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Down syndrome suppression of tumor growth and the role of the calcineurin inhibitor DSCR1

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Abstract

The incidence of many cancer types is significantly reduced in individuals with Down syndrome1–4 and it is proposed that this broad cancer protection is conferred by the elevated expression of one or more of the 231 supernumerary genes on the extra copy of chromosome 21. One such gene is the Down syndrome candidate region-1 (*Dscr1*, *RCAN1*), which encodes a protein that suppresses vascular endothelial growth factor (VEGF)-mediated angiogenic signalling via the calcineurin pathway5–10. Here we show that DSCR1 is elevated in Down syndrome individuals and a mouse model of Down syndrome. Further, we show that the modest elevation in

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Author Contributions

K.H.B., A.Z., R.C.L. and C.B. performed the experiments, analyzed the data and generated the figures. I.H.P., M.W.L performed experiments. Y.O., R.S., T.M., W.C.A. and Z.G. generated the transgenic mouse models in this manuscript and analyzed data. G.Q.D. designed experiments and analyzed data. S.S.Y., J.R.K. and R.R. contributed to pilot experiments. J.F. and S.R. generated the hypotheses. S.R. designed and performed experiments, analyzed the data and wrote the manuscript. K.H.B., A.Z., R.C.L. contributed equally to this study.

expression afforded by a single extra transgenic copy of *Dscr1* is sufficient to confer significant suppression of tumor growth in mice and that such resistance is a consequence of a deficit in tumor angiogenesis arising from suppression of the calcineurin pathway. We also provide evidence that attenuation of calcineurin activity by DSCR1 together with another chromosome 21 gene DYRK1A, may be sufficient to dramatically diminish angiogenesis. These data provide a mechanism for the reduced cancer incidence in Down syndrome and identifies the calcineurin signalling pathway and its regulators DSCR1 and DYRK1A as potential therapeutic targets in cancers arising in all individuals.

Down syndrome (DS), is the most common genetic cause of mental retardation in humans occurring in 1 out of 700 live births. Epidemiological studies suggest that while individuals with DS have increased risk of leukemia, they have a considerably reduced incidence of most solid tumors1–4. In the largest study to date involving 17,800 DS individuals, the mortality from cancers was <10% of expected4. Such data imply that one or more of the 231 trisomic genes on chromosome 21 is responsible for protecting these individuals against cancer. Of note, DS individuals also exhibit a reduced incidence of other angiogenesis-related diseases such as diabetic retinopathy11 and atherosclerosis12, suggesting that cancer protection in the DS population may be due in part, to angiogenesis suppression.

The *Dscr1* gene lies on chromosome 21 and encodes a negative regulator of VEGF-calcineurin signalling in the endothelium5–10. Previous studies have demonstrated that gross over-expression of DSCR1 in endothelial cells blocks VEGF-mediated angiogenic responses *in vitro*7–10. We examined DSCR1 protein expression in DS human fetal tissues and observed a consistent 1.8-fold increase in DSCR1 levels in tissues from DS embryos, as well as elevated expression of DSCR1 fetal isoforms5, compared with those from age-matched non-DS fetuses (Fig. 1A). Substantial synteny exists in gene identity and order between much of human chromosome 21 and the mouse13. The Ts65Dn mouse model of DS is trisomic for 104 of the 231 genes on human chromosome 21 including DSCR114 (Supplemental Fig. 1A). We probed tissues from Ts65Dn mice to ascertain whether DSCR1 was also upregulated in the Ts65Dn mouse and found a 1.7-fold increase in DSCR1 protein expression compared with diploid littermates (Supplemental Fig. 1B). Thus, DSCR1 expression in Ts65Dn mice is elevated in an analogous fashion to DSCR1 expression in DS fetal tissues.

We hypothesized that inhibition of tumorigenesis in the DS population may be partially due to suppression of tumor angiogenesis, thus predicting that the inhibitory effect of *Dscr1* trisomy on tumor growth occurs within the host tumor microenvironment. To establish whether the Ts65Dn DS mouse, like DS individuals, exhibits generalized protection from cancers, we assayed the growth of two transplantable tumor models – Lewis lung carcinoma and B16F10 melanoma cells. We observed considerable growth suppression of both Lewis lung and B16F10 tumor cells in Ts65Dn mice relative to littermate controls (Fig. 1B), correlating with a significant decrease in microvessel density (Fig. 1C). Endothelial cells isolated from Ts65Dn mice demonstrated upregulation of *Dscr1* mRNA in contrast to diploid littermates (Supplemental Fig. 1C) and were noticeably less responsive to VEGF-mediated proliferation *in vitro* (Supplemental Fig. 1D), further implicating an angiogenic

defect in these mice. Thus, trisomy for orthologs of half the genes on human chromosome 21 was sufficient to slow ectopic tumor growth.

To validate that the compromised angiogenesis we observed in murine models of DS extended to human cells carrying trisomy 21, we compared microvessel density in teratomas derived from DS induced pluripotent stem (iPS) cells versus those from cytogenetically normal iPS cells from a healthy volunteer15. These iPS cell lines were inoculated intramuscularly into immunodeficient mice ($Rag2^{-/-}\gamma c^{-/-}$), and angiogenesis assessed in the resulting tumors using a human-specific antibody to the endothelial marker CD3116. Microvessel density was significantly reduced in teratomas derived from Down syndrome iPS cells compared to iPS cells from the normal control (Fig. 1D and Supplemental Fig. 1E).

