Supporting Information

Zhang *et al*. 10.1073/pnas.0809763105

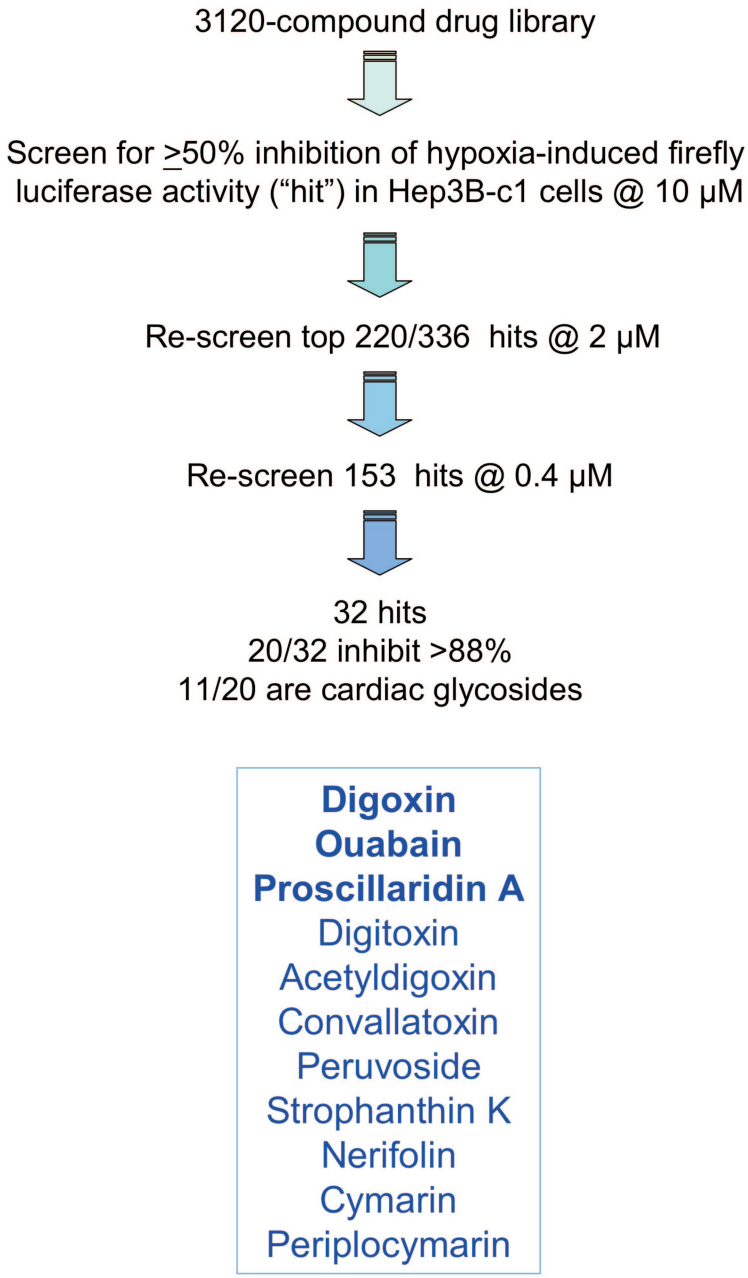

Fig. S1. Screen for drugs that inhibit HIF-1 transcriptional activity. Hep3B cells were stably cotransfected with: p2.1, a plasmid containing firefly luciferase coding sequences downstream of a 67-bp hypoxia response element and the SV40 early region promoter; and pSV-Renilla, a plasmid containing *Renilla* luciferase coding sequences downstream of the SV40 early region promoter. The ratio of firefly/*Renilla* luciferase activity in cells exposed to nonhypoxic (20% O_2) or hypoxic (1% O_2) culture conditions was determined. A drug library was screened for compounds that reduced the firefly/*Renilla* luciferase activity in hypoxic cells, resulting in the identification of 11 members of the drug class known as cardiac glycosides (*Box*) that specifically inhibited HIF-1-dependent transcriptional activity by >88% at a concentration of 0.4 μM. Three members of this class (digoxin, ouabain, and proscillaridin A) were chosen for further study.

