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siRNA induces *gelsolin* gene transcription activation in human esophageal cancer cell

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Recent studies show that targeting gene promoter or 3′ terminal regions of mRNA with siRNA induces target gene transcription. However, the ability of exon-targeting siRNA to affect transcription has yet to be demonstrated. We designed and synthesized siRNA against various exons in the gelsolin gene (*GSN*) to knockdown GSN transcript in KYSE150 and KYSE450 cells. Surprisingly, we found that siGSN-2, targeting the GSN twelfth exon, induced GSN gene transcription detected by real time RT-PCR. An siGSN-2 co-precipitation assay was performed and H3 histone, previously shown to correlate with gene transcription, was detected in the siGSN-2 pull-down pellet. However, H3 histone was not detected in an siGSN-1-precipitated pellet, which resulted in GSN knockdown. In addition, siGSN-2 decreased stress fibers, lamellipodia and filopodia, demonstrating that siGSN-2 induced GSN transcription activation and exerted biological function. In conclusion, our finds reveal siRNA, which is derived from target gene exon, can form the complex with H3 histone to be involved in the regulation of gene expression.

S mall dsRNAs, including siRNA and microRNA, down-regulate homologous mRNA in cells to result in target gene post-transcriptional silencing[1]. The target gene mRNA template is cleaved and degraded via the Dicer endoribonuclease activated by the RNA-induced silencing complex (RISC) composed of siRNA and multiple proteins, including SND1, AEG-1, Ago and other proteins[2,3]. MicroRNA binding to RISC results in gene mRNA degradation if its seed region completely matches the mRNA template, or it inhibits gene mRNA translation[4,5]. However, some small dsRNAs, known as RNA activation (RNAa), up-regulate gene transcription by mechanisms distinct from RNAi. For example, an siRNA targeting CpG-rich regions or CpG islands in gene promoters activates gene expression[6]. Li et al. showed an siRNA targeting a region outside gene promoter CpG islands also adheres to RNAi rules activating gene transcription[7], and an endogenous microRNA-373 induces expression of genes with complementary promoter sequences[8]. Yue et al. identified a non-coding transcript that overlaps the 3′ terminus of the progesterone receptor (PR) gene and found that small RNAs complementary to sequences beyond the 3′ terminus of PR mRNA modulate PR expression by recruiting Argonaute 2 to the non-coding transcript to further induce chromatin changes at the PR promoter[9].

Gelsolin, encoded by the *GSN* gene, can sever, cap and nucleate actin filaments, and thus plays a critical role in maintaining the normal cellular motility and morphology[10–12]. Gelsolin is a downstream effector of RAC that regulates organization of actin filaments and gelsolin knockdown induces RAC overexpression and increases amounts of F-actin in stress fibers and lamellipodia[13,14]. However, gesolin up-regulation inhibits RAC expression and reduces amounts of F-actin[15]. Gelsolin plays a key role as a tumor suppressor in human bladder cancer cells[16]. In the early stages of both human and animal tumor carcinogenesis, down-regulation of gelsolin has been widely observed[17]. Gelsolin down-regulation may be the mechanism through which tumor cells evade apoptotic signaling pathways[18]. Gelsolin may later become up-regulated to enhance metastasis. Therefore, gelsolin plays a key role in human cancer cells. However, the delineating biological function of gelsolin in human cancer cells is still unclear.

