

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

**Birc2/lap1 regulates endothelial cell integrity and blood vessel homeostasis.**

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/92786> since

*Publisher:*

Nature America Incorporated:345 Park Avenue South, 6th Floor:New York, NY 10010:(888)331-6288,

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

# Birc2 (clap1) regulates endothelial cell integrity and blood vessel homeostasis

Massimo M Santoro<sup>1,2</sup>, Temesgen Samuel<sup>3,4</sup>, Tracy Mitchell<sup>1,5</sup>, John C Reed<sup>3</sup> & Didier Y R Stainier<sup>1</sup>

**Integrity of the blood vessel wall is essential for vascular homeostasis and organ function<sup>1,2</sup>. A dynamic balance between endothelial cell survival and apoptosis contributes to this integrity during vascular development and pathological angiogenesis<sup>3–6</sup>. The genetic and molecular mechanisms regulating these processes *in vivo* are still largely unknown. Here, we show that Birc2 (also known as clap1) is essential for maintaining endothelial cell survival and blood vessel homeostasis during vascular development. Using a forward-genetic approach, we identified a zebrafish null mutant for *birc2*, which shows severe hemorrhage and vascular regression due to endothelial cell integrity defects and apoptosis. Using genetic and molecular approaches, we show that Birc2 positively regulates the formation of the TNF receptor complex I in endothelial cells, thereby promoting NF- $\kappa$ B activation and maintaining vessel integrity and stabilization. In the absence of Birc2, a caspase-8-dependent apoptotic program takes place that leads to vessel regression. Our findings identify Birc2 and TNF signaling components as critical regulators of vascular integrity and endothelial cell survival, thereby providing an additional target pathway for the control of angiogenesis and blood vessel homeostasis during embryogenesis, regeneration and tumorigenesis.**

Vascular development is regulated by proangiogenic and antiangiogenic factors in the immediate environment of endothelial cells. A positive balance of angiogenic factors stimulates endothelial cell proliferation, migration and survival, leading to the formation of new vessels, whereas a prevalence of antiangiogenic factors shifts the equilibrium to vessel quiescence or, under certain circumstances, to endothelial cell apoptosis and vessel regression<sup>5,6</sup>. Abnormalities in the control of these processes can contribute to embryonic death and to a variety of vascular and immune diseases (for example, diabetic retinopathies, atherosclerosis and systemic lupus erythematosus)<sup>3,4,7,8</sup>. Thus, elucidation of the genetic control of endothelial cell survival and apoptosis is critical to better understanding vascular biology and endothelial angiogenesis in normal and pathological conditions.

To identify the molecular pathways involved in endothelial cell survival and vascular homeostasis, we used a forward genetic approach in zebrafish, a model organism that has a number of advantages for investigating vascular development and homeostasis<sup>9</sup>. In the course of a large-scale *N*-ethyl-*N*-nitrosourea mutagenesis screen for vascular mutants<sup>10</sup>, we identified the mutation *tomato* (*tom*). The *tom* mutation is recessive and shows complete penetrance and expressivity. *tom* mutants show distinct vascular hemorrhage and blood pooling in several tissues in the context of a wild-type body morphology (Fig. 1a,b). To examine whether the hemorrhage is associated with vascular defects, we crossed the mutation into the *Tg(flk1:GFP)<sup>s843</sup>* line, a transgenic line expressing the green fluorescent protein (GFP) in endothelial cells throughout the developing vasculature<sup>10</sup>. Fluorescence microscopy analyses showed that the hemorrhagic phenotype in *tom* mutants was accompanied by specific blood vessel regression starting between 54 and 60 hours post-fertilization (hpf) (Fig. 1c,d and Supplementary Fig. 1 online). Endothelial regression and cell fragmentation were observed in the brain and trunk vasculature of *tom* mutants (Fig. 1e and Supplementary Movie 1 online).

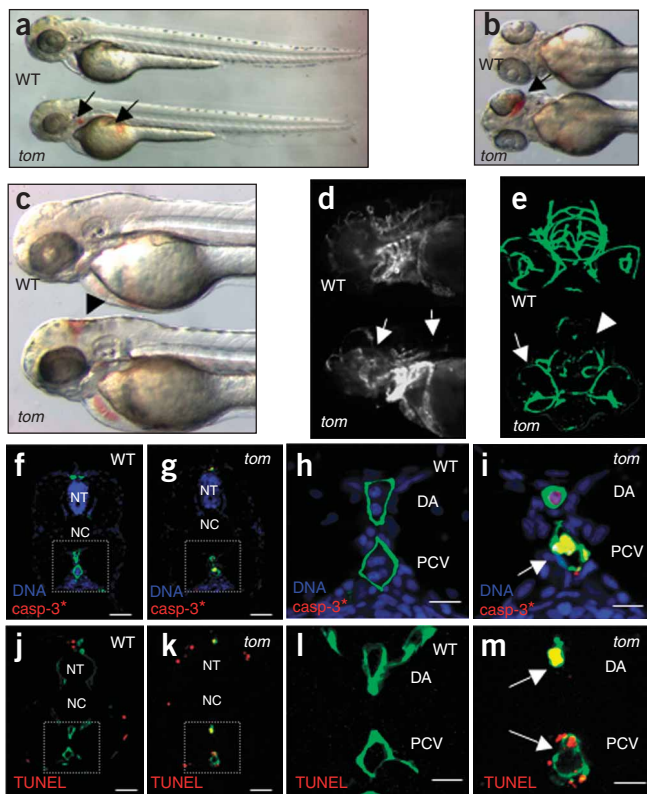
To test whether the vascular regression was due to apoptotic cell death, we examined the expression of apoptotic markers in wild-type and *tom* mutant embryos (Fig. 1f–m). Indeed, endothelial cells in *tom* mutants were positive for active caspase-3 and TUNEL staining, and showed typical morphological features of apoptotic cells at this stage. Notably, no other embryonic tissues showed specific apoptosis. These data indicate that the apoptotic program is activated specifically in endothelial cells in *tom* mutant embryos and suggest that *tom* may regulate endothelial cell survival and vascular integrity.

