**SUPPLEMENTAL MATERIAL**

**Rostama et al.** DLL4/Notch and BMP9 interdependent signaling induces human endothelial cell quiescence via $P27^{KIP1}$ and thrombospondin-1

## Supplemental Materials and Methods

**Reversibility ("washout") experiment** – HAEC stimulated with ligand for 24h (as described) were washed and trypsinized, and plated in new 6-well plates and allowed to grow for 24h with fresh complete media. BrdU was performed as described.

**Ligand stimulation** - Recombinant human TGFβ1 (R&D Systems, Minneapolis, MN, USA) was reconstituted in sterile PBS to 2μg/mL, and was used to stimulate cells at a final concentration of 1ng/mL alone, or in DLL4-coated dishes for 24h, with PBS serving as a vehicle control for both ligands.

**Senescence-associated β-galactosidase assay** – Ligand stimulated or aged positive control HAEC (passage 19) were washed with sterile PBS twice and fixed in 4% neutral buffered formalin phosphate for 3 minutes, and washed again with PBS. 20mg/mL X-gal (5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside, Amresco, Solon, OH, USA) stock solution was prepared by dissolving in dimethylformamide. Cells were incubated with fresh X-gal working solution (20mM citric acid, 40mM dibasic sodium phosphate, 150mM sodium chloride, 2mM magnesium chloride, 5mM potassium ferricyanide, 5mM ferrocyanide, 1mg/mL X-gal from stock solution, pH=6.00) for 16h at 37°C in a dry, non-atmosphere controlled incubator. Cells were washed twice with PBS, imaged under phase contrast microscopy, and counted using ImageJ software (NIH).

**TGFβ-associated kinase 1 (TAK1) inhibitor** – (5Z)-7-Oxozeaenol (TAK1 inhibitor, Calbiochem) was resuspended in DMSO at 725μg/mL (2mM) stock concentration. During ligand stimulation, HAEC were simultaneously incubated with 200nM TAK1 inhibitor or DMSO control.

**Inflammatory Cytokine Simulation experiment** – Determination of a suitable marker for endothelial "activation" (i.e. response to an inflammatory molecule) was determined by treating HAEC (passage 6) with 100ng/mL E-Coli 055:B5 lipopolysaccharide (LPS, in water, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or vehicle for 8h. Whole cell lysates were immunoblotted for VCAM1, MCP1, and ICAM1, and ICAM1 was chosen for its robust induction. To determine the effects of ligand stimulation on activation, HAEC were stimulated with ligands for 24h (as described) to induce proliferative quiescence, followed by 8h stimulation with vehicle (water), LPS (as described), or 100μg/mL polyinosinic:polycytidylic acid (PIC, in water, EMD Millipore, Billerica, MA, USA), a mimic of double-stranded RNA/viral infection. Whole cell lysates were immunoblotted for ICAM1, and band intensity was quantified using ImageJ software (NIH).

**Excess growth factor addition** – 50,000 HAEC were plated onto 6-well plates in ligand-stimulation conditions (as described). After allowing cells to attach for 2h, complete medium was removed and the cells were washed twice with PBS. The media was replaced with serum free endothelial basal medium, including fresh BMP9. Growth factors were added to the media individually at 30ng/mL for the duration of the 24h incubation. Recombinant human VEGF-$A_{165}$, EGF and FGF2 were purchased from Peprotech (Cat. # 100-20, 100-15, 100-18c, respectively). Recombinant human IGF was generously provided by Dr. Anyona Guntur.

