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NOXIN as a cofactor of DNA polymerase-primase complex could promote hepatocellular carcinoma

Zhuang-Zhuang Zhang[1,2], Jian Huang[2], Yu-Ping Wang[1,2], Bing Cai[3] and Ze-Guang Han[1,2,4]

[1] Key Laboratory of Systems Biomedicine (Ministry of Education) and Collaborative Innovation Center of Systems Biomedicine of Rui-Jin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China
[2] Shanghai-MOST Key Laboratory for Disease and Health Genomics, Chinese National Human Genome Center at Shanghai, Shanghai, China
[3] Department of Hepatobiliary Surgery, Wuxi People's Hospital of Nanjing Medical University, Jiangsu, China
[4] Shanghai Center for Systems Biomedicine, Shanghai Jiao Tong University, Shanghai, China

Oncogene activation or inactivation of tumor suppressor genes are crucial to tumor initiation and progression. DNA copy number amplification is one of many mechanisms that activate oncogenes in many tumors, including hepatocellular carcinoma (HCC). Although it has been known that some oncogenes such as *c-myc* amplification is involved in HCC pathogenesis, more oncogenes with DNA copy amplification contribute to HCC initiation and progression remain to be characterized. Here, we identified NOXIN as a novel potential oncogene with DNA copy number amplification by Single Nucleotide Polymorphism microarray-based genome-wide DNA copy number analysis of 43 human HCC samples. We identified the focal DNA gain and amplification region containing *NOXIN* on chromosome 11q14.1 and NOXIN overexpression significantly associated with HCC progression. We then assessed the role of NOXIN in HCC cells. NOXIN overexpression promoted cellular proliferation, colony formation, cellular migration and *in vivo* tumorigenicity, whereas NOXIN knockdown attenuated these effects. Interestingly, NOXIN overexpression accelerated the G1-S phase transition by enhancing DNA synthesis. Furthermore, we found that NOXIN interacts with DNA polymerase α, suggesting that NOXIN may promote *de novo* DNA synthesis by promoting DNA polymerase-primase complex formation. These collective data indicated that NOXIN overexpression, as a result of genomic DNA gain or amplification, promotes HCC tumorigenesis by accelerating DNA synthesis and cell cycle progression, where NOXIN functions as a cofactor of DNA polymerase-primase complex by associating with DNA polymerase α.

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide. New evidence-based therapies for HCC are urgently needed. Like other malignancies, HCC is a genetic disease that progresses in a stepwise fashion from early dysplastic lesions to malignant and metastatic tumors with increased genomic instability. Genomic

Key words: cell cycle, DNA copy number, DNA polymerase α, NOXIN

Abbreviations: AFP: α-fetoprotein; CCK: cell-counting kit; CGH: comparative genomic hybridization;; CNVs: copy number variants; DNA pol α: DNA polymerase α; FACs: fluorescence-activated cell sorting; HCC: hepatocellular carcinoma; HMM: hidden Markov model; LCM: laser capture microdissection; LOH: loss of heterozygosity; NOXIN: nitric oxide-inducible gene protein; P-Rb: phosphorylated retinoblastoma gene protein; PRCP: prolylcarboxypeptidase; PRIM2A: DNA primase 58 kDa subunit; PVTT: portal vein tumor thrombus; Rb: retinoblastoma gene protein; RPA: replication protein A; SNP: single nucleotide polymorphism; TMAs: tissue microarrays

Additional Supporting Information may be found in the online version of this article.

Conflict of Interest: The authors have no conflicts to disclose.

Z.-Z.Z., J.H. and Y.W. contributed equally to this work

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Correspondence to: Ze-Guang Han MD, Key Laboratory of Systems Biomedicine (Ministry of Education) of Rui-Jin Hospital, Shanghai Jiao Tong University School of Medicine; Shanghai-MOST Key Laboratory for Disease and Health Genomics, Chinese National Human Genome Center at Shanghai, 351 Guo Shou-Jing Road, Shanghai 201203, China, Tel.: [862150801325], Fax: +[86-21-5080-0402], E-mail: hanzg@sjtu.edu.cn.

What's new?

DNA copy number amplification mechanism which contributes to the activation of oncogenesis one of the most common genetic aberrations found in human hepatocellular carcinoma (HCC). Here, the authors found that NOXIN overexpression, as a result of genomic DNA gain or amplification, promotes HCC tumorigenesis by accelerating DNA synthesis and cell cycle progression. The data also indicate that NOXIN functions as a cofactor of the DNA polymerase-primase complex by associating with DNA polymerase α. This study for the first time suggests NOXIN as a potential oncogene for HCC and provides new insight in how oncogenes may contribute to HCC.

instability includes chromosomal instability, such as amplification, duplication, deletion, rearrangements, translocation, number (aneuploidy and polyploidy) and microsatellite instability.[1,2] Oncogenes are known to be activated by DNA copy number amplification, point mutations and chromosomal translocations, whereas tumor suppressor genes can be inactivated by allelic deletion.[3,4] DNA copy number amplification was one of the most common genetic aberrant in humane HCC and therefore, based on DNA copy number aberration analyses, to identify potential cancer-related genes may facilitate understanding HCC pathogenesis and lay a solid foundation for development of new treatments for HCC.[5,6]

Previous studies using traditional methodologies, such as the comparative genomic hybridization (CGH) assay, have revealed frequent DNA copy number gains at 1q, 8q, 7q, 17q and 20q and losses at 4q, 8p, 13q, 16q and 17p, in human HCC samples.[2,7,8] Some candidate oncogenes or tumor suppressor, such as CHD1L (1q21)[9] and DLC1 (8p22),[10] were identified using these traditional methodologies. However, low resolution of these methodologies makes it difficult to define fine tumor genomic aberrations. High-resolution genome-wide DNA copy number analyses with array-based CGH have recently been applied in tumors to obtain a comprehensive view of whole genome copy number changes and identify candidate cancer genes.[11,12] Based on high-resolution array-based CGH, candidate cancer-promoting genes, such as TAGLN2 on 1q21-q25, MDM4 on 1q32.1, SNRPE on 1q32, SPP1 on 4q22, VEGFA on 6p21, PEG10 on 7q21, Jab1 on 8q13, HEY1 on 8q21, BOP1 on 8q24 and EEF1A2 on 20q13.33, were ascribed based on their amplification or gains of genomic DNA.[2,8,13–17] Many candidate tumor suppressor genes with allelic deletions, including TRIM35 on 8p21.2, DLC1 on 8p22, CRYL1 on 13q12.11 and Spry2 on 13q31.1,[2,6,18,19] have also been proposed in human HCCs.

