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Airn Regulates Igf2bp2 Translation in Cardiomyocytes

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Abstract

Rationale—Increasing evidence indicates the presence of long noncoding RNAs (lncRNAs) in various cell types. *Airn* is an imprinting gene transcribed from the paternal chromosome. It is in antisense orientation to the imprinted, but maternally-derived, *Igf2r* gene, on which *Airn* exerts its regulation in *cis*. Although *Airn* is highly expressed in the heart, functions aside from imprinting remain unknown.

Objective—Here, we studied the functions of *Airn* in the heart, especially cardiomyocytes.

Methods and Results—Silencing of *Airn* via siRNAs augmented cell death, vulnerability to cellular stress, and reduced cell migration. To find the cause of such phenotypes, the potential binding partners of *Airn* were identified via RNA pull-down followed by mass spectrometry, which indicated Igf2bp2 and Rpa1 as potential binding partners. Further experiments showed that *Airn* binds to Igf2bp2 to control the translation of a number of genes. Moreover, silencing of *Airn* caused less binding of Igf2bp2 to other mRNAs and reduced translation of Igf2bp2 protein.

Conclusions—Our study uncovers a new function of *Airn* and demonstrates that *Airn* is important for the physiology of cardiomyocytes.

Keywords

lncRNA; cardiomyocytes; gene expression; noncoding RNA; transcriptome

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DISCLOSURES
None.

Subject Terms

Basic Science Research; Developmental Biology; Functional Genomics; Gene Expression and Regulation; Ischemia

INTRODUCTION

Most of the human genome is transcribed; however, only a minor fraction is translated into proteins. Many of these untranslated RNAs are classified as noncoding RNAs (ncRNAs), including those longer than 200 nucleotides (nt) called “long ncRNAs (lncRNAs)”. To date, numerous lncRNAs being identified in the heart; however, few of them have been functionally studied.[1, 2] It is speculated that lncRNAs exert various functions to control the development, homeostasis, and pathophysiological processes in the heart. Thus, lncRNAs might be a key to unravel the regulatory networks controlling cardiomyocyte differentiation and function.

Airn (“antisense Igf2r RNA”, also known as “*Air*”) is an imprinted gene transcribed from the paternal chromosome.[3, 4] In the human and mouse genomes, *Airn* is located in antisense orientation to the imprinted, but maternally-derived, “insulin-like growth factor 2 receptor (*Igf2r*)” gene. *Airn* can regulate nearby protein-coding genes *Igf2r*, *Slc22a2*, and *Slc22a3* in *cis*.[5–7] In murine hearts, there is no expression difference between male and female mice.[8] Although *Airn* is capped, polyadenylated, a majority of *Airn* transcripts evade co-transcriptional splicing to give rise to a mature 118 kb transcript, which resides in the nucleus but is highly unstable, whereas the spliced *Airn* isoforms are as stable as other mRNAs (i.e., *Igf2r*) and are exported into the cytoplasm.[4] Although the function of *Airn* as an imprinting gene is known, up until now, the functions of *Airn* isoforms are largely unknown, especially in the heart. Here, we uncover functional roles of *Airn* isoforms in cardiomyocytes using the murine cardiomyocytic HL-1 cell line.

METHODS

All data and materials have been made publicly available at the Gene Expression Omnibus and can be accessed at GSE87223. An extended methods section is available in the Online Data.

HL-1 cells were cultured according to the original publication.[9] Primer and siRNA sequences are provided in Online Table I. Microarray experiment was performed as previously done.[10–12] Heteroscedastic two-tail Student’s t-test was applied to calculate a p-value.

Adult male mice were subjected to non-reperfused, coronary artery occlusion to induce heart failure[13] and in accordance with the University of Louisville Animal Care and Use Committee.