**Mouse heart vessel density –** Formalin fixed hearts were cut equatorially through the ventricles, approximately 3mm cranial to the apex. The lower portions of the hearts were paraffin-embedded, sectioned, and immunohistochemically stained for VE-Cadherin to identify blood vessels of the ventricular walls and septum. The area around the ventral intersection of the septum and walls of the left and right ventricles was analyzed, and vessels with lumen circumferences larger than 10μm were counted. Vessel density was calculated as a ratio of vessel number per $mm^2$ of tissue. N=8 WT, 8 *Dll4+/-*
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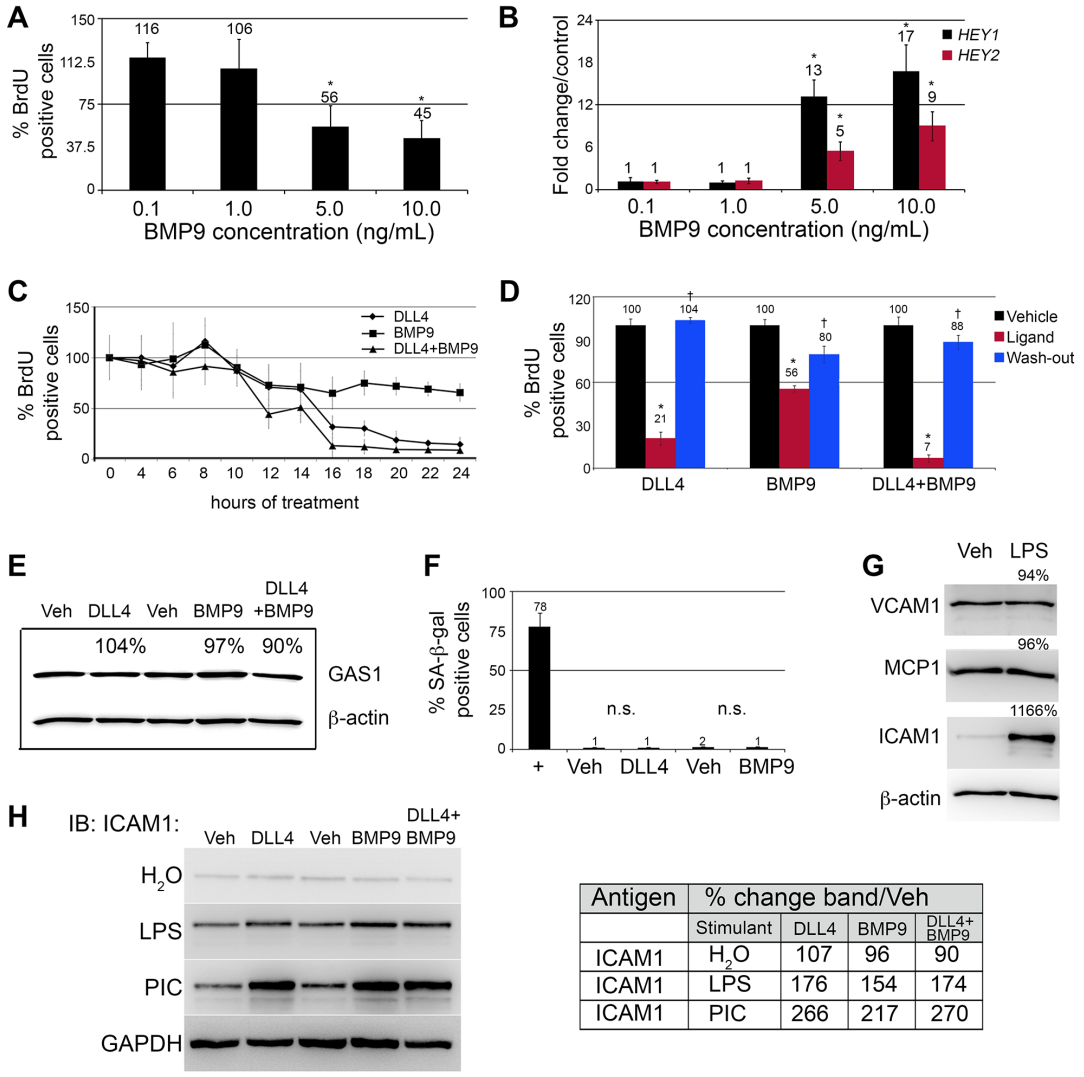
| Antibody target | Host | Company | Catalog number |
|---|---|---|---|
| P14 | Mouse | Cell Signaling | 2407 |
| P15 | Rabbit | Cell Signaling | 4822 |
| P16 | Rabbit | Cell Signaling | 4824 |
| Cyclin E1 | Mouse | Cell Signaling | 4129 |
| Cyclin E2 | Rabbit | Cell Signaling | 4132 |
| CDK2 | Rabbit | Cell Signaling | 2546 |
| GAS1 | Rat | R&D Systems | MAB2636 |
| V5 | Mouse | Invitrogen | R960-25 |
| pSMAD1/5/9 | Rabbit | www.mmcri.org/antibody | VLI31-5 |
| VEGFR2 | Rabbit | Cell Signaling | 2479 |
| VEGFR1 | Rabbit | Cell Signaling | 2893 |
| NRP1 | Goat | Santa Cruz | SC7239 |
| EGFR1 | Rabbit | Cell Signaling | 4267 |
| IGFR1 | Rabbit | Genetex | GTX111666 |
| FGFR1 | Rabbit | Cell Signaling | 9740 |
| FGFR2 | Rabbit | Genetex | GTX112047 |
| Total ERK1/2 | Rabbit | Cell Signaling | 4695 |
| pERK1/2 | Rabbit | Cell Signaling | 4370 |
| Total AKT | Rabbit | Cell Signaling | 9272 |
| pAKT | Rabbit | Cell Signaling | 4060 |
| Total Rb | Mouse | Cell Signaling | 9309 |
| pRb | Rabbit | Cell Signaling | 9308 |
| HRas | Rabbit | Cell Signaling | 3965 |
| FLAG tag | Mouse | Sigma | F3165 |
| TGF$\beta$1 | Rabbit | Cell Signaling | 3711 |
| ICAM1 | Mouse | Genetex | GTX78387 |
| VCAM1 | Rabbit | Genetex | GTX110684 |
| MCP1 | Rabbit | Rockland | 200-401-B60S |
| VE-Cadherin | Rabbit | www.mmcri.org/antibody | VLI-37 |
| GAPDH | Goat | Genscript | A00191 |

Table 1. Specifications of antibodies used for supplemental immunoblot assays.

