

1 **A Key Cytoskeletal Regulator of Ubiquitination Amplifies TGF β Signaling During Mouse**
2 **Developmental Vascular Patterning**

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22

23 **Abstract**

24 Vascular development involves *de novo* formation of a capillary plexus, which is then pruned and
25 remodeled by angiogenic events. Cytoskeletal remodeling and directional endothelial migration are
26 essential for developmental and pathological angiogenesis. Smad-dependent TGF β signaling controls
27 vascular patterning and is negatively regulated by microtubules. Here we show that a positive regulator
28 of TGF β signaling is essential for developmental vascular patterning and microtubule stability.
29 Rudhira/BCAS3 is known to bind microtubules and to play a nodal role in cytoskeletal remodeling and
30 directional endothelial cell (EC) migration *in vitro*. We demonstrate that the molecular and cellular
31 function of Rudhira is deployed at critical steps in vascular patterning. We generated the first floxed
32 mice for *rudhira* and find that global or endothelial knockout of *rudhira* results in mid-gestation lethality
33 due to aberrant embryonic and extra-embryonic vessel patterning and defective cardiac morphogenesis.
34 *Rudhira* null yolk sac ECs show random and retarded migration. Yolk sac transcriptome analysis revealed
35 key mediators of angiogenic processes and TGF β receptor signaling were perturbed in *rudhira* null
36 mutants. Molecular and biochemical analyses showed that *rudhira* depletion reduced microtubule
37 stability but increased expression of pathway inhibitors leading to high levels of SMAD2/3 ubiquitination
38 and reduced activation. These effects were not rescued by exogenous TGF β . However, TGF β treatment
39 of wild type ECs increased Rudhira expression. Further, exogenous Rudhira, which promotes directional
40 cell migration, caused increased SMAD2/3 nuclear translocation and reduced inhibitor levels. Therefore,
41 we propose that Rudhira and TGF β signaling are mutually dependent. Rudhira has a dual function in
42 promoting TGF β signaling, possibly by sequestering microtubules and simultaneously preventing
43 SMAD2/3 ubiquitination to permit EC migration and vascular patterning. TGF β signaling and aberrant
44 human Rudhira (Breast Cancer Amplified Sequence 3, *BCAS3*) expression are both associated with
45 tumour metastasis. Our study identifies a cytoskeletal, cell type-specific modulator of TGF β signaling
46 important in development and cancer.

47

48 **Author Summary**

49 Remodeling and fine patterning of the blood vasculature requires controlled and co-ordinated
50 endothelial cell (EC) migration. This is achieved by the tight regulation of complex signaling pathways in
51 developmental and adult vascular patterning. The TGF β pathway, in particular, is important for this
52 process. Here we show that the cytoskeletal protein Rudhira plays a critical role in promoting TGF β
53 signaling in EC. We generated “knockout” mice and show that Rudhira is crucial for EC migration in
54 mouse developmental vascular patterning. In the absence of Rudhira there is increased degradation of
55 TGF β pathway effectors resulting in reduced signaling. This affects target gene expression and
56 angiogenic processes, especially extracellular matrix remodeling and EC migration. As a result, the
57 hierarchical vascular pattern is not achieved and embryos die mid-gestation. We propose that Rudhira
58 plays a pivotal role in amplifying TGF β signaling, which is critical for vascular remodeling in multiple
59 normal and pathological contexts.

60

61 **Introduction**

62 Vertebrate blood vessel formation involves *de novo* differentiation of endothelial cells (ECs) to form a
63 primary plexus (1). This is resolved into a branched hierarchical network by EC migration and inhibition
64 of proliferation, basement membrane reconstitution and recruitment of smooth muscle cells and
65 pericytes to stabilize the network (2-4). Perturbation of the balance between pro- and anti-angiogenic
66 cues leads to endothelial activation and cytoskeletal changes resulting in sprouting, migration and
67 maturation (5, 6). This process is spatially and temporally orchestrated during development and adult
68 neo-angiogenesis. Recent studies have elegantly elucidated the cellular dynamics of vessel regression
69 and pruning (7, 8). EC migration and tube formation are key steps during angiogenesis, however,
70 molecular mechanisms that operate are incompletely defined. Random and retarded EC migration
71 results in an unpatterned, often leaky vasculature (9, 10).

72 A primary response of EC to angiogenic stimuli, flow induced shear stress or signals that induce pruning,
73 is the reorganization of the cytoskeleton, resulting in activation of various signaling pathways (11-13).
74 The loss of EC polarity, actin reorganization and extracellular matrix remodeling are key outcomes of
75 cytoskeletal remodeling, resulting in directional cell migration essential for angiogenesis. The
76 cytoskeleton also induces changes in cell shape and gene expression through a complex network of
77 signaling pathways by influencing transcription factor activity (14).

78 The TGF β pathway is sensitive to cytoskeletal rearrangements and regulates EC proliferation,
79 differentiation, survival and migration (15). Multiple components of this pathway crosstalk with other
80 signaling pathways to collaboratively pattern the vasculature (16). Mouse knockouts of TGF β pathway
81 components have demonstrated that a functional TGF β pathway is essential for cardio-vascular
82 development. Knockout of many of the ligands, receptors, effectors or regulators results in vascular
83 developmental abnormalities and often causes embryonic death (17). In the embryonic yolk sac,

84 paracrine TGF β signaling does not affect EC specification or differentiation but regulates gene
85 expression for assembly of robust vessels (18). Microtubules bind to and negatively regulate Smad
86 activity (19). There is limited understanding of how cytoskeletal elements regulate the TGF β and other
87 signaling pathways and bring about changes in EC shape, polarity and directed migration. While several
88 mouse mutant models of vascular patterning defects have been reported, those that perturb the
89 cytoskeleton and associated proteins show pre-gastrulation lethality on one hand or display mild
90 developmental phenotypes on the other (20, 21).

91 Here we investigate the role of an endothelial cytoskeletal protein Rudhira/BCAS3 in regulating
92 developmental vascular remodeling. Rudhira is a WD40 domain-containing microtubule-binding protein
93 expressed in vascular endothelial cells during mouse embryonic development (22). Rudhira promotes
94 directional EC migration *in vitro* by rapidly re-localizing to the leading edge and mediating Cdc42
95 activation and filopodial extension (23).

96 We report that global or EC-specific *rudhira* deletion results in mid-gestation lethality due to aberrant
97 vascular patterning. Knockout ECs fail to show directed migration. In *rudhira* mutants, hierarchical
98 patterning of the embryonic and extra-embryonic vasculature is lost. Expectedly, this has a major effect
99 on gene expression as seen by yolk sac transcriptome and protein expression studies. While several
100 processes and signaling pathways involved in vascular patterning are perturbed, extracellular matrix
101 remodeling and TGF β signaling are majorly affected. We show that Rudhira does not induce, but
102 promotes TGF β signaling by suppressing expression of the inhibitor *smurf2*, thereby preventing
103 SMAD2/3 ubiquitination. Further, Rudhira shows increased association with microtubules upon TGF β
104 induction. Thus Rudhira provides cytoskeletal control of several angiogenic processes by modulating
105 TGF β signaling. This indicates that normal Rudhira function is required to aid the EC cytoskeleton in
106 regulating gene expression and cell migration. The identification and mechanistic deciphering of genes

107 such as *rudhira*, provide us a pivot on which molecular partnerships with both ubiquitous ‘essential’ or
108 ‘redundant’ players can be understood and deciphered in the EC context.

109 **Results**

110 **Endothelial *rudhira* is vital for embryonic development**

111 *Rudhira* is expressed primarily in early embryonic vascular development and neo-angiogenesis, but its
112 role *in vivo* is not known. Hence we generated *rudhira* floxed mice (Fig. 1A and S1A Fig.) and crossed
113 them to *CMV-Cre* for ubiquitous deletion (*rudhira*^{flox/flox}; *CMVCre*⁺ abbreviated to *rudh*^{-/-}) or *Tie-2-Cre* for
114 tissue-specific ablation (*rudhira*^{flox/flox}; *TekCre*⁺ abbreviated to *rudh*^{CKO}) of the *rudhira* locus (see Materials
115 and Methods, and S1 Fig.). While heterozygotes were viable, ubiquitous or endothelial deletion of
116 *rudhira* gave no live homozygous pups (S1A and S1B Tables). This indicates that endothelial deletion of
117 *rudhira* causes recessive embryonic lethality. Analysis of embryos from E8.5 to E11.5 showed a reduced
118 number of homozygous mutant embryos (S1A and S1B Tables) as identified by genotyping (Fig. 1B),
119 transcript (Fig. 1C) and protein (S1B and S1C Fig.) expression, suggesting that lethality occurred between
120 E9.0 and E11.5.

121 Since *rudhira* expression may be transient or undetectable in some migrating cells we also analysed the
122 effect of globally deleted *rudhira* (*rudh*^{-/-}) on development from E7.5 onwards, a stage before *rudhira*
123 expression is initiated (22). At E7.5 *rudhira* mutant embryos were indistinguishable from controls with
124 respect to morphology as well as primitive streak formation as seen by Brachyury expression (S1D Fig.).
125 E8.5 mutant embryos showed unpatterned dorsal aorta as detected by Flk1 staining (S1E Fig.). By E9.5,
126 mutant embryos were growth retarded (Fig. 1D and 2A) with defects including reduced somite number
127 (19±2 at E9.5 in mutants compared to 25±2 in controls; n= 10) but expressed lineage markers of
128 ectoderm (Nestin), mesoderm (Brachyury) and endoderm (AFP) (S1F Fig.). *Rudhira* is known to have
129 restricted expression during vasculogenesis and primitive erythropoiesis (22). However, we did not find
130 any significant change in the expression of vascular (CD34, Flk1, Ephrin, SMA, PECAM) and early
131 hematopoietic (β-globin, c-kit, GATA-1) markers by semi-quantitative RT-PCR (S1F Fig.) showing that the

132 two lineages are specified in *rudhira* null embryos. This suggests that *rudhira* is not essential for lineage
133 specification and early vascular differentiation. Hence, we reasoned that the growth retardation and
134 morphological defects seen in *rudhira* null embryos likely arise from defects in vascular rearrangement
135 and patterning.

