Human Krüppel-related 3 (HKR3) is a novel transcription activator of alternate reading frame (ARF) gene

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Running title: HKR3, a transcription activator of ARF

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Background: HKR3, one of the POK family proteins, is uncharacterized in its biological function.

Result: HKR3 activates transcription of ARF by interfering with FBI-1 binding on ARF promoter.

Conclusion: HKR3 inhibits cell proliferation by activating transcription of ARF.

Significance: HKR3 is a novel transcription activator of ARF, antagonizing proto oncogene FBI-1.

HKR3 (Human Krüppel-related 3) is a novel POK (POZ-domain Krüppel-like zinc-finger) family transcription factor. Recently, some of the POK (POZ-domain Krüppel-like zinc finger) family proteins have been shown to play roles in cell cycle arrest, apoptosis, cell proliferation, and oncogenesis. We investigated whether HKR3, an inhibitor of cell proliferation and an uncharacterized POK family protein, could regulate the cell cycle by controlling expression of genes within the p53 pathway (ARF-MDM2-TP53-p21WAF/CDKN1A). HKR3 potently activated the transcription of the tumour suppressor gene ARF by acting on the proximal promoter region (bp, -149~+53), which contains Sp1 and FBI-1 binding elements (FREs). HKR3 interacted with the co-activator p300 to activate ARF transcription, which increased the acetylation of histones H3 and H4 within the proximal promoter. Oligonucleotide pull-down assays and ChIP assays revealed that HKR3 interferes with the binding of the proto-oncogenic transcription repressor FBI-1 to proximal FREs, thus derepressing ARF transcription.

The regulation of cell proliferation is a complex process involving numerous transcription factors and regulatory proteins. Furthermore, cell proliferation can be controlled by dynamic regulation of oncogene and tumour suppressor gene expression, in response to various signals. Recently, some of the POK (POZ-domain Krüppel-like zinc finger) family proteins have been shown to play roles in cell cycle arrest, apoptosis, cell proliferation, oncogenesis, or tumour suppression by acting either as oncoproteins or tumour suppressors. POK family proteins, such as...
ZBTB2, ZBTB5, ZBTB7A(FBI-1), ZBTB7C(KR-POK) and ZBTB8A(BOZF1), were shown to regulate cell cycle and proliferation through the regulation of CDKN1A, a gene member of the p53 pathway (ARF-MDM2-TP53-CDKN1A). CDKN1A encodes the protein p21, which is a negative regulator of the cell cycle (1-5).

In particular, interactions between certain POK family proteins and co-regulators (e.g., BCoR, NCoR, SMRT, p300 and p120) are important for the transcriptional regulation of target genes (6, 7). While the biological functions of ZBTB4, ZBTB7C(FBI-1), ZBTB16(PLZF), ZBTB27(BCL6) and ZBTB33(KAISO) are relatively well studied, the other POK family proteins remain little investigated.

ARF (alternative open reading frame) is a tumor suppressor protein that is encoded by an alternate reading frame of the INK4a/ARF locus (CDKN2A) (8, 9). Expression of ARF is upregulated during replicative senescence and also by oncogenes. Animals lacking ARF function are highly tumour-prone. The Ink4a/Arf locus is frequently mutated in various human cancers (8, 9). ARF, which mostly accumulates in the nucleolus, forms a complex with Mdm2, a multifaceted protein that opposes the function of p53 by blocking its transcriptional activation domain, facilitating its nuclear export, and catalyzing its ubiquitination and proteosomal degradation (10-12). Ectopic ARF stabilizes p53 and causes cells to arrest in G1 and G2 phases, accompanied by increased expression of p53 target genes such as p21 and MDM2, resulting in cell cycle arrest or apoptosis (13, 14). A loss of ARF or p53 promotes cell proliferation (15).

ARF has also been reported to arrest cell cycles in S-phase in a p53-independent manner (16, 17). ARF induces cell-cycle arrest in mouse embryonic fibroblasts lacking p21 or p53, probably by inducing additional downstream effectors (18-20).

The molecular mechanism by which ARF transcription is regulated is quite complex and involves a cascade of molecular events that vary, depending on the cell types and species (9). ARF expression is induced by activation of oncogenes like Ras or c-Myc (21, 22). Ras activates Arf expression in MEFs by increasing DMP1 (cyclin D-binding Myb-like protein) binding to the Arf promoter (23). AP1 (e.g, c-Jun and Fra-1) also cooperates with Ras in the transcriptional activation of the ARF promoter (24). However regulation of human ARF expression in response to oncogenic signals is different from that of murine Arf. Oncogenic Ras cannot induce ARF expression in human cells (25). Other oncogenes like E2F-1 and c-Myc can induce ARF expression, but each trigger expression other genes that promote G1 phase progression and apoptosis (26).

ARF is negatively regulated by various factors like Bmi1, TBX3, p53, and FBI-1. These factors can bind directly to the Arf promoter and repress its transcription (30-33), e.g., p53 binds to the Arf promoter and represses Arf transcription by recruiting both histone deacetylases (HDACs) and polycomb group (PcG) proteins (32). FBI-1 (Factor Binding to IST, ZBTB7A), recently defined as a proto-oncoprotein, also represses ARF expression, which aids in cellular transformation by oncoproteins, such as c-MYC, RAS, and E2F-1 (33-35).

