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Endothelial–cell FAK targeting sensitizes tumours to DNA–damaging therapy

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Abstract

Chemoresistance is a serious limitation of cancer treatment¹. Until recently, almost all the work done to study this limitation has been restricted to tumour cells². Here we identify a novel molecular mechanism by which endothelial cells regulate chemosensitivity. We establish that specific targeting of focal adhesion kinase (FAK; also known as PTK2) in endothelial cells is sufficient to induce tumour-cell sensitization to DNA-damaging therapies and thus inhibit tumour growth in mice. The clinical relevance of this work is supported by our observations that low blood vessel FAK expression is associated with complete remission in human lymphoma. Our study shows that deletion of FAK in endothelial cells has no apparent effect on blood vessel

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function *per se*, but induces increased apoptosis and decreased proliferation within perivascular tumour-cell compartments of doxorubicin- and radiotherapy-treated mice. Mechanistically, we demonstrate that endothelial-cell FAK is required for DNA-damage-induced NF- κ B activation *in vivo* and *in vitro*, and the production of cytokines from endothelial cells. Moreover, loss of endothelial-cell FAK reduces DNA-damage-induced cytokine production, thus enhancing chemosensitization of tumour cells to DNA-damaging therapies *in vitro* and *in vivo*. Overall, our data identify endothelial-cell FAK as a regulator of tumour chemosensitivity. Furthermore, we anticipate that this proof-of-principle data will be a starting point for the development of new possible strategies to regulate chemosensitization by targeting endothelial-cell FAK specifically.

Despite encouraging initial responses to DNA-damaging chemotherapies and radiotherapy, many tumours become resistant to treatment¹. Previous work has concentrated on understanding resistance by focusing on mechanisms within tumour cells². Although recent evidence suggests that the tumour stroma can regulate chemoresistance, the underlying molecular mechanisms are largely unknown^{3–9}.

FAK is a non-receptor tyrosine kinase and regulator of cell migration, proliferation and survival¹⁰. FAK can also regulate transcription via its scaffolding functions in the nucleus¹¹. Although some studies have implicated a role for endothelial-cell FAK in tumour growth and angiogenesis^{12–14}, its role in the regulation of chemoresistance has not been identified.

Here we demonstrate that targeting endothelial-cell FAK, in established tumours, is sufficient to sensitize tumour cells to DNA-damaging therapies. *Pdgfb-iCre^{ER};Fak^{fl/fl}* mice were injected subcutaneously with mouse melanoma (B16F0) or lung carcinoma (CMT19T) cell lines. At 7 days after tumour-cell inoculation, endothelial-cell FAK deletion was induced, generating ECFAK^{KO} mice (Extended Data Fig. 1). Mice were then treated with one of two forms of DNA-damaging therapies: doxorubicin or radiation. Similarly treated *Pdgfb-iCre^{ER}*;non-floxed mice or *Fak^{fl/fl}* mice (ECFAK^{WT}) were used as controls for endothelial-cell FAK expression. Loss of endothelial-cell FAK did not affect B16F0 or CMT19T tumour growth in placebo-treated or non-irradiated mice (Fig. 1a, b), nor did it affect tumour angiogenesis, blood vessel perfusion, or endothelial-cell apoptosis *in vivo* (Extended Data Fig. 2). In contrast to deleting endothelial-cell FAK before tumour development¹⁴, here our data indicate that endothelial-cell FAK deletion after tumour growth has begun is not sufficient to affect blood vessel density, results that are supported by other studies^{15,16}. Moreover, we go on to show that doxorubicin or radiation therapy in ECFAK^{WT} mice was not sufficient to affect B16F0 or CMT19T tumour growth, respectively, indicating that these tumour types are not sensitive to such forms of therapy *in vivo* (Fig. 1c, d). In contrast, endothelial-cell FAK deletion resulted in sensitizing B16F0 tumours to doxorubicin, causing a significant delay in tumour growth when compared with similarly treated ECFAK^{WT} mice (Fig. 1c). Likewise, endothelial-cell FAK deletion in mice bearing CMT19T tumours sensitized tumours to radiation therapy, also leading to a significant decrease in tumour growth rates (Fig. 1d). Despite elevated numbers of γ H2AX-positive tumour-cell nuclei (an indicator of DNA damage) in ECFAK^{KO} when compared with ECFAK^{WT} mice after treatment (Extended Data Fig. 3a), no changes in tumour blood vessel permeability, doxorubicin delivery, tumour hypoxia or CD45-positive immune-cell

infiltration were observed between genotypes (Extended Data Fig. 3b–e). These data suggest that loss of endothelial-cell FAK enhances tumour-cell responses to DNA damage without affecting the delivery function of blood vessels. Indeed, using other mouse models of cancer—experimental metastasis to the lung, using either tail-vein injection of B16F10 melanoma or EuMycBCL2 lymphoma—we show that loss of endothelial-cell FAK is sufficient to sensitize tumours to doxorubicin and significantly extend median survival (Extended Data Fig. 4). Together, these data demonstrate that endothelial-cell FAK deletion alone is sufficient to sensitize tumours to DNA-damaging therapies.

The clinical relevance of our results is also apparent in human cancer. Chemotherapy can induce complete remission and be curative in some subsets of lymphoma. However, many patients are resistant to doxorubicin-containing chemotherapy, and either fail to achieve remission or relapse and subsequently show progression months or even years later^{17,18}. We asked whether disease progression in lymphoma patients correlated with altered blood-vessel FAK expression. First, sections of human lymphoma samples, all taken at diagnosis, were analysed for the percentage of FAK-positive blood vessels. Results showed that, after doxorubicin-containing treatment, a high percentage of FAK-positive blood vessels was associated significantly with subsequent disease progression, while a low percentage of FAK-positive blood vessels correlated significantly with complete remission (Fig. 1g). This result is unlikely to be due to a direct effect of doxorubicin treatment on FAK expression levels because, although doxorubicin can affect FAK localization in endothelial cells, suggesting a possible change in function, it does not affect FAK expression levels (Extended Data Fig. 5). Second, using matched samples taken at diagnosis and at relapse, endothelial-cell FAK expression was elevated significantly at relapse when compared with expression levels at diagnosis (Fig. 1h–j). Overall, our data indicate a substantial correlation between chemoresistance and endothelial-cell FAK levels in human cancer.

