The zinc transporter ZIP12 regulates the pulmonary vascular response to chronic hypoxia

The typical response of the adult mammalian pulmonary circulation to a low oxygen environment is vasoconstriction and structural remodelling of pulmonary arteries, leading to chronic elevation of pulmonary artery pressure (pulmonary hypertension) and right ventricular hypertrophy. Some mammals, however, exhibit genetic resistance to hypoxia-induced pulmonary hypertension1–3. We used a congenic breeding program and comparative genomics to exploit this variation in the rat and identified the gene Slc39a12 as a major regulator of hypoxia-induced pulmonary vascular remodelling. Slc39a12 encodes the zinc transporter ZIP12. Here we report that ZIP12 expression is increased in many cell types, including endothelial, smooth muscle and interstitial cells, in the remodelled pulmonary arterioles of rats, and humans susceptible to hypoxia-induced pulmonary hypertension. We show that ZIP12 expression in pulmonary vascular smooth muscle cells is hypoxia dependent and that targeted inhibition of ZIP12 inhibits the rise in intracellular labile zinc in hypoxia-exposed pulmonary vascular smooth muscle cells and their proliferation in culture. We demonstrate that genetic disruption of ZIP12 expression attenuates the development of pulmonary hypertension in rats housed in a hypoxic atmosphere. This new and unexpected insight into the fundamental role of a zinc transporter in mammalian pulmonary vascular homeostasis suggests a new drug target for the pharmacological management of pulmonary hypertension.

We have reported previously that the Fisher 344 (F344) rat strain is resistant to hypoxia-induced pulmonary hypertension compared with the Wistar Kyoto (WKY) strain3. Linkage analysis of an F2 population derived from inbred WKY × F344 rats identified a quantitative trait locus (QTL) on chromosome 17 (ref. 2). On the basis of this observation, we next conducted ten successive microsatellite-guided backcrosses of offspring with WKY rats and derived two congenic strains in which the original QTL was dissected and represented as partly overlapping regions of a donor F344 genome interposed onto the genetic background of the WKY recipient strain (Extended Data Figs 1 and 2). Resistance to hypoxia-induced pulmonary hypertension was detected in one of the congenic strains (R47A; Fig. 1a–d and Extended Data Fig. 2). Three subcongenic strains (SubA, SubB and SubC) were derived by further backcrosses of R47A onto the WKY background and the congenic interval was fine-mapped to a region of 8.28 megabase pairs (Mbp) containing an estimated 65 genes (rat chromosome 17: positions 85,072,475–93,347,784) (Fig. 1 and Extended Data Fig. 2a). Whole-genome sequencing (greater than 20× coverage) of the WKY and F344 parental strains revealed 13 non-synonymous coding SNPs affecting 9 genes within the refined congenic interval, and 6 indels resulting in frameshift mutations in 4 genes (Extended Data Table 1). Polymorphic examination of the 13 SNPs and 6 indels in 2 additional rat strains susceptible to hypoxia-induced pulmonary hypertension (the spontaneously hypertensive and fawn-hooded rat strains, respectively) excluded 5 SNPs and 5 indels and narrowed the genes of interest to 7 (Slc39a12, St8sia6, Cubn, Nmt2, Dclre1c, Hspa14 and Cdnf) (Fig. 1e and Extended Data Table 1). Further polyphen analysis allowed us to exclude five listed genes (St8sia6, Cubn, Nmt2, Dclre1c and Cdnf) as the non-synonymous coding changes were predicted to be benign. We identified Slc39a12, with a loss of thymidine at position 88,575,534 leading to a frameshift mutation in exon 11, as the highest priority candidate gene for further investigation.

Slc39a12 encodes the solute carrier 39 zinc transporter family (ZIP1–14) member 12 (ZIP12) and has high specificity for zinc. The ZIP family tightly regulates cellular zinc homeostasis in numerous cell types by promoting zinc uptake from the extracellular space or release from intracellular compartments. The rat Slc39a12 gene contains 12 exons and the ZIP12 protein comprises 688 amino acids with a secondary structure comprising 8 transmembrane domains. In the F344 strain, the frameshift mutation in Slc39a12 induces a stop-codon predicting a carboxy (C)-terminal truncated ZIP12 protein of 553 amino acids (Extended Data Fig. 3a). This affects the conserved zinc transporting aqueous cavity between transmembrane domains IV and V, resulting in the loss of the metalloprotease motif (HEXPHE), which would be expected to lead to a reduction in zinc transport.

A pathognomonic histological signature of chronic hypoxia-induced pulmonary hypertension is thickening of the pulmonary vascular media (owing to hyperplasia and hypertrophy of smooth muscle cells) and the muscularization of previously un muscularized pulmonary arterioles. We found that ZIP12 messenger RNA (mRNA) levels were very low and ZIP12 protein was undetectable by immunohistochemistry in the pulmonary vasculature of adult WKY rats housed in a normal oxygen atmosphere, but WKY rats exposed to hypoxia showed markedly increased lung ZIP12 mRNA levels and pronounced ZIP12 expression in remodelled pulmonary arterioles (Fig. 2a, b and Extended Data Fig. 3b). ZIP12 expression was evident in vascular smooth muscle but also other cell types (endothelial and interstitial cells) known to contribute to structural changes seen in hypoxic lungs. In contrast, and consistent with a frameshift mutation in Slc39a12 predicting a C-terminal truncated protein, ZIP12 was undetectable with an antibody directed at the C terminus of the protein in the lungs of chronically hypoxic F344 rats (Fig. 2b and Extended Data Fig. 3b).