HKR3 is a relatively uncharacterized POK family protein with a POZ-domain and 11 zinc finger domains. HKR3 is ubiquitously expressed in human tissues (36). The HKR3 gene is mapped to human chromosome 1p36, which is commonly rearranged (leiomyoma and leukemias) or deleted in various cancers (neuroblastoma, melanoma, Merkel cell carcinomas, pheochromocytoma and breast and colon carcinomas) (36). A correlation between deletions or rearrangements of HKR3 and human cancer suggest a role for HKR3 as a potential tumour suppressor (36). However, a more fine deletion of the 1p36 region (1...
Mbp), which is observed in human neuroblastoma, does not contain HKR3. Based on this observation, authors suggested that HKR3 may not be a tumour suppressor (37). Little is known about the functions of the HKR protein at the molecular, cellular, or genetic levels. It is not certain whether HKR3 can play a role as a tumour suppressor or not. By bimolecular fluorescence complementation (BiFC) assays of protein-protein interactions among POK family proteins, we found that HKR3 interacts with FBI-1, a potent repressor of ARF transcription (38). We then further investigated functions of HKR3 in the regulation of ARF expression. We found that HKR3, by acting on the proximal ARF promoter, activates ARF transcription, which is reversed by the proto-oncoprotein FBI-1.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies and Reagents—pcDNA3.1-HKR3-His, pcDNA3.0-FLAG-FBI-1 and the expression plasmids of various p300 polypeptide fragments were prepared by cloning cDNA fragments into pcDNA3.1 or pcDNA3.0 (Invitrogen, Carlsbad, CA, USA). Various promoter-luciferase gene fusion constructs, such as pGL2-TP53-Luc, pGL2-ARF-Luc, pGL2-MDM2-Luc and pGL2-CDKN1A-Luc, were reported elsewhere (1). A series of pGL2-ARF-Luc plasmids were prepared by cloning the human ARF promoter DNA fragments (bp, -500~+28; bp, -309~+28; bp, -164~+28; bp, -106~+28; bp, -82~+28; bp, -52~+28; bp, +5~+28) into a pGL2-basic vector (Promega, Madison, WI, USA). The pGL2-ARF-Luc -500 bp construct, with a mutation at FRE #4 (bp, -41~37, GGGGG to AAAAA), was prepared by site-directed mutagenesis. The pGL2-CDKN1A-Luc plasmid was kindly provided by Dr. Yoshihiro Sowa of the Kyoto Perpetual University of Medicine (Kyoto, Japan). Dr. Kyung-Sup Kim of Yonsei University provided the pcDNA3.0-HA-p300 expression plasmid. To prepare recombinant GST-POZ$_{HKR3}$, GST-ZF$_{HKR3}$, GST-POZ$_{FBI-1}$ and GST-ZF$_{FBI-1}$ proteins, cDNA fragments encoding the HKR3 POZ domain (a.a. 1-140), HKR3 zinc fingers (a.a. 291-600), FBI-1 POZ domain (a.a. 1-120), and FBI-1 zinc fingers (a.a. 376-489) were cloned into pGEX4T3 or pGEX4T1. All plasmid constructs were verified by DNA sequencing.

Antibodies against GAPDH, ARF, p300, His-tag, FLAG-tag, acetylated Ac-H3, and Ac-H4 were purchased from Millipore, Sigma-Aldrich, Thermo Fisher Scientific or Santa Cruz Biotechnology. To obtain a rabbit polyclonal antibody against the HKR3 protein, a white rabbit was immunized subcutaneously with recombinant GST-ZF$_{HKR3}$ (a.a. 291-600) polypeptides 6 times at 2-week intervals. Blood was collected, incubated at 37 °C for 90 min, and centrifuged. IgG was purified from the serum (10 ml) using an Econo-Pac serum IgG purification Kit (Santa Cruz, CA, USA), and was further incubated with Affi-Gel 10 beads conjugated with GST- ZF$_{HKR3}$. The beads were then collected and washed, and the column was eluted to obtain the affinity-purified antibody.

Most of the chemical reagents were purchased from Sigma-Aldrich and Invitrogen.

Cell cultures—HEK293 embryonic kidney and HCT116 colon cancer, and A459 lung cancer cells were maintained at 37 °C in a humidified incubator with 5% CO$_2$ in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 units/ml penicillin. HDFn (Human Dermal Fibroblast, neonatal) cells (Invitrogen, Carlsbad, CA, USA) were grown at 37 °C in a humidified incubator with 5% CO$_2$ in Medium 106 supplemented with low serum growth supplement (LSGS) and 100 units/ml penicillin.

Transcriptional analysis of ARF, MDM2, TP53 and CDKN1A promoters—Reporter plasmids (pGL2-MDM2-Luc, pGL2-TP53-Luc, pGL2-CDKN1A-Luc and various pGL2-ARF-Luc plasmids) and pcDNA3.1-HKR3-His, pcDNA3.1-HKR3-ΔPOZ-His, pcDNA3-HA-p300, pcDNA3-FLAG-FBI-1 or pcDNA3 control vector in various combinations were transiently cotransfected...
into HEK293 cells using Lipofectamine with Plus reagent (Invitrogen, Carlsbad, CA, USA). After 36 h of incubation, the cells were harvested and analyzed for luciferase activity using a Microplate LB 96V luminometer (EG&G Berthold). Reporter activity was normalized to co-transfected β-galactosidase activities or protein concentration to normalize the transfection efficiency. β-galactosidase activity was measured using a SpectraMax 250 ELISA reader (Molecular Device Co., Sunnyvale, CA, USA) at 420 nm. The assays were repeated 3 times.

