Oncofetal protein IGF2BP1 regulates IQGAP3 expression to maintain stem cell potential in cancer

Highlights
- Oncofetal protein IGF2BP1 is a key stabilizer of IQGAP3 mRNA
- IGF2BP1's targets SRF and FOXM1 act as additional regulators of IQGAP3 expression
- IGF2BP1-SRF-FOXM1-IQGAP3 network contributes to cancer stemness
Oncofetal protein IGF2BP1 regulates IQGAP3 expression to maintain stem cell potential in cancer

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SUMMARY
We reported earlier that IQGAP3 is an important stem cell factor in rapidly proliferating isthmus stem cells in the stomach and that IQGAP3 expression is robustly induced in terminally differentiated chief cells and de-differentiated cells following tissue damage. The elevated IQGAP3 expression in cancer and its association with metastasis suggest a fundamental role for IQGAP3 in proliferating cancer stem cells. What causes IQGAP3 upregulation in cancer is unclear. Here, we show that IGF2BP1 and IQGAP3 expression levels are highest in the blastocyst, with both decreasing during adulthood. This suggests that IQGAP3, like IGF2BP1, is an early developmental gene that is aberrantly upregulated upon re-expression of IGF2BP1 during carcinogenesis. IGF2BP1 binds and stabilizes m6A-modified IQGAP3 transcripts. Downstream targets of IGF2BP1, namely SRF and FOXM1, also upregulate IQGAP3 expression. These multiple layers of IQGAP3 regulation, which may safeguard against inappropriate stem cell proliferation, present additional drug targets to inhibit IQGAP3-driven malignant growth.

INTRODUCTION
The study of stem cell regulators is necessary to understand how proliferation and multipotency are maintained in stem cells and how they are hijacked in cancer. Our recent work identified cytoskeletal protein IQGAP3 (IQ motif containing GTPase-activating protein 3) as a highly specific marker of proliferating stem cells in the stomach corpus (Matsuo et al., 2021). We further found rapid upregulation of IQGAP3 expression, which was accompanied by massive dedifferentiation of mature lineages, upon tissue damage (Matsuo et al., 2021). Our observation that the depletion of IQGAP3 led to differentiation indicated a strong requirement for IQGAP3 in the maintenance of stem cell potential and proliferation (Matsuo et al., 2021).

IQGAP3 is a scaffold protein with multiple protein binding modules, such as the calponin homology domain (CHD) for F-actin-binding, the WW domain that interacts with ERK½, calmodulin-binding IQ motifs, Ras GTPase-activating protein-related domain (GRD) that interacts with Rac1/Cdc42, and the RasGAP binding domain at the C-terminus (RGCT) (Hedman et al., 2015; Nojima et al., 2008). These domains allow IQGAP3 to integrate diverse biological pathways for important roles in cell adhesion, motility, proliferation, and signal transduction (eg. RAS-ERK cascade and Rho-Rac1) (Nojima et al., 2008; Wang et al., 2007). The Bgee database (Swiss Institute of Bioinformatics) revealed that IQGAP3 expression is highest during mammalian embryonic/fetal development when compared to the adult. Moreover, IQGAP3 is essential for cell proliferation and motility—through FGFR1-Ras signaling—during zebrafish embryonic development (Fang et al., 2015), thereby reinforcing the notion that IQGAP3 is necessary for development across species.

IQGAP3 is overexpressed in several types of human malignant tumors including gastric, colorectal, liver, pancreatic, lung, ovarian, and breast cancers (Dongol et al., 2020; Hu et al., 2016; Que et al., 2018; Skawran et al., 2008; Wu et al., 2019; Xu et al., 2016; Yang et al., 2014). Importantly, high expression levels of IQGAP3
are associated with poor prognosis in different cancer types (Koon et al., 2004; Kumar et al., 2017; Skawran et al., 2008; Wu et al., 2015). Our finding that IQGAP3 is involved in the maintenance of stem cell properties in human embryonic and undifferentiated gastric cancer cells (Matsuo et al., 2021) suggests that IQGAP3 could be a potential therapeutic target for cancer treatment.

The mechanisms underlying the tight association of IQGAP3 expression with proliferating stem cells and cancer cells are unclear. Our study aims to delineate the regulation of IQGAP3 expression in the pluripotent embryonic cancer cell line model NTERA-2 and the undifferentiated human gastric cancer cell line, HGC-27. We found that IQGAP3 RNA stability is enhanced by the oncofetal protein IGF2BP1 (insulin-like growth factor 2 mRNA-binding protein 1) in an N6-methyladenosine (m6A) RNA methyltransferase METTL3/14-dependent manner. IGF2BP1 has earlier been shown to bind to m6A-modified mRNAs, thereby stabilizing the RNA of cancer-associated genes such as MYC, E2F1, and Serum Response Factor (SRF) (Degrauwe et al., 2016; Huang et al., 2018; Muller et al., 2019, 2020). SRF regulates gene expression through cooperation with ternary complex factors (TCFs) and with myocardin-related transcription factors (MRTFs) (Clark and Graves, 2014). SRF-TCF complexes modulate the transcription of genes involved in proliferation and response to growth factors while SRF-MRTF drives the transcription of genes related to cytoskeleton regulation, cell adhesion, and migration (Clark and Graves, 2014; Esnault et al., 2014; Leitner et al., 2011). More recently, SRF has been shown to destabilize cellular identity through its regulation of cytoskeletal genes (Ikeda et al., 2018). We found that SRF strongly upregulates IQGAP3 expression in NTERA-2 and HGC-27. We also identified FOXM1 (forkhead box protein M1) RNA as a novel direct target of IGF2BP1 and as an upstream regulator of IQGAP3. Together, our study suggests that the aberrant upregulation of IQGAP3 in cancer may be owing to the re-expression of IGF2BP1 in malignant tissues and its interplay with SRF and FOXM1. IGF2BP1, SRF, FOXM1, and IQGAP3 may therefore form a critical axis for tumorigenesis and cancer stem cell viability of undifferentiated cancer cells, such as HGC-27.