Slc39a12 is highly conserved across species and transcribed constitutively in many tissues (http://www.biogps.org). To investigate

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The pulmonary vascular response to hypoxia in the F344 rat is influenced by a region of chromosome 17 containing Slc39a12. a. A genetic map of three sub-congenic strains (SubA, SubB and SubC) derived from the R47A congenic strain (originally derived from a WKY × F344 cross) backcrossed with the WKY parental strain. The refined congenic region (orange) of 8.28 Mbp containing 65 genes is within the SubB strain. b-d. SubB exhibits attenuated pulmonary hypertension after 2 weeks exposure to a 10% O2 atmosphere compared with WKY, SubA and SubC rats. b, mean pulmonary artery pressure (mPAP); c, right ventricular hypertrophy (ratio of mass of right ventricle to left ventricle plus septum, RV/LV + sep.) \((n = 17\) WKY, 15 F344, 14 R47A, 8 SubA, 10 SubB, 10 SubC); d, vascular muscularization \((n = 6\) per group). Dotted line indicates mean measurements from all the rats in a normal oxygen atmosphere \((21%\) O2; mPAP = 14.7 ± 0.3 mm Hg, right ventricular hypertrophy = 0.270 ± 0.004%; percentage muscularization = 34.2 ± 0.36%; for actual values in rat strains see Extended Data Fig. 2c–e). Values are expressed as mean ± s.e.m. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) compared with WKY after one-way analysis of variance (ANOVA) followed by Bonferroni correction for multiple testing. e. The genes of interest \((Slc39a12, St8sia6, Cubn, Nmt2, Dcbre1c, Hspa14 and Ccdnf) identified within the SubB congenic interval. The frameshift mutation in Slc39a12 introduces a stop-codon, resulting in a truncated protein.

Slc39a12 encodes a zinc transporter, ZIP12, which is upregulated in pulmonary vascular tissue from mammals exposed to chronic hypoxia. a. ZIP12 mRNA levels in control and hypoxic WKY rat lungs \((n = 6\) per group). b. Prominent ZIP12 immunostaining in remodeled pulmonary arteries in WKY (red arrows) but not F344 rats exposed to hypoxia. c. No ZIP12 staining was detected in pulmonary arteries of low-altitude (normoxia control, CO calf) calves and sea-level humans, yet prominent ZIP12 immunostaining was observed in the remodelled pulmonary arteries of calves with severe pulmonary hypertension (Hx calf), in cattle naturally susceptible to pulmonary hypertension at altitude (‘Brisket disease’, BD) as well as Kyrgyz highlanders residing above 2,500 m. d. Design of the luciferase reporter vector pGL4.10 containing a 5’ region of ZIP12 that includes an HRE encoding for both HIF-1α- and HIF-2α-binding motifs or a mutant HRE sequence where the 5’-AGCGT-3’ motif has been replaced by 5’-AGCAG-3’ (mHRE). e. Human pulmonary artery smooth muscle cells (HPASMCs) transfected with the ZIP12 HRE reporter vector demonstrated significantly increased luciferase activity after exposure to hypoxia, but not in the cells transfected with the mutant HRE vector \((n = 6\) per group, replicated twice). f. Increased levels of HIF-1α and HIF-2α bound to the ZIP12 HRE assayed by ChIP–qPCR of chromatin from HPASMCs cultured in normoxia and hypoxic conditions \((n = 3\) per group, replicated twice). Data are calculated as the percentage of input levels, with the dotted line marking percentages below mock immunoprecipitation (IP). Values are expressed as mean ± s.e.m. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\) compared with normoxic control after one-way ANOVA followed by Bonferroni correction for multiple testing. NS, not significant.
Figure 3 | ZIP12 knockdown inhibits hypoxia-induced increase in intracellular labile zinc concentration and proliferation of HPASMCs. 

A. Representative wide-field microscope images of HPASMCs transfected with eCALWY-4 probe. Hypoxia exposure produced a striking increase in intracellular free zinc (resulting in decreased FRET)\(^1\). This was inhibited by transfection with ZIP12 siRNA. TPEN-mediated Zn\(^{2+}\) chelation was used to derive maximum fluorescence and 100\(\mu\)M ZnCl\(_2\) in the presence of the Zn\(^{2+}\) ionophore and pyrithione (ZnPyr) was used to derive the minimum fluorescence. 

B. Representative traces showing the changes in fluorescence ratio of the eCALWY-4 probe. Steady-state fluorescence intensity ratio of citrine/cerulean (R) was measured, then maximum and minimum ratios were determined to calculate free Zn\(^{2+}\) concentration using the formula: 

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\text{[Zn}^{2+}\text{]}_\text{cyt} = K_d \times \frac{(R_{\text{max}} - R)(R - R_{\text{min}})}{
\end{equation}

where the dissociation constant (K\(_d\)) the relevance of our observations to other susceptible animal species, as well as humans, we examined ZIP12 expression in whole lung samples of (1) neonatal calves housed in a normal atmosphere or exposed to hypobaric hypoxia for 2 weeks (barometric pressure = 445 mm Hg (1 mm Hg = 133.3 Pa), equivalent to 4,500 m altitude, 12% O\(_2\)), (2) older (yearling) cattle with naturally occurring pressure sphere or exposed to hypobaric hypoxia for 2 weeks (barometric altitude, 12% O\(_2\)), (3) animal species, as well as humans, we examined ZIP12 expression in whole lung samples of (1) neonatal calves housed in a normal atmosphere or exposed to hypobaric hypoxia for 2 weeks (barometric pressure = 445 mm Hg (1 mm Hg = 133.3 Pa), equivalent to 4,500 m altitude, 12% O\(_2\)), (2) older (yearling) cattle with naturally occurring pressure sphere or exposed to hypobaric hypoxia for 2 weeks (barometric altitude, 12% O\(_2\)), (3)result of prolonged pasturing at high altitude (2,438–3,505 m) and (3) human subjects at sea level and Kyrgyz highlanders residing above 2,500 m. ZIP12 expression, which is undetectable by immunohistochemistry in healthy bovine and human lung exposed to a normal atmospheric oxygen environment (Fig. 2c), was clearly visible in the remodelling pulmonary vessels from chronic hypoxia exposure, indicating that ZIP12 upregulation in pulmonary vasculature is a common response to hypoxia (Fig. 2c).