Previous studies mainly focused on chromosomal regions with frequent DNA copy number gains or losses in larger populations of HCC patients. However, in smaller cohorts of HCC patients, DNA copy number aberrations exist in a limited genomic region, where certain cancer genes may be crucial for HCC initiation and progression due to their genomic instability. In this study, we performed DNA copy number analyses of 43 paired HCC samples and identified *NOXIN* on chromosome 11q14.1 as a candidate cancer-promoting gene with increased DNA copy numbers in approximately one-third of HCCs.

NOXIN, also called C11orf82, identified as a nitric oxide-inducible gene, is strongly induced by a wide range of stress signals: γ- and UV irradiation, hydrogen peroxide, adriamycin and cytokines. *NOXIN* gene does not have sequelogs in the genome and encodes a highly serine-rich protein.[20,21] In this work, our data revealed that NOXIN overexpression, as a result of genomic DNA amplification, promotes HCC tumorigenesis by accelerating DNA synthesis and cell cycle progression by interacting with DNA polymerase α.

Material and Methods

Tissue specimens

All HCCs were obtained from HCC patients *via* surgery and with informed consent. Specimens were frozen at −80 °C for DNA/RNA extraction. The project and protocol were approved by the ethics committee of the Chinese National Human Genome Center at Shanghai.

Single nucleotide polymorphism (SNP) array analyses

SNPs were genotyped using NSP and STY, in parallel (GeneChip® Mapping 500 K ARRAY, Affymetrix, Santa Clara, CA). Array experiments were conducted according to recommended instructions. More detail was described in Supporting Information Material and Methods.

Data analyses

More detail was described in Supporting Information Material and Methods.

Immunohistochemical staining

TMAs containing 90 paired HCC primary tumors and matched nontumor tissues was used for immunohistochemical staining.[22] The TMAs were stained using a mouse anti-NOXIN antibody (1:50; Sigma).

Construction of recombinant plasmids and adenoviral vectors

More detail was described in Supporting Information Material and Methods.

Tumor xenograft experiments

Experiments were performed as described previously.[23] Cells (2×10^6 per mouse) expressing NOXIN were injected into two upper flanks of nude mice. We monitored the tumor

sizes weekly and quantified tumor size. Tumor weights were measured at the time of sacrifice.

Coimmunoprecipitation (co-IP) assays

Reciprocal co-IP experiments and Immunoblotting assays were performed using anti-NOXIN antibodies (Sigma-Aldrich, St. Louis, MO), DNA pol α (Santa Cruz Biotechnology, Santa Cruz, CA) and the DNA primase 58 kDa subunit (PRIM2A; Santa Cruz Biotechnology, Santa Cruz, CA) to detect the endogenous interactions in Focus cells. An anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO) was used to detect FLAG-tagged NOXIN endogenous interactions with DNA pol α in HEK293T cells. More detail is provided in the Supporting Information Material and Methods section.

Statistical analyses

The *Chi*-square test was used to evaluate the associations between NOXIN expression and clinicopathologic parameters. Student's *t*-test was used to compare significant differences between the different groups. The Mann-Whitney U test was used for tumor volume comparisons between groups. p values <0.05 were considered to be significant.

More detail is provided in Supporting Information Material and Methods. All cell lines involved in present study were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences within 4 years and Hep3B, PLC/PRF/5, Hep-SK-1, Huh-7, HEK293, 7721, Focus and YY8103 cell lines were reauthenticated with DNA (STR) profiling.

Results

DNA copy number analyses in human HCCs

A SNP array-based CGH assay was performed to explore CNVs in 43 paired HCCs, including tumors and matched nontumor tissues (Fig. 1*a*). The raw data (accession number: GSE49317) revealed that the mean call rates in HCC specimens and matched non-tumor tissues were 97.62 ± 1.3 and 22.22 ± 2.84, respectively. The heterozygous call rates were 95.63 ± 2.82 and $19.33 \pm 4.1\%$, respectively. Based on the criteria, copy numbers ≥ 2.7 for gains or amplification and ≤ 1.3 for losses or deletion, we found nearly all chromosomes were affected by DNA copy number alterations. The higher copy number frequency gains (at least 30% of HCCs) occurred on chromosomes 1q, 5p, 6p, 7q, 8q, 10p, 17q 19q, 20q and 22q, while loss of heterozygosity (LOH) indicating monoallelic deletion often involved chromosomes 1p, 4q, 8p, 9p, 10q, 13p, 13q, 14q, 16p, 16q and 17p (Fig. 1*b*). In present study, the recurrent gains at 1q, 7q, 8q, 17q, 20q and losses at 4q, 8p, 16q, 17p, were consistent with previous studies.[24,25]

The NOXIN locus is amplified in human HCCs

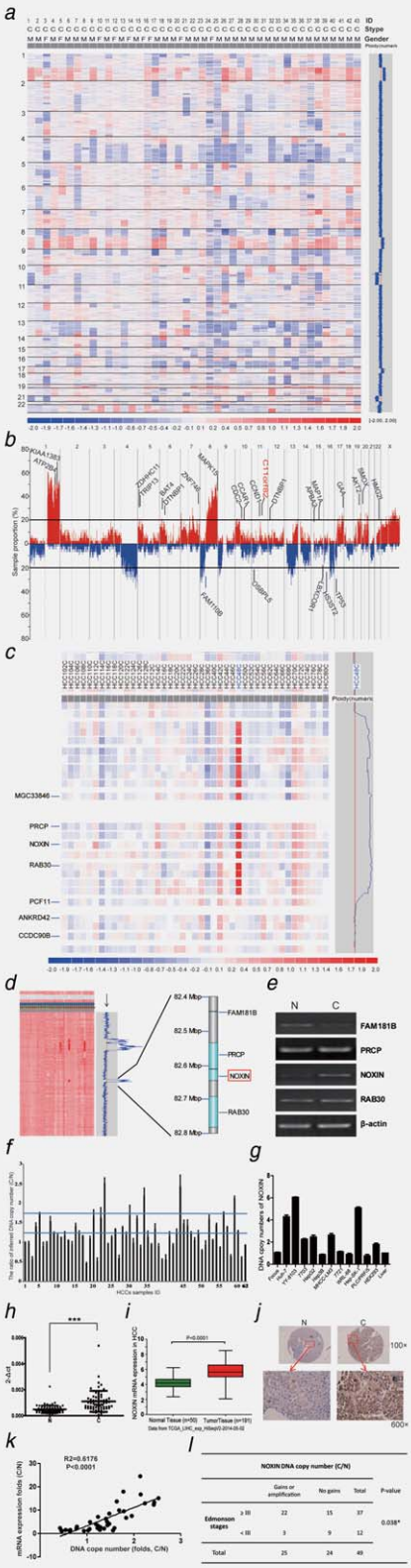
Some oncogenes harbor on chromosomal regions with DNA copy number amplifications in tumors. In this study, we analyzed significant DNA copy number amplifications in each HCC sample. As illustrated in Figure 1*b*, many DNA copy number amplification regions with high incidence occurred in these HCC samples, which could affect some known oncogenes (Supporting Information Table S1). However, most of these regions were too large to define the novel candidate oncogenes with DNA copy number amplification. Thus, in this study, we focused on the focal genomic DNA amplification regions with relative high incidence ($\geq$10%) containing novel few genes that have not related to HCC.