136

137 **Rudhira functions in extraembryonic vascular development**

138 Impaired development and embryonic lethality between E8.5-E11.5 is often the result of aberrant and
139 functionally impaired extra-embryonic vasculature (24). Moreover, Rudhira is strongly expressed in the
140 yolk sac vasculature (22). Hence we analysed extraembryonic structures of mutant embryos, such as
141 yolk sac and placenta which connect the maternal and fetal vasculature. Mutant yolk sacs were pale and
142 lacked major blood vessels (Fig. 1D). Immunostaining for the blood vessel marker PECAM showed that
143 *rudh*^{-/-} yolk sac vessels were irregular and fused, unlike the finely patterned honey-comb like vascular
144 network seen in control littermates (Fig. 1E, 1I-K). Thus mutants could form a primitive vascular plexus
145 which, however, did not undergo angiogenic remodeling. Primitive erythrocytes were dispersed all over
146 the mutant yolk sac indicating an unpatterned and leaky vasculature. Histological analyses of yolk sac
147 showed congested capillaries lined by thinner endothelium (Fig. 1F, arrowhead) as compared to controls
148 (Fig. 1F, arrow). To test whether the yolk sac vessel patterning requires endothelial *rudhira* or is due to
149 non-specific effects we analysed endothelial-deleted *rudh*^{CKO} yolk sac. Although of less severity than in
150 the global knock-out, endothelial deletion of *rudhira* resulted in reduced branching from major vessels,
151 vessel fusion and loss of branch hierarchy at both E10.5 and E11.5 (Fig. 1G-H and I-K). These results
152 indicate that *rudhira* is essential for remodeling the yolk sac vascular network. Aberrant vascular
153 remodeling in *rudhira* mutant embryos is likely the primary cause of death.

154 **Fig. 1. Rudhira is essential for vascular patterning in extraembryonic tissues.** (A) Schematic showing
155 strategy for generation of floxed allele of *rudhira* at exon 6. White rectangles indicate exons of the
156 *rudhira* locus; black and white triangles indicate *loxP* and *frt* sequences respectively. 5'P and 3'P
157 represent the probes that were used for Southern blot analyses. Arrows depict the positions of primers
158 for routine genotyping of mice and embryos. (B) Genomic DNA PCR analyses showing genotype of
159 control (+/+), heterozygous knock-out (+/-) and homozygous knock-out (-/-) embryos. (C) RT-PCR
160 analysis showing *rudhira* mRNA expression in control (+/+) and homozygous knock-out (-/-) embryos.
161 *GAPDH* RNA was used as a loading control. (D-H) Yolk sac morphology of control and *rudh*^{-/-}
162 (*rudhfl/fl;CMVCre*+) at E9.5 in whole mount unstained (D), immunostained for PECAM/CD31 (E) and
163 sectioned and stained with hematoxylin-eosin (F). en: endoderm, m: mesoderm. (G, H) Yolk sac
164 vasculature marked by PECAM staining in control and *rudh*^{CKO} (*rudhfl/fl;TekCre*+) at E10.5 and E11.5. (I-
165 K) Graphs showing quantitation of number of secondary vessels, branch points and lumen size in control
166 and *rudh*^{-/-} (*rudhfl/fl;CMVCre*+) at E9.5 and control and *rudh*^{CKO} (*rudhfl/fl;TekCre*+) at E10.5 and E11.5.

167
168 Placental circulation is vital for nourishment and development of the embryo. Improper development of
169 the labyrinth, the feto-maternal interface, results in poor fetal invasion and causes growth retardation
170 (25). Morphological analyses showed that *rudhira* null embryos have a smaller placenta (S2A, S2B and
171 S2M Fig.) and with abnormal histology (S2A and S2B Fig.) as compared to controls of the same stage.
172 Control placenta showed a distinct chorionic plate, labyrinth, spongiotrophoblast and decidual layers.
173 *Rudh*^{-/-} placenta lacked stratified layers with a greatly reduced labyrinth and chorionic plate composed
174 mostly of trophoblast giant cells. Fetal blood vessels could not invade into the placenta of *rudh*^{-/-} and
175 contained fewer Ly76+ fetal erythrocytes as compared to controls where maternal (arrows) and fetal
176 (arrowheads) blood cell pockets co-existed (S2C and S2D Fig.). Upon endothelial deletion of *rudhira* with
177 *Tie2-Cre* (*rudh*^{CKO}), placental thickness was reduced in mutant embryos at both E10.5 and E11.5 (S2E,

178 S2F, S2I, S2J and S2M Fig.). Fetal vessel invasion was comparable to control at E10.5 (S2G and S2H Fig.)
179 and was found to be affected only at E11.5 (S2K and S2L Fig.) although not as severely as in the global
180 knock-out. This could explain the absence of significant growth retardation in conditional mutant
181 embryos.

182 Taken together, these findings suggest that *rudhira* is essential for fetal vessel invasion into the
183 developing placenta. Further, growth retardation in *rudh*^{-/-} embryos is likely a result of defective
184 placental circulation.

185

186 **Rudhira plays a key role in cardiovascular development and tissue patterning**

187 Since *rudh*^{-/-} embryos survived to E9.5 and *rudh*^{CKO} survived to E11.5, we analyzed both genotypes for
188 embryonic development and vascular patterning (Fig. 2A). Whole mount immunostaining of *rudh*^{-/-}
189 embryos with anti-PECAM1 antibodies showed striking defects in the morphology and vasculature of the
190 head, heart and intersomitic vessels (ISVs) (S3 Fig.). While control embryos had a well formed vascular
191 network comprising major vessels giving rise to intricate secondary and tertiary branches (S3 Fig.
192 arrows), *rudhira* mutants showed completely disorganized head vasculature with defective vessel
193 sprouting, reduced capillaries and impaired branching of intersomitic vessels (ISV) that failed to sprout
194 into fine capillaries (S3 Fig. arrowheads). Histological analysis as well as immunostaining for
195 cardiovascular markers showed that both *rudh*^{-/-} and *rudh*^{CKO} embryos had collapsed, smaller heart
196 chambers, reduced endocardium development and a fused atrio-ventricular canal. Dorsal aorta was
197 discontinuous with a pronounced decrease in the lumen and intersomitic vessels were improperly
198 patterned (Fig. 2B-D). The endothelial lining was disorganized in all tissues and ECs seemed to have
199 impaired or random migration and were unable to form organized vessels. Similar phenotypes were
200 observed in *rudh*^{CKO} at E10.5 and E11.5 (Fig. 2B-D).

201 **Fig. 2. Absence of Rudhira leads to cardiovascular defects.** Control and *rudh*^{-/-} embryos at E9.5 or
202 control and *rudh*^{CKO} at E10.5 and E11.5 were analyzed as indicated. (A) Unstained embryos, (B, C)
203 Histological analysis showing comparison of heart and dorsal aorta (DA). (D) Immunostaining analysis of
204 heart, dorsal aorta (DA) and intersomitic vessels (ISV) using myocardial marker SMA, primitive erythroid
205 marker Ly76 and vascular markers PECAM and Flk1. Arrows indicate the normal vascular patterning and
206 arrowheads point to irregular and discontinuous vasculature. TS: Transverse section, LS: Lateral section,
207 V: Ventricle, At: Atrium. Nuclei are marked by DAPI (Blue). Black or white dotted lines mark the
208 boundary of DA. (E) Graph shows quantitation of dorsal aorta width in the thoracic region. Results
209 shown are a representative of at least three independent experiments with at least three biological
210 replicates. Scale bar: (A) 500 μm; (B, C) 100 μm; (D) E9.5 heart: 100 μm, E10.5 and E11.5 heart: 200 μm,
211 E9.5 DA: 20 μm, E10.5 and E11.5 DA: 50 μm, ISV: 50 μm.

212

213 To test whether *rudhira* null ECs also show slow and random migration, we cultured yolk sac ECs and
214 tested them in a wounding assay (Fig. 3A). *Rudhira* null ECs also showed reduced migration rate and
215 decrease in directed migration (Fig. 3B and 3C). Taken together, our results demonstrate a key role for
216 Rudhira in directed cell migration, essential for vascular remodeling during developmental angiogenesis.

217

218 **Fig. 3. Rudhira depletion causes impaired cell migration and promotes yolk sac transcriptome**
219 **expression.** (A-C) Migration tracks (A) of control and *rudh*^{-/-} yolk sac endothelial cells subjected to
220 wounding assay. Quantification of the rate of migration (B) and directionality (C) compared between
221 control and *rudh*^{-/-} yolk sac endothelial cells. At least 30 cells were analysed per genotype. (D)
222 Representation of differentially expressed genes in *rudh*^{-/-} by volcano plot. (E-H) Transcriptome analysis.
223 (E) Venn diagram showing number of genes (unique probe sets) dysregulated in embryo and yolk sac

224 upon *rudhira* deletion. (F) Unsupervised hierarchical clustering of differentially expressed gene changes
225 in *rudh*^{-/-} embryo (Emb) and yolk sac (YS) at E9.5 compared to controls. Each row represents a gene.
226 Expression intensities are displayed from green (low expression) to red (high expression). Lines on the
227 left represent the similarity between genes with the most similar expression profiles clustered together
228 with the shortest branches and represented in the dendrogram to illustrate their relationship. (G)
229 Unsupervised hierarchical clustering of differentially expressed gene changes in *rudh*^{-/-} yolk sac at E9.5
230 compared to controls. Each row represents a gene, and column represents the tissue. (H) Histogram
231 showing significantly enriched Gene Ontology (GO) and Pathways ($p < 0.05$) harboring differentially
232 expressed genes in the embryo and yolk sac upon *rudhira* deletion. (I) Model depicting *rudhira/BCAS3*
233 gene regulatory pathway. Cytoscape V 8.0 was used to visualize the network. Results shown are a
234 representative of two biological and two technical replicates for microarray and at least three
235 independent experiments with at least three biological replicates for other experiments. Statistical
236 analysis was carried out using one-way ANOVA. Error bars (in B and C) indicate mean \pm SD. *** $p < 0.001$.

