The Forkhead box F1 transcription factor inhibits collagen deposition and accumulation of myofibroblasts during liver fibrosis

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ABSTRACT

Hepatic fibrosis is the common end stage to a variety of chronic liver injuries and is characterized by an excessive deposition of extracellular matrix (ECM), which disrupts the liver architecture and impairs liver function. The fibrous lesions are produced by myofibroblasts, which differentiate from hepatic stellate cells (HSC). The myofibroblast’s transcriptional networks remain poorly characterized. Previous studies have shown that the Forkhead box F1 (FOXF1) transcription factor is expressed in HSCs and stimulates their activation during acute liver injury; however, the role of FOXF1 in the progression of hepatic fibrosis is unknown. In the present study, we generated αSMACreER:Foxf1fl/fl mice to conditionally inactivate Foxf1 in myofibroblasts during carbon tetrachloride-mediated liver fibrosis. Foxf1 deletion increased collagen depositions and disrupted liver architecture. Timp2 expression was significantly increased in Foxf1-deficient mice while MMP9 activity was reduced. RNA sequencing of purified liver myofibroblasts demonstrated that FOXF1 inhibits expression of pro-fibrotic genes, Col1α2, Col5α2, and Mmp2 in fibrotic livers and binds to active repressors located in promoters and introns of these genes. Overexpression of FOXF1 inhibits Col1α2, Col5α2, and MMP2 in primary murine HSCs in vitro. Altogether, FOXF1 prevents aberrant ECM depositions during hepatic fibrosis by repressing pro-fibrotic gene transcription in myofibroblasts and HSCs.

KEY WORDS: FOXF1, Hepatic fibrosis, Myofibroblast, Hepatic stellate cell, Carbon tetrachloride liver injury

INTRODUCTION

The liver is the body’s filter and insulins can result from a variety of infectious, toxic and metabolic agents. Hepatic fibrosis is the common end stage to a multitude of liver diseases (Civan, 2016) and is characterized by an excessive deposition of extracellular matrix (ECM) and collagen (Cheng and Mahato, 2007). Novel animal models of hepatic fibrosis are greatly needed to identify molecular mechanisms responsible for the disease pathogenesis and for the development of therapeutic agents. Hepatic stellate cells (HSC) reside in the space of Disse and are characterized by their storage of lipids when in the quiescent state (Yin et al., 2013; Croci et al., 2013). During fibrogenesis, quiescent HSCs differentiate into myofibroblasts (MF) in response to cytokine signaling from damaged hepatocytes and immune cells after liver insult. MFs secrete ECM and collagen to encapsulate the site of injury and shield the liver from plaguing insults (Cheng and Mahato, 2007). While HSCs and MFs make up only a small number of cells in liver tissue, they are the main contributors of ECM and collagen during liver repair and fibrogenesis (Brenner et al., 2012; Fausther et al., 2013). The TGF-β and PDGF signaling pathways play key roles in hepatic fibrosis and HSC activation (Makarev et al., 2016). TGF-β signaling stimulates cellular transdifferentiation of HSCs to MFs (Hellerbrand et al., 1999; Bachem et al., 1993), whereas PDGF signaling induces cellular proliferation in fibrotic foci (Wong et al., 1994; Kinmann et al., 2002).

The Forkhead Box F1 (FOXF1) transcription factor is expressed in human and murine HSCs and is important in regulating stellate cell activation after acute liver injury (Kalinichenko et al., 2003). In the advanced disease state of hepatocellular carcinoma (HCC), which is associated with significant fibrotic depositions, FOXF1 expression has been shown to be significantly decreased (Hodo et al., 2013). Foxf1−/− mice are embryonic lethal due to severe developmental abnormalities in the yolk sac and allantois (Mahlapuu et al., 2001). Murine haploinsufficiency of Foxf1 causes lung hypoplasia, loss of alveolar capillaries in the lung and gall bladder agenesis (Kalinichenko et al., 2002; Bolte et al., 2018), and was associated with delayed lung and liver repair. After acute liver injury by carbon tetrachloride (CCL4), Foxf1−/− mice exhibited diminished activation of HSCs and delayed liver repair, indicating that FOXF1 is essential for liver repair after acute liver injury (Kalinichenko et al., 2003). Foxf1 siRNA delivered to mice through nanoparticles prevented activation of HSCs and subsequent collagen deposition after cholestatic liver injury (Abshagen et al., 2015). While these studies have shown that FOXF1 is required for activation of HSCs after acute liver injury, the role of FOXF1 in MFs and in the progression of fibrotic responses remains unknown.