To better understand the regulation of ZIP12 by hypoxia, we exposed human pulmonary vascular smooth muscle cells in culture to hypoxia (2% O\(_2\)). Increased hypoxia-inducible factor (HIF) protein and ZIP12 gene expression was observed in hypoxic cells; mRNA levels of other zinc transporters, ZIP6, ZIP7, ZIP10 and ZnT8, were unchanged (Extended Data Fig. 4a, b). Further examination of the Scl39a12 gene using HOMER analysis\(^8\) revealed a hypoxia response element (HRE) encoding both HIF-1\(\alpha\)- and HIF-2\(\alpha\)-binding motifs (Fig. 2d) located 1 kb downstream of the ZIP12 transcription start site (human (hg19) chr10: 18,241,879–18,241,887). We cloned a 1.5 kb fragment of the 5\(^\prime\) region of ZIP12 containing this HRE into the luciferase reporter vector, pGL4.10 (Fig. 2d). Human pulmonary vascular smooth muscle cells transfected with the ZIP12 HRE reporter vector demonstrated significantly increased luciferase activity after exposure to hypoxia, while the luciferase activity of cells transfected with the mutant HRE vector (a substitution of the 5\(^\prime\)-ACGTG-3\(^\prime\)- motif by 5\(^\prime\)-AGCAG-3\(^\prime\); Fig. 2d) remained at basal normoxia levels (Fig. 2e). Chromatin immunoprecipitation (ChiP) followed by quantitative PCR (qPCR) confirmed the enrichment of both HIF-1\(\alpha\) and HIF-2\(\alpha\) bound to this ZIP12 HRE after hypoxia exposure (Fig. 2f).

We then explored the contribution of ZIP12 to the regulation of intracellular zinc levels in human pulmonary vascular smooth muscle cells. Intracellular labile zinc measured using a genetically encoded fluorescence resonance energy transfer (FRET)-based zinc probe, eCALWY-4 (ref. 10), exhibited a striking increase in cells exposed to hypoxia for 48 h, which was markedly reduced by inhibiting ZIP12 expression with a targeted short interfering (si)RNA (Fig. 3a–e). ZIP12 siRNA inhibition of hypoxia-induced proliferation in HPASMCs (n = 5 per group, replicated twice). *P < 0.05, **P < 0.001 compared with control group; #P < 0.05 compared with hypoxia group; scr, scrambled siRNA control.

for eCALWY-4 is 630 pM, the maximum ratio (R\(_{\text{max}}\)) was obtained upon intracellular zinc chelation with 50 \(\mu\)M TPEN and the minimum ratio (R\(_{\text{min}}\)) was obtained upon zinc saturation with 100 \(\mu\)M ZnCl\(_2\) in the presence of the Zn\(^{2+}\) ionophore pyrithione (5 \(\mu\)M) (ref. 10). c. Quantification of intracellular zinc levels (n = 10 each group). d. Chronic hypoxia (48 h) increases ZIP12 mRNA levels in HPASMCs, which is inhibited by Slc39a12 siRNA (n = 5 per group). e. Representative immunoblot of ZIP12 demonstrating inhibition of hypoxia-stimulated ZIP12 protein expression by Slc39a12 siRNA in HPASMCs (n = 3). f, ZIP12 siRNA inhibits hypoxia-induced proliferation in HPASMCs (n = 5 per group, replicated twice). *P < 0.05, **P < 0.001 compared with control group, #P < 0.05 compared with hypoxia group; scr, scrambled siRNA control.
in Slc39a12 in the hypoxia-susceptible WKY rat strain. A mutant line was generated containing a frame-shift resulting in a truncated ZIP12 protein with loss-of-function (Extended Data Fig. 5). Inter-cross of heterozygous animals generated homozygous (ZIP12+/−) heterozygous (ZIP12+/−) and wild-type (WT) rats that were then exposed to hypoxia (10% O2) for 2 weeks. ZIP12+/− rats demonstrated lower pulmonary artery pressures, reduced right ventricular hypertrophy and less vascular remodelling than WT rats (Fig. 4a–c and Extended Data Fig. 6a–d) with ZIP12+/− rats exhibiting an intermediate phenotype. WT rats resembled WKY rats after exposure to hypoxia, showing markedly increased lung ZIP12 expression in the remodelled pulmonary arteries, in contrast to the absence of expression in ZIP12+/− rats (Fig. 4d, e). Comparison of the ZIP12−/− response to hypoxia with the WKY and F344 parental strains revealed that mutation of Slc39a12 was responsible for about 50% of the resistance observed in the F344 strain, highlighting the importance of Slc39a12 as a hypoxia-susceptibility gene but also suggesting that other genes yet to be identified might also contribute.

Systemic blood pressure and cardiac output in the hypoxic ZIP12+/− rats was similar to that of WT rats (Extended Data Fig. 6e–g), signifying that the reduced pulmonary artery pressures in the ZIP12+/− rat in chronic hypoxia is due to reduced pulmonary vascular resistance (PVR; mean pulmonary artery pressure = PVR × cardiac output). Both vascular tone and structural remodelling contribute to PVR, and increased pulmonary vascular tone precedes the structural changes. ZIP12 expression may increase PVR by increasing pulmonary vascular tone. Zinc thiolate signalling has been reported to mediate the constriction of pulmonary microvascular endothelial cells in acute hypoxia through activation of protein kinase C and inhibition of myosin light chain phosphatase, inducing stress fibre formation and endothelial cell contraction12. We have shown that ZIP12-targeted siRNA attenuates stress fibre formation in human pulmonary vascular smooth muscle cells cultured in hypoxia (Extended Data Fig. 6h, i). However, given the time-dependent induction of ZIP12 expression in pulmonary vasculature by hypoxia, the main contribution of ZIP12 is likely to be in regulating the response to chronic rather than acute hypoxia. In further support of a direct effect on structural remodelling of pulmonary arterioles, we investigated angiogenesis ex vivo using pulmonary arteriole rings dissected from ZIP12−/− and WT rats. Vascular outgrowth from ZIP12−/− vessels in response to hypoxia was attenuated compared with vessels from WT rats (Extended Data Fig. 6j, k).