In the present study, during examination of the biological function of gelsolin, we designed several siRNAs targeting various exons in the *GSN* gene and found that an siRNA targeting the eleventh exon decreased *GSN* transcription. However, an siRNA targeting the twelfth exon caused *GSN* transcription up-regulation. We
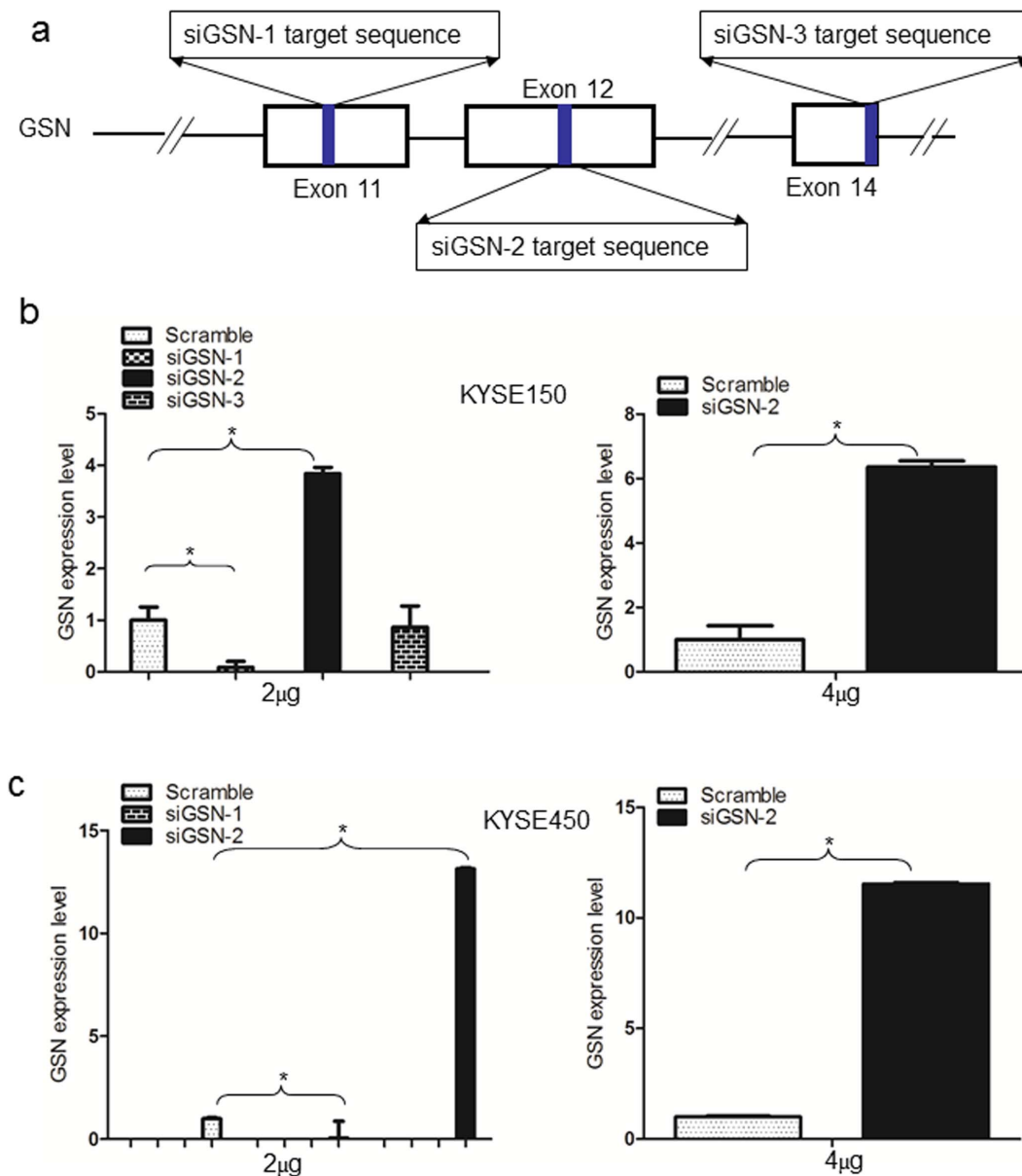
Figure 1 | GSN transcript was up-regulated with siGSN-2 transfection. (a) Schematic diagram of siGSNs against target sequence of *GSN* gene exons. (b) *GSN* transcripts individually detected by real-time RT-PCR at 48 h post-transfection with 2 µg or 4 µg siGSN(−1, −2, −3) into KYSE150 cells cultured in 6-well plate. (c) *GSN* transcripts were individually detected by real time RT-PCR at 48 h post-transfection with 2 µg and 4 µg siGSN (−1, −2, −3) into KYSE450 cells cultured in 6-well plate. Results are mean ± standard error (SE). *$p \leq 0.05$.

investigated the possibility that the twelfth exon by siRNA targeting activates gene transcription through mechanisms distinct from those of targeting gene promoters or 3′ terminal regions of mRNA.