237

238 **Rudhira regulates expression of the angiogenesis network**

239 Rudhira regulates actin dynamics which is known to affect nuclear transcription. We performed whole
240 transcriptome-based analysis of gene expression in *rudhira* knockout yolk sac and embryos at E9.5 to
241 determine its effect on vascular remodeling. While Rudhira expression is primarily in the endothelium,
242 our studies *in vitro* ((23) and this report) indicated that changes in Rudhira expression and localization
243 are rapid and depend on inter-cellular and cell-ECM interactions. Hence we chose to analyze intact
244 tissue with minimal manipulation to understand how Rudhira affects the transcriptome. While embryos
245 at E9.5 have a diverse set of derivatives of all three germ layers, yolk sacs are primarily made of
246 primitive endoderm and mesoderm, the latter comprising mainly endothelial and hematopoietic
247 lineages. Hence we separately analyzed *rudh*^{-/-} embryos and yolk sacs to avoid confounding effects of

248 possible mosaic deletion of *rudhira* in the *Tie2-Cre* conditional knockout. We then extensively validated
249 the data by quantitative PCR-based expression analysis of yolk sac RNA and endothelial cell line RNA.

250 Volcano plot based method was used to visualize the transcripts that are two-fold differentially
251 expressed in yolk sac (Fig. 3D). 3291 unique probes showed 2-fold or greater statistically significant
252 changes in gene expression (S2 Table). Of these 546 were downregulated and 334 upregulated in
253 embryo and 566 downregulated and 1960 upregulated in yolk sac (Fig. 3E). 29 downregulated genes and
254 43 upregulated ones were common between embryo and yolk sac (Fig. 3E and S3 Table). Unsupervised
255 hierarchical cluster analysis showed that genes with similar expression patterns were clustered together
256 with branch distance proportional to their similarity in expression pattern. A distinct sub set showed
257 reciprocal expression between embryo and yolk sac (Fig. 3F). Interestingly the majority of clustered
258 genes were mainly upregulated in the yolk sac (Fig. 3F and 3G), while the embryo had a more balanced
259 distribution in each cluster (Fig. 3F). To define how changes in gene expression caused by *rudhira*
260 depletion may influence vascular development and remodeling, we functionally annotated the data
261 using DAVID (Database for Annotation, Visualization and Integrated Discovery) and found that genes
262 linked to many biological pathways were enriched. Key deregulated biological categories were identified
263 (Fig. 3H).

264 Analysis of the entire data set showed greater variation between duplicates of embryo than yolk sac,
265 possibly because of heterogeneity in the embryonic tissue. Hence for further analysis we focused on
266 yolk sac data as it is also the primary site of vascular remodeling and shows early *Rudhira* expression.
267 Significant expression changes were seen in genes that relate to a range of processes or pathways,
268 which could impact on vascular development and remodeling (Fig. 3H and S4 Fig. and S5 Table). Gene
269 ontology analysis of the common genes identified principal biological processes affected by the loss of
270 *rudhira* with a Z score above 2.5. Amongst signaling pathways, negative regulation of the transforming
271 growth factor beta (TGF β) signaling pathway was identified as significantly perturbed. Other pathways

272 implicated in vascular development such as Wnt, JAK/STAT and Notch signaling showed changed levels
273 of a few genes but expression of the majority of the pathway components was unaffected. Important
274 regulators of cellular processes such as angiogenesis/blood vessel remodeling, extracellular matrix,
275 regulation of proteolysis, negative regulation of peptidase activity and cell projection organization were
276 identified. Further, key molecular families involved in cytoskeletal remodeling, cell adhesion, cell
277 migration and TGF β and VEGF pathways (all important during angiogenesis) were connected by Rudhira/
278 BCAS3 allowing us to identify the Rudhira network in angiogenesis (Fig. 3I and S6 Table).

279

280 **Identification of regulatory networks and nodes regulated by Rudhira**

281 A total of 140 genes from cluster analysis were enriched in the key gene ontology (GO) and pathways
282 identified (S5 Table) with a significance criterion of $p < 0.05$. Further, we were able to associate GOs and
283 pathways known to co-operate during vascular development and remodeling namely adhesion,
284 angiogenesis, cytoskeleton, ECM organisation, peptidase activity and TGF β signaling (Fig. 3I). Genes
285 differentially expressed in these six processes were subjected to unsupervised hierarchical clustering to
286 identify molecular signatures (Fig. 4A, 5A and S4 Fig.). An interaction network of significant GO terms
287 was assembled into a GO map to depict the relationship among prominent functional categories (Fig. 4B,
288 5B and S4 Fig.). Subsequent verification of expression data was carried out for key genes known to
289 mediate these processes (Fig. 4C, 5C, 5D and S4 Fig.). 51 out of the 3407 genes that showed significant
290 variation from control in the knockout yolk sac were validated by qPCR on cDNA generated from *rudhira*
291 knockdown and non-silencing control endothelial cell line RNA (Fig. 4C, 5C, 5D and S4 Fig.) and 70% of
292 these (36/51) agreed with array data. Changes in expression level of selected candidates were further
293 validated using cDNA generated from fresh E9.5 yolk sac RNA (S4F Fig.).

294 **Fig. 4. Rudhira depletion affects extra cellular matrix (ECM) organization around blood vessels.** (A)
295 Unsupervised hierarchical clustering and (B) regulatory network of the extracellular matrix (ECM)
296 organization biological process showing genes significantly deregulated in *rudh*^{-/-} yolk sac compared to
297 control. Edge weighted spring embedded layout was used to visualize the network (see methods). (C)
298 Graph shows relative transcript levels of selected *MMP* genes from the ECM network that were
299 validated by qRT PCR on wild type (WT), non-silencing control (NS) and *rudhira* knockdown (KD) lines
300 (SVEC). (D-G) Yolk sac sections from control and *rudh*^{CKO} at E10.5 showing (D) live *in situ* gelatin
301 zymography (FITC, green), (E-G) co-expression of vascular marker PECAM with (E) CollagenIV (ColIV), (F)
302 Laminin, (G) Fibronectin (Fn). Nuclei are marked by DAPI (Blue). Error bars indicate standard error of
303 mean (SEM). Results shown are a representative of at least three independent experiments with at least
304 three biological replicates. Statistical analysis was carried out using one-way ANOVA. Scale bar: (D-G) 20
305 μm (see also S5 Fig.). ** $p < 0.01$, *** $p < 0.001$.

306

307 **Endothelial Rudhira regulates extracellular matrix (ECM) organization**

308 Yolk sac transcriptome analysis revealed several processes important for angiogenesis that were
309 perturbed upon *rudhira* depletion. Extracellular matrix was an over-represented functional category, in
310 which genes for matrix components and ECM proteases are aberrantly expressed (Fig. 4A and 4B).
311 Controlled ECM remodeling is important for cell migration. Mutations that affect the dynamic regulation
312 and crosstalk between the cell and ECM are also known to affect tissue patterning and homeostasis (26).
313 *MMP10*, *MMP21* and *MMP25* were aberrantly expressed while *TIMPs* (Tissue inhibitors of
314 metalloproteases) were not significantly changed in *rudh*^{-/-} yolk sacs. Expression of a large number of the
315 serine protease inhibitors was aberrant with eight members downregulated and eleven upregulated
316 (Fig. 4B and S2 Table). Interestingly several members of the extracellular *Serpina* clade were
317 downregulated (6/8) whereas the intracellular *Serpinb* clade was upregulated (8/9). Serpinb clade

318 members inhibit Granzyme (Gzm) activity (27). In the *rudh*^{-/-} yolk sac *Gzmd* and *Gzmg* transcripts were
319 upregulated suggesting a loss of balance between production of proteases and protease inhibitors. A
320 disintegrin and metallopeptidase domain (Adam) class of endopeptidases namely *Adam11*, *Adam24*,
321 *Adam32*, *Adam7*, *Adamts12*, and *Adamts15* were all significantly upregulated in *rudh*^{-/-} yolk sacs. This
322 suggested that matrix organization or degradation may be affected.

323 ECM degradation is an essential step in angiogenesis and vascular patterning (28, 29). Collagen type IV is
324 a major component of the vascular basement membrane. Of 11 collagen family members affected by
325 loss of *rudhira*, 7 were downregulated (Fig. 4B). *In situ* zymography on live *rudh*^{CKO} yolk sac and embryo
326 sections at E10.5 using dye quenched (DQ) gelatin showed absence of or significant decrease in signal
327 around vessels indicating reduced matrix degradation as compared to controls (Fig. 4D and S5A Fig.).
328 Sections were stained for collagen and laminin post zymography (S5A and S5D Fig.). Both *rudh*^{CKO} yolk
329 sacs and embryos showed highly disorganized collagen around the vessels (Fig. 4E and S5B Fig.). Further,
330 *Rudhira* expression in vessels of control embryos overlapped with collagen staining (S5B and S5C Fig.).
331 Co-staining of fixed sections of yolk sac and embryos with endothelial marker PECAM and ECM markers
332 showed that the organization of the matrix components collagen, fibronectin and laminin was disrupted
333 (Fig. 4E-G, S5 Fig.). These results indicate that *Rudhira* is essential for maintaining proper ECM
334 organisation. TGF β signaling plays a major role in regulating genes involved in ECM deposition and
335 degradation. As levels of several transcripts of the TGF β pathway were altered in *rudhira* mutant yolk
336 sac (Fig 5A, 5B and 5H) we analysed this pathway further.