RESULTS

Airn isoforms are differentially expressed in tissues and cardiomyocytes

To study *Airn* isoforms (Online Figure I), we compared expression profiles among 10 murine tissues using a primer pair specifically targeting each isoform (Figure 1A). Of all the isoforms, *Airn-001* expression was most similar to the unspliced form of *Airn,* which is the most studied and is involved in imprinting of nearby protein-coding genes. Other isoforms (*Airn-002*, *-003* and *-006*) showed high expression in certain tissues, such as brain and kidney, brain and liver, and lung, respectively, which is consistent with previous reports[14, 15] (Figure 1A). Based on these results and the high expression of *Airn* in the heart,[4] we focused on unspliced *Airn* and *Airn-001* in this study.

Previous studies show that many lncRNAs were dysregulated following myocardial infarction.[1, 2] To identify changes in expression of unspliced *Airn* and *Airn-001*, we used hearts from mice subjected to non-reperfused myocardial infarction and from sham-operated mice. When whole hearts were compared, there is a slight tendency of down-regulation for both unspliced *Airn* and *Airn-001* in the infarcted hearts compared to the control 4 weeks post operation (Figure 1B). When the expression patterns of unspliced *Airn* and *Airn-001* were examined spatially, their expressions were down-regulated in the non-infarcted regions of infarcted hearts compared to the similar regions in the sham-operated hearts, whereas no change was detected in the infarct region, which consists of mainly dead cardiomyocytes (Figure 1C; Online Figure II).

Although the above expression profiling is informative, there remains a question of whether *Airn* is specifically expressed in cardiomyoyctes or not. To this end, we analyzed the published single-cell RNA-seq data of embryonic hearts (Figure 1D, E).[16] In all compartments of the heart, *Airn* expression was recorded, which showed slightly higher expression in the left compared to right atrium and ventricle (Figure 1F). When cell types are compared, in the atrium, *Airn* is preferentially expressed in fibroblasts, whereas in the left ventricle, the expression of *Airn* is higher in cardiomyocytes compared to cell types.

To study the function of *Airn* in cardiomyocytes, we employed HL-1 cells.[9] Because HL-1 is a cell line, we first quantified the expressions of unspliced *Airn* and *Airn-001* in HL-1 cells by comparing to embryonic and adult cardiomyocytes, which indicated the comparable level of expression for unspliced *Airn* to adult cardiomyocytes than embryonic ones (Figure 1G). In the case of *Airn-001,* its expression is lower in HL-1 cells but higher than that of embryonic cardiomyocytes. Next, the subcellular localization of *Airn-001* was investigated, which showed its preferential localization in the nucleus, similar to its unspliced form (Figure 1H). Because a previous study showed that *Airn* isoforms are more stable than the unspliced form,[4] we investigated the stability of *Airn-001* in comparison to its unspliced form by suppressing RNA synthesis by actinomycin D (Figure 1I) and 5,6-Dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) (Figure 1J). As result, *Airn-001* is as stable as the protein-coding gene *Gapdh,* whereas unspliced *Airn* is rapidly degraded.

Next, *Airn* was knocked down in HL-1 cells with siRNAs (Figure 2A) and evaluated by microarrays (Figure 2B). When a threshold of 1.4-fold and $p<0.05$ were applied, 45 up- and

29 down-regulated genes were identified (Online Table II), which includes genes whose Gene Ontology terms associated with apoptosis (Online Table III). To confirm the molecular profiling, assays to quantify cell death were conducted via Annexin V staining using flow cytometry (Figure 2C) and reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Figure 2D). Silencing *Airn* showed slightly increased apoptosis, which was associated with caspase activation as detected by Western blotting (Figure 2E). Furthermore, necrosis was significantly induced upon silencing of *Airn*, which was determined by lactate dehydrogenase (LDH) release (Figure 2F). In line with these observations, when the cells were challenged with hydrogen peroxide (H_2O_2), doxorubicin (Doxo), DRB, and cycloheximide (CHX), fewer cells survived upon silencing of *Airn* compared to controls (Figure 2G). Interestingly, similar data were obtained for silencing of highly abundant lncRNAs *Malat1* and *Neat1* (Online Figure III). These results are in line with changes in expression of apoptotic genes after silencing of *Airn* compared to the control (Figure 2H). These data suggest that *Airn* is important for cell survival. Given that migration of cardiomyocytes is important for the proper development of the heart and regeneration upon injury in mice and zebrafish,[17, 18] cell migration was measured via scratch assay, which showed reduced migration upon silencing of *Airn* (Figure 2I; Online Figure IV). Taken together, these results indicate that silencing of *Airn* affects the physiology of cardiomyocytes.