The underlying mechanisms through which ZIP12 affects hypoxic responses remain to be defined. Excess intracellular zinc concentrations mediated by upregulation of ZIP family members have been observed in a variety of tumour tissues and linked to cell proliferation and survival13–15. Zinc is a structural component of a large variety of intracellular proteins, including enzymes and transcription factors. Zinc-binding motifs have been identified in drug targets for pulmonary hypertension, for example phosphodiesterase type 5 (PDE5) and histone deacetylases16,17. Reduced ZIP12 expression and intracellular labile zinc levels would be expected to inhibit PDE5 activity18, and we have previously shown that PDE5 inhibition attenuated pulmonary vascular smooth muscle proliferation in culture19.

Following on from our demonstration that ZIP12 is hypoxia inducible and a key regulator of the pulmonary vascular response to chronic alveolar hypoxia exposure, we examined lung ZIP12 expression in other presentations of pulmonary hypertension where tissue hypoxia is an important driver of pathology. Again, in contrast to healthy lungs, ZIP12 expression was clearly evident in lung tissues from chronic iron-deficient rats20 and rats exposed to monocrotaline, as well as in patients with idiopathic pulmonary arterial hypertension (IPAH)21 (Fig. 4f), prominent in the remodelled pulmonary vasculature as identified by co-staining with smooth muscle actin (Fig. 4g). HIF activation in these tissues was confirmed by upregulation of c-onic anhydrase IX, a recognized HIF-regulated biochemical signature...
of tissue hypoxia (Extended Data Fig. 7). Interestingly, the F344 rat strain has previously been reported to exhibit some resistance to monocrotaline-induced pulmonary hypertension; this was recapitulated in the ZIP12−/− rat (Extended Data Fig. 8). These data signal a fundamental role for ZIP12 in the regulation of pulmonary vascular homeostasis in hypoxic stress, relevant to the pathogenesis of pulmonary hypertension beyond that associated with life in a low-oxygen atmosphere. The current treatments for pulmonary hypertension centre on the pharmacological manipulation of signalling mechanisms used by vasoactive factors and have limited therapeutic benefit. Our observations open a new avenue of research into the therapeutic potential of ZIP12 inhibition and suppressed excursions of intracellular free zinc as a novel strategy for preventing or treating pulmonary hypertension.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 5 December 2014; accepted 3 June 2015.
Published online 10 August 2015.

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Acknowledgements This research was supported by successive grants from British Heart Foundation to M.R.W. and L.Z. (PG/95170, PG/98018, PG/200137, PG/04/035/16912, PG/12/61/29818, PG/10/59/28478 and RG/10/16/28575). G.A.R. was supported by a Wellcome Trust Senior Investigator Award (WT098424AA), MRC Programme Grant (MR/J0003042/1) and a Royal Society Research Merit Award. T.A. acknowledges support from European Research Council Advanced Grant ERC-2010-AdG, number 268880. We thank A. I. Garcia-Diaz for advice on genotyping and discussions on the rat genetic map.

Author Contributions L.Z. and M.R.W. were principal investigators on grants from the British Heart Foundation, developed concepts and supervised the project. L.Z., M.R.W. and E.O. designed and implemented the experiments. T.A. gave conceptual advice on the congenic program and whole-genome sequencing. L.Z., M.R.W., T.A., K.M., E.O. and S.S.A. analysed whole-genome sequence data and performed the Polyphen analysis. L.Z., E.O. and O.D.D., with the support of B.M. and Z.W., generated the ZIP12 transgenic rat. L.Z., E.C. and L.W. performed immunohistochemistry and immunofluorescence. E.O. performed statistical analysis. L.Z., M.R.W. and E.O. performed histology and histopathology. C.-N.C. and E.O. performed ChIP–PCR. J.P.-C. and E.O. cloned the HRE motif. O.D.D. conducted the congenic program and whole-genome sequencing. L.Z., M.R.W., T.A., K.M., E.O. and O.D.D. conducted the congenic breeding program. S.S.A. analysed whole-genome sequence data and performed the Polyphen analysis. L.Z., E.O. and O.D.D., with the support of B.M. and Z.W., generated the ZIP12 transgenic rat. L.Z., E.C. and L.W. performed immunohistochemistry and immunochemistry. E.O. conducted the in vitro cell culture experiment. C.A. and E.O. performed the angiogenesis assay. G.R. supervised and E.O. and P.L.C. conducted the intracellular labile zinc measurement experiments. C.N.C. and E.O. performed ChIP–PCR. J.P.-C. and E.O. cloned the HRE construct and performed luciferase reporter assays. M.G.F., K.R.S. and A.A. provided cattle and human lung sections. E.O. performed statistical analysis. L.Z., M.R.W. and E.O. interpreted the data and wrote the manuscript. T.A., G.R., J.F., S.S.A. and K.D. edited the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to L.Z. (l.zhao@imperial.ac.uk).
Animals. Inbred Wistar-Kyoto (WKY, Charles River) and Fischer 344 rats (F344, Harlan) were used as original strains. Animals were maintained at a constant temperature (20–22 °C) with a 12-h on/12-h off light cycle, with food and water ad libitum. All experiments were conducted under the project licence PPL70/7425 in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. To ascertain the pulmonary artery pressure phenotype, male rats (WKY, F434, congenic and sub-congeneric strains, ZIP12 transgenic rats) aged 10–12 weeks were studied in batches, with the parental WKY strain included as an internal control in each batch studied. Sample sizes were chosen on the basis of experience of pulmonary artery pressure variation in the parental strains. A sample size of at least n = 5 per group was predicted to detect a difference in mean pulmonary arterial pressure2 of 5 mm Hg (SD = 3) with 95% power with 95% confidence. Additional animals were studied to obtain sufficient tissue for supportive analyses.

Generation of congenic and sub-congeneric strains. To investigate the involvement of the chromosome 17 QTL in the pulmonary hypertension (PH) phenotype, we introgressed the F344 chromosome QTL segment into the WKY genetic background by repeated backcrossing2. We produced a congenic rat strain, R47A (WKY.F344-D17Got91/D17Rat51), which contains 15 Mb from the F344 donor region that maps to the distal end of the QTL on the WKY background.