337

338 **Regulation of TGF β signaling by *Rudhira***

339 TGF β pathway was one of the major over-represented categories as levels of several of the TGF β
340 pathway molecules were altered in *rudhira* mutant yolk sac (Fig 5A, 5B and 5I). The TGF β pathway is

341 essential for vascular patterning and angiogenesis. Deletion of TGF β 1 leads to delayed wound healing
342 (30). Ablation of TGF β RI or TGF β RII results in embryonic lethality with severe patterning defects (31, 32).
343 A significant decrease in expression of *TGFb2*, *TGFb3* and *TGFbr2* was seen in the microarray analysis
344 and subsequent validation by qRT-PCR (Fig 5A-D and Fig 5I). TGF β antagonists like *Smurf1*, *Smurf2* and
345 *Smad7* were upregulated in *rudhira* knockdown ECs (Fig 5D). TGF β is produced by the yolk sac ECs and is
346 also available as a paracrine signal from the yolk sac endoderm and embryonic tissue (18). This activates
347 the SMAD2 effector which leads to the differentiation of vascular smooth muscles cells and promotes
348 their association with endothelial cells, to establish a properly patterned vasculature. Reduced pSMAD2
349 levels in *Smad2/3* and *endoglin* mutants co-relate with an unpatterned vasculature and mid-gestation
350 lethality (18, 33). To assess pSMAD2 levels in the absence of Rudhira, we stained control and *rudh*^{CKO}
351 yolk sacs for pSMAD2. Mutant yolk sacs had fewer pSMAD2 positive endothelial cells (Fig. 5E, arrows).
352 Upon induction of live yolk sacs with exogenous TGF β , control yolk sacs showed increased pSMAD2,
353 whereas *rudh*^{CKO} yolk sacs continued to show low pSMAD2 signal in the endothelial cells (Fig. 5E,
354 arrowheads). Similarly, *rudhira* knockout embryo-derived endothelial cells or *rudhira* knockdown
355 endothelial cell line (SVEC KD) treated with TGF β also failed to activate pSMAD2 as compared to controls
356 (Fig. 5F and Fig. 5G respectively, and S6A Fig.). This suggests that Rudhira is essential for TGF β pathway
357 activation via pSMAD2.

358

359 **Fig. 5. Rudhira depletion deregulates TGF β signaling machinery essential for angiogenesis.** (A, B)

360 Unsupervised hierarchical clustering computed with Pearson Uncentered algorithm with average linkage
361 rule and regulatory networks of differentially expressed genes and biological processes significantly
362 deregulated in *rudh*^{-/-} yolk sac compared to control. Network maker program (Bionivid Technology Pvt
363 Ltd) was used to identify the nodes and edges that form the regulatory circuit and Cytoscape V 8.0 was

364 used to visualize the network. Edge weighted spring embedded layout was used. (C-D) Graphs show
365 representative positive regulators (C) and negative regulators (D) of the TGF β pathway that were
366 validated by qRT PCR on wild type (WT), non-silencing control (NS) and *rudhira* knockdown (KD)
367 endothelial cell (EC) lines (SVEC). (E-F) Untreated and TGF β treated control and *rudh*^{CKO}
368 (*rudhfl/fl;TekCre+*) yolk sacs (E) or yolk sac derived cells (F) were analyzed for the expression of
369 phosphorylated SMAD2 (pSMAD2) by immunostaining. Samples were co-stained with endothelial
370 markers PECAM (E) or VCAM (F). Graphs show mean fluorescence intensity for pSMAD2. n = 3 yolk sacs.
371 (G) Untreated and TGF β treated non-silencing control (NS) or *rudhira* knockdown (KD) SVEC lines were
372 analyzed for expression of TGF β pathway molecules by immunoblot of cell lysates. Graph shows
373 pSMAD2/SMAD2 ratio. (H) Graphs showing relative migration of control (NS) and *rudhira* knockdown
374 (KD) SVEC lines plated on collagen or gelatin- coated dishes and induced with TGF β . Error bars indicate
375 standard error of mean (SEM). Results shown are a representative of at least three independent
376 experiments with at least three biological replicates. Statistical analysis was carried out using one-way
377 ANOVA. Scale bar: (E) 20 μ m *p<0.05, **p<0.01, ***p<0.001. (I) KEGG TGF β Pathway. Genes in the TGF β
378 pathway from KEGG database mapped based on fold change in *rudh*^{-/-} yolk sac in comparison to control
379 to understand pathway regulation. Green: downregulated, Red: upregulated. # indicates negative
380 regulators and antagonists of TGF β pathway. Blue circles indicate genes whose expression level was
381 validated in this study or processes that were found to be affected.

382

383 **Rudhira functions downstream of TGF β receptor activation**

384 To test whether provision of TGF β could rescue the cell migration defect seen upon *rudhira* depletion,
385 we cultured SVEC knockdown cells in the presence of exogenous TGF β . Provision of exogenous TGF β in a
386 cell monolayer wounding assay did not rescue the migratory defects of *rudhira*-depleted endothelial

387 cells migrating on gelatin (Fig. 5H). This suggested that the TGF β pathway requires Rudhira for activating
388 processes that promote cell migration. Since loss of *rudhira* results in an inability to activate TGF β -
389 mediated Smad2 signaling, this could lead to an inability to migrate, resulting in angiogenic defects.
390 Further, *rudhira* depletion cell autonomously affects TGF β -dependent migration. Alternatively, since
391 Rudhira affects multiple signaling pathways, TGF β alone may not be sufficient to rescue the effect of
392 *rudhira* deletion.

393

394 **Rudhira is required for microtubule stability**

395 Microtubules (MT) bind to TGF β pathway effectors Smad2 and Smad3 thereby preventing their
396 phosphorylation and activation (19). Binding of Smads to MT is independent of TGF β stimulation. TGF β
397 triggers dissociation of the Smad complex from MT and increases Smad2 phosphorylation and nuclear
398 translocation. Our earlier work (23) showed that Rudhira interacts with MT, which prompted us to
399 investigate whether Rudhira is required for MT-regulated TGF β signaling. Rudhira depletion led to
400 reduced alpha-tubulin as well as acetylated tubulin levels in SVEC endothelial cell line as well as *rudhira*
401 null primary ECs (Fig. 6A,B). MTs containing acetylated and detyrosinated tubulin are known to re-
402 localize towards the leading edge of a migrating cell (34). In a monolayer wounding assay Rudhira-
403 depleted cells showed reduced acetylated tubulin staining, which was not localized towards the leading
404 edge (Fig. 6C). Further, upon TGF β stimulation, wild type ECs (SVEC) showed increased co-localization of
405 Rudhira with MT, suggesting increased binding (Fig. 6D). This suggests a role for Rudhira in regulating
406 MT architecture and MT- regulated TGF β signaling.

407

408 **Rudhira augments TGF β pathway activation**

409 To test whether Rudhira alone is sufficient to activate the TGF β pathway, we transfected endothelial
410 cells (SVEC) with a Rudhira overexpression construct (*Rudh2AGFP*) and analyzed the status of TGF β
411 pathway activation. Upon ligand binding to the TGF β receptor, Smad2/3 gets activated and translocates
412 to the nucleus (35, 36). Rudhira overexpression alone was not sufficient to activate TGF β signaling and
413 cause Smad2/3 nuclear localization (Fig. 6E). However, upon addition of TGF β 1 to the culture, Rudhira
414 overexpressing cells showed markedly increased pathway activation as seen by increased nuclear
415 Smad2/3 in *Rud2AGFP* transfected cells compared to un-transfected or vector control cells. Further,
416 Rudhira overexpression is unable to overcome the receptor-level inhibition of TGF β pathway using the
417 small molecule inhibitor SB431542 (SB) that binds to the Alk5 co-receptor, thereby blocking pathway
418 activation (Fig. 6E). This confirms that Rudhira can augment pathway activity but not substitute for TGF β
419 receptor activation. It also indicates that Rudhira functions downstream of the TGF β receptor. This is in
420 agreement with the cytoskeletal localization of Rudhira.

421

422 **Fig. 6. Rudhira stabilizes microtubules and augments TGF β pathway activation.** Non-silencing control
423 (NS) or *rudhira* knockdown (KD) ECs were analysed for microtubule organization and stability. (A-C)
424 Expression of α -Tubulin and acetylated-Tubulin (Ac-Tubulin) by immunoblot of cell lysates (A) and
425 localisation of acetylated-tubulin by immunostaining of fixed yolk sac cells (B) and SVEC NS and KD cells
426 (C). (D) Colocalisation analysis of Rudhira and microtubules on TGF β induction. Graph shows
427 quantitation of Rudhira and MT colocalisation from at least 20 cells. (E) SVEC were transfected with
428 plasmid constructs for expression of GFP or Rudh2AGFP, and treated with either TGF β or TGF β +
429 SB431542 (TGF β inhibitor) and analysed for SMAD2 by immunostaining. Graph shows quantitation of
430 SMAD2/3 nuclear fluorescence intensity. Compare arrows (transfected cell) and arrowheads
431 (untransfected cell). (F) qRT PCR analysis of *smurf2* transcript levels in wild type (WT), GFP or

432 Rudhira2AGFP expressing endothelial cells (SVEC). Error bars indicate standard error of mean (SEM).
433 Results shown are a representative of at least three independent experiments with at least three
434 biological replicates taken into account. Statistical analysis was carried out using one-way ANOVA. Scale
435 bar: (B-E) 20 μm . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

436
437 Interestingly, Rudhira overexpression also led to a decrease in *smurf2* levels (Fig. 6F and S7C Fig.). This
438 indicates that Rudhira augments TGF β pathway activation by increasing SMAD2/3 activation, possibly
439 due to a reduction in inhibitor levels. To test whether the converse is true, i.e. whether TGF β affects
440 Rudhira expression, we treated wild type SVEC cultures with TGF β 1. There was a significant increase in
441 Rudhira transcript and protein levels upon TGF β induction, as detected by qPCR and
442 immunofluorescence staining (S7A, B Fig.). This indicates that Rudhira and the TGF β pathway are
443 mutually dependent for their expression and function.

444

445 **Rudhira inhibits Smurf- mediated TGF β pathway attenuation**

446 Our analysis indicated that Rudhira is not an inducer but a promoter of the TGF β signaling pathway in
447 endothelial cells that acts downstream of receptor-level activation but upstream to SMAD activation.
448 KEGG pathway analysis of the yolk sac transcriptome showed that a majority of the genes involved in
449 TGF β signaling were deregulated upon *rudhira* loss. Especially, several antagonists (*noggin*, *folliculin*,
450 *bambi*) and negative regulators (*sara*, *smurf1/2*, *smad6/7*) of the pathway were upregulated (Fig. 5I).