We observed the genome-wide copy number of each patient, as illustrated in Supporting Information Figures 1*a* and 1*b*. Interestingly, we found the focal genomic region on 11q14.1 with DNA copy gains (copy numbers ≥ 2.7) in 6/43 (13.95%) and amplification (copy numbers ≥ 4) in one case, as shown by the zoom-in Figure 1*c*. The boundaries of the aberrant event in the sample with DNA amplification is between 81.789 and 82.492 MB distance from the end of short arm. The amplification region only harbored four genes, including *FAM181B*, *prolylcarboxypeptidase (PRCP)*, *NOXIN* (*C11orf82*) and *RAB30* (Fig. 1*d*). Subsequently, we performed reverse transcript PCR to determine the expression levels of these genes in the paired samples from this patient. The results indicated that, only *NOXIN* was obviously upregulated which is consistent with DNA copy gains, whereas the remaining three genes were either unchanged or decreased (Fig. 1*e*), implying that *NOXIN* upregulation could be ascribed to genomic amplification of the locus.

In this work, we also try to look for potential causes for accounting for the flanking genes not elevated, in spite of their DNA copy number amplification in HCC samples. We suspected that epigenetic mechanism such as DNA methylation was involved in the regulation of these flanking genes. Here, we analyzed the promoters of four genes localized on the focal amplification regions to define whether there are CpG islands in those regions (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) and found that less CpG islands in NOXIN promoter, as compared with that in RAB30, PRCP and FAM181B promoters (Supporting Information Fig. S1C). This implied that the epigenetic mechanism such as DNA methylation level in these promoter regions may suppress the expression of the flanking genes *RAB30*, *PRCP* and *FAM181B*, not *NOXIN*, in HCC, although their DNA copy numbers were increased in the same HCC samples.

Real time-PCR was performed to further evaluate DNA copy numbers of the *NOXIN* locus in an additional 62 paired HCC specimens, as previously described.[26] The results indicated that there were DNA copy number gains of the NOXIN locus ($\geq$ 1.5 fold) in 12/62 (19.35%) HCCs, of which four cases (6.45%) showed amplification ($\geq$ 2 fold; Fig. 1*f*).

To strengthen our finding, we also analyzed *NOXIN* DNA copy number variation in HCC samples deposited in public COSMIC database (http://cancer.sanger.ac.uk/cosmic/gene/analysis?ln=C11orf82). Among 71 HCC samples with DNA copy number analysis, DNA copy number gains of the *NOXIN* locus ($\geq$ 3 fold) occurred in 55/71 (77.46%; data not



shown). These data suggested that *NOXIN* locus DNA copy number gains or amplification had occurred in some HCCs.

Moreover, we further evaluated the DNA copy numbers of *NOXIN* locus in 12 HCC cell lines, as previously described.[26] The results indicated that the DNA copy numbers of NOXIN locus ($\geq$ 2.5 fold) amplified in Huh-7, YY-8103 and Hep-SK-1 cell lines, but not in Focus, Hep3B, WRL-68 and PLC/PRF/5 cells, as compared with normal human liver (Fig. 1*g*). These collective data implied that DNA copy number of *NOXIN* amplified.

NOXIN expression levels are correlated with HCC clinical progression

Real time-PCR was performed to evaluate *NOXIN* expression levels in other independent cohort of 64 paired HCC samples. The data revealed that the mRNA levels of *NOXIN* were significantly upregulated in 36 (56.25%) HCC specimens (Fig. 1*h* and Supporting Information Fig. S1D). To strengthen our finding, we also analyzed the *NOXIN* mRNA level in HCC samples based on public TCGA dataset. As shown in Figure 1*i*, mRNA levels of *NOXIN* were significantly upregulated in HCC specimens as compared with nontumor livers ($p < 0.0001$).

Subsequently, immunohistochemical staining of the tissue array containing another cohort of 90 paired HCC samples,

Figure 1. DNA copy number amplifications and NOXIN upregulation in human HCCs. (*a*) DNA copy number analyses of 43 HCC specimens using SNP 5.0 arrays. Each chromosome is displayed in genomic order. (*b*) Significant chromosomal regions with DNA copy number gains and losses. Some of the genes were labeled. (*c*) A zoom-in picture of the amplification region on chromosome 11q14.1 for each sample. Some genes localized on the region were labeled. (*d*) A small amplification region on chromosome 11q14.1 containing four genes, FAM181B, PRCP, NOXIN and RAB30, exhibited DNA copy number amplification. DNA copy number alterations of chromosome 11 in all 43 samples (left), DNA copy number alterations of chromosome 11 in one patient (middle) and the amplified genomic region containing four genes (right) are shown, respectively. (*e*) The mRNA expression levels of these four genes were evaluated using RT-PCR in the paired HCC specimens. (*f*) DNA copy numbers of NOXIN in 62 paired HCC specimens and their matched nontumoros livers detected by Real-time PCR. The results revealed the ratio (y axis) of the inferred DNA copy numbers of NOXIN from HCCs to nontumorous liver. (*g*) DNA copy numbers of NOXIN in 12 cell lines detected by Real-time PCR. The results revealed the ratio (y axis) of the inferred DNA copy numbers of NOXIN from HCC cell lines to normal human liver. (*h*) The expression levels of NOXIN in 64 paired HCC and nontumor tissues were determined using Real time-PCR. ***, $p < 0.001$. C, HCC specimen; N, nontumorous liver. (*i*) The expression level of NOXIN in normal liver tissues ($n = 50$) and HCC tumors ($n = 191$), based on TCGA dataset. $p < 0.0001$. (*j*) Representative immunohistochemical staining of NOXIN in one patient from an independent set of 90 paired HCC specimens. (*k*) Correlation between NOXIN DNA copy numbers and mRNA expression levels evaluated with regression analysis. C, HCC; N, matched nontumor tissues. (*l*) The correlation of *NOXIN* DNA copy number and Edmonson histology grade was analyzed by *Chi*-square test. $p = 0.038$. C, HCC specimen; N, nontumorous liver.

