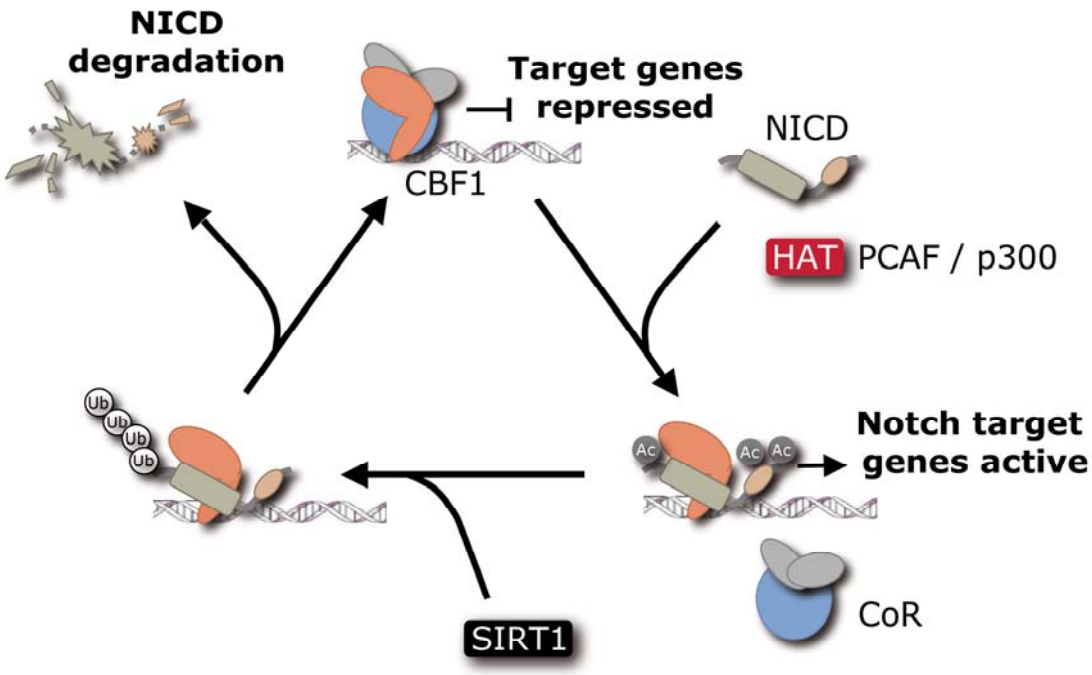
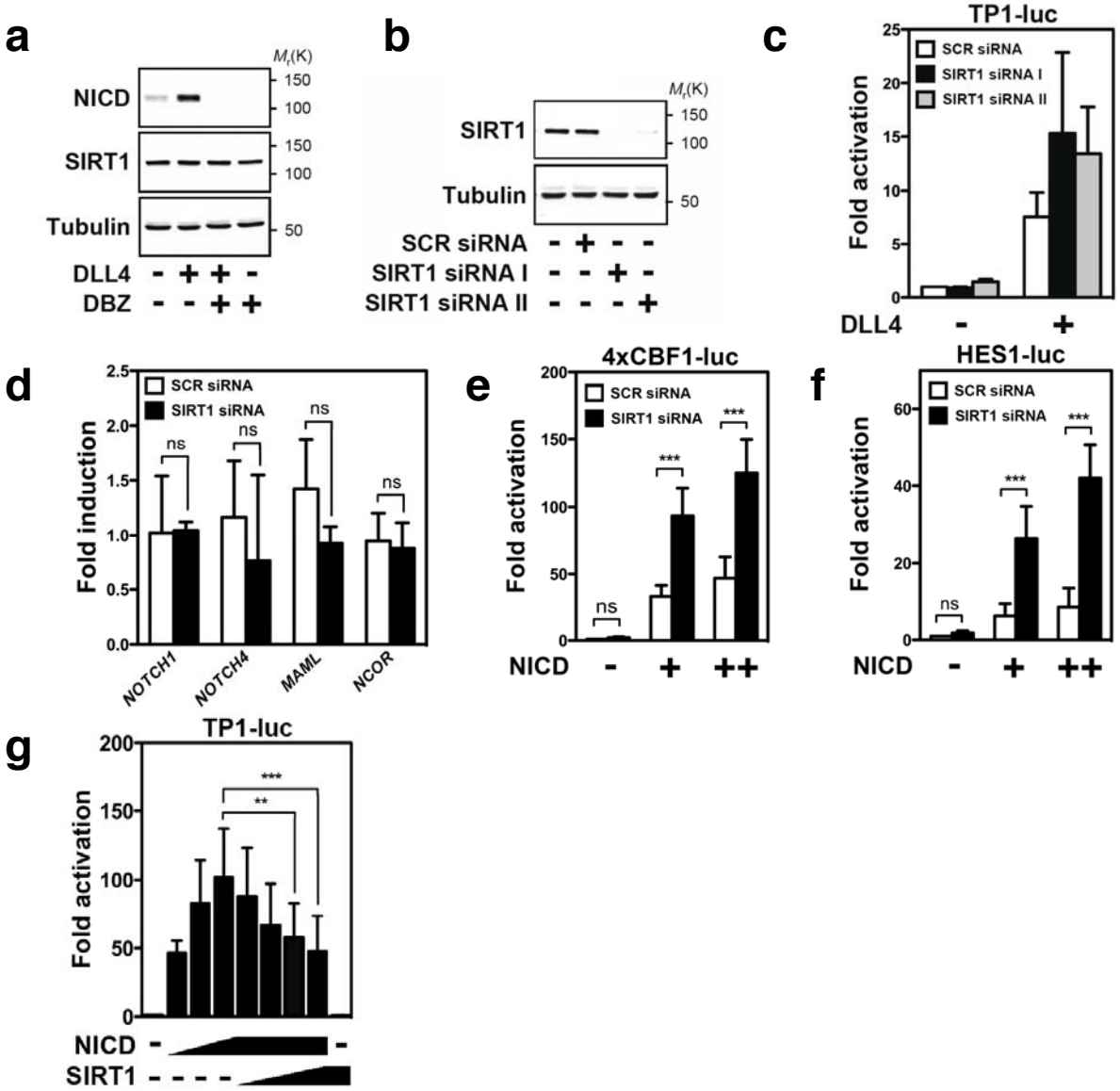
Supplementary Figure 1 Guarani et al.



