM_204553.3).

RT-PCR and qPCR analysis

Total RNAs from DF1 cells (GNO30, Cell Library of the Chinese Academy of Sciences) were isolated using RNAiso Plus reagent (Takara) and reverse transcribed to cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). PCR was performed using Taq MasterMix (CWBio). The PCR conditions were: 5 min at 94°C, 30-33 cycles at 94°C.
for 30 s, 60°C for 30 s, 72°C for 30 s, followed by 72°C for 10 min. qPCR was performed using a LightCycler 480 system (Roche Applied Science) with the UltraSYBR Mixture (CWBIO). Gene expression levels were normalized to β-actin. Data were quantified using the delta-delta CT method. Each experiment was repeated at least three times. Primer sequences are listed in Table S2.

**Virus production and in vivo infection**

Lentiviruses were produced in HEK293T cells using the standard protocol as described (Chu et al., 2014). Lentiviral supernatant was concentrated by precipitation using PEG 6000 (8.5% final concentration) and 0.4 M NaCl for 2 h at 4°C, followed by centrifugation at 9600 g for 15 min. The virus was re-suspended with PBS containing 8 μg/ml hexadimethrine bromide (Sigma-Aldrich) to enhance the infection efficiency. Sample collection and processing, and documentation of feather morphology, have been described previously (Yue and Xu, 2017).

**Electroporation**

DF1 cells were cultured with DMEM containing 10% fetal bovine serum (Hyclone). Electroporation was performed using the CTX-1500A electroporator (Celetrix LLC). Briefly, 1×10^6 DF1 cells were mixed with 10 μg plasmid in 130 μl electrolytic buffer and loaded into an electroporation tube. The parameters were: 700v voltage, 30 ms pulse width, 1 pulse. The cells were then washed once at 6 h post-seeding and fresh medium was added. Cells were collected 48 h later and total RNAs were extracted using RNeasy Plus (Takara).

**Wnt reporter assay**

Wnt activation was monitored using the luciferase reporter pGL3-7TCS. DF1 cells were transfected with 0.5 μg RNAi constructs, 0.5 μg pGL3-7TCS and 0.2 μg CMV-LacZ using the TransIntra EL Transfection Reagent (Transgene Biotech). The transfected cells were lysed 48 h later and luciferase activity was measured using a luciferase assay kit, normalized to β-galactosidase expression (Beyotime). Each experiment was repeated three times.

**Statistics**

Data analysis was performed using GraphPad Prism 6.01. All data were represented as mean±s.d. based on at least three repeats. Group differences were assessed using Student’s t-test. P<0.05 was considered statistically significant (*P<0.05, **P<0.01, ***P<0.001; n.s., not significant).

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Data availability**

RNA-seq data have been deposited in GEO under the accession number GSE118374.

**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup doi:10.1242/dev.162388

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