Table 1. Correlation of NOXIN expression with clinicopathological features of two cohorts of HCC patients

Clinicopathological parameters	Numbers of patients	Upregulation		χ^2	*p* Value
		Yes	No		
A cohort of 72 HCC samples examined by immunohistochemistry staining					
Gender					
Male	61	35	26		
Female	11	3	8	3.3891	0.0656
Age					
≤50	22	10	12		
>50	50	25	25	0.12641	0.7222
PVTT					
Absence	42	14	28		
Presence	30	19	11	6.3441	0.0118
Edmonson stages					
< II	20	7	13		
≥ II	52	32	20	4.0981	0.0429*
Another cohort of 49 HCC samples examined by quantitative RT-PCR					
Gender					
Male	39	29	10		
Female	10	6	4	0.80411	0.3699
Age					
≤50	17	13	4		
>50	32	20	12	0.9853	0.3209
Size					
<3 cm	13	10	3		
≥3 cm	36	25	11	0.2618	0.6089
Edmonson stages					
< III	12	5	7		
≥ III	37	31	6	7.2421	0.0071*

* Significant statistic value $P<0.05$.

revealed that NOXIN was significantly elevated in 43 (47.78%) HCC specimens (Fig. 1*j* and Supporting Information Figs. S1F and 1G). We also analyzed the correlation between *NOXIN* DNA copy numbers and its transcript levels in 40 overlapping HCC samples by integrating two cohorts of 62 and 64 paired HCC samples. The data indicated that higher *NOXIN* mRNA levels were significantly associated with DNA copy number gains or amplification ($R^2 = 0.6167$, $p < 0.0001$; Fig. 1*k*). The similar results were also observed in other cell lines (Supporting Information Table S2 and Fig. S1E).

To explore the clinical significance of NOXIN in HCCs, we statistically analyzed the correlation between its expression levels and clinicopathologic features in the two cohorts of HCC patients based on the integrity of the clinical data. Seventy-two of 90 paired HCC samples contained in TMAs and 49 of the above 64 paired HCC samples were further statistically analyzed. Interestingly, NOXIN expression level was significantly correlated with Edmonson histology grades or portal vein tumor thrombus (PVTT), but not tumor size, gender and age (Table 1). Subsequently, we also further analyzed the correlation of *NOXIN* DNA copy number and Edmonson histology grade by Chi-square test and the data demonstrated that *NOXIN* DNA copy gains or amplification was significantly and positively associated with Edmonson histology grade (Fig. 1*l*).

These collective data implied that increased NOXIN expression may be involved in HCC progression.

NOXIN overexpression promotes cellular proliferation, colony formation, cellular migration and *in vivo* tumorigenicity

To explore the role of NOXIN in HCC behavior, we transiently transfected the recombinant plasmid pcDNA3.1b-

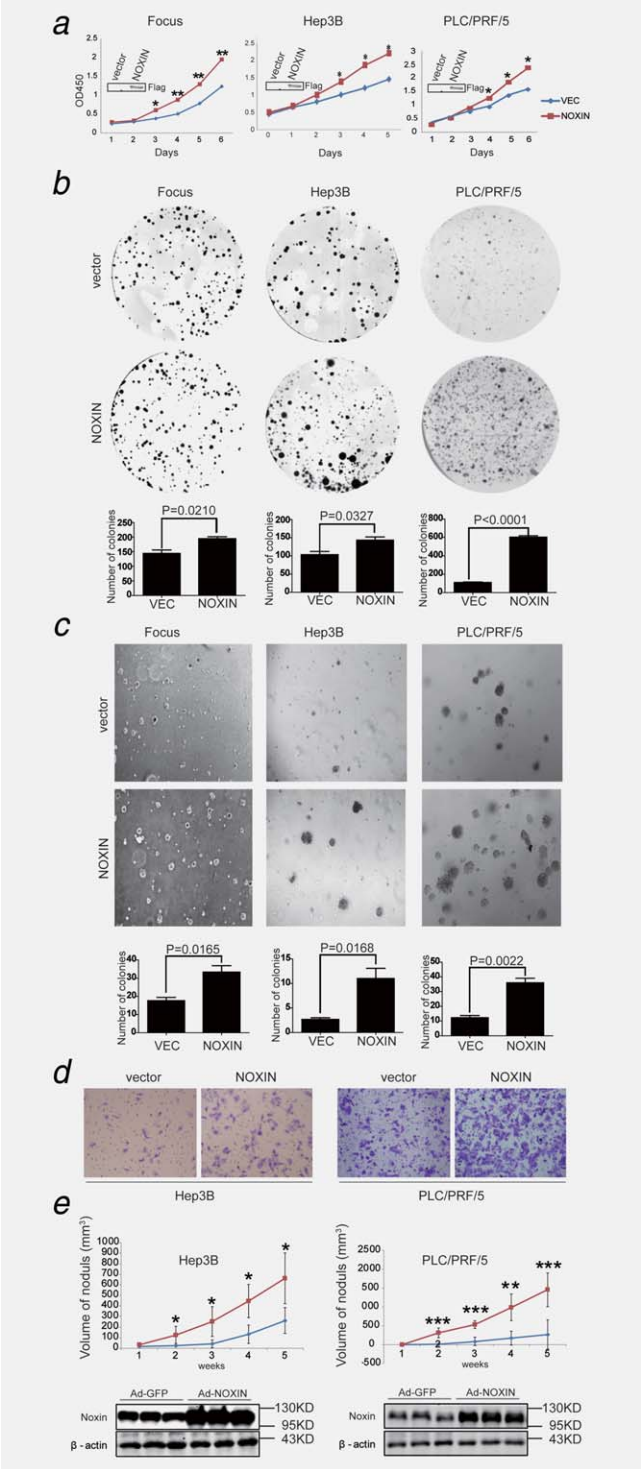


Figure 2. NOXIN overexpression promotes cellular proliferation, colony formation, cell migration and *in vivo* tumorigenicity. Ectopic NOXIN promotes cell proliferation (*a*), colony formation (*b*) and anchorage-independent colony formation (*c*) in Focus, Hep3B and PLC/PRF/5 cells. (*d*) Ectopic NOXIN promoted cell migration in Hep3B and PLC/PRF/5 cells. (*e*) Ectopic NOXIN promoted the *in vivo* tumorigenicity of Hep3B and PLC/PRF/5 cells in a tumor xenograft model in nude mice ($n = 8$ and 7, respectively). Ectopic NOXIN expression in these xenograft tumors was evaluated using an immunoblotting assay (low). *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

NOXIN into Focus, Hep3B and PLC/PRF/5 cells with low NOXIN level but no DNA copy gains (Supporting Information Fig. S1G). The results indicated that enforced NOXIN expression significantly promoted cell growth and colony formation (Figs. 2*a* and 2*b* and Supporting Information Figs. S2A and 2B). Additionally, NOXIN overexpression significantly enhanced the anchorage-independent growth of Focus, Hep3B and PLC/PRF/5 cells. We observed similar effects of NOXIN in HCC-LM3 and WRL-68 cells (Fig. 2*c* and Supporting Information Fig. S2C).