Results

Up-regulation of *GSN* expression by si*GSN*-2. During the process of exploring endogenous GSN protein biological functions, we tempted to make GSN level knockdown by RNA interference. Therefore, we designed three siRNAs and the target sequence of si*GSN*-1, si*GSN*-2 or siGSN-3 against GSN exon was individually depicted (Figure 1a). 2 µg si*GSN*-1, si*GSN*-2 or siGSN-3 was individually transfected into KYSE150 cells cultured in 6-well plate and *GSN* level was detected by real time RT-PCR. Transfection of siGSN-1 resulted in *GSN* down-regulation. However, si*GSN*-2 transfection up-regulated *GSN* expression. siGSN-3 transfection did not alter *GSN* transcript expression. To confirm that siGSN-2 results in *GSN* up-regulation, we increased the siGSN-2 amount to

4 µg to treat KYSE150 cells cultured in 6-well plate. At this siRNA concentration, *GSN* was still up-regulated (Figure 1b). To further demonstrate that siGSN-2 induced GSN expression up-regulation, siGSN-2 was added to KYSE450 cells cultured in 6-well plate and siGSN-1 was selected as control. The result showed that *GSN* was down-regulated with 2 µg si*GSN*-1, whereas *GSN* was up-regulated with 2 µg siGSN-2. Similarly, 4 µg si*GSN*-2 up-regulated *GSN* compared to the scramble (Figure 1c).

si*GSN*-2-mediated up-regulation of *GSN* expression involves transcription. Since si*GSN*-2 induced *GSN* up-regulation, we first speculated that si*GSN*-2 was involved in *GSN* transcription. Therefore, we transfected cells with biotin-labeled siGSN-2 and pulled down the potential siGSN-2-containing protein complexes (Figure 2a). To determine if transfection with biotin-labeled si*GSN*-1 still resulted in *GSN* knockdown and biotin-labeled siGSN-2 resulted in *GSN* expression up-regulation, *GSN* mRNA
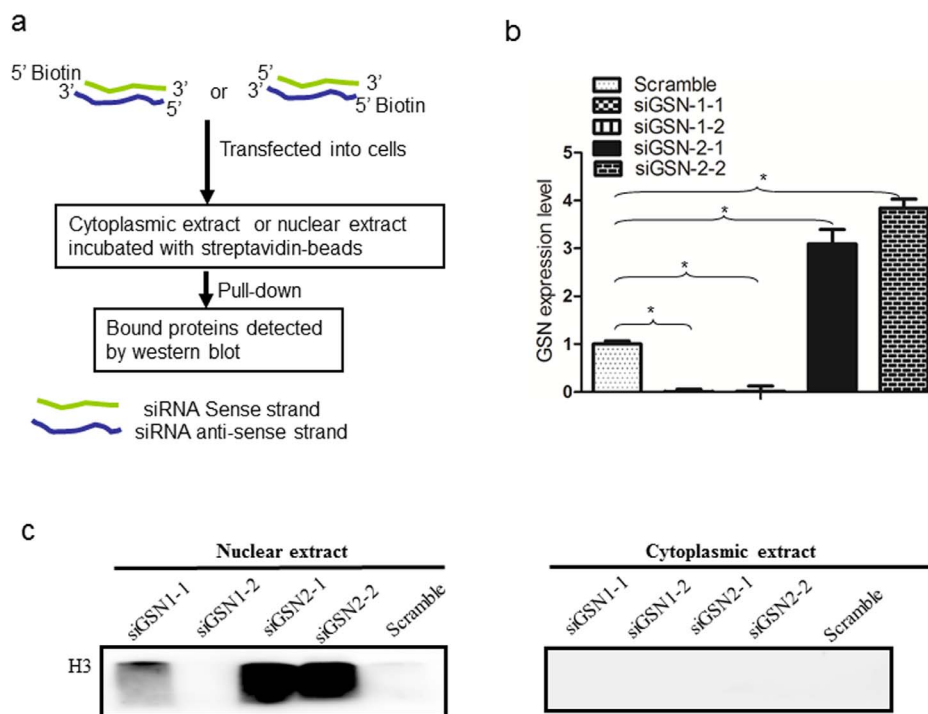
Figure 2 | Histone H3 copreciptates with siGSN. (a) Schematic diagram of the siGSN co-precipitation assay. (b) siGSN (−1–1, −1–2, −2–1, −2–2) labeled with biotin was individually transfected into KYSE450 cells, and *GSN* transcripts were detected by real time RT-PCR at post-transfection 48 h. Results are expressed as mean ± SE. *p ≤ 0.05. (c) Histone H3 was detected in the siGSN (−1–1, −1–2, −2–1, −2–2) co-precipitated complexes from cytoplasmic or nuclear extracts probed by mouse anti-H3 antibody by western blot.