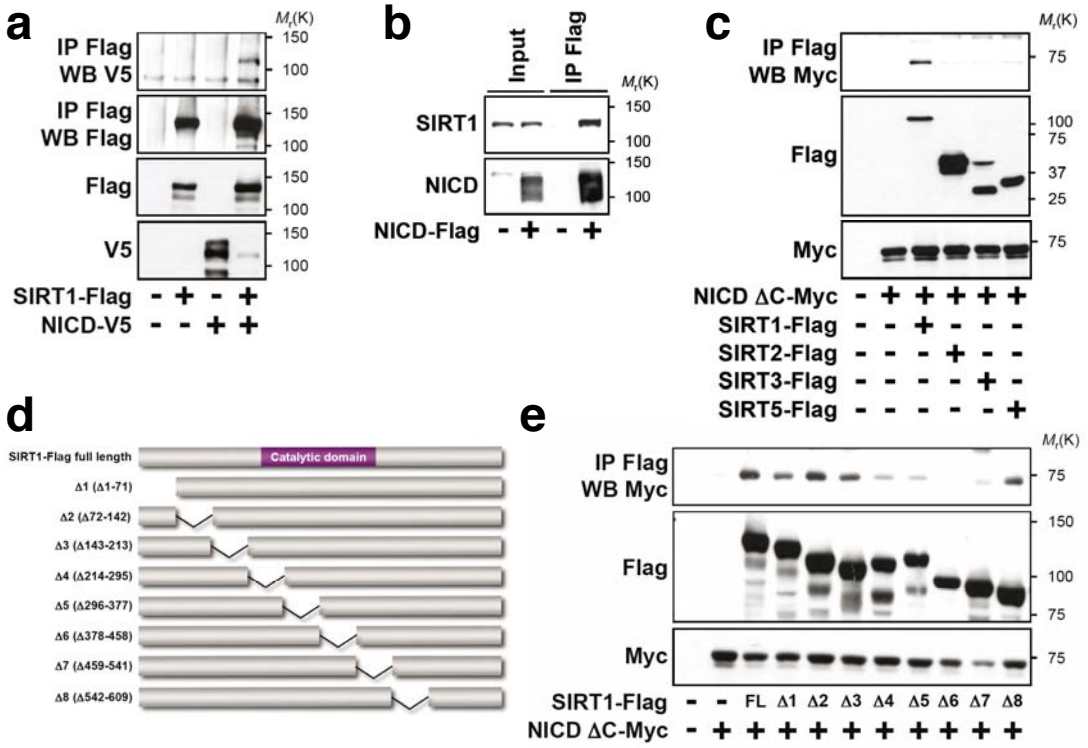
Supplementary Figure 1 – A model for the regulation of Notch signalling by reversible acetylation and SIRT1.
Theoretical model illustrating the regulatory role of SIRT1 in the Notch pathway. Upon release of the NICD, the NICD enters the nucleus, where it forms a complex with CBF1, Mastermind and histone acetyltransferases (HATs, e.g. PCAF, p300) to induce Notch target gene expression. HATs acetylate not only histones, but also the NICD itself to stabilize the otherwise short-lived NICD protein during target gene transcription. SIRT1, in turn, binds to the NICD complex and promotes NICD deacetylation and proteasomal degradation, thereby limiting Notch signalling responses. CoR, nuclear corepressors; Ac, acetyl group; Ub, ubiquitin.

Supplementary Figure 2 Guarani et al.



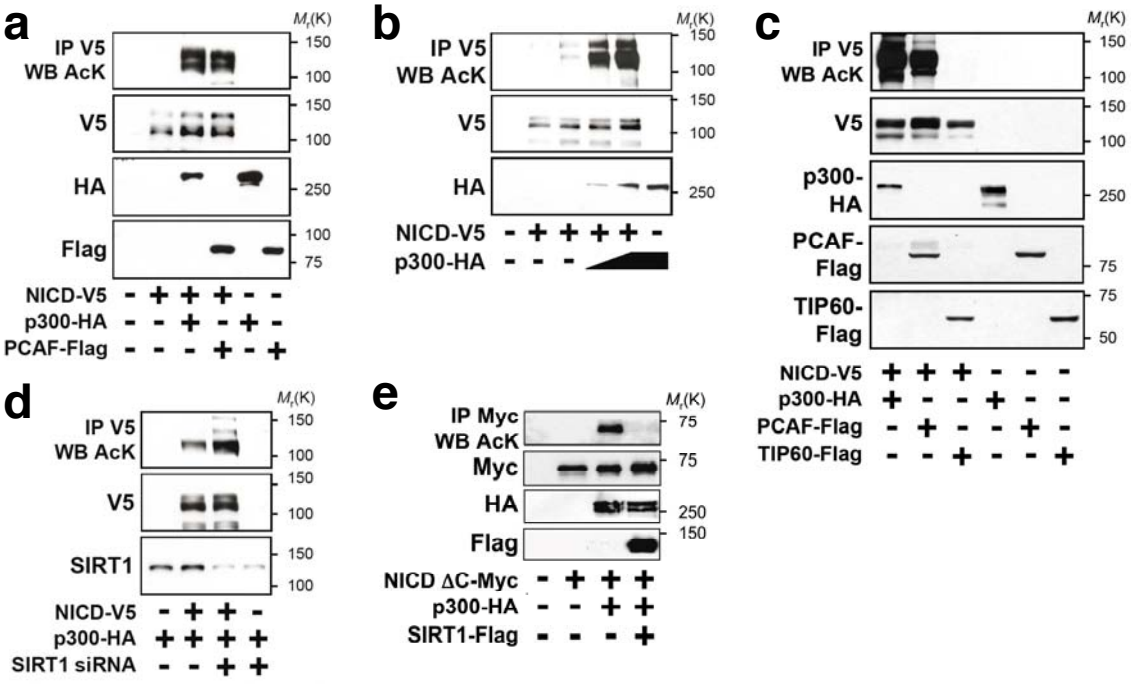
Supplementary Figure 2 – SIRT1 limits DLL4/Notch signalling in endothelial cells.
a, HUVECs were pretreated with DBZ or solvent (DMSO) and than replated on DLL4 or solvent for 6 hours. **b**, SIRT1 expression in protein extracts obtained from HUVECs, which were either left untreated, transfected with scrambled siRNA or two different SIRT1 siRNAs (I and II). Protein extracts were analyzed by immunoblotting with antibodies targeting NICD (**a**), SIRT1 and tubulin (**a**,**b**). **c**, HUVECs were transfected with 2 different SIRT1 siRNAs (I or II) or scrambled control siRNA and 24 hours later with the TP1-luciferase and pGL4-TK-Renilla-luciferase reporters. TP1-luciferase activity was measured in response to DLL4 stimulation after additional 24 hours. **d**, Relative expression levels of the mRNA transcripts of *NOTCH1*, *NOTCH4*, *NCOR* and *MAML* in HUVECs, which were transfected with a SIRT1-specific siRNA or a scrambled oligonucleotide siRNA (n = 3). Expression profiles were assessed with an Affymetrix gene chip expression assay as described[8]. **e**-**f**, Luciferase activity was measured in scrambled and SIRT1 siRNA-transfected HUVECs, which were subsequently transfected with increasing amounts of NICD and the indicated Notch reporters (4xCBF1, HES1), (n = 4). **g**, Luciferase activity was assayed in HUVECs, which were transfected with TP1-luciferase, NICD, and increasing amounts of SIRT1, (n = 5). All luciferase measurements were performed after 24 hours of DLL4 stimulation or NICD overexpression. All values are expressed as mean ± s.d. ***P*<0.01, ****P*<0.001; ns, not significant.

Supplementary Figure 3 Guarani et al.



Supplementary Figure 3 – Interaction of SIRT1 with NICD.
a, SIRT1-Flag was immunoprecipitated from SIRT1-Flag and NICD-V5 cotransfected HEK293 cells and immune complexes analyzed for the presence of NICD-V5. **b**, NICD-Flag was immunoprecipitated from HEK 293 cells, which were transfected with pcDNA and NICD-Flag 36 hours before. Flag-immunoprecipitates were analyzed for the presence of endogenous SIRT1 by immunoblotting using an anti-SIRT1 antibody. **c**, HEK293 cells were transfected with NICD ΔC-Myc and either Flag-SIRT1, 2, 3 or 5. To avoid protein degradation of NICD by SIRT1, we used the NICD mutant lacking parts of the C-terminus (NICD ΔC) as described in Fig. 1i. Immunoprecipitations were carried out with an antibody to Flag and immunoprecipitates analyzed for the presence of NICD (Myc) by immunoblotting. **d-e**, full-length or serial deletions (**d**) of SIRT1-Flag were immunoprecipitated from HEK293 cells coexpressing NICD ΔC-Myc and immune complexes analyzed for the presence of NICD ΔC-Myc by immunoblotting with antibodies to Myc.