Airn binds to Igf2bp2 and Rpa1

To identify the mechanism by which *Airn* affects cardiomyocyte survival and function, we employed RNA pull-down experiment by labeling full-length *Airn-001* with biotin and mixing it with cellular lysates of HL-1 cells to identify proteins that bind to *Airn-001* via mass spectrometry analysis (Figure 3A). Among nine potential binding partners, two proteins (Igf2bp2 and Rpa1) were selected for further validation due to their subcellular localizations and the availability of working antibodies. When Western blotting experiments were performed after RNA pull-down, the binding of Rpa1 was observed for both sense and antisense *Airn-001* (Figure 3B), suggesting that Rpa1 is not specifically bound to *Airn-001*, but nonspecifically binds to RNA. In contrast, Igf2bp2 preferentially binds to sense *Airn-001* (Figure 3C). The binding of Igf2bp2 is further confirmed by RNA immunoprecipitation (RIP) followed by RT-PCR. Unspliced *Airn* and *Airn-001* showed preferential binding to Igf2bp2 (Figure 3D), which confirms that *Airn* binds to Igf2bp2.

Igf2bp2 protein binds various mRNA in cardiomyocytes

To understand the causal relationship between *Airn* and targets of Igf2bp2, RIP assay followed by microarray (RIP-chip) was performed. Compared to the control, there were significantly fewer Igf2bp2-bound genes upon silencing of *Airn* (2,228 and 83 genes, respectively) at the threshold value of 1.5-fold enrichment and $p<0.05$ over anti-IgG antibody as negative control (Figure 4A, Online Table IV), suggesting that *Airn* assists the binding of Igf2bp2 to mRNAs.

To further validate these findings, expressions of Igf2bp2-bound mRNAs were quantified. If, indeed, *Airn* affects the binding of Igf2bp2 to these mRNAs, their gene expression should not change upon silencing of *Airn*, while the level of their proteins should be reduced. When

the expression of the selected genes were quantified, 5 genes (*Bgn*, *Emp1*, *Fbln5*, *Inhba* and *Ogn*) among 8 genes tested did not show statistically significant changes upon silencing of *Airn* at the mRNA level (Figure 4B, C). At the protein level, biglycan (*Bgn*) and inhibin beta-A (*Inhba*) were reduced to a similar extent by silencing of *Airn* or *Igf2bp2* (Figure 4D). Of note, although the secretion of Bgn was not affected by silencing of *Airn* or *Igf2bp2*, the reduction in the secreted level of Inbha was observed (Online Figure V). These results suggest that their translation is inhibited due to the reduced expression of *Airn* that is necessary for the functionality of Igf2bp2 protein as a translation regulator.

The reduction of Igf2bp2-bound genes raises a question whether the expression of *Igf2bp2* is altered upon silencing of *Airn*. Although no reduction of *Igf2bp*2 was observed upon silencing of *Airn* compared to the control at the mRNA level (Figure 4E), to our surprise, silencing of *Airn* caused a reduction of Igf2bp2 protein (Figure 4F). To further confirm this finding, surface sensing of translation (SUnSET) assay[19] was performed to monitor global protein synthesis. In this assay, puromycin is used to label newly synthesized peptides. Upon silencing of *Airn*, the amount of puromycin-labelled peptides is similar to the negative control (treated with CHX to block translation) than other conditions (Figure 4G). Based on these results, we conclude that *Airn* affects the translation (but not transcription) of Igf2bp2, which binds to many mRNAs to control their translation efficiencies.