Moreover, the enforced NOXIN overexpression also significantly promoted Hep3B and PLC/PRF/5 cell migration compared to the empty vector, as indicated by Transwell assays (Fig. 2*d*), implying that NOXIN may contribute to tumor metastasis, which may contribute to PVTT in HCC specimens.

To investigate the effects of NOXIN overexpression on HCC tumorigenicity, Hep3B (2×10^6 cells) and PLC/PRF/5 (2×10^6 cells) cells transfected with recombinant adenovirus harboring *NOXIN* (Ad-*NOXIN*) and GFP (Ad-GFP) were injected into the opposite flanks of the same athymic nude mice. After 5 weeks observation, the results revealed that cells expressing ectopic NOXIN formed larger tumors. Ectopic NOXIN expression were verified by Western blotting (Fig. 2*e* and Supporting Information Figs. S2D–2F). These data indicated that NOXIN overexpression could significantly promote HCC cell proliferation and *in vivo* tumorigenicity.

NOXIN knockdown inhibits cell growth, cell migration and increases apoptosis

To further investigate the effects of NOXIN on HCC cells, we used chemically synthesized siRNAs to knock down endogenous NOXIN expression in HCC cell lines with not only relatively higher NOXIN levels but also DNA copy gains and selected the cells with stable knockdown of NOXIN. These siRNAs or shRNAs efficiently knocked down endogenous NOXIN (Fig. 3*a*) and significantly inhibited the cell growth and colony formation of YY-8103 and HuH-7 cells (Figs. 3*b* and 3*c*). NOXIN knockdown also significantly suppressed the anchorage-independent colony formation of HuH-7 and YY-8103 cells (Fig. 3*d*). However, as indicated by Typan blue dye assay, NOXIN knockdown increased cell death of YY-8103 and HuH-7 cells, especially after doxorubicin treatment and ultraviolet irradiation (Fig. 3*e*). Furthermore, TUNEL assay showed that NOXIN knockdown increased cell apoptosis (Fig. 3*f*). Moreover, Transwell assays showed that NOXIN knockdown suppressed the cellular migration in YY-8103 and Huh-7 cells (Fig. 3*g*). These data indicated that NOXIN was essential for HCC cell growth, survival and migration.

NOXIN promotes the G1-S transition during cell cycle progression by enhancing DNA synthesis

To find out what effects of higher NOXIN expression on cell proliferation, we performed Gene Set Enrichment Analysis

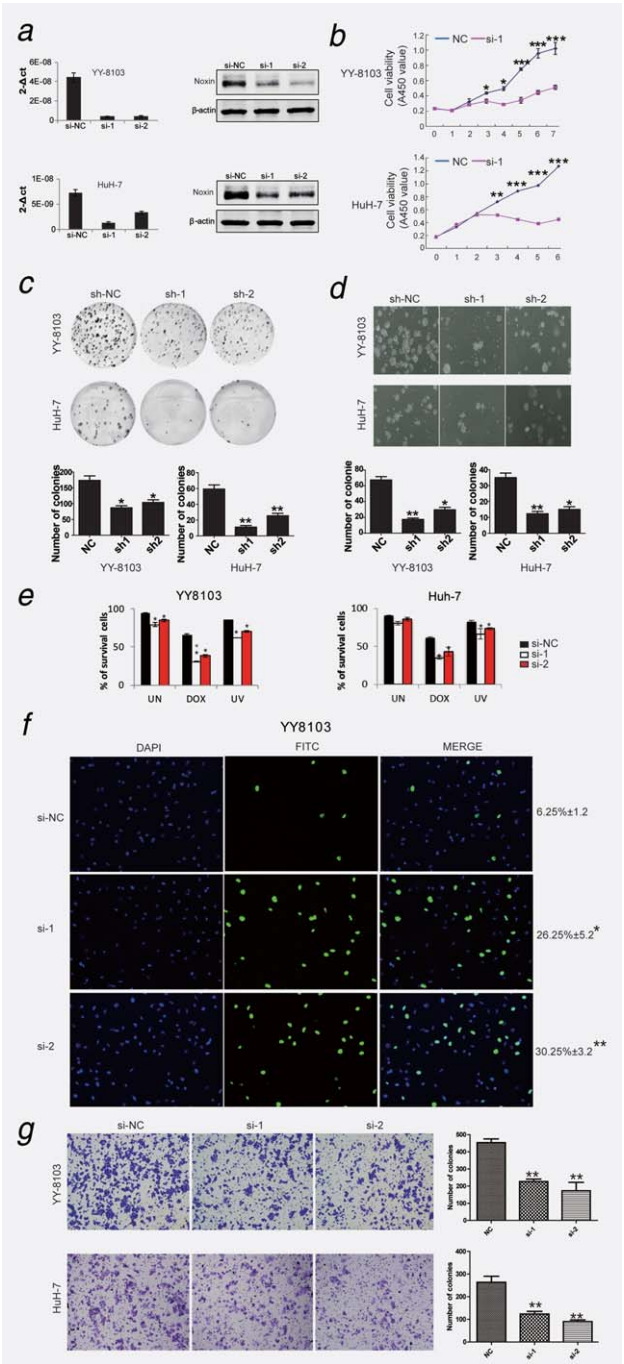


Figure 3. NOXIN knockdown suppresses HCC cell proliferation and colony formation. (*a*) The efficiency of si-1 and si-2 in knocking down endogenous NOXIN in YY-8103 and HuH-7 cells was evaluated using Real-time PCR and western blotting. The synthesized siRNAs or shRNA against NOXIN inhibited cellular proliferation (*b*) and colony formation (*c*), respectively. (*d*) shRNA-mediated NOXIN knockdown also suppressed anchorage-independent colony formation in soft agar. (*e*) Rate of Cell survival was assessed by Typan blue dye assay when YY-8103 and HuH-7 cells were knocked down with siRNAs, combination with doxorubicin (Dox) treatment and ultraviolet (UV) irradiation. (*f*) Apoptotic cells were measured by TUNEL assay when YY-8103 cells were knocked down with siRNAs. (*g*) NOXIN knockdown suppressed the cellular migration in YY-8103 and Huh-7 cells with transwell assay. *, $p < 0.05$ and **, $p < 0.01$.