Previous reports have suggested that chemotherapy and/or radiation therapy may result in perivascular chemoresistant niches that actually protect tumour cells from apoptosis^{7,19}. However, the regulators of this process within the endothelium *in vivo* have not yet been identified. We show, at 48 h post-treatment cessation, that the number of blood vessels within apoptotic perivascular tumour-cell niches, detected by cleaved caspase 3 staining, was enhanced significantly in doxorubicin-treated ECFAK^{KO} mice when compared with similarly treated ECFAK^{WT} or placebo-treated mice (Fig. 2a). Furthermore, tumour-cell proliferation, detected by Ki67 staining, was reduced in perivascular zones of doxorubicin-treated ECFAK^{KO} mice when compared with controls (Fig. 2b). Similar results were observed for radiotherapy-treated ECFAK^{KO} mice (Fig. 2c, d). No differences between ECFAK^{WT} and ECFAK^{KO} mice in non-treated groups were observed (Fig. 2a–d). These results suggest that, upon DNA damage, endothelial cells may provide protective paracrine signals to tumour cells, which are absent when endothelial-cell FAK is deleted. To confirm this, we show that although conditioned media from untreated wild-type and FAK-null endothelial cells has no apparent effect on tumour-cell survival, conditioned media from either doxorubicin or irradiated wild-type endothelial cells protects cultured tumour cells from DNA damage over time and allows for tumour-cell growth. In contrast, conditioned media from either doxorubicin-treated or irradiated FAK-null endothelial cells confer

chemo- and radio-sensitivity to tumour cells, reducing their survival *in vitro* (Fig. 2e, f). Together, these results demonstrate a novel role for endothelial-cell FAK in tumour-cell sensitization to doxorubicin treatment or radiotherapy by the release of paracrine signals.

We next sought to identify the molecular basis for these endothelial-cell effects by FAK. Activation and nuclear translocation of the NF- κ B family of transcription factors is known to mediate cellular responses to DNA-damaging therapies²⁰; however, a role for FAK-dependent NF- κ B functions in endothelial-cell responses to chemotherapy has not been defined previously. In NF- κ B luciferase reporter assays, doxorubicin-treated FAK-null endothelial cells exhibited significantly reduced levels of NF- κ B activity when compared with similarly treated wild-type control cells (Fig. 3a). Corroborating these results, while doxorubicin-treated wild-type endothelial cells induced nuclear translocation of the p65 sub-unit of NF- κ B, this was significantly blocked in FAK-null endothelial cells at 4, 24 and 48 h after doxorubicin treatment (Fig. 3b, c and Extended Data Fig. 6). These data are supported by increased levels of phosphorylated p65 (Ser 536) in nuclear fractions from wild-type, but not FAK-null, endothelial cells after doxorubicin treatment (Fig. 3d) and decreased levels of phosphorylated-I κ B α in cytosolic fractions of doxorubicin-treated FAK-null endothelial cells (Extended Data Fig. 7). Moreover, nuclear localization of p65 *in vivo*, an indicator of NF- κ B activity, was evident *in vivo* in the tumour endothelium of ECFAK^{WT}, but not ECFAK^{KO}, mice that had been treated with doxorubicin (Fig. 3e). Thus, our data indicate a novel role for endothelial-cell FAK in doxorubicin-induced control of NF- κ B activation *in vitro* and *in vivo*.

Although other signalling pathways may be involved, FAK-dependent regulation of the NF- κ B pathway has been shown to be a major regulator of cytokine production in tumour cells^{21–26}. However, the regulation of cytokine production in endothelial cells, especially after DNA-damaging treatment, is not known. Therefore, we next examined whether endothelial-cell FAK deficiency affected doxorubicin-induced cytokine production. Cytokine protein array analysis revealed that doxorubicin stimulation induced an increase in production of several cytokines in wild-type endothelial cells when compared with untreated controls. In contrast, doxorubicin-induced responses were not increased after DNA-damaging therapy in FAK-null endothelial cells (Fig. 4a). A reduction in fold-increase of cytokine levels, some similar to those found after doxorubicin treatment, was also observed after irradiation of FAK-null endothelial cells when compared with irradiated wild-type controls (Extended Data Fig. 8). Cytokine levels were similar between untreated wild-type and FAK-null endothelial cells (Extended Data Fig. 9a). An indicator of cytokine and interleukin activity is phosphorylation of the transcription factor STAT3. We show that doxorubicin treatment enhances the percentage of tumour cells with phosphorylated (p)-STAT3 in ECFAK^{WT} mice, but not tumour cells of similarly treated ECFAK^{KO} mice (Extended Data Fig. 9b), corroborating our results of reduced cytokine production in doxorubicin-treated ECFAK^{KO} mice. These data provide *in vivo* evidence for decreased cytokine effects in doxorubicin-treated ECFAK^{KO} when compared with similarly treated ECFAK^{WT} mice.

To expand the mechanistic basis of chemosensitization in ECFAK^{KO} mice, we tested whether inactivation of the NF- κ B signalling pathway in wild-type endothelial cells is

sufficient to mimic FAK-null endothelial cells. Wild-type endothelial cells were transfected with the super-repressor, non-phosphorylatable mutant form of I κ B α , I κ B α SR, which has been shown previously to inhibit the NF- κ B pathway²⁷. Inhibition of the NF- κ B pathway by I κ B α SR transfection of wild-type endothelial cells reduced DNA-damage-induced cytokine production when compared with similarly treated mock-transfected controls (Fig. 4b). These data indicate that inhibition of the NF- κ B pathway is sufficient to mimic the reduced NF- κ B signalling effect in FAK-null endothelial cells after DNA damage. Indeed, conditioned media from doxorubicin-treated, I κ B α SR-transfected wild-type endothelial cells was sufficient to sensitize cultured tumour cells to doxorubicin, when compared with mock-transfected wild-type endothelial cells *in vitro*. Furthermore, conditioned medium from doxorubicin-treated I κ B α SR-transfected wild-type endothelial cells was able to reduce tumour-cell survival, phenocopying the chemosensitization responses conferred by FAK-null endothelial cells (Fig. 4c). Lastly, intratumoral administration of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (15 ng) or interleukin (IL)-6 (3 ng), were sufficient to induce similar tumour growth rates in ECFAK^{WT} and ECFAK^{KO} mice, reversing the chemosensitization phenotype in ECFAK^{KO} mice (Extended Data Fig. 10). Together, our results provide proof-of-principle that a decrease in endothelial-cell FAK and a subsequent decrease in DNA-damage-induced NF- κ B-dependent endothelial-cell cytokine production controls tumour-cell chemosensitization.

Overall, our data indicate that, upon DNA damage, loss of endothelial-cell FAK is sufficient to sensitize tumour cells to chemotherapy by suppressing NF- κ B activation and subsequent cytokine production (Fig. 4d, e). These data establish a new concept in the regulation of chemoresistance. Specifically, our data point to a role for endothelial-cell FAK in the regulation of chemotherapy responses, and provide a starting point for the development of new approaches to improve response to DNA-damaging therapies by specifically targeting endothelial-cell FAK.

Methods

Mice

Pdgfb-iCre^{ER};Fak^{fl/fl} mice¹⁴ were maintained on a mixed C57BL/6/1 (ref. 14) background, or on a pure C57 background for Eumyc experiments. Both male and female mice were used, aged 6–24 weeks old.