Subsequently, we generated three sub-congenic strains: Sub-A (WKY.F344-D17Got91/D17Rat47), Sub-B (WKY.F344-D17Rat47/D17Rat51) and Sub-C (WKY.F344-D17Rat131/D17Rat51). These three recombination events divided the R47A congenic interval into three smaller and overlapping sub-congeneric intervals (Extended Data Fig. 1).

Microsatellite genotyping of congenic rats. Congenic and sub-congenic rats were genotyped using simple sequence length polymorphism markers (Extended Data Table 2). To reduce the unknown regions between the markers, rats were also genotyped using primers specifically designed to amplify known regions containing insertions or deletions in one of the two parental strains (Extended Data Table 2). Genomic DNA was isolated from rat ear clippings using hot sodium hydroxide and Tris (HotSHOT) extraction24. Forward primers were fluorescently labelled with 6-FAM. PCR products together with the fluorescent size marker (ROX 400HD, Applied Biosystems) were diluted in formamide and run on a 3730xl DNA Analyzer (Applied Biosystems). Results were analysed using GeneMapper version 3.7 software (Applied Biosystems).

Illumina genome sequencing library preparations. Five micrograms of male WKY/Ncrl (two animals) and F344/Ncrl (one animal) rats were used to construct paired-end whole-genome libraries with 300–500 bp insert size. Genomic DNA was prepared by standard phenol chloroform extraction followed by treatment with hot sodium hydroxide and Tris (HotSHOT) extraction24. Forward primers were fluorescently labelled with 6-FAM. PCR products together with the fluorescent size marker (ROX 400HD, Applied Biosystems) were diluted in formamide and run on a 3730xl DNA Analyzer (Applied Biosystems). Results were analysed using GeneMapper version 3.7 software (Applied Biosystems).

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HEPES, 25 mM NaHCO3, which was warmed, bubbled with 95% O2/5% CO2, set to pH 7.4 and contained 3 mM glucose. Imaging of zinc using eCALWY sensors was performed as optimized before.26,27 Briefly, cells were maintained at 37°C throughout with a heating stage (MC60, LINKAM, Scientific Instruments), and KHB buffer was perfused (1.5–2 mL min−1) with additions as stated in the figures. Images were captured at 433 nm monochromatic excitation wavelength (Polychrome IV, Till Photonics) using an Olympus IX-70 wide-field microscope with a ×40/1.35 numerical aperture oil immersion objective and a Zyla sCMOS camera (Andor Technology) controlled by Micromanager software.28 Acquisition rate was 20 images per minute. Emitted light was split and filtered by a Dual-View beam splitter (Photometrics) equipped with a 505dcx dichroic mirror and two emission filters (Chroma Technology D470/24 for cerulean and D535/30 for citrine).

Image analysis used ImageJ software29 with a homemade macro, and the fluorescence emission ratios were derived after subtracting background. Steady-state fluorescence intensity ratio of citrine/cerulean (R) was measured, then maximum and minimum ratios were determined to calculate free Zn2+ concentrations using the following formula: [Zn2+]free = Kd × (Rmax − R)/(R − Rmin). The maximum ratio (Rmax) was obtained upon intracellular zinc chelation with 50 μM TPEP and the minimum ratio (Rmin) was obtained upon zinc saturation with 100 mM ZnCl2 in the presence of the Zn2+-ionophore pyrithione (5 μM) (ref. 10).

HIF-motif analysis and cloning. HOMER30 was used to scan for HIF-1α and HIF-2α recognition motifs in the region 2 kb upstream and 1.5 kb downstream of the ZIP12 transcription start site. Results with a HOMER score <6.5 were discarded. A 5′ region of ZIP12 gene containing these motifs (HRE) (human (hg19) chromosome 10; positions 18,240,587–18,242,100) was cloned into the multicloning site of pGL4.10, which encodes the luciferase reporter gene luc2, by Gibson Assembly (NEB, E2611S). Three nucleotide substitutions in the core of the predicted HIF1α/2α-binding site motif were created by site-mutagenic PCR to produce a disabling mutant (Fig. 2d).

Transfection and luciferase assay. HPASMCs were seeded in 24-well plates at 70–80% confluence. Cells were transfected with 300 ng of each plasmid together with 2 ng of Renilla plasmid using Lipofectamine 2000 (Life Technologies), exposed to hypoxia and lysed according to the manufacturer’s conditions. Luciferase activity was measured using Dual-Luciferase Reporter Assay Chemistry (Promega) as previously described.31 Experiments were repeated in two cell lines, n = 6 per line.

ChIP and PCR. Specific protein–DNA interactions were examined by ChIP followed by qPCR (Chromatin Immunoprecipitation Assay Kit, Millipore). Protein–DNA crosslinks were achieved by fixation with 1% formaldehyde for 10 min at room temperature. DNA–protein complexes from 2 × 106 cells were sheared to lengths between 200 and 500 base pairs by sonicator (Bioruptor). The pre-cleared samples were incubated with 10 μg of HIF-1α or HIF-2α specific antibody (Novus Biologicals), or without antibody (as a negative control) overnight, followed by immunoprecipitation by Protein A Agarose/Salmon Sperm DNA (50% Slurry). The crosslinks were reversed by heating at 65°C overnight, followed by Proteinase K digestion at 45°C for 2 h. DNA was then recovered with QIAquick PCR purification kit (Qiagen) for qPCR to prove affinity against ZIP12 promoter region (Fig. 2d). Experiments were conducted in two separate cell lines (n = 3 each) and gave the same result.

Quantitative PCR was performed as previously described in the methodology, using 1 μl of DNA sample, and using the forward primer 5'-TTTCCCAACTCTGGGCTCTAT-3' and the reverse primer 5'-ACGGACGAAAAAGCTTGC-3'. C terminus values were normalized compared with the values detected in the starting non-immunoprecipitated DNA sample (input). Protein–DNA affinity was confirmed when normalized C terminus values were above the basal levels measured in the negative control.