RESULTS

IGF2BP1 binds and stabilizes m6A-modified IQGAP3 RNA in NTERA-2 cells

To identify the regulatory factors of IQGAP3 gene expression, we first queried the public database The Encyclopedia of RNA Interactomes (ENCORI) (http://starbase.sysu.edu.cn/) (Li et al., 2014). This interface provides access to the enhanced crosslinking and immunoprecipitation (eCLIP) datasets, which are used for the identification of target sites of RNA binding proteins (Van Nostrand et al., 2016). Distinct enrichment of IGF2BP1 at the 3’ untranslated region (3’UTR) of IQGAP3 RNA from the human liver hepatocellular carcinoma cell line HepG2 and the multipotential, hematopoietic malignant cells K562 cell line were observed (Figure S1A).

IGF2BP1 is expressed mainly during embryonic development, with likely roles in cell migration, metabolism, and stem cells (Degrauwe et al., 2016). After birth, IGF2BP1 expression drops to very low or negligible levels in most tissues (Degrauwe et al., 2016). Importantly, it is re-expressed in a broad range of cancer types and typically associated with poor prognosis (Degrauwe et al., 2016). We compared the RNA expression levels of Iqgap3 and Igf2bp1 in the mouse blastocyst stage and various adult tissues and found stronger co-expression of both in the blastocyst compared to adult tissues (Figure 1A). It would seem that Iqgap3 and Igf2bp1 play prominent roles in embryonic development. Interestingly, Iqgap3 expression is higher in the adult stomach than in tissues such as adult brain, heart, kidney, and liver, whereas Igf2bp1 levels are negligible in all adult tissues (Figure 1A). We had earlier detected high IQGAP3 expression specifically in rapidly proliferating isthmus stem cells in the adult stomach corpus (Matsuo et al., 2021). It is possible that, while markedly reduced in the adult, IQGAP3 expression is still maintained in proliferative tissues with high regeneration capacity. By contrast, IGF2BP1 expression and function appear to be mainly fetal. As IGF2BP1 is expressed in pluripotent and various cancer cell lines, we selected the pluripotent human testicular embryonal carcinoma cell line NTERA-2 to further explore the oncofetal association between IQGAP3 and IGF2BP1.

We introduced FLAG-tagged IGF2BP1 into NTERA-2 cells and used FLAG-affinity beads to immunoprecipitate FLAG-IGF2BP1-ribonucleoprotein complexes for RNA immunoprecipitation sequencing (rIP-seq). Motif analysis revealed the enrichment of various motifs resembling the known IGF2BP1 consensus binding motif 5’-GGAC-3’ at the IGF2BP1 peaks (Figure 1B). Gene ontology (GO) analysis of the IGF2BP1 binding sites indicated significant enrichment for genes involved in Rho GTPase signaling, insulin receptor signaling, organelle organization, developmental growth, positive regulation of epithelial
Figure 1. IGF2BP1 binds to 3' UTR of IQGAP3 in an m^6A-dependent manner in NTERA-2 cells

(A) Comparison of Igf2bp1 and Iqgap3 mRNA expression in mouse blastocyst and various adult mouse tissues. Statistical significance was determined by the one-way ANOVA method.

(B) Motif enrichment in IGF2BP1 binding sites in RIP-seq.

(C) GO pathway enrichment analysis of IGF2BP1 binding target genes identified by RIP-seq.

(D) Distribution of IGF2BP1 binding sites across MYC and SRF as identified by RIP-seq in NTERA-2 overexpressed with FLAG-IGF2BP1.
cell proliferation, and negative regulation of differentiation (Figure 1C). As expected, RIP-seq showed enriched IGF2BP1 binding at the previously identified targets MYC and SRF mRNA, thereby indicating the specificity of our screen (Figure 1D). Importantly, the IGF2BP1 peaks observed throughout the IQGAP3 RNA—with strong enrichment at the 3’ UTR—confirmed IQGAP3 as a high-confidence RNA target of IGF2BP1 (Figure 1E). It is interesting that of the three IQGAP family members, IQGAP3 mRNA showed the strongest binding by IGF2BP1: IGF2BP1 binding of IQGAP1 RNA was 2-fold less than that in IQGAP3, while the binding of IGF2BP1 to IQGAP2 RNA was negligible (Figure S1B). Intriguingly, we also detected the interaction between IGF2BP1 and FOXM1 RNA (Figure S2A). FOXM1 is a transcription factor that has been strongly linked to cell proliferation as well as stem cell self-renewal (Liao et al., 2018). In the same vein, we also identified RNA of pluripotency factors NANOG and KLF4 as IGF2BP1 binding targets (Figure S1C).

To validate the RIP-seq data, we performed RIP of endogenous IGF2BP1 in NTERA-2 cells. RIP-quantitative PCR (RIP-qPCR) confirmed strong enrichment of IQGAP3 RNA in IGF2BP1 immunoprecipitates, relative to the HIST2H3A antibody which served as negative control (Figure 1F). Similarly, MYC and SRF RNA were also bound by IGF2BP1, which served as positive controls for the RIP-qPCR (Figure 1F). IGF2BP1 preferentially binds m6A-modified RNA. Therefore, we performed methylated RNA immunoprecipitation with m6A antibody followed by qPCR. We confirmed that IQGAP3 RNA was modified by m6A methylation at levels comparable to that of SRF and MYC RNA (Figure 1G).

The heterodimer comprising methyltransferases METTL3 and METTL14 catalyzes m6A methylation of RNA (Liu et al., 2014). We found that knockdown (KD) of METTL3/L14 abolished the ability of IGF2BP1 to interact with IQGAP3 RNA, thus indicating that m6A modification of IQGAP3 RNA is necessary for binding to IGF2BP1 (Figure 2A). Likewise, the interactions of IGF2BP1 with its known targets MYC and SRF RNA were strongly impaired after METTL3/L14 knockdown (Figure 2A). Of note, IQGAP3, MYC, and SRF protein levels were depleted after METTL3/L14 knockdown, suggesting that m6A may be involved in regulating the protein abundance of its target genes through the modulation of RNA levels (Figure 2B).