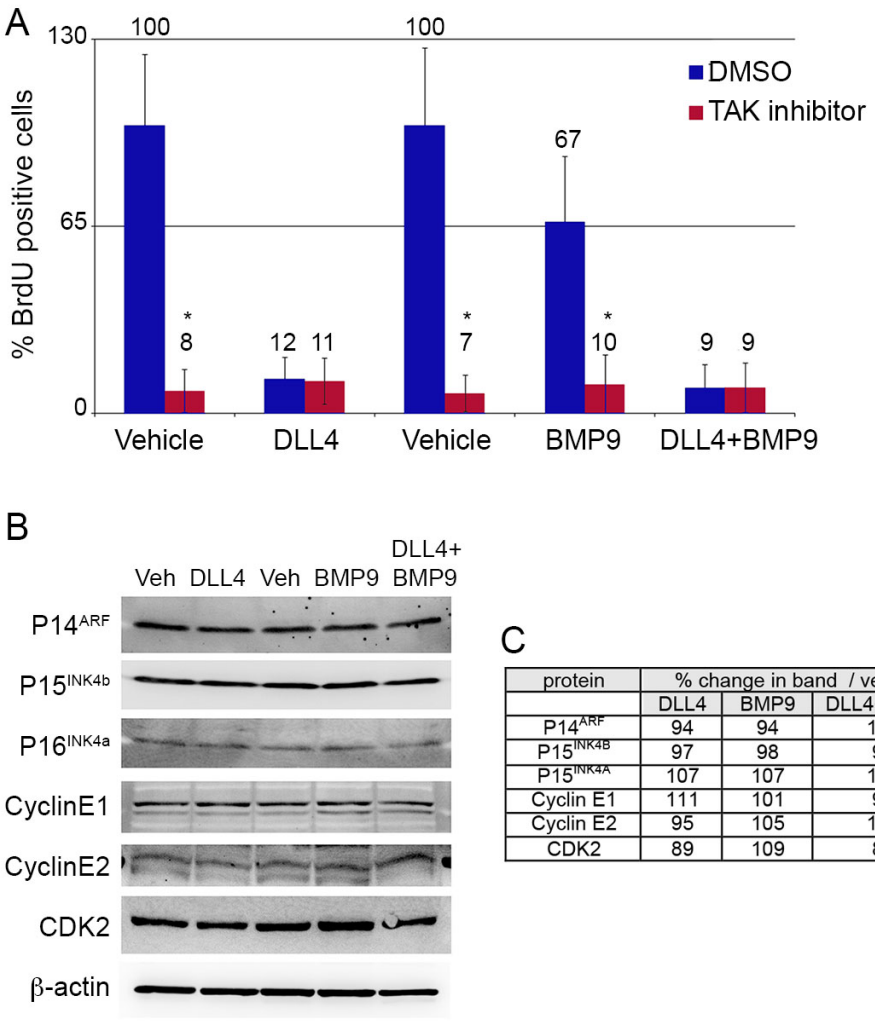
**Supplemental Figure I**




| Antigen | % change band/Veh | | | |
|---|---|---|---|---|
| | Stimulant | DLL4 | BMP9 | DLL4+ BMP9 |
| ICAM1 | $H_2O$ | 107 | 96 | 90 |
| ICAM1 | LPS | 176 | 154 | 174 |
| ICAM1 | PIC | 266 | 217 | 270 |

**Supplemental Figure I. DLL4 and BMP9 suppress proliferation reversibly, and do not cause senescence**. A) Primary human aortic endothelial cells (HAEC) were treated with increasing amounts of BMP9, and grown for 24h. Proliferation was measured by quantifying BrdU incorporation relative to vehicle. Graphed are means ± SD. B) HAEC were treated with BMP9 and total mRNA was collected for qPCR to quantify *HEY1* and *HEY2* transcript normalized to *PPIA.* C) HAEC were treated with ligands as indicated, and proliferation determined by BrdU incorporation. Graphed are means ± SD. D) HAEC were treated as described, with an additional group where cells were collected after 24h of ligand treatment and then grown in new plates, in the absence of ligands for 24h. E) HAEC were treated with ligand for 24h. Cell lysates were collected for immunoblot to detect growth arrest-specific protein 1 (GAS1). Numbers indicate normalized protein levels as a percentage of vehicle. F) HAEC were treated with ligands as indicated, and fixed after 24h for detection of senescence-associate β-galactosidase activity (SA-β-gal). HAEC at passage 19 were used as a positive control for senescence (+). G) HAEC were treated with 100ng/ml bacterial lipopolysaccharide (LPS) or vehicle ($H_2O$) for 8h and lysates were collected for immunoblot for markers of endothelial inflammation. Numbers indicate normalized protein as a percentage of vehicle. H) HAEC were treated with ligands as indicated for 24h to induce quiescence, followed by 8h stimulation with vehicle ($H_2O$), 100ng/ml bacterial lipopolysaccharide, or 100µg/ml polyinosinic:polycytidylic acid (PIC, a mimic of double-stranded RNA/viral infection). Cell lysates were collected and immunoblotted for ICAM1. Quantified immunoblot bands are represented in tabular form as a percentage of vehicle. (*) signifies statistical significance with $p<0.05$. (†) signifies statistically significant reversal of effects ($p<0.05$).