Discussion

In this study, we uncovered the role of *Airn* other than imprinting in cardiomyocytes; that is, *Airn* binds to Igf2bp2 protein and control translation of Igf2bp2 as well as other genes. Compared to protein-coding genes, lncRNAs are less sequence-conserved among species[20]; however, some well conserved lncRNAs do exist (e.g. *MALAT1*[21]), especially those that are involved in genomic imprinting (e.g. *Meg3*[22, 23]). Given that *Airn* is an imprinting gene and conserved in humans,[24] the question is whether a similar function exists in humans. Indeed, human *AIRN* is highly expressed in the heart (Online Figure VIA). Upon its silencing by siRNA in human embryonic kidney cells 293 (HEK-293) (Online Figure VIB), the survival of cells was reduced when treated with H_2O_2 (Online Figure IIIC), which indicates the similar phenotype as silencing of mouse *Airn*. Further investigation is necessary to understand the exact mechanism of reduced survival upon silencing of human *AIRN.*

Although its polymorphisms are linked to type 2 diabetes,[25] the function of Igf2bp2 (also known as “IMP2”) in cardiomyocytes is unknown. There are studies of Igf2bp2 in related cell types (i.e. myoblasts and skeletal muscle), reporting the following: (i) It is highly expressed in myoblasts, where Igf2bp2 controls cell adhesion and motility via direct binding to the mRNA of *Lims2* as well as that of *Trim54* for the stabilization of microtubules;[26] and (ii) Under the transcriptional control via Hmga2, Igf2bp2 contributes to myoblast proliferation by promoting the translation of various mRNAs, including *Myc*, *Sp1* and *Igf1r*, but not *Igf2.*[27] Here, we confirmed that Igf2bp2 directly binds to various mRNAs in cardiomyocytes (Figure 4A). Furthermore, through the interaction with *Airn* and its isoforms, the translation of Igf2bp2 protein itself is controlled (Figure 4E, F). Thus, we conclude that *Airn* lies in the upstream of Igf2bp2 to control the translation of many mRNAs, which include genes involved in apoptosis directly related to cell survival. Taken

together, our study uncovered a novel function of *Airn* in controlling translation of various protein-coding genes through the regulation of Igf2bp2 protein synthesis in cardiomyocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

lncRNA	long noncoding RNA
NGS	next generation sequencing
NcRNA	noncoding RNA
Nt	nucleotides

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NOVELTY AND SIGNIFICANCE

What Is Known?

- The majority of the mammalian genome is transcribed as RNA, but only a minor part corresponds to exons of protein-coding genes.
- The number of long noncoding RNAs (the noncoding transcripts longer than 200 nucleotides) is more than protein-coding genes, but their functions are largely unknown.
- *Airn* ("antisense Igf2r RNA", also known as "*Air*") is an imprinted gene transcribed from the paternal chromosome, but besides imprinting, its functions remain unknown.

What New Information Does This Article Contribute?

- Silencing of *Airn* augmented cell death, vulnerability to cellular stress, and reduced cell migration.
- *Airn* binds to the RNA-binding protein Igf2bp2 to control the translation of a number of genes via reducing the translation of Igf2bp2 protein.

Although lncRNAs are present in the heart, their functions remain largely unknown. Of the few predicted functions, binding to macromolecules (e.g., nucleic acids or proteins) is of great interest because lncRNAs may function as molecular scaffolds or switches to activate or to inhibit biological processes. Recent evidence indicates that dysregulation of lncRNAs alters the transcriptome and leads to cardiac dysfunction. Because the number of lncRNAs exceeds that of protein-coding genes, more systematic study of lncRNAs in the heart is needed to uncover the causal relationship between lncRNAs and cardiac health and disease. Here, we show that the lncRNA *Airn* has a function besides its well known function as an imprinting gene. Our findings on the translational control of a number of genes via the interaction between *Airn* and Igf2bp2 have a broad translational impact as its manipulation may allow for post-transcriptional control of various protein-coding genes and their protein products. Although a majority of lncRNAs are not conserved across species, *Airn* is one of the few well defined lncRNAs that are conserved between humans and mice. These findings could significantly advance the understanding of human lncRNAs and their function in the heart.