Supplementary Figure 4 Guarani et al.



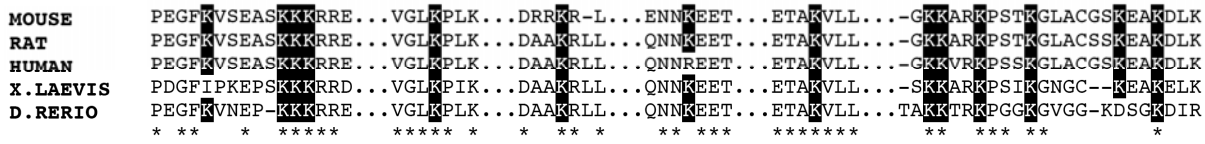
Supplementary Figure 4 – NICD is acetylated by the histone acetyltransferases PCAF and p300 and deacetylated by SIRT1. **a**, HEK293 cells were cotransfected with pcDNA, NICD-V5, p300-HA, PCAF-Flag or combinations thereof. **b,** NICD-V5 was cotransfected with increasing amounts of p300-HA and immunoprecipitated with an anti-V5 antibody. **c**, HEK293 cells were transfected with NICD-V5, p300-HA, PCAF-Flag, TIP60-Flag and combinations thereof. **d**, HEK293 cells were transfected with SIRT1 or scrambled siRNA and either pcDNA, NICD-V5, p300-HA, or combinations thereof. **e,** HEK293 cells were cotransfected with NICD-Myc ΔC, p300-HA, SIRT1-Flag, or combinations thereof. NICD was immunoprecipitated with antibodies to V5 (**a**-**d**) or Myc (**e**) and acetylation assessed by immunoblotting using antibodies specific to acetylated lysines.

Supplementary Figure 5 Guarani et al.

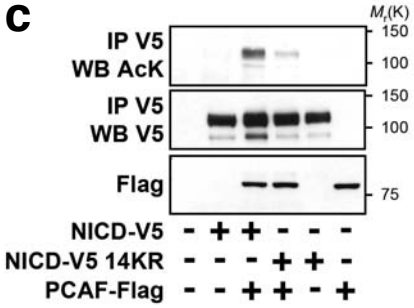
a

Postition	Sequence (Probability)	Mascot Score	Score Diff	PTM Score	Mass Error [ppm]
1764	RQHGQLWFPEGFK(1)VSEASK	60,86	84,98	192,14	0,13
1770	VSEASK(1)K(0.987)K(0.013)	42,53	53,02	154,74	-0,49
1771	VSEASK(0.998)K(0.998)K(0.003)	42,53	27,72	154,74	0,35
1772	VSEASK(0.973)K(0.513)K(0.513)	42,53	0,00	154,74	0,52
1785	EPLGEDSVGLK(1)PLK	58,83	39,42	170,32	-0,29
1935	DMQNNK(1)EETPLFLAAR	125,48	47,86	92,44	-2,14
2050	EGSYETAK(1)VLLDHFANR	128,69	87,48	128,69	0,06
2068	YSRSDAAK(1)R	154,74	45,32	154,74	0,10
2146	SATQGK(1)K(1)AR	57,01	118,18	118,18	0,82
2147	SATQGK(1)K(1)AR	57,01	118,18	118,18	0,82
2150	K(1)PSTK(1)GLACGSK	78,96	128,76	235,98	-0,95
2154	KPSTK(1)GLACGSK	101,54	71,66	260,00	-0,25
2161	GLACGSK(1)EAK	101,54	78,38	260,00	0,09
2164	GLACGSK(0.278)EAK(0.722)	63,17	4,15	196,39	1,10

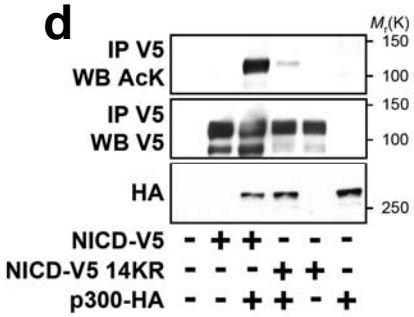
b



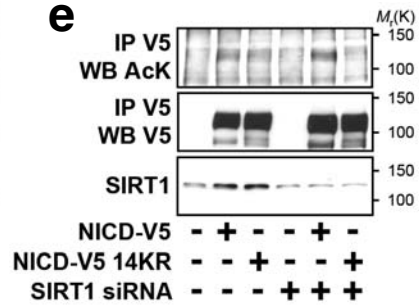
c



d

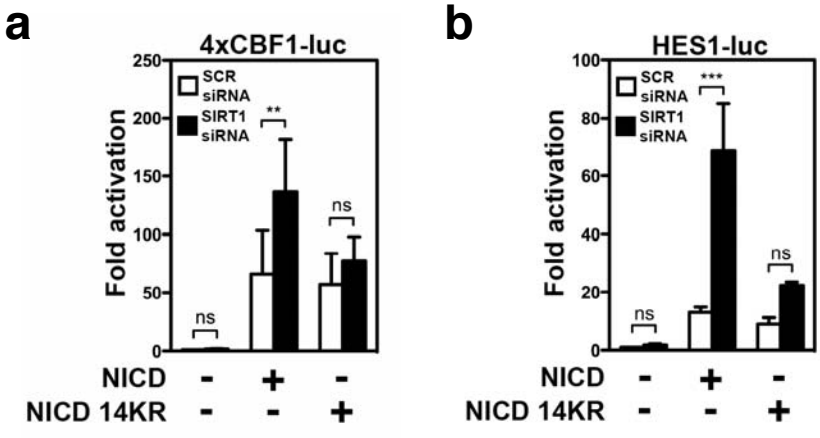


e



Supplementary Figure 5 – Mapping of the acetylated lysine residues in the murine Notch1 intracellular domain (NICD).
a, Sequences including flanking amino acid residues and Mascot scores of identified acetylated peptides of the NICD are shown. **b,** Selected sequence alignment of Notch1 orthologs from mouse, rat, human, xenopus laevis and danio rerio showing the conservation of the acetylated lysine residues identified in the murine NICD. Conserved acetylated lysines are coloured in black, identical residues are highlighted with an asterisk (*). **c**-**d**, HEK293 cells were cotransfected with pcDNA, NICD-V5 or NICD-V5 14KR as well as PCAF-Flag (**c**) or p300-HA (**d**). **e**, HEK293 cells were cotransfected with scrambled or SIRT1 siRNA and pcDNA, NICD-V5 or NICD-V5 14KR. After immunoprecipitation with antibodies to V5 (**c**-**e**), acetylation of the V5-immunoprecipitates was assessed by immunoblotting using antibodies specific to acetylated lysines.

