Supporting Information

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SI Materials and Methods

Cell Culture. Three MCHs (14F, 14T, and 14U), seven NPC cell lines (HONE1, HK1, HNE1, CNE1, CNE2, C666-1, and SUNE1), a tetracycline transactivator tTA-producing cell line (HONE1-2), and the immortalized nasopharyngeal epithelial cell line NP460 were cultured as described previously (1–3). HUVECs (Lonza) were cultured as described previously (4).

NPC Tissue Specimens. Paired normal nasopharyngeal and NPC biopsy specimens from 60 NPC patients were collected from Queen Mary Hospital between 2006 and 2008, as described previously (5, 6). This study was approved by the University of Hong Kong's from the Institutional Review Board. Fiberoptic nasopharyngoscopy was used to obtain the paired normal and tumor biopsy materials. Tumor tissues were obtained directly from the site of tumor growth. If tumors localized to one side, the normal tissue samples were taken from the contralateral side having a normal mucosal appearance and no evidence for contact bleeding. For patients having bilateral tumor involvement, normal tissues were taken from the nasal cavity.

Real-Time qPCR Analysis. qPCR was performed as described previously (7) in an Applied Biosystems Step-One Plus analyzer using TaqMan PCR core reagent kits, *CRIP2*- and *GAPDH*-specific primers and probes, and SYBR-green PCR core reagent kits (Applied Biosystems). The universal probe library (Roche Diagnostics), was used to confirm the expression of *BclXL* and *survivin* expression according to manufacturer's instructions. Primer sequences for the angiogenesis and apoptosis-related proteins are listed in Table S2. The IL-6 qPCR primer was designed as described previously (8).

Western Blot and IHC Staining Analyses. Western blot analysis was performed as described previously (9). Primary antibodies for *CRIP2* (1:1,000; ab83489; Abcam), IκBα and phosphorylated p65 (1:1,000; 2859 and 3033, respectively; Cell Signaling Technology), histone (1:2,500; sc-8030; Santa Cruz Biotechnology), and α-tubulin (1:10,000; Calbiochem) were used. IHC staining analysis was performed using a CD34 antibody (1:40; sc-18917; Santa Cruz Biotechnology), as described previously (10).

In Vivo Tumorigenicity Assay. The in vivo tumorigenicity assay was performed as described previously (3). In brief, a total of 1×10^7 of cells were injected s.c. into both flanks of three athymic BALB/c Nu/Nu 6–8-wk old nude mice (six sides). Tumor sizes were measured weekly. The in vivo inhibition of tetracycline-induced gene expression was performed by addition of doxycycline to the drinking water, as described previously (2).

HUVEC Tube Formation Assay. The HUVEC tube formation assay was performed as described previously (4). In brief, 50 μL of growth factor-reduced matrigel (BD Biosciences) was coated on each well of a 96-well culture plate. The conditioned medium (±dox) was collected by incubating the vector-alone and *CRIP2*-expressing clones with DMEM without serum for at least 24 h. HUVEC cells were mixed with 100 μL of conditioned media from different cell lines and 1% FBS. The mixture was then seeded into the well coated with matrigel. The cells were then incubated for four to five hours for tube-like structure formation. The images were captured using an inverted microscope (400× magnification) (Nikon Instruments). The total tube length from three different fields was measured using SPOT software (Diagnostic Instrument).

In Vivo Matrigel Plug Angiogenesis Assay. A total of 10^7 cells mixed with 50 μL of DMEM and 250 μL of Matrigel (BD Biosciences) were injected s.c. into one site for each of five nude mice. The gel plugs were excised after 7 d postinjection. The gel plugs were then fixed with formalin and paraffin embedded. Histological sections were stained with anti-CD34 antibody as described in the previous section. The CD34-positive staining of vascular endothelial cells was analyzed by ImageScope v10 software (Aperio).