**Supplemental Figure II**

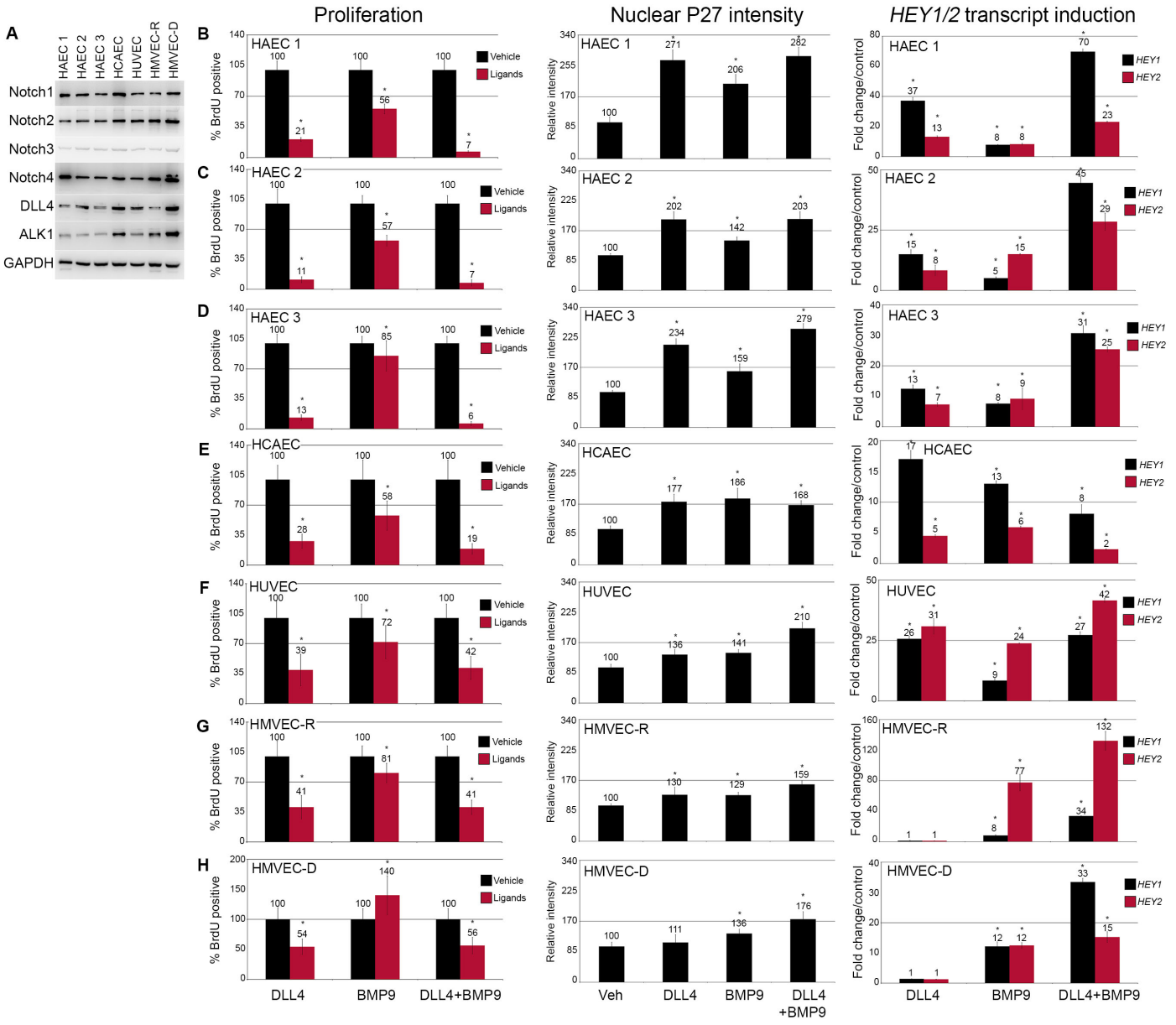



C

| protein | % change in band / vehicle | | |
|---|---|---|---|
| | DLL4 | BMP9 | DLL4+BMP9 |
| $P14^{ARF}$ | 94 | 94 | 101 |
| $P15^{INK4B}$ | 97 | 98 | 96 |
| $P15^{INK4A}$ | 107 | 107 | 108 |
| Cyclin E1 | 111 | 101 | 99 |
| Cyclin E2 | 95 | 105 | 118 |
| CDK2 | 89 | 109 | 86 |

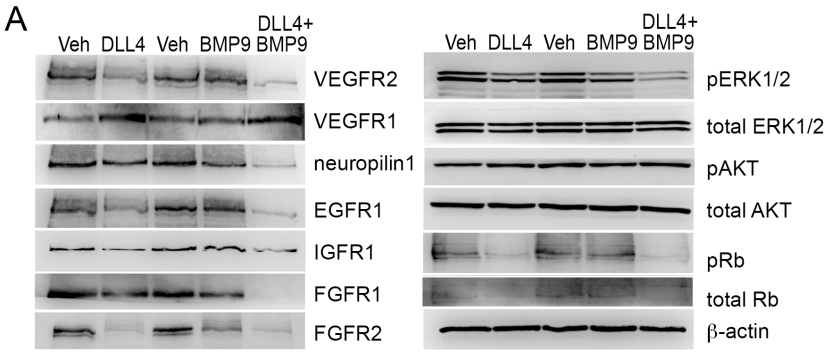
**Supplemental Figure II. TAK1 and other cell cycle regulators are not specific to ligand stimulation.** A) Primary human aortic endothelial cells (HAEC) were plated on control dishes or those coated with 20pmols DLL4, or treated with 5ng/ml BMP9 on control or DLL4-coated dishes. In addition, cells were treated with vehicle (DMSO) or 200nM (5Z)-7-Oxozeaenol, a TAK1 inhibitor. Cell proliferation was quantified using BrdU incorporation. Graphed are means ±SD. B) HAEC were plated on control dishes or those coated with 20pmols DLL4, or treated with 5ng/ml BMP9 on control or DLL4-coated dishes, and cell lysates collected after 24h. Cell lysates were used for immunoblot to detect the indicated cell cycle regulators. C) Quantified immunoblot bands are represented in tabular form as a percentage of vehicle. (*) signifies statistical significance with $p<0.05$.