**Knockdown of HKR3 mRNA by siRNA**—Four siRNA constructs against HKR3 mRNA were purchased from Dharmacon (Lafayette, CO, USA) and synthesized from Bioneer (Bioneer, Daejon, South Korea). The siRNA sequences were as follows, siHKR3-1, 5’-GAUCUUUGGCCUCUUGUG-3’, 5’-CAACAAAGGCGCAAAGAUC-3’, siHKR3-2, 5’-GCUAAGUUAACCACGAC-3’, 5’-UGUCGGGUAAACUUGUAGC-3’. Each siRNA (100 pmole) was transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After transfection, the cells were harvested, total RNA was isolated using TRIzol, and the samples were analyzed for knockdown by RT-qPCR.

**Quantitative chromatin immunoprecipitation (ChIP-qRT-PCR) assays**—Cells were treated with formaldehyde (final 1%) to cross-link proteins to the ARF promoter. The remaining ChIP procedures have been reported elsewhere (1-3). As a negative control for the ChIP-qRT-PCR assays, IgG was used. qRT-PCR of chromatin immunoprecipitated DNA was carried out using oligonucleotide primer sets designed to amplify the proximal promoter region (bp, -149~+53; forward, 5’-ATCTTCCACCCCTCAGCGGCGGCG-3’; reverse, 5’-ATCTTCCACCCCTCAGCGGCGGCG-3’). Also, in some ChIP-qRT-PCR assays, the 3’-UTR of the ARF promoter was used as a negative control for protein-DNA interaction. Forward, 5’-ACGCCCTAAGCGCACATTCC-3’ and reverse, 5’-CCTAGTTGCAAAATGCTTGTCATG-3’ were used.

For ChIP-reChIP assays of HKR3 and p300 binding at the ARF proximal promoter, the first ChIP samples using anti-HKR3 antibody or anti-p300 antibody were diluted 10 times with dilution buffer (15mM Tris-HCl, pH 8.1, 1% Triton X-100, 1mM EDTA, 150mM NaCl) and immunoprecipitated using anti-HKR3 or anti-p300 antibodies. Negative control ChIP assays were carried out using a rabbit IgG antibody.

**Immunoprecipitation assays**—HEK293 cells (transfected with expression vector, if necessary) were washed, pelleted, and resuspended in a lysis buffer supplemented with protease inhibitors (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100). Cell lysates were precleared, and the supernatant was incubated overnight with anti-HKR3, FBI-1, His or FLAG antibody on a rotating platform at 4 ºC, followed by incubation with protein A-Sepharose Fast Flow beads (Santa Cruz, CA, USA). The beads were collected, washed, and resuspended in equal volumes of 5x SDS loading buffer (1 M Tris-HCl, pH 6.8, 50% glycerol, 10% SDS, 2-mercaptoethanol, 1% bromophenol blue). Immunoprecipitated proteins were separated by 10-15% SDS-PAGE.

**Biotin-streptavidin oligonucleotide pull-down assay**—Cellular extracts or recombinant protein (GST-fusion protein) were incubated with biotinylated double stranded oligonucleotides for 12 h. The mixtures were incubated with streptavidin-agarose beads (Thermo Fisher Scientific, Waltham, MA, USA) for 4 h, washed with HKMG buffer five times, and precipitated by centrifugation. The precipitated protein-biotin-streptavidin oligonucleotide complexes were then analyzed by western blot. The sequences of the oligonucleotides...
are the following (note that only the top strands are shown): ARF promoter \textit{FRE}#1, 5'-GATCAGATGGTGGGATGGGGGTCGACACAGG-3'; \textit{FRE}#2, 5'-GATCGAGTGGGGATGGGGGTCGACACAGG-3'; \textit{FRE}#3, 5'-GATCGAGTGGGGATGGGGGTCGACACAGG-3'; \textit{FRE}#4, 5'-GATCGAGTGGGGATGGGGGTCGACACAGG-3'.

**GST fusion protein purification and pull-down assays**—Recombinant GST, GST-POZHKR3, GST-ZFHKR3, GST-POZFBI-1 and GST-ZFZBFBI-1 fusion proteins were expressed in \textit{E. coli} BL21 (DE3) cells and purified by affinity chromatography using glutathione-agarose-4 beads (Peptron, Daejeon, South Korea). p300, four polypeptide fragments of p300, FBI-1 or HKR3 were prepared using TNT Quick-coupled Transcription/Translation Extract (Promega, Madison, WI, USA). GST-fusion protein-agarose bead complexes were incubated with \textit{in vitro} translated $^{35}$S-labeled p300, four p300 polypeptide fragments, FBI-1 or HKR3 at 4°C overnight in HEMG buffer. The reaction mixtures were precipitated, the pellets were washed, and the bound proteins were separated by 10~15% SDS-PAGE. Gels were then exposed to X-ray film using an image-intensifying screen (Kodak).

**Fluorescence-activated cell sorting (FACS) analysis**—Cells were fixed with 70% ethanol, washed with PBS, and cellular DNA was then stained with propidium iodide (100 $\mu$g/ml) for cell cycle analysis. A cell cycle profile and forward scatter were determined using a Becton Dickinson FACS Calibur, and the data was analyzed using ModFit LT 2.0 (Verity Software House, Topsham, ME, USA).

**MTT assays**—To investigate the effect of HKR3 on cell viability, cells were plated onto 6-well dishes at 30~50% confluency and incubated. The cell viability of each sample was determined by measuring the conversion of the tetrazolium salt MTT (Sigma-Aldrich, St. Louis, MO, USA) to formazan (absorbance at 570 nm).