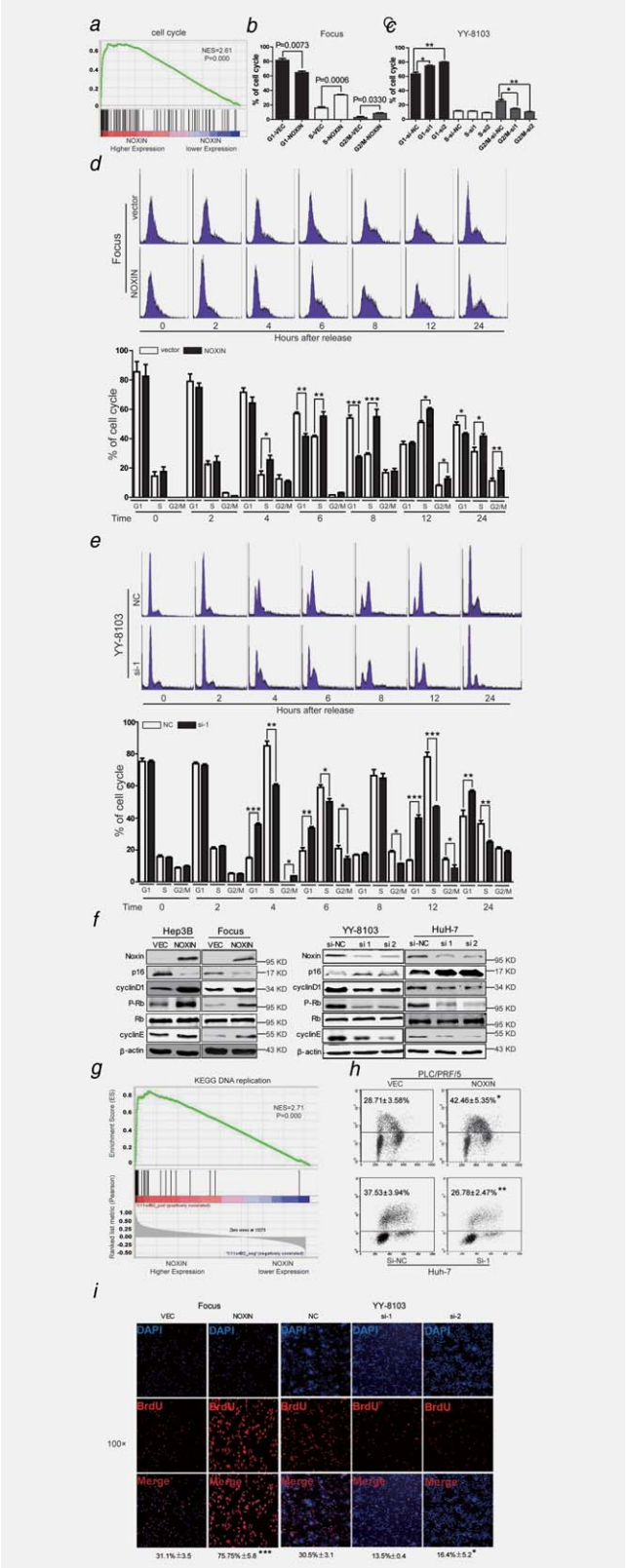
(GSEA) in HCC samples with higher *NOXIN* expression by analyzing cell cycle pathways of KEGG database. Interestingly, we found that higher NOXIN expression was positively correlated with cell cycle pathways in HCC patients, based on TCGA PAAD dataset (Fig. 4*a*). We then analyzed cell cycle progression of HCC cells with NOXIN overexpression or knockdown using flow cytometry to explore the cellular mechanism underlying NOXIN-induced cell proliferation. After serum starvation, Ad-*NOXIN* infected Focus and HepG2 cells exhibited increased S phase cell population (Fig. 4*b* and Supporting Information Fig. S3A). Contrarily, NOXIN knockdown reduces the S phase cell population and lead to G1 arrest in YY-8103 and HuH-7 cells (Fig. 4*c* and Supporting Information Fig. S3B). These data suggested that NOXIN participates in cell cycle progression.

To confirm these observations, we synchronized the HCC cells with hydroxyurea and monitored cell cycle progression. Expectedly, ectopic NOXIN promoted Focus cells to enter into S phase. Conversely, NOXIN knockdown led to significant G1 arrest of YY8103 cells (Figs. 4*d* and 4*e* and Supporting Information Fig. S3C). These data suggested that NOXIN overexpression may promote cell cycle progression by enhancing the G1-S transition.

We analyzed some known cell cycle-related factors, including cyclin D1, cyclin E, p16, RB and phosphorylated RB (pRB), to further explore the role of NOXIN in cell cycle progression. In Hep3B and Focus cells ectopically expressing NOXIN, cyclin D1, cyclin E and pRB were significantly upregulated, consisted with promoting cell cycle progression. Conversely, following NOXIN knockdown, cyclin D1, cyclin E and pRB were significantly decreased, but p16 was upregulated (Fig. 4*f* and Supporting Information Fig. S3D), supporting the idea that S phase entry was delayed. These collective data indicated that NOXIN indeed promotes the G1-S phase transition.

However, it should be pointed out that NOXIN knockdown also showed the increase of phosphorylated H2AX, a marker of DNA damage, as well as P53 and downstream molecules P21 and Bax levels (Supporting Information Fig. S3E), which could be responsible for the NOXIN knockdown-induced apoptosis. Interestingly, ectopic NOXIN decreased P53 level and its downstream P21 and Bax expression but did not affect the phosphorylated H2AX (Supporting Information Fig. S3E), indicating that the role of NOXIN in tumor may partially through P53 pathway.

To further explore the role of NOXIN in the G1-S phase transition, we also performed GSEA of KEGG DNA replication in HCC samples with higher *NOXIN* expression *versus* lower *NOXIN* expression based on TCGA PAAD dataset, implying that higher NOXIN expression was positively correlated with DNA replication in HCC samples (Fig. 4*g*). To verify the hypothesis, *de novo* DNA synthesis was assessed using BrdUrd-incorporating assay as NOXIN overexpression or knockdown in HCC cells. Flow cytometry analysis demonstrated that NOXIN overexpression increased the DNA synthesis and S-phase cell proportion in PLC/PRF/5 cells,



whereas NOXIN knockdown decreased DNA synthesis and S-phase cell proportion Huh-7 cells (Fig. 4*h*). To further confirm the results, an immunofluorescence assay was performed. The result showed that ectopic NOXIN overexpression enhanced the BrdUrd-incorporating cell proportion in Focus, PLC/PRF/5, SMMC-7721 and WRL-68 cells, whereas NOXIN knockdown attenuated the effects in YY-8103, HuH-7 and Hep-SK-1 cells (Fig. 4*i* and Supporting Information Figs. S3F and 3G). These data implied that NOXIN may promote the G1-S phase transition by increasing *de novo* DNA synthesis.