was detected by real time RT-PCR at 48 h post-transfection. The results showed that *GSN* was down-regulated in si*GSN*-1-1- or si*GSN*-1-2- treated KYSE450 cells, respectively. However, *GSN* was up-regulated in siGSN-2-1 or siGSN-2-2 treated KSYE450 cells, respectively (Figure 2b). The results demonstrated that biotin labeling did not significantly alter the effects of our siRNAs on *GSN* expression. A previous study showed that histone H3 played an important role during gene transcription[20]. Therefore, we first speculated that histone H3 might exist in the biotin pull-down complexes from nuclear and cytoplasmic extracts. Histone H3 was co-precipitated with siGSN-2-1 and siGSN-2-2 in nuclear extracts. However, histone H3 was not detected in the cytoplasmic extract. Following transfection and pull down of complexes after siGSN-1-biotin or scramble-biotin transfection, histone H3 was not detected from either nuclear or cytoplasmic extracts (Figure 2c).

Redistribution of the actin cytoskeleton is inhibited by siGSN-2.
Since GSN up-regulation results in a reduction of F-actin filaments[15], to further demonstrate siGSN-2 involvement in up-regulation of GSN expression, siGSN-2 was transfected into cells and then F-actin was observed. We observed that siGSN-2 inhibited F-actin redistribution and made stress fibers formation impeded and meanwhile, made lamellipodia and filopodia formation become less (Figure 3a). The result of western blot analysis further demonstrated that GSN protein was slightly up-regulated with siGSN-2 transfection (Figure 3b).

Discussion
Although promoter-targeting and 3′UTR-targeting siRNAs have been shown to be capable of transcriptional activation[7,9], there are still no reports of exon-targeting siRNAs with similar capability. In this present study, we report that siGSN-2 targeting the twelfth exon of gene GSN up-regulates *GSN* transcript.

The RNA-induced silencing complex (RISC), which is a multiprotein complex composed of a bound siRNA and the RNA endoribonuclease

Dicer complex, induces target gene transcript degradation[21]. In the present study, siGSN-1 induces *GSN* transcript knockdown and forms a RISC complex distinct from the complex composed of siGSN-2 and accessory proteins that induce *GSN* transcription up-regulation. Lysine methylation of H3K4, H3K36 and H3K79 correlates with transcription activation[22–24]. Our detection of histone H3 in the siGSN-2-biotin pull-down complexes indicates that siGSN-2 stimulates H3K4, H3K36 and H3K79 lysine residue methylation. However, the exact nature of the lysine residue modification is still not clear. In fact, we reasoned that siGSN-2 was involved in the transcription activation through the interaction between siGSN-2 and H3 protein (Figure 2c). H3 protein was expressed in the nuclei and the interaction between siRNA and H3 protein had been reported. Li L.C et al. reported that siRNA targeting gene promoter caused target gene expression up-regulation by inducing H3 histone demethylation in the promoter region[7].

