SUPPLEMENTARY INFORMATION

FOXR2 Interacts with MYC to Promote Its Transcriptional Activities and Tumorigenesis

Xu Li[1,4], Wenqi Wang[1,4], Yuanxin Xi[2,3], Min Gao[1], MyKim Tran[1], Kathryn E. Aziz[1], Jun Qin[2], Wei Li[2,3], and Junjie Chen[1,*]

This PDF file contains Supplemental Experimental Procedures, Supplementary Figures S1 and S2, and Supplementary Table S1.

Supplementary figures and table

Figure S1 is related to Figure 3 and shows that FOXR2 binds to MYC target promoters and promotes MYC transcriptional activities.

Figures S2 is related to Figure 4 and shows that FOXR2 facilitates MYC's activities and it is overexpressed in several breast cancer cell lines.

Table S1 is related to Figure 1 and shows the high-confidence candidate interacting protein (HCIP) lists of transcription factors used in this study, and additional information estimated by the HCIP lists.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Constructs and small hairpin RNAs (shRNAs)

cDNAs encoding all the genes in this study, including FOXR2, MYC, and MAX, were obtained from the hORFV5.1 library and Open Biosystems. cDNAs were subcloned into the pDONR201 vector (Invitrogen) as entry clones and subsequently transferred to gateway-compatible destination vectors for the expression of C-terminal SFB-, MYC-, GFP-, or HA-FLAG (for retrovirus packaging)–tagged fusion proteins. Point or deletion mutants were generated using sequential PCR methods and verified by sequencing. GIPZ lentiviral non-silencing control shRNA and FOXR2 shRNA target sets were obtained from the shRNA and ORFeome core facility at The University of Texas MD Anderson Cancer Center (Houston, Texas). The shRNA sequences were as follows:

FOXR2 shRNA-1$^{\#}$ (V2LHS_27943): 5′-TTACTGGAGAGATAGGTGG-3’;

FOXR2 shRNA-2$^{\#}$ (V3LHS_334199): 5′-AAGACACGAGTCTCCTCCC-3′;

FOXR2 shRNA-3$^{\#}$ (V3LHS_404756): 5′-CTAACTTTACTTAACACCA-3′;

control shRNA: 5′-TCTCGCTTGGGCGAGAGTAAG-3′.

Cell culture, transfection, retrovirus, and lentivirus packaging

HEK293T, MDA-MB-231, MDA-MB-435, MDA-MB-468, HS578T, HBL100, MCF-7 and Huh7 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum. A549 cells were cultured in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum. ZR75.1, HCC1937, BT549, H358, H460, H1650, H2279, PC9, SNU-182, SNU-398 and SNU-449

cells were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum. MCF10A, HMLE and SUM52PE cells were cultured in Dulbecco's modified Eagle's medium/F12 supplemented with 5% horse serum, 10 μg/ml insulin, 20 μg/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 0.1 μg/ml choleratoxin. WI-38, Hep3B and HepG2 cells were cultured in Eagle's Minimum Essential Medium, supplemented with 10% fetal bovine serum. NHBE, THLE-2 and THLE-3 cells were cultured in Lonza's BEGM medium. All the culture mediums were supplemented with 1% penicillin and streptomycin.

Constructs encoding C-terminal SFB-tagged proteins were transfected into HEK293T cells using polyethylenimines. Constructs encoding HA-FLAG–tagged proteins were packaged into retroviruses by co-transfecting them with the packaging plasmid PCL into HEK293T cells. Forty-eight hours after transfection, the supernatant was collected and used to infect MCF10A cells. shRNAs and constructs encoding SFB-tagged FOXR2, MYC, and MAX were packaged into lentiviruses by co-transfecting them with packaging plasmids pMD2G and pSPAX2 into HEK293T cells. Forty-eight hours after transfection, the supernatant was collected and used to infect MCF10A or MDA-MB-468 cells. Infections were repeated twice with an interval of 24 hr to achieve maximal infection efficiency. Cell lines that stably expressed SFB-tagged proteins or were infected with shRNAs were selected with medium containing 2 μg/ml puromycin. The expression of exogenous proteins was confirmed by immunoblotting and immunostaining.