Tumour growth, doxorubicin and irradiation treatment

Mouse melanoma (B16F0, ATCC; mycoplasma free) or mouse lung carcinoma (CMT19T, CR-UK Cell Production; mycoplasma free) cells (1×10^6) were injected subcutaneously in the flank of *Pdgfb-iCre^{ER};Fak^{fl/fl}* mice and wild-type control mice (*Pdgfb-iCre^{ER}*;non-floxed or *Fak^{fl/fl}*). Simultaneously, animals were given a soy-free diet (Harlan) to reduce oestrogen levels and increase tamoxifen sensitivity. At days 7 and 8 after tumour inoculation, once tumour growth had begun, all mice were injected intraperitoneally (i.p.) with 150 μ l of 10 mg ml⁻¹ of tamoxifen (Sigma, T5648) diluted in 10% ethanol in peanut oil (Sigma) to induce endothelial-cell FAK deletion. From day 8 onwards, all animals were fed with tamoxifen-containing diet (TAM400, Harlan). All animals with B16F0 subcutaneous

tumours were injected i.p. with 8 mg kg⁻¹ of doxorubicin (Accord Healthcare) or PBS as a negative control on days 9, 11 and 13 after tumour-cell inoculation. Alternatively, animals with subcutaneous CMT19T tumours were irradiated with 5Gy of γ -irradiation on day 10 after tumour injection. For both tumour types calliper measurements were taken over time and animals were killed when tumours reached the maximum size allowed by UK Home Office regulations.

FAK and PECAM staining in human non-Hodgkin lymphoma samples

Biopsy samples from non-Hodgkin lymphoma patients, either before (that is, at diagnosis) or after doxorubicin-based chemotherapy (that is, from relapsed patients), were analysed. Formalin-fixed samples were de-waxed; rehydrated; blocked in 10% goat serum; incubated in rabbit anti-FAK antibody (Cell Signaling, 3285) and mouse anti-human CD31 (Leica, CD31-1A10-CE-S) in 0.5% goat serum in PBS overnight at 4 °C; incubated in biotinylated anti-rabbit (DAKO) and Alexa 546 anti-mouse (Invitrogen) diluted 1:100 in 0.5% goat serum in PBS; washed in PBS; and finally processed using streptavidin-HRP/fluorescein kit (TSA Fluorescence Systems). The levels of endothelial-cell FAK were quantitated with Image J software and the mean fluorescence intensity of FAK per pixel was measured.

Immunostaining

Sections of human lymphoma or mouse tumours were immunostained for endothelial cells using either anti-PECAM antibody (MEC13.3; BD Biosciences, 553370) or rat anti-endomucin (V.7C7; SantaCruz, SC-65495) in combination with either rabbit anti-FAK antibody (Cell Signaling, 3285), anti-cleaved caspase 3 (Cell Signaling, 9661), rabbit anti-Ki67 (Abcam, AB15580), or rabbit anti-p65 subunit of NF- κ B (D14E12; Cell Signaling, 8242S). See later for details.

Blood vessel and CC3 immunostaining in mouse tumour sections

Snap-frozen mouse tumour sections were fixed in acetone for 10 min at -20 °C; rehydrated in PBS for 10 min; blocked in 5% goat serum diluted in PBS for 1 h at room temperature; washed once in PBS; incubated overnight at 4°C with rat anti-mouse PECAM antibody (MEC13.3; BD Biosciences, 553370) or rabbit anti-cleaved caspase 3 (Cell Signaling, 9661), both diluted 1:100 in 0.5% goat serum in PBS; washed three times in PBS; incubated with anti-rat Alexa 546 (Invitrogen) and anti-rabbit Alexa 488 (Invitrogen) for 1 h at room temperature; washed in PBS; and finally mounted in ProLong Gold with DAPI (Invitrogen). Blood vessels indirect contact with cleaved caspase-3-positive tumour cells were quantified as a percentage of total vessels within five fields of view using a $\times 40$ objective.

NF- κ B and Ki67 staining in mouse tumours

Sections from fixed tumours were de-waxed and rehydrated in descending concentrations of ethanol. A3 % hydrogen peroxide diluted in methanol incubation was carried out for 15 min at room temperature between the 100% ethanol immersions. Sections were washed in PBS; microwaved in 10 mM Nacitrate buffer (pH6.0) for 20 min; blocked for 1 h at room temperature in 10% goat serum diluted in PBS; and incubated at 4 °C overnight with the primary antibodies rabbit anti-p65 NF- κ B (D14E12; Cell Signaling, 8242S) or rabbit anti-

Ki67 (Abcam, AB15580), together with the vessel marker rat anti-endomucin (V.7C7; Santa Cruz, SC-65495), both diluted 1:100 in 1% goat serum in PBS); washed three times in PBS; incubated for 1 h at room temperature with secondary fluorescent antibodies (goat anti-rat Alexa 546 and goat anti-rabbit Alexa 488); washed in PBS and finally mounted in ProLong Gold with DAPI (Invitrogen). Ki67-positive tumour cells, within a perivascular distance of 50 μ m from PECAM-positive blood vessels, were quantified as a percentage of perivascular DAPI-positive nuclei.

Microscopy was carried out on a LSM510META or LSM710META confocal microscope (Zeiss).

Endothelial-cell culture

Primary mouse lung endothelial cells (MLECs) were isolated from *Pdgfb-iCre^{ER};Fak^{fl/fl}* or *Pdgfb-iCre^{ER}*; non-floxed adult mice as described previously²⁸. After a negative sort with rat anti-CD16/CD32 (Serotec, MCA2305EL), cells were immortalized with polyoma middle T (PmT) virus by incubating them over 2 consecutive days for 4 h with supernatant from GgP+E packaging cells²⁹. Cells were grown in Mouse Lung Endothelial Cell Media²⁸ supplemented with 500 nM 4-hydroxytamoxifen (4-OHT). Two positive sorts using rat anti-ICAM2 (Serotec, MCA 2295EL) and sheep anti-rat IgG magnetic beads (Dynabeads) were carried out as described previously²⁸. Tamoxifen-treated endothelial cells isolated from *Pdgfb-iCreER;Fak^{fl/fl}* mice gave rise to FAK-depleted endothelial cells (FAK-null) and those isolated from *Pdgfb-iCreER*; non-floxed mice gave rise to FAK wild-type endothelial cells.

Doxorubicin-induced cytokine production *in vitro*

Wild-type and FAK-null endothelial cells were treated with 7.5 μ g ml⁻¹ mitomycin C (Roche) for 2 h. After plating equal numbers of endothelial cells, cells were cultured for 24 h in full MLEC medium and then the medium was changed for MLEC medium supplemented with 500 nM 4-OHT with, or without, 0.125 μ M doxorubicin to generate conditioned medium, which was collected after 48 h. The conditioned media from wild-type and FAK-null endothelial cells were harvested and filtered through a 0.22 μ m filter to remove cell debris before being added to B16F0 tumour cells, plated in 96-well plates. MTS assessment of cell survival was carried out at 3 and 4 days as described later.