451

452 **Fig. 7. Rudhira inhibits Smurf- mediated inhibition of TGF β pathway.** (A) qRT PCR analysis of negative
453 regulators of TGF β pathway in non-silencing control (NS) and *rudhira* knockdown (KD) endothelial cell
454 (EC) lines (SVEC) upon TGF β treatment. (B) Untreated and TGF β treated non-silencing control (NS) or

455 *rudhira* knockdown (KD) SVEC lines were analysed for Smad2/3 ubiquitination by immunoprecipitation
456 (IP) of Smad2/3 and immunoblotting for ubiquitin. Cell extracts were resolved by SDS-PAGE and either
457 Coomassie- stained or immunoblotted for indicated proteins. (C) *in situ* Proximity ligation assay (PLA) for
458 Smad2/3 and Ubiquitin on control and *rudh*^{-/-} yolk sac cultured cells with or without TGFβ treatment.
459 PLA dots represent ubiquitinated Smad2/3. Graph shows the quantitation of PLA dots per cell. (D)
460 Schematic representation of the role of Rudhira in vascular patterning. The status of TGFβ signaling and
461 microtubules in the presence (wild type) or absence (Rudhira mutant) of Rudhira is shown. Boxed insets
462 show blood vessel (red) branching and ECM (blue lining) in wild type and *rudhira* mutant yolk sacs. Error
463 bars (A-C) indicate standard error of mean (SEM). Results shown are a representative of at least two
464 independent experiments with at least two biological replicates. Statistical analysis was carried out using
465 one-way ANOVA. *p<0.05, **p<0.01, ***p<0.001.

466
467 The TGFβ pathway is regulated at multiple steps by a complex combination of activators, inducers and
468 inhibitors (37). Ubiquitin- mediated proteasomal degradation is essential for fine regulation of TGFβ
469 pathway activity in the basal as well as activated states. The E3 ubiquitin ligases for SMADs, namely
470 Smurf1 and Smurf2 (SMAD specific E3 Ubiquitin ligases) are important regulators of the pathway. They
471 control SMAD2/3 levels by ubiquitination and targeting for proteasomal degradation (38, 39). These
472 inhibitors are also targets of TGFβ pathway activation.

473 Since Rudhira depletion results in increased *Smurf1/2* and inhibitory Smad (*Smad6* and *Smad7*) levels,
474 we checked the effect of exogenous TGFβ addition on these inhibitors. While control cells show a sharp
475 decrease in the levels of these inhibitors upon addition of TGFβ, Rudhira- depleted cells showed no
476 reduction in their levels, suggesting a loss of control on the negative regulators of TGFβ signaling (Fig.
477 7A). To test whether increased inhibitor transcript levels co-relate with function, we assayed for levels of
478 ubiquitinated-SMAD2/3 by immuno-pulldown of SMAD2/3 followed by immunoblotting for Ubiquitin.

479 Rudhira depletion indeed results in increased SMAD2/3 ubiquitination, which is markedly increased on
480 TGF β - mediated activation of the pathway (Fig. 7B). To further check the status of SMAD2/3
481 ubiquitination *in vivo*, we performed a Proximity Ligation Assay (PLA) for SMAD2/3 and Ubiquitin in
482 primary endothelial cell cultures derived from E9.5 control and *rudh*^{-/-} yolk sacs. PLA is a robust and
483 sensitive assay to identify post-translational modifications in proteins specifically and at a single
484 molecule resolution (40). Rudhira knockout cells showed significantly increased SMAD2/3 ubiquitination
485 *in vivo* (marked by an increase in the number of PLA dots), both in the presence or absence of
486 exogenous TGF β (Fig. 7C). This indicates that Rudhira promotes TGF β pathway activation by negatively
487 regulating inhibitors and thereby checking SMAD2/3 ubiquitination.

488

489 Discussion

490 A balance of signal sensing and response is essential to maintain homeostasis and depends on the
491 nature of the ECM and the responsiveness of the cytoskeleton, two very important parameters in
492 determining cell phenotype during development and disease (29, 41, 42). The cytoskeletal response to
493 signals would determine whether a cell can change its shape, divide or adhere. The cytoskeleton affects
494 nuclear stability, which impinges on chromosome scaffolds and ultimately on gene expression (43). The
495 generic role of the cytoskeleton in cell phenotype is acknowledged and even obvious, but there are only
496 a few examples linking its organization to primary determinants of cell fate, as few tissue-specific
497 cytoskeletal components are known. Context-specific cellular responses are likely to depend on the
498 presence of such components, which use ubiquitous elements to shape context-dependent outputs. We
499 therefore chose to analyse the function of Rudhira, a cytoskeletal protein expressed predominantly in
500 the vasculature.

501

502 We report here that global or endothelial-specific deletion of *rudhira* resulted in mid-gestation lethality
503 with severe defects in cardiovascular patterning and tissue morphogenesis. Based on a transcriptome
504 analysis we identified key steps in blood vessel remodeling such as cell adhesion, migration and
505 modulation of extracellular matrix components that are regulated by the action of Rudhira. We show
506 the essential function of Rudhira in directing endothelial cell migration during development and
507 describe, for the first time, a regulatory network mediated by Rudhira with reference to its interacting
508 partners at both binary (regulatory) as well as physical levels (Fig. 3I). We propose a model by which
509 Rudhira/BCAS3, an endothelial cell cytoskeletal component, can regulate microtubule-mediated TGF β
510 signaling (Fig. 7D).

511

512 In ECs TGF β signaling modulates the transcriptome in a manner that would promote EC migration during
513 angiogenesis (17). Also, in the yolk sac, paracrine TGF β - signaling regulates gene expression and ECM
514 production, deposition and remodeling, required for assembly of robust vessels (18). However, the
515 absence of Rudhira hampers TGF β signaling, negatively affecting endothelial cell migration. Expectedly,
516 *rudhira* null ECs show a disorganized ECM. Matrix degradation is known to support angiogenesis in
517 multiple ways such as by physical removal of barriers to migration, release of sequestered growth
518 factors or by exposing cryptic protein sequences that function in angiogenesis (28, 44). MMP activity is
519 essential for releasing TGF β for growth factor signaling, which promotes cytoskeletal remodeling seen in
520 mesenchymal migration. In the absence of Rudhira at least some of these angiogenesis promoting
521 events do not occur, resulting in a disorganized matrix, possibly not permissible for directional
522 migration. Though gelatin degradation is reduced upon *rudhira* depletion, expression of the cognate
523 MMPs (2, 9 etc.) is not altered. We see significant increase in *MMP10*, *MMP21*, *MMP25* and reduced
524 level of *MMP28* transcripts suggesting that this should cause increased degradation. However, MMPs

525 are also regulated by TGF β signaling. The reduced matrix degradation seen in *rudhira* CKO yolk sacs is in
526 concordance with reduced TGF β signaling.

527

528 Cardiac defects are seen from early development in *rudhira* mutants, suggesting impaired circulation.
529 From E8.5 in the mouse yolk sac, blood flow dictates vessel fusion and directional cell migration
530 resulting in vascular remodeling (45). Hence cardiac defects seen in *rudhira* mutants could also
531 contribute to the vascular remodeling abnormalities and aberrant TGF β signaling.

532

533 Rudhira overexpression in endothelial cells promotes TGF β pathway activation. Further, addition of
534 TGF β induces Rudhira expression. This, in addition to other observations, indicates a positive feedback
535 of Rudhira on TGF β signaling and *vice versa*. In the absence of Rudhira this feedback is lost and it affects
536 expression of TGF β targets and the endothelial cell transcriptome. The upstream sequence of *rudhira*
537 bears binding sites for the SMAD family of transcription factors (Smad3, Smad4), Twist subfamily of class
538 B bHLH transcription factors, Pax (Paired box factors), Brachyury and ELK1 (member of ETS oncogene
539 family). Further, our analysis shows that Rudhira functions after receptor activation but before or at the
540 level of SMAD2/3 activation. This suggests that Rudhira may function to regulate transcription in
541 response to TGF β activation, possibly by interaction with other cytoskeletal and signaling components.

542

543 Rudhira localization is cytoskeletal and is dynamic in migrating cells that change shape and adhesion
544 properties. Microtubules regulate TGF β signaling by binding to and preventing activation of SMADs (19).
545 Thus our analysis provides a platform for testing cell type-specific cross talk between TGF β signaling and
546 the dynamic cytoskeleton in normal development as well as pathological situations such as tumor

547 metastasis. An increase in MT and Rudhira co-localization upon TGF β stimulation suggests that Rudhira
548 might serve to sequester microtubules thus inhibiting MT and Smad2/3 association and promoting
549 SMAD2/3 phosphorylation (Fig. 6D). While targeting the interaction of MT with Smads would allow
550 regulation of the TGF β pathway, the ubiquitous nature of these molecules is likely to result in
551 widespread and possibly undesirable effects of therapeutic intervention. Rudhira being restricted to the
552 vasculature could provide a suitable tissue-specific target to regulate pathological angiogenesis and
553 TGF β pathway activation.

554

555 The presence of cell adhesion assemblies is dependent on the underlying matrix and on whether the
556 surface is rigid as in 2D cell culture or embedded within a 3D ECM (46). Elucidating mechanisms by
557 which Rudhira governs cell migration will be crucial to understanding tumor cell invasion and metastasis.
558 Indeed the upregulation of Rudhira in metastatic tumors underscores its importance (47). Interestingly,
559 TGF β cross talks with several other signaling pathways and is a drug target for multiple diseases. Since
560 TGF β is also a key inducer of the epithelial/endothelial to mesenchymal transition, it is likely that the
561 cytoskeletal remodeling in these transitions is mediated by Rudhira. Further studies on the role of
562 Rudhira in normal and tumor EMT will be informative.

563

564 Given the large number of signals and their varying levels that EC encounter, it is unlikely that they
565 respond only to one or the same concentration all across the organism. A robust response over a range
566 of signals is likely mediated by molecules that can crosstalk with a wide variety of processes. The
567 cytoskeleton is ideally positioned for this role. EC respond to a variety of signals resulting in a limited
568 repertoire of cytoskeletal changes, which in turn determine cell phenotype. The presence or absence of
569 cell type-specific components such as Rudhira could provide active decisive control of EC behaviour in

570 response to a varying milieu of signals. Our analysis indicates that in endothelial cells Rudhira remodels
571 actin (23) and affects TGF- β mediated gene expression (this report), which in turn results in matrix
572 degradation, making the ECM permissive to cell migration. Thus our study opens up new avenues for
573 developing strategies to regulate the vascular pattern in development and disease. This could be
574 generally applicable to various cell and tissue types and identification of new components awaits further
575 investigation.