Cancer correlation and gene alteration analysis

The cancer correlation were generated using the non-self HCIPs identified in our studies, weighted by the spectra counts, and searched in the Knowledge Base provided by Ingenuity pathway software (Ingenuity Systems, www.ingenuity.com), which contains findings and annotations from multiple sources including the Gene Ontology database, to estimate the significance of these correlations. Transcription factors were searched for their alteration case numbers and rates in multiple TCGA databases (Cerami et al., 2012; Gao et al., 2013) using their HCIP sets. Mutation: the mutation rates. Amplification or Deletion: the non-mutational alteration rates. Protein sequences were downloaded from the UniProt Consortium (www.uniprot.org). The cancer correlations were generated using the HCIPs identified in our studies and weighted by the spectra counts. We next searched in the Knowledge Base provided by Ingenuity pathway software (Ingenuity Systems), which contains findings and annotations from multiple sources, including the Gene Ontology database, to estimate the significance of these correlations.

SUPPLEMENTARY FIGURES

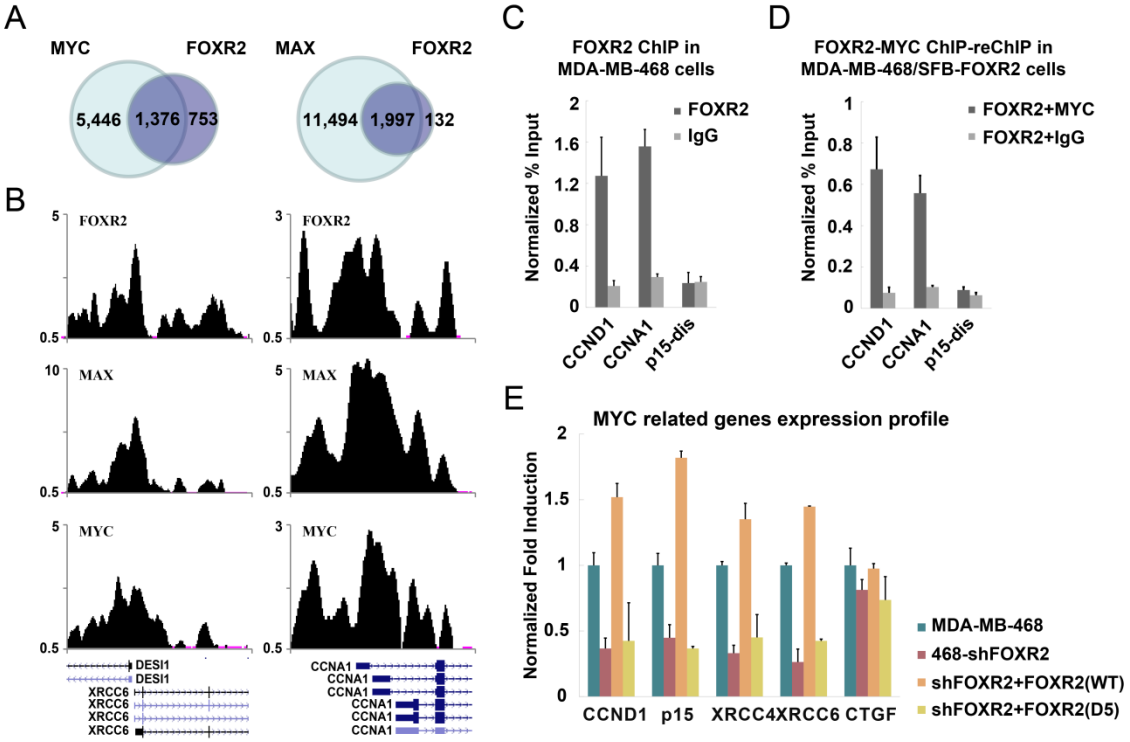

Figure S1

Figure S1. FOXR2 binds to MYC target promoters and promotes MYC transcriptional activities.