In the present study, we generated a novel genetic mouse model to conditionally delete Foxf1 from MFs (αSMACreER:Foxf1fl/fl). During chronic liver injury, deletion of Foxf1 in MFs exacerbated hepatic fibrosis, increased collagen deposition and stimulated expression of profibrotic genes in the liver tissue. Our studies indicate that Foxf1 expression in MFs is critical to prevent MF accumulation and collagen deposition during liver fibrosis.

RESULTS

Deletion of Foxf1 in αSMA-positive cells exacerbates CCL4-induced hepatic fibrosis

Previous studies demonstrated that FOXF1 is present in HSCs in murine developing and adult livers (Kalinichenko et al., 2003; Kim et al., 2005). Consistent with these studies, FOXF1 staining was
detected in livers of e12.5-e17.5 mouse embryos as well as in mesenchyme of stomach and intestine (Fig. S1). In adult mice, FOXF1 is specifically expressed in the liver parenchyma but not in endothelial or smooth muscle cells surrounding the portal vein or hepatic artery (Kalinichenko et al., 2003) (Fig. 1A; Fig. S1), and FOXF1 staining co-localized with desmin (DES) (Fig. 1A), a known marker of HSCs (Yokoi et al., 1984). To investigate the role of Foxf1 in liver fibrosis, we utilized a conditional knockout approach. Transgenic mice containing a tamoxifen-inducible aSMA-CreER transgene and two Foxf1-flxed alleles (aSMA-CreER;Foxf1fl/fl) were generated by breeding aSMA-CreER and Foxf1fl/fl mice (Fig. 1B,C). Hepatic fibrosis was induced by chronic liver injury using multiple administrations of CCl4, which is known to increase fibrotic depositions and disrupt liver architecture (Mederacke et al., 2013). While αSMA was not detected in parenchyma of quiescent livers, αSMA staining was increased after CCl4 injury. FOXF1 was detected in MFs of control livers but not in aSMA-CreER;Foxf1fl/fl livers (Fig. 2A). Quantitative counts of FOXF1-expressing cells demonstrated that the number and percentage of FOXF1+ MFs (FOXF1−SMA) were reduced whereas the number and percentage of FOXF1−SMA+ MFs (FOXF1−SMA+) were elevated in injured aSMA-CreER;Foxf1fl/fl livers compared to controls (Fig. S3).

**FOXF1 expression is decreased in hepatic myofibroblasts of aSMA-CreER;Foxf1−/− mice**

Since FOXF1 is expressed in HSCs in the liver (Kalinichenko et al., 2003), we examined the efficiency of Foxf1 deletion in our experimental model, using immunostaining for FOXF1 and DES. Without CCl4 treatment, FOXF1 was observed in cell nuclei of DES-positive stellate cells in Foxf1fl/fl and aSMA-CreER;Foxf1fl/fl livers (Fig. 2A). After CCl4 and Tam treatment, FOXF1 staining was reduced in DES-positive cells of aSMA-CreER;Foxf1−/− livers but not in Foxf1fl/fl livers (Fig. 2A). We also immunostained liver sections for FOXF1 and αSMA, a marker of MFs (Rockey et al., 2013). While αSMA was not detected in parenchyma of quiescent livers, αSMA staining was increased after CCl4 injury. FOXF1 was detected in MFs of control livers but not in aSMA-CreER;Foxf1−/− livers (Fig. 2B). Quantitative counts of FOXF1-expressing cells demonstrated that the number and percentage of FOXF1+ MFs (FOXF1−αSMA−) were reduced whereas the number and percentage of FOXF1−αSMA+ MFs (FOXF1−αSMA+) were elevated in injured aSMA-CreER;Foxf1−/− livers compared to controls (Fig. S3).

