

Hypoxia-induced DNp73 stabilization regulates Vegf-A expression and tumor angiogenesis similar to TAp73

Iqbal Dulloo¹, Phang Beng Hooi¹, and Kanaga Sabapathy^{1,2,3,*}

¹Division of Cellular & Molecular Research; Humphrey Oei Institute of Cancer Research; National Cancer Centre; Singapore; ²Cancer and Stem Cell Biology Program; Duke-NUS Graduate Medical School; Singapore; ³Biochemistry; Yong Loo Lin School of Medicine; National University of Singapore; Singapore

P73, the homolog of p53, exists in 2 major forms: either as a pro-apoptotic TAp73 or an amino-terminally truncated DNp73, the latter lacking the first transactivation domain. While TAp73s tumor suppressive functions have been established, DNp73 is an anti-apoptotic protein conferring chemoresistance and is associated with poor survival. However, both forms are variably overexpressed in many human cancers. In this context, we have recently demonstrated that TAp73 is stabilized by hypoxia, a tumor-relevant condition that is associated with cell survival, via HIF-1 α -mediated suppression of Siah1 E3 ligase that degrades TAp73. Consequently, hypoxic signals lead to TAp73-mediated activation of several angiogenic genes and blood vessel formation, thereby supporting tumorigenesis. We show here that, similar to TAp73, DNp73 is stabilized by hypoxia in a HIF-1 α -dependent manner, which otherwise is degraded by Siah1. Moreover, DNp73 is capable of inducing the expression of Vegf-A, the prototypic angiogenic gene, and loss of DNp73 expression results in reduction in tumor vasculature and size. These data therefore indicate a common mode of regulation for both p73 forms by hypoxia, resulting in the promotion of angiogenesis and tumor growth, highlighting common functionality of these antagonistic proteins under specific physiological contexts.

Introduction

Homology among proteins often reflects common functionality, though

structural variations due to truncations or addition of other domains could not only lead to loss of such common functions, but also to the acquisition of novel or even opposing ones. One group of proteins with such characteristics is the p53 family of tumor suppressors, which include the p63 and p73 proteins.^{1,2} Among them, while p53 is a classical tumor suppressor, p73 exists as either a tumor suppressor (the TAp73 form), or as the anti-apoptotic DNp73 form, the latter due to truncation of the amino-terminal transactivation domain.³ Expectedly, TAp73 has been shown to exhibit tumor suppressive properties in both cellular and animal model systems, whereas DNp73 expression is often associated with resistance to apoptosis and therapy, due to its ability to inhibit both p53 and TAp73.^{4,5} Consistently, cell death-inducing stress signals often lead to the stabilization and activation of TAp73, with the concomitant destabilization of DNp73,⁶⁻⁸ thus allowing apoptosis to ensue. However, clinical data are not entirely consistent with TAp73 being a classical tumor suppressor. Both TAp73 and DNp73 are often overexpressed in a variety of human cancers, without being mutated.^{4,9-12} Two possible scenarios could explain these clinical observations: firstly, DNp73 could negate the tumor suppressive properties of TAp73 when both are co-overexpressed, thereby providing a survival advantage. However, this reasoning does not explain all the cases where only TAp73 may be overexpressed.^{9,10} Alternatively, both TAp73 and DNp73 may have other common functions consistent with the promotion of carcinogenesis. We and others have previously demonstrated that TAp73

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© Iqbal Dulloo, Phang Beng Hooi, and Kanaga Sabapathy

*Correspondence to: Kanaga Sabapathy; Email: cmrksb@nccs.com.sg

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can indeed support cellular survival in combination with activator-protein (AP)-1 factors such as c-Jun, that are often activated in cancers,^{13,14} and via the transcriptional activation of genes involved in G1-S and G2-M progression,¹⁵ suggesting that contextual settings could dictate TAp73s functions. Additionally, growth promoting signals such as serum stimulation has been shown to activate TAp73.¹⁶ We have therefore been investigating if other physiologically relevant stimuli can promote p73s pro-proliferative properties. In this context, we have recently shown that hypoxia, a condition that is prevalent in cancers and contributes to chemo-resistance and cellular survival,¹⁷⁻¹⁹ leads to the stabilization of the TAp73 form, which allows the activation of the angiogenic transcriptional program to promote angiogenesis, and hence supports tumorigenesis.²⁰ Given that the DNp73 form

shares high sequence and structural homology to TAp73 and its role in cellular survival, we have examined if DNp73 is also subjected to comparable regulation by hypoxia, and its influence on angiogenesis. The results suggest a common mode of activation with similar consequential effects on tumor angiogenesis and growth.