We next ascertained whether IGF2BP1 regulates IQGAP3 mRNA turnover in an m6A-dependent manner. NTERA-2 cells were treated with control siRNA, siRNA targeting IGF2BP1 or METTL3/L14, followed by actinomycin D to inhibit the transcription. Cells were harvested at various time durations and assayed for IQGAP3 mRNA by RT-qPCR. We found that IGF2BP1 knockdown was associated with a significant reduction of IQGAP3 mRNA half-life (Figure 2C). Similarly, METTL3/L14 knockdown also resulted in reduced IQGAP3 mRNA half-life. We also found that the half-lives of known IGF2BP1 targets MYC and SRF mRNAs were reduced upon the knockdown of IGF2BP1 or METTL3/L14 (Figures 2D and 2E). Therefore, our results indicate that IGF2BP1 bound and stabilized m6A modified IQGAP3 mRNA (Figure 2C).

We further examined whether IGF2BP1 controls the stability of IQGAP3, MYC, and SRF mRNAs in another cell type, HEK293T. Similar to NTERA-2, knockdown of IGF2BP1 in HEK293T led to a dramatic reduction in the half-life of IQGAP3 mRNA (Figure S3A). The half-lives of MYC and SRF mRNAs were also reduced after the knockdown of IGF2BP1 in HEK293T cells (Figures S3B and S3C). Of note, the mRNA decay process in HEK293T cells follows the one-phase decay phenomenon (non-linear) while the linear regression analysis was conducted to generate the line that best fits the data of NTERA-2 cells. Moreover, the half-lives of IQGAP3, MYC, and SRF mRNAs in HEK293T cells were shorter compared to that of NTERA-2 cells.

The IGF2BP1-IQGAP3-SRF-FOXM1 network regulates the pluripotent transcriptome

We recently reported the specific expression of IQGAP3 in rapidly proliferating isthmus stem cells of the stomach corpus (Matsuo et al., 2021). Moreover, we showed that IQGAP3 knockdown in NTERA-2 cells was associated with the reduction of the mRNA of Yamanaka factors NANOG, KLF4, SOX2, and MYC (Matsuo et al., 2021). Therefore, in addition to its critical role in proliferation (Nojima et al., 2008), we
Figure 2. IGF2BP1 stabilizes IQGAP3 RNA in a METTL3/14 dependent manner in NTERA-2 cells

(A) Binding of IGF2BP1 to IQGAP3, SRF, and MYC mRNAs after the depletion of m^6^A via METTL3/14 knockdown (KD). Data bars represent mean values with standard deviation (n = 3). Statistical analysis was conducted using two-tailed Student’s t-test (**** for P-value of <0.0001, *** for P-value of <0.001 and NS for not significant).

(B) Immunoblot showing IQGAP3, SRF, and MYC protein expression upon METTL3/14 KD.

(C–E) Half-life analyses of IQGAP3, (D) MYC and (E) SRF mRNAs after IGF2BP1 (left) or METTL3/14 (right) KD. Each data point represents mean values with a standard deviation of 3 independent experiments. Linear regression model was used to fit the data points. See also Figure S3.
Figure 3. The IGF2BP1-IQGAP3-SRF-FOXM1 network regulates the pluripotent transcriptome

(A) NTERA-2 cells were treated with retinoic acid (RA). qPCR and immunoblots show the mRNA and protein expression of IQGAP3, IGF2BP1, SRF, FOXM1, and other stem cell factors, respectively. C and D represent control and differentiated cells, respectively. Data bars represent mean values with SD (n = 3).

(B–D) mRNA and protein expression levels of IQGAP3 and stem cell markers after IGF2BP1, SRF or FOXM1 KD in NTERA-2 cells. Differentiation status was assessed by GFAP expression. Data bars represent mean values with SD (n = 3).
We hypothesize that IQGAP3 is necessary for the maintenance of pluripotency and/or stem cell properties. Indeed, the induction of differentiation in NTERA-2 cells through treatment with retinoic acid (RA) led to the dramatic reduction of mRNA and protein levels of both IQGAP3 and IGF2BP1, with concomitant decrease of pluripotency factors (Figure 3A). The expression of neuronal differentiation marker GFAP was strongly induced, thereby confirming differentiation onset (Figure 3A). We next knocked down IGF2BP1 in NTERA-2 cells. qPCR and Western blot analysis revealed drastic reductions of the pluripotency/stem cell markers (i.e. OCT4, CD44v9, SOX2, NANOG, KLF4), reminiscent of RA-induced differentiated cells (Figure 3B). Importantly, IGF2BP1 knockdown in NTERA-2, similar to IQGAP3 knockdown, was associated with morphological changes resembling differentiation (Figure 3E). Of note, FOXM1 and SRF expression levels were reduced following IGF2BP1 depletion and onset of differentiation.

Our RIP-seq data revealed IGF2BP1 binding sites within the FOXM1 RNA, with the highest peaks at the 3’ UTR. We further validated the RIP-seq data by performing IGF2BP1 RIP-qPCR assay to show strong enrichment of IGF2BP1 on FOXM1 RNA (Figure S2B). The FOXM1 RNA bound by IGF2BP1 was enriched with m6A, as shown by m6A-IP (Figure S2C). Depletion of m6A by METTL3/14 knockdown resulted in reduced binding of IGF2BP1 to FOXM1 RNA (Figure S2D). It was reported that FOXM1 expression increases at the start of the S-phase and that FOXM1 induces cell proliferation (Laoukili et al., 2005). Moreover, FOXM1 has been described as an oncofetal protein that regulates stem cell renewal (Bella et al., 2014). All these traits are reminiscent of IQGAP3, which is induced by serum and associated with stem cell potential (Matsuo et al., 2021; Nojima et al., 2008). Moreover, the gene expression analysis of The Cancer Genome Atlas (TCGA) dataset which comprises 407 stomach adenocarcinoma samples revealed a strong positive correlation of IQGAP3 mRNA expression with that of FOXM1 (Figure S2E). The UCSC genome browser ChIP-seq public database showed small but clear FOXM1 binding in proximity to the H3K4me3 mark at the IQGAP3 promoter (data not shown).