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**Fig. 1. Hepatic fibrosis is increased after CCl4 injury in mice with FOXF1 deficiency.** (A) FOXF1 co-localizes with DES in hepatic stellate cells in adult mice. (B) Diagram demonstrates aSMA-CreER transgene with LoxP sites flanking the Foxf1 Exon 1 (encoding DNA-binding domain). (C) DNA gel shows genotypes of Foxf1fl/fl and aSMA-CreER;Foxf1fl/fl mice. (D) Diagram illustrates CCl4 and tamoxifen (Tam) treatment protocol. (E,F) H&E and Masson’s Trichrome staining show fibrotic depositions after five weeks of CCl4 treatment. Fibrosis was increased in livers from aSMA-CreER;Foxf1−/− mice. White dashed lines indicate fibrotic lesion boundaries. (G) Collagen deposition was quantitated using the Sircol assay. n=2 mice per group in week 0; n=4 mice per group in week 5. (H) qRT-PCR analysis demonstrates significant increases in Col1a1 and Col3a1 mRNAs in livers from aSMA-CreER;Foxf1−/− mice. n=3 mice per group in week 0; n=5 mice per group in week 5. Untreated livers from Foxf1fl/fl and aSMA-CreER;Foxf1fl/fl mice were used as normal controls. mRNAs were normalized to Actb. *P<0.05, **P<0.01, ***P<0.001.
FOXF1 protein and mRNA were increased in CCl4-treated Foxf1fl/fl livers and purified HSCs (Fig. 2C,D; Fig. S3) but not in the αSMACreER;Foxf1−/− livers (Fig. 2C,D). The loss of FOXF1 in αSMACreER;Foxf1−/− livers occurred in periporal regions while pericentral regions were unaffected (Fig. 2E). The αSMA-CreER transgene allows for the maintained presence of FOXF1 for HSC activation (Kalinichenko et al., 2003) and only deletes FOXF1 after αSMA is expressed in MFs. Thus, αSMA-CreER transgene effectively deletes Foxf1 from hepatic MFs during CCl4-mediated chronic liver injury.

**Deletion of Foxf1 reduces MMP9 activity in CCl4-injured livers**

Histological staining with Sirius Red/Fast Green showed a significant increase in collagen accumulation in αSMACreER; Foxf1−/− livers after five weeks of CCl4 treatment (Fig. 3A; Fig. S4). Increased fibrosis in Foxf1-deficient livers was confirmed by immunostaining for DES and αSMA (Fig. 3B,C). To examine the consequences of extended CCl4 treatment, we treated mice with αSMA-CreER effectively deletes Foxf1 from hepatic MFs during CCl4-mediated chronic liver injury.

Since MMP9 plays an important role in collagen degradation after liver injury (Duarte et al., 2015), we evaluated mRNA expression of Mmp9 and its inhibitor, Timp2, in liver tissue. Timp2 mRNA was increased in CCl4-injured αSMACreER;Foxf1−/− livers compared to controls (Fig. 3F). Although Mmp9 mRNA was unchanged (Fig. S8), evaluation of MMP9 activity through zymography demonstrated a significant decrease in enzymatic activity of MMP9 in αSMACreER;Foxf1−/− livers after CCl4 treatment (Fig. 3G,H). Mmp8, Mmp13, Mmp16, Timp1 and Timp3 mRNA levels were not affected in Foxf1-deficient livers (Fig. S8). Thus, Foxf1 deletion from MFs increases Timp2 mRNA and reduces MMP9 activity in fibrotic livers.