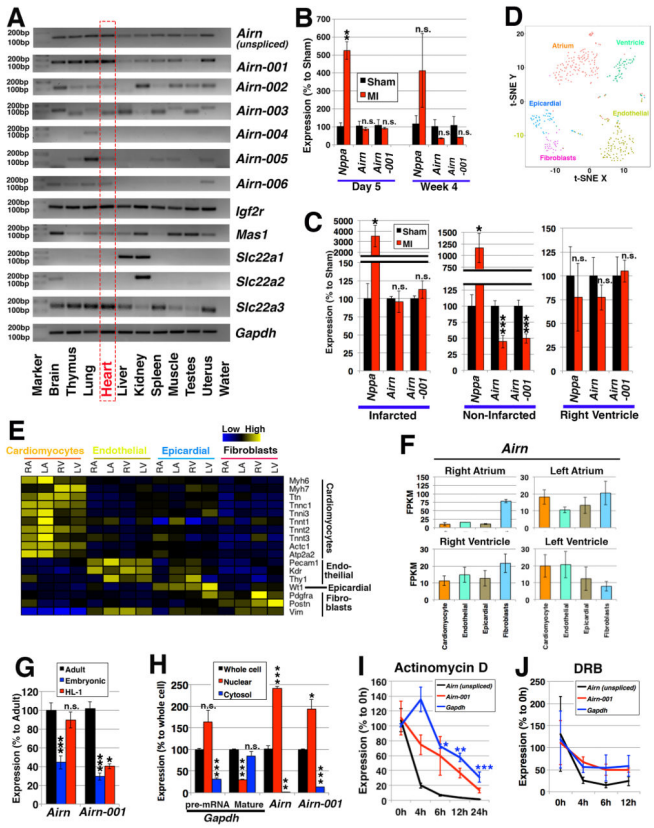

Figure 1. *Airn* and its isoforms

(A) Expression profiling of *Airn* and its isoforms in mouse tissues. (B, C) Expression in myocardial infarction (MI). (B) The time-course data of 5 days and 4 weeks post surgery comparing “Sham” control and “MI” mice. n=3 mice for sham (day 5) and n=4 mice for all the other conditions. The expressions were normalized to those of sham samples using *Gapdh* as internal control. (C) Expression in different regions of hearts 4 weeks after the operation. One heart was divided into 3 regions: “Infarcted”; “Non-Infarcted” (i.e., border and remote regions); and “Right Ventricle”. n=5 mice. The expressions were normalized to those of sham samples using *Rn18s* as internal control. (D-F) Expression profiling of single-cell RNA-seq data of E10.5 mouse hearts. (D) 5 clusters of cells were defined: cardiomyocytes from atrium or ventricle, endothelial cells, epicardial cells, and fibroblasts. (E) Expressions of marker genes. (F) Expression patterns of the *Airn* gene. (G) Expression of unspliced *Airn* (“*Airn*”) and *Airn-001* in “adult” cardiomyocytes compared to those of “embryonic” cardiomyocytes and “HL-1” cells. n=4 samples for adult cardiomyocytes and n=3 samples for others. (H) Subcellular localization in HL-1 cells. As controls, primer pairs for *Gapdh* of its pre-mRNA (targeting the intron between its exon 2 and 3) and mature RNA (targeting exons 4 and 5) were used. Statistical calculations were made against the expressions in the whole cell. n=3. (I, J) Stability of mRNAs upon the treatment with (I) actinomycin D at 4, 6, 12 and 24 hours; and (J) DRB at 4, 6 and 12 hours. n=3. The expressions were normalized using those of *Rn18s*. *, ** and *** represent p<0.05, 0.01 and 0.005, respectively. “n.s.” represents “not statistically significant”.