We and others have shown that SRF is a direct target of IGF2BP1 (this work and Muller et al., 2020). SRF is a well-established transcription regulator of mitogen-activated genes (e.g. cytoskeletal genes) (Posern and Treisman, 2006). More recently, SRF was reported to repress cell-type-specific genes and promote pluripotency (Ikeda et al., 2018). We queried the publicly available UCSC genome browser ChIP-seq database using the IGV tool (Kent et al., 2002; Robinson et al., 2011) and observed the occupancy of SRF, which overlapped with its consensus binding sequence, at the IQGAP3 gene promoter in HCT-116 and MCF-7 cells (Figures S4A and S4B). The SRF binding site was enriched with the H3K4me3 epigenetic mark, which indicates active transcription. We, therefore, performed ChIP-qPCR using SRF antibody and confirmed the SRF binding site between 200 and 450 base pairs upstream of IQGAP3 promoter in NTERA-2 cells (Figures S4C and S4D). Unfortunately, our ChIP-seq experiment did not show SRF binding to IQGAP3, suggesting highly dynamic or transient interaction (data not shown).

It would seem that IGF2BP1 regulates SRF and FOXM1 mRNA, which in turn influences IQGAP3 gene activity. Therefore, we performed siRNA knockdown of SRF and FOXM1 in NTERA-2 cells. Similar to NTERA-2 singly treated with IQGAP3 and IGF2BP1 siRNA, SRF and FOXM1 siRNA led to reductions in the expression of the core pluripotency markers NANOG, KLF4, SOX2, and MYC, whereas GFAP levels were increased (Figures 3C and 3D). Moreover, the morphology of cells after IQGAP3, IGF2BP1, SRF, or FOXM1 knockdown resembled that of differentiated NTERA-2 cells, suggesting that the NTERA-2 cells had undergone differentiation after IQGAP3, IGF2BP1, SRF, or FOXM1 knockdown (Figure 3E).

Taken together, our results are indicative of an IGF2BP1-SRF-FOXM1-IQGAP3 cooperative in maintaining pluripotency in NTERA-2 cells. To further investigate the biological significance of this network, NTERA-2 cells treated with control siRNA (siC), siRNA targeting IQGAP3 (siIQGAP3), IGF2BP1 (siIGF2BP1), SRF (siSRF), or FOXM1 (siFOXM1) were subjected to RNA sequencing (RNAseq)-based transcriptome profiling. Gene set enrichment analysis (GSEA) of the RNAseq data revealed blastocyst formation among the
top-ranked enriched gene signatures which were downregulated in siGF2BP1-treated cells (Figure 3F). IGF2BP1 knockdown, therefore, promoted a more differentiated and less proliferative state in NTERA-2 cells. Similarly, GSEA of siSRF cells showed that genes related to blastocyst formation were downregulated (Figure 3F). Given that SRF destabilizes cell identity (Ikeda et al., 2018), and that SRF is a downstream target of IGF2BP1, it is not surprising that SRF knockdown is associated with a shift to the differentiation state. The GSEA of siFOXM1 also revealed the downregulation of blastocyst formation, suggesting the shutdown of embryogenesis upon FOXM1 downregulation (Figure 3F). Analysis of siIQGAP3 revealed the downregulation of genes associated with self-renewal (Figure 3F). These gene signatures indicate that IQGAP3 plays regulatory roles in the determination of cell fates and stemness.

The GSEA of siGF2BP1, siSRF, siFOXM1, and siIQGAP3 indicated the induction of the differentiated state, in agreement with the observations obtained from changes in cell morphology and RT-qPCR analysis after siRNA treatment. We next queried the shared gene targets of IGF2BP1, SRF, FOXM1, and IQGAP3. Of the 2755 genes that were downregulated in siGF2BP1, siSRF, siFOXM1, and siIQGAP3 cells, 74 genes were common targets (Figure 3G and Table S3). Gene ontology analysis (GO) of these 74 genes revealed signaling pathways regulating pluripotency of stem cells, cellular response to retinoic acid, and let-7 inhibition of ES cell reprogramming as the top-ranked program, reflecting the transition of NTERA-2 cells toward the differentiated state upon knockdown of IGF2BP1, SRF, FOXM1, and IQGAP3 (Figure 3H). Moreover, pathways that have been highly associated with IQGAP3 and SRF such as the positive regulation of MAPK cascade, regulation of cell-cell adhesion, and regulation of actin cytoskeleton are among the top significant pathways, highlighting the important roles of SRF and IQGAP3 in this network (Figure 3H). Interestingly, the significant associations of these shared differentially expressed genes with tissue regeneration and degradation of extracellular matrix is reminiscent of the notion that “cancer is a wound that never heals” (Ge et al., 2017). We queried the TCGA dataset and observed that IQGAP3 expression is significantly increased in tumor lesions, compared to adjacent normal, in most of the tissues (Figures S5A and S5B). Moreover, IQGAP3 overexpression is associated with poor survival across all TCGA cancer datasets, and a similar pattern is observed for IGF2BP1 (Figure S5C).

Inhibition of the IGF2BP1-SRF-FOXM1-IQGAP3 signaling axis promotes differentiation in undifferentiated HGC-27 gastric cancer cells

We, therefore, examined the interplay of IGF2BP1, SRF, FOXM1, and IQGAP3 in regulating stem cell potential in cancer and the possibility of inhibiting this interplay for cancer treatment. Undifferentiated gastric cancer cell line HGC-27 was treated with siRNA targeting IGF2BP1, SRF, FOXM1, and IQGAP3. Similar to NTERA-2 cells, knockdown of IGF2BP1, SRF, FOXM1, or IQGAP3 resulted in reduced stemness and a more differentiated state. qPCR and Western blot analysis revealed drastic reductions in stem cell markers, such as OCT4, SOX2, NANOG, and CD44v9 (Figures 4A–4C). In gastric cells, KLF4 was reported to reduce self-renewal and promote differentiation (Miao et al., 2020). Accordingly, KLF4 levels remained unchanged in the differentiated cells. Moreover, differentiated cell marker Pepsinogen C (PGC) was induced in siGF2BP1, siSRF, siFOX1M, and siIQGAP3, compared to control (siC) cells. To ascertain the proliferative capacity of the stem cells in control (siC) and IGF2BP1, SRF, FOXM1, and IQGAP3 siRNA-treated HGC-27 cells, we next performed the tumor spheroid assay. The treated cells were assessed for growth in serum-free and non-adherent conditions as only stem cells can proliferate in this environment (Johnson et al., 2013). In agreement with the gene expression data reported here (Figures 4A–4C) and previously reported data (Matsuo et al., 2021), siGF2BP1, siSRF, siFOX1M, and siIQGAP3 treated cells showed a significant reduction in size and viability, relative to control (Figure 4D). Together, the siRNA treatments showed that the individual depletion of IGF2BP1, SRF, FOXM1, and IQGAP3 was sufficient to reduce stemness and promote the differentiation of HGC-27.