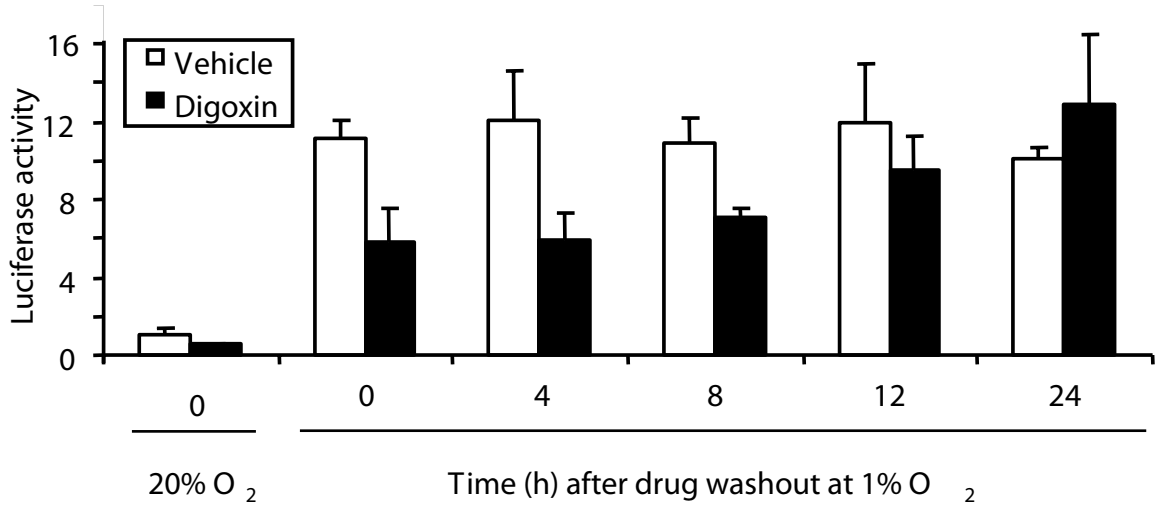

Fig. S2. Restoration of HIF-1 activity after drug washout. Hep3B-c1 cells were incubated at 20% or 1% O_2 for 24 h in the presence of vehicle or digoxin, followed by replacement of media without vehicle or digoxin, respectively, and incubation at the same O_2 concentration for an additional 0–24 h, after which the *Renilla*/firefly luciferase activity ratio was determined. The results were normalized to those from vehicle-treated cells incubated at 20% O_2. (Bars show mean and SD; $n = 3$.)