Gelsolin, a well-known actin-binding protein, can sever, cap and nucleate actin filaments, and plays a critical role in maintaining the normal cellular motility and morphology, and gelsolin up-regulation inhibits actin filament elongation. The fact that siGSN-2 up-regulates *GSN* expression is further verified by siGSN-2 inducing the reduction of stress fibers, lamellipodia and filopodia formation in KYSE450 cells. Also, the result of western blot showed that siGSN-2 induced GSN protein expression up-regulation, which caused the reduction of stress fibers, lamelipodia and filopodia formation of cells (Figure 3b).

In a previous report, siRNA-mediated target gene transcription activation was complementary to the promoter or 3′UTR sequence[7,9]. However, in the present study, siGSN-2, derived from the twelfth *GSN* exon, is not complementary to the *GSN* promoter sequence. One possible mechanism could be that siGSN-2 is just used as a nuclear acid scaffold for an siGSN-2 complex which up-regulates GSN transcription activation, making it unnecessary for siGSN-2 to directly bind to the GSN promoter to activate GSN transcription. In fact, Small RNAs serving as the scaffold have been previously reported[25].
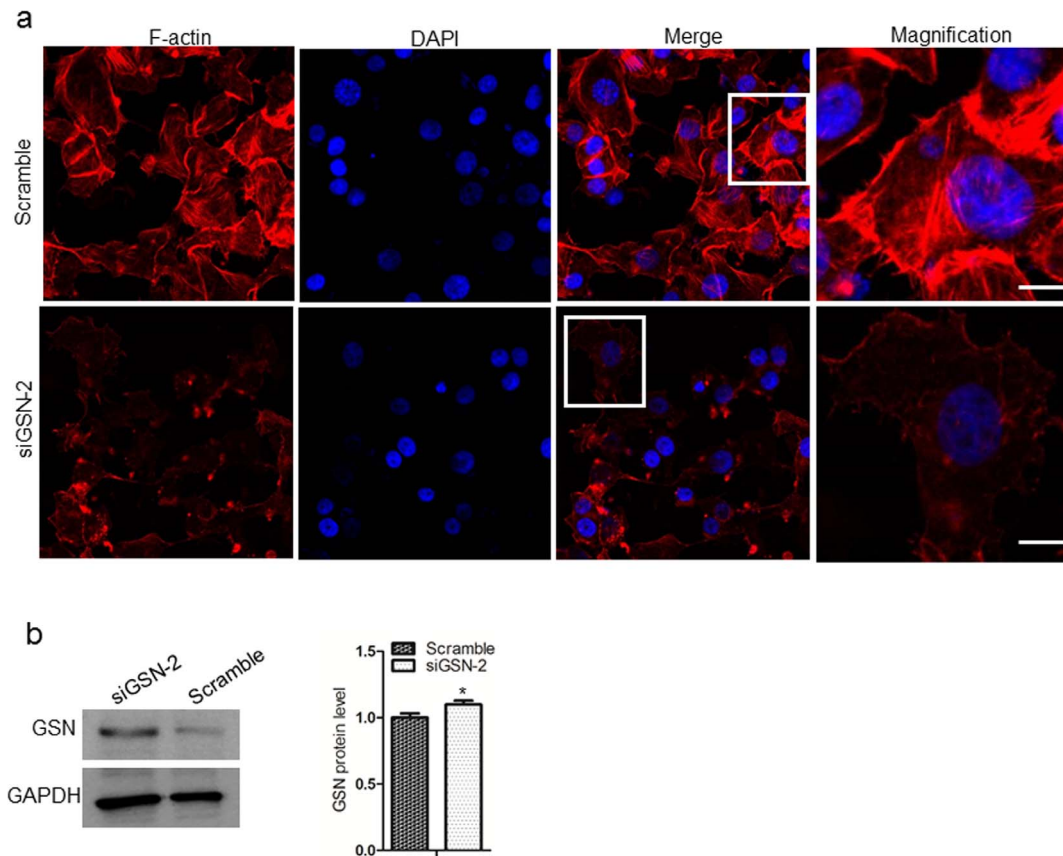
Figure 3 | KYSE450 cell morphological alteration was observed by immunofluorescence staining and GSN protein was slightly up-regulated following 2 µg siGSN-2 or control siRNA (scramble) post-transfection 48 h. (a) F-actin was labeled with Alexa594-conjugated mouse anti-actin antibody. Stress fiber, lamellipodia and filopodia were observed by laser confocal microscopy. Photos were observed at 40× optical magnification with a 2× digital zoom. Scale bar = 20 µm. (b) GSN protein was detected by western blot after 2 µg siGSN-2 or control siRNA (scrambled) transfection into KYSE 450 cells cultured in 6-well plate for 48 h (Left) and the bands of GSN protein was analyzed by Image J software (Right).