DISCUSSION

IQGAP3 expression is elevated in most, if not all, cancer cells (Dongol et al., 2020; Jinawath et al., 2020; Kumar et al., 2017; Matsuo et al., 2021; Monteleon et al., 2015; Shi et al., 2017; Wu et al., 2015, 2019; Xu et al., 2016). This indicates the importance of IQGAP3 as a cancer driver gene and its involvement in the fundamental aspect of carcinogenesis. That IQGAP3 is essential for cell proliferation and maintenance of stemness suggests that it is mainly associated with proliferating cancer stem cells. Depletion of IQGAP3 reduced the proliferation of breast and gastric cancer cell lines (Hu et al., 2016; Oue et al., 2018). Moreover, IQGAP3 has been shown to regulate metastasis and EMT (Jinawath et al., 2020; Shi et al., 2017). IQGAP3 is therefore a likely target for the treatment of aggressively proliferating cells as well as metastasis in most cancers. However, what causes the specific increase of IQGAP3 expression in cancer is unclear.
Cell cycle regulator E2F1 was shown to transactivate the IQGAP3 promoter (Lin et al., 2019). More recently, YAP was reported to promote the binding of B-MYB to the IQGAP3 promoter and enhancer, thereby upregulating IQGAP3 transcription (Leone et al., 2021). Here, we identified regulators of IQGAP3 expression, which may explain its specific expression in proliferating stem cells and cancer cells. We report the importance of IGF2BP1-SRF-FOXM1-IQGAP3 signaling axis in maintaining stem cell properties in a pluripotent stem cell line NTERA-2 and an undifferentiated gastric cancer cell line HGC-27. IGF2BP1 has long been implicated in cell metabolism and stem cell maintenance during early development (Degrauwe et al., 2016). We show that IGF2BP1 and IQGAP3 expression levels are highest in the blastocyst stage and that expression of both showed corresponding decreases during adulthood. The re-expression of IGF2BP1 during malignant transformation is likely to increase IQGAP3 mRNA stability. On the other hand, the differences between the Iqgap3 and Igf2bp1 expression levels in adult mouse stomach tissues suggest that there are mechanisms other than IGF2BP1, which regulate the IQGAP3 expression, especially in rapidly proliferating tissues similar to stomach. E2F1 or YAP, previously reported to have a role in the transcriptional regulation of IQGAP3, may be accountable for these mechanisms.

Moreover, IGF2BP1-mediated increase of SRF and FOXM1 mRNA levels afford a further layer of IQGAP3 regulation, namely a link to mitogenic stimulation. SRF is a major regulator of actin cytoskeleton genes (Miano et al., 2007). It is, therefore, not surprising that IQGAP3, with its activities in the reorganization of cytoskeletal architecture and cell proliferation (Nojima et al., 2008; Wang et al., 2007), is an SRF downstream gene. Although the public dataset and our ChIP-qPCR showed SRF binding at or near its consensus sites at the IQGAP3 promoter, our inability to use ChIP-seq to show SRF binding to the IQGAP3 gene indicates that its binding is transient. FOXM1 is important for proliferation, self-renewal, and tumorigenesis (Liao et al., 2018). Similar to IQGAP3, FOXM1 is found mainly in highly proliferative cells, such as stem/progenitor cells, regenerating tissues, and cancer cells (Liao et al., 2018; Matsuo et al., 2021).

The similarities between robust proliferation during tissue damage repair/regeneration and cancer have long been observed. IGF2BP1 plays important role in the tissue repair and homeostasis of the colonic...
epithelium (Chatterji et al., 2021; Hamilton et al., 2015; Manieri et al., 2012; Singh et al., 2020). Although our earlier study on stomach tissues did not show the upregulation of IGF2BP1 after tamoxifen-induced tissues damage, FOXM1 and SRF levels were increased, suggesting that both may be contributing to the strong IQGAP3 upregulation in the stomach during tissue repair.

Our findings, which linked SRF and FOXM1 to the upregulation of IQGAP3 gene expression, raise many interesting questions: are they critical for IQGAP3’s ability to maintain self-renewal and drive proliferation in cancer cells? FOXM1 and SRF have been linked to chemoresistance (Okada et al., 2013; Whitson et al., 2018). Do they, in response to external stimuli, activate IQGAP3 to stimulate proliferation in rare quiescent cancer stem cells and thus confer chemotherapy resistance? Clearly, more research is required to determine the mechanistic basis of the associations between IQGAP3, SRF, and FOXM1.

Undifferentiated cancer cells are regarded to be more malignant than well-differentiated cancers, which possess limited tumorigenic potential. Undifferentiated cancer cells, with stem-like properties, are responsible for aggressive metastasis. Drugs that target this IGF2BP1-SRF-FOXM1-IQGAP3 axis will be therapeutically attractive as inducers of terminal differentiation in cancer stem cells. Given the commonality of elevated IQGAP3 in many cancer types, our work suggests that, in addition to identifying drug inhibitors of IQGAP3, inhibitors of IGF2BP1 binding activity (Mahapatra et al., 2017), SRF/Rho/MRTF activity (Leal et al., 2019) and FOXM1 transcription (Petrovic et al., 2010; Sleiman et al., 2011)—some of which are in clinical trials—may potentially be used to deplete IQGAP3 and improve cancer treatment.

Limitations of the study
A limitation was that our study was performed using only two cell types, namely the pluripotent embryonic cancer cell line model NTERA-2 and the undifferentiated human gastric cancer cell line HGC-27. To assess the commonality of the IGF2BP1-SRF-FOXM1-IQGAP3 network, more cell types including adult stem cells as well as undifferentiated cancer cell lines from diverse tissues should be studied. Moreover, the comparison of protein expression levels between IGF2BP1, SRF, FOXM1, and IQGAP3 in tumor tissue microarrays may be needed. Nevertheless, this proof-of-concept study reveals the hitherto unknown relationship of IGF2BP1, SRF, FOXM1, and IQGAP3 and suggests avenues for future mechanistic studies on the regulation and pharmacological inhibition of IQGAP3 in cancer stem cells.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:
**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105194.