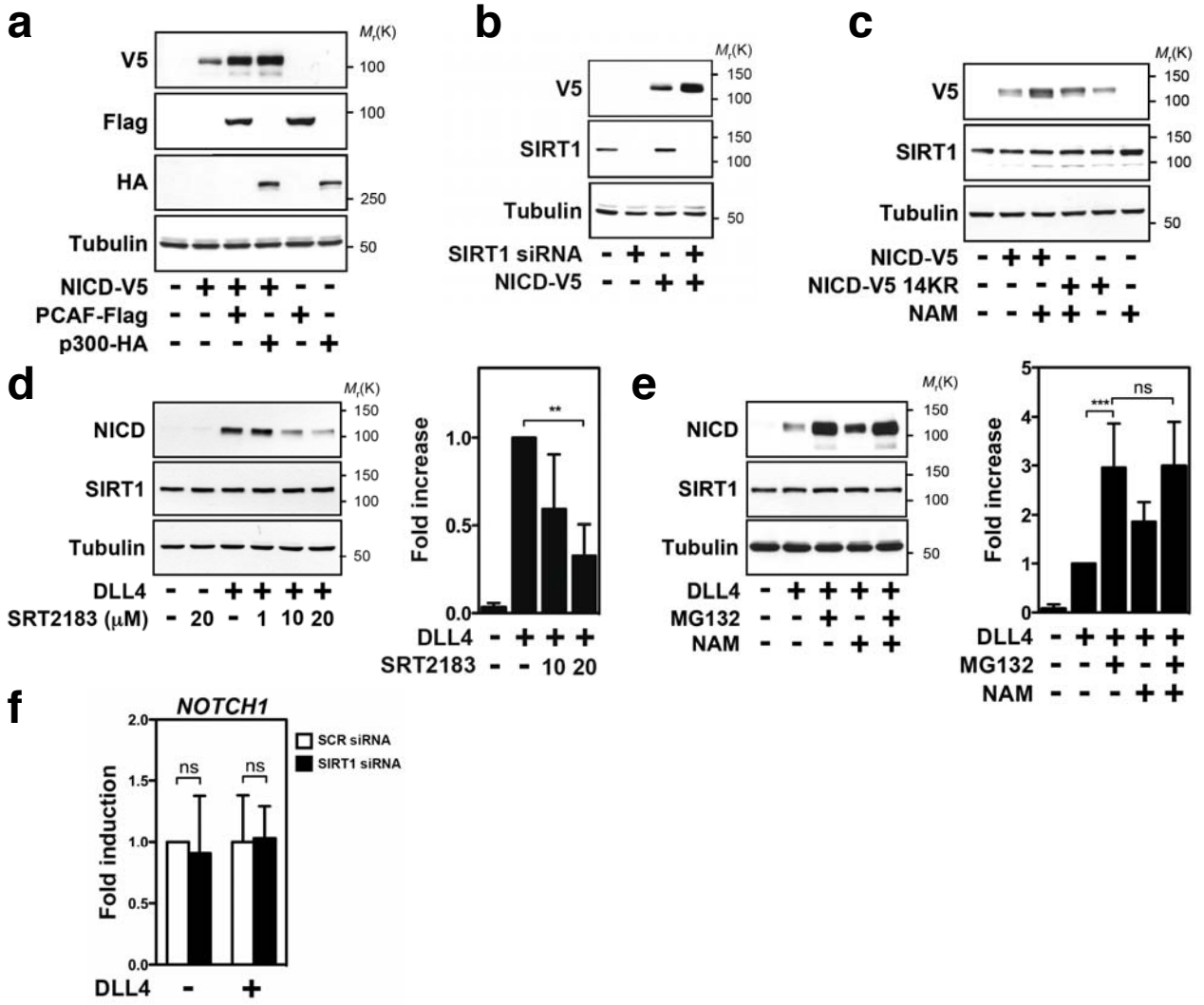
Supplementary Figure 6 Guarani et al.



Supplementary Figure 6 – The acetylation-defective NICD mutant is resistant to the SIRT1 siRNA-induced increase in Notch activity.

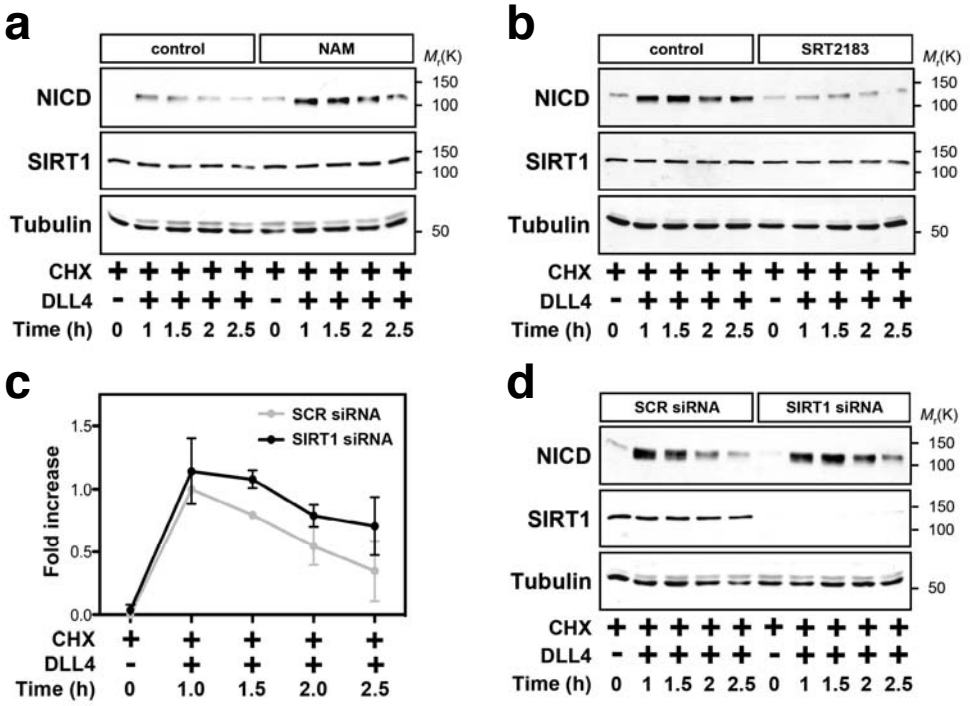
a-b, Luciferase activity was measured in scrambled and SIRT1 siRNA-transfected HUVECs, which were cotransfected with NICD-V5 or NICD-V5 14KR and the 4xCBF1- (**a**) or the HES1- (**b**) Notch reporter gene, (n = 4). Luciferase measurements were performed 24 hours after NICD overexpression. All values are expressed as mean ± s.d. ***P*<0.01, ****P*<0.001; ns, not significant.

Supplementary Figure 7 Guarani et al.