Results

DNp73, like TAp73, is stabilized by hypoxia

We first determined if DNp73 could be stabilized by hypoxia. Since endogenous DNp73 expression is not detectable by conventional methods, we transfected the plasmids of both the α and β forms of TAp73 and DNp73 in p53 null H1299 cells followed by overnight exposure to

hypoxia – ascertained by the upregulation of *vegf-A* and *glut-1*, key hypoxic target genes – which led to a significant increase in steady-state of both these forms (Fig. 1A), that occurred in a time-dependent manner (Fig. 1B). Similar effects were observed by treatment with deferoxamine (DFX) or dimethylxaloylglycine (DMOG), prolyl hydroxylase inhibitors that mimic hypoxic conditions (Fig. 1C). Elevation of DNp73 β following hypoxia correlated with increased half-life ($T_{1/2}$) from ~6 h to >9 h as measured by cycloheximide pulse-chase (Fig. 1D), similar to TAp73 as reported earlier.²⁰ Moreover, the hypoxia-mediated upregulation of DNp73 β was gradually reversed upon re-oxygenation (Fig. 1E), highlighting the oxygen-mediated regulation of DNp73 expression. These data therefore demonstrate that hypoxia stabilizes DNp73, in a similar fashion to TAp73.

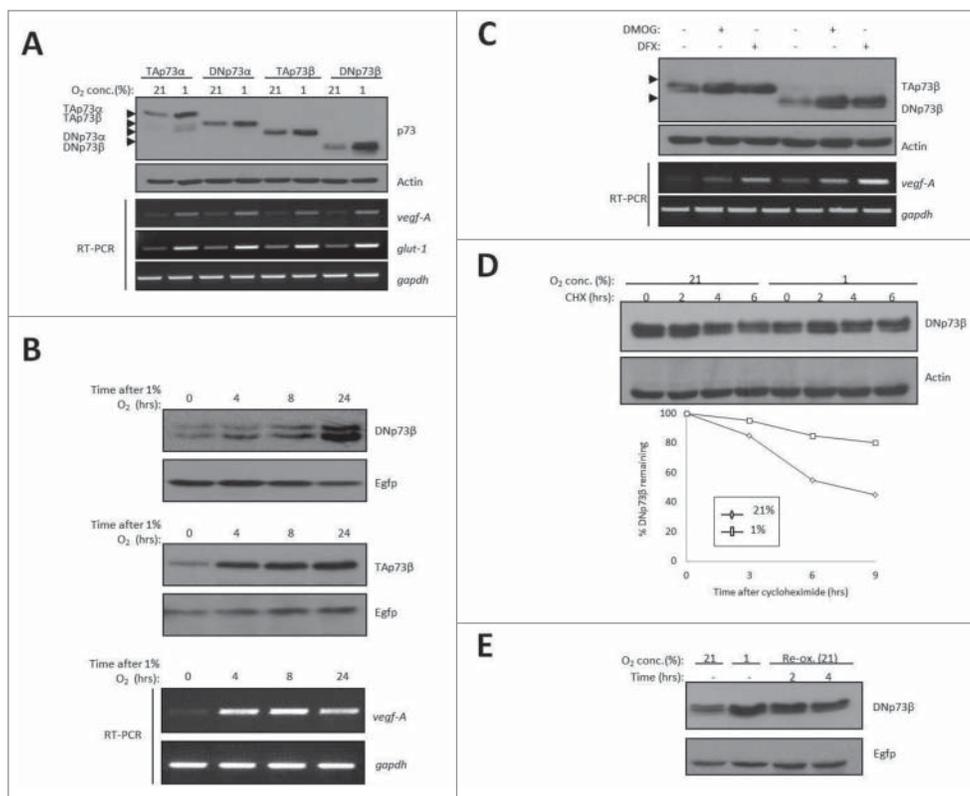


Figure 1. Hypoxia. Induces the stabilization of DNp73. (A-C) Levels of transfected DNp73 α and DNp73 β , as well as TAp73 α and TAp73 β in H1299 cells were assessed by immunoblot upon exposure to 1% O₂ overnight (A) or for the indicated time points (B), or in the presence of DMOG (1 mM) and DFX (250 μ M) (C). Egfp levels are shown for equal transfection efficiency. Endogenous *vegf-A* and *glut-1* expression relative to *gapdh* was measured in the same experiment by real-time qPCR analysis (lower panels). Arrowheads indicate position of the respective p73 forms. Part of (A) is reproduced from Dulloo et al., 2015.²⁰ (D) Half-life of DNp73 β in H1299 cells was measured in the presence of 1% O₂ upon addition of 50 μ g/ml cycloheximide (CHX) for the indicated time-periods (upper panel) and quantified (lower panel). (E) Levels of transfected DNp73 β in H1299 cells were assessed upon exposure to 1% O₂ for 24 hours, and upon re-oxygenation (Re-ox.) at 21% O₂ for 2 hours and 4 hours.

DNp73 stabilization is dependent on HIF-1 α and SIAH1