Irradiation-induced cytokine production *in vitro*

Wild-type and FAK-null endothelial cells were treated with 7.5 μ g ml⁻¹ mitomycin C (Roche) for 2 h. Equal numbers of endothelial cells were plated in full MLEC medium for 24h and irradiated with 5Gy of X-rays using a RS2000 biological irradiator. Non-irradiated wild-type and FAK-null endothelial cells were used as controls. Cells were cultured for a further 72 h with MLEC medium supplemented with 500 nM 4-OHT to generate conditioned medium. The conditioned media from wild-type and FAK-null endothelial cells were harvested and filtered through a 0.22 μ m filter to remove cell debris. Three-thousand CMT19T cells per well were plated in 96-well plates and were irradiated with 5 Gy. Endothelial cells conditioned medium was applied to CMT19T tumour cells 8h after

irradiation. Cells underwent MTS assessment of cell survival at days 4 and 5 as described later.

Conditioned media production from I κ B α SR-transfected cells

Wild-type endothelial cells were transfected using the Nucleofector electroporation system (Lonza) with either the empty vector (mock control) or I κ B α SR. The next day, cells were treated with 7.5 $\mu\text{g ml}^{-1}$ mitomycin C for 2h (Sigma). Equal numbers of cells were plated in complete medium supplemented with 4-OHT. After 24 h the medium was replaced by fresh complete medium supplemented with 4-OHT $\pm$ 0.125 μM doxorubicin. This medium was collected after 48 h incubation at 37 °C and applied to B16F0 cells and tumour-cell survival was assessed using the MTS assay as described later.

MTS assay for tumour-cell survival

In vitro chemosensitivity was assessed using the CellTiter 96 AQueous One Solution Reagent (Promega). Assays were done by incubating each well, containing tumour cells, with 20 μl of reagent in 100 μl OptiMEM (Invitrogen) for 90 min. Plates were read at 490 nm, with absorbance corrected relative to blank wells containing reagent only.

Immunostaining endothelial cells

Wild-type or FAK-null endothelial cells (4×10^4) were grown for 48h in MLEC medium supplemented with 500 nM 4-OHT with or without 0.125 μM doxorubicin. Cells were then serum starved for 4h in OptiMEM (Invitrogen) with or without 0.125 μM of doxorubicin.

For NF- κ B detection cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature; washed in PBS three times; and permeabilized in 0.5% NP40 in PBS for 10 min at room temperature; blocked with 0.1% BSA/0.2% Triton X-100 for 10min at room temperature; washed three times in PBS; incubated for 1 h at room temperature in anti-p65 NF- κ B antibody (Cell Signaling); washed three times in PBS; incubated with Alexa-546-conjugated anti-rabbit diluted 1:100 in PBS; washed three times in PBS; and, finally, mounted in Prolong Gold with DAPI.

Cytokine arrays

Wild-type and FAK-null endothelial cells or transfected wild-type endothelial cells were grown in normal MLEC media supplemented or not with 0.125 μM doxorubicin (Accord Healthcare) or irradiated (5Gy). After 48 h of serum starvation, whole-cell lysates were extracted at 48 h (for doxorubicin-treated endothelial cells) and 72 h (for irradiated endothelial cells). Mouse cytokine arrays (Proteome Profiler ARY006, R&D Systems) were processed according to the manufacturer's instructions using 100 μg of lysates (in 3% SDS, 60 mM sucrose, 65 mM Tris-HCl pH 6.8) per membrane. Pixel analysis was used for quantification with Image J software.

Western blot analysis of nuclear fraction p65

Wild-type and FAK-null endothelial cells were serum starved for 18 h in OptiMEM supplemented with 2% FCS and tamoxifen. The cells were stimulated with 0.125 μM

doxorubicin in the same medium without tamoxifen. Cells were scraped in hypotonic buffer and left on ice for 5min. After centrifugation for 10 min at 500g the supernatants were collected as cytosolic extracts. The pellets were washed once in hypotonic buffer, then resuspended and sonicated in nuclear extraction buffer and centrifuged to remove debris. The following antibodies were used for western blot analysis: anti-phospho-p65 NF- κ B (S536; Cell Signaling, 3033), anti-p65NF- κ B (D14E12; Cell Signaling, 8242).

NF- κ B activation assay

Wild-type and FAK-null cell lines were co-transfected with 2 μ g each of the 3 $\times$ kB ConA NF- κ B reporter firefly luciferase plasmid and an internal control plasmid expressing *Renilla* luciferase (pRL-TK, Promega), using the Basic Endothelial Cell kit and T23 program on the Nucleofector II (Lonza) according to the manufacturer's instructions. After 24 h, cells were treated with 0.125 μ M of doxorubicin, and a further 48 h later cells were actively lysed with Passive Lysis Buffer (Promega) and assayed using the Dual Luciferase Reporter Assay system (Promega) in a Lumat LB9507 luminometer (Berthold Technologies)³⁰. Results shown are the mean plus s.e.m. of at least three independent experiments and were normalized to the expression of the internal control plasmid. Statistical analysis was performed using Prism 5 (GraphPad).

FAK immunostaining in mouse tumour sections

Tumours were fixed, paraffin embedded and processed as described earlier. For detection of FAK, a rabbit anti-FAK antibody (Cell Signaling, 3285) was used. For FAK staining, amplification of signal was achieved by incubating samples after the primary antibody incubation with anti-rabbit biotinylated (DAKO, E0353) and anti-rat Alexa 546 (Invitrogen) diluted 1:100 in 1% goat serum in PBS. After three washes in PBS, the sections were stained with a streptavidin-HRP/fluorescein kit (TSA Fluorescence Systems).

Blood vessel density

Snap-frozen sections of tumours were immunostained for PECAM to detect blood vessels as described earlier. Blood vessel density was calculated by counting the total number of blood vessels across entire midline sections from age-matched, size-matched tumours. Blood vessel density is presented as the number of blood vessels per mm² of tumour section.

Blood vessel perfusion and permeability

Pdgfb-iCre^{ER};non-floxed mice were injected subcutaneously with 1×10^6 B16F0 tumour cells. At 7 and 8 days after tumour inoculation, all mice were injected i.p. with 150 μ l of 10 mg ml⁻¹ tamoxifen. From day 8 onwards, all mice were fed with tamoxifen containing diet. On days 9,11 and 13 after tumour-cell inoculation, mice were injected with PBS. On day 14, mice were injected with 100 μ l of PE-conjugated anti-PECAM antibody (Biolegend) via the tail vein 10 min before killing the mice to analyse blood vessel perfusion. One minute before mice were killed, they were also injected via the tail vein with Hoechst dye (4 μ g ml⁻¹) to analyse blood vessel permeability. Tumours were dissected and immediately snap-frozen. Cryosections were air-dried then fixed in -20 °C acetone for 10 min. Sections were then rehydrated in PBS and washed once with water and mounted with Prolong Gold. The level

of blood vessel perfusion was calculated as the percentage of tumour blood vessels that were positive for PE-PECAM over total blood vessel counts. Permeability was analysed by counting the numbers of Hoechst positive nuclei per field of view.