576

577 **Materials and Methods**

578

579 **Generation and validation of *rudhira* knockout mice**

580 All animals were maintained and experiments performed according to the guidelines of the animal
581 ethics committees. *Rudhira* floxed mice (Accession No. CDB0664K:
582 <http://www2.clst.riken.jp/arg/mutant%20mice%20list.html>) were generated as described, validated by
583 genotyping and crossed to *Cre* mice to generate knockout mice (see Supporting Information and Fig. 1
584 and S1 Fig.).

585

586 **RT-PCR and qRT-PCR**

587 RNA from E9.5 embryos was isolated using TRIzol reagent (Invitrogen). Reverse transcription was
588 performed using 2 µg of DNase treated RNA and Superscript II (Invitrogen, Carlsbad, CA) according to
589 manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was carried out using EvaGreen (BIO-RAD,
590 CA) in Biorad-CFX96 Thermal cycler (BIO-RAD, CA). Primers used are provided in S7 Table.

591

592 **Immunostaining, Immunohistochemistry, Microscopy and analysis**

593 Embryos were dissected at desired stages between E7.5 to E11.5, fixed in 4% paraformaldehyde and
594 processed for cryosectioning or immunostaining using standard procedures (48). Primary antibodies
595 used were against *Rudhira* (23), PECAM1, CD34, Flk1, Ly76 (BD Biosciences), Brachyury (Santa Cruz
596 Biotechnology), laminin, fibronectin, Smooth Muscle Actin, acetylated tubulin, α -tubulin (Sigma
597 Chemical Co. USA), SMAD2/3, pSMAD2 (Cell Signaling Technologies, USA). Secondary antibodies were
598 coupled to Alexa-Fluor 488 or Alexa-Fluor 568 or Alexa-Fluor 633 (Molecular Probes). Cryosections were

599 stained with haematoxylin and eosin using standard protocols. Images were acquired using a stereo
600 zoom (SZX12 Olympus) or inverted (IX70, Olympus) microscope, confocal microscopes (LSM 510 Meta
601 and LSM 700, Zeiss) and a motorized inverted microscope with fluorescence attachment (IX81,
602 Olympus). For details, see Supporting Information. Co-localization analysis was done using co-
603 localization plugin in ImageJ (NIH, USA).

604

605 **TGF β induction and analysis**

606 Live E10.5 and E11.5 CKO yolk sacs cut into two pieces or embryo-derived cells were washed in PBS and
607 induced with 0 ng/ml (uninduced) or 10 ng/ml of TGF β in DMEM for 2 h and then fixed and stained for
608 pSMAD2. At least two each of the control and CKO E10.5 yolk sacs were taken for analysis. Non-silencing
609 control (NS) or *rudhira* knockdown (KD) ECs were induced with 0 or 10 ng/ml of TGF β in DMEM for 2 h
610 and analysed by immunofluorescence or western blotting for SMAD2/3 and pSMAD2. Non-silencing
611 control (NS) or *rudhira* knockdown (KD) ECs were induced with 0 or 10 ng/ml of TGF β in DMEM for 48 h
612 and analysed by qPCR for indicated genes or immunofluorescence for SMAD2/3.

613

614 **Western blot analysis and immuno-precipitation**

615 50 μ g lysate from control or knockdown cell lines of SVEC was used for Western blot analysis by
616 standard protocols. Primary antibodies used were: SMAD2/3, pSMAD2 (Cell Signaling Technologies,
617 USA), GAPDH, acetylated tubulin, α -tubulin (Sigma Chemical Co., USA), Ubiquitin (Clone FK2, Biomol)
618 and BCAS3 (Bethyl Labs, USA). HRP conjugated secondary antibodies against appropriate species were
619 used and signal developed by using Clarity Western ECL substrate (Biorad, USA). Western blot intensities
620 were normalised to GAPDH and quantification was carried out using ImageJ. For immuno-precipitation

621 studies, Protein G sepharose beads coated with SMAD2/3 antibody were incubated with cell lysates for
622 4 hours, clarified by centrifugation and extensively washed. Equal volumes of sample were loaded and
623 resolved by SDS polyacrylamide gel and further taken for western blot analysis.

624

625 ***In situ* PLA reaction (Duolink assay)**

626 *In situ* PLA reaction was performed on yolk sac primary cells. The cells were cultured, fixed,
627 permeabilised and stained with primary antibodies for SMAD2/3 and Ubiquitin as mentioned earlier.
628 Thereafter, the protocol for PLA as recommended by manufacturer (Duolink, USA) was followed. Post
629 PLA, nuclei were counterstained with DAPI.

630

631 ***In situ* zymography**

632 Unfixed embryos or yolk sacs were overlaid with tissue freezing medium, snap frozen in liquid nitrogen,
633 cryosectioned at 10 μ m, collected onto slides and overlaid with zymography solution [20 μ g/ml DQ
634 gelatin (Life Technologies, USA) in 50mMTris-HCl, 150mMNaCl, 5mM CaCl₂]. The slides were incubated
635 at 37°C in the dark in a moist chamber for 15 min (yolk sac) or 2 h (embryos), then rinsed with ultrapure
636 water (Milli-Q, Millipore) and fixed with 4% paraformaldehyde. Multiple sections from at least four
637 embryos per genotype were analysed.

638

639 **Endothelial cell culture, adhesion and migration assays**

640 E9.5 embryos or yolk sacs were washed in phosphate buffered saline (PBS), minced and dissociated in
641 0.2% collagenase type IV (GIBCO/BRL) at 37°C for 5 minutes, washed in culture medium, pelleted,

642 resuspended in culture medium (DMEM, 20% fetal calf serum, 1X Glutamax, 1X antibiotics and 50 µg/ml
643 Endothelial Cell Growth Supplement (ECGS) (Sigma Chemical Co., USA) and plated onto 0.1% gelatin
644 coated dishes. Confluent monolayers were incubated with 5µg/ml Dil-Ac-LDL (Invitrogen) for 4 hours to
645 mark endothelial cells, then scratched and monitored for cell migration in real time by video microscopy
646 (see Supporting Information). For assessing effects on cell migration on different substrates, SVEC plated
647 on desired matrix components were allowed to adhere and form a monolayer for 24 h before wounding.
648 For assaying the effect of TGFβ on migration, 5ng/ml TGFβ was added to cultures 6 h before scratching
649 and continuously provided up to 12h post-wounding. Wound width at 0 h and 12 h post-wounding was
650 measured and quantified as described before (23).

651

652 **Microarray analysis**

653 Stage-matched E9.5 embryos of control and ubiquitous knockout littermates were used for microarray
654 analysis as detailed in Supporting Information.

655

656 **Quantification and Statistical analyses**

657 Quantification and statistical analysis are described in Supporting Information.

658

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664

665 **Author Contributions**

666 M.S.I. conceived of the project and directed the work. M.S.I., R.S., D.J., J.C.P., M.J., G.B., P.B. designed
667 and performed animal experiments, cell biology, and imaging. R.S., M.V., M.S.I., D.J. analyzed
668 transcriptome data. M.S.I. designed floxed mice and T.A., and H.K generated floxed mice. M.S.I., R.S.,
669 M.V., K.V.R. wrote the manuscript. All authors reviewed and made comments on the manuscript.

670

671 **Disclosures**

672 Madavan Vasudevan is Co-Founder & Director, Bionivid Technology Pvt Ltd.

673

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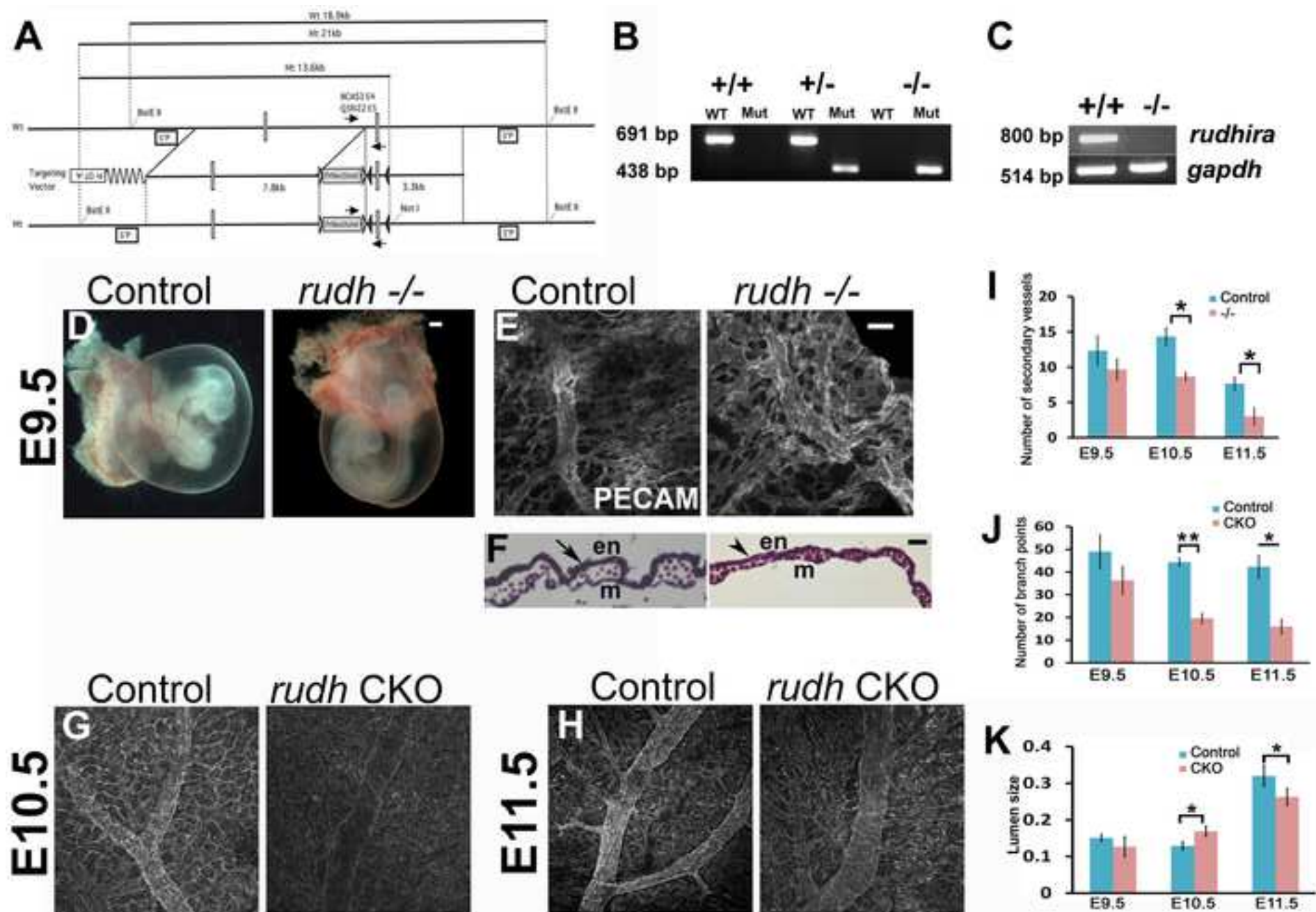