By positional cloning, we determined that *tom* encodes baculoviral IAP repeat-containing protein-2, Birc2 (also called clap1, cellular inhibitor of apoptosis 1) (Fig. 2a). Sequence analysis showed that the *tom* mutant allele contains a nonsense mutation in *birc2* that introduces a premature stop codon in the protein at amino acid position 29, likely generating a null allele (Fig. 2b). To further test whether Birc2 regulates vascular integrity, we knocked down Birc2 expression using an antisense morpholino oligonucleotide. *birc2* morphants showed the hemorrhagic phenotype and vascular apoptosis

<sup>1</sup>Department of Biochemistry and Biophysics, Programs in Developmental Biology, Genetics and Human Genetics, and Cardiovascular Research Institute, University of California San Francisco, San Francisco, California 94158, USA. <sup>2</sup>Department of Environmental and Life Sciences, University of Piemonte Orientale “A. Avogadro,” Alessandria, 15100, Italy. <sup>3</sup>Burnham Institute for Medical Research, La Jolla, California 92037, USA. <sup>4</sup>Tuskegee University, College of Veterinary Medicine, Nursing and Allied Health, Pathobiology, Pattersen Hall, Tuskegee, Alabama 36088, USA. <sup>5</sup>Osteoarthritis Research Group, Wyeth Research, 200 Cambridge Park Drive, Cambridge, Massachusetts 02140, USA. Correspondence should be addressed to M.M.S. (massimo.santoro@ucsf.edu) or D.Y.R.S. (didier.stainier@biochem.ucsf.edu).

Received 16 April; accepted 7 August; published online 14 October 2007; doi:10.1038/ng.2007.8





**Figure 1** Vascular integrity and endothelial survival are defective in *tom* mutants. (a,b) Wild-type (WT) and *tomato* (*tom*) mutant embryos at 60 hpf. Vascular hemorrhage is visible at several locations in *tom* mutants (arrows). (c,d) Bright-field (c) and fluorescent (d) micrographs of head vasculature in *Tg(flk1:GFP)<sup>S843</sup>* WT and *tom* mutant embryos at 60 hpf. *tom* mutants show hemorrhage (arrowhead) due to vascular integrity defects and regression (arrows) as detectable in the *Tg(flk1:GFP)<sup>S843</sup>* background. (e) Confocal projections of WT and *tom* mutant embryo head vasculature (dorsal view, anterior to the bottom) showing endothelial breakdown and regression in *tom* mutants, in particular in the hindbrain (arrowhead) and eye capillary network (arrow) at 60 hpf. (f–m) Confocal transverse sections of *Tg(flk1:GFP)<sup>S843</sup>* WT (f,h,j,l) and *tom* mutant (g,k,i,m) embryos stained for DNA (blue), active caspase-3 (red; f–i) or TUNEL (red; j–m) at 60 hpf. Endothelial cells in *tom* mutants are positive for active caspase-3 and TUNEL. No other embryonic tissues show specific apoptosis in *tom* mutants; however, some TUNEL-positive cells are visible in the skin of both WT and *tom* mutant embryos (j–k). h,i,l and m show a magnification of the areas outlined in f,g,j and k, respectively. Endothelial cells within the dorsal aorta and posterior cardinal vein are positive for active caspase-3 and TUNEL (arrows), and feature cell fragmentation, characteristic of cells undergoing programmed cell death. Sections shown are at the level of the tenth somite. NT, neural tube; NC, notochord; DA, dorsal aorta; PCV, posterior cardinal vein. Scale bars, 20  $\mu$ m.