**Annexin-V staining**—An Annexin-V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) was used to detect apoptosis by flow cytometry. All cells were harvested (including detached cells) and processed according to the manufacturer’s instructions. Cells were washed and resuspended with 1X binding buffer at a concentration of 1x10$^6$ cells/ml, and 100 ml of the solution (1x10$^6$ cells) was transferred to a 5 ml culture tube. In all, 5 $\mu$l of Annexin-V-fluorescein isothiocyanate and 5 $\mu$l of propidium iodide were added to the solution, followed by gentle vortexing of the cells and incubation for 15 min at room temperature in the dark. In total, 400 $\mu$l of 1X binding buffer was added to each tube. Cell apoptosis was determined using a FACS Calibur (BD Biosciences, San Jose, CA, USA) instrument within 1 hr. Data was analyzed using BD CellQuest Pro software (BD Biosciences, San Jose, CA, USA).

**Subcellular fractionation**—The nuclear extract Kit (Active Motif, Carlsbad, CA, USA) was used to separate cellular cytoplasmic and nuclear fractions. Cells were resuspended in 1X hypotonic buffer and incubated for 15 min on ice, followed by adding detergent and vortexing. The cytoplasmic fraction (supernatant) was collected following centrifugation. The nuclear pellet was resuspended in complete lysis buffer and the nuclear fraction (supernatant) then recovered by centrifugation.

**Statistical analysis**—Student’s $t$-test was used for the statistical analyses. P-values of <0.05 were considered statistically significant.

**RESULTS**

**HKR3 suppresses cell proliferation and decreases the number of cells in S-phase**—HKR3 has a POZ domain at the N-terminus (a.a. 4~119) and eleven C$_2$H$_2$ type Krüppel-like zinc finger motifs in the C-terminal half of the protein (Fig. 1A). HKR3 was detected in the nucleus by immunocytochemistry (data not shown). We then investigated whether HKR3 could regulate cell cycle progression using flow cytometry and MTT assays in HKR3- or siRNA-transfected
HEK293 cells. MTT assays revealed that HKR3 significantly decreased cell proliferation, while siRNA knockdown of HKR3 mRNA increased cell proliferation in HEK293 cells (Fig. 1B). Flow cytometry indicated that HKR3 induced cell cycle arrest and substantially decreased the number of HEK293 cells in S-phase (49.7% versus 30.2%). The knockdown of endogenous HKR3 mRNA by two HKR3 siRNAs resulted in an increased number of cells in S-phase (49.7 versus 67.4, a 69.6% increase), and a concomitant decrease in the number of cells in G0 and G1 phases (Fig. 1C). Ectopic expression or knockdown of endogenous HKR3 mRNA by two HKR3 siRNAs resulted in an increased number of cells in S-phase (49.7 versus 67.4, a 69.6% increase), and a concomitant decrease in the number of cells in G0 and G1 phases (Fig. 1C). Ectopic expression or knockdown of HKR3 did not induce apoptosis in HEK293 cells (Fig. 1D). These results suggest that HKR3 inhibits cell proliferation via cell cycle arrest, but not by apoptosis.

**HKR3 potently activates transcription of the ARF gene**—Recently, we found that some POK proteins regulate the cell cycle by controlling the p53 pathway (1-5). Accordingly, we investigated whether HKR3 inhibited cell proliferation by controlling expression of genes within the ARF-p53 pathway. Among the genes of ARF-p53 pathway tested, the transcription of ARF was most effectively activated by ectopic HKR3 in HEK293 cells. HKR3 relatively weakly activated transcription of TP53 and CDKN1A by 1.5-5-fold (Fig. 2A). We next investigated whether HKR3 inhibits cell proliferation through upregulation of ARF. In ARF-null A549 cells, ectopic HKR3 expression or knockdown of HKR3 showed little effect on cell proliferation (Fig. 2B).

ARF is an upstream regulator of the p53 pathway. Because the regulation of ARF expression can significantly affect p53 stability and thereby, p21 expression, we investigated whether knockdown or ectopic expression of HKR3 affects mRNA expression of endogenous ARF. RT-qPCR and western blot analysis of HEK293 cells transfected with either control pcDNA3 or pcDNA3.1-HKR3-His showed that HKR3 increased endogenous ARF expression (Fig. 2, C and D). Alternatively, the same analyses of HEK293 cells transfected with siRNA targeting HKR3 mRNA resulted in decreased endogenous ARF transcription (Fig. 2, E and F). In normal human primary HDFn cells (human dermal fibroblasts), HKR3 also increased ARF expression, suggesting that HKR3 increases ARF expression both in immortalised (HEK293 and normal (HDFn) human cells (Fig. 2, G, H and I).

HKR3 activates ARF by acting on the short proximal promoter (bp, -52~+5) containing FBI-1 and Sp1 binding sites—Because HKR3 significantly increased the transcription of ARF, we mapped the regulatory elements responsible for the transcriptional activation of ARF by HKR3. Eight ARF reporter gene fusion plasmid constructs, differing in length of their 5' upstream sequences, were prepared. Transient transfection and reporter assays in HEK293 cells showed that ectopic HKR3 increased the transcription of all the ARF promoter constructs. However, a distinct decrease in transcription was observed in the constructs lacking two GC-rich regions: a distal (bp, -540~309; GC content, 68%) and a proximal (bp, -52~+5; GC content, 68.4%) region (Fig. 3A). These results suggest that HKR3 likely activates transcription by acting on distal and proximal regions.