To our knowledge, this is the first report that siRNA, derived from target gene exon sequence, involves in target gene transcription activation by mechanisms distinct from those siRNAs from promoter or 3′UTR in regulation of gene expression.

Method

Cell culture. KYSE150 and KYSE450 human esophageal cancer cell lines were kindly provided by Dr. Ming-Zhou Guo (Department of Gastroenterology & Hepatology, Chinese PLA General Hospital, Beijing, China). KYSE150 and KYSE450 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and grown under a 5% CO_2 atmosphere at 37°C.

siRNA transfection. siRNA against *GSN* were individually transfected into KYSE150 cells or KYSE450 cells by X-tremeGene siRNA transfection reagent (Roche, USA) and *GSN* mRNA transcripts were detected by real time RT-PCR at 48 h post-transfection. Primers and siRNA oligoes were individually synthesized by BGI and GenePharma (China) and the sequence for the primers and siRNA as follows: si*GSN*-1: sense 5′-GACAGUGCAGCUGAGGAACGGAAAU-3′; antisense 5′-AUUUCCGUUCCUC-AGC-UGCACUGUC-3′. si*GSN*-2: sense 5′-GAAAGGAGGU-GUGGCAUCAG-GAUUC-3′; antisense 5′-GAAUCCUGAUGCCACACCUCCUUUC-3′.si*GSN*-3:sense5′-AUCUUUGUCUGGAAAGGCAAGCAGG-3′; antisense 5′-CCUGCU-UGCCUUUCCAGACAAAGAU-3′. Scramble siRNA: sense 5′-UUCUCCGAAC-GUGUCACGU-3′; antisense 5′-ACGUGACACGUUCGGAGAA-3′. Real time RT-PCR primers for *GSN* were: forward 5′-CGTGCCCACCAACCTTTA-3′; reverse 5′-TCATTGCCCAGCCAGTAG-3′. Real time RT-PCR primers for *β-Actin* were: forward 5′-AGCGAGCATCCCCCAAAGTT-3′; reverse: 5′-GGGCACGAAGGCT-CATCATT-3′.

Biotin labeling of si*GSN*. Biotin labeling of si*GSN* was performed by GenePhama (GenePharma, Shanghai, China). The 5′ end of the si*GSN*-1 sense strand, labeled with biotin, was designated as si*GSN*-1-1, and that of the si*GSN*-1 antisense strand, labeled with biotin, was designated si*GSN*-1-2. Similarly, the 5′ ends of the si*GSN*-2 sense and antisense strands labeled by biotin were denoted si*GSN*-2-1 and si*GSN*-2-2,

respectively. The 5′end of scrambled RNA labeled with biotin was used as negative control.