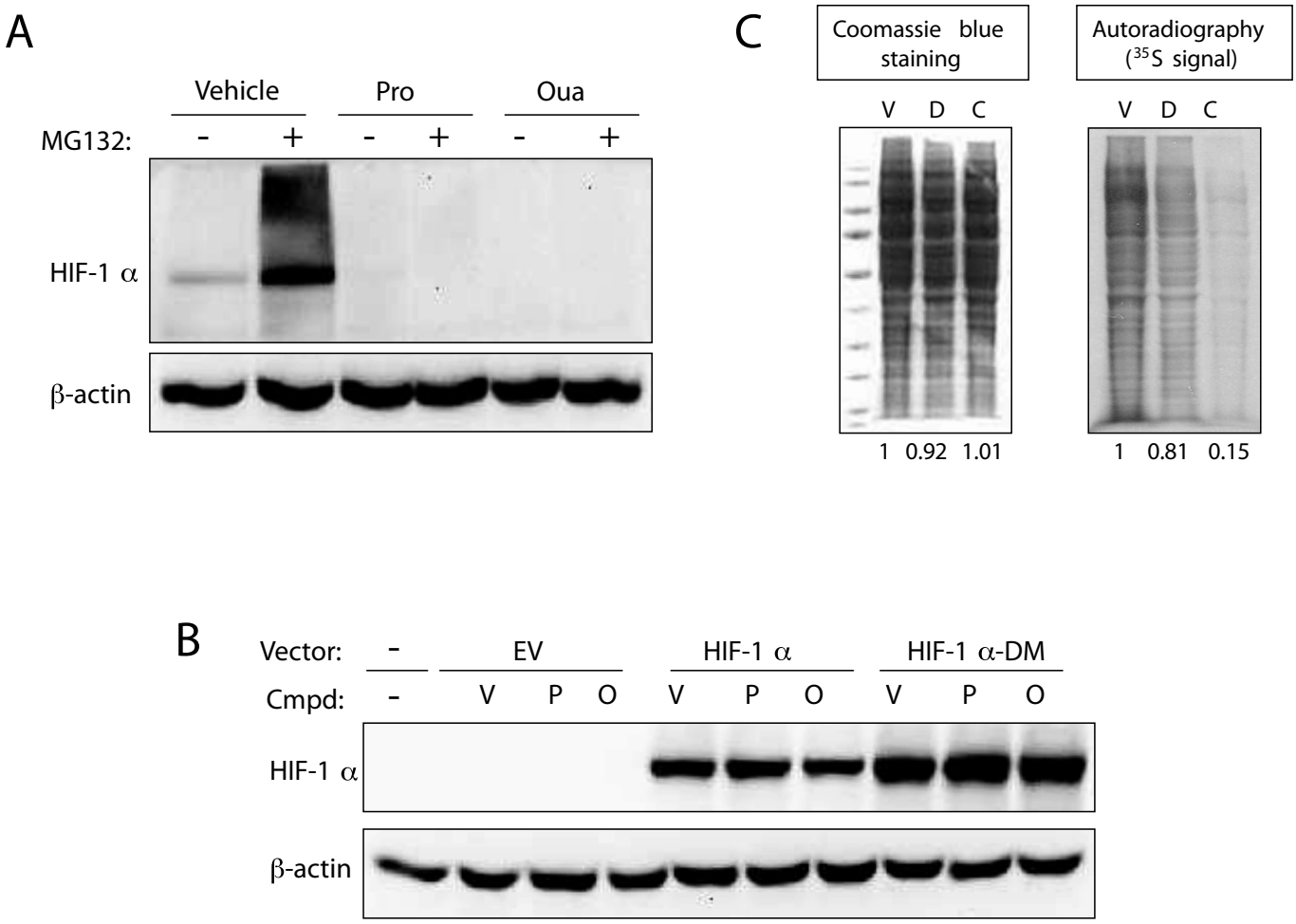

Fig. S3. Cardiac glycosides inhibit HIF-1α protein synthesis. (*A*) Hep3B cells were treated with vehicle, proscillaridin A (Pro), or ouabain (Oua) in the absence (−) or presence (+) of 5 μM MG132 and cell lysates were subjected to immunoblot assays by using anti-HIF-1α and anti-β-actin antibodies. (*B*) 293T cells were transfected with empty vector (EV) or expression vector encoding FLAG epitope-tagged HIF-1α that was wild type or double mutant (DM) because of Pro-to-Ala substitutions at residues 402 and 564 of the protein. The cells were cultured in the presence of vehicle (V), proscillaridin A (P), or ouabain (O) for 24 h, and cell lysates were subjected to immunoblot assays by using anti-FLAG antibody. (*C*) Hep3B cells were pretreated for 4 h with vehicle (V), 100 nM digoxin (D), or 20 μg/ml cycloheximide (C) and [^{35}S]methionine/cysteine was added for 1 h. Aliquots of cell lysate were analyzed for total protein by Coomassie blue staining (*Left*) and for de novo protein synthesis by ^{35}S-autoradiography (*Right*). Signal intensity was determined by densitometry.

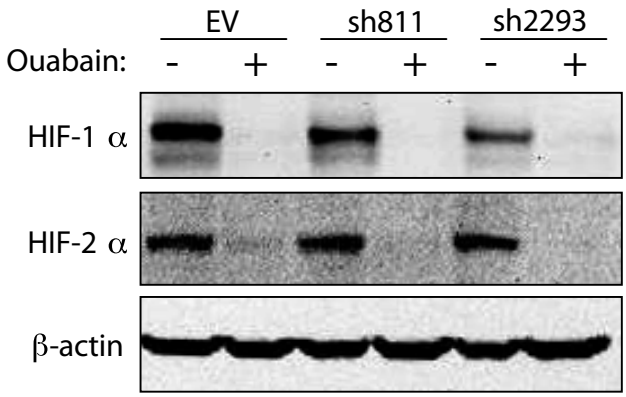

Fig. S4. Na^+/K^+ ATPase α1 subunit knockdown in ouabain-treated cells. 293T cells were transiently transfected with empty vector (EV) or expression vectors encoding two different short hairpin RNAs against the Na^+/K^+ ATPase α1 subunit (sh811, sh2293) and treated with vehicle (−) or 100 nM ouabain at 1% O_2 for 24 h. Whole cell lysates were subjected to immunoblot assays for HIF-1α, HIF-2α, and β–actin.