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**AUTHOR CONTRIBUTIONS**

Y.I., K.M., and L.S.H.C. conceptualized and supervised the project. K.M., Y.X.T., and N.A.M. performed the in vitro experiments, M.M.H.M. did the RNA extraction from blastocysts, and E.J.S. and N.N. performed the mouse blastocyst and tissue extraction. Y.L. and H.Y. analyzed the sequencing data. All authors interpreted the data. Y.I., K.M., and L.S.H.C. wrote the article with inputs from all co-authors.

**DECLARATION OF INTERESTS**

All authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| SRF                 | Cell Signaling Technology | 5147 |
| FOXM1               | Cell Signaling Technology | 20459 |
| IQGAP3              | Proteintech | 25930-1-AP |
| IGF2BP1             | Cell Signaling Technology | 8482 |
| IGF2BP1             | MBL | RN007P |
| NANOG               | Cell Signaling Technology | 4903 |
| OCT4                | Abcam | ab19857 |
| KLF4                | Abcam | ab106629 |
| CD44v9              | Cosmo Bio | LKG-M001 |
| GFAP                | Proteintech | 16825-1-AP |
| PGC                 | Abcam | ab93886 |
| GAPDH               | Cell Signaling Technology | 2118 |
| SOX2                | Proteintech | 11064-1-AP |
| cMYC                | Cell Signaling Technology | 5605 |
| Rabbit IgG          | Cell Signaling Technology | 2729 |
| m6A                 | Sigma-Aldrich | MABE1006 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| All-Trans Retinoic Acid | Sigma-Aldrich | R2625 |
| Actinomycin D       | Sigma-Aldrich | A9415 |
| Formaldehyde        | Sigma-Aldrich | 252549 |
| **Critical commercial assays** |        |            |
| RNeasy kit          | Qiagen | 74104 |
| iScript cDNA synthesis kit | Bio-Rad | 1708890 |
| iTaq Universal SYBR green kit | Bio-Rad | 1725150 |
| QIAquick PCR purification kit | Qiagen | 28106 |
| CellTiter-GLO       | Promega | G9681 |
| Pierce BCA Protein Assay kit | Thermo Fisher Scientific | 23225 |
| **Deposited data**  |        |            |
| RNA seq and RNA-IP seq data | This paper | GSE196406 |
| **Experimental models: Cell lines** |        |            |
| NTERA-2             | ATCC | CRL-1973 |
| HGC-27              | CellBank Australia, the European Collection of Cell Cultures | 9402256 |
| HEK293T             | ATCC | CRL-3216 |
| HEK293FT            | ATCC | CRL-11268 |
| **Oligonucleotides** |        |            |
| siRNAs targeting IGF2BP1, SRF, FOXM1, and IQGAP3 | Horizon Discovery | Refer to Table S1 |
| siRNAs targeting METTL3 and METTL14 | IDT | Refer to Table S1 |
| Primers for qPCR of human target genes in knockdown experiments | IDT | Refer to Table S2 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yoshiaki Ito (yoshi_ito@nus.edu.sg).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- Raw data of RNA seq and RNA-IP seq are available on GEO: GSE196406.
- This study does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture
NTERA-2, human malignant pluripotent embryonal carcinoma cell line, obtained from ATCC (NTERA-2 cl.D1, Cat#CRL-1973, Lot#70004008) were cultured in DMEM (Dulbecco’s Modified Medium, Nacalai Tesque) with 10% FBS (Fetal bovine serum) at 37°C in a humidified 5% CO2 incubator according to the manufacturer’s instructions. Cells were maintained at high density and the medium was changed every 2–3 days. On confluency, cells were passaged at 1:4 ratio. For differentiation of NTERA-2, cells were treated with 10 μM of all-trans retinoic acid (Sigma-Aldrich, Cat#R2625) every 3–4 days for 28 days. HGC-27, human gastric cancer cell line, obtained from CellBank Australia, the European Collection of Cell Cultures (Cat#94042256), and HEK293T and HEK293FT cells obtained from ATCC were cultured respectively in RPMI 1640 (Nacalai Tesque) or DMEM (Dulbecco’s Modified Medium, Nacalai Tesque) with 10% FBS at 37°C in a humidified 5% CO2 incubator according to the manufacturer’s instructions. Cells were maintained at high density and the medium was changed every 2–3 days. On confluency, cells were passaged at 1:10 ratio when the confluency reached 80–90%.

METHOD DETAILS

siRNA-based knockdown of target genes
ON-TARGET plus SMARTpool siRNAs targeting human FOXM1, IGF2BP1, IQGAP3, SRF, METTL3/14 and a non-targeting siRNA pool (Dharmacon, Cat#D001810-10-50) as the control siRNA were used for the knockdown experiments. At 24 h after seeding in a 6-well culture plate, cells were transfected with 35 nM of siRNAs using jetPRIME (Polyplus Transfection) according to the manufacturer’s protocol. At 48 h after transfection, cells were reseeded into a 6-well plate and a 6 cm dish which were then transfected in a similar
manner at 6–8 h after seeding, and cells were harvested at 72 h later for RNA and protein quantification. The details of siRNA sequences are as described in Table S1.

**RNA extraction and qPCR**

Total RNA from the cell pellets was extracted using RNeasy Kit (Qiagen) with the removal of genomic DNA using the RNase-free DNase Set (Qiagen) according to the manufacturer’s instructions. Complementary DNA (cDNA) was prepared from 1 μg of total RNA extracted using iScript reverse transcription supermix for RT-qPCR (Bio-Rad). qPCR was performed using iTaq Universal SYBR green kit (Bio-Rad) and QuantStudio3 Real-Time PCR systems (Thermo Fisher Scientific), and the primers used are listed in Table S2. The fold-change in the expression levels of target genes in treated cells compared to control cells was calculated using the 2^{-ΔΔCt} method where GAPDH was used as a housekeeping gene.