Given that Ts65Dn mice are trisomic for 104 genes, we next determined whether 3 copies of only *Dscr1* would be sufficient to suppress tumorigenesis. We generated a *Dscr1* transgenic mouse targeting a myc-tagged *Dscr1* cDNA driven by its native promoter to the *Hprt* locus (Supplemental Fig. 2A, B). Expression of the targeted third copy of *Dscr1* was verified by Western blot analysis on isolated endothelial cells (Supplemental Fig. 2C) and quantitative PCR analysis demonstrated a 2.4-fold increase in *Dscr1* mRNA relative to littermate controls (Supplemental Fig. 2D). To ensure that one extra copy of *Dscr1* was sufficient to restrain VEGF-calcineurin signalling, we examined NF-ATc1 subcellular localization as a measure of calcineurin activation (Fig. 2A). After VEGF treatment, endothelial cells isolated from *Dscr1* transgenic mice exhibited predominantly cytoplasmic NF-ATc1 localization while wild-type endothelial cells displayed the expected nuclear localization (Fig. 2A). *Dscr1* transgenic endothelial cells also exhibited decreased sensitivity to VEGF relative to wild-type endothelial cells, as assessed by VEGF-induced proliferation (Fig. 2B).

Substantial growth inhibition of Lewis lung carcinoma and B16F10 melanoma cells was observed in *Dscr1* transgenic mice (Fig. 2C) with a corresponding decrease in microvessel density (Fig. 2D) as compared with tumors from wild-type littermates. Quantification of endothelial cells in tumors isolated from *Dscr1* transgenic and wild-type mice by flow cytometry showed a significant decrease in $CD31^{+}CD45^{-}$ cells in tumors from *Dscr1* transgenic mice (Supplemental Fig. 3A). Additionally, many of the CD31 reactive microvessels in tumors isolated from *Dscr1* transgenic mice lacked functional lumens as evidenced by the absence of co-staining with circulating FITC-lectin and CD31-positivity (Supplemental Fig. 3B). Immunostaining with CD31 and Myc antibodies confirmed specific expression of the DSCR1 transgene in tumor endothelium of *Dscr1* transgenic animals (Fig. 2E). Thus, a single extra copy of *Dscr1* appears to be sufficient to blunt host angiogenic responses and suppress tumor angiogenesis and tumor growth. A subset of tumors initiate growth by coopting existing host vessels17 but the progressive growth and elaboration of new vessels accompanying tumor expansion requires neoangiogenesis. To ascertain whether excess DSCR1 suppresses initial vessel cooption or subsequent angiogenesis, *Dscr1* transgenic and wild-type mice were inoculated with reduced numbers of Lewis lung carcinoma cells to generate slowly growing tumors. DSCR1 elevation inhibited the extended growth phase of transplanted tumors and not just their initial expansion (Supplemental Fig. 3C), supporting the notion that excess DSCR1 suppresses tumor angiogenesis.

To confirm that tumor protection in the Ts65Dn mouse was specifically due to *Dscr1* trisomy, we crossed the Ts65Dn mice to *Dscr1*$^{+/-}$ mice18 generating Ts65Dn/*Dscr1*$^{+/-}$ animals with 2 copies of *Dscr1* but maintaining trisomy for the other 103 genes (Fig. 3A). After validating both segmental trisomy and *Dscr1* status (Supplemental Fig. 4A and B), we compared flank tumor growth in diploid mice to that in Ts65Dn mice with 2 or 3 copies of *Dscr1*. Reduction to 2 *Dscr1* copies in Ts65Dn/*Dscr1*$^{+/-}$ mice significantly abrogated the tumor protection observed in Ts65Dn parental mice (Fig. 3B). Loss of the protective effect against tumor growth was mirrored by a corresponding increase in microvessel density in tumors from Ts65Dn/ *Dscr1*$^{+/-}$ mice relative to their Ts65Dn littermates (Fig. 3C), confirming the pivotal role played by *Dscr1* in tumor suppression in the Ts65Dn DS mouse model. Together, our data provide strong support for the notion that 1 extra copy of *Dscr1* is necessary for maximal suppression of tumor growth via inhibition of tumor angiogenesis.

Increased dosage of *Dscr1* might suppress angiogenesis by down-regulating expression of calcineurin-NFAT-dependent targets in endothelial cells. Cyclooxygenase 2 (COX-2) has been identified as a calcineurin-dependent gene and an important mediator of the angiogenic response to VEGF19. Quantitative PCR and Western blot analysis of COX-2 expression in *Dscr1* transgenic endothelial cells demonstrated a substantial decrease in COX-2 levels relative to control littermates (Fig. 4A, B) suggesting that a modest increase in DSCR1 expression suppresses expression of COX-2 and likely other VEGF-responsive targets. While *Dscr1* trisomy plays a significant role in preventing tumor angiogenesis in our DS mouse model, there are clearly other chromosome 21 genes that contribute to the tumor suppressive effects observed in the Ts65Dn mouse. Since increased dosage of *Dscr1* attenuates VEGF-calcineurin-NFAT signalling, we examined the role of *Dyrk1A*, another chromosome 21 gene known to regulate NFAT signalling and contribute to other Down syndrome phenotypes20, 21. After confirmation of *Dyrk1A* expression in endothelial cells (Supplemental Fig. 4C), we examined the effects of *Dyrk1A* upregulation with *Dscr1* trisomy after over-expression of *Dyrk1A* into *Dscr1* transgenic endothelial cells (Fig 4C). VEGF-mediated endothelial proliferation was dramatically inhibited as a consequence of increased expression of both DYRK1A and DSCR1 (Fig. 4D) implying that upregulation of *Dyrk1A* may contribute to the remaining tumor suppression observed in the Ts65Dn-*Dscr1*$^{+/-}$ mouse with 2 copies of *Dscr1*. Collectively, our data demonstrate that the modest excess of DSCR1 afforded by a single extra copy, impedes VEGF-calcineurin signalling in endothelial cells. The consequent suppression of tumor angiogenesis and, ultimately, tumor growth provides a mechanistic basis for the remarkable protection from solid tumors observed in DS individuals. Of note, host *Dscr1* copy number influences the growth and vascularization of allografted tumor cell lines confirming the cell intrinsic tumor suppressive effect of *Dscr1* dosage by modulating angiogenesis within the host tumor microenvironment.