**Deletion of Foxf1 does not influence cellular proliferation in fibrotic livers**

We evaluated proliferation markers to investigate if the increased fibrosis in αSMACreER;Foxf1−/− livers was due to an expansion of the stromal cells. While cellular proliferation was increased after CCl4 treatment, there were no significant differences in the number of proliferating hepatocytes or non-hepatocytes between Foxf1fl/fl and αSMACreER;Foxf1−/− livers (Fig. 4A–C; Fig. S9). Hepatocytes and non-hepatocytes were identified through distinct morphological appearances (Malarkey et al., 2005) from high magnification images. mRNAs of proliferation-specific genes Foxm1, Ccnb1, Ccn1, and AurKB (Wang et al., 2009; Kalin et al., 2011; Ren et al., 2013) were unchanged between Foxf1fl/fl and αSMACreER;Foxf1−/− livers (Fig. 4D). Proliferating HSCs and MFs were detected in
CCL4-treated livers by co-localization of Ki-67 with DES (Fig. 4E) and αSMA (Fig. S9); however, there were no changes in the number of Ki-67-positive HSCs and MFs after deletion of Foxf1 (Fig. 4F).

Protein levels of proliferation-specific genes FOXM1 and CCND1 were unaltered in αSMACreER;Foxf1−/− livers compared to controls (Fig. 4G). Thus, Foxf1 deletion does not affect proliferation of HSCs and MFs after chronic CCL4 liver injury.

RNA-seq analysis identified direct FOXF1 target genes critical for ECM deposition and hepatic fibrosis

In order to identify FOXF1 target genes, RNA-seq (GEO accession GSE123726) was performed on primary hepatic stromal cells (containing MFs and HSCs) isolated from CCL4-treated Foxf1fl/fl and αSMACreER;Foxf1−/− livers. Purified cells expressed Des and Acta2, but lacked hepatocyte (Nikoozad et al., 2014) and Kupffer cell (Yang et al., 2013) markers (Fig. S10). Foxf1 mRNA was lost in isolated aSMACreER,Foxf1−/− stromal cells (Fig. 5A), a finding consistent with efficient deletion of Foxf1 from CCL4-treated livers. The RNA-seq was used to compare differential gene expression patterns between the Foxf1fl/fl and aSMACreER,Foxf1−/− stromal cells. The differential gene expression in the two groups are represented in a heat map (Fig. 5B). Gene ontology demonstrated that increased functional pathways for the aSMACreER,Foxf1−/− mice were related to ECM regulation, while decreased functional pathways included normal liver functions and metabolism (Fig. 5C).

RNA-seq analysis was cross referenced with FOXF1 ChIP-seq analysis (GEO accession GSE100149). 905 genes were common between RNA-seq and ChIP-seq (Fig. 5D), which include 74 genes related to ECM deposition and fibrosis. ChIP-seq proximity analysis revealed that 20 of these ECM genes had FOXF1 binding sites within 2KB of the transcription start site (Fig. 5E). Interestingly, Col1α2, Col5α2 and Mmp2 were among the 20 ECM-related genes that had FOXF1 binding sites within the gene loci (Fig. S11, Table S1). COL1α2 and COL5α2 are common ECM components in fibrotic livers (Mak et al., 2016), whereas MMP2 is a collagenase that is increased during liver fibrosis and associated with disease progression (Benyon et al., 1996). Expression of Col1α2, Col5α2 and Mmp2 mRNAs were increased in CCL4-treated aSMACreER,Foxf1−/− livers as shown by RNA-seq and qRT-PCR (Fig. 5E, Fig. 6D), suggesting a negative regulation by FOXF1. The presence of gene silencing histone methylation marks H3K9me3 and H3K27me3 (Dong and Weng, 2013; Bernstein et al., 2006) in...
FOXF1-binding promoter regions (Fig. 6A–C) is consistent with negative regulation of these genes by FOXF1. In order to confirm the regulation of Col1α2, Col5α2 and Mmp2 by FOXF1, we overexpressed FOXF1 in isolated murine HSCs (Fig. 6E). Lentiviral-mediated overexpression of FOXF1 decreased Col1α2 and Mmp2 in vitro (Fig. 6F). Thus, FOXF1 negatively regulates expression of pro-fibrotic genes in MFs. Altogether, FOXF1 expression in myofibroblasts is essential to inhibit liver fibrosis after chronic liver injury (Fig. 6G).