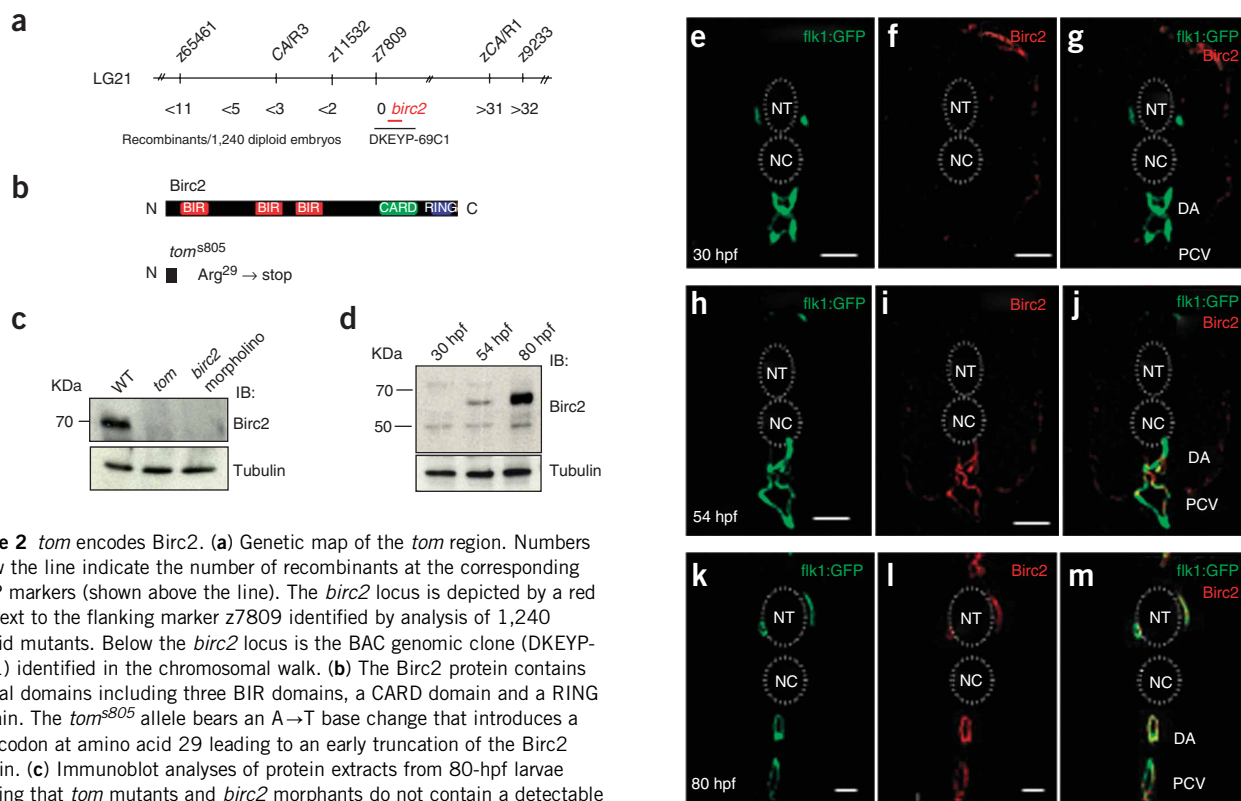
observed in *tom* mutants (Supplementary Fig. 2 online). Using a specific Birc2 antibody, we carried out immunoblot analyses on protein lysates from wild-type and *tom* mutant embryos, as well as *birc2* morphants (Fig. 2c). We found that *tom* mutants and *birc2* morphants did not express detectable levels of Birc2. Together, these results indicate that *tom* is a null mutation in *birc2* and that Birc2 regulates endothelial cell survival and vascular integrity in zebrafish embryos.

To further understand Birc2 function, we analyzed Birc2 expression in developing zebrafish embryos. By *in situ* hybridization, we detected *birc2* transcripts in the vasculature starting at 48 hpf (Supplementary Fig. 3a,b online). By immunoblot analyses of embryo lysates, we observed that Birc2 expression begins by 54 hpf (Fig. 2d). By immunofluorescence analyses, we detected Birc2 protein specifically in the vasculature also starting at 54 hpf (Fig. 2e–m). Birc2 is present in the cytosol of endothelial cells but not in their nuclei (Supplementary Fig. 3c–e). This temporal expression pattern of Birc2 correlates with the onset of vascular apoptosis and regression that is detectable in *tom* mutants starting between 54 and 60 hpf.

Genomic and phylogenetic analyses reveal that—in contrast to mammalian genomes, which contain two closely related *BIRC* genes called *BIRC2* (*cIAP1*) and *BIRC3* (*cIAP2*)—lower-vertebrate genomes contain only one *birc2* (*ciap1*) gene. This finding highlights the usefulness of the zebrafish model, and the *tom* mutant in particular, in exploring the functional role of *birc2* *in vivo*. Birc2 belongs to a family of inhibitor of apoptosis proteins (IAPs): members of this family regulate apoptosis induced by various stimuli<sup>11</sup>. Mammalian *BIRC2* and *BIRC3* have been identified as components of the tumor necrosis factor receptor (TNFR) signaling complex through binding to TNFR-associated factor 2 (TRAF2)<sup>12,13</sup>. TNFR belongs to the superfamily of death receptors and regulates numerous cellular functions, including cell survival and differentiation and apoptosis<sup>14–16</sup>. TNFR

activates cell survival and differentiation or apoptosis by assembling two molecularly and spatially distinct signaling complexes. Within a few minutes of TNF binding to TNFR, a plasma membrane-bound signaling complex, termed complex I, forms. This complex contains the receptor itself, receptor interacting protein 1 (RIP1), TRAF2, BIRC2, BIRC 3 and the TNFR-associated death domain (TRADD). Complex I initiates signals that lead to the rapid activation of NF- $\kappa$ B through polyubiquitination of NF- $\kappa$ B essential modulator (NEMO) and recruitment of the IKK ‘signalsome’. Later on, possibly during receptor internalization, TRADD and RIP1 dissociate from the receptor and recruit fas-associated via death domain (FADD) and caspase-8 into a secondary complex, called complex II, that mediates caspase-8 activation and cell death<sup>17</sup>.

To investigate whether zebrafish Birc2 modulates endothelial cell survival by regulating TNFR signaling, we first tested whether zebrafish Birc2 can bind TRAF2. To this end, we performed TRAF2 co-immunoprecipitation assays using HEK293 cell lines expressing zebrafish Birc2. Zebrafish Birc2, but not Birc4 (another member of the IAP family and potent inhibitor of effector caspases<sup>18</sup>), binds endogenous mammalian TRAF2 (Fig. 3a). We also found that zebrafish Traf2 can bind Birc2 (Supplementary Fig. 4 online), indicating that the mechanisms of TRAF2 and Birc2 interactions are conserved among different vertebrate species. Thus, we investigated whether Birc2 could regulate endothelial cell survival in a TRAF2-dependent manner by engineering a Birc2 point mutant, called Birc2-E66A/R67A, which renders Birc2 defective for TRAF2 binding (owing to a GluArg  $\rightarrow$  AlaAla mutation in BIR1 domain)<sup>19</sup>. Recent studies indicate that recruitment of Birc2 to complex I through TRAF2 interaction promotes NEMO polyubiquitination and subsequent NF- $\kappa$ B activation via the E3-ubiquitin ligase activities in the complex<sup>20,21</sup>. Therefore, we also generated a second mutant, called Birc2-H617A, that renders Birc2 defective in its E3-ubiquitin ligase activity (owing to a His  $\rightarrow$  Ala mutation in its RING domain)<sup>19,22</sup>. We first confirmed by *in vivo* expression that these Birc2 variants were defective in TRAF2 binding (the E66A/R67A mutant) and in ubiquitin ligase activity (the H617A mutant) (Supplementary Fig. 4). We then tested whether the variants could rescue the *tom* mutant phenotype (Fig. 3b). Although wild-type Birc2 rescued the phenotype, the mutant constructs did not rescue the vascular apoptosis and hemorrhagic phenotypes in *tom* mutants, suggesting that both the TRAF2-binding and E3-ligase activity of