## Supplemental Figure III



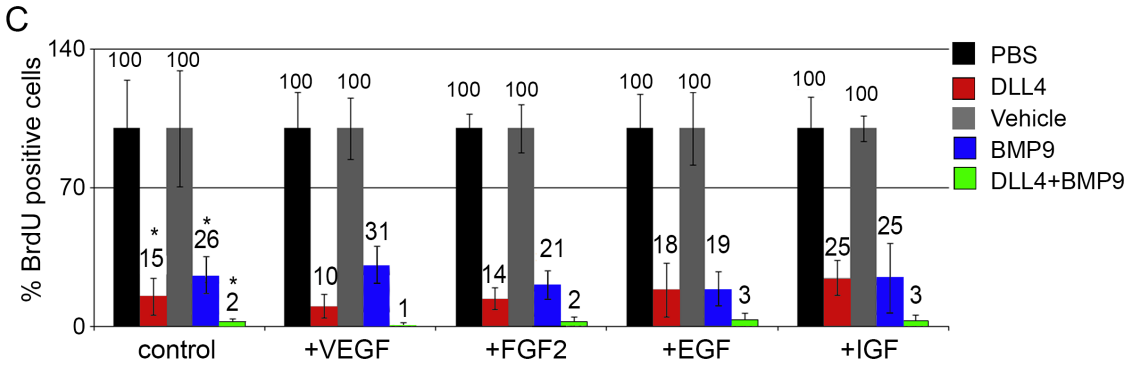

**Supplemental Figure III. Primary single-donor human endothelial cells from various tissues express similar Notch- and BMP-related proteins, and exhibit similar ligand responses.** A) Primary single-donor human endothelial cells from aorta (HAEC), coronary artery (HCAEC), umbilical vein (HUVEC), retinal microvasculature (HMVEC-R), and dermal microvasculature (HMVEC-D) were grown without ligand stimulation, and cell lysates were collected and immunoblotted for Notch- and BMP9-related proteins. Exposure times for all antibody targets were identical (60 seconds), except for GAPDH (2 seconds). B-H) Various human endothelial cells were stimulated with ligands for 24h as indicated to determine consistency of response to DLL4 and BMP9 among various human sources of endothelial cells. Column 1: BrdU incorporation was used to calculate proliferation relative to vehicles. Graphed are means ±SD. Column 2: Immunofluorescent staining was used to quantify levels of $P27^{KIP1}$, relative to vehicles. Graphed are means ±SD. Column 3: Total mRNA was collected for qPCR and primers were used to amplify *HEY1* and *HEY2* transcripts, normalized to *PPIA* and quantified as fold change relative to vehicle. Graphed are means ±SD. (*) signifies statistical significance with $p<0.05$.

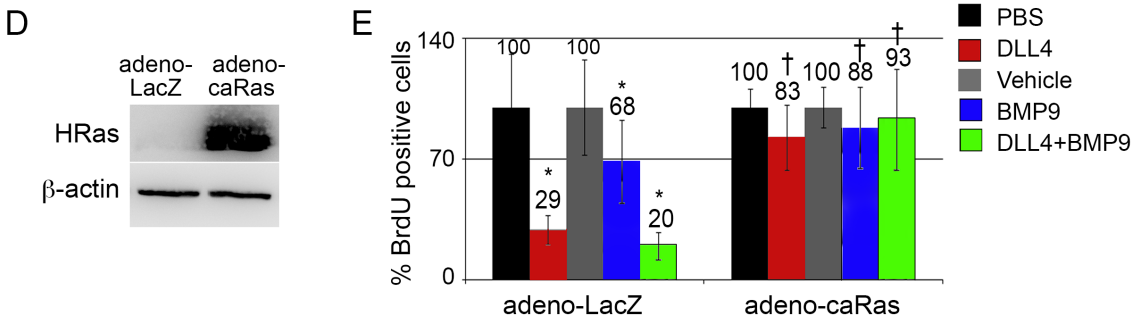
**Supplemental Figure IV**




B

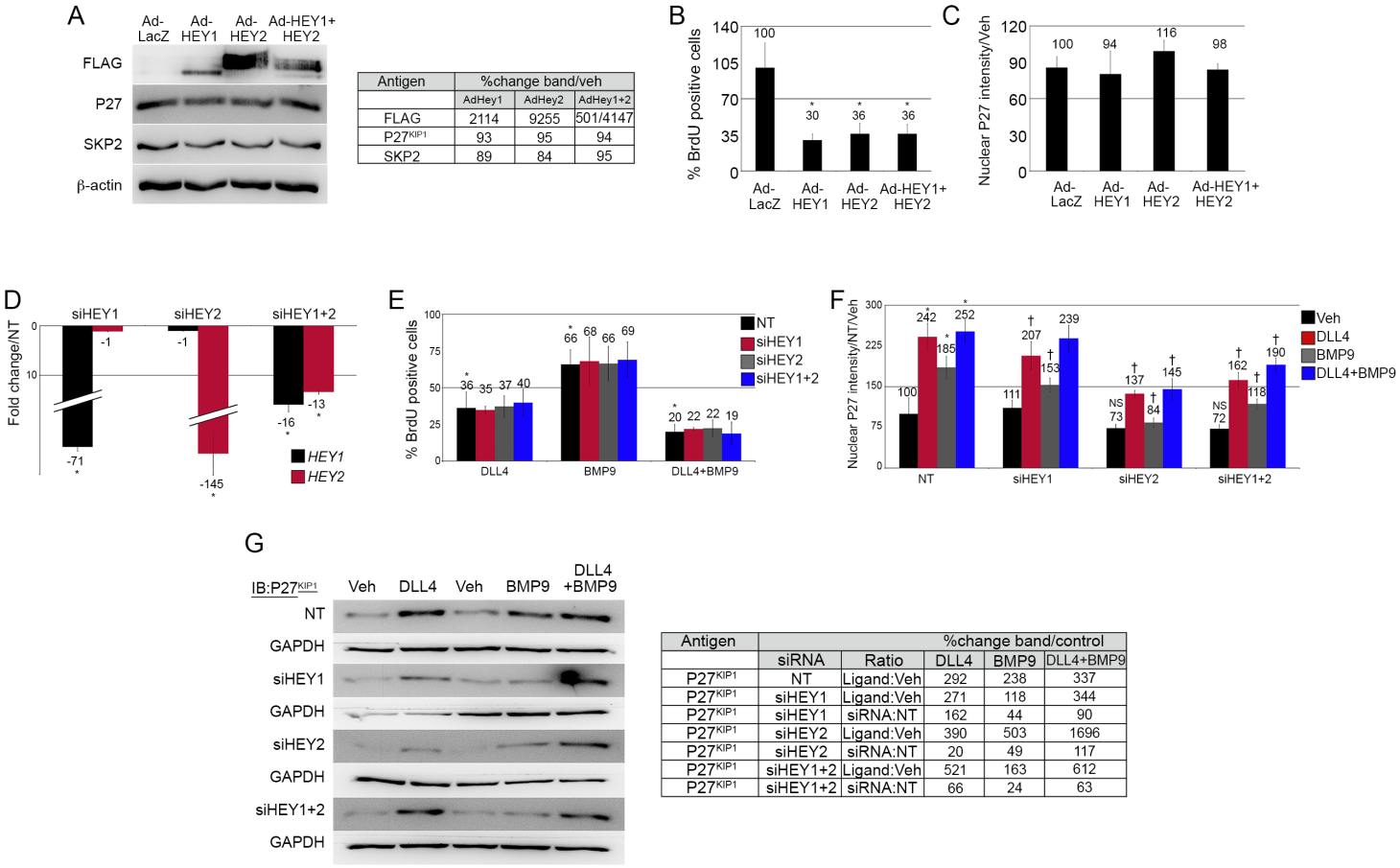
| Antigen | % change band/veh | | |
|---|---|---|---|
| | DLL4 | BMP9 | DLL4+BMP9 |
| VEGFR2 | 54 | 96 | 31 |
| VEGFR1 | 177 | 153 | 280 |
| neuropilin1 | 66 | 80 | 22 |
| EGFR1 | 58 | 81 | 24 |
| IGFR1 | 56 | 91 | 54 |
| FGFR1 | 70 | 66 | 16 |
| FGFR2 | 14 | 46 | 17 |
| pERK1 | 61 | 61 | 26 |
| pERK2 | 47 | 55 | 27 |
| Tot ERK1 | 94 | 96 | 101 |
| Tot ERK2 | 78 | 98 | 108 |
| pAKT | 127 | 80 | 103 |
| Tot AKT | 99 | 103 | 105 |
| pRb | 29 | 86 | 29 |
| Tot Rb | 82 | 84 | 59 |