**DISCUSSION**

Myofibroblast activation is a key mechanism in the development of hepatic fibrosis. However, transcriptional regulation of myofibroblasts during liver fibrogenesis remains poorly characterized. In the present study, we found that the deletion of Foxf1 in MFs during chronic CCl4-mediated injury exacerbated liver fibrosis, increased collagen deposition and stimulated expression of pro-fibrotic genes. ECM-related proteins were identified as novel FOXF1 transcriptional targets, suggesting that FOXF1 plays an important role in the regulation of ECM and collagen deposition during the progression of hepatic fibrosis.

Previous studies have focused on the role of FOXF1 in acute liver injury using a single CCl4 administration to Foxf1+/- mice. These studies demonstrated that FOXF1 is necessary for HSC activation to promote liver repair (Kalinichenko et al., 2003). CCl4-treated Foxf1+/- mice exhibited diminished collagen depositions and increased mortality after the liver injury (Kalinichenko et al., 2003). A recently published model of Foxf1-silencing using a lipid-based nanoparticle system to deliver Foxf1 siRNA to the liver demonstrated attenuated collagen deposition when Foxf1 siRNA was delivered 48 h prior to bile duct ligation (Abshagen et al., 2015). It is likely that Foxf1 siRNA inhibited FOXF1 signaling in hepatic stellate cells, decreasing their activation and subsequent collagen depositions into the liver tissue, a finding consistent with previous studies using Foxf1+/- mice (Kalinichenko et al., 2003). Recently, a model of chronic hepatic injury using CCl4-injections,
similar to the present study, was unsuccessful in silencing Foxf1 expression using the same lipid based system to deliver Foxf1 siRNA (Abshagen et al., 2015, 2017). This method involved four weeks of IP CCl4-injections before two weeks of treatment with Foxf1 siRNA (Abshagen et al., 2017). It is possible that the lack of Foxf1 silencing was due to inability of nanoparticles to target hepatic MFs. In the current study, we utilized a conditional genetic mouse model to delete Foxf1 in MFs during CCl4-induced hepatic fibrosis which shares multiple histological similarities with human disease (Masugi et al., 2018; Bataller and Brenner, 2005). Interestingly, the loss of Foxf1 in MFs resulted in increased collagen deposition, causing severe fibrotic lesions between hepatic portal triads in αSMACreER;Foxf1−/− livers. Our studies suggest that FOXF1 inhibits production of collagen and ECM during the progression of liver fibrosis. Increased fibrosis in Foxf1-deficient mice was associated with the appearance of liver tumors, a finding consistent with increased tumor formation in patients with liver cirrhosis (EASL-EORTC et al., 2018). Our studies suggest that maintaining Foxf1 expression can be beneficial in patients with advanced liver fibrosis to inhibit fibrotic responses and decrease the risk of liver tumorigenesis.

In the present study, collagens were significantly increased in αSMACreER;Foxf1−/− livers after chronic CCl4-treatment. Desmin and αSMA were both increased in αSMACreER;Foxf1−/− livers; however, there were no differences in the number of proliferating cells between Foxf1fl/fl and αSMACreER;Foxf1−/− livers. Previously, FOXF1 has been shown to stimulate cell proliferation in lung endothelial cells (Ren et al., 2014; Bolte et al., 2017) and in rhabdomyosarcoma tumor cells (Milewski et al., 2017). Surprisingly, we found that deletion of Foxf1 from MFs does not affect their proliferation during liver fibrogenesis. It is possible that FOXF1 requires additional co-activator or co-repressor proteins (that are not present in MFs) to regulate cellular proliferation. Additionally, we found an increase in Timp2 expression with a decrease in MMP9 activity in αSMACreER;Foxf1−/− livers. Since it is well-known that TIMPs and MMPs regulate ECM depositions to...
balance the scarring and healing processes during fibrosis (Duarte et al., 2015), it is possible that the loss of Foxf1 alters the TIMP/MMP balance to allow accumulation of collagens without the degradation mechanisms necessary for proper wound healing. Interestingly, MMP9 has been implicated in HSC to MF transdifferentiation (Han et al., 2007) in addition to its roles in ECM degradation (Duarte et al., 2015; Kurzepa et al., 2014). Therefore, transdifferentiation (Han et al., 2007) in addition to its roles in ECM degradation mechanisms necessary for proper wound healing.