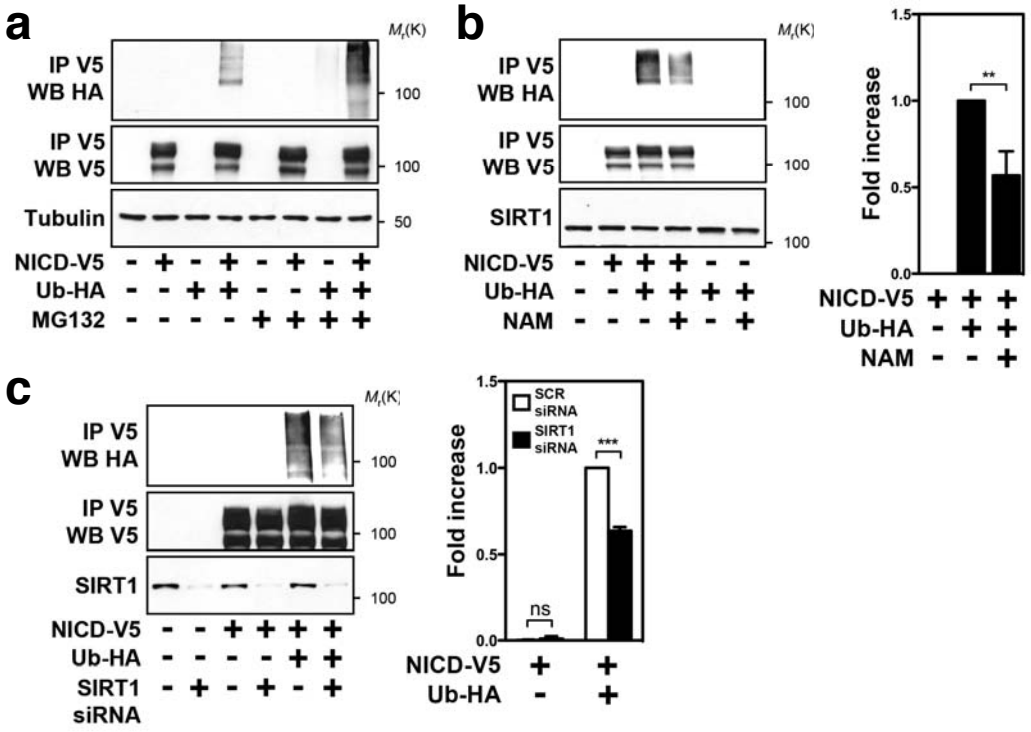
Supplementary Figure 7 – SIRT1 regulates proteasomal degradation of NICD in endothelial cells.
a, HEK293 cells were cotransfected with pcDNA, NICD-V5, PCAF-Flag, p300-HA or combinations thereof. 24 hours after transfection protein extracts were analyzed by immunoblotting with antibodies targeting V5, Flag, HA and tubulin. **b**, NICD-V5 protein expression in HUVECs transfected with scrambled or SIRT1 siRNA and pcDNA or NICD-V5. NICD protein expression was detected with antibodies against V5. **c**, NICD-V5 protein expression in HEK293 cells transfected with pcDNA, NICD-V5 or NICD-V5 14KR and treated with NAM or solvent. **d**, HUVECs were cultured in absence or presence of DLL4 and treated with 1, 10 or 20 μM SRT2183 or DMSO for 6 hrs, (n = 3). **e**, HUVECs were cultured on solvent or DLL4 and treated with MG132, NAM, DMSO, or combinations thereof for 6 hours. Protein extracts were analyzed by immunoblotting with antibodies targeting NICD, SIRT1 and tubulin. Relative quantifications of NICD protein levels in **d** and **e** are shown on the right of the respective panel. **f**, *NOTCH1* mRNA expression in HUVECs transfected with scrambled and SIRT1 siRNA and stimulated with or without DLL4 for 6 hours, (n = 4). All values are expressed as mean ± s.d., ***P*<0.01, ****P*<0.001; ns, not significant.

Supplementary Figure 8 Guarani et al.



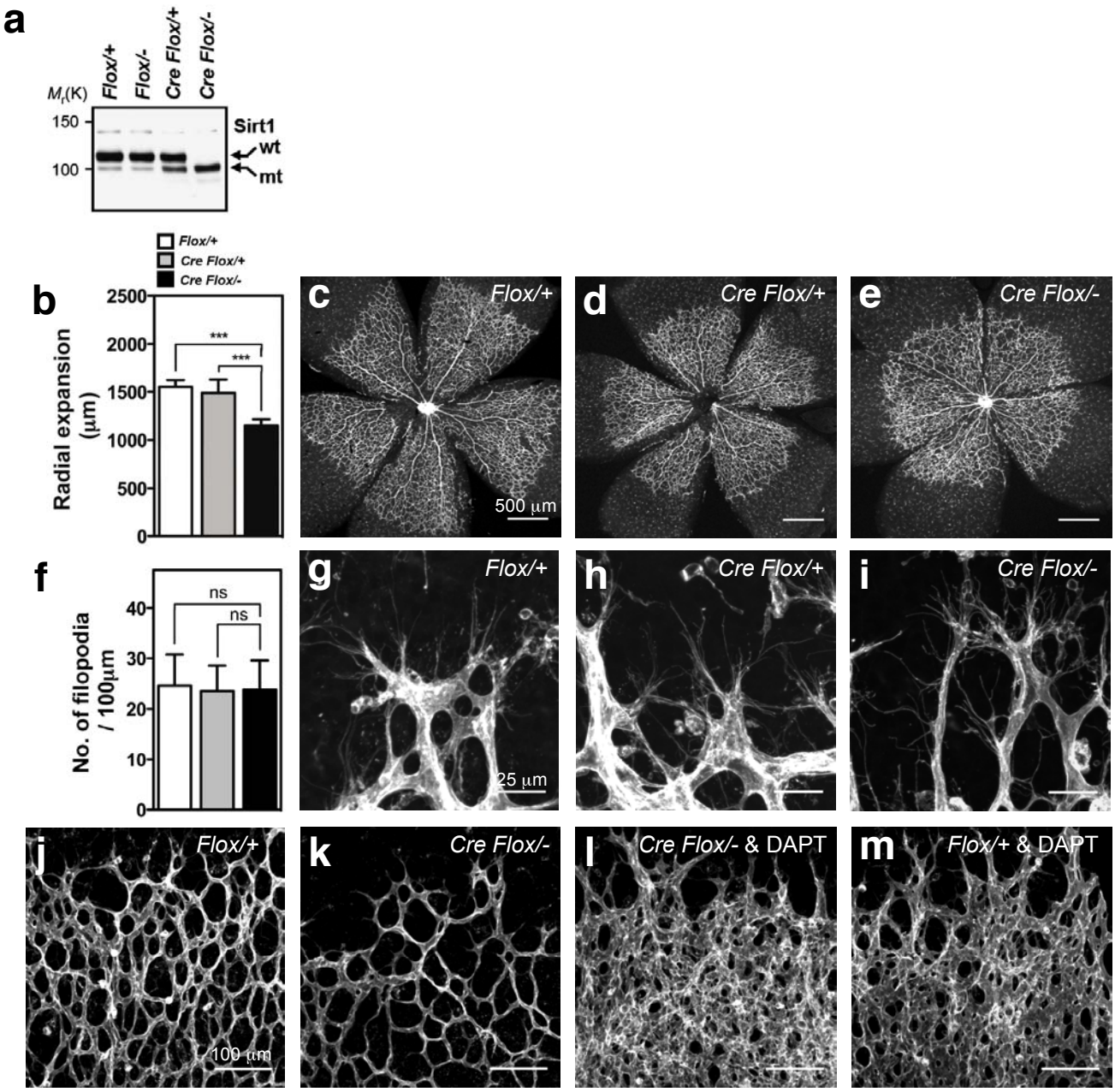
Supplementary Figure 8 – SIRT1 regulates NICD protein stability in endothelial cells.
a-b, HUVECs were pretreated with cycloheximide and then with NAM or SRT2183. After replating on DLL4- or solvent-coated dishes cells were lysed at the indicated time points. Protein extracts were analyzed by immunoblotting with antibodies targeting NICD, SIRT1 and tubulin. To better illustrate the differences in NICD protein levels, immunoblots with different exposure times are shown in **a** and **b**. **c-d**, HUVECs were transfected with SIRT1 or scrambled siRNA. After 24 hours, cells were pretreated with cycloheximide and than replated on solvent or DLL4. NICD protein levels were detected by Western blotting. The graph illustrates the quantification of NICD by densitometry, n=3.

Supplementary Figure 9 Guarani et al.