**Figure 2** *tom* encodes Birc2. (a) Genetic map of the *tom* region. Numbers below the line indicate the number of recombinants at the corresponding SSLP markers (shown above the line). The *birc2* locus is depicted by a red bar next to the flanking marker z7809 identified by analysis of 1,240 diploid mutants. Below the *birc2* locus is the BAC genomic clone (DKEYP-69C1) identified in the chromosomal walk. (b) The Birc2 protein contains several domains including three BIR domains, a CARD domain and a RING domain. The *tom*<sup>s805</sup> allele bears an A→T base change that introduces a stop codon at amino acid 29 leading to an early truncation of the Birc2 protein. (c) Immunoblot analyses of protein extracts from 80-hpf larvae showing that *tom* mutants and *birc2* morphants do not contain a detectable level of Birc2 protein. Anti-tubulin immunoblot serves as protein control for loading. (d) Time-course analysis of Birc2 expression in zebrafish. Immunoblot analyses of protein extracts from 30-, 54- and 80-hpf embryos showing that Birc2 expression begins by 54 hpf. Anti-tubulin immunoblot serves as protein control for loading. (e–j) Confocal transverse sections of *Tg(flk1:GFP)<sup>S843</sup>* embryos at 30 (e–g), 54 (h–j) and 80 (k–m) hpf stained for Birc2 (red). Birc2 is expressed and co-localizes with GFP-positive endothelial cells starting at 54 hpf. Sections shown are at the level of the 18th somite. NT, neural tube; NC, notochord; DA, dorsal aorta; PCV, posterior cardinal vein. Scale bars, 20  $\mu$ m.

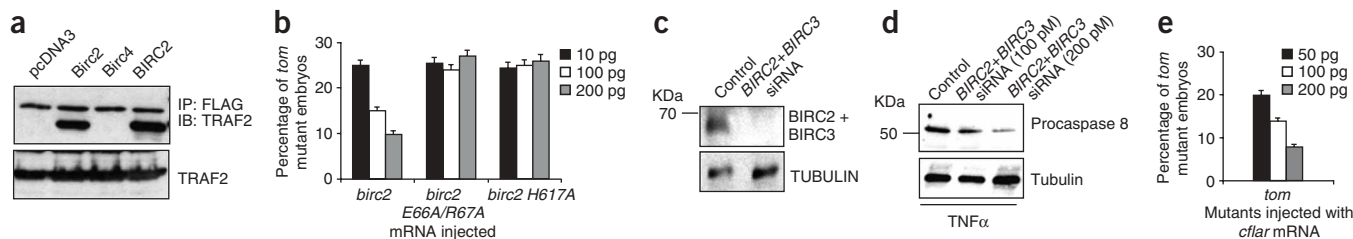
Birc2 are critical in promoting endothelial cell survival. These data suggest that Birc2 regulates endothelial cell survival *in vivo* by modulating TRAF2-dependent signaling and, possibly, by ubiquitinating target proteins in the TNFR-associated complex, such as NEMO and TRAF2<sup>21</sup>.

Impairment of complex I formation and signaling promotes TNFR-dependent complex II formation and subsequent apoptosis<sup>17</sup>. To test whether Birc2 is required to prevent TNFR-mediated formation of complex II (and thus, caspase-8 activation), we depleted endogenous *BIRC2* and *BIRC3* in human primary endothelial cells (HUVECs) with siRNAs. siRNAs specific for *BIRC2* and *BIRC3* reduced expression of their respective proteins (Fig. 3c). Cells were then stimulated with TNF $\alpha$ , and protein lysates from the stimulated cells were tested for procaspase-8 by immunoblotting (Fig. 3d). We observed that compared to control-transfected cells, cells depleted of *BIRC2* and *BIRC3* showed greater procaspase-8 cleavage and activation upon TNF $\alpha$  exposure. These data suggest that in endothelial cells exposed to certain extracellular stimuli, the absence of *BIRC2* and *BIRC3* can promote a caspase-8-dependent apoptotic program, possibly mediated by complex II formation. Following these observations, we hypothesized that endothelial cell apoptosis in *tom* mutants could be initiated by complex II formation and caspase-8 activation. To test this hypothesis, we overexpressed Casp8 and FADD-like apoptosis regulator (Cflar), a potent inhibitor of caspase-8, in *tom* mutants. Cflar binds to FADD and caspase-8 through a homotypic interaction and blocks caspase-8 recruitment into complex II and its subsequent activation<sup>16,23,24</sup>. Cflar overexpression rescued endothelial cell death

in *tom* mutants (Fig. 3e). These data indicate that endothelial cell apoptosis in *tom* mutants is associated with caspase-8 activation and possibly TNFR signaling. To further test this hypothesis, we treated *tom* mutants with a cell-permeable caspase-8-specific inhibitor, IETD-fmk. This treatment also rescued endothelial cell death in *tom* mutants (Supplementary Fig. 5 online), supporting the hypothesis that caspase-8 activation may mediate endothelial cell apoptosis and vessel regression in *tom* mutants. Because there is no evidence that *BIRC2* or *BIRC3* suppresses caspases directly<sup>25</sup>, we hypothesize that Birc2 most likely functions by preventing apoptosis promoted by formation of the TNFR-mediated complex II.