Interestingly, MMP9 has been implicated in HSC to MF transdifferentiation (Han et al., 2007) in addition to its roles in ECM degradation (Duarte et al., 2015; Kurzepa et al., 2014). Therefore, decreased MMP9 activity can contribute to increased liver fibrosis in aSMACreER;Foxf1−/− mice. Surprisingly, FOXF1 was increased in activated HSCs compared to quiescent HSCs. It is possible that FOXF1 is differentially regulated in HSCs compared to hepatic MFs, and that after liver injury, FOXF1 protects HSCs from differentiating into MFs through transcriptional repression of profibrotic genes.

Consistent with increased fibrosis in Foxf1-deficient livers, RNA-seq analysis revealed increased ECM-related functional pathways in a purified stromal cell population. Comparison with FOXF1 ChIP-seq data revealed 20 novel transcriptional targets of FOXF1, which include Col1a2, Col5a2 and Mmp2, expression of which was increased in Foxf1-deficient cells.

Col1a2 is one of the most abundant ECM components in the liver along with COL1α1 and COL3α1 (Lai et al., 2011). COL5α2 is highly expressed with Collagens 1 and 3 and is important in regulating the assembly and structure of these collagens in the fibrotic matrix (Moriya et al., 2011). MMP2 acts as a collagenase, known to be activated during hepatic fibrosis (Benyon et al., 1996).

In addition to increased mRNA levels of the genes in FOXF1-deficient cells, we found multiple FOXF1 binding sites within their gene promoter region and introns, suggesting direct transcriptional repression. This hypothesis is supported by the presence of H3K4me3 and H3K9ac, histone modifications associated with transcriptional repression. This hypothesis is supported by the presence of H3K4me3 and H3K9ac, histone modifications associated with transcriptional repression (Dong and Weng, 2013; Rea et al., 2000), at FOXF1 binding sites. In summary, we have developed a novel genetic mouse model to study the role of FOXF1 in MFs during chronic liver injury. Using this model, we demonstrated that Foxf1 expression in MFs is necessary to inhibit hepatic fibrosis and maintain the balance of collagen depositions, through transcriptional repression of pro-fibrotic genes.

**MATERIALS AND METHODS**

**Mice**

The Foxf1−/− mouse line was previously generated and bred into the C57Bl/6 mouse background (Ren et al., 2014; Cai et al., 2016). Foxf1−/− mice were bred with aSMA-CreER mice (Jackson Laboratory, 029925; Wendling et al., 2009) to generate aSMACreER;Foxf1−/− mice (Black et al., 2018). aSMACreER;Foxf1−/− mice were bred with Foxf1−/− mice and male pups were genotyped and used for all experiments at the age of 6–8 weeks. The following primers were used for genotyping: aSMA-CreER sense: 5′-TGCAACGAGTGATGAGGTTCGC 3′ and anti-sense: 5′-GATCCGGGAATTTCGGCTATACG 3′; aSMA-WT sense 5′-GGTTTCTATTGCAGACATTC 3′ and anti-sense: 5′-GGTTTCTATTGCAGACATTC 3′; Foxf1−/− sense: 5′-TGCACCAAAACCTCGGCTACTAAGCAT 3′; Foxf1−/− sense: 5′-TGCACCAAAACCTCGGCTACTAAGCAT 3′.
and anti-sense: 5′-TTCAGATCTGAGAGTGGCAGCTTC-3′. Foxf1<sup>fl/fl</sup> littersmates were used as controls. To activate the conditional Foxf1 knockout, tamoxifen (Tam) was given via intraperitoneal injection (40 mg/kg of body weight; Sigma-Aldrich) three days in a row at the beginning of each week starting at week 2 over the course of the chronic liver injury period. Hepatic injury was induced by intraperitoneal injections of carbon tetrachloride (CCL<sub>4</sub>; 1 μl/g of body weight 20% v/v; Sigma-Aldrich; diluted in sunflower seed oil) three times a week every other day over the course of the chronic liver injury period. The levels of aminotransferases AST and ALT, proteins albumin and globulin, and direct and indirect bilirubin were determined by serological analysis of blood serum as previously described (Sun et al., 2017; Ren et al., 2010). All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Cincinnati Children’s Research Foundation and the NIH IACUC Guidebook. All experiments were covered under our animal protocol (IACUC2016-0038). The Cincinnati Children’s Research Foundation Institutional Animal Care and Use Committee is an AAALAC and NIH accredited institution (NIH Insurance #8310801).