Since human chromosome 21 harbors over 200 genes, it would be surprising if *Dscr1* were the only chromosome 21 gene implicated in tumor suppression in DS individuals. Indeed our data suggests that increased dosage of *Dyrk1A*, appears to act in concert with *Dscr1* to suppress tumor angiogenesis by further attenuating VEGF-calcineurin-NFAT signalling in endothelial cells. By disrupting the balance of NFAT phosphorylation, DYRK1A blocks

transactivation of NFAT-dependent target genes20, 21. Other trisomy 21 genes may inhibit tumor development via cancer cell autonomous mechanisms. A recent study using the Ts65Dn mouse on the *APC*^{min} mice background demonstrated that 3 copies of the *Ets2* proto-oncogene reduced the incidence of spontaneous intestinal tumors22. However, examination of a spontaneous tumor model prevents the distinction of *Ets2* dosage effects on tumor initiation versus progression. In contrast, our work specifically investigates suppression of tumor progression as a consequence of *Dscr1* trisomy. Additional studies are necessary to determine the magnitude and synergy of these genes in suppressing tumor growth in both the Ts65Dn mouse and the DS population.

Our studies implicate DSCR1-dependent inhibition of the VEGF-calcineurin-NFAT pathway in endothelial cells as a key component of the reduced cancer incidence in DS individuals. Hence, it is reasonable to speculate that cyclosporin A and FK506, immunosuppressive drugs that specifically inhibit calcineurin23, would also suppress tumor angiogenesis. Surprisingly, numerous clinical studies indicate that a significant increase in cancer incidence is a serious complication of transplant recipients receiving long-term immunosupressive therapy24. The mechanism behind this increased rate of cancer is not yet understood, however, such studies point to an important distinction between the calcineurin-inhibitory action of DSCR1 and those of cyclosporin A and FK506.

The cancer protection observed in DS individuals is remarkable given the heterogeneous mechanisms utilized amongst tumors. While DS individuals have less exposure to environmental and other factors that contribute to tumor incidence, the implication remains that one or more of the trisomic genes on chromosome 21 exert an anti-neoplastic effect, presumably by modulating some fundamental aspect of tumor initiation and/or progression. Microscopic avascular tumors represent the earliest stages of human neoplasias and are commonly observed in many organs upon autopsy25. This data suggests that progression into macroscopic tumors, not tumor initiation, may be rate limiting in human cancers. Such progression is critically dependent upon interactions between the incipient tumor and its microenvironment – most notably, activation of the “angiogenic switch”. The pivotal role played by DSCR1 in tumor angiogenesis makes it a compelling candidate for cancer protection in the DS population. Our data implicates VEGF-calcineurin-NFAT as a critical signalling axis in endothelial cells and that maximal suppression of calcineurin requires increased expression of both *Dscr1* and *Dyrk1A.* Additional studies will be required to explore whether angiogenesis in general may also be impaired in the Ts65Dn mouse and in DS individuals. Finally, our studies in the Ts65Dn mouse and transgenic tri-allelic *Dscr1* mouse demonstrate the *in vivo* relevance of a modest excess of *Dscr1* arising from trisomy is sufficient to negatively regulate tumor growth by dampening VEGF-calcineurin signalling. By analogy, we conclude that elevated expression of *Dscr1* in individuals with trisomy 21 is likely a significant contributor to the decreased cancer incidence in this population. It is perhaps, inspiring that the Down syndrome population provides us with novel insight into mechanisms that regulate cancer growth and, by so doing, identify potential targets for tumor prevention and therapy.

Methods Summary

Western blot analysis

Fourteen week-old human fetal kidney and liver tissue were isolated from control and DS tissue, lysed in RIPA buffer and quantified for protein concentration. Tissue lysates were separated by SDS-PAGE, probed with anti-DSCR1 mAb18 and detected via chemiluminescence. Blots were stripped and re-probed with beta actin. DSCR1 levels were quantified by densitometric analysis in the linear range and compared to beta actin. Ts65Dn mice, diploid littermates and *Dscr1*-null mice were sacrificed between 8–12 weeks of age, their brains dissected and probed as described above. Human microvascular endothelial cells were lysed and probed with anti-DYRK1A mAb.