As TAp73 was regulated by the HIF-1 α /Siah1 axis,²⁰ and given the high similarity of both these major p73 forms, we next examined if this will also be the case with DNp73. However, to rule out the involvement of cullin-based Von Hippel-Lindau (VHL) E3 ligase complex, which is a critical regulator of HIF-1 α expression,²¹ we utilized cells inducibly expressing the dominant-negative C111S mutant Nedd8-conjugating enzyme Ubc12 (dnUbc12), capable of inhibiting all cullin-based ligases,²² as shown by the increase of endogenous p27 levels (Fig. 2A), in which DNp73 was transfected. Like TAp73, hypoxia-mediated upregulation and re-oxygenation-mediated reversal of DNp73 β steady-state levels were not affected by dnUbc12 expression, ruling out a role for VHL in DNp73 regulation by hypoxia. We next tested the role of HIF-1 α in DNp73

stabilization. Silencing of *hif-1 α* led to a decrease in the levels of DNp73 even in normoxic conditions, and the increase upon hypoxia was compromised, similar to TAp73 (Fig. 2B). In addition, co-expression of wild-type Siah1 (Siah1-WT), but not the catalytic mutant (Siah1- Δ RING), with DNp73 β led to the marked reduction of the latter's steady-state levels (Fig. 2C). Moreover, silencing of *siah1* upregulated the basal level of DNp73 and rescued the decrease in its level observed in the presence of *hif-1 α* siRNA (Fig. 2D). Thus, these data indicate that DNp73 is stabilized by HIF-1 α , which acts upstream of Siah1 to negate its destabilizing activity on DNp73 under hypoxic conditions.

DNp73 regulates Vegf-A expression and angiogenesis

Consequential effect of DNp73 stabilization by hypoxia was evaluated by the

analysis of *vegf-A* expression, the prototypic angiogenic gene regulated by hypoxia. Real-time qPCR analysis of endogenous *vegf-A* upon transfection of various p73 plasmids revealed that similar to TAp73 β and HIF1- α , DNp73 β was capable of inducing *vegf-A* expression (Fig. 3A). However, DNp73 β was incapable of transactivating *Mdm2* efficiently, unlike TAp73 β , highlighting that the amino-terminal transactivation domain of TAp73 might not be involved in *vegf-A* regulation. Similar results were obtained using the *vegf-A* promoter linked to a luciferase reporter gene (Fig. 3B). To evaluate if DNp73 binds to the same site as TAp73 on *vegf-A* promoter,²⁰ we performed chromatin immunoprecipitation using anti-p73 antibody in cells transfected with either TAp73 β or DNp73 β . As shown in Figure. 3C, there was specific binding of both TAp73 β and DNp73 β onto the same site on the *vegf-A* promoter, which does not contain a HIF1- α binding