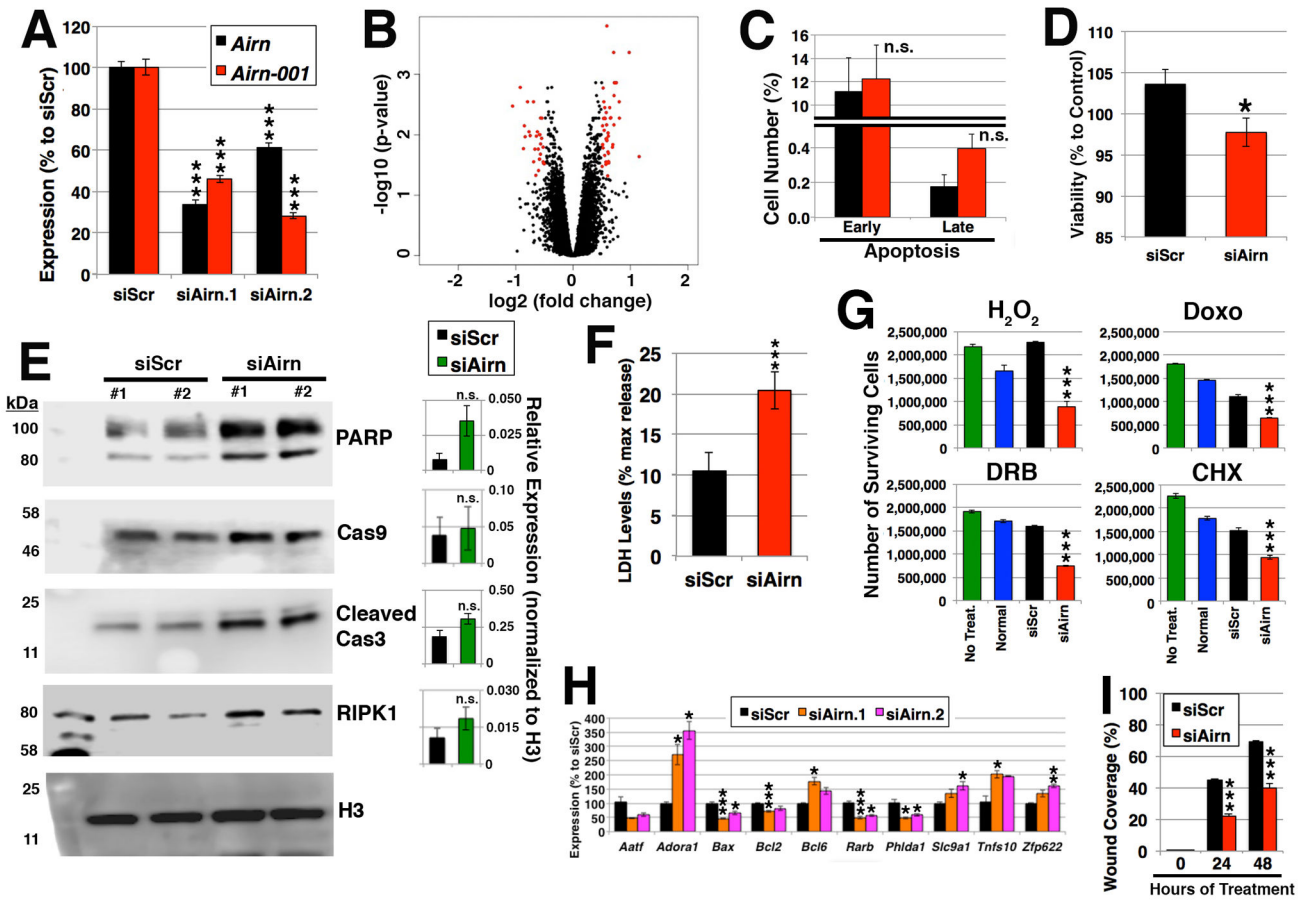

Figure 2. Silencing of *Airn-001*

(A) Efficiency of knockdown for two siRNAs against *Airn* (siAirn.1 (n=6) and siAirn.2 (n=5)) in comparison to the control siRNA (siScr (n=6)). (B) Volcano plot of microarray results. Genes that were selected with threshold values of 1.4-fold and FDR<0.05 are colored in red. n=6 for siScr, n=4 for siAirn.1, and n=4 for siAirn.2. (C, D) Quantification of apoptosis via (C) flow cytometry for Annexin V compared to the total number of cells measured. n=2 (each with three technical replicates); and (D) MTT assay. The data were normalized to the untreated cells. n=3 (each with 24 technical replicates). (E) Western blotting of marker proteins of apoptosis. PARP, Cas9, cleaved Cas3, and RIPK1. Anti-histone H3 antibody was used as loading control. The representative blotting images are shown. The quantification of each corresponding band was normalized to that of H3 and represented as relative expression in each bar graph next to the corresponding blotting image (n=6 samples). (F) LDH assay. n=5 (each with 16 technical replicates). A total of 80 wells were counted. (G) Numbers of