Tumor models

Six-ten week old mice were inoculated with 1–5 × 10^5 Lewis lung carcinoma or B16F10 melanoma cells in HBSS into the subcutaneous flank region as previously described6. Human iPS cell-derived teratomas were generated as previously described15.

Immunohistochemistry

Mice were euthanized and tumors harvested and fixed in neutral-buffered formalin for paraffin embedding. Sections were deparaffinized and epitopes unmasked as previously described6. Sections were immunostained with rat anti-CD31 mAb or anti-Myc pAb overnight at room temperature followed by incubation with goat anti-rat Alexa 594 and goat anti-rabbit Alexa 488, stained with Hoechst and analyzed using Axio Vision 4.0 software (Carl Zeiss Vision).

Endothelial cell isolation, immunofluorescence and proliferation

Four-week old *Dscr1* transgenic or littermate control mice were euthanized, lungs removed, and endothelial cells isolated as previously described6. For immunofluorescence endothelial cells were plated on gelatin coated coverslips, fixed with paraformaldehyde, blocked, permeabilized and incubated with anti-NF-ATc1 mAb followed by goat anti-mouse Alexa 594. For proliferation assays endothelial cells were plated in triplicate in tissue culture wells coated with 0.2% gelatin as previously described6 and treated with the indicated concentrations of VEGF.

Statistical analysis

Data are shown as mean± s.e.m or s.d. as denoted. *P* values were calculated using Student's *t*-test.

Methods

Generation of Ts65Dn mice and Ts65Dn-*Dscr1* heterozygous mice

Two-month old diploid and Ts65Dn mice age 2–4 months old were obtained by breeding Ts65Dn females with C57BL/6C3H F1 males or acquired from Jackson Laboratories (Bar Harbor, ME). Mice were karyotyped with chromosomal spreads from the blood26, and

metaphase chromosomal spreads were prepared and karyotypes evaluated. All protocols were approved by the Children's Hospital or USUHS Institutional Animal Care and Use Committee. Experiments were performed without prior knowledge of genotypes.

The Ts65Dn/*Dscr1*$^{+/-}$ mice that were diploid for the *Dscr1* gene and trisomic for the rest of the Ts65Dn segment of Chr.16 was generated as follows: Males heterozygous for the *Dscr1* gene on a C57Bl/6 background were bred with Ts65Dn females. Litters were genotyped by fluorescence in situ hybridization (FISH) and PCR screening using tail DNA as previously described6, 27. In brief, nuclei of blood leukocytes stained by DAPI show red spots that reveal presence of chromosomes 16 and Ts65Dn segment detected by FISH with a probe produced by labeling of BAC 480C6 (Research Genetics), containing a fragment of Ts65Dn segment) with biotin-dUTP or digoxigenin-dUTP (Boehringer Mannheim) in a nick-translation reaction.

Dscr1 transgenic mice

A 593 bp cDNA fragment encoding the inducible murine *Dscr1* isoform (*Dscr1.Ex4*) was subcloned with a 5' 6-*myc* epitope into a a hypoxanthine phosphoribosyltransferase (*Hprt*) targeting vector with the *Dscr1.Ex4* native 2 Kb promoter28. Allowing generation of single-copy, site-specific recombination at the *Hprt* locus reducing the risk of non-specific phenotypes due to site of insertion29. This construct was targeted into the *Hprt* locus by electroporation into *Hprt*-deficient embryonic stem cells (BK4 cells). Two independent recombinant embryonic stem cell clones screened by PCR and Southern blot were injected into blastocysts with both lines demonstrating germline transmission. *Dscr1* transgenic mice were backcrossed onto a C57Bl/6 background for 6 generations.

Western blot analysis

Fourteen week-old human fetal kidney and liver tissue were isolated from control and DS tissue obtained from therapeutic miscarriages according to an approved IRB protocol at UCLA. Tissues were lysed in RIPA buffer and protein quantified by the Bio Rad DC Protein Assay. Five μg of tissues were, separated by SDS-PAGE, probed with anti-DSCR1 (clone 14B4) mAb18 and detected via chemiluminescence (ECL, Amersham). Blots were stripped and re-probed with beta actin. Levels of DSCR1 were quantified by densitometric analysis in the linear range and compared to beta actin. Ts65Dn mice, diploid littermates and *Dscr1*-null mice were sacrificed between 8–12 weeks of age, their brains dissected and probed as described above.

Human microvascular endothelial cells (Cambrex) were cultured as previously described6, lysed, separated by SDS-PAGE and probed with anti-DYRK1A pAb (Abcam). Primary mouse endothelial cells from the indicated genotypes were lysed, separated by SDS-PAGE and probed with anti-Myc (9E10) mAb or anti-COX2 mAb (Alexis).

Immunohistochemistry and microvessel density

Mice were euthanized and tumors harvested and fixed in neutral-buffered formalin for paraffin embedding. Paraffin embedded sections were deparaffinized and epitopes unmasked as previously described6. Sections were immunostained with rat anti-CD31 mAb

(1:50; Pharmingen) overnight at room temperature followed by incubation for 2 hours with goat anti-rat Alexa 594 conjugated secondary antibody (1:1000; Molecular Probes), and analyzed using Axio Vision 4.0 software. Microvessel density was quantified after CD31 immunostaining of 5 sections per tumor, 5 mice per cohort and quantified as previously described6.