Quantitative PCR with reverse transcription. RNA was extracted from lungs using RNeasy Mini Kit (Qiagen). PCR was performed with an ABI 7500 Sequence Detection System (Applied Biosystems). Quantitative PCR was performed using a two-step protocol starting with complementary DNA (cDNA) synthesis using the Invitrogen M-MLV Reverse transcriptase (Promega), followed by PCR using the Power SYBR Green PCR Master Mix (Applied Biosystems). A total of 100 ng of cDNA per sample was used. All samples were amplified using biological triplicates with two technical replicates per sample. The 7500 Sequence Detection System software (Applied Biosystems) was used to obtain Ct values. Results were analysed using the comparative Ct method.32 Samples were normalized to a reference gene, Ubc (for rat samples) or Cyclophilin (for human cell samples), to account for cDNA loading differences.

Western blot. Frozen rat tissues (lungs) and cell pellets were homogenized in RIP buffer (50 mM tris–HCl, pH 8.0, 150 mM sodium chloride, 1.0% Igepal, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate) (Sigma) supplemented with protease inhibitor cocktail (Roche). Western blotting was Mini-PROTEIN TGX Prestain Gels (Bio-rad) following the manufacturer’s suggestions. Blots were incubated for 1 h at room temperature with Anti-ZIP12 (1:10,000); Anti-HIF1α (1:1,000, Novus Biological) or Anti-HIF2α (1:1,000, Novus Biological). Proteins were detected by Clarity western ECL substrate (Bio-rad). Optical densities of individual bands were measured using ImageJ software and protein expressions were standardized with vinculin.

Statistical analysis. Data are presented as mean ± s.e.m. Data were tested for normality using a Kolmogorov–Smirnov test. All data were confirmed as normally distributed with similar variance between comparator groups. Data were analysed using one-way ANOVA followed by Bonferroni post-test adjustment for multiple comparisons or unpaired t-test. Graphpad Prism was used for all statistical analysis.

Other bioinformatics analyses. The Ensemble database33 was mined with the BioMart tool34 to identify all transcribed elements in the confidence interval region. The search was limited to chromosome 17 between positions 85,072,475 and 93,347,758. PolyPhen analysis was used to predict the possible impact of described SNP on amino-acid substitution on the structure and function of a human protein.35

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Extended Data Figure 1 | Generation of congenic and sub-congenic strains.

Congenic rat lines were produced by introgression of the F344 chromosome 17 QTL segment onto the WKY genetic background by repeated backcrossing. Congenic rat strain R47A (WKY.F344-D17Got91/D17Rat51) contains 15 Mbp from the F344 donor region that maps to the distal end of the QTL on a WKY background. Three sub-congenic strains, SubA (WKY.F344-D17Got91/D17Rat42), SubB (WKY.F344-D17Rat47/D17Rat51) and SubC (WKY.F344-D17Rat131/D17Rat51), were produced containing separate fragments of the R47A donor region by backcrossing (R47A × WKY) F1 with WKY parental rats. Three recombination events within the R47A congenic interval divided the congenic interval into three smaller and overlapping sub-congenic intervals (Fig. 1 and main text).
Extended Data Figure 2 | Dissection of QTL and cardiovascular phenotype of rat strains. **a**, An illustrative genetic map showing the relationship of the congenic strains (R42, R47A), subcongenic strains (SubA, SubB, SubC) and Sgc39a12 to the original QTL (defined by a LOD score > 3 (ref. 2)) on a physical map of chromosome 17 (using Rat Genome Assembly version 5.0). **b**, The hypoxia-resistant F344 phenotype tracks with the congenic R47A line. Rats were kept in 10% O2 for 2 weeks and right ventricular hypertrophy was significantly attenuated in the congenic R47A strain (0.32 ± 0.03, n = 13, **P < 0.01) compared with WKY rats (0.37 ± 0.03, n = 15), whereas congenic R42 rats (0.36 ± 0.03, n = 17) were similar (NS) to WKY rats. In normoxia, WKY, F344, R47A, SubA, SubB and SubC rats show no significant differences in (c) mPAP, (d) right ventricular hypertrophy and (e) vascular muscularization (n = 8 each group); f, systemic blood pressure (SBP) is similar in all strains in both normoxia and hypoxic conditions. g, F344, R47A and SubB rats exhibit attenuated pulmonary vascular remodelling after 2 weeks exposure to a 10% O2 atmosphere compared with WKY, SubA and SubC rats (n = 6 each group). Values are expressed as the mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 compared with WKY (percentage of fully muscularized and partly muscularized vessels); ##P < 0.01, ###P < 0.001 compared with WKY (percentage of non-muscularized vessels) after one-way ANOVA followed by Bonferroni correction for multiple testing.
Extended Data Figure 3 | Hypoxia-induced pulmonary vascular remodelling in parental strains. a, Upper panel sequence shows the WKY protein sequence (688aa); lower panel shows the truncated F344 protein sequence (553aa). Stars (*) mark the mutated amino acids compared with WKY protein. Dotted line indicates the C-terminal truncated region in F344. The grey square highlights the metalloprotease motif. b, Prominent ZIP12 immunostaining is seen in remodelled pulmonary arterioles in the chronically hypoxic WKY rat alongside vessels with a double elastic lamina (stained with elastic Van Gieson) but not F344 lungs exposed to hypoxia. Red arrow, vessel with double elastic lamina; blue arrow, vessel with single elastic lamina.
Extended Data Figure 4 | ZIP12 upregulation in response to hypoxia exposure and measurements of intracellular labile zinc concentration and proliferation of HPASMCs in normoxic conditions. a, Upregulation of ZIP12 in HPASMCs exposed to hypoxia, in contrast to other zinc transporters (*n* = 6). b, Representative western blots demonstrating increased HIF-1α and HIF-2α expression in HPASMCs after exposure to hypoxia for 24 h. c, Confocal laser scanning images of HPASMCs transfected with eCALWY-4 probe. Intracellular free zinc was not affected by transfection with ZIP12 siRNA in normoxia. d, Representative traces showing the changes in fluorescence ratio using the eCALWY-4 probe. e, Quantification of intracellular zinc levels (*n* = 10). f, ZIP12 siRNA did not affect proliferation of HPASMCs in normoxic conditions (*n* = 5).
Extended Data Figure 5 | Design of specific Slc39a12 ZFN and confirmation of mutant line.  
a, CompoZr Custom Zinc Finger Nucleases (Sigma-Aldrich) for the rat Slc39a12 gene were designed to target exon 8.  
b–d, Cel-I surveyor assay and gene sequencing confirmed NHEJ-induced mutations in at least one pup (mutant 77).  
e, The 4-bp (AGTT) deletion followed by 2-bp insertion (TA) into mutant 77 caused a frame-shift in coding, introducing a stop codon leading to a truncated protein. Red star refers to stop codon.  
c, We subsequently genotyped next generation litters using SwaI (cutting point: 5' 'ATTTAAT-3'), showing 100% digestion for homozygous pups (−/−), 50% for heterozygous (+/−) and no DNA digestion for WT rats (+/+).
**RESEARCH LETTER**