Human Angiogenesis Antibody Array Analysis. Conditioned media of the vector-alone and *CRIP2*-stable clones were used for angiogenesis antibody array analysis. The conditioned media were hybridized with a human angiogenesis antibody array (RayBiotech), as described in the manufacturer's manual.

VEGF ELISA Analysis. VEGF protein levels in the vector-alone and *CRIP2*-stable clones were detected using the Quantikine Human VEGF immunoassay system (R&D Systems) (4). Absorbance was detected using the Labsystems Multiskan MS Plate Reader (Thermo Fisher Scientific).

Subcellular Fractionation. Subcellular fractionation was performed as described previously (11). In brief, cells were seeded onto a 150-mm culture plate. Cells were then scraped from the plate, and subcellular fractionation was performed to obtain the nuclear, cytoplasmic, and membrane fractions. The α-tubulin and histone were used as positive controls of the cytoplasmic and the membrane and nuclear fractions, respectively.

Coimmunoprecipitation (Co-IP). Cells were seeded on a 150-mm culture plate for each co-IP reaction. The nondenaturing lysis buffer [20 mM Tris HCl (pH 8), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 2 mM EDTA] was used for cell lysis. The cell lysate was then immunoprecipitated with *CRIP2* and IgG antibodies.

Statistical Analysis. The results of in vitro assay represent the arithmetic mean ± SE of triplicate determinations of at least two independent experiments. Student *t*-test was used to determine the confidence levels in group comparisons. The χ^2 and Fisher's exact tests were used to analyze significant differences of *CRIP2* gene expression observed by qPCR. A P value < 0.05 was considered statistically significant.

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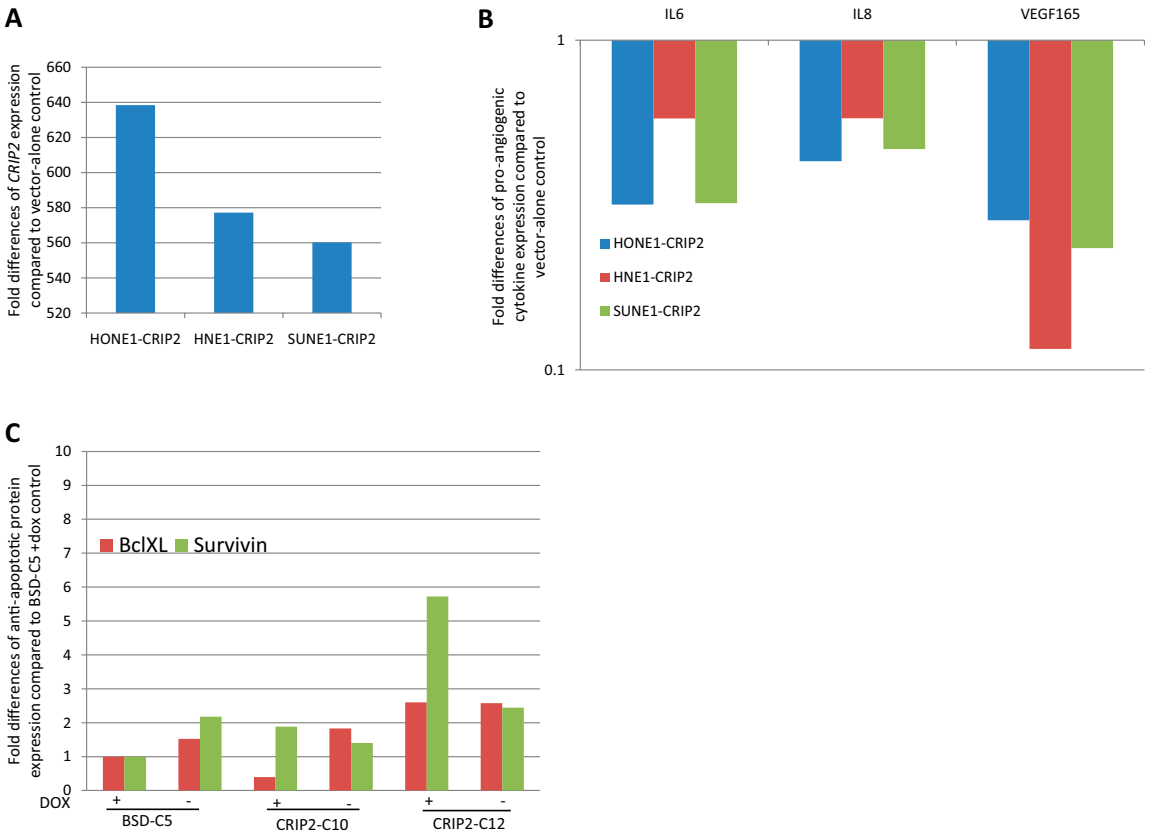