surviving cells. n=4 technical replicates. (H) qRT-PCR for apoptotic genes upon silencing of *Airn* compared to siScr. n=3 samples. (I) Scratch assay. The recovered areas were quantified. n=3. *, ** and *** represent p<0.05, 0.01 and 0.005, respectively. “n.s.” represents “not statistically significant”.

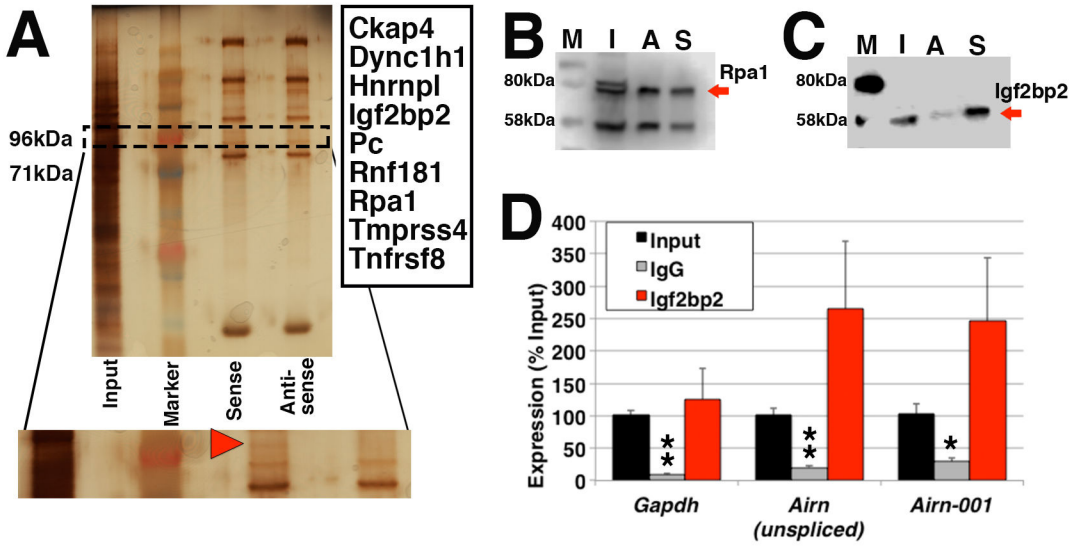

Figure 3. Binding partners

(A) Silver-stained SDS-PAGE gel after RNA pull-down assay. The band specific to the sense sample but missing in the antisense sample was used for mass spectrometry analysis. In addition, the similar region in the antisense sample was analyzed by mass spectrometry. As result, 9 proteins listed were detected only in the sense *Airn-001* compared to antisense one (negative control) in two idependent assays. (B, C) Western blotting after RNA pull-down assay for (B) Rpa1; and (C) Igf2bp2 “M” stands for marker; “I” for 5% input; “A” for antisense; and “S” for sense probe for *Airn-001*. Representative image from 5 independent assays. (D) RIP using anti-Igf2bp2 antibody. The expression values were normalized to those of the input. * and ** represent $p<0.05$ and 0.01, respectively. n=3.