**A.** EVG

**B.** Vascular Remodelling

**C.** Proliferation Ki-67 score (Hypoxia)

**D.** Ki67

**E.** Systemic Blood Pressure

**F.** Cardiac Output

**G.** Pulmonary Vascular Resistance

**H.** Volume of Actin fibers per cell normalized to Normoxia

**I.** Control siRNA

**J.** Pulmonary Arteriole Angiogenesis

**K.** Normoxia

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Extended Data Figure 6 | ZIP12 knockout attenuated hypoxia-induced pulmonary vascular remodelling. a, Representative lung sections from WT and ZIP12−/− rats 2 weeks after hypoxia exposure. Elastic van Gieson staining showing double elastic lamina (red arrow) in WT but single elastic laminae (blue arrow) in ZIP12−/− rats. b, Genetic disruption of ZIP12 in WKY rat attenuated pulmonary vascular remodelling after exposure for 2 weeks to a 10% O2 atmosphere compared with WT rats (n = 5 each group). *P < 0.01 compared with WT (percentage of fully muscularized vessels); ###P < 0.001 compared with WT (percentage of non-muscularized vessels) after one-way ANOVA followed by Bonferroni’s multiple comparison test. c, Ki67 staining showing reduced proliferation in hypoxic ZIP12−/− rat lungs compared with the WT strain. *P < 0.01 compared with WT. d, Representative sections from hypoxic WT and ZIP12−/− rats lungs showing differences in staining with the proliferation marker, Ki67. e–g, Genetic disruption of ZIP12 in WKY rat did not influence (e) systemic blood pressure (SBP) or (f) cardiac output (CO) but attenuated hypoxia-induced increases in (g) PVR (n = 7 each group). Values are expressed as the mean ± s.e.m. *P < 0.05, **P < 0.01 compared with normoxic rats, #P < 0.05 compared with WT hypoxic rats after one-way ANOVA followed by Bonferroni correction for multiple testing. h, ZIP12-targeted siRNA inhibition attenuates stress fibre formation in HPASMCs in hypoxia (n = 5 each group). ***P < 0.01 compared with normoxia control group, #P < 0.05 compared with hypoxia control group. i, Representative pictures of actin stress fibre in HPASMCs. j, Ex vivo angiogenesis studies demonstrated that vascular outgrowth from ZIP12−/− pulmonary vessels in response to hypoxia was attenuated compared with vessels from WT rats (n = 12 each group, 2 rings per rat, 6 ZIP12−/− and 6 WT rats). *P < 0.05 compared with normoxia WT group; #P < 0.05, ###P < 0.001 and ####P < 0.001 compared with hypoxia ZIP12−/− group. k, Representative pictures of pulmonary arteriole ring outgrowth at day 6.
Extended Data Figure 7 | Carbonic anhydrase (CAIX) expression.

a. Representative sections demonstrating increased CAIX expression in remodelled pulmonary arterioles in the lungs of rats exposed to alveolar hypoxia (2 weeks), monocrotaline (MCT, 3 weeks) or a chronic iron-deficient diet (4 weeks).

b, c. No CAIX staining was detected in pulmonary arteries of low-altitude (normoxia control, CO calf) calves and sea-level humans, but prominent CAIX immunostaining was observed in the remodelled pulmonary arteries of calves with severe pulmonary hypertension (Hx calf), in cattle with naturally occurring pulmonary hypertension (‘Brisket disease’, BD) as well as patients with IPAH.
Extended Data Figure 8 | Genetic disruption of ZIP12 in WKY rat attenuated monocrotaline-induced pulmonary hypertension. a, mPAP, (b) right ventricular hypertrophy and (c) pulmonary arteriole muscularization (n = 5 each group). Values are expressed as the mean ± s.e.m. *P < 0.05, **P < 0.01 compared with WT monocrotaline group after unpaired Student’s t-test. d, Representative lung sections from WT and ZIP12−/− rats 3 weeks after monocrotaline injection. Elastic van Gieson staining showing double elastic lamina (red arrow) in WT but single elastic laminae (blue arrow) in ZIP12−/− rats.
Extended Data Table 1 | Frameshift and non-synonymous coding mutations in the refined congenic interval of F344 and the other hypoxia-susceptible strains, WKY, spontaneously hypertensive (SHR) and fawn-hooded (FHH) rat strains