RT-PCR, real-time PCR and western blot. RNA was extracted with Trizol, and RT-PCR and real-time PCR were performed using PrimeScript® RT reagent Kit with gDNA Eraser and SYBR® Premix Ex Taq™ (DRR047A and DRR081A; Takara, Dalian, China) according to the manufacturer's instructions. Briefly, the contaminated DNA in 1 µg total RNA was firstly removed at 42°C for 2 min as following conditions: 10 µl reaction volume including 1 µg total RNA, 1 µl gDNA Eraser, 2 µl buffer and then cDNA was synthesized as 37°C, 15 min; 85°C, 5 sec. 1 µl cDNA was used as real-time PCR template and real-time PCR was performed by 7300 real-time PCR system (Life technology, American) as following conditions: 95°C, 30 sec; 95°C, 5 sec; 60°C, 31 sec; total 40 cycles. β-actin was used as internal control and water as PCR negative control (NTC). The fold changes of GSN mRNA level were calculated with $2^{-\Delta\Delta Ct}$ method. Total proteins prepared in RIPA buffer, were separated in SDS-polyacrylamide gelelectrophoresis (SDS-PAGE) and transferred onto PVDF membrane (Millipore, USA) and rabbit anti-human GSN primary antibody and HRP-conjugated secondary antibody were from Sigma-Aldrich Company and the bands of GSN protein was analyzed by Image J software. GAPDH was used as internal control and GAPDH primary antibody was from Santa Cruz Biotechnology Company.

siRNA co-precipitation assay. The equivalent number of KYSE450 cells was inoculated in four 6-well plates and siGSN-1-1, siGSN-1-2, siGSN-2-1, siGSN-2-2 or scramble siRNA, were individually transfected into KYSE450 cells. All the cells from each transfection were individually collected together at post-transfection 48 h, and cytoplasm and nuclei were isolated according to http://www.lifetechnologies.com/cn/zh/home/references/protocols/cell-and-tissue-analysis/elisa-protocol/elisa-sample-preparation-protocols/nuclear-extraction-method-.html. Briefly, the cell pellet was suspended in 500 µl 1× hypotonic buffer solution (20 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2) and placed on ice for 15 min; The cell suspension was centrifuged at 3000 g for 10 min, after which 25 µl NP-40 was added to the mixture. The supernatant (cytoplasmic extract) was used for siGSN-1-1, siGSN-2-1 and scramble RNA co-precipitation. The nuclear pellet was resuspended in 100 µl ice-cold nuclear extraction buffer mix (Biovision, San Francisco, USA) and then vortexed

every 10 min for 1 h at 4°C, then nuclear extract was collected by centrifugation at 16000 g for 10 min. The nuclear extract was used for siGSN-1-2, siGSN-2-2, and scramble RNA co-precipitation. siGSN-1-1, siGSN-1-2, siGSN-2-1 and siGSN-2-2 in the cytoplasm or nuclear extract co-precipitation were performed by the binding of biotin to streptavidine coupled on the magnetic beads according to Lehrbach[19].

Immunofluorescence. KYSE 450 cells were seeded on cover slides in the 6-well plate and 2 µg siGSN-2 was transfected into KYSE450 cells after 24 h, and F-actin was stained with Alexa594-conjugated mouse anti-actin antibody (Cytoskeleton, California, USA) at 48 h post-transfection, and stress fibers, lamellipodia and filopodia formation were observed with a laser confocal microscope (Olympus, Tokyo, Japan).

Statistical analysis. Statistical analyses were performed using SPSS 13.0 for windows (IBM, Chicago, IL, USA). A p value of less than 0.05 was considered statistically significant and each value was two-tailed.

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Acknowledgments

Author contributions

G.W.H. and L.D.L. performed the experiments and prepared the figures 1–3. E.M.L. and L.Y.X. conceived and designed the experiments and involved in the writing of the manuscript and the decision to submit the manuscript for publication.

Additional information