Supplementary Data

Supplementary Methods

Cell culture

Neonatal mouse cardiomyocytes were isolated from 1-day old 129sv mice. Neonatal hearts were pre-digested O.N. at 4°C in 0.5 mg/mL Trypsin (Sigma) Hank's Balanced Salt Solution followed by 4 subsequent dissociation cycles with 240 U/mL Collagenase type II (Worthington) in HBSS. Fibroblasts were removed by 2 rounds of pre-plating for 2 h on plastic tissue culture dishes. Cardiomyocytes were plated on gelatin/fibronectin-coated dishes and maintained in DMEM-M199 medium, 10% Horse Serum (Gibco) and 5% FBS (Gibco). Final cultures contained >90% cardiomyocytes as determined by immunofluorescence staining for sarcomeric alpha-actinin (A5044 Sigma; 1:400).

Neonatal mouse cardiac fibroblasts collected by pre-plating, were maintained in DMEM 10% FCS for 48 hours, starved for 24 hours in DMEM 1% FCS and stimulated with 10 ng/mL TGFβ1 (Peprotech) for 36 hours for RNA analysis and 48 hours for immunofluorescence. Cells were then fixed in 3% PFA for IF or lysed with trizol reagent for RNA extraction.

Immunofluorescence for fibronectin was performed using rabbit polyclonal antibody [1] together with TRITC-conjugated phalloid from Sigma.

Growth curves were generated by seeding 80,000 cells/well in 6-well plates. At the indicated times, cells were fixed and stained with crystal violet, extracted with acetic acid, diluted, and the optical density at 595 nm was determined. Values were normalized to the optical density at day 0. Within an experiment, each point was determined in triplicate .

Immunoprecipitation and Western blots

For immunoprecipitation experiments, hearts were homogenized in lysis buffer 50 mM HEPES, 100 mM NaCl, 0.1% Na-Deoxycholate, 0.1% Triton X-100, 1 mM EGTA, 2 mM EDTA,

containing Roche Complete protease inhibitor cocktail, 10 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4. Protein extracts were clarified with 3 sequential centrifugations at 14000 rpm, at 4°C. Immunoprecipitation experiments were performed for 2 hours at 4°C using 5 mg of total heart protein extract, 5 μg of anti-AKT and 20 μL of protein G sepharose (GE). After immunoprecipitation, beads were washed 10 times and resuspended in Laemmli buffer. Immunoprecipitated samples were analyzed by Western blotting to detect co-precipitated proteins. Band quantification was performed using Quantity One software (Bio-Rad).

For Western blot analysis of phospho-proteins and total protein levels, hearts were lysed in 1% Triton X-100 TBS plus phosphatases and protease inhibitors as indicated above.

For Western blot analysis the following antibodies were used: ERK1/2 (#9102), MEK1/2 (#9122), Phospho-T202/Y204 ERK1/2 (#9101), Phospho-S217/S221 MEK1/2 (#9121), JNK1/2 (#9252), Phospho-T183/Y185 JNK 1/2 (#9251), p38 (#9212), Phospho-T180/Y182 p38 (#9211), BclXL (#2762), BAD (#9292) (1:1000, Cell Signalling), IQGAP1 (Sc-10792), c-RAF (Sc-133), b-RAF (Sc-166), p53 (Sc-126) (1:1000, Santa Cruz), Vinculin (hVIN-1) (1:5000, Sigma), β1D integrin [2] were used 1μg/ml.

Monoclonal antibodies against IQGAP1 (clone 7B1/F4) were produced in our laboratory by immunizing mice with a mouse IQGAP1 recombinant fragment (amino acids residues 301-389) fused to GST and its reactivity was characterized in Western blot and immunoprecipitation.

Histological analyses

Hearts were collected at the indicated time, fixed in PBS 4% PFA and embedded in paraffin. Serial 4 μm heart sections were cut and stained with hematoxylin and eosin and cardiomyocyte cross-sectional areas were measured using MetaMorph Software.

The area of fibrosis was measured on heart sections stained for 1h at RT with Picrosirius red solution (0.5% Sirius red in picric acid saturated aqueous solution) using MetaMorph Software.