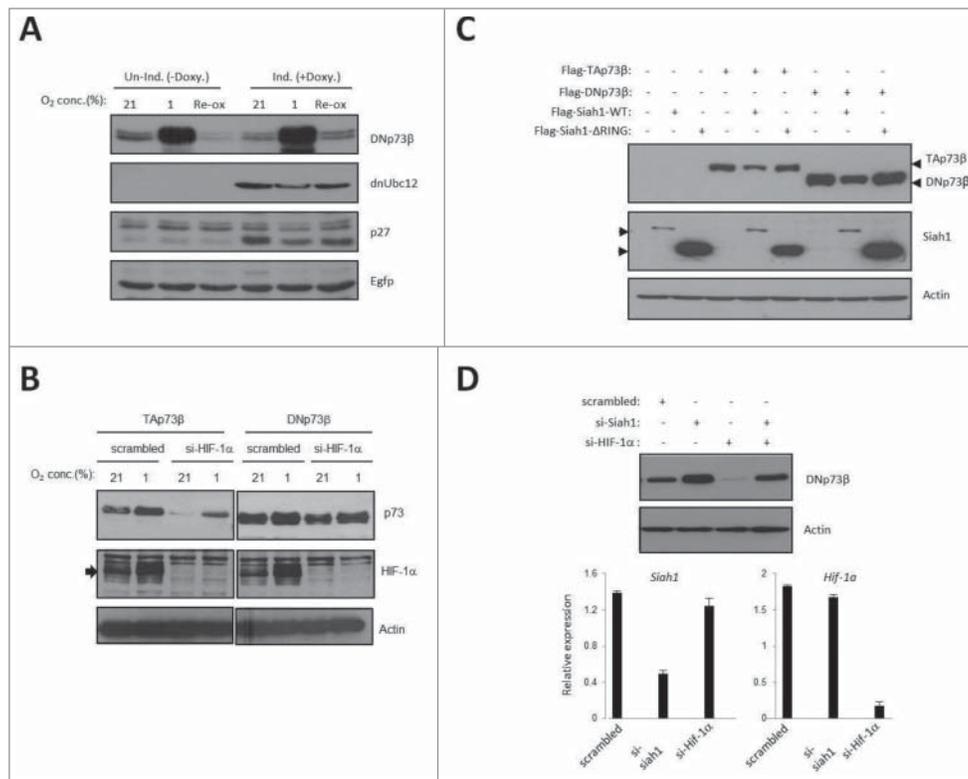


Figure 2. HIF-1 α and Siah1 regulate DNp73 levels. **(A)** Effects of 1% O₂ and re-oxygenation (Re-ox.) with 21% O₂ on transfected DNp73 in HEK293 cells inducibly expressing the dominant negative form of Ubc12 (dnUbc12) were assessed, as described.²⁰ **(B)** Levels of TAp73 β and DNp73 β upon hypoxia were determined after silencing the expression of *hif-1 α* in H1299 cells. Arrowhead indicates HIF-1 α . **(C)** Effects of co-expression of either wild-type or Δ RING mutant Siah1 on TAp73 β and DNp73 β levels in H1299 cells are shown. Arrowheads indicate position of the respective p73 forms. Arrowheads indicate position of the respective Siah proteins. **(D)** Effect of siRNA-mediated *siah1* silencing alone or after co-silencing with *hif-1 α* on transfected DNp73 β levels in H1299 cells. Efficiency of knockdown for both siRNAs is shown by real-time qPCR in the lower panel.