**Supplemental Figure 4. Growth factor receptors are suppressed by ligand activation, but quiescence is not overcome with excess growth factors.** A) Primary human aortic endothelial cells (HAEC) were stimulated with DLL4 or BMP9 ligands for 24h. Cell lysates were collected for immunoblot and probed with antibodies against growth factor receptors and the ERK, AKT, and Rb pathways. B) Quantified immunoblot bands are represented in tabular form as a percentage of vehicle. C) In addition to ligands, cells were treated with saturating concentrations of different growth factors individually, including VEGF-$A_{165}$ (30ng/mL), FGF2 (30ng/mL), EGF (30ng/mL), and IGF1 (30ng/mL), under serum free conditions for 24h. Proliferation was assessed by BrdU incorporation. Groups with added cytokines were compared to ligands alone with no additional factors added to the basal media (control). Graphed are means ± SD. Additional growth factors were unable to reverse the quiescence-promoting effects of DLL4 and BMP9. D-E) Cells were transduced with adenoviral constructs for LacZ (adeno-LacZ) or constitutively active Ras (adeno-caRas), cell lysates were immunoblotted to verify overexpression of Ras (D, 2108% of adenoLacZ), and proliferation was measured by BrdU incorporation (E). Constitutively active Ras potently reversed ligand-mediated endothelial quiescence. (*) signifies statistical significance of P<0.05. (†) signifies statistically significant reversal of effects (p<0.05).