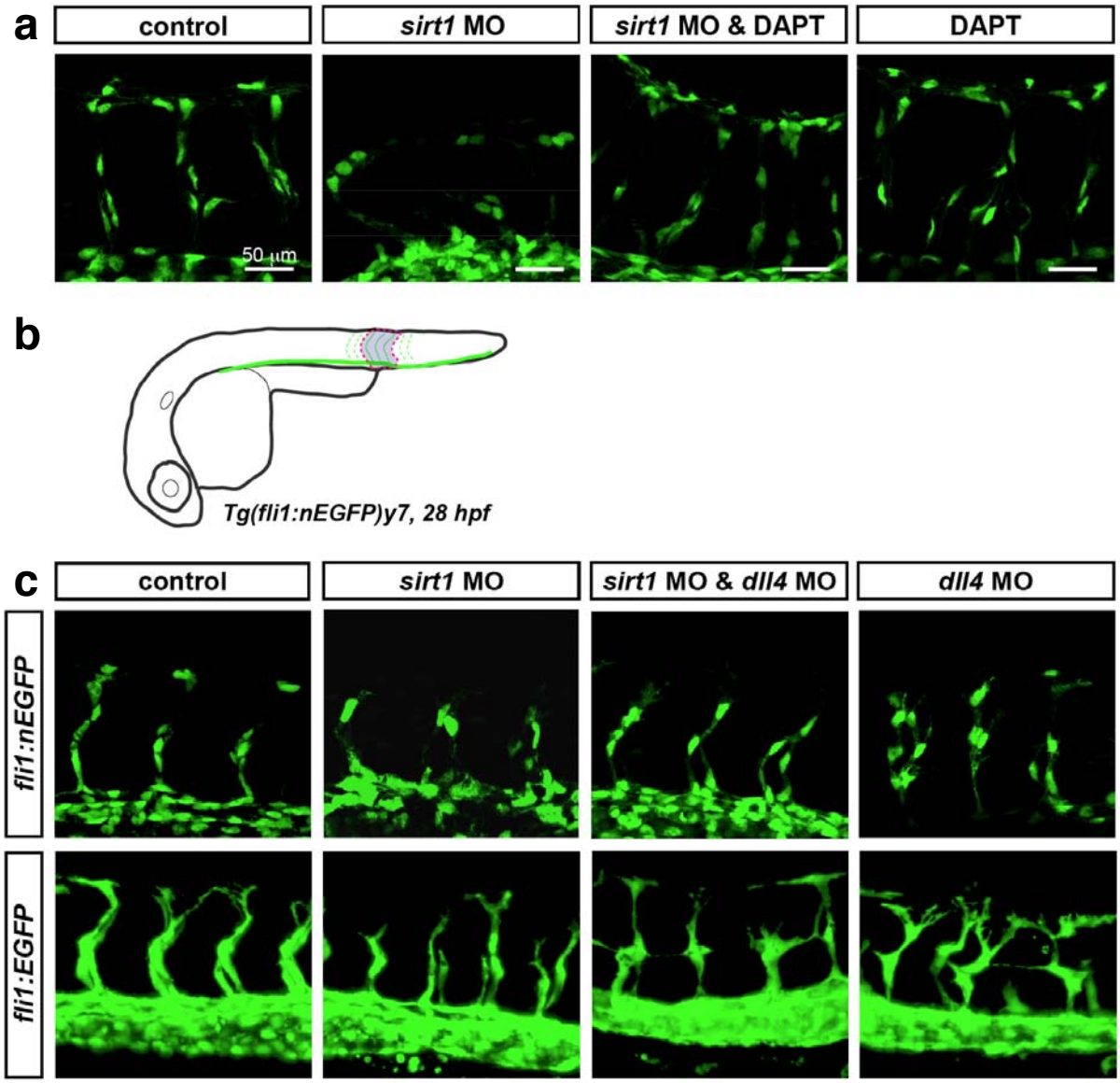
Supplementary Figure 9 – Inhibition of SIRT1 impairs the ubiquitination of NICD.
a, HEK293 cells were cotransfected with V5-tagged NICD and HA-tagged ubiquitin. 24 hours later transfected cells were treated with the proteasomal inhibitor MG132 or solvent control (DMSO) for 6 hours. Cells were lysed and V5-tagged NICD was immunoprecipitated using a V5 antibody. The amounts of NICD present in the precipitate were determined with a V5 antibody, tubulin served as loading control. **b**, HEK293 were cotransfected with NICD-V5 and Ub-HA. Cells were treated with NAM and NICD immunoprecipitated. **c,** HEK293 cells were transfected with SIRT1 or scrambled siRNAs and NICD-V5 and Ub-HA. Ubiquitination of NICD was assessed with an antibody for the HA epitope (**a**-**c**). Relative quantifications of NICD ubiquitination are shown on the right of the panel. All experiments n ≥ 3. Error bars, mean ± s.d. ***P*<0.01, ****P*<0.0001, ns, not significant.

Supplementary Figure 10 Guarani et al.



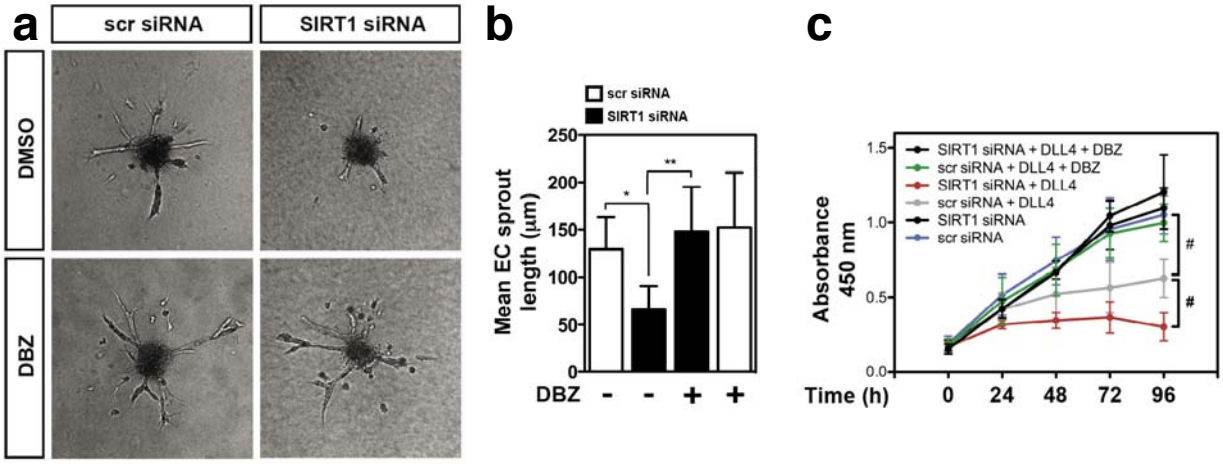
Supplementary Figure 10 – Inactivation of *Sirt1* in ECs leads to impaired retinal angiogenesis.
a, Western blot analysis with an antibody against Sirt1 showing that the floxed exon 4 of the *Sirt1* allele is efficiently deleted in endothelial cells isolated from the *Tie2Cre;Sirt1 flox/-* mutant mice giving rise to a mutant protein lacking deacetylase activity. **b**, Bar graph shows mean distance of radial vessel expansion in *Sirt1 flox/+*, *Tie2Cre;Sirt1 flox/+* and *Tie2Cre;Sirt1 flox/-* retinas, (n = 8-9). **c-e**, Images of whole-mount P5 *Sirt1 flox/+*, *Tie2Cre;Sirt1 flox/+* and *Tie2Cre;Sirt1 flox/-* retinas stained with isolectin B4. **f**, *Sirt1 flox/+*, *Tie2Cre;Sirt1 flox/+* and *Tie2Cre;Sirt1 flox/-* retinas have similar numbers of filopodia protrusions. Quantification of filopodia/100 mm vessel length is shown, n = 6-8. **g-i**, Representative high magnification images of tip cells of whole-mount P5 *Sirt1 flox/+*, *Tie2Cre;Sirt1 flox/+* and *Tie2Cre;Sirt1 flox/-* retinas stained with isolectin B4. **j-m**, Images of the vascular front of whole-mount P5 *Sirt1 flox/+* and *Tie2Cre;Sirt1 flox/-* retinas treated with DAPT or vehicle for 40 hours before dissection and stained with isolectin B4. All values are expressed as mean ± s.d. ***P<0.001, ns, not significant.