Quantification of microvessel density in tumors by flow cytometry

Tumor-bearing mice were anesthetized with avertin and the vasculature perfused for 3 minutes at 120 mm Hg pressure with saline from a 18-gauge cannula inserted into the aorta via an incision in the left ventricle. Tumors were dissected, minced with scissors, and then digested in PBS supplemented with 2 mg/ml of collagenase/dispase (Roche) for 45 minutes at 37°C. Dissociated cells were resuspended with PBS containing 10% FBS and 10^6 cells were stained with anti-mouse CD31-FITC conjugated antibody plus anti-mouse CD45-PerCP conjugated antibody (BD Pharmingen) for 15 minutes on ice. Cells were washed in PBS, examined in a FACScan flow cytometer, and analyzed using CellQuest software (BD Biosciences).

FITC-lectin perfusion

Mice were anesthetized with isofluorane. FITC-labeled Lycopersicon esculentum lectin (100 μg in 100 μl of 0.9% NaCl; Vector Laboratories, Burlingame, CA) was injected into the femoral vein and allowed to circulate for 3 minutes before perfusion of fixative. The chest was opened and the vasculature perfused for 3 minutes at a pressure of 120 mm Hg with fixative (4% paraformaldehyde in PBS) from a 18-gauge cannula inserted into the aorta via an incision in the left ventricle. The right atrium was incised to create a route for the fixative to escape. After removal, tissues were processed for immunohistochemistry.

Immunofluorescence

10×10^5 endothelial cells/ml were plated onto gelatin-coated glass coverslips overnight. Cells were fixed with 4% paraformaldehyde for 10 min followed by blocking in 3% milk in TBS-T for 45 min. Anti-VEGF-R2 mAb (1:500; Cell Signaling) or anti-mouse NF-ATc1 (1:200) or isotype matched control antibodies was added for 1 hr. Cells were washed with PBS-T before the addition of rabbit anti-mouse-Alexa594 (1:500; Molecular Probes) for 30 min protected from light. Nuclei were stained with 1% Hoechst dye for 1 minute. Cells were washed with PBS-T, mounted and imaged on a fluorescence microscope.

Quantification of *Dscr1* and *Cox-2* mRNA in endothelial cells

Total RNA was isolated from 5×10^5 endothelial cells using the RNeasy kit (Qiagen). cDNA was generated from 5 ug total RNA using SuperScript III First –Strand Synthesis system (Invitrogen) and subjected to real-time PCR with PerfeCTa™ SYBR Green SuperMix (Quanta Biosciences) using the RNA Engine Opticon Monitor 2 System (MJ Research Inc). *Dscr1* was amplified with forward primer: 5'-AGCTCCCTGATTGCTTGTGT-3'and reverse primer: 5'-AGGAACTCGGTCTTGTGCAG-3'. COX-2 was amplified with forward primer: 5'-CCCCCACAGTCAAAGACACT-3'and reverse primer: 5'-AGTTGCTCATCACCCCACTC-3'. GAPDH was amplified with forward primer: 5'-

TGAAGGTCGGTGTGAACGGATTTGG-3' and reverse primer: 5'-CATGTAGGCCATGAGGTCCACCAC-3'.

Retroviral expression of DYRK1A

Dyrk1A cDNA was obtained from Open Biosystems, excised with EcoR1 and cloned into pBABE. DYRK1A retrovirus was generated by transfecting GPG293 cells as previously described30. Virus was used to infect 2×10^5 human microvascular endothelial cells or primary mouse endothelial cells by serial incubations with DYRK1A virus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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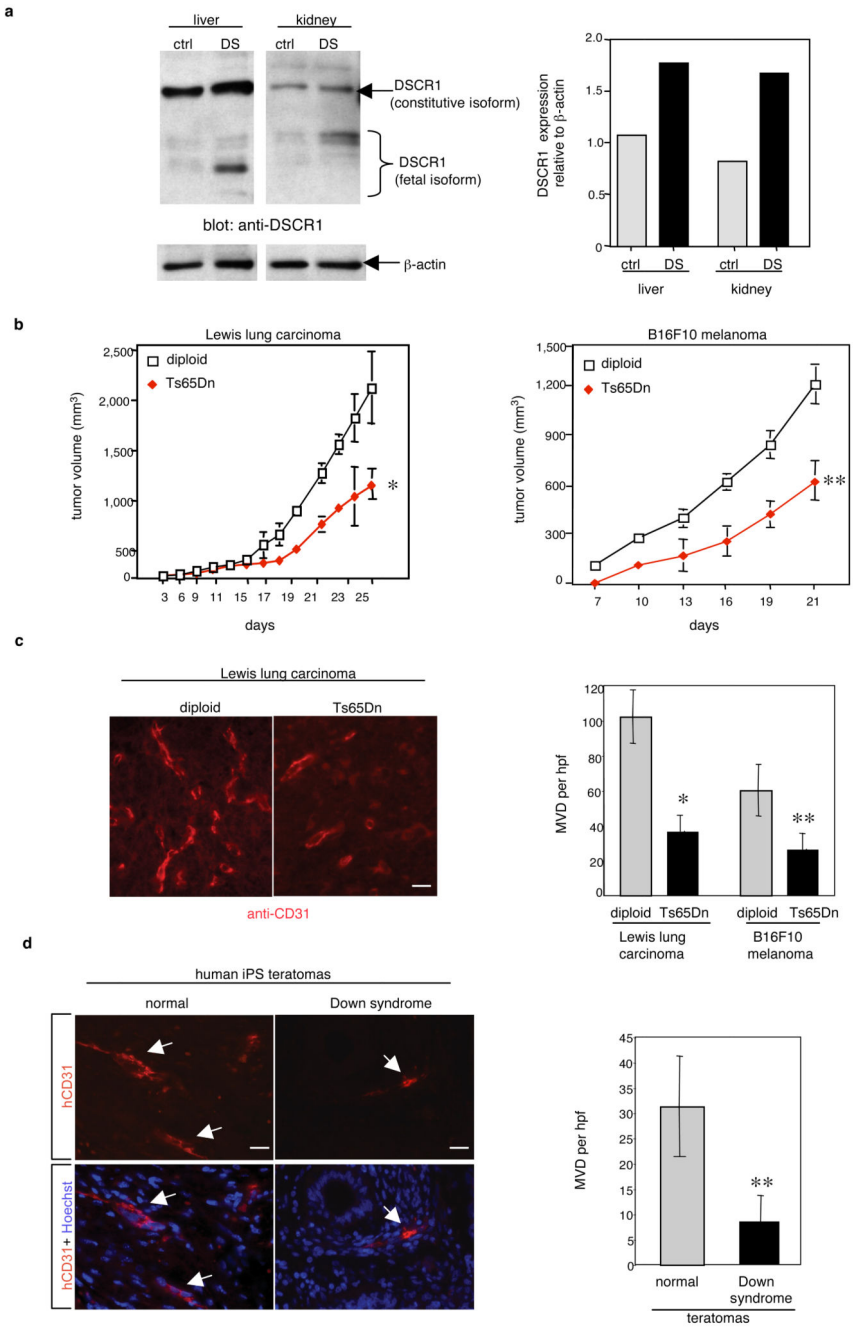