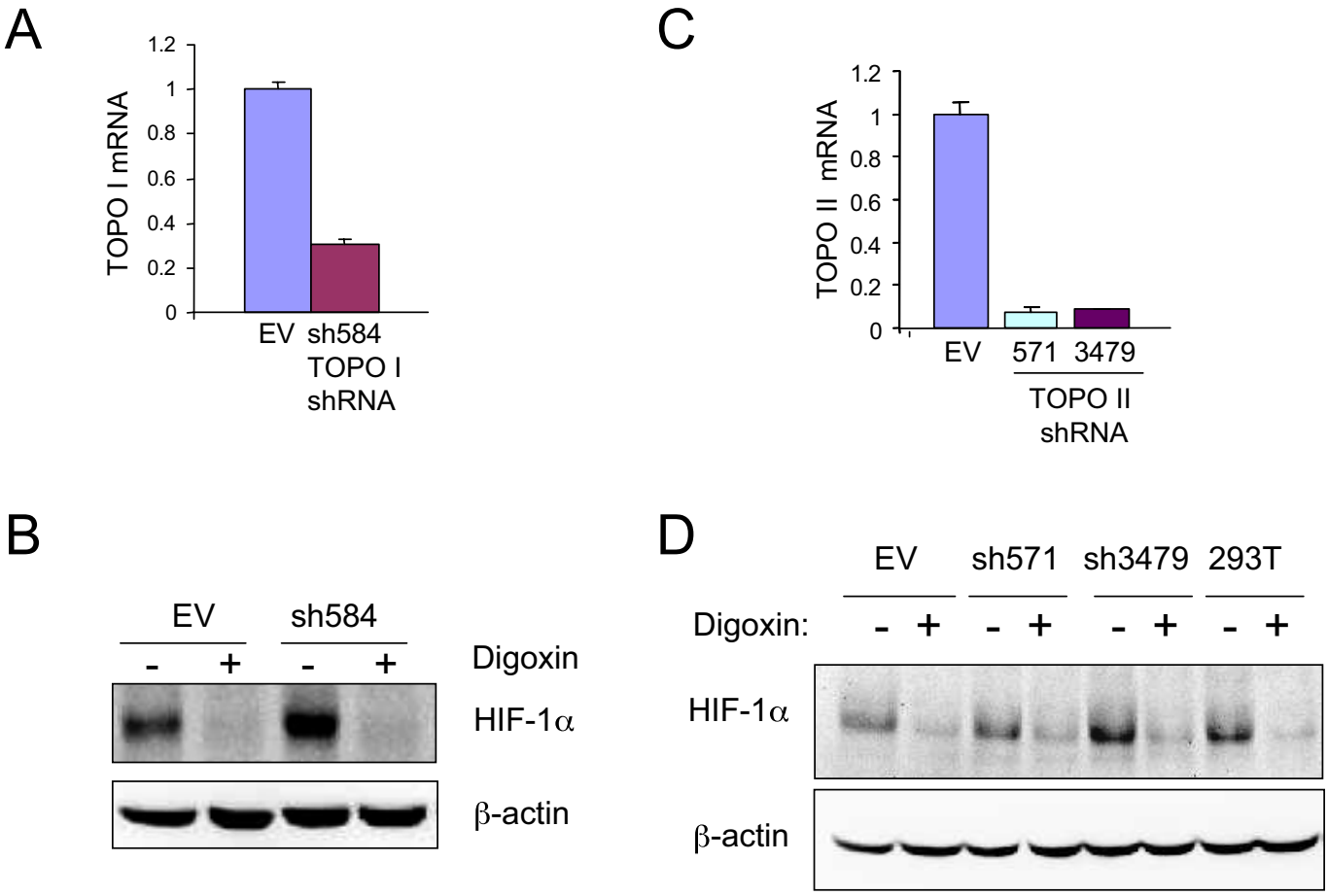

Fig. S5. Knockdown of topoisomerase I or II does not affect HIF-1α levels in the presence or absence of digoxin. 293T cells were transfected with empty vector (EV) or expression vector encoding short hairpin RNA against topoisomerase I (sh584; TOPO I shRNA) or topoisomerase II (sh571 and sh3479; TOPO II shRNA). The expression of TOPO I (*A*) and TOPO II (*C*) mRNA was determined by quantitative real-time RT-PCR 72 h after transfection and normalized to the result from cells transfected with EV. The experiment was repeated and nontransfected 293T cells and transfected cells