Supplementary Figure 11 Guarani et al.



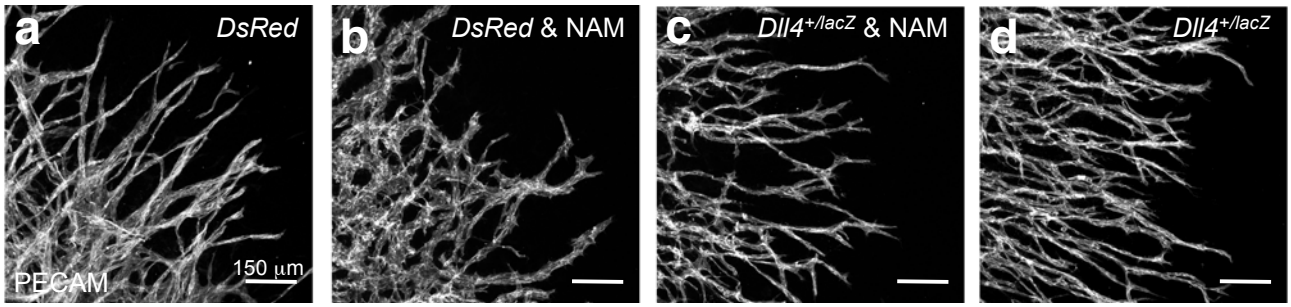
Supplementary Figure 11 – Inhibition of Notch signalling partially reverses the blood vessel defects in *sirt1*-deficient zebrafish embryos.
a, Lateral views of the trunk region (anterior is on the left) of 28 hpf fixed *Tg(fli1:nEGFP)y7* zebrafish embryos injected with a splice blocking morpholino against *sirt1* (*sirt1* MO) or control and treated with DAPT or DMSO. **b**, Schematic drawing of a zebrafish embryo. The region used for endothelial cell counts in the *Tg(fli1:nEGFP)y7* line is illustrated between the two red dotted lines. **c**, Lateral views of the trunk region (anterior is on the left) of 28 hpf fixed *Tg(fli1:nEGFP)y7* or *Tg(fli1:EGFP)y7* zebrafish embryos injected with a splice blocking morpholino against *sirt1* (*sirt1* MO), a *dll4* morpholino *(dll*4 MO) or combinations thereof.

Supplementary Figure 12 Guarani et al.



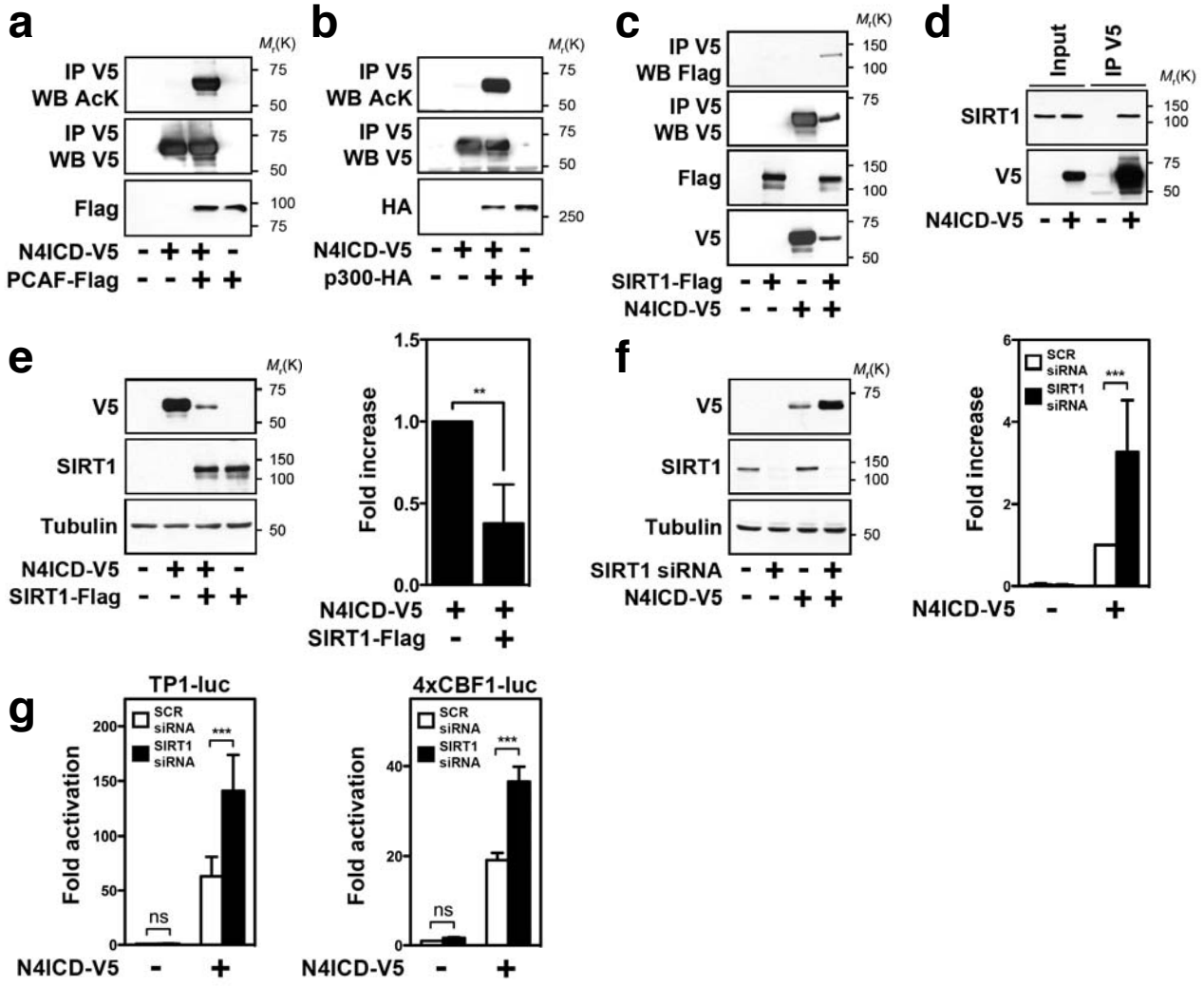
Supplementary Figure 12 – Impaired sprout elongation in SIRT1-deficient ECs is reversed by γ-secretase inhibition.
a-b, Representative images and statistical summary of 3D *in vitro* angiogenesis assays with SIRT1 or scrambled siRNA-transfected ECs treated with DMSO or DBZ. The mean sprout length was quantified, (n = 4). **c**, Cell growth of SIRT1 or scrambled siRNA-transfected HUVECs cultured on solvent or DLL4 and treated with DBZ or DMSO was assayed after 0, 24, 48, 72 and 96 hours, (n = 4). All values are expressed as mean ± s.d. **P*<0.05, ***P*<0.01, #*P*<0.001.

Supplementary Figure 13 Guarani et al.