Figure 1. *Dscr1* expression is upregulated in Down syndrome tissues and tumor angiogenesis is suppressed in Down syndrome models

(**a**) Increased DSCR1 expression in human fetal Down syndrome (DS) tissues versus age-matched control (ctrl) tissues relative to β-actin. (**b**) Tumor growth is suppressed in the Ts65Dn Down syndrome mouse model. Values are mean ± s.e.m. n=10–12, *p<0.03; **p <0.01. (**c**) Microvessel density (MVD) per high-powered field (hpf) of tumors is quantified by anti-CD31 immunofluorescence. Bar, 20 μM. Values are mean ± s.e.m. *p <0.02; **p<0.01. (**d**) Angiogenesis in tumors from induced pluripotent stem cells (iPS) isolated

from Down syndrome or cytogenetically normal cells was quantified by human specific anti-CD31 immunofluorescence. Arrows, hCD31-positive vessels. Values are mean ± s.e.m. n=3–6, **p<0.01.

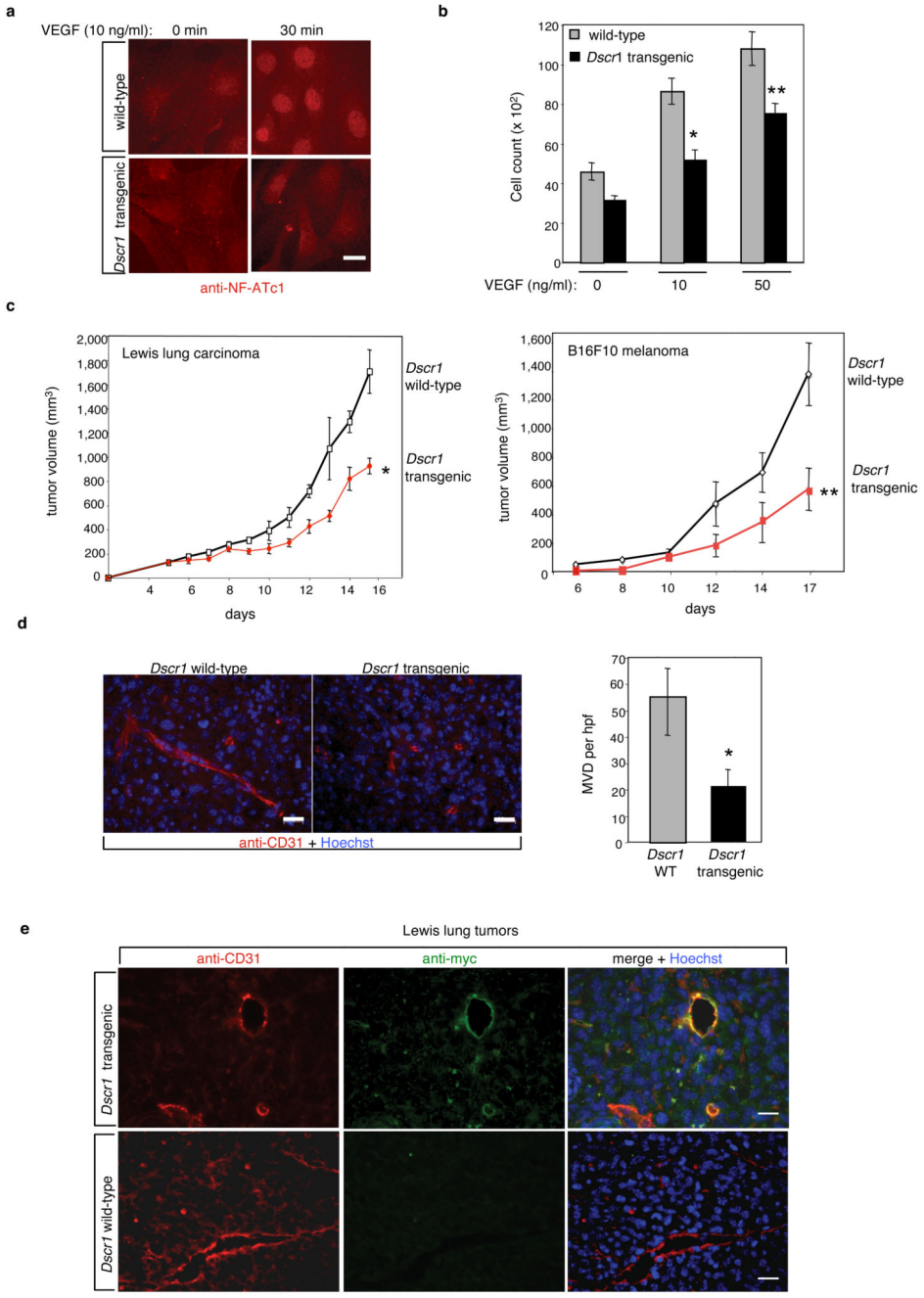