(**A**) Chromatin IP-sequencing (ChIP-seq) assay was performed in MDA-MB-468 cells using endogenous antibodies against FOXR2, MYC or MAX. Overlap between FOXR2 and MAX or MYC target genes were evaluated. (**B**) Two examples of overlapping FOXR2, MAX and MYC peaks on XRCC6 and CCNA1 promotors were shown. (**C**) A chromatin IP (ChIP) assay was performed in MDA-MB-468 cells using FOXR2 antibody or control IgG. The recovery of MYC downstream gene promoter regions was examined by real-time PCR. All the promoters except the control p15-distal promoter have significantly enriched in the FOXR2 ($P < 0.001$) but not IgG immunoprecipitants. (**D**) A

ChIP-reChIP assay was performed in MDA-MB-468 cells overexpressing SFB-tagged FOXR2 using streptavidin-beads, eluted with biotin and re-IPed with MYC-antibody or control IgG. The recovery of MYC downstream gene promoter regions was examined by real-time PCR. All the promoters except the control p15-distal promoter have significantly enriched in the MYC ($P < 0.001$) but not IgG immunoprecipitants. (**E**) MYC target gene expression profiles were evaluated by RT PCR in MDA-MB-468, MDA-MB-468-shFOXR2, MDA-MB-468-shFOXR2+SFB-FOXR2 and MDA-MB-468-shFOXR2+SFB-FOXR2(D5) cells. mRNA levels were determined by real-time RT-PCR and normalized with GAPDH. CCND1, p15, XRCC4 and XRCC6 expression levels have been significantly changed in MDA-MB-468-shFOXR2 and MDA-MB-468-shFOXR2+SFB-FOXR2(D5) cells ($P < 0.01$), comparing with MDA-MB-468 cells.

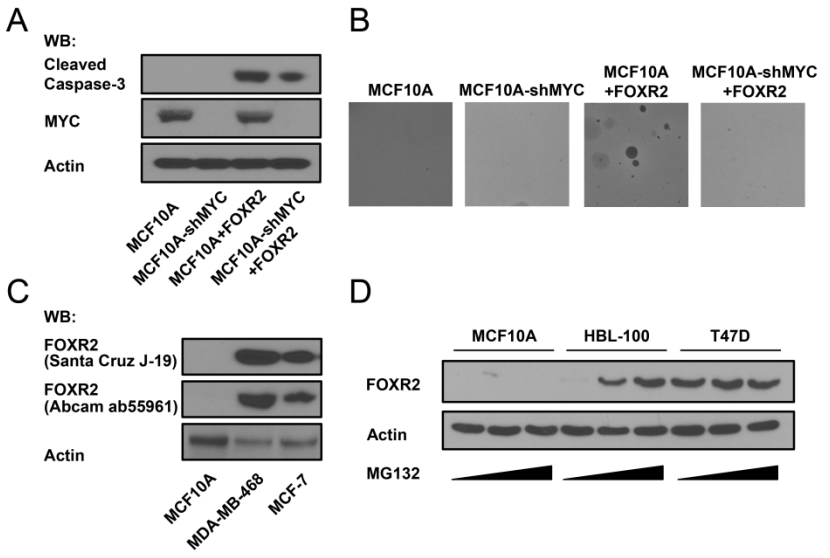

Figure S2

Figure S2. FOXR2 facilitates MYC's activities and it is overexpressed in several breast cancer cell lines.

(**A**) MCF10A cells were infected in six-well plates with lentivirus encoding MYC shRNA to generate MCF10A-shMYC cells. MCF10A and MCF10A-shMYC cells were infected in six-well plates with retroviruses encoding FOXR2 to generate MCF10A -FOXR2 and MCF10A-shMYC-FOXR2 cells. Lysates prepared from these cells were immunoblotted with the antibodies as indicated. (**B**) Soft agar colony formation of MCF10A cells stably expressing the indicated proteins was assessed and presented. (**C**) Immunoblotting in MDA-MB-468 and MCF-7 cell lines using two different FOXR2 antibodies, with MCF10A cells as the negative control. (**D**) MCF10A, HBL-100 and T47D cells were treated with vehicle, 5 μM or 50 μM MG132. Cell lysates were immunoblotted with the indicated antibodies.

SUPPLEMENTARY TABLE LEGENDS

Table S1. Cancer correlation and alteration of transcription factor HCIP datasets. The high-confidence candidate interacting protein lists of transcription factors used in this study, and the involvement in cancer related processes estimated by the HCIPs; total HCIP amplification/deletion cases and relative HCIP amplification/deletion rates; total HCIP mutation cases and relative HCIP amplification/deletion rates.

References

Cerami, E., Gao, J., Dogrusoz, U., Gross, B.E., Sumer, S.O., Aksoy, B.A., Jacobsen, A., Byrne, C.J., Heuer, M.L., Larsson, E.*, et al.* (2012). The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov *2*, 401-404.

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