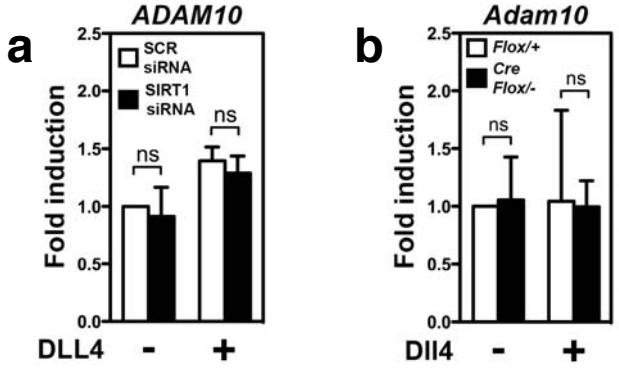
Supplementary Figure 13 – Pharmacological Sirt1 inhibition normalizes branching behaviour in *Dll4* heterozygote EBs.
a-d, overview of vascular sprouts from EBs of *DsRed* and *Dll4*$^{+/lacZ}$ ES cells treated with NAM or solvent. Vascular sprouts were identified by Pecam1 immunostaining.

Supplementary Figure 14 Guarani et al.



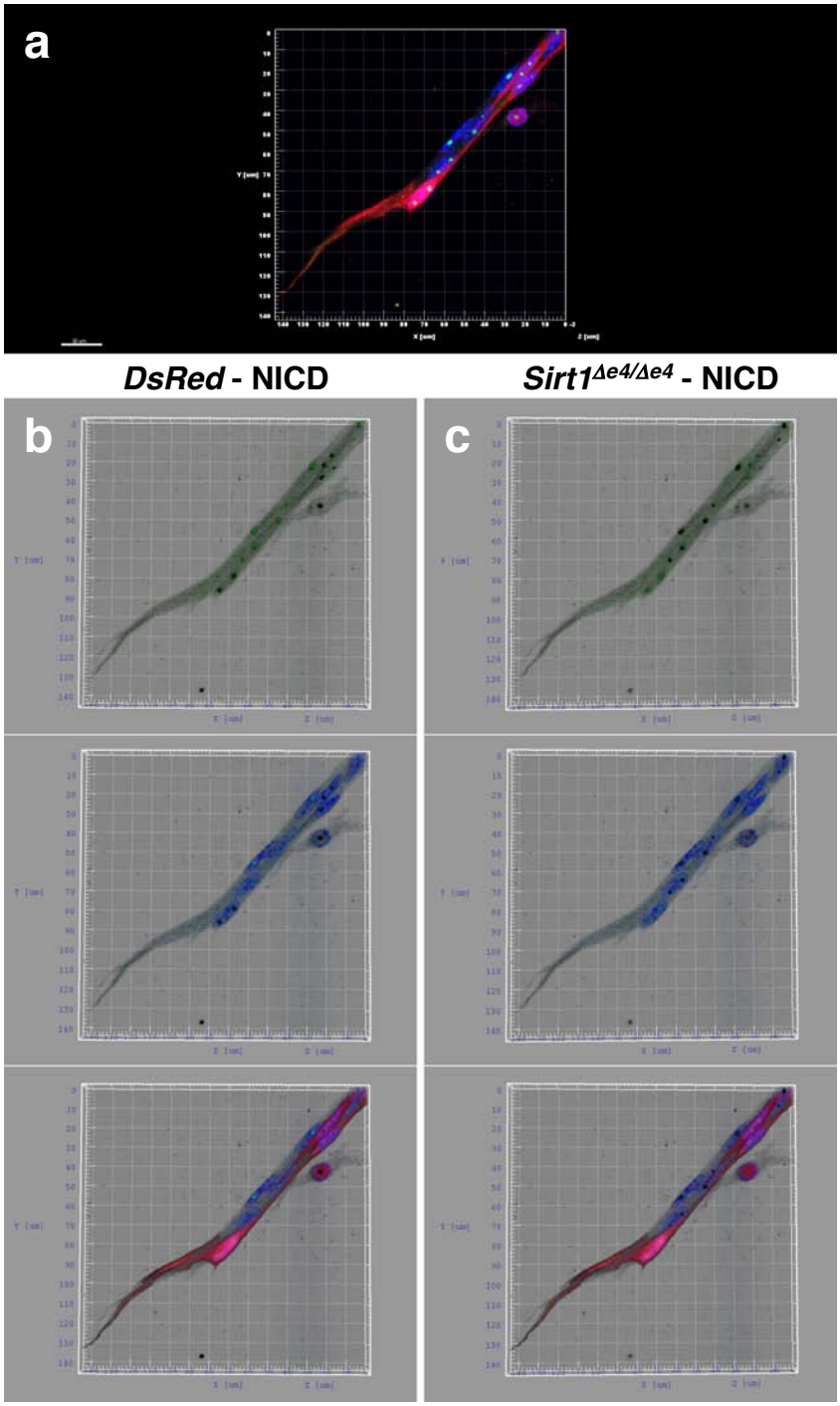
Supplementary Figure 14 – The Notch4 intracellular domain (N4ICD) is a reversibly acetylated protein regulated by SIRT1. **a-b**, HEK293 cells were cotransfected with N4ICD-V5, PCAF-Flag or p300-HA. N4ICD-V5 was immunoprecipitated with a V5 antibody and acetylation assessed by immunoblotting using antibodies to acetylated lysines. **c**, N4ICD-V5 was immunoprecipitated from SIRT1-Flag and N4ICD-V5 cotransfected HEK293 cells and immune complexes analyzed for the presence of SIRT1-Flag. **d**, N4ICD-V5 was immunoprecipitated from HEK 293 cells, which were transfected with pcDNA or N4ICD-V5. V5-immunoprecipitates were analysed for the presence of endogenous SIRT1 by immunoblotting using a SIRT1- specific antibody. **e**, HEK293 cells were cotransfected with N4ICD-V5 and SIRT1-Flag and N4ICD-V5 protein levels assessed by immunoblotting 24 hours after transfection using an antibody to the V5 epitope. **f**, N4ICD-V5 protein expression in HUVECs transfected with scrambled or SIRT1 siRNA and pcDNA or N4ICD-V5. Relative quantifications of N4ICD-V5 protein levels in **e** and **f** are shown on the right of the respective panel, (n = 5) and (n = 4), respectively. **g**, Luciferase activity was measured in scrambled and SIRT1 siRNA-transfected HUVECs, which were subsequently transfected with N4ICD-V5 and the indicated Notch reporters (TP1, 4xCBF1), (n = 4). All values are expressed as mean ± s.d., ***P*<0.01, ****P*<0.001; ns, not significant.

Supplementary Figure 15 Guarani et al.



Supplementary Figure 15 – *ADAM10* mRNA expression is not regulated by SIRT1 in endothelial cells.
a, Analysis of *ADAM10* mRNA levels in scrambled or SIRT1 siRNA-transfected HUVECs stimulated with DLL4 or solvent for 24 hours, (n = 3). **b**, qPCR of *Adam10* mRNA expression in ECs derived from *Sirt1 flox/+ and Tie2Cre;Sirt1 flox/-* mice and cultured in the presence or absence of Dll4, (n = 5). All values are expressed as mean ± s.d. ns, not significant.

Supplementary Figure 16 Guarani et al.



Supplementary Figure 16 – Quantification analysis of NICD levels in EB sprouts.
a-**c**, For the quantification of NICD protein levels, complete 1024 x 1024, 12 bit images of vascular sprouts were collected with a 63x objective lens using the Laser Scanning Microscope 710 (Zeiss). The same settings were used for all images during image aquisition. Images were processed using the Imaris 7.0.0 software. To identify nuclear NICD spots we used ”surface” function in Imaris. Measurements of the number of spots, the volume and total pixel intensity of each spot were made and statistics were analyzed using Prism 5.0c software.