Figure 2. Targeted *Dscr1* transgenic mice with three copies of *Dscr1* show inhibition of tumor growth

(**a**) NF-ATc1 nuclear import after VEGF treatment is suppressed in endothelial cells from *Dscr1* transgenic mice. Bar, 5 μM. (**b**) VEGF-induced proliferation of endothelial cells from *Dscr1* transgenic mice is significantly inhibited. *p<0.02; **p<0.03. (**c**) Tumor growth is suppressed in *Dscr1* transgenic mice. n=8–12 mice per group, *p<0.05; **p<0.04. (**d**) Microvessel density (MVD) per high-powered field (hpf) of tumors is quantified by anti-CD31 immunofluorescence. Bar, 20 μM. Values are mean ± sem. *p<0.01. (**e**) Tumors from

Dscr1 transgenic and wild-type mice are co-immunostained with anti-CD31 to detect endothelial cells and anti-Myc to detect the *Dscr1* transgene. Bar, 20 μM.

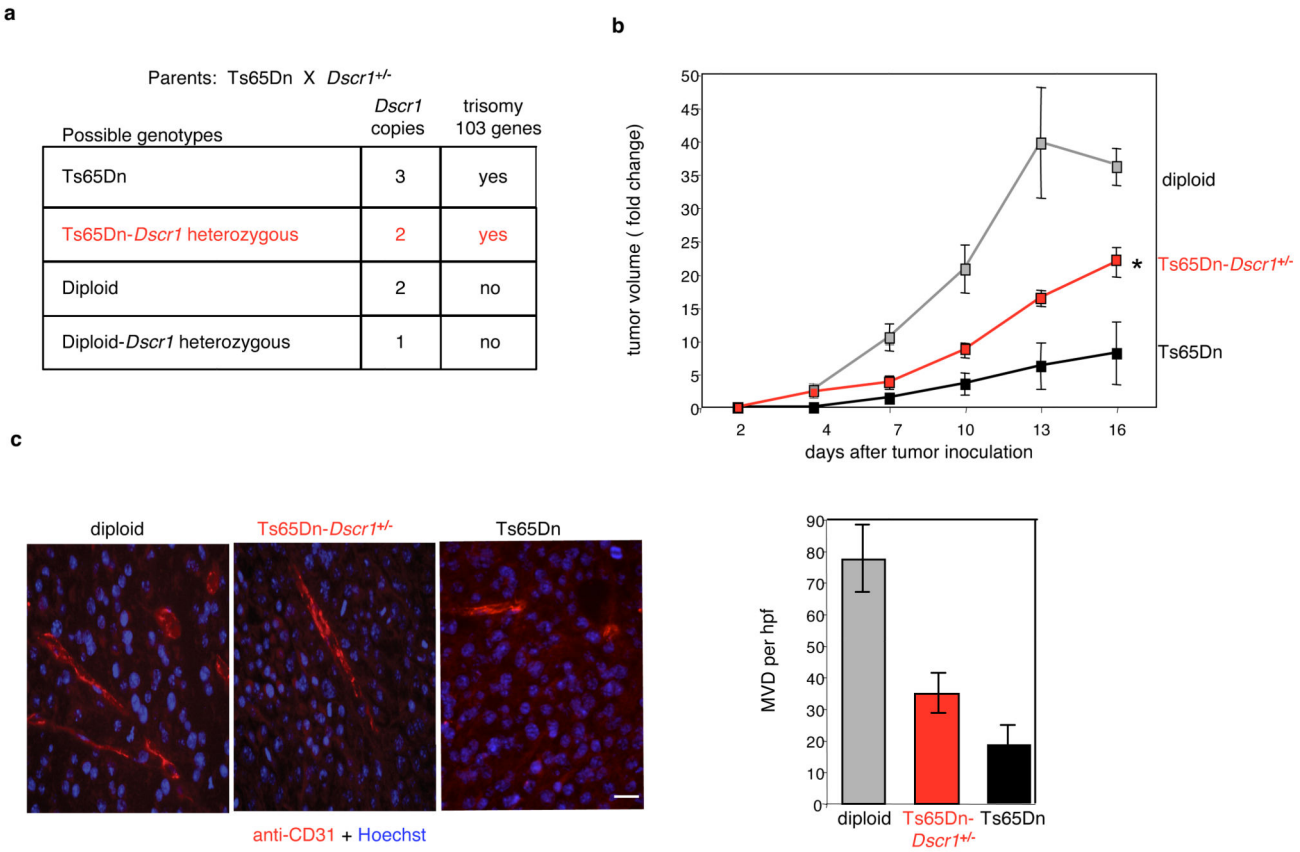

Figure 3. Trisomic expression of *Dscr1* is necessary for significant suppression of tumor growth in the Ts65Dn Down syndrome mouse model