were treated with vehicle (−) or 100 nM digoxin (+) for the last 24 h at 1% O_2. The effect of TOPO I (*B*) or TOPO II (*D*) knockdown on HIF-1α and β-actin protein expression was determined by immunoblot assay.

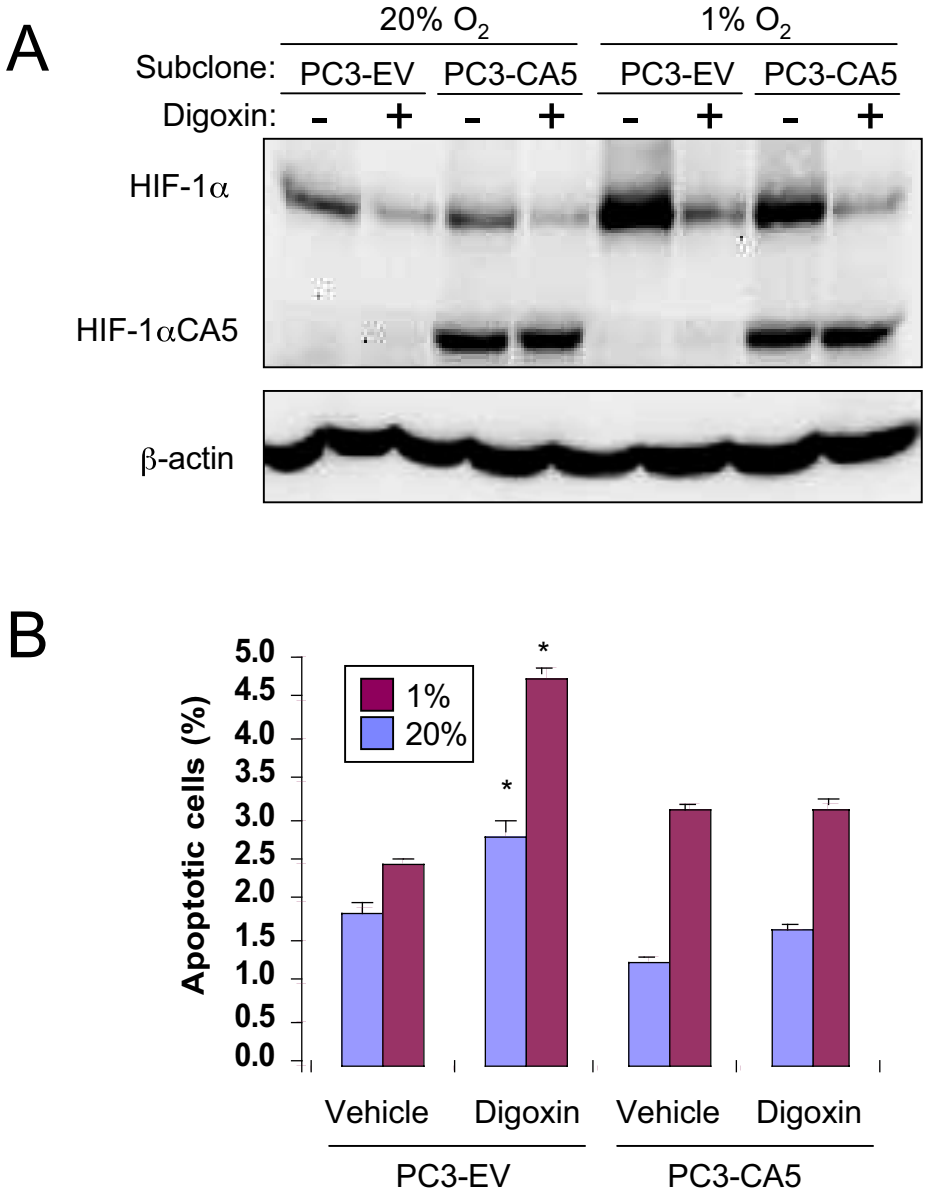

Fig. S6. Effect of digoxin on PC3 cell growth. (*A*) PC3 subclones were cultured for 24 h in the presence of vehicle (−) or digoxin (+) and immunoblot assays were performed. (*B*) PC3 subclones were cultured for 72 h in the presence of vehicle or digoxin, and the percentage of apoptotic cells was determined based on detection of annexin V. (*, $P < 0.05$ vs. vehicle.)