**Immunoblot analysis**

Cell pellets were first lysed by resuspending of the cell pellets with the lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycholate and 1% NP-40) supplemented with Halt phosphatase inhibitor (Thermo Fisher Scientific), Complete protease inhibitor (Merck) and PMSF (Sigma-Aldrich) and incubating at 4°C for 45 min. After centrifugation of the lysates at 13500 rpm for 15 min at 4°C, the supernatant of each lysate was collected and the protein concentrations were measured using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Cat # 23225) according to the manufacturer’s protocol. 40 μg of protein was loaded into each well of 8% SDS/PAGE. The antibodies used listed in key resources table.

**Tumour spheroid assay**

For tumour spheroid assay, 1000 cells per well were seeded in an ultra-low attachment round bottom 96-well plate (Thermo Fisher Scientific, Cat # 174929), and cultured in normal growth medium. Cells were treated with siRNAs targeting genes of interest using reverse transfection method where suspension of cells were treated with 35 nM of siRNAs prior to seeding and jetPRIME (Polypus Transfection) was used according to the manufacturer’s protocol. Spheroid growth viability was determined on 5 days after seeding using CellTiter-GLO (Promega, Cat #G9681). Spheroid size was measured by using ImageJ software.

**Mouse tissue extraction for gene expression analysis**

Blastocysts and adult mouse tissues samples were derived from C57BL/6 mouse. For adult mouse tissue samples, 16-week old mice were sacrificed and tissues (brain, heart, kidney, liver and stomach) were extracted. Tissues samples were washed in ice-cold PBS for 3 times, and processed for RNA extraction using Trizol reagent (Invitrogen). After adding Trizol, aqueous phase was collected, and RNA was precipitated at –20°C for overnight using isopropanol and glycogen. Precipitated RNA was then cleaned up using ethanol. cDNA synthesis and qPCR analysis were done as described above.

**RNA Immunoprecipitation**

RNA immunoprecipitation (RIP) was performed with some modifications (Kwok et al., 2021). Briefly, cells seeded in a 10-cm plate were cross-linked by UV at 375 J/cm² and harvested by scraping. Total cell extracts were prepared using polysome lysis buffer (100 mM KCl, 5 mM MgCl₂, 10 mM Hepes (pH 7) and 0.5% NP-40) supplemented with 1 x protease inhibitor, 200 units/ml Rnase out (Invitrogen, Cat #10777019) and 200 units/ml SUPERase IN (Ambion, Cat #AM2694), and 10% of the lysate was kept as an input. IGF2BP1 (MBL, Cat # RN007P) or rabbit IgG antibody (CST, Cat#2729) were incubated with Protein A/G Dynabeads® (Life Technologies, Cat #10015D) in 5% BSA (Bovine serum albumin) in PBS for 2 h at 4°C followed by an overnight incubation with 400 μg of cellular extracts at 4°C. Antibodies were used at the dilution of 1:75 for one RIP reaction. Reaction mixture was then washed for 5 times with NT2 buffer (50 mM Tris-HCL (pH 7.0), 150 mM NaCl, 10 mM MgCl₂, 0.05% (v/v) NP-40) and RNA was eluted by incubating beads with 200ul of 1%SDS-TE buffer supplemented with 20μg of Proteinase K at 55°C for 30mins. RNA was extracted using Trizol reagent (Invitrogen), precipitated with ethanol and cleaned up using Phenol:Chloroform:Isoamyl Alcohol. For RIP-qPCR assay, cDNA was generated from input and IP samples using iScript cDNA synthesis kit (BioRad) and the transcript enrichment of known target genes and genes of interest were examined by doing real-time qPCR with iTaq Universal SYBR green kit (Bio-Rad) and QuantStudio3 Real-Time PCR systems (Thermo Fisher Scientific). Primers used in the experiments were listed in Table S2.
For RIP-sequencing, NTERA-2 cells overexpressed with FLAG-IGF2BP1 and FLAG-affinity beads (Sigma Aldrich) were used, and RIP was performed as described above. RNA samples were sent to Beijing Genomics Institute (BGI) for cDNA synthesis, PCR amplification, transcriptome library preparation and sequencing.

Half-life analysis of RNA stability
Cells were treated with 5 μg/ml actinomycin D (Sigma, Cat# A9415) and harvested at 0 (no treatment), 30, 60, 120, 240 and 360 min after treatment. The extraction of total RNA from the cell lysate, cDNA synthesis, and the measurement of mRNA levels of target genes and housekeeping genes were done as described previously. The expression levels of target genes were calculated using the 2-ΔΔCT method where GAPDH was used as a housekeeping gene. The mRNA levels of target genes at different time points were normalized to the amount of mRNA at time 0. Using the GraphPad Prism 7.03 software, the linear regression analysis was done to generate the line that best fits the data from NTERA-2 cells, while the non-linear curve (one-phase decay) was used for the data from HEK293T cells, and the half-life of mRNA was calculated. For statistical analysis, area under curve (AUC) was calculated and compared between the lines that best fits the data using GraphPad Prism 7.03.

m^6^A-IP
m^6^A-IP was done with some modifications from previously reported method (Dominissini et al., 2012). Total RNA was extracted using TRIzol (Invitrogen) and DNase I (QIAGEN) treatment was done to eliminate genomic DNA contamination. 150 ng of total RNA as an input, and 3 μg of RNA was used to incubate with 10 μg of m^6^A antibody (Sigma-Aldrich, Cat# MABE1006) or rabbit IgG antibody (CST, Cat#2729) conjugated to Protein A/G Dynabeads® (Life Technologies, Cat #10015D) in IP buffer (50 mM Tris-HCL (pH 7.4), 750 mM NaCl and 0.5% NP-40) supplemented with 200 units/ml Rnase out (Invitrogen, Cat #10777019) and 200 units/ml SUPERase IN (Ambion, Cat #AM2694). After overnight incubation at 4°C, RNA-antibody complexes were pelleted and washed with IP buffer for 3 times followed by the isolation of RNA with Trizol reagent (Invitrogen) and the RNA precipitation with ethanol. RNA was then cleaned up using Phenol: Chloroform:isoamyl Alcohol method. cDNA was generated from input and IP samples using iScript cDNA synthesis kit (BioRad) and the transcript enrichment of known target genes and genes of interest were examined by doing real-time qPCR with iTaq Universal SYBR green kit (Bio-Rad) and QuantStudio3 Real-Time PCR systems (Thermo Fisher Scientific). Primers used in the experiments were listed in Table S2.