(**a**) Possible genotypes arising from mating Ts65Dn and *Dscr1*$^{+/-}$ mice. (**b**) Suppression of B16F10 tumor growth in Ts65Dn Down syndrome mice is relieved upon loss of the third copy of *Dscr1* (Ts65Dn-*Dscr1*$^{+/-}$). Values are mean± sem, n=4–8 per group, *p<0.01. (**c**) Microvessel density (MVD) per high-powered field (hpf) is quantified by anti-CD31 immunofluorescence of tumors harvested from the indicated mice at comparable volumes (200–400 mm^3). Bar, 20 μM. Values are mean ± sem.

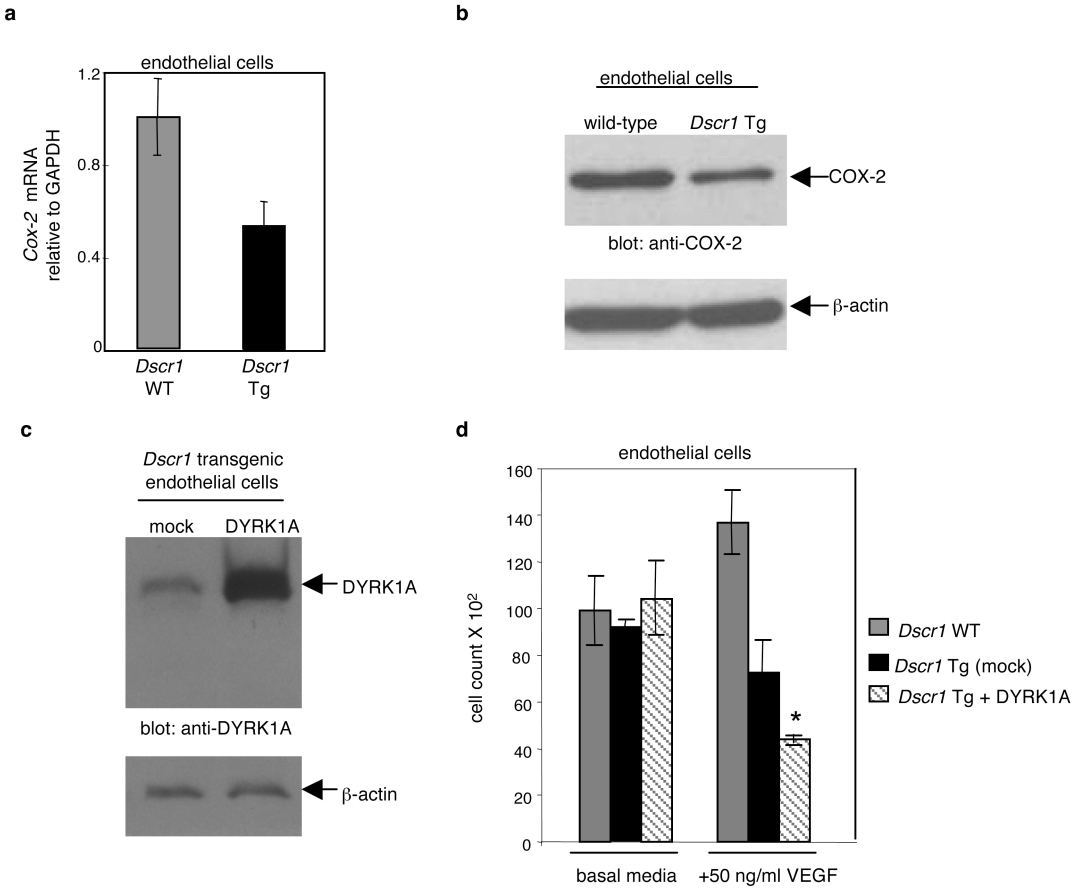

Figure 4. Calcineurin suppression by DSCR1 and DYRK1A attenuates endothelial cell activation

(**a, b**) Cyclooxygenase-2 (COX-2) mRNA (**a)** and protein expression (**b**) was quantified by qPCR and Western blot analysis in endothelial cells isolated from *Dscr1* wild-type (WT) and transgenic (Tg) mice. (**c**) Western blot analysis of endothelial cells isolated from *Dscr* transgenic mice and probed for DYRK1A after either mock infection (mock) or retroviral infection with *Dyrk1A* (DYRK1A). (**d**) Endothelial cells isolated from *Dscr1* transgenic (Tg) mice with DYRK1A over-expression (+DYRK1A) demonstrate an even greater suppression of VEGF-mediated proliferation as compared to wild-type (WT) and DSCR1 over-expression alone (mock). Values are mean ± sem, *p<0.01.