## Supplemental Figure V

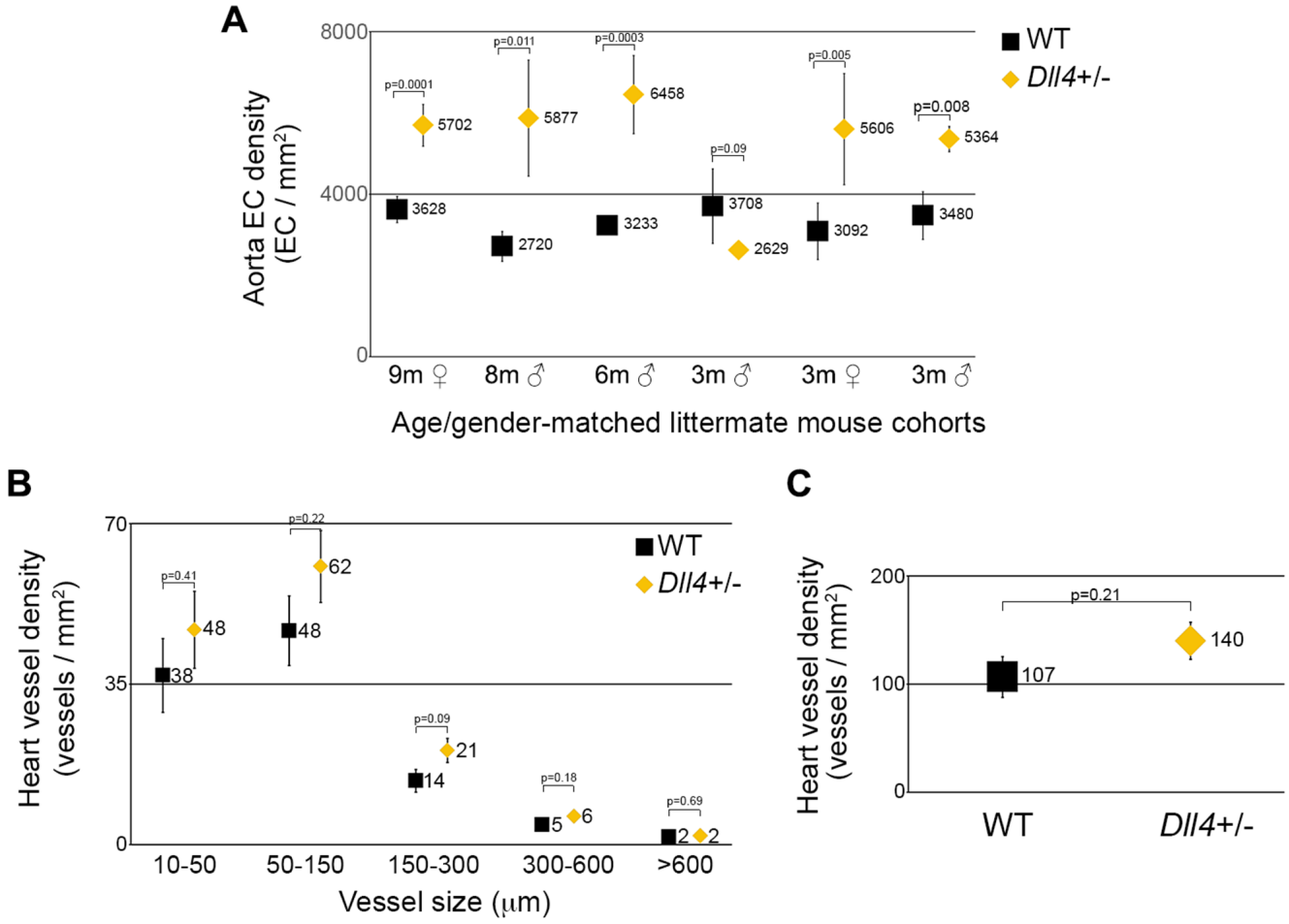



| Antigen | %change band/veh | | |
|---|---|---|---|
| | AdHey1 | AdHey2 | AdHey1+2 |
| FLAG | 2114 | 9255 | 501/4147 |
| $P27^{KIP1}$ | 93 | 95 | 94 |
| SKP2 | 89 | 84 | 95 |

| Antigen | %change band/control | | | | |
|---|---|---|---|---|---|
| | siRNA | Ratio | DLL4 | BMP9 | DLL4+BMP9 |
| $P27^{KIP1}$ | NT | Ligand:Veh | 292 | 238 | 337 |
| $P27^{KIP1}$ | siHEY1 | Ligand:Veh | 271 | 118 | 344 |
| $P27^{KIP1}$ | siHEY1 | siRNA:NT | 162 | 44 | 90 |
| $P27^{KIP1}$ | siHEY2 | Ligand:Veh | 390 | 503 | 1696 |
| $P27^{KIP1}$ | siHEY2 | siRNA:NT | 20 | 49 | 117 |
| $P27^{KIP1}$ | siHEY1+2 | Ligand:Veh | 521 | 163 | 612 |
| $P27^{KIP1}$ | siHEY1+2 | siRNA:NT | 66 | 24 | 63 |

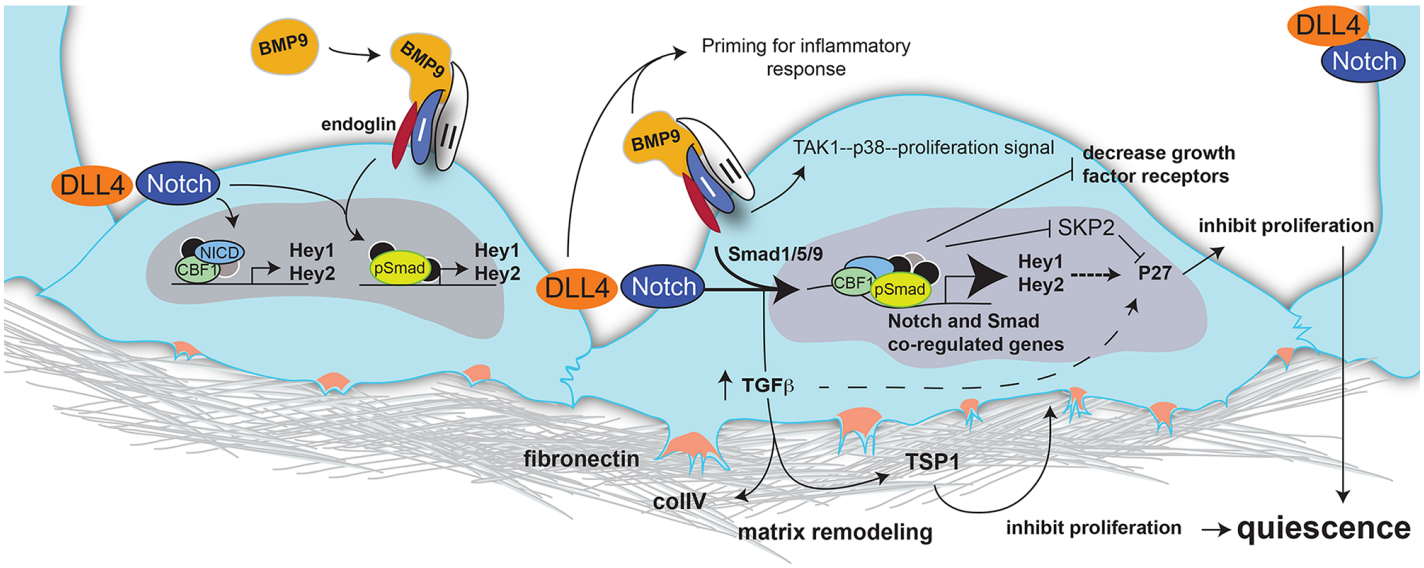
**Supplemental Figure V. *HEY2* contributes to the induction of $P27^{KIP1}$ by DLL4 and BMP9.** A) Primary human aortic endothelial cells (HAEC) were transduced with adenoviral control LacZ (AdLacZ) or FLAG-tagged *HEY1* (AdHEY1) or *HEY2* (AdHEY2) constructs or both combined (using half the viral load of each individual construct) for 6h, then grown for an additional 42h, and cell lysates were immunoblotted for FLAG to confirm overexpression. Overexpression was also confirmed by qPCR (data not shown). Quantified immunoblot bands are represented in tabular form as a percentage of vehicle. B) HAEC were transduced with adenoviral *HEY* constructs and proliferation measured by BrdU incorporation. Graphed are means ±SD. C) HAEC transduced with AdHEY1, AdHEY2 or both were fixed and immunofluorescently stained to quantify $P27^{KIP1}$relative to AdLacZ. Graphed are means ±SD. D) Endogenous *HEY1, HEY2* or both (at half concentration) were targeted using siRNA, with non-targeting (NT) siRNA as control. Total RNA was collected for qPCR after 48h. Graphed are means ±SD. E) HAEC with *HEY* knockdown were stimulated with ligands for 24h, and proliferation assessed by BrdU incorporation. Graphed are means ±SD. F) HAEC with *HEY* knockdown were stimulated with ligands for 24h, fixed and immunofluorescently stained for $P27^{KIP1}$. Graphed are means ±SD. G) In a similar experiment, lysates were collected and immunoblotted for $P27^{KIP1}$. Graphed are means ±SD. Quantified immunoblot bands are represented in tabular form comparing siRNA bands as a percentage of vehicle or NT bands (ratio). (*) signifies statistical significance with $p<0.05$. (†) signifies statistically significant reversal of effects ($p<0.05$).