Fig. S1. (*A*) qPCR analysis of *CRIP2* expression in three *CRIP2* down-regulated cell lines after transfection. The expression fold changes were compared with corresponding vector-alone control. (*B*) qPCR analysis of proangiogenic protein expression in transiently transfected NPC cell lines. The expression fold changes were compared with corresponding vector-alone control. (*C*) qPCR analysis of antiapoptotic proteins. The fold changes were compared with the BSD-C5 (+dox) control.

Table S1. Tumorigenicity assays of vector-alone (BSD-C5) and *CRIP2*-expressing clones CRIP2-C9, -C10, and -C12 in nude mice

Transfectant cell lines	Identification	dox	Tumor formation, no. of tumors/no. of sites	Days to appearance of tumors	*P* value*	
BSD-C5	HONE1-2 × pETE-Bsd	+	6/6	21–28	—	
		–	6/6	21–28	0.29	
CRIP2-C9	HONE1-2 × pETE-Bsd-*CRIP2*	+	6/6	14–21	0.221	
		–	4/6	35–42	0.002	0.009†
CRIP2-C10	HONE1-2 × pETE-Bsd-*CRIP2*	+	6/6	14–21	0.05	
		–	0/6	35–42	0.001	0.0004†
CRIP2-C12	HONE1-2 × pETE-Bsd-*CRIP2*	+	6/6	21–28	0.473	
		–	4/6	>42	0.000	0.009†

**P* value obtained by comparison with BSD-C5 (+dox) at 6 wk after injection.
†*P* value obtained by comparison of tumor sizes after treatment with or without dox.

Table S2. qPCR primer sequences of angiogenesis-related genes

Gene	Product size	Annealing temperature, °C		Primer sequence
GAPDH	220	60	Forward:	GAAGGTGAAGGTGGAGTC
			Reverse:	GAAGATGGTGATGGGATTTC
IL6	238	60	Forward:	GATGAGTACAAAAGTCCTGATCCA
			Reverse:	CTGCAGCCACTGGTTCTGT
IL8	242	60	Forward:	GGCCGTGGCTCTCTTGGCAG
			Reverse:	GCACCCAGTTTTCCTTGGGGTCC
VEGF165	74	60	Forward:	TGTGAATGCAGACCAAAGAAAGA
			Reverse:	TGCTTTCTCCGCTCTGAGC
angiogenin	191	60	Forward:	TGTCCTGCCCGTTTCTGCGG
			Reverse:	CCGGCCCTGTGGTTTGGCAT
MCP1	231	60	Forward:	TTCCCCAAGGGCTCGCTCAG
			Reverse:	GGGTTTGCTTGTCCAGGTGGTCC
uPAR	139	60	Forward:	CTGCCCTCGCGACATGGGTC
			Reverse:	TCCCAGGGCGCACTCTTCCA
BclXL	102	60	Forward:	AGCCTTGGATCCAGGAGAA
			Reverse:	AGCGGTTGAAGCGTTCCT
survivin	86	60	Forward:	GCCCAGTGTTTCTTCTGCTT
			Reverse:	CCGGACGAATGCTTTTTATG