γ H2AX immunostaining on mouse tumour sections

For mice used and treatment schedules see earlier. On day 14, tumours were removed and snap-frozen. Cryosections were fixed in acetone for 10 min at -20°C and rehydrated in PBS for 10 min. Staining was performed using the M.O.M. Fluorescein kit (Vector Labs). Sections were incubated for 1 h at room temperature with anti-mouse phosphohistone γ H2AX (Ser 139) antibody (Millipore, clone JBW301). Nuclei of the tumour cells with γ H2AX foci were quantified as a percentage of total nuclei.

Doxorubicin delivery

Pdgfb-iCre^{ER};Fak^{fl/fl} mice and wild-type control mice (*Pdgfb-iCre^{ER}*;non-floxed) were given a subcutaneous injection of 1×10^6 B16F0 cells, then treated with tamoxifen (as described earlier) to generate ECFAK^{KO} and ECFAK^{WT} mice. To assess delivery of doxorubicin to the tumours, mice were injected with 20 mg kg^{-1} doxorubicin over 1 min, 5 min before euthanasia. Under terminal anaesthesia mice were perfused with 4% paraformaldehyde. Perfused tumours were removed and fixed overnight in 4% paraformaldehyde, then transferred to 70% ethanol. Tumours were embedded in paraffin, sectioned, rehydrated and counter-stained with DAPI. Tumour sections were analysed using the Zeiss Axioplan microscope and images were captured using Axiovision Rel.4 software.

Tumour hypoxia

For mice used and treatment schedules see earlier. On day 14, 1 h before mice were killed, tumour-bearing mice were injected with 60 mg kg^{-1} pimonidazole hydrochloride (HypoxyprobeTM-1 HPI, diluted in ddH₂O to a final concentration of 10 mg ml^{-1}) intravenously via the tail vein. Tumours were processed immediately after cervical dislocation. Cryosections were thawed, rehydrated and fixed for 10 min in -20°C acetone then incubated with 1:10 anti-pimonidazole antibody to identify hypoxic areas. Sections were then washed and mounted with ProLong Gold with Antifade plus DAPI (Invitrogen, P36930). Images were taken with a Zeiss microscope and Axioplan camera. The total tumour area and pimonidazole-positive areas were measured using Axiovision Rel. 4 software (Zeiss). The percentage hypoxic area for each tumour section was then calculated.

CD45 infiltration

For mice used and treatment schedules see earlier. On day 14, tumours were harvested and snap-frozen. Cryosections were fixed in acetone for 10 min at -20°C ; rehydrated in PBS for 10 min; blocked in 5% goat serum diluted in PBS for 1 h at room temperature; washed once in PBS; incubated overnight at 4°C with rat anti-mouse CD45 antibody (Serotec, MCA1388) diluted 1:100 in 0.5% goat serum in PBS; washed three times in PBS; incubated with anti-rat Alexa 488 (Invitrogen) for 1 h at room temperature; washed in PBS and finally mounted in ProLong Gold with DAPI (Invitrogen). CD45 was quantified as a percentage of total DAPI area within five fields of view using a $\times 40$ objective.

Experimental metastasis survival experiments

For experimental metastasis assays, 0.5×10^6 B16F10 and EumycBCL2 cells (obtained from S. Hallam and T. Hagemann) were injected via the tail vein of *Pdgfb-iCre^{ER};Fak^{fl/fl}* mice and control mice (*Pdgfb-iCre^{ER};non-floxed*). When tumours had grown, at either days 7 and 8 (for mice with B16F10) or days 10 and 11 (for EumycBCL2) after tumour-cell inoculation, mice were given tamoxifen to induce endothelial-cell FAK deletion in *Pdgfb-iCre^{ER};Fak^{fl/fl}* but not *Pdgfb-iCre^{ER};non-floxed* mice, generating ECFAK^{KO} and ECFAK^{WT} mice, respectively. Mice were then treated with placebo or doxorubicin at days 12, 13 and 14 (for mice with B16F10) or days 11, 13 and 15 (for EumycBCL2) after tumour-cell inoculation. Survival was recorded for each animal.

Phospho-STAT3 immunostaining

Pdgfb-iCre^{ER};Fak^{fl/fl} mice and wild-type control mice (*Pdgfb-iCre^{ER};non-floxed*) were given a subcutaneous injection of 1×10^6 CMT19T tumour cells and the treatment schedule was performed as described earlier. On day 10 after tumour-cell inoculation, mice were irradiated, or not, with 5 Gy γ -irradiation. Tumours were harvested when they had reached the maximum legal size allowed by the UK Home Office regulations, and snap-frozen immediately. Cryosections were permeabilized with ice-cold methanol for 10 min, followed by one PBS wash. Slides were blocked (5% normal goat serum, 0.3% Triton X-100 in PBS) for 60min at room temperature then incubated with phospho-STAT3 (Tyr705) (Cell Signaling; 1:30 dilution in 1% BSA, 0.3% Triton-X 100 in PBS) primary antibody overnight at 4 °C. The slides were washed three times with PBS and incubated with fluorescent-conjugated secondary antibody (1:100 dilution in 1% BSA, 0.3% Triton-X 100 in PBS). Slides were rinsed in PBS three times and once in water containing DAPI (1:10,000) and then mounted with coverslips using Prolong Gold Anti-fade Reagent.

Endothelial apoptosis

Apoptosis was measured in tumour sections by double immunostaining for either cleaved caspase 3 (CC3) or TdT-mediated dUTP nick end labelling (TUNEL) and PECAM. Immunostaining was performed as described earlier. Endothelial apoptosis was calculated by counting the percentage of tumour blood vessels that were also CC3-positive or TUNEL-positive.

Primary endothelial-cell culture and nuclear translocation of p65

Wild-type and FAK-null primary endothelial cells were generated as described previously²⁸. Wild-type and FAK-null primary endothelial cells were grown for 24 h or 48 h with MLEC supplemented with 500 nM 4-hydroxytamoxifen with or without doxorubicin (0.125 μ M or 0.25 μ M). Immunostaining for p65 was performed as described earlier.

Cultured endothelial-cell FAK expression analysis

Endothelial cells were prepared as described earlier. For FAK detection, cells were fixed with ice-cold acetone for 5 min at -20 °C. The fixed cells were then blocked for 30min at room temperature in 5% goat serum in PBS and washed once with PBS, then incubated with mouse anti-FAK antibody (77/FAK; BD Biosciences, 610088) diluted 1:100 in PBS. This

was followed by three washes in PBS and the cells were then incubated for 1 h at room temperature with Alexa-488-conjugated anti-mouse (Invitrogen) diluted 1:100 in PBS. After three final washes in PBS the coverslips were mounted in Prolong Gold (Invitrogen).

Western blotting

Wild-type and FAK-null endothelial cells were grown and lysed under the same conditions as described earlier. Anti-FAK (BD Biosciences) was used to detect FAK expression levels. Western blot analysis was processed with 50 µg of total cell lysate as described previously¹⁴. The following antibodies were used for western blot analysis of cytosolic fractions: phospho-IκBα (S32, Cell Signaling, 2859), IκBα (Santa-Cruz Biotechnology, sc-371).