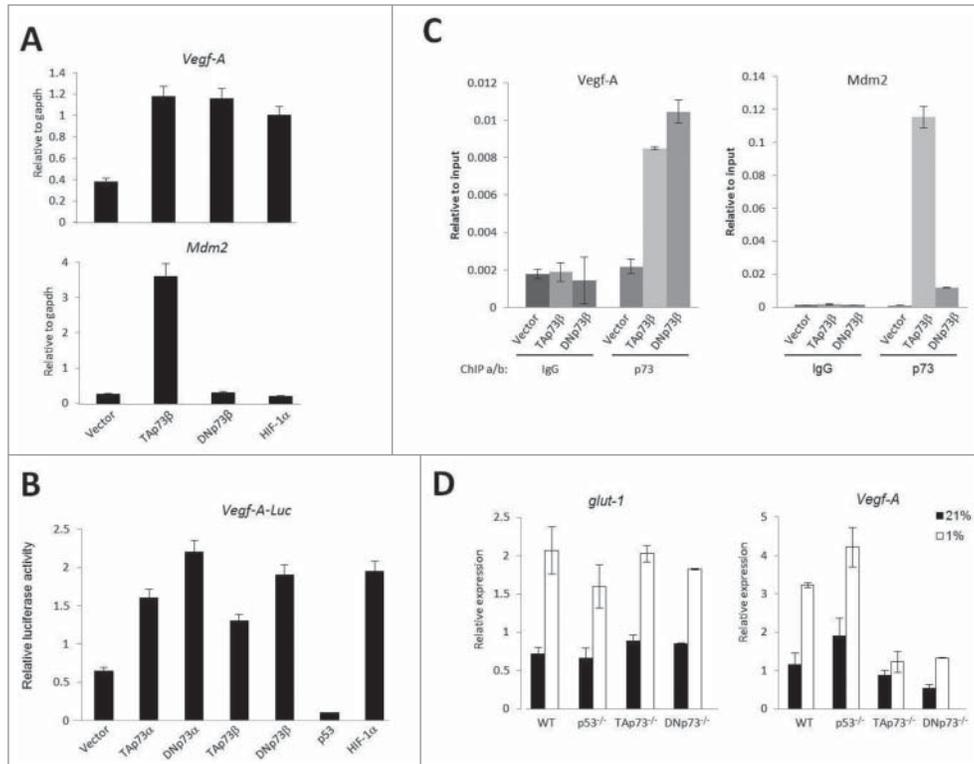


Figure 3. Vegf-A expression is induced by DNP73β. (A) *Vegf-A* and *Mdm2* levels were determined by real-time qPCR analysis after transfection of indicated plasmids in H1299 cells. (B) Effect of indicated plasmids on Vegf-A promoter luciferase construct (Vegf-A-Luc) was assessed by luciferase assay in H1299 cells. (C) Chromatin immunoprecipitation (ChIP) analyses with anti-p73 and anti-IgG antibodies on promoters of *Vegf-A* and *Mdm2* were carried out using H1299 cells inducibly expressing either TAp73β or DNp73β after 8 hours induction. Real-time qPCR was used to quantify the fragments pulled-down with anti-p73 and IgG antibodies, relative to input. (D) *Vegf-A* and *glut-1* levels were determined by real-time qPCR analysis in wild-type, p53^{-/-}, TAp73^{-/-} and DNp73^{-/-} MEFs upon exposure to 1% O₂ for 4 hours.

site,²⁰ whereas TAp73β was much more proficient in *Mdm2* promoter binding than DNp73. Furthermore, endogenous *vegf-A* expression in mouse embryonic fibroblasts lacking DNp73 was reduced both at the basal level and particularly upon exposure to hypoxia (Fig. 3D), without any effect on *glut-1* expression, altogether highlighting DNp73's role in the specific regulation of *vegf-A* expression, likely in a manner independent of HIF1-α promoter binding site.

We finally examined the effects of DNp73 absence on *in vivo* tumor angiogenesis. Using E1a/Ras transformed DNp73^{-/-} and DNp73^{+/-} MEFs, which were injected into nude mice, we observed a reduction in the tumor volume/size due to the absence of DNp73 (tumor size [mm³] - DNp73^{-/-} vs. DNp73^{+/-} MEFs: 384.91 vs. 1031.37) (Fig. 4A). Histological analysis of these tumors for CD34 staining to detect blood vessels revealed a correspondingly significant

reduction in blood vessel density DNp73 (Fig. 4B). Collectively, these data indicate that similar to TAp73, DNp73 is also an important regulator of *vegf-A* expression and efficient blood vessel formation in tumors.

Discussion

The data presented here, together with that in our recent publication,²⁰ alludes to a scenario in which both TAp73 and DNp73 are stabilized by hypoxia through a mechanism involving HIF-1α-mediated Siah1 suppression, leading to the subsequent activation of *vegf-A* and an array of angiogenic genes, thereby resulting in enhanced tumor blood vessel formation. These data thus highlight a common pro-survival role for both these p73 forms, which in other contexts such as in response to DNA damage,²³⁻²⁵ are differentially regulated to perform opposite

functions on cell survival. It is interesting to note that although TAp73 is often induced by multiple stress signals leading to its stabilization, DNp73 is often degraded by similar stimuli,²³⁻²⁵ with the net effect of controlling cellular proliferation and inducing death. Therefore, it has always been expected that this inverse correlation is maintained for the tumor suppressive effects of TAp73 to be felt. However, human tumors often exhibit elevated TAp73, DNp73 or both, insinuating that other possible scenarios can occur. Our data indicate that tumor-associated hypoxia is a condition that is exceptional to the reciprocal regulation of TAp73 and DNp73; positively regulating both p73 forms, thereby supporting survival.