NOXIN interacts with DNA polymerase α and functions as a cofactor for the DNA polymerase-primase complex

To explore the molecular mechanism by which NOXIN contributes to the G1-S phase transition by enhancing *de novo* DNA synthesis, we analyzed the functional domain of NOXIN protein. NOXIN contains two potential nuclear localization signals, replication protein A (RPA) domain, DNA-PK phosphorylation consensus sequence and Med29 domain (Fig. 5*a*). RPA is a conserved heterotrimeric single-stranded DNA-binding protein. RPA plays essential roles in DNA replication.[27] RPA has been reported to initiate DNA synthesis by physically interacting with DNA polymerase-primase complex.[28,29] Possibly, NOXIN may promote DNA synthesis and cell cycle progression by interacting with DNA pol α *via* RPA domain. To verify this hypothesis, we first observed the co-localization of endogenous NOXIN and DNA pol α in YY-8103, SMMC-7721, HuH-7 and Focus cells using immunofluorescence assay. Both NOXIN and DNA pol α colocalized within the nucleus (Fig. 5*b* and Supporting Information Fig. S4*a*), implying NOXIN could directly or indirectly associate with DNA pol α.

We subsequently performed co-IP assays in Focus, YY-8103 and Hep-SK-1 cells using anti-NOXIN and anti-DNA pol α antibodies. The results indicated that endogenous

Figure 4. NOXIN enhances the G1-S phase transition during cell cycle progression and DNA synthesis. (*a*) GSEA analysis of KEGG cell cycle pathways in HCC patients with higher *NOXIN* expression *versus* lower *NOXIN* expression based on TCGA PAAD datasets. NES, normalized enrichment score. $p < 0.001$ (*b*) FCAS analysis revealed that ectopic NOXIN increased the S phase population of Focus cells and (*c*) NOXIN knockdown increased the G1 phase population of YY8103 cells. (*d*) The delineation revealed cell cycle progression after releasing from synchronous status. Ectopic NOXIN significantly promoted G1-S transition (bottom). (*e*) NOXIN knockdown resulted in significant G1 arrest in YY8103 cells (bottom). (*f*) The expression levels of cell cycle-related proteins examined by immunoblotting assay. (*g*) DNA replication in HCC patients with higher *NOXIN* expression *versus* lower *NOXIN* expression based on TCGA PAAD datasets. NES, normalized enrichment score. $p < 0.001$. (*h*) Flow cytometry analysis on BrdUrd-incorporated S-phase cell population when NOXIN overexpression in PLC/PRF/5 cells (upper) or NOXIN knockdown in HuH-7 cells (lower). (*i*) BrdUrd-incorporated population in HCC cells, as analyzed using an immunofluorescence assay following NOXIN overexpression in Focus cells (left) or knockdown in YY8103 cells (right). *, $p < 0.05$ and **, $p < 0.01$.