GM-CSF and IL-6 intratumoral injections

B16F0 subcutaneous tumours were grown in *Pdgfb-iCreER;Fak^{fl/fl}* mice and wild-type control mice (*Pdgfb-iCreER*;non-floxed), and given tamoxifen after tumour growth had begun and treated with doxorubicin as described earlier. On days 9, 10, 11, 12 and 13 after tumour inoculation, half the animals were injected intratumorally with 100 µl of either 15 ng ml⁻¹ or 30 ng ml⁻¹ of recombinant mouse GM-CSF or IL-6 diluted in PBS (PeproTech) — concentrations that mimic the levels of wild-type endothelial-cell GM-CSF and IL-6 production. Controls were injected with 100 µl of PBS. Tumours were measured twice a week and the animals were killed when tumours reached maximum legal size.

Statistical analysis

Results are presented as means ± s.e.m. for at least 2–3 independent experiments, unless otherwise stated. The sample sizes used were based on level of changes and consistency expected. Statistical significance was reported as appropriate. For animal experiments, animals were excluded from the analysis if tumour volume breached the Home Office legal size limit. Sample sizes were chosen on the basis of the level of changes expected. No randomization methods were used. During animal experiments the investigator was blinded to the genotype of the animals under study. *P* values were calculated with the two-tailed Student's *t*-test unless otherwise stated. *P* < 0.05 was considered statistically significant.

Ethical regulations

All animals were used according to the UK Home Office regulations. Human lymphoma samples were obtained with signed informed consent from patients and Ethical Committee approval.

Extended Data

Extended Data Figure 1. Loss of endothelial-cell FAK in established tumours

Pdgfb-iCreER;Fak^{fl/fl} mice¹⁴ and wild-type control mice (*Pdgfb-iCreER*;non-floxed) were injected subcutaneously with B16F0-melanoma cells. At day 7 after tumour-cell injection, once tumour growth was established, mice were given tamoxifen to induce, or not, endothelial-cell FAK deletion (generating ECAFAK^{KO} and ECAFAK^{WT} mice) and tumours continued to grow until they reached the legal size limit at day 24 after tumour-cell injection. Immunofluorescence staining of tumour sections from ECAFAK^{WT} and ECAFAK^{KO} mice for FAK (green) and endomucin (red) shows efficient deletion of endothelial FAK in tumour blood vessels when FAK deletion is induced after tumour growth has begun. DAPI staining is shown in blue. Endothelial-specific FAK deletion *in vivo* was confirmed for all experiments. Representative images are given for a minimum of 5 mice per genotype. Scale bar, 75 μ m.

Extended Data Figure 2. Loss of endothelial-cell FAK in established tumours does not affect tumour blood vessel density, perfusion or endothelial apoptosis

a–c, *Pdgfb-iCreER;Fak^{fl/fl}* mice and wild type control mice (*Pdgfb-iCreER*;non-floxed) were injected subcutaneously with B16F0-melanoma cells. At day 7 after tumour-cell injection, once tumour growth was established, mice were given tamoxifen to induce, or not, endothelial-cell FAK deletion (generating ECFAK^{KO} and ECFAK^{WT} mice, respectively) and tumours continued to grow until they reached the legal size limit at day 24 after tumour-cell injection. Blood vessels were analysed histologically in midline tumour sections. **a**, Tumour blood vessel density was not affected by the deletion of FAK after tumour growth had begun. Immunofluorescence of endomucin-stained blood vessels and quantitation of number of blood vessels per mm² of tumour section are given. **b**, In an ante-mortem procedure, tumour burdened ECFAK^{KO} and ECFAK^{WT} mice were injected intravenously with PE-conjugated PECAM antibody. Midline sections were immunostained to detect endomucin-positive vessels. Examination of the percentage of endomucin-positive blood

vessels that are PE-PECAM-positive gives an indication of blood vessel perfusion. Tumour blood vessel perfusion was not affected significantly by the deletion of FAK after tumour growth had begun. **c**, Double immunostaining for tumour endothelial cells (PECAM), and either cleaved caspase 3 (CC3) or TUNEL, and DAPI in tumour sections from ECF^{WT} and ECF^{KO} mice. Apoptotic tumour cells are clearly visible (arrowheads). In contrast, apoptosis is not detectable in the endothelium of either genotype (arrows). Quantitation of the percentage of CC3-positive or TUNEL-positive tumour endothelial cells showed no significant difference between genotypes. Bar charts show means $\pm$ s.e.m. $n = 5-7$ mice per group. NS, not significant, Student's *t*-test. Scale bars, 100 μ m.

Extended Data Figure 3. Increased tumour-cell DNA damage without changes in blood vessel permeability, doxorubicin delivery, hypoxia or CD45 infiltration in ECF^{KO} mice

a, Quantitation of γ H2AX immunostaining indicates that the level of DNA damage in the tumour-cell compartment is increased in treated ECF^{KO} mice when compared with

ECFAK^{WT} mice. Bar chart shows mean percentage of γ H2AX-positive tumour cell nuclei $\pm$ s.e.m. $n = 3$ mice per group. **b**, Mice were injected via the tail vein with Hoechst dye and PE-PECAM, in an ante-mortem process, and tumour sections were analysed for blood vessel permeability. Representative images of tumour sections showing Hoechst uptake in tumour cells and PE-PECAM-positive blood vessels are given. Bar chart shows mean number of Hoechst-positive nuclei per field of view for tumours grown in ECFAK^{WT} and ECFAK^{KO} mice + s.e.m. $n = 8$ mice per group. **c**, Mice were injected via the tail vein with doxorubicin (20mg kg^{-1}) and analysed for levels of autofluorescent doxorubicin delivery. Representative images of autofluorescent doxorubicin are given. Bar chart shows mean percentage of doxorubicin-positive area proportional to tumour section area + s.e.m. $n = 7$ mice per group. **d**, Mice were treated or not with doxorubicin at 9, 11 and 13 days, injected via the tail vein with pimonidazole at day 14 and killed 1 h thereafter. Tumour sections were immunostained to detect hypoxia using an anti-pimonidazole antibody. Bar chart shows percentage hypoxic tumour section area + s.e.m. $n = 7$ mice per genotype. **e**, Mice were treated or not with doxorubicin as in c and tumour sections were immunostained for CD45-positive immune cells. Bar chart shows mean CD45 infiltration as a percentage of CD45-positive cell area over tumour section area + s.e.m. $n = 4\text{--}6$ mice per group. NS, not significant, Student's *t*-test. Scale bar, 100 μm .