Pathway enrichment analysis of target genes
For the pathway enrichment analysis of IGF2BP1’s target genes identified by RNA-IP sequencing, the target genes with the most statistically significant enrichment scores were selected and analysed using Metascape analysis tool (Zhou et al., 2019). The details of the analysis could be found on the webpage (https://metascape.org). Briefly, for each given gene list, pathway and process enrichment analysis has been carried out using the different ontology sources including KEGG Pathway, GO Biological Processes, Reactome Gene Sets, Canonical Pathways and WikiPathways. Gene ontology terms with p value < 0.01, a minimum count of 3, and an enrichment factor >1.5 (the enrichment factor is defined as the ratio between the observed counts and the counts expected by chance) are grouped into clusters. The term with the most statistically significant value within a cluster is taken to represent the cluster. "Log10(P)" means the p value in log base 10, and "Log10(q)" means the multi-test adjusted p value in log base 10.

For the pathway enrichment analysis of commonly downregulated genes in all 4 different knockdown gene sets (downregulated genes in NTERA-2 cells after IGF2BP1, IQGAP3, SRF or FOXM1 knockdown), commonly downregulated genes were firstly defined using the publicly available tool called Draw Venn Diagram (ugent.be). The 74 commonly downregulated genes were then analyzed using Metascape analysis tool as described above.

Chromatin IP (ChIP)
Chromatin IP was performed with some modifications of previously published method (Lee et al., 2006). Briefly, cells were fixed with 1% formaldehyde for 10 min at room temperature (RT) and quenched by glycine (final concentration 125 mM) for 5 min at RT. Cells were washed 3 times by ice-cold PBS supplemented with 1 mM PMSF, collected by scraping and then spinning down at 1500 rpm for 5 min. Nuclear extraction was done and nuclear extracts were resuspended in shearing buffer (1 mM EDTA, 10 mM Tris-HCl pH 7.6, 0.1% SDS) followed by sonication with ME220 Focused-ultrasonicator (Covaris) to achieve DNA fragments of
sizes between 200 and 500 bp. Sonicated samples were then centrifuged at 13,000 rpm for 10 min to remove the cell debris and 1 mL of supernatant was incubated with 3 μg of desired antibody conjugated with Protein A/G Dynabeads (Life Technologies, Cat #10015D) for overnight at 4 °C. Antibodies were listed in key resources table. Reaction mixture was then washed with ice-cold low salt wash buffer, high salt wash buffer, LiCl wash buffer and TE buffer supplemented with proteinase inhibitor. Bound DNA was then eluted by incubating with elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) at 65 °C for 30 min where tubes were quickly vortexed every 7–8 min. The eluted DNA-protein complexes were then treated with RNase A and proteinase K followed by incubating the reaction for overnight at 65 °C for reverse cross-linking. DNAs were then purified by using QIAquick PCR purification kit (Qiagen). qPCR was done to measure the enrichment of the genes of interest bound by the target proteins.

TCGA and GTEX dataset analysis
For the analysis of the IQGAP3 mRNA expression in different human cancer types, IQGAP3 mRNA expression data available in TCGA Pan-cancer Atlas dataset was downloaded via UCSC Xena analysis tool (http://xena.ucsc.edu) (Goldman et al., 2020). The expression data for three subtypes which are adjacent normal, primary tumor, and metastatic tumor tissue samples in 33 different cancer types (n = 10,833) were then analyzed using GraphPad Prism 7.03 software.

To compare IQGAP3 mRNA expression levels between normal and tumor samples in 18 different human tissues, IQGAP3 mRNA expression data available in TCGA and GTEX datasets were downloaded from UCSC Xena analysis tool (http://xena.ucsc.edu) (Goldman et al., 2020). The expression data derived from the normal and tumor tissue samples (n = 11493) were analysed using GraphPad Prism 7.03 software.

GEPIA (http://gepia.cancer-pku.cn/) was used to analyse the association between the expression levels of IQGAP3 and the overall survival in months across 33 different human cancer types available on TCGA datasets (Tang et al., 2017). GEPIA uses the Mantel-Cox test to calculate the hazards-ratio, and high-expression and low-expression cohorts were defined with the cut-off threshold set at 50%. Percent survival was calculated as the percentage of patients in the defined cohort who remained alive for a given period in months. Hazards ratio of greater than 1 was defined as high and p value of less than 0.05 was defined as statistical significance.

RNA-seq analysis and GSEA
RNA-seq reads were mapped by STAR to the human reference genome GRCh37 with reference gene annotation GENCODE 26 (Dobin et al., 2013; Harrow et al., 2012). Reads with low mapping quality <20 were not used for further analysis. Next, PCR duplicates were removed in the paired-end alignments using SAMtools. Gene expression levels of individual genes/transcripts were generated using featureCounts (Liao et al., 2014). Cross-sample normalization of reads counts was based on total mappable counts using RPKM. Normalized read counts were then applied to perform GSEA analysis to identify significantly enriched pathways/gene sets between the wildtypes and their corresponding knockdowns. The full version of MSigDB v7.2 was employed as GSEA dataset database.

RIP-seq analysis
Reads from the RIP-Seq samples and the corresponding controls were mapped against human reference genome GRCh37 by STAR with GENCODE 26 for transcriptome annotation. First, peak calling was performed by MACS2 to identify RIP binding enriched regions (Zhang et al., 2008). For reads mapped to multiple exons or peaks presented in neighboring exons, the peaks were merged together. Sequences covered by the reproducible peaks of the duplicated samples were extracted for motif finding. The upstream and downstream sequences of reproducible peaks were also extracted and served as the background sequences in the motif finding. Motif discovery is performed with MEME suite using differential enrichment mode (Bailey et al., 2009; Bailey and Elkan, 1994).

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analyses were done by using Student’s t-tests (two-tailed) and all experiments were conducted at least for three replicates as indicated in the figure legends.
For the overall survival curves, log rank analysis method was used to determine statistical significance. Data were presented as mean with standard deviation and p-value of <0.05 was considered as a statistical significance. For RNA-seq, three sets of RNA samples were used, and for RIP-seq, two sets of RNA samples were used. All western blot and brightfield images were representative of three independent experiments.