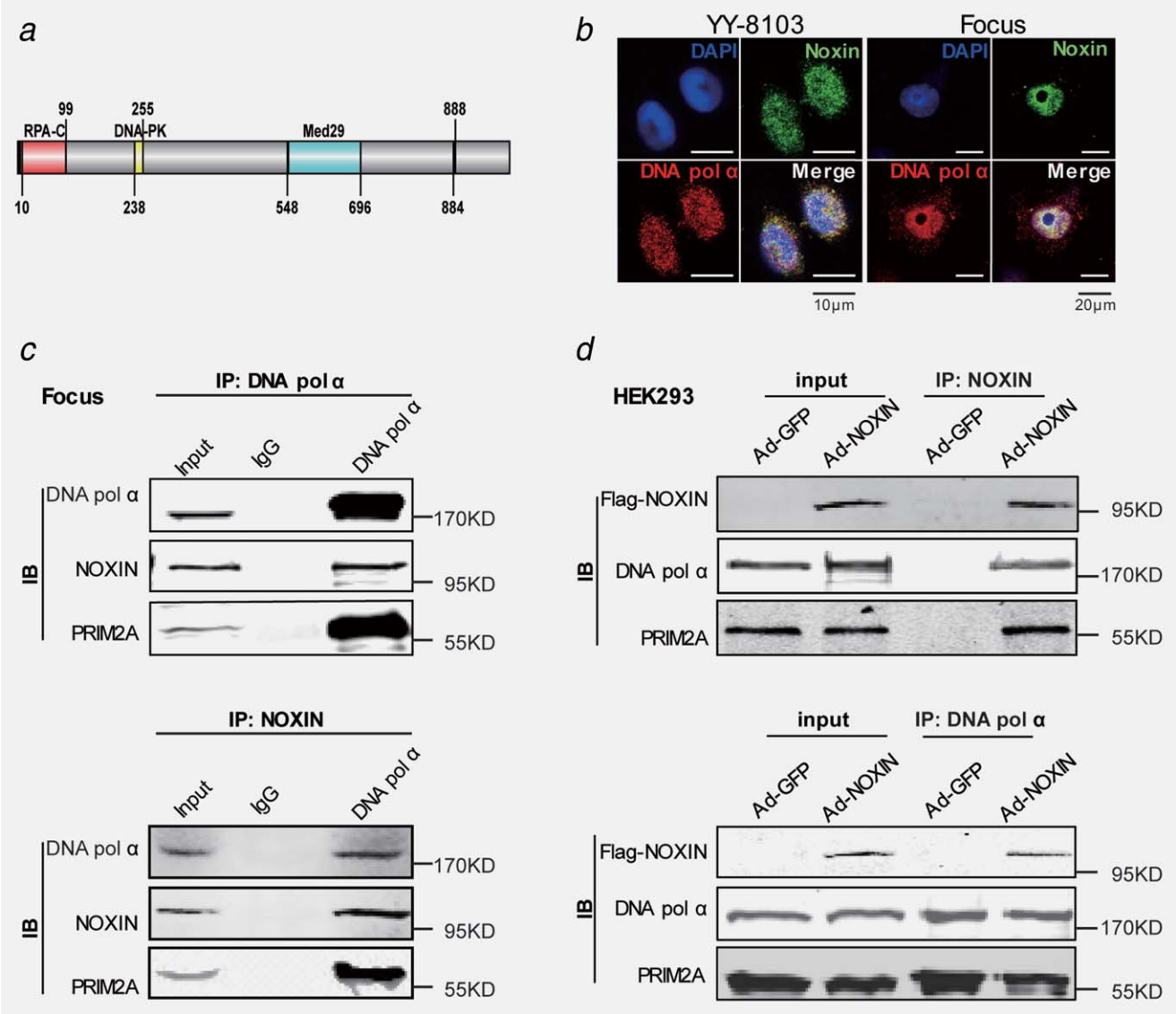


Figure 5. NOXIN is associated with DNA pol α and PRIM2A. (*a*) Schematic illustration of NOXIN structure. NOXIN contains two potential nuclear localization signals (dark), an RPA C-terminal domain (red) and a DNA-PK phosphorylation consensus sequence (yellow) and Med29 domain (green). (*b*) Colocalization of endogenous NOXIN and DNA pol α in nucleus using immunofluorescence assay in YY8103 (left) and Focus (right) cells. (*c*) co-IP assay revealed that endogenous NOXIN and DNA pol α are reciprocally immunoprecipitated. (*d*) FLAG-tagged NOXIN reciprocally immunoprecipitates endogenous DNA pol α (upper), in HEK293 cells. PRIM2A was also immunoprecipitated in Focus and HEK293 cells. IP, immunoprecipitation; IB, immunoblotting.

NOXIN and DNA pol α were reciprocally immunoprecipitated by these antibodies (Fig. 5*c* and Supporting Information Fig. S4B), suggesting that NOXIN may directly associate with DNA pol α. We transfected Ad-NOXIN into HEK293 cells and then performed reciprocal co-IP assays with anti-FLAG and DNA pol α antibodies. The results indicated that ectopic NOXIN immunoprecipitates DNA pol α and that, reciprocally, endogenous DNA pol α also immunoprecipitates FLAG-tagged NOXIN (Fig. 5*d*). These collective data indicated that NOXIN physically associates with DNA pol α.

Both DNA pol α and primase, also known as PRIM2A (p58), are two key enzymatic components of the DNA polymerase-primase complex that are responsible for DNA replication in eukaryotic cells.[30–32] Interestingly, PRIM2A was also immunoprecipitated by NOXIN and DNA pol α in reciprocal co-IP experiments (Figs. 5*c* and 5*d*), suggesting that NOXIN may function as a component of the DNA polymerase-primase complex to enhance DNA replication in HCC cells. Furthermore, we investigated the effects of NOXIN overexpression or knockdown on DNA polymerase α in PLC/PRF/5 and HuH-7 cells, respectively, by a real-time polymerase assay. The result indicated that ectopic NOXIN overexpression enhanced DNA polymerase α-primerase complex enzyme activity in PLC/PRF/5 cells,

whereas NOXIN knockdown attenuated the effect in HuH-7 cells (Supporting Information Fig. S4C).

These collective data suggested that NOXIN influences on DNA synthesis and G1-S transition, as consequence of the formation of DNA polymerase-primase complex and the subsequently increased DNA polymerase α enzyme activity.

Discussion

In this study, we employed SNP array-CGH to screen for potential HCC-associated genes with DNA copy number alterations. In addition to certain chromosomal regions with frequent DNA copy number aberrations (which were highly concordant with previous observations), we identified a limited DNA amplification region on 11q14.1, which contained four genes that have not previously been associated with cancers. Thus, these data provided us with an opportunity to identify new cancer genes.

Interestingly, chromosome 11q amplification has also been detected in many tumors, including breast cancer,[33,34] invasive gastric cancer[35] and HCC[2,36,37] using array-CGH approaches. Previous studies indicated that CCND1 amplification on 11q13 was closely associated with tumor initiation and progression. Recently, other genes located on the 11q amplification region were identified as potential oncogenes, including GAB on 11q14 in breast cancer,[34] cIAP1 and YAP on 11q22 in HCC.[21,38] In this study, we identified NOXIN as a potential oncogene in HCC based on DNA copy number amplification and overexpression, which promote cell proliferation and *in vivo* tumorigenicity.

NOXIN was previously identified to be a nitric oxide-inducible gene and is strongly induced by a wide range of stress signals, including γ- and UV irradiation, hydrogen peroxide, adriamycin and cytokines.[20] The previous study revealed that ectopic NOXIN overexpression arrested the cell cycle at G1, whereas loss or downregulation of NOXIN led to increased cell death. However, our data showed that NOXIN overexpression promotes DNA synthesis and the G1-S phase transition (Fig. 4), whereas NOXIN knockdown increased cell death of YY-8103 and HuH-7 cells (Figs. 3*e* and 3*f*), which is consistent with the previous study and a most recent report.[21,39] These different results following overexpression of NOXIN may due to a number of factors, including the gene itself (human *vs*. mouse), types of transfected cells, conditions of transfection, degree of overexpression of the ectopically introduced gene and other reasons.

Although the previous work described the cellular phenotype induced by NOXIN overexpression or knockdown, they did not completely clarify the molecular mechanism involved in cell cycle. In this work, in addition to cellular phenotype, we explore the molecular mechanism by which NOXIN functions as a cofactor of DNA polymerase-primase complex by physically associating with DNA polymerase α (Fig. 5 and Supporting Information Fig. S4D), a key enzyme in the DNA polymerase-primase complex that is responsible for DNA synthesis. Here, we proposed that NOXIN is involved in the DNA polymerase-primase complex through its functional domain RPA that interacts with DNA pol α. In addition, the finding that NOXIN knockdown led to DNA damage and then activated P53-Bax proapoptotic signalling and cell apoptosis (Supporting Information Fig. S3E) also supported the notion that NOXIN functions as a cofactor of DNA polymerase-primase complex.

NOXIN has not been previously demonstrated to be associated with HCC. Here, a set of experiments in HCC cell lines revealed that NOXIN overexpression promoted cellular proliferation, colony formation, cellular migration and *in vivo* tumorigenicity (Figs. 2 and 3), suggesting for the first time that NOXIN may be a potential oncogene in HCC. Normal cellular division requires temporal control of DNA replication, called as replication-timing program. Cancer cells with genetic or epigenetic alterations, which deregulate key pathways that control cell growth and proliferation, are characterized by replication-timing aberrations. Aberrant DNA replication timing is associated with altered gene expression, mutagenesis and genomic instability,[40,41] which contribute to tumor formation. In the present study, we identified NOXIN as an enhancer of DNA replication-timing program. Acceleration of the G1-S phase transition and DNA synthesis may significantly promote tumor progression.[40–43] Our data revealed that NOXIN expression levels were significantly correlated with HCC grade and PVTT (Table 1), suggesting that NOXIN overexpression is positively correlated with HCC progression.

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