One of the main signaling pathways activated by TNFR through complex I is the IKK–NF- $\kappa$ B pathway. This pathway blocks complex II formation through Cflar expression<sup>26,27</sup>. To further test the hypothesis that Birc2 may promote TNFR-dependent complex I formation in endothelial cells, we examined whether Birc2 was required for TNFR-mediated NF- $\kappa$ B activation by analyzing the IKK–NF- $\kappa$ B signaling pathway in HUVECs deficient in both *BIRC2* and *BIRC3*. Cells transfected with *BIRC2* and *BIRC3* siRNAs had a reduction in IKK $\alpha$ , IKK $\beta$  and NF- $\kappa$ B phosphorylation and activation in response to TNF $\alpha$  stimulation (Fig. 4a). As a control, these cells were also stimulated with IL1 $\beta$ , a cytokine known to induce NF- $\kappa$ B activation through a signaling complex other than TNFR complex I<sup>28</sup>. Unlike TNF $\alpha$ , IL1 $\beta$  stimulation resulted in equivalent levels of IKK $\alpha$  and  $\beta$  and NF- $\kappa$ B activation in *BIRC2*- and *BIRC3*-deficient and control cells, suggesting that Birc2 specifically promotes TNFR-dependent complex I formation and NF- $\kappa$ B activation in endothelial cells. We





**Figure 3** Birc2 regulates TNFR-TRAF2 signaling and death receptor-mediated caspase-8 activation in endothelial cells. **(a)** Co-immunoprecipitation of TRAF2 with zebrafish Birc2 in HEK293T cells. Above, TRAF2 co-immunoprecipitates with transfected zebrafish Birc2 and human BIRC2, but not zebrafish Birc4. Upper bands are IgG. Below, TRAF2 expression in whole cell lysates. **(b)** Embryos from *tom*<sup>s805</sup> heterozygote intercrosses injected at the one-cell stage with the indicated amount of mRNA encoding Birc2, Birc2-E66A/R67A or Birc2-H617A. Histogram shows the percentage of *tom* mutants identified 72 hpf after injection. Birc2, but not Birc2-E66A/R67A or Birc2-H617A, rescued *tom* mutants in a dose-dependent manner. Error bars, s.d. **(c)** BIRC2/3 expression in siRNA-transfected HUVECs. Transfection of specific siRNA for BIRC2 and BIRC3 (BIRC2/3 siRNA) reduces endogenous BIRC2/3 expression in HUVECs. **(d)** Knock-down of BIRC2/3 promotes a TNF $\alpha$ -dependent activation and degradation of procaspase-8 in HUVECs. siRNA-transfected cells were stimulated with TNF $\alpha$  for 1 h and evaluated for procaspase-8 cleavage and activation. Anti-tubulin immunoblot serves as protein control for loading. **(e)** Embryos from *tom*<sup>s805</sup> heterozygote intercrosses injected at the one-cell stage with the indicated amount of mRNA encoding Cflar. Histogram shows the percentage of *tom* mutants identified 72 hpf after injection. Cflar rescues *tom* mutants in a dose-dependent manner. Error bars, s.d.

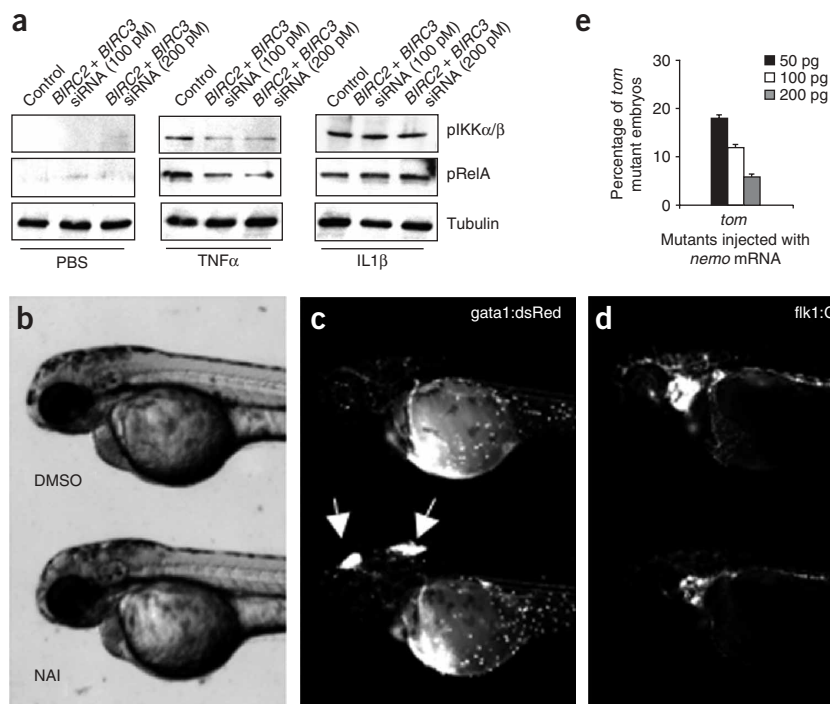
further assessed whether loss of BIRC2 and BIRC3 expression correlates with endothelial cell apoptosis. BIRC2- and BIRC3-deficient HUVEC cells stimulated with TNF $\alpha$  showed a specific apoptotic phenotype (**Supplementary Fig. 6** online), confirming that impairment of TNFR complex I promotes an apoptotic response in endothelial cells.