**Supplemental Figure VI**




**Supplemental Figure VI. Haploinsufficiency of a *Dll4* allele increases aortic endothelial density, but vessels of the heart wall remain unchanged.** A) Thoracic aortas of *Dll4+/-* mice and WT littermates were fixed, mounted enface, stained with Hoechst nuclear dye and endothelial density measured by immunofluorescent imaging. Graphed are means ± SEM. B-C) Equatorial ventricular sections of paraffin embedded hearts of *Dll4+/-* mice and WT littermates were immunohistochemically stained for VE-cadherin to visualize endothelial cells. Vessels at the ventral intersection of the septum and ventricular walls were counted and lumen circumferences measured. Vessel density was calculated relative to tissue surface area. N=8 mice/group. Graphed are means ± SEM.

**Supplemental Figure VII**




**Supplemental Figure VII. Summary of DLL4 and BMP9 pathways to quiescence.** Schematic overview of the cooperative signals between DLL4/Notch1 and BMP9/ALK1. Notch and ALK-mediated signals result in transcriptional regulation of gene targets, and in human endothelial cells, Notch signaling is required for BMP9 activity (left). (Right) Our studies show that cooperative signaling between these pathways result in co-regulation of transcriptional targets, such as *Hey* genes, and result in the induction of $P27^{KIP1}$, TGFβ1, and TSP1, and the suppression of growth factor receptors. Collectively, these molecular changes contribute to the quiescence phenotype, and also a heightened sensitivity to inflammatory stimuli that would lead to endothelial cell activation. In our mouse *Dll4+/-* model, decreased Dll4 protein led to a compensatory increase in pSmad1/5 signaling.

**Supplemental Table I**

A

| Antigen | %change band/veh | | |
|---|---|---|---|
| | DLL4 | BMP9 | DLL4+BMP9 |
| $P18^{INK4C}$ | 55 | 98 | 54 |
| $P19^{INK4D}$ | 18 | 95 | 90 |
| $P21^{CIP1}$ | 25 | 50 | 11 |
| $P27^{KIP1}$ | 381 | 234 | 400 |
| P53 | 69 | 48 | 41 |
| pP53 (S10) | 60 | 60 | 32 |
| Cyclin D1 | 33 | 64 | 27 |
| Cyclin D3 | 52 | 122 | 49 |
| CDK4 | 44 | 79 | 78 |
| CDK6 | 131 | 177 | 190 |
| Ki-67 | 14 | 46 | 8 |

B

| Antigen | %change band/veh | | |
|---|---|---|---|
| | DLL4 | BMP9 | DLL4+BMP9 |
| P27KIP1 | 170 | 193 | 221 |
| pP27 (S10) | 437 | 121 | 164 |
| SKP2 | 12 | 74 | 30 |
| pP27 (T157) | 13 | 42 | 21 |

C

| Antigen | %change band/control | | | | |
|---|---|---|---|---|---|
| | siRNA | Ratio | DLL4 | BMP9 | DLL4+BMP9 |
| $P27^{KIP1}$ | NT | Ligand:Veh | 340 | 397 | 2130 |
| $P27^{KIP1}$ | siP27 | siRNA:NT | 6 | 6 | 2 |

**Supplemental Table I.** A) Quantification of immunoblots in Fig. 3A, B) quantification of immunoblots in Fig. 3B, and C) quantification of immunoblots in Fig. 3D.