Figure 1

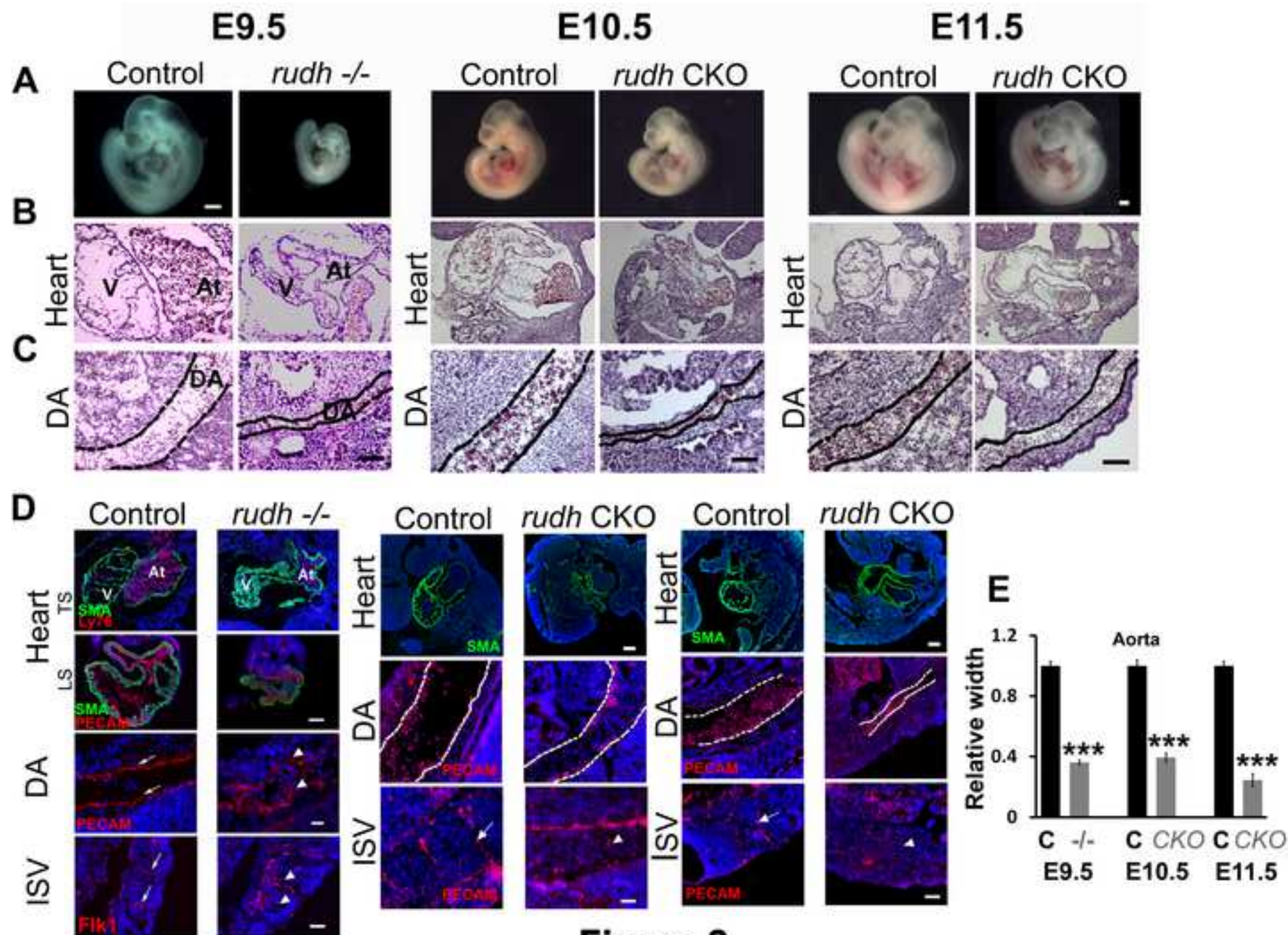


Figure 2

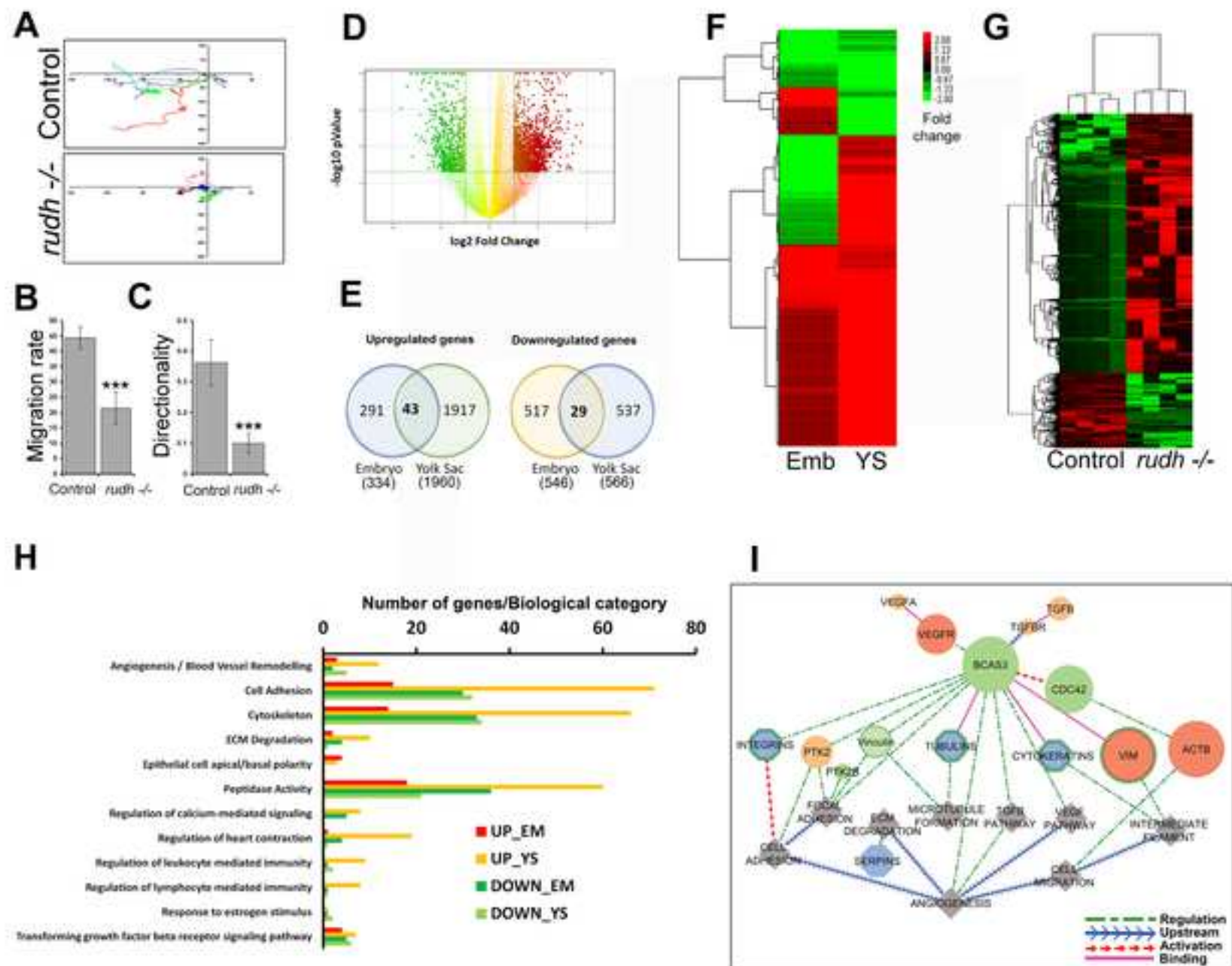


Figure 3

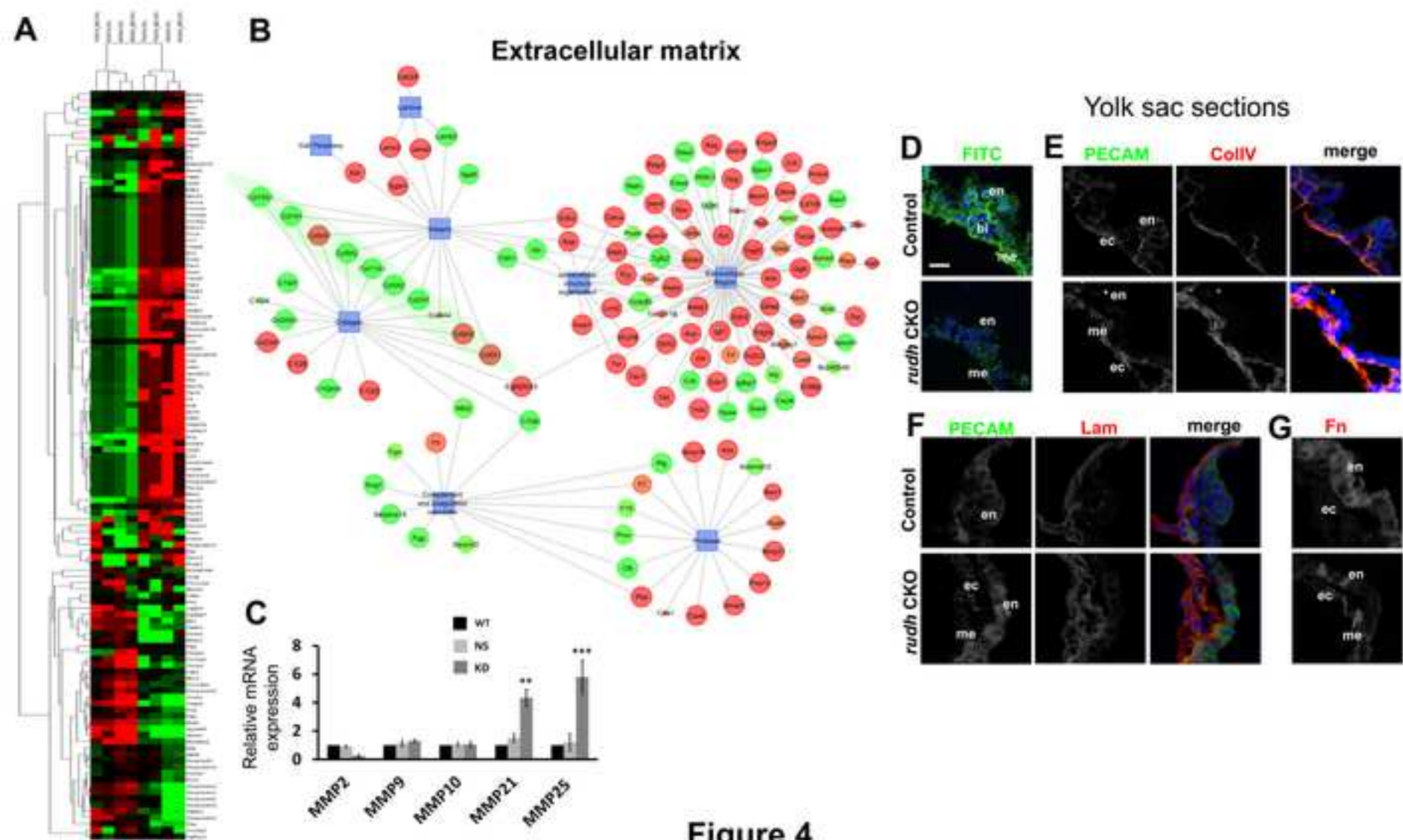