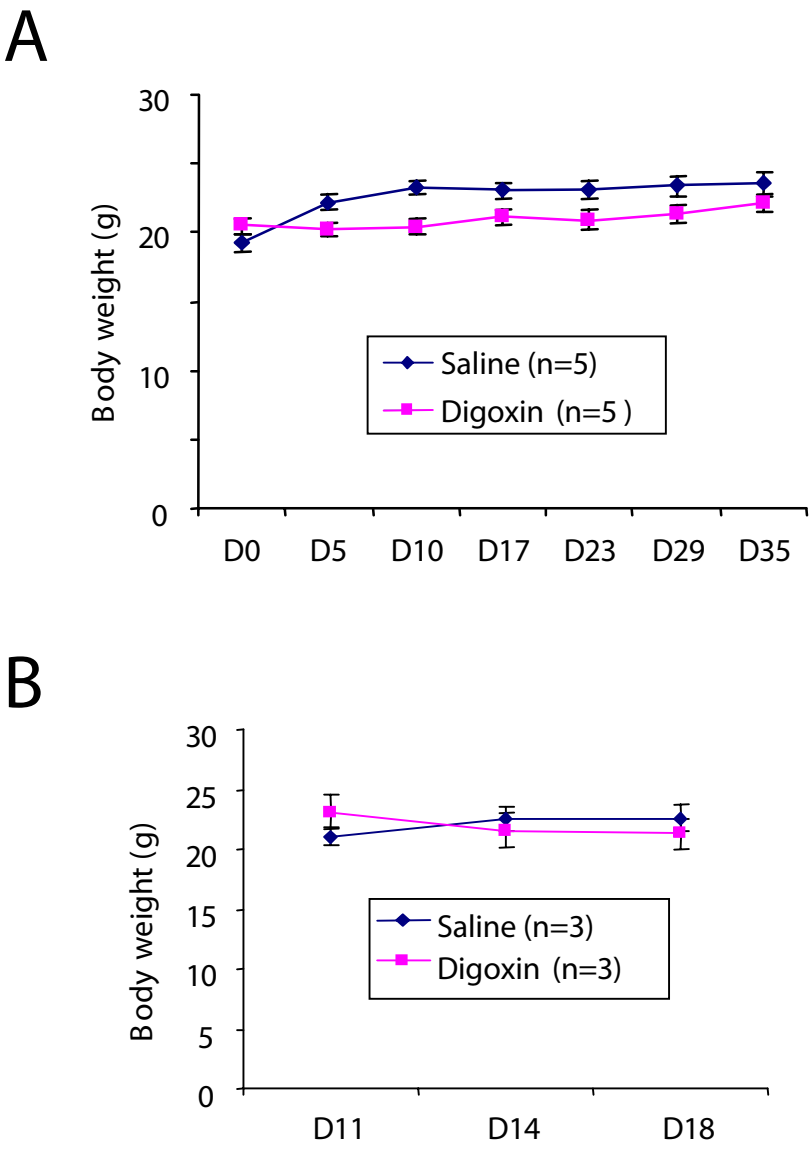

Fig. S7. Analysis of body weight in tumor-bearing mice. The body weight (mean ± SEM) of the mice bearing tumor xenografts, (*A*) analyzed in main text Fig. 7*A* and (*B*) analyzed in main text Fig. 7*D*, are shown.