Because HKR3 can activate ARF transcription, we investigated whether HKR3 binds to the ARF promoter and which promoter region is involved in HKR3 binding, using ChIP assays. A ChIP assay of HEK293 cells expressing ectopic HKR3 showed that ectopic HKR3 increased the transcription of all the ARF promoter constructs. However, a distinct decrease in transcription was observed in the constructs lacking two GC-rich regions: a distal (bp, -540~309; GC content, 68%) and a proximal (bp, -52~+5; GC content, 68.4%) region (Fig. 3A). These results suggest that HKR3 likely activates transcription by acting on distal and proximal regions.

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HKR3 interacts with the transcription co-activator p300 to activate transcription of ARF—Transcriptional activators often recruit coactivator complexes containing HAT (histone acetyltransferase) proteins
such as PCAF and p300/CBP (39, 40). HAT proteins acetylate the histones of nucleosomes in close proximity to their recruitment sites, thereby opening nucleosome structures to activate transcription (41). Co-immunoprecipitation and western blot assays of HEK293 cells transfected with a HKR3 expression vector revealed that HKR3 interacts with p300 in vivo (Fig. 4A). GST fusion protein pull-down assays also showed that the GST-POZHKR3 and GST-ZFHKR3 domains interact with p300 through the HAT domain of p300 in vitro, suggesting that HKR3 directly interacts with p300 (Fig. 4B).

Transient transfection and transcription analyses of pGL2-ARF-Luc in HEK293 cells indicated that ectopic HKR3 or p300 activates the ARF promoter, most likely by interacting with endogenous p300 or HKR3. HKR3 and p300 potently activated transcription when they were co-transfected (Fig. 4C). A HKR3 mutant lacking the POZ domain, however, did not activate transcription, suggesting the importance of POZ domain in ARF transcriptional activation. The HKR3 mutant (POZ deletion, ΔPOZ) localized to the nucleus, similar to wild-type HKR3 (Fig. 4D). ChIP assays showed that p300 recruited by HKR3 significantly increased the acetylation of histones 3 and 4 at the proximal promoter of ARF, thereby opening the promoter region for subsequent transcription initiation (Fig. 4, E and F). HKR3 and p300 could not bind to an ARF 3’ UTR (bp, +26463→+26554) negative control region, nor increase acetylation levels of histones 3 and 4 in the ARF 3’ UTR region changed (Fig. 4, E and F). These results demonstrate that p300 is involved in the transcriptional activation of ARF by HKR3 by facilitating histone acetylation in its proximal promoter region.

HKR3 directly interacts with the proto-oncogene FBI-1 and decreases the transcriptional repression of ARF by FBI-1—Interestingly, the proximal promoter region to which HKR3 binds is also the region where the proto-oncogene FBI-1 exerts its repressor activity (33). FBI-1 regulates the transcription of target genes through interactions with co-repressor proteins and other transcription factors. Previously, using the BiFC system we developed (38), we found that the POZ domain of HKR3 interacts with the proto-oncogene FBI-1 (unpublished data). Co-immunoprecipitation and western blot analysis of cell lysates prepared from HEK293 cells transfected with HKR3-His expression vectors showed a physical interaction between the FBI-1 and HKR3 proteins (Fig. 5A). GST-fusion protein pull-down assays demonstrated that both the POZ and zinc finger domains of HKR3 interact directly with FBI-1. The assays also showed that both the POZ and zinc finger domains of FBI-1 interact with HKR3 (Fig. 5B).

HKR3 competes with FBI-1 for binding to the proximal region containing FBI-1 binding sites—Previous studies have shown that FBI-1 can bind to four sites within the ARF promoter (33), and three of those four potential FBI-1 response sites (FREs) are within the ARF proximal promoter region. Because HKR3 is expected to bind the proximal promoter and activate transcription of ARF, we investigated whether FBI-1 binding to the proximal promoter was affected by HKR3. Oligonucleotide pull-down assays using total lysates from FBI-1- and/or HKR3-expressing cells showed binding competition between FBI-1 and
HKR3 at the three FREs (#2~#4) of the proximal promoter region (Fig. 6B). HKR3 could not bind to FRE#1, as shown by ChIP assays (Fig. 3B). Also, FBI-1 binding in FRE#1 was not affected by HKR3 (Fig. 6B). Oligonucleotide pull-down assays using recombinant GST-POZHKR3 or GST-ZFHKR3 indicated that only ZFHKR3 bound directly to FRE#2, 3 and 4 (Fig. 6C). We also investigated whether HKR3 and FBI-1 compete with each other to bind to FREs. ChIP assays of the ARF promoter in HEK293 cells that were transiently co-transfected with pcDNA3-FLAG-FBI-1, and increasing amounts of pcDNA3.1-HKR3-His, revealed that binding of FBI-1 to the ARF promoter decreased with increasing HKR3 expression (Fig. 7B). Conversely, in cells transfected with pcDNA3.1-HKR3-His and increasing amounts of pcDNA3-FLAG-FBI-1, binding of HKR3 to the ARF promoter was decreased with increasing FBI-1 expression (Fig. 7C). HKR3 and/or FBI-1 could not bind to the negative control ChIP #2 3' UTR region (bp, +26463~+26554) (Fig. 7, B and C). These results indicate that FBI-1 and HKR3 compete with each other for FRE sites #2, #3, and #4. Although HKR3 can compete with FBI-1 to decrease FBI-1 binding, ectopic FBI-1 decreases HKR3 binding more effectively, as evidenced by transient transcription assays and oligonucleotide pull-down assays.