Extended Data Figure 4. Endothelial-cell FAK deletion enhances the mean survival of doxorubicin-treated mice in experimental metastasis models of melanoma and lymphoma
a–c, *Pdgfb-iCre^{ER};Fak^{fl/fl}* mice and wild-type control mice (*Pdgfb-iCre^{ER}*;non-floxed) were injected via the tail vein (intravenously) with either B16F10 melanoma cells (**a, b**) or EumycBCL2 lymphoma cells (**c**) to establish experimental metastasis models. After tumour growth was established endothelial-cell FAK deletion was induced, or not, by treatment of *Pdgfb-iCre^{ER};Fak^{fl/fl}* and *Pdgfb-iCre^{ER}*;non-floxed mice with tamoxifen (generating ECFK^{KO} and ECFK^{WT} mice, respectively). Mice were then either treated with placebo (**a**) or doxorubicin (**b, c**) and survival of the mice was recorded. Data show that endothelial-cell FAK deletion, after tumour growth was established, had no effect on survival per se (**a**). In contrast, in both the B16F10 and EumycBCL2 experimental metastasis assays, the deletion of endothelial-cell FAK was sufficient to significantly extend median survival after treatment with doxorubicin (**b, c**). Dashed lines represent median survival. Timelines for tamoxifen and doxorubicin treatment are given in the horizontal bars below each graph. *n* =

10–20 mice per genotype per test. * $P = 0.0209$, ** $P = 0.0055$, Gehan–Breslow–Wilcoxon Test. NS, not significant.

Extended Data Figure 5. Alterations in FAK distribution but not expression levels in doxorubicin-stimulated wild-type endothelial cells *in vitro* and *in vivo*

a, Double immunofluorescence staining of cultured wild-type (WT) and FAK-null endothelial cells, with or without doxorubicin treatment, for FAK (green) and DAPI (blue). Staining confirms that FAK is not detectable in FAK-null endothelial cells. In contrast FAK is redistributed in doxorubicin-treated wild-type endothelial cells when compared with untreated wild-type controls. Experiments are representative of three repeats. **b**, Western blot analysis confirms that FAK levels are not significantly changed in doxorubicin-treated wild-type endothelial cells. HSC70 acts as a loading control. Bar chart represents mean densitometric readings of FAK levels relative to controls. $n = 3$. **c**, FAK expression levels are not different after doxorubicin treatment *in vivo*. Image J analysis of endothelial-cell

FAK intensity was performed on tumour sections from PBS or doxorubicin-treated ECFAK^{WT} mice stained for endomucin and FAK. Graph shows average FAK fluorescence pixel intensity levels in endomucin-positive endothelium for individual mice, with means per group $\pm$ s.e.m. $n = 13$ –15 mice per treatment group. NS, not significant, Student's *t*-test. Scale bar, 50 μ m.

Extended Data Figure 6. FAK deficiency inhibits doxorubicin-induced p65 nuclear localization in primary endothelial cells

a, Doxorubicin-induced p65 nuclear translocation is inhibited in FAK-null primary endothelial cells *in vitro*. Wild-type (WT) and FAK-null primary lung endothelial cells were treated for 24 h with tamoxifen and 0.25 μ M doxorubicin. Immunostaining for p65 (red) was performed. DAPI staining is shown in blue. Arrows indicate cytoplasmic p65; arrowheads indicate nuclear p65. Scale bar, 50 μ m. **b**, Bar charts show the percentage of endothelial cells with nuclear p65 (fold increase) after doxorubicin treatment (0.125 μ M or 0.25 μ M) for 24 h (T24) or 48 h (T48). $n = 91$ –205 cells per test group. *** $P < 0.0001$, Student's *t*-test. NS, not significant.

Extended Data Figure 7. Phosphorylation of IκBα is reduced in doxorubicin-treated FAK-null endothelial cells

Wild-type (WT) and FAK-null endothelial cells were treated with 0.125 μM doxorubicin for the time indicated. Representative western blot of cytosolic phospho-S32-IκBα and total IκBα. Bar chart shows mean densitometric readings from two biological replicates.

Extended Data Figure 8. Loss of endothelial-cell FAK inhibits the production of irradiation-induced endothelial cytokines

Wild-type (WT) and FAK-null endothelial cells were treated with 5 Gy irradiation. Seventy-two hours later, whole-cell lysates were extracted and used in proteome profiler cytokine arrays. Bar chart shows the fold difference in cytokine expression between irradiated and non-irradiated wild-type and FAK-null endothelial cells + s.e.m., $n = 4$ experimental repeats. † $P = 0.06$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t -test.

Extended Data Figure 9. Cytokine production is similar in untreated wild-type and FAK-null endothelial cells and DNA damage does not increase tumour-cell p-STAT3 expression in ECFAK^{KO} mice

a, Wild-type (WT) and FAK-null endothelial-cell whole-cell lysates were extracted and used in proteome profiler cytokine arrays. Bar chart shows baseline cytokine expression in untreated wild-type and FAK-null endothelial cells + s.e.m., $n = 4$ experimental repeats. NS, not significant. **b**, *Pdgfb-iCre^{ER};Fak^{fl/fl}* mice and control mice (*Pdgfb-iCre^{ER}*;non-floxed) were injected subcutaneously with CMT19T tumour cells (day 0). At 7–8 days post-inoculation, that is, once tumour growth was established, mice were given tamoxifen to induce endothelial-cell FAK deletion in *Pdgfb-iCre^{ER};Fak^{fl/fl}* but not *Pdgfb-iCre^{ER}*;non-floxed mice, generating ECFAK^{KO} and ECFAK^{WT} mice, respectively. Thereafter CMT19T bearing mice were given 5 Gy gamma irradiation (day 10). Immunostaining for p-STAT3 in tumour sections revealed that although irradiation increased the percentage of p-STAT3-positive perivascular tumour cells this was not evident in ECFAK^{KO} mice. Representative

images of double immunostaining for p-STAT3 and PECAM are given. Bar chart shows mean percentage of p-STAT3-positive perivascular tumour cells + s.e.m. $n = 6$ mice per group. *** $P < 0.005$, Student's t -test. NS, not significant.

Extended Data Figure 10. *In vivo* rescue of chemosensitization phenotype

Mouse melanoma B16F0 cells (1×10^6) were injected subcutaneously in the flank of *Pdgfb-iCre^{ER};Fak^{fl/fl}* mice and control mice (*Pdgfb-iCre^{ER}*;non-floxed). Seven days after tumour-cell injection, that is, once tumour growth was established, these mice were given tamoxifen to induce endothelial-cell FAK deletion in *Pdgfb-iCre^{ER};Fak^{fl/fl}* but not *Pdgfb-iCre^{ER}*;non-floxed mice, generating ECFAK^{KO} and ECFAK^{WT} mice, respectively. Intratumoral injection of a low dose of recombinant GM-CSF (15 ng) (top graph), or IL-6 (3 ng) (bottom graph), restored doxorubicin-treated tumour growth in ECFAK^{KO} mice to wild-type levels. Top graph shows mean tumour volumes over time + standard deviation. Bottom graph shows mean tumour volumes over time + s.e.m. $n = 10$ –18 mice per group. NS, not significant, Student's t -test.