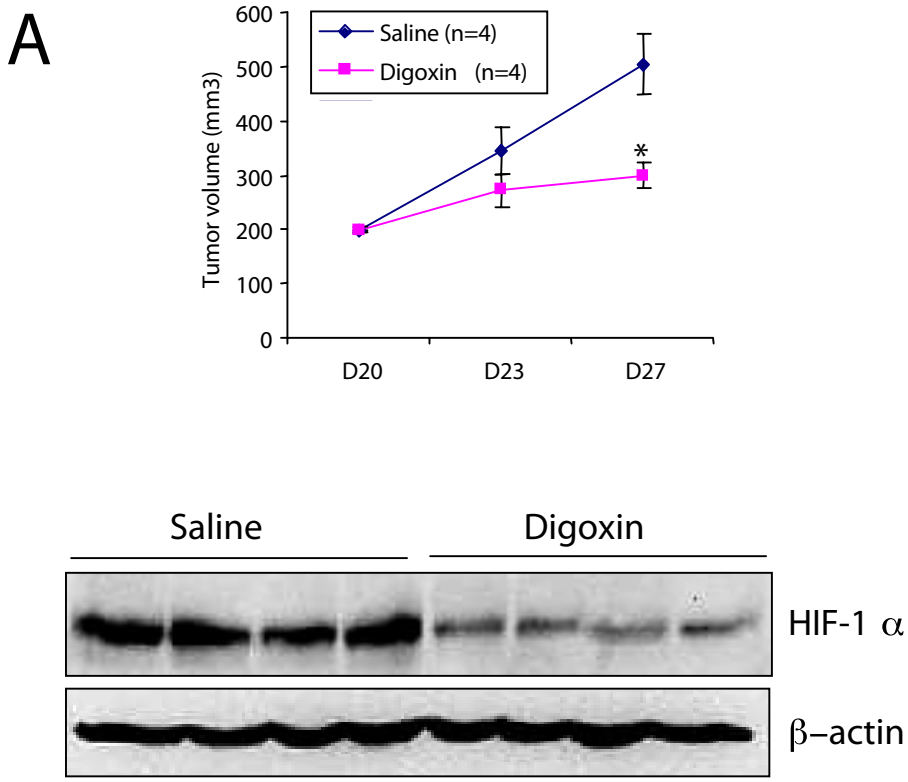

Fig. S8. Delayed treatment of PC3 tumor-bearing mice. Mice were treated from day 20 to day 27 after s.c. implantation of PC3 cells with daily i.p. injections of digoxin (2 mg/kg) or saline ($n = 4$ mice each). Tumor volumes were measured on days 20, 23, and 27 (*A*). The tumors were harvested on day 27, and aliquots of tissue lysates were subjected to immunoblot assays (*B*).

Table S1. Primers for quantitative real-time RT-PCR assays

Name	Nucleotide sequence
Hs-HK1-FWD	TGGAGTCCGAGGTTTATG
Hs-HK1-REV	TTTGGATTGTTGGCAAGG
Hs-HK2-FWD	CCAGTTCATTCACATCATCAG
Hs-HK2-REV	CTTACACGAGGTCACATAGC
Hs-Glut1-FWD	CGGGCCAAGAGTGTGCTAAA
Hs-Glut1-REV	TGACGATACCGGAGCCAATG
Hs-HIF-1α-FWD	CCACAGGACAGTACAGGATG
Hs-HIF-1α-REV	TCAAGTCGTGCTGAATAATACC
Hs-VEGF-FWD	CTTGCCTTGCTGCTCTAC
Hs-VEGF-REV	TGGCTTGAAGATGTACTCG
Hs-TOPO I-FWD	AATGCGAACTTAGGCTGTTACAC
Hs-TOPO I-REV	CTTCTTCTTCACAACATCAAC
Hs-TOPO II-FWD	ATGAACAAGTAAACCACAGG
Hs-TOPO II-REV	GAAACGGCTGAGGCTGCTAC
Hs-ATP1A1-FWD	CTCTGTGCTTTTCTCTCTG
Hs-ATP1A1-REV	CTTCTTTCTTCAGTTCATCC

Table S2. Targeting sequences for short hairpin RNAs

Name	GenBank accession no.	Targeting sequence
HsTOPO I-sh584	NM_003286	CGAAGAAAGAAGAGGAACA
HsTOPO II-sh571	NM_001067	AAACAGACATGGATGGATA
HsTOPO II-sh3479		GAAAGAGTCCATCAGATTT
ATP1A1-sh811	NM_000701	GATTCGAAATGGTGAGAAA
ATP1A1-sh2293		GGTCGTCTGATCTTTGATA