NF- $\kappa$ B signaling regulates endothelial cell integrity and vascular homeostasis<sup>29,30</sup>. We thus examined whether NF- $\kappa$ B signaling regulates vessel development in zebrafish by treating embryos with two different NF- $\kappa$ B inhibitors, NF- $\kappa$ B activation inhibitor (NAI) (**Fig. 4b–d** and **Supplementary Movie 2** online) and BAY11-7082 (data not shown). Confirming the essential function of NF- $\kappa$ B activation in vascular homeostasis, chemical inhibition of NF- $\kappa$ B activation provoked vascular leakage, hemorrhage and vascular regression in zebrafish embryos. We obtained the same phenotype by inhibiting the upstream IKK $\alpha$ –IKK $\beta$  complex with the specific IKK kinase inhibitor wedelolactone

(data not shown). Notably, endothelial differentiation was not affected in NAI-treated embryos as assessed by the expression of the arterial-specific marker EphrinB2a and the venous-specific marker *flt4* (data not shown). Additionally, confocal microscopy analyses of NAI-treated and *tom* mutant embryos indicated that NF- $\kappa$ B inhibition altered vessel morphology of the dorsal aorta and cardinal vein (**Supplementary Fig. 7** online). The absence of cell death in NAI-treated embryos suggests that the vascular leakage and integrity defects observed in *tom* mutants precede endothelial cell death.

To further test the genetic link between *birc2* and the IKK–NF- $\kappa$ B pathway, we overexpressed zebrafish NEMO in *tom* mutants. NEMO overexpression, which is required for NF- $\kappa$ B activation, can rescue endothelial cell death in *tom* mutants (**Fig. 4e**), providing further evidence that *birc2* mutants have defects in NF- $\kappa$ B activation. In summary, NF- $\kappa$ B inhibition and Birc2 deficiency in zebrafish alter

**Figure 4** NF- $\kappa$ B activation is regulated by Birc2 in endothelial cells and regulates vascular homeostasis in zebrafish embryos. **(a)** BIRC2/3 siRNA-transfected HUVECs show a reduction in IKK $\alpha$ / $\beta$  and NF- $\kappa$ B phosphorylation and activation after stimulation for 15 min with TNF $\alpha$ , but not with IL1 $\beta$ . Anti-tubulin immunoblot serves as protein control for loading. **(b–d)** Pictures of 54-hpf *Tg(flk1:EGFP)<sup>s843</sup>;Tg(gata1:DsRed)<sup>sd2</sup>* embryos treated with 0.1% dimethylsulfoxide (DMSO) or 100 nM NAI (NF- $\kappa$ B activator inhibitor) from 15-somite stage onwards. **(c)** Chemical inhibition of NF- $\kappa$ B caused severe hemorrhage that is visible as blood pooling in the *Tg(gata1:DsRed)* line, in which all the red blood cells express DsRed. **(d)** NAI-treated embryos also showed vascular regression as seen in the *Tg(flk1:GFP)<sup>s843</sup>* background. **(e)** Embryos from *tom*<sup>s805</sup> heterozygote intercrosses injected at the one-cell stage with the indicated amount of mRNA encoding zebrafish NEMO. Histogram shows the percentage of *tom* mutants identified 72 hpf after injection. NEMO rescues *tom* mutants in a dose-dependent manner. Error bars, s.d.



blood vessel homeostasis by inducing vascular leakage and causing endothelial cell integrity defects. These data highlight the specific and critical role of the Birc2–NF- $\kappa$ B pathway in vascular stability and endothelial cell survival *in vivo*.

Vascular cells within a blood vessel are exposed to a unique mixture of survival (proangiogenic: for example, VEGF, FGF, VE-cadherin, integrins) and apoptotic (antiangiogenic: for example, Fas, TSP-1, endostatin) signals produced by the surrounding tissues. Endothelial cells assimilate and respond to these environmental cues by remaining viable or committing suicide by initiating apoptosis. Therefore, closely regulated molecular control of endothelial cell survival, integrity and apoptosis is essential for both embryonic and adult angiogenesis. By using a combination of genetic and biochemical methods, we have shown that Birc2 (clap1) plays an essential role in regulating endothelial cell survival in the microenvironment of blood vessels *in vivo*. In this context, Birc2 appears to function by modulating death-receptor signaling in endothelial cells, possibly by regulating the formation and function of TNFR complexes. It is also possible that Birc2 plays a part in Fas and CD40 receptor complexes and signaling, whose function in angiogenesis is well established. Although the roles of IAPs in regulating apoptosis have been extensively studied, the *in vivo* function of vertebrate BIRC2 and BIRC3 has remained largely unknown. The *in vivo* significance of several *in vitro* BIRC-protein interactions has also remained elusive. Our results now identify an *in vivo* function for vertebrate BIRC2 and BIRC3 and reveal the importance of these proteins in vascular biology, specifically in vascular integrity. Studies of the genetic and biochemical mechanisms regulating vascular development and integrity are important as means to better understand the molecular basis of development, as well as to design new therapeutic strategies for endothelial cell-associated diseases, such as tumor angiogenesis, retinopathies and atherosclerosis.

## METHODS

**Zebrafish strains, mapping, genotyping and constructs.** Embryos and adult fish were raised and maintained under standard laboratory conditions. We used the following lines: *tom*<sup>s805</sup>, *Tg(flk1:GFP)*<sup>s843</sup> and *Tg(gata1:DsRed)*<sup>sd2</sup>. We mapped the *tom* mutation to linkage group 21 using a set of SSLP markers. For fine mapping, 1,240 mutant embryos were tested with SSLP markers in the critical interval (Fig. 2a). *tom*<sup>s805</sup> mutants were genotyped by polymerase chain reaction (PCR) amplification followed by restriction enzyme digestion with *Sfi*I. We isolated and sequenced *birc2* cDNA from wild-type and *tom* mutant embryos. Full-length *birc2*, *birc4*, *clar* and *nemo* were subcloned into the pCS2+ vector for mRNA injection. We used the QuikChange site-directed mutagenesis kit (Stratagene) to introduce specific point mutations in *birc2*. *Birc4*, *Birc2*, *Birc2 E66A/R67A* and *Birc2 H617A* cDNAs were also subcloned in pcDNA3-Flag plasmids for transfection experiments. All these constructs were transfected in HEK cell lines and tested for comparable levels of expression (data not shown).