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Figure 1. Endothelial-cell FAK deletion sensitizes cancer cells to DNA-damaging therapies *in vivo*

a–f, *Pdgfb-iCre^{ER};Fak^{fl/fl}* and control mice were injected subcutaneously with B16F0 or CMT19T tumour cells (day 0), given tamoxifen (Tam.; from day 7 onwards) to generate ECFAK^{KO} and ECFAK^{WT} mice, respectively, and subsequently treated or not with DNA-damaging therapy. **a, b**, In untreated mice tumour growth did not differ between genotypes. **c, d**, DNA-damaging therapy significantly inhibited tumour growth in ECFAK^{KO} mice when compared with ECFAK^{WT} controls. Graphs show mean tumour volumes $\pm$ standard error of the mean (s.e.m.). $n = 9$ ECFAK^{WT} and 15 ECFAK^{KO} mice per test. Horizontal bars represent procedure timelines. Dox., doxorubicin; Irrad., irradiation. **e, f**, Representative images of tumours at experimental endpoints. **g–j**, Immunofluorescence staining analysis for endothelial-cell FAK in PECAM-positive blood vessels in human lymphoma sections. **g**, At diagnosis, a reduced percentage of FAK-positive blood vessels correlates with subsequent achievement of complete remission, but an increased percentage of FAK-positive blood vessels correlates with subsequent disease progression. Bar chart shows the mean percentage of FAK-positive blood vessels $\pm$ s.e.m. $n = 16$ biopsy samples taken at diagnosis, 7 of which achieved complete remission and 9 of which subsequently progressed after treatment. Blood vessels were counted from triplicate tissue microarray (TMA) samples. **h**, Endothelial-cell FAK expression was significantly higher in relapsed lymphoma when compared with

endothelial-cell FAK expression at diagnosis in matched patient samples. Scatter plot shows mean endothelial-cell FAK fluorescence pixel intensity per sample $\pm$ s.e.m. $n = 13$ matched patient biopsies. **i, j**, Representative images of human lymphoma taken at diagnosis and relapse (that is, after treatment including doxorubicin) immunostained for PECAM (red), FAK (green) and 4',6-diamidino-2-phenylindole (DAPI; blue). Arrowheads indicate low FAK expression; arrows indicate high FAK expression. Scale bars, 5 mm (**e, f**); 50 μ m (**j**). $*P < 0.05$, $**P < 0.01$, Student's t -test. NS, not significant.

Figure 2. Loss of endothelial-cell FAK sensitizes tumour cells to DNA-damaging therapy *in vivo* and *in vitro*

a–d, Double immunostaining of B16F0 (**a, b**) and CMT19T (**c, d**) tumour sections from mice treated or not with doxorubicin (Dox.) or irradiation (Irrad.) for the apoptotic marker cleaved caspase 3 (CC3; green; **a, c**) or the proliferation marker Ki67 (green; **b, d**), and the endothelial marker PECAM (red). DAPI (blue) provides a nuclear marker. Bar charts show quantitation at 48 h post-treatment cessation of the mean number of blood vessels that are within CC3-positive tumour cell niches + s.e.m. (**a, c**; $n = 3$ mice per group) or the percentage of Ki67-positive perivascular tumour cells + s.e.m. (**b, d**; $n = 5$ mice per group). **e**, Conditioned media from untreated (–) and doxorubicin-treated (+) endothelial cells were applied to B16F0 cell cultures and tumour-cell survival was measured. $n = 9$ technical replicates. **f**, Conditioned medium from non-irradiated (–) or irradiated (+) endothelial cells was applied to irradiated CMT19T cells and tumour-cell survival was measured in MTS assays at 4 and 5 days. Bar charts show mean tumour-cell survival + s.e.m. according to

corrected absorbance readings of MTS assays. $n = 12$ technical replicates. $A_{490 \text{ nm}}$, absorbance at 490 nm. WT, wild type. Arrowheads indicate CC3-positive perivascular tumour cells; arrows indicate Ki67-positive perivascular tumour cells. Scale bars, 100 μm (**a**, **c**); 50 μm (**b**, **d**). $\dagger P = 0.1$, $*P < 0.05$, $***P < 0.001$, Student's t -test.

Figure 3. FAK deficiency inhibits doxorubicin-stimulated endothelial-cell p65 activity, phosphorylation and nuclear translocation

a, Luciferase assays indicate that NF-κB activity is significantly reduced in doxorubicin-stimulated FAK-null endothelial cells. $n = 4$ experimental repeats. Dox., doxorubicin. **b**, Immunofluorescence detection of p65 (red), DAPI (blue) in wild-type (WT) and FAK-null immortalized endothelial cells with and without doxorubicin treatment. Arrows indicate cytoplasmic p65; arrowheads indicate nuclear p65. **c**, Bar chart shows mean percentage of endothelial cells with nuclear p65 $\pm$ s.e.m. $n = 188$ –385 cells per group. **d**, Western blot analysis of nuclear fractions of wild-type and FAK-null endothelial cells after doxorubicin treatment. Bottom, bar chart shows mean densitometry readings over time. p-p65, phosphorylated p65. $n = 2$ experimental repeats. **e**, Tumour sections from doxorubicin-treated ECFAK^{WT} and ECFAK^{KO} mice were immunostained for endomucin (red), p65 (green) and DAPI (blue) and the percentage of endothelial cells with nuclear p65 was assessed. Right, bar chart shows mean percentage of endothelial cells with nuclear p65 *in vivo* $\pm$ s.e.m. $n = 34$ –89 endothelial cells per tumour and 4 tumours per group. Scale bars, 50 μ m (**b**); 20 μ m (**e**). * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$, Student's *t*-test.

Figure 4. Loss of endothelial-cell FAK inhibits doxorubicin-induced, NF- κ B-dependent production of endothelial cytokines

a, Quantitation of fold difference in cytokine expression between doxorubicin-treated and non-treated wild-type (WT) and FAK-null endothelial cells + s.e.m. $n = 3$ experimental repeats. **b**, Quantitation of the fold difference in cytokine expression between doxorubicin-treated and non-treated mock- and I κ B α SR-transfected wild-type endothelial cells $\pm$ s.e.m. $n = 4$ experimental repeats. **c**, Conditioned media from doxorubicin-treated mock- or I κ B α SR-transfected wild-type endothelial cells were applied to doxorubicin-treated B16F0 cells and cell survival was measured. Conditioned medium from doxorubicin-treated, I κ B α SR-transfected wild-type endothelial cells mimics the effects of conditioned medium from doxorubicin-treated mock-transfected FAK-null endothelial cells. Bar charts show mean tumour-cell survival as corrected absorbance readings from MTS, one-step cell survival assays + s.e.m. $n = 10$ technical repeats. **d**, **e**, Schematic representation of the role of

endothelial-cell FAK in tumour-cell sensitization to DNA-damaging therapy. $*P<0.05$,
 $**P<0.01$, $***P<0.01$, Student's *t*-test.

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