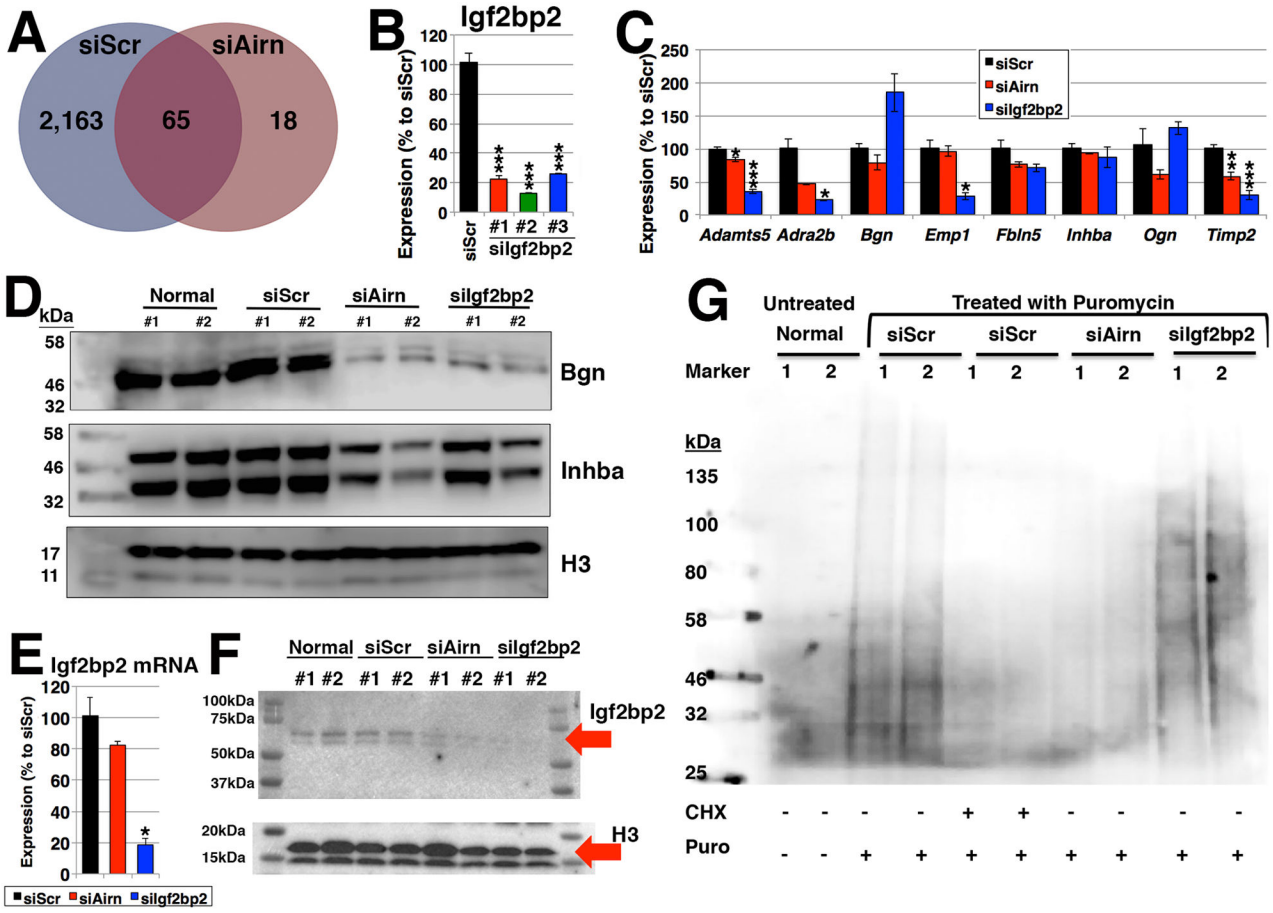

Figure 4. Binding of Igf2bp2

(A) Venn diagram of Igf2bp2-bound genes upon silencing of *Airn*. RIP-chip experiments (n=4 samples) were performed using anti-Igf2bp2 antibody and anti-IgG antibody as negative control. The thresholds of 1.5-fold enrichment and FDR<0.05 were applied. (B) Efficiency of *Igf2bp2* knockdown. n=3. (C) qRT-PCR. n=3. (D) Western blotting using anti-Bgn and -Inhba antibodies. (E, F) Expression of *Igf2bp* at the levels of (E) RNA (n=3) and (F) protein (n=2 and representative images from four independent assays). (G) SUnSET. The addition of CHX is used as a negative control. Representative image from 3 independent assays. *, ** and *** represent p<0.05, 0.01 and 0.005, respectively. “n.s.” represents “not statistically significant”.