Figure 4

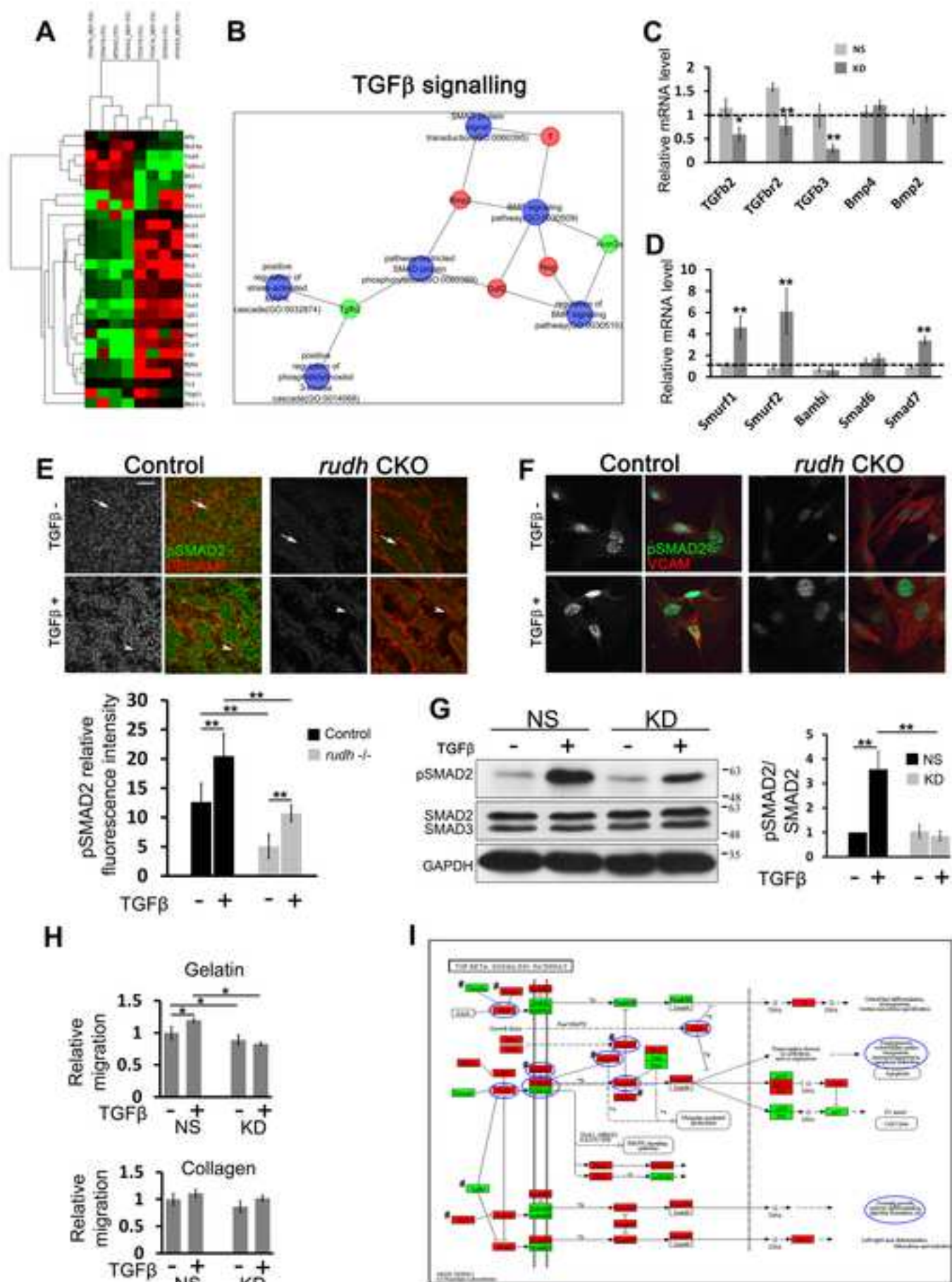


Figure 5

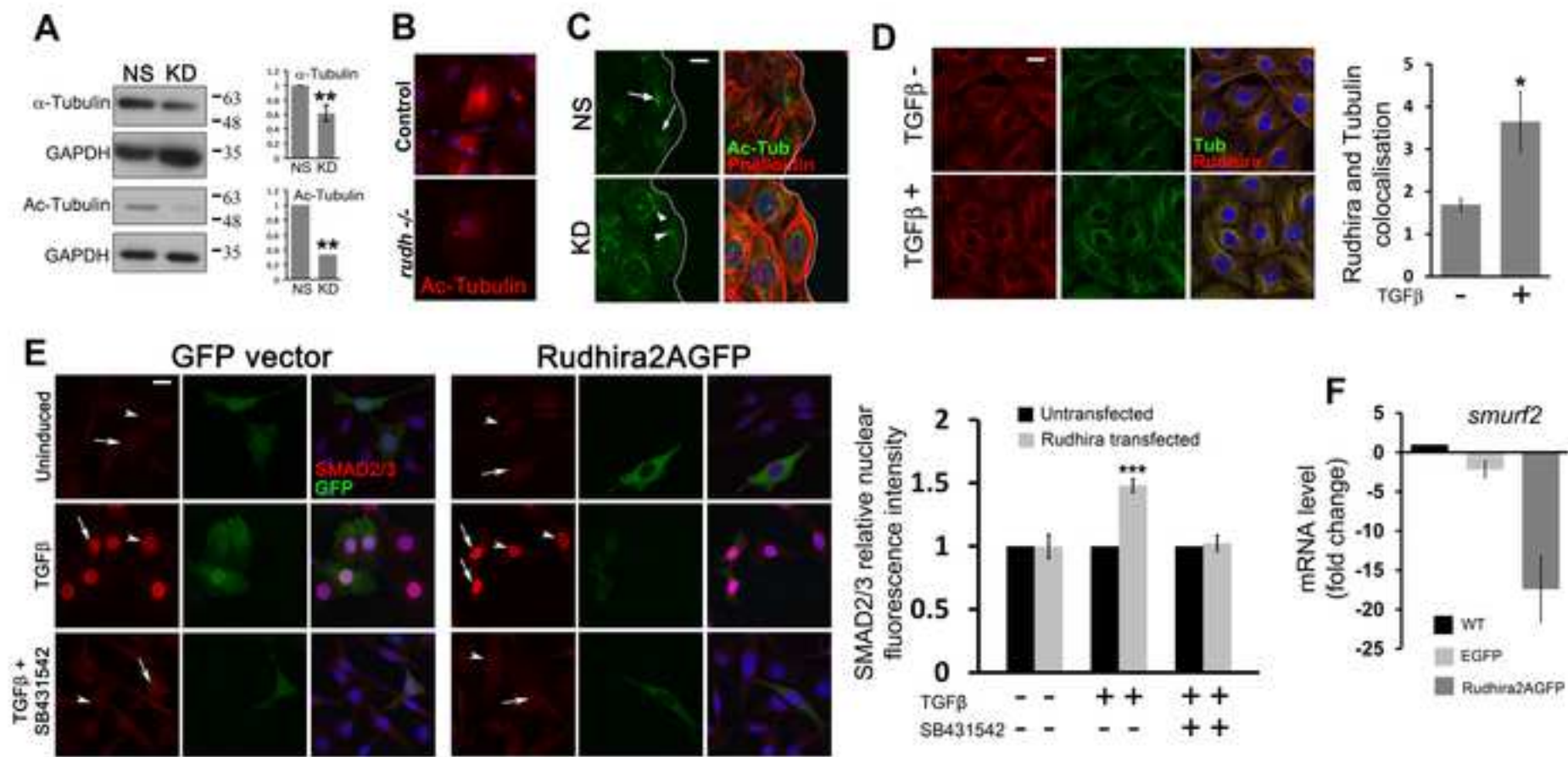


Figure 6