| Gene Name | ENSEMBL gene ID | ENSEMBL transcript ID | SWRNA ID | Chromosome | Start | End | Reference Alte | Alternative Alte | Consequences | cDNA position | CDU position | Polypeptide Position |
|-----------|----------------|----------------------|----------|------------|-------|-----|----------------|----------------|--------------|--------------|--------------|---------------------|
| Conf      | ENST00000008936 | ENST00000008936      |          |            |       |     | G A 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 275           | 275          | 77                  |
| Hgna1     | ENST00000008936 | ENST00000008936      |          |            |       |     | G C 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 478           | 478          | 160                 |
| Hgna2     | ENST00000008936 | ENST00000008936      |          |            |       |     | A G 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 864           | 864          | 160                 |
| Hgna3     | ENST00000008936 | ENST00000008936      |          |            |       |     | A G 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 1071          | 1071         | 160                 |
| Dck2      | ENST00000008936 | ENST00000008936      |          |            |       |     | G A 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 1262          | 1262         | 160                 |
| Dck3      | ENST00000008936 | ENST00000008936      |          |            |       |     | G A 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 1363          | 1363         | 160                 |
| Dgk9      | ENST00000008936 | ENST00000008936      |          |            |       |     | G A 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 1363          | 1363         | 160                 |
| Dgk10     | ENST00000008936 | ENST00000008936      |          |            |       |     | G A 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 1363          | 1363         | 160                 |
| Fdnav1    | ENST00000008936 | ENST00000008936      |          |            |       |     | G A 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 1363          | 1363         | 160                 |
| Fdnav2    | ENST00000008936 | ENST00000008936      |          |            |       |     | G A 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 1363          | 1363         | 160                 |
| Fdnav3    | ENST00000008936 | ENST00000008936      |          |            |       |     | G A 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 1363          | 1363         | 160                 |
| Fdnav4    | ENST00000008936 | ENST00000008936      |          |            |       |     | G A 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 1363          | 1363         | 160                 |
| Fdnav5    | ENST00000008936 | ENST00000008936      |          |            |       |     | G A 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 1363          | 1363         | 160                 |
| Fdnav6    | ENST00000008936 | ENST00000008936      |          |            |       |     | G A 2 0 0 0 0 0 | 0              | Non-Synonymous Coding | 1363          | 1363         | 160                 |

Genotypes: 0 = Homozygous reference allele; 1 = Homozygous alternate allele; 2 = Heterozygous; 3 = Ambiguous. Gene name = strain-specific gene name. Bold = deletion variant.
Extended Data Table 2 | Polymorphism markers for congenic strain genotyping

| Rat chr 17 Marker | Genetic map SHRSP × BN | Physical map position | FORWARD | PRIMERS | REVERSE | Expected size (bp) | Genotyping information |
|------------------|------------------------|-----------------------|---------|---------|---------|-------------------|------------------------|
| D17Rat41         | 38.27                  | 80234337 - 80234511   | GCTTTCCTTTCCACCTTC  | GCTTTAAGGTTGTTGGCTAG | 171 157 WW WW WW WW WW |
| D17Rat44         | 40.89                  | 81612362 - 81612488   | CAGACAAACCCCTCAGCT  | AGCAGAAAGAAACAGCAGA | 133 121 WW WW WW WW WW |
| D17Got91         | 38.27                  | 82277435 - 82277611   | CACGACACACACATCACAC  | CTTCTCATGTGATGGAGTGTGTG | 160 176 FF FF WW WW |
| D17Rat43         | 40.89                  | 82337230 - 82337376   | CACGTACCTGCTGCTGTCT  | GAGAAGAAGCTGAGAGAGCA | 150 124 FF WW WW WW |
| D17Rat42         | 40.33                  | 82505422 - 82505562   | CAGCCTACCTTAAGCTACTTC  | CACAAAGGCTAAATACCTC | 141 121 FF WW WW WW |
| D17Rat62         | 42.35                  | 83479938 - 83480058   | GAAGAGCTGAGAGACTTGTTC  | CACAGCTACCTGCTACCTGCTACT | 145 121 WW WW WW |
| D17Rat46         | 42.33                  | 83600152 - 83600282   | TGGGTCTTCTTATCTGCAGC  | GCTACACCACACACACATTC | 135 125 FF WW WW WW |
| D17Rat47         | 43.34                  | 85072353 - 85072475   | CCTGCTTCCCTCTGTTAAC  | TGCTATAGAAGTAAAGCTCAA | 114 126 FF FF FF WW |
| Del83862103      | 58.62060 - 85862261    | ACCATAGACACAGCATGATGT  | ACAGGCTGCTGCTCCTGCA | 203 193 FF WW WW WW |
| In85923365       | 85923262 - 85923495    | ACCCTTTGCTGCTGCTTACAT  | AAACCTGGAAGCACCACCA | 235 243 FF WW WW WW |
| D17Got93         | 86032700 - 86032904    | CACATAGACACAGCATGATGT  | ACAGGCTGCTGCTCCTGCA | 227 215 FF WW WW WW |
| D17M4B           | 45.19                  | 87465135 - 87465345   | GGGCCCTTATGCTGTAAGA  | CTTAGCTACTAGGAGAAGGG | 193 195 FF WW WW WW |
| D17Rat60         | 45.19                  | 88268817 - 88269054   | GGGCCCTTATGCTGTAAGA  | CTTAGCTACTAGGAGAAGGG | 193 195 FF WW WW WW |
| D17Rat48         | 45.19                  | 88667790 - 88667952   | CAGAAGCCTACCTGCTACATA  | TTTGGTTCTTTTCTTGCTATGG | 166 156 FF WW WW WW |
| Del83931756      | 89391713 - 89391951    | TCCATGTTTTATCCCGGAAGCG  | ACCTTGATGCATGCTCATGAGCC | 230 238 FF WW WW WW |
| In90455808       | 90455697 - 90455921    | AAGTACCTGCTTTCCAAAGGA  | TCCAGTCTTTCTCTCTGTCAC | 233 225 FF WW WW WW |
| D17Rat131        | 47.54                  | 93347784 - 93347990   | TTAAGAAGGCAACAGCAAGGA  | TCCCTAATAAAAGAAAGGGAAGA | 203 213 FF WW WW FF |
| D17Rat51         | 47.54                  | 96587775 - 96587905   | TCCACTGCTTCATCCATTTT  | ACATGCAGAAGAAGACATCTT | 144 148 FF WW WW FF |