We were also able to demonstrate by ChIP assays that FBI-1 knockdown increased endogenous HKR3 binding, while HKR3 knockdown increased endogenous FBI-1 binding to the ARF promoter (Fig. 8B). Also, no binding of endogenous HKR3 or FBI-1 was observed in the ChIP #2 (bp, +26463~+26554) negative control region (Fig. 8B). We examined whether endogenous HKR3 and p300 could bind to the ARF promoter simultaneously by ChIP-reChIP. HKR3 bound to the ARF proximal promoter to recruit p300 to activate ARF transcription and knockdown of FBI-1, resulting in a significant increase in p300 recruitment and acetylation of histones H3 and H4 (Fig. 8, C and D).

**DISCUSSION**

Previously, we reported that POK family proteins, such as FBI-1, ZBTB2 and ZBTB5, regulate cell cycle and proliferation by repressing CDKN1A gene expression (1-3). Because very little is known about the biological functions of HKR3, we investigated a possible role for HKR3 in regulation of the cell cycle. HKR3 repressed cell proliferation by decreasing the number of cells in S-phase. Overall, our data demonstrated that HKR3, a putative transcriptional activator of ARF, is a negative regulator of cell proliferation.

The molecular mechanism of the transcriptional activation of ARF and the regulation of cell proliferation by HKR3 is intriguing. HKR3 activates ARF expression by acting on two major 5' upstream regulatory elements (bp, -52 to +5; bp -500 to -309) of the ARF promoter (Fig. 3A). Because ChIP assays indicated that HKR3 binds only to proximal, but not the distal, promoter regions, HKR3 appears to activate transcription by directly affecting molecular events at the proximal region, while also exhibiting indirect secondary effects (e.g., increasing transcriptional activator binding or decreasing transcriptional repressor binding) at the distal region.

The ARF proximal promoter region is a direct target of transcriptional regulation by Sp1 and FBI-1. Sp1 activates transcription by acting on three FRE elements (FRE #2, bp -95~90; FRE #3, bp -58~52; FRE #4, bp -41~35) of the ARF promoter. FBI-1 represses transcription by binding competitively against Sp1 at GC-boxes or FREs (3). FBI-1 was reported to facilitate cellular transformation by repressing ARF expression, and FBI-1 was therefore proposed to be a proto-oncogene (33). Intriguingly, oligonucleotide pull-down assays showed that these particular sites are also bound by HKR3, which leads to ARF transcriptional activation. Thus, HKR3 and FBI-1 can compete with each other to bind to these sites.

Accordingly, we tested the function of one FBI-1 binding site (FRE#4), which is also a binding site for Sp1 and HKR3 and is
located within the minimal proximal promoter element (bp, -52~+5). This element was shown to be important in transcriptional activation by HKR3 (Fig. 3A). When we introduced a mutation in FRE#4, ARF transcriptional activation by HKR3 considerably decreased, suggesting that HKR3 activates ARF transcription by acting on this specific element (data not shown).

Transcriptional regulation of ARF may be determined by the cellular context of Sp1, FBI-1, and HKR3 expression. In cells with low FBI-1 and HKR3 expression, the proximal ARF promoter is likely to be occupied by Sp1 and express ARF at a basal level (Fig. 9A). When FBI-1 expression level is high, as in many cancer cells, the proximal promoter FREs and the distal FRE#1 are bound by FBI-1, which represses ARF transcription by interacting with corepressor HDAC complexes. This may be how cancer cells with high FBI-1 expression evade signalling pathways that activate ARF (Fig. 9B). In cells that express high HKR3 and FBI-1, the proteins may compete with each other at the FRE#2, 3, and 4, while FBI-1 binds to FRE#1 (Fig. 9B). Accordingly, the level of ARF transcription may be determined by occupancy of the proximal FREs by HKR3 or FBI-1.

The POZ domain is a protein-protein interaction motif. Transcriptional repressors, such as PLZF, ZBTB2, FBI-1, Kr-pok and BCL6, can repress transcription through interactions between the POZ domain and corepressors, such as SMRT, NCoR, BCoR, and mSin3A (6, 7). Corepressor-HDAC complexes recruited by these repressors deacetylate histones of nearby nucleosomes (6). We found that HKR3 interacts with the coactivator p300 through a direct molecular interaction between its POZ and/or ZF domains and the HAT domain of p300, which is the first intriguing observation for a POZ domain protein. That molecular interaction results in acetylation of histones within nucleosomes juxtaposed to the HKR3/p300 binding sites.

FBI-1 has been shown to be oncogenic by repressing the tumour suppressor ARF, which in turn lowers the expression of the p53 tumour suppressor gene (33). Overexpressed FBI-1 causes cancer in the thymus, liver and spleen in a transgenic mouse model (33). HKR3, by contrast, decreases the transcriptional repression of ARF by FBI-1 on the proximal promoter via modulation of FBI-1 DNA binding activity or competition with FBI-1 for target sites shared by the two factors.

Although our study can be considered novel and comprehensive, the physiologic relevance of HKR3 to the regulation of ARF transcription remains somewhat uncertain because little is known about the function of HKR3.

In summary, we found that HKR3 is a transcription factor that inhibits cell proliferation and activates transcription of ARF. The activation of ARF involves molecular interactions of HKR3 with the transcription coactivator p300 and inhibition of ARF promoter binding by FBI-1.