***In situ* hybridization, morpholinos and mRNA injection and chemical treatment.** Whole-mount *in situ* hybridization was performed as previously described<sup>10</sup>. To generate a *birc2 in situ* probe, 630 base pairs at the 3' end of the gene were cloned into pCS2+. We used a splicing morpholino against the exon 1–intron 1 junction of *birc2* (Supplementary Methods). We injected *Tg(flk1:GFP)*<sup>s843</sup> embryos at the one-cell stage with 4 ng of morpholino and assayed them between 48–72 hpf. Embryos from *tom*<sup>s805</sup> heterozygote intercrosses were injected at the one- or two-cell stage with the indicated amount of mRNA encoding for the different proteins. We also included a control mRNA for H2B-cherry (50 pg) in each injection. Injected embryos were scored for vascular apoptosis and integrity defects at 60–72 hpf and genotyped. We injected at least 100 eggs for each experimental point. Data are representative of three independent experiments for each construct. We treated embryos with the indicated amount of 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (NAI), BAY11-7082, wedelolactone and Z-IETD-fmk (Calbiochem). As a

control, embryos from the same batch were treated with DMSO. Embryos were treated from the 15-somite stage until they were harvested for fixation at the indicated time.

**Cell culture, *in vitro* binding, immunoblot and immunohistochemistry.** We cultured primary human endothelial cells (HUVECs) and HEK293 cells in appropriate media according to the manufacturer's protocols (Clonetics and ATCC). We used the TNT Quick Coupled *In vitro* Transcription and Translation kit (Promega) for *in vitro* translation. Zebrafish TRAF2 were *in vitro* translated in the presence of <sup>35</sup>S-methionine. For *in vitro* binding assays, *in vitro*-translated products were co-incubated with cell lysates from HEK293T cells transfected with Flag-tagged wild-type or mutant Birc2, to allow protein-protein interaction. We immunopurified formed protein complexes using beads coated with anti-Flag antibodies. After washing to remove unbound proteins, bound proteins were detected by SDS-PAGE and autofluorography. The *ex vivo* ubiquitination assay to examine Birc2 E3 activity was performed as described<sup>19</sup>. In the current ubiquitination assay, *in vitro* translated TRAF2 was added as E3 substrate to the cell lysates and co-immunoprecipitated with Birc2, and then subjected to the ubiquitination assay. Control, BIRC2 and BIRC3 siRNA were from siGENOME collection of Dharmacon. We transfected HUVECs with the same amount of control or BIRC2/3 siRNA with oligofectamine reagent according to the manufacturer's protocols (Invitrogen). Cells were then stimulated with TNF $\alpha$  or IL1 $\beta$  and total cell extracts analyzed for immunoblotting. After 48 h, we lysed transfected cells with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 20 mM DTT, 10 mM sodium pyrophosphate, 20 mM NaF, 1 mM sodium orthovanadate and protease inhibitors. We carried out co-immunoprecipitation experiments as previously described<sup>19</sup>. Protein extracts were prepared by placing equal numbers (typically 40–70) of animals in 0.5 ml of lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100 and 0.1% SDS) containing a cocktail of protease and phosphatase inhibitors (Roche) and sonicating them for 10 s. Insoluble materials were removed by centrifugation. Proteins were separated by SDS-PAGE gels under reducing conditions, transferred to nitrocellulose membranes, and stained with antibodies. Embryos were fixed overnight with 2% paraformaldehyde and embedded in low melting agarose. We cut embedded embryos with a Leica vibratome into 250  $\mu$ m sections. Sections were processed in PBDT (1% BSA, 1% DMSO and 0.1% Triton X-100 in PBS, pH 7.3). We mounted processed samples in Vectashield (Vector Laboratories) and acquired the images using a Zeiss LSM5 Pascal confocal microscope and Zeiss confocal projection software. We used the following antibodies and dilutions: rabbit anti-Birc2 (PTG) at 1:1,000 for immunoblotting and 1:100 for immunofluorescence, mouse IgG anti- $\beta$ -catenin (Sigma) at 1:100, mouse IgG anti-zona occludin-1 (BD Transduction Laboratory) at 1:200, rabbit anti-tubulin (Abcam) 1:1000, rabbit anti-active caspase-3 and mouse anti-caspase-8 (Calbiochem) at 1:100 and phosphoIKK $\alpha$ / $\beta$  (ser176/180) and phosphoNF- $\kappa$ B p65 (ser536) (Cell Signaling) at 1:1,000. DNA was visualized with TO-PRO3 and f-actin with phalloidin (Molecular Probes). The TUNEL cell death assay was carried out using the "In Situ Cell Death Detection Kit" TMR red (Roche) according to manufacturer's instructions.

Note: Supplementary information is available on the Nature Genetics website.

## ACKNOWLEDGMENTS

We would like to thank members of the Stainier laboratory for invaluable support and E. Kratz and P. Eimon for discussion and reagents. Support for this research came from a Human Frontier Science Program Fellowship (M.M.S.), Cardiovascular Research Institute National Institutes of Health Training Grant (T.M.) and grants from the US National Institutes of Health (AG-15402 (J.C.R.); HL-54737 (D.Y.R.S.)), American Heart Association and Packard Foundation (D.Y.R.S.).