It is noteworthy that upregulation of both these members by hypoxia lead to transactivation of the canonical angiogenic gene, *vegf-A*. Although DNp73 lacks the amino-terminal transactivation domain,^{1,3} it has been shown to retain some

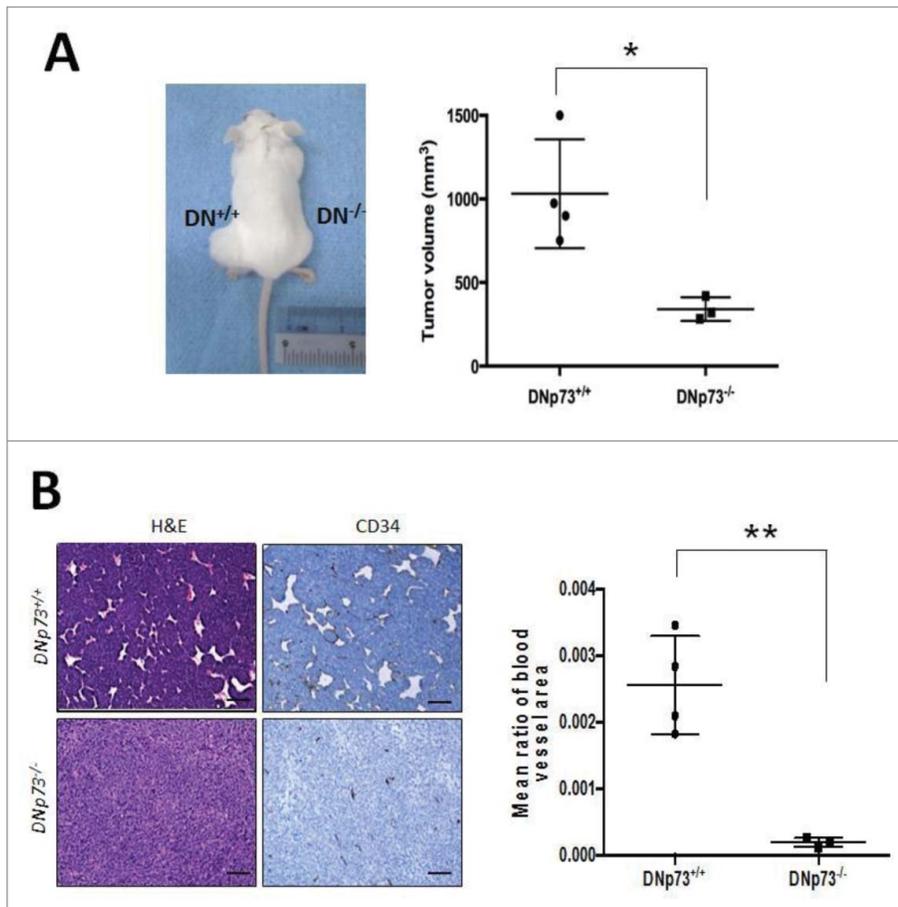


Figure 4. DNp73 supports tumor formation and angiogenesis. **(A and B)** SCID mice were injected subcutaneously on either flanks with E1a/Ras transformed DNp73^{+/+} and DNp73^{-/-} cells and tumors were analyzed for tumor growth and blood vessel formation. Graph shows the size of tumors derived from each mouse [each dot represents a mouse/tumor; n = 4 (WT), n = 3 (KO)] with a representative mouse shown **(A)**. Tumors were stained for H&E and CD34 **(B)**. Scale bars: 100 μ m. Image is representative of all tumors analyzed. *p < 0.05 **p < 0.01.

transactivation potential due to the presence of a putative second transactivation domain, which includes the N-terminal 13 unique amino acid residues and the PXXP motif, and was shown to weakly activate targets such as *p21*, *14-3-3* and *Gadd45*.²⁶ Additionally, it has been reported that the C-terminal end of p73 within amino acid residues 381 to 399, common in both TAp73 and DNp73, can function as a putative transactivation domain required for preferential regulation of genes involved in cell cycle progression.²⁷ We have also recently shown that both TAp73 and DNp73 are capable of inducing the transactivation of caspase-2S, an anti-apoptotic member of the caspase family,²⁸ alluding to a distinct transactivation domain common to both these forms that is required for the regulation of

specific target genes. An interesting aspect that emerges from these findings is that the dominant-negative effect of DNp73 on TAp73 might be lost in growth-promoting conditions such as hypoxia. Whether stimuli-specific protein modifications and/or recruitment of interactors enable this phenomenon is currently under investigation.