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**FIGURE LEGENDS**

**FIGURE 1. HKR3 decreases cell proliferation and the number of cells in S-phase.** A, Structure of the human HKR3 protein. B, MTT assays. HEK293 cells transfected with either control pcDNA3 or pcDNA3.1-HKR3 expression vector were grown for 1-6 days and analyzed for cell growth. Alternatively, the cells were transfected with either negative control siRNA or HKR3 siRNA and cultured for 1 to 4 days. All assays were performed in triplicate. *, p<0.05. C, Flow cytometry of HEK293 cells cotransfected with pcDNA3 and siRNA negative control (N.C.), pcDNA3.1-HKR3 and siRNA N.C. or pcDNA3 and HKR3#1 siRNA or HKR3#2 siRNA. Cells were cultured, stained with propidium iodide, and analyzed by flow cytometry. Western blot analysis of the HEK293 cell lysates transfected with various combination of the expression vector and the siRNA is shown on the right. GAPDH, control

**FIGURE 2. HKR3 increases the expression of ARF in HEK293 and HDFn cells.** A, Transient transfection and transcription assays. Expression vectors of HKR3 and various reporter plasmids were transiently co-transfected into HEK293 cells and luciferase activities were measured. All assays were performed in triplicate. Error bars indicate standard deviations. *, p<0.05. B, Flow cytometry analysis of ARF-null A549 cells transfected with various combinations of the expression vectors and siRNAs indicated. Cells were cultured for 36 h, stained with propidium iodide, and analysed by flow cytometry. C, RT-qPCR analysis of HKR3 and ARF mRNA expression in HEK293 cells transiently transfected with a HKR3-His expression vector. *, p<0.05. D, Western blot analysis of endogenous ARF expression in cell lysates of HEK293 cells transiently transfected with HKR3-His expression vector. GAPDH, control. E, RT-qPCR analysis of HKR3 and ARF mRNA expression in HEK293 cells transfected with siRNA negative control (N.C.) or HKR3 siRNA. *, p<0.05. F, Western blot analysis of the cell lysates prepared from HEK293 cells transfected with siRNA negative control (N.C.) or two HKR3 siRNAs. GAPDH, control. G and H, RT-qPCR analysis of HKR3 and ARF mRNA expression in HDFn cells transiently transfected with HKR3-His expression vector or HKR3 siRNA. *, p<0.05. I, Western blot analysis of endogenous ARF expression in the HDFn cell lysates transiently transfected with HKR3-His expression vector or two HKR3 siRNAs.

**FIGURE 3. HKR3 activates ARF by acting on its proximal promoter element (bp, -52 to +5) containing both FBI-1 and Sp1 binding sites.** A, Transient transcription assays of ARF. HEK293 cells were transiently co-transfected with pGL2-ARF-Luc reporter plasmid (with a variable upstream sequence, shown left) and HKR3 expression vector and analyzed for luciferase activity. All assays were performed in triplicate. Error bars represent standard deviations. *, p<0.05. B, ChIP assays of HKR3 binding to endogenous ARF promoter. Structure of endogenous ARF promoter containing the distal (ChIP#1: bp, -540 to -300) and
proximal (ChIP#2: bp, -149~+53) regulatory regions is shown above. Arrows indicate the locations of the ChIP primer binding sites flanking the HKR3-responsive promoter elements. Open Circle, Sp1 binding sites; Filled circle, FBI-1 binding sites. *, p<0.05.

**FIGURE 4.** HKR3 interacts with the co-activator p300 and activates transcription of ARF by acetylating histones at the proximal promoter. A, Co-immunoprecipitation of HKR3 and p300. Cell lysates prepared from the HEK293 cells transfected with a HKR3-His expression vector were immunoprecipitated using anti-His and analyzed for p300 by western blotting. GAPDH, control. B, GST-fusion protein pull-down assays of the interaction between HKR3 and p300. Recombinant GST protein, GST-POZHKR3, and GST-ZFHKR3 were incubated with in vitro synthesized [35S]-methionine-labeled polypeptide fragments (domain #1, 2, 3 and 4) of p300, pulled-down, resolved by 10% SDS-PAGE, and analyzed by autoradiography. C, Transient transcription assays. pGL2-ARF-Luc (-500 bp) and expression vectors of HKR3, HKR3ΔPOZ (POZ deletion) and p300 were co-transfected into HEK293 cells as indicated, and the luciferase activity of cell lysates was measured. All assays were performed in triplicate. Error bars indicate standard deviations. *, p<0.05. D, Nuclear localization of HKR3ΔPOZ. W, whole cell lysate; C, cytoplasm; N, nucleus; tubulin, cytoplasm marker protein; HDAC1, nucleus marker protein. E, ChIP assays of HKR3-His and/or p300 binding to the proximal promoter (bp, -149~+53) of endogenous ARF in HEK293 cells. The 3’-UTR region (bp, +26463~+26554) of ARF was also used as a negative control for binding of His-tagged HKR3 and p300. *, p<0.05. F, ChIP assays of histone acetylation at the proximal promoter of endogenous ARF. The cells were transfected with pcDNA3.1-HKR3-His expression vector and immunoprecipitated with the antibodies indicated. Oligonucleotides (ChIP#2) used in ChIP assays are depicted in Fig. 3B. The 3’-UTR region (bp, +26463~+26554) of ARF was also used as a negative control for histone acetylation. *, p<0.05.