Published online at <http://www.nature.com/naturegenetics>

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions>

1. Red-Horse, K., Crawford, Y., Shojaei, F. & Ferrara, N. Endothelium-microenvironment interactions in the developing embryo and in the adult. *Dev. Cell* **12**, 181–194 (2007).
2. Coultas, L., Chawengsaksophak, K. & Rossant, J. Endothelial cells and VEGF in vascular development. *Nature* **438**, 937–945 (2005).

3. Winn, R.K. & Harlan, J.M. The role of endothelial cell apoptosis in inflammatory and immune diseases. *J. Thromb. Haemost.* **3**, 1815–1824 (2005).
4. Carmeliet, P. Angiogenesis in life, disease and medicine. *Nature* **438**, 932–936 (2005).
5. Duval, H., Harris, M., Li, J., Johnson, N. & Print, C. New insights into the function and regulation of endothelial cell apoptosis. *Angiogenesis* **6**, 171–183 (2003).
6. Dimmeler, S. & Zeiher, A.M. Endothelial cell apoptosis in angiogenesis and vessel regression. *Circ. Res.* **87**, 434–439 (2000).
7. Tricot, O. *et al.* Relation between endothelial cell apoptosis and blood flow direction in human atherosclerotic plaques. *Circulation* **101**, 2450–2453 (2000).
8. Rajagopalan, S. *et al.* Endothelial cell apoptosis in systemic lupus erythematosus: a common pathway for abnormal vascular function and thrombosis propensity. *Blood* **103**, 3677–3683 (2004).
9. Weinstein, B.M. Plumbing the mysteries of vascular development using the zebrafish. *Semin. Cell Dev. Biol.* **13**, 515–522 (2002).
10. Jin, S.W. *et al.* A transgene-assisted genetic screen identifies essential regulators of vascular development in vertebrate embryos. *Dev. Biol.* **307**, 29–42 (2007).
11. Deveraux, Q.L. & Reed, J.C. IAP family proteins—suppressors of apoptosis. *Genes Dev.* **13**, 239–252 (1999).
12. Rothe, M., Pan, M.G., Henzel, W.J., Ayres, T.M. & Goeddel, D.V. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell* **83**, 1243–1252 (1995).
13. Shu, H.B., Takeuchi, M. & Goeddel, D.V. The tumor necrosis factor receptor 2 signal transducers TRAF2 and c-IAP1 are components of the tumor necrosis factor receptor 1 signaling complex. *Proc. Natl. Acad. Sci. USA* **93**, 13973–13978 (1996).
14. Ashkenazi, A. & Dixit, V.M. Death receptors: signaling and modulation. *Science* **281**, 1305–1308 (1998).
15. Chen, G. & Goeddel, D.V. TNF-R1 signaling: a beautiful pathway. *Science* **296**, 1634–1635 (2002).
16. Eimon, P.M. *et al.* Delineation of the cell-extrinsic apoptosis pathway in the zebrafish. *Cell Death Differ.* **13**, 1619–1630 (2006).
17. Micheau, O. & Tschopp, J. Induction of TNF receptor 1-mediated apoptosis via two sequential signaling complexes. *Cell* **114**, 181–190 (2003).
18. Eckelman, B.P., Salvesen, G.S. & Scott, F.L. Human inhibitor of apoptosis proteins: why XIAP is the black sheep of the family. *EMBO Rep.* **7**, 988–994 (2006).
19. Samuel, T. *et al.* Distinct BIR domains of cIAP1 mediate binding to and ubiquitination of tumor necrosis factor receptor-associated factor 2 and second mitochondrial activator of caspases. *J. Biol. Chem.* **281**, 1080–1090 (2006).
20. Tang, E.D., Wang, C.Y., Xiong, Y. & Guan, K.L. A role for NF-kappaB essential modifier/ I kappa B kinase-gamma (NEMO/IKKgamma) ubiquitination in the activation of the I kappa B kinase complex by tumor necrosis factor-alpha. *J. Biol. Chem.* **278**, 37297–37305 (2003).
21. Sebban, H., Yamaoka, S. & Courtis, G. Posttranslational modifications of NEMO and its partners in NF-kappaB signaling. *Trends Cell Biol.* **16**, 569–577 (2006).
22. Li, X., Yang, Y. & Ashwell, J.D. TNF-R1I and c-IAP1 mediate ubiquitination and degradation of TRAF2. *Nature* **416**, 345–347 (2002).
23. Irmeler, M. *et al.* Inhibition of death receptor signals by cellular FLIP. *Nature* **388**, 190–195 (1997).
24. Micheau, O., Lens, S., Gaide, O., Alevizopoulos, K. & Tschopp, J. NF-kappaB signals induce the expression of c-FLIP. *Mol. Cell. Biol.* **21**, 5299–5305 (2001).
25. Eckelman, B.P. & Salvesen, G.S. The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. *J. Biol. Chem.* **281**, 3254–3260 (2006).
26. Baud, V. & Karin, M. Signal transduction by tumor necrosis factor and its relatives. *Trends Cell Biol.* **11**, 372–377 (2001).
27. Aggarwal, B.B. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat. Rev. Immunol.* **3**, 745–756 (2003).
28. Hayden, M.S. & Ghosh, S. Signaling to NF-kappaB. *Genes Dev.* **18**, 2195–2224 (2004).
29. De Martin, R., Hoeth, M., Hofer-Warbinek, R. & Schmid, J.A. The transcription factor NF-kappa B and the regulation of vascular cell function. *Arterioscler. Thromb. Vasc. Biol.* **20**, E83–E88 (2000).
30. Kisseleva, T. *et al.* NF-kappaB regulation of endothelial cell function during LPS-induced toxemia and cancer. *J. Clin. Invest.* **116**, 2955–2963 (2006).