A recent publication using cells deficient for both TAp73 and DNp73 also alluded to the requirement for p73 in vasculogenesis, especially in the context of development.²⁹ Moreover, knockdown of DNp73 relative to that of TAp73, was noted to have a more profound effect, supporting the data that both forms contribute to the angiogenic process, perhaps to varying degrees. However, loss of either TAp73 or DNp73 alone appears to be

sufficient to retard tumor growth and angiogenesis,^{20,30} suggesting that while both proteins have a role in promoting angiogenesis, they may not be able to compensate for the loss of the other. This alludes to the possibility of TAp73 and DNp73 having both overlapping as well as distinct targets that are critical in regulating the angiogenic process, highlighting probable non-redundant functions of TAp73 and DNp73 in tumorigenesis, as well as non-overlapping functions that could be spatially and temporally controlled. Nonetheless, common growth promoting signals appear to similarly regulate both the major p73 forms, to enable the concerted activation of target genes to promote survival, such as in cancers. Thus, understanding the similarity and dichotomy in the regulation of both these forms under specific physiological contexts will enable better strategies to be developed for their modulation in pathological contexts.

In conclusion, work from us and others demonstrate a common role for both the TAp73 and DNp73 forms in promoting tumor growth and angiogenesis, suggesting that these proteins could serve as targets as well as a biomarker for anti-angiogenic therapy.

Materials and Methods

Cells and reagents

Cells lines used were as follows: human cell lines - p53 null H1299 lung adenocarcinoma cells; H1299 cells stably expressing TAp73 β or DNp73 β ; HEK293 human embryonic kidney cells that stably express the mutant dnUbc12-HA under tet-on system. Mouse fibroblasts - DNp73^{+/+} and DNp73^{-/-} (gift from Dr. Tak Mak) stably expressing E1a and Ras constructs, p53^{-/-} and wild-type MEFs. Two μ g/ml of doxycycline (Sigma-Aldrich) was used for induction. All cells were tested for mycoplasma contamination. Chemicals used: dimethylxaloylglycine, deferoxamine (Sigma-Aldrich).

Hypoxia was induced by incubating cells in a modular incubator chamber (Billups-Rothenberg) containing 1% O₂ at 37°C for 24 hours, unless otherwise stated. Re-oxygenation was carried out by

placing the hypoxic cells back at 21% O₂, for indicated time points. For half-life determination under hypoxia, medium containing 50 µg/ml cycloheximide was de-gassed 2 hours before adding to hypoxic cells.

Plasmids, siRNAs and transfection

Plasmids used in this study: pCDNA3.1, Flag-TAp73β, TAp73β, Flag-DNp73β, DNp73β, p53, β-gal, Egfp. Vegf-A-luc was a gift from Dr. Fatima Mehta-Grigoriou. Wild-type *Siah1* and ΔRING mutant *Siah1* Flag-tagged plasmids were gifts from Dr. Shu-Ichi Matsuzawa. Two × 10⁵ cells were transfected with the various plasmids (0.2–0.5 µg of DNA) using Lipofectamine Plus-Reagent (Life Technologies). Cells were generally collected 24 hours after transfection for luciferase analyses and immunoblotting. siRNA transfections were performed using Transmessenger transfection kit (Qiagen) and cells were harvested 72 hours post transfection. Target sequence of siRNAs used: human *hif-1α*: 5' CTAACGGACA-CAGTGTGT 3'; human *Siah1*: 5'GATAG GAACACGCAAGCAA 3'.