**FIGURE 5.** HKR3 interacts with FBI-1 in vitro and in vivo and inhibits transcriptional repression of ARF by FBI-1. A, Co-immunoprecipitation and western blotting assays of cell extracts from HKR293 cells transfected with pcDNA3.1-HKR3-His. Cell lysates were immunoprecipitated using either an anti-FBI-1 antibody or an anti-HKR3 antibody, and the precipitates analyzed by western blotting using anti-FBI-1 or anti-HKR3 antibodies. GAPDH, control. B, The structures of recombinant proteins GST-POZHKR3, GST-ZFHKR3 GST-POZFBI-1 and GST-ZFFBI-1 and in vitro GST-fusion protein pull-down assays. Recombinant GST, GST-POZHKR3, and GST-ZFHKR3 were incubated with [35S]-methionine-labeled FBI-1 prepared by in vitro transcription and translation. Alternatively, recombinant GST, GST-POZFBI-1, or GST-ZFFBI-1 were incubated with [35S]-methionine-labeled HKR3 by in vitro transcription and translation. The reaction mixtures were pulled down and resolved by 10% SDS-PAGE. The gel was exposed to X-ray film. Input, 10% of [35S]-labeled FBI-1 or HKR3 added in binding reactions. C, Transcriptional regulation of the ARF by HKR3 and FBI-1. HEK293 cells were transiently co-transfected with ARF reporter plasmid and the expression plasmids of HKR3, HKR3ΔPOZ or/and FBI-1 and luciferase activity was measured. All assays were performed in triplicate. Error bars indicate standard deviations. *, p<0.05.

**FIGURE 6.** HKR3 competes with FBI-1 at the proximal region of ARF, including three FBI-1 binding sites, in vitro. A, Diagram of the endogenous ARF promoter. Sp1 and FBI-1 binding sites are indicated. +1, Tsp, transcription start site. B, Oligonucleotide pull-down assays. Whole cell lysates of the HEK293 cells transfected with pcDNA3.1-HKR3-His and/or pcDNA3-FLAG-FBI-1 were incubated with streptavidin agarose beads conjugated to biotinylated oligonucleotide probes: FRE#1 (bp, -303~273), FRE#2 (bp, -130~104), FRE#3 (bp, -103~76), FRE#4 (bp, -53~26). Proteins bound to the probes were precipitated by centrifugation and analyzed by western blotting using the antibodies indicated. C,
Oligonucleotide pull-down DNA binding assays of recombinant GST, GST-POZHKR3, GST-ZFHKR3 to the FRE#1, 2, 3 and 4. Recombinant proteins were incubated with streptavidin agarose beads conjugated to biotinylated oligonucleotide FRE probes. The precipitates were analyzed by western blotting using an anti-GST antibody.

**FIGURE 7.** HKR3 competes with FBI-1 at the proximal region of ARF with three FBI-1 binding sites in HEK293 cells. A, Structures of the endogenous ARF gene. The oligonucleotide primers spanning three FREs and the 3’ UTR used in ChIP assays are indicated by arrows. B and C, ChIP assays examining binding competition between HKR3 and FBI-1 at the proximal promoter region (bp, -149~+53; ChIP #1). HEK293 cells were co-transfected with an FBI-1 expression vector and increasing amounts of HKR3 expression vector or vice versa. The cells were fixed with formaldehyde, and chromatin was immunoprecipitated with the indicated antibodies and measured by qRT-PCR. The 3’-UTR region (bp, +26463~+26554; ChIP #2) of ARF and IgG antibody were used as negative controls for the ChIP-qRT-PCR assays. *, p<0.05.

**FIGURE 8.** Change of p300 recruitment and histone acetylation status by the expression of HKR3 and FBI-1. A, Western blot of the HEK293 cell lysates prepared from the cells transfected with HKR3 siRNA and/or FBI-1 siRNA. GAPDH, control. B, Knock-down of FBI-1 and HKR3 expression and ChIP assays of HKR3 and FBI-1 binding at the endogenous ARF proximal promoter (bp, -149~+53; ChIP #1). The 3’ UTR region (bp, +26463~+26554; ChIP #2) of the ARF promoter and IgG antibody were used as negative controls for ChIP-qRT-PCR assays. *, p<0.05. C, ChIP-reChIP assays of HKR3 and p300 binding at the at the endogenous ARF proximal promoter (bp, -149~+53; ChIP #1) by knockdown of either HKR3 and/or FBI-1. *, p<0.05. D, ChIP assays of histone modification at the endogenous ARF proximal promoter (bp, -149~+53; ChIP #1). The cells were transfected with control siRNA, HKR3 siRNA and/or FBI-1 siRNA and immunoprecipitated with the antibodies indicated. Oligonucleotides used in ChIP assays are as depicted in Fig. 7A. *, p<0.05.

**FIGURE 9.** Hypothetical model of transcriptional regulation of ARF expression by HKR3, p300, and FBI-1. A, In cells that express low levels or no HKR3 and FBI-1, ARF is expressed at a low level by transcriptional activation by Sp1. B, Transcription of ARF is determined by the relative levels of HKR3 and FBI-1. In cancer cells with high FBI-1 expression, FBI-1 binds to distal FRE#1 and proximal FREs within the ARF promoter. FBI-1 represses the transcription of ARF by recruiting a co-repressor-HDAC complex. When HKR3 expression is induced at a high enough level, HKR3 competes with FBI-1 to bind to the proximal FREs. The FRE#4 element is particularly important in transcriptional activation by HKR3. HKR3 interacts with the co-activator p300 to activate transcription of ARF. The relative abundance of HKR3 and FBI-1 may determine the promoter occupancy and thus the transcription level of ARF. FBI-1 binds at FRE#1 in the presence or absence of HKR3, of which the functional significance for transcription is unclear. C, In the cells that express HKR3 but not FBI-1, HKR3 binds to the proximal FRE#2, 3 and 4, recruits the p300 co-activator complex, acetylates histones around the proximal promoter and activates the transcription of ARF.
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Human Kruppel-related 3 (HKR3) is a novel transcription activator of alternate open reading frame (ARF) gene

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