**Histology and immunohistochemistry**

Paraffin-embedded liver sections were used for H&E staining, immunohistochemistry (IHC), or immunofluorescence (IF) as previously described (Ren et al., 2010; Kalinichenko et al., 2003; Wang et al., 2003). The following antibodies were used for immunostaining: FOXF1 (1:1000 IHC, 1:200 IF; R&D Systems), DES (1:500 IHC, 1:100 IF; Santa Cruz Technologies), αSMA (1:10,000 IHC, 1:5000 IF; Sigma-Aldrich), Ki-67 (1:1000 IHC, 1:200 IF; Thermo Fisher Scientific), Ki-67 (1:1000 IF; BD Biosciences), and PH3 (1:10,000 IHC; Santa Cruz Technologies). Antibody-antigen complexes were detected using biotinylated secondary antibodies followed by avidin-biotin-horseradish peroxidase complex and 3,3′diaminobenzidine substrate (Vector Labs) as previously described (Kalinichenko et al., 2003; Ren et al., 2010; Wang et al., 2012). Sections were counterstained with Nuclear Fast Red (Vector Labs). For immunofluorescence imaging, secondary antibodies conjugated with Fluorescent Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen/Molecular Probes) were counterstained with Nuclear Fast Red (Vector Labs). For staining, cells were selected for gene set enrichment analysis using ToppGene Suite. Hierarchical clustering was performed by Ward’s method using Euclidean distance metric. RNA-seq data are available at GEO accession GSE123726. RNA-seq data were compared to previously published ChIP-seq data (GEO accession GSE100149) using a two-way Venn diagram.

**Statistical analysis**

Statistical significance differences in measured variables between control and experimental groups were assessed with a Student’s t-test (two-tailed) or one-way analysis of variance (ANOVA) with Bonferroni post hoc test as appropriate. P<0.05 was considered to be significant, with P<0.05 indicated by *, P<0.01 indicated with **, P<0.001 indicated with ***, and P<0.0001 indicated with ****. Values for all measurements were expressed as mean±s.e. of mean.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Conceptualization: H.M.F., V.V.K.; Methodology: H.M.F.; Software: H.M.F.; Validation: H.M.F., N.D., V.V.K.; Formal analysis: H.M.F., C.B., N.D., A.S., Y.Z., C.R.G., T.V.K., V.V.K.; Investigation: H.M.F., C.B., A.S., Y.Z., C.R.G.; Resources: H.M.F.; Data curation: H.M.F.; Writing - original draft: H.M.F., V.V.K.; Writing - review & editing: H.M.F., C.B., N.D., A.S., Y.Z., C.R.G., T.V.K., V.V.K.; Visualization: H.M.F., V.V.K.; Supervision: H.M.F., V.V.K.; Project administration: H.M.F., V.V.K.; Funding acquisition: T.V.K., V.V.K.

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**Data availability**

The RNA sequencing microarray data presented in this article has been uploaded to Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information (NCBI) and the accession number is GSE123726.
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