Luciferase assay

Cells were transiently transfected with 0.2 µg of the relevant plasmids along with the reporter construct (Vegf-A-luc) and 0.1 µg of β-galactosidase construct to normalize for transfection efficiency. Luciferase assays were performed in triplicates as described.¹³

Real-time qPCR analyses

Total RNA was prepared from cells using TRIZOL Reagent (Life Technologies) and converted into single-strand cDNA using Superscript II Reverse Transcriptase (Life Technologies). Real-time quantitative RT-PCR (qPCR) was carried out using Rotor-Gene 6000 (Corbett Life Science) and Maxima[®] SYBR Green (Fermentas) according to manufacturers' protocol. Primers for RT-PCR used in this study are: mouse/human- GLUT1-Fwd: 5' TGGATGTCCTATCT GAGCATCGTG 3'; GLUT1-Rev: 5' CTTTGAAGTAGGTGAAGATGAA GA 3'; VEGFA-Fwd: 5' ATGC-CAAGTGGTCCCAGGCTGCAC 3'; VEGFA-Rev: 5' TGTGCTGTAG-GAAGCTCATCTCTC 3'. human-

MDM2-Fwd: 5' GAATCATCGGACT-CAGGTACATC 3'; MDM2-Rev: 5' TCTGTCTCACTAATTGCTCT CCT 3'.

Immunoblotting

Immunoblot analysis was performed essentially as described.⁶ Typically, 50 µg of protein lysate were used for immunoblot. Detection was done using enhanced Chemiluminescent reagent (Amersham) or Super signal West Dura (Pierce). Antibodies used in this study are: anti-p73 (ER15; 1:1000; Calbiochem), anti-p73 (GC15; 1:1000; Calbiochem), anti-FLAG (M2; 1:2000; Stratagene), anti-p27 (C-19; 1:500; Santa Cruz Biotechnology), anti-Actin (AC-74, 1:5000; Sigma-Aldrich), anti-Egfp (B-2; 1:2000; Santa Cruz Biotechnology), anti-HIF-1α (1:250; BD Biosciences).

Chromatin immunoprecipitation

Inducible cells were fixed with 1% formaldehyde for 10 mins at room temperature, and the reaction was stopped by addition of glycine to final concentration of 125 mM for 15 mins. Cells were then processed as described.²⁰ For all CHIP experiments, real-time qPCR analyses were performed. Relative occupancy values were calculated after normalization with CHIP input DNA. All CHIP experiments were repeated at least 3 times. The primers used for real-time qPCR to quantify the CHIP-enriched DNA are: MDM2-Fwd: 5' GATCGCAGGTGC-CTGTCCGGTCACTA 3'; MDM2-Rev: 5' GGTCTACCCTCCAATCGCCACT-GAACACA 3'; -1162-VEGFA-Fwd: 5' CTACAGACGTTCCCTTAGTGCTG 3'; -800-VEGFA-Rev: 5' CTCATCTGGC-CTGCA GACATCA 3'

Mouse xenograft studies

E1a/RAS transformed MEFs (2 × 10⁶ per 0.1 ml of PBS) were injected subcutaneously into both the left and right flanks of 5-weeks old immunodeficient SCID female mice. Tumors were allowed to grow for 5 weeks and tumor volume was measured prior to harvest (max tumor size of 1500 mm⁻³). Tumors were formalin-fixed and used for immunohistochemistry studies.

Numbers of mice for each group are indicated in figure legends. Tumor volume was calculated with the equation V (in mm⁻³) = $a \times b^2 \times 0.5$, where a is the longest diameter and b is the shortest diameter. All animal experiments were carried with the approval of the Singhealth Institutional Animal Care and User Committee (IACUC).

Immunohistochemistry

Paraffin sections were processed using Bond Epitope Retrieval Buffer, Bond Leica Automated Staining and Bond Refine Kit (Leica Microsystems). Antibody used for mouse tissues: anti-CD34 (MEC14.7; Santa Cruz Biotechnology), at a dilution of 1:100. All slides were counterstained with haematoxylin. Species- and subtype-matched antibodies were used as negative controls.

Quantification of blood vessels size was done using ImageJ software. Values were calculated based on the mean size of 5 randomly chosen CD34-stained blood vessels for each tumor in each genotype and based on pixel numbers.

Statistical Analysis

Results were expressed as mean ± standard deviation (s.d.) of 3 or more biological replicates and representative data are shown from 3 independent experiments, unless otherwise indicated. Student's t -test was used to determine if the observed difference was statistically significant. All analyzes were done using GraphPad Prism. * $p < 0.05$; ** $p < 0.01$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author Contributions

ID designed the experiments, performed the research work and analyzed the data. BHP performed the research work and analyzed the data. KS designed the experiments, analyzed the data, supervised the project and wrote the manuscript with the co-authors input. All authors reviewed the manuscript.

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