Capillary density was determined by Alexa488-labeled lectin from Griffonia Simplicifolia (1:50;

Invitrogen) staining on serial sections. Images were captured using a DMI3000B Leica fluorescence/optical microscope provided of a Leica DFC340FX camera (Leica Microsystems, Wetzlar, Germany) and processed for quantitative analyses with the Leica Application Suite (LAS V3.3) Image Analysis.

Cardiomyocyte apoptosis was assayed using In Situ Cell Death Detection Kit TMR red (Roche), following manufacturer's instructions. TUNEL positive nuclei of cardiomyocytes, identified by α-actinin co-staining, were counted on entire tissue slices at 400X magnification.

To asses inflammatory cell infiltrate, heart sections were probed with anti-CD18 (BMA Biomedicals) specific antibodies as described by [3]. Positive cells were counted over the entire section area at 400X magnification.

RNA extraction and Real Time PCR

Frozen heart samples were homogenized in 1 mL of Trizol reagent (Invitrogen) and total RNA was purified following manufacturer's protocol. cDNA was synthesized from 200 ng of total RNA using M-MLV reverse transcriptase and random hexadeoxynucleotides (Promega) as primers for the first strand synthesis. Singleplex TaqMan real time PCR was performed using Universal Probe Library System (Roche) and specific primers for each target (see table below). 18S rRNA was used as endogenous control to normalize target expression levels. Amplification was performed on 384 well-plates using 7900 HT Fast Real Time system (Applied Biosystems). Relative quantification of gene expression levels was analyzed using RQ Manager software (Applied Biosystems).

Gene name	**Gene ID**	**Left primer**	**Right Primer**
ColIa1 (Collagen I)	NM_007742.3	catgttcagctttgtggacct	gcagctgacttcagggatgt
ColIIIa1 (Collagen III)	NM_009930.2	tcccctggaatctgtgaatc	tgagtcgaattggggagaat
Fn1 (Fibronectin)	NM_010233.1	cggagagagtgcccctacta	cgatattggtgaatcgcaga
Tgfb1 (TGF β)	NM_011577.1	tggagcaacatgtggaactc	cagcagccggttaccaag
Mmp9 (MMP9)	Nm_013599.2	acgacatagacggcatcca	gctgtggttcagttgtggtg
Nppb (BNP)	NM_008726.4	gtcagtcgtttgggctgtaac	agacccaggcagagtcagaa
Nppa (ANP)	NM_008725.2	cacagatctgatggatttcaa	cctcatcttctaccggcatc
Vegfa (VEGF)	NM_001025250.3	aacgatgaagccatggagt	aggtttgatccgcatgatct

Ctgf (CTGF)	NM_010217.2	tgacctggaggaaaacattaaga	agcctgtatgtcttcacactg
Hif1a (HIF1a)	NM_010431.2	gcactagacaaagttcacctgaga	cgctatccacatcaaagcaa
Myh6 (αMHC)	NM_010856.4	cgcatcaaggagctcacc	cctgcagccgcattaagt
Myh7 (βMHC)	NM_080728.2	cgcatcaaggagctcacc	ctgcagccgcagtaggtt
Bad (Bad)	NM_007522.2	ggagcaacattcatcagcag	tacgaactgtggcgactcc
Bcl2l1 (BclXL)	NM_009743.4	tgaccacctagagccttgga	tgttcccgtagagatccacaa
Trp53 (p53)	NM_001127233.1	acgcttctccgaagactgg	agggagctcgaggctgata

Supplementary Table

Table 1

	WT		IQGAP-/-	
	basal	1 week AB	basal	1 week AB
E/e'	20.69 ± 1.13	23.98 ± 0.41	21.34 ± 1.25	24.15 ± 1.22

Blood flow Doppler peak (E) at mitral inflow to the tissue movement Doppler peak (e') at the lateral mitral annulus (E/e') ratio in wild type (WT) and IQGAP1-null (IQGAP1-/-) mice in basal condition and after 1 week AB.

Supplementary Figure Legends

Figure S1 Fibrosis and capillary density were comparable between IQGAP1-null and wild type mice after 12 weeks of AB.

A) Picrosirius Red staining of sham-operated and 12 week-banded hearts from IQGAP1-null and wild type mice. Scale bar: 50 μm.

B) Isolectin and DAPI staining of 12 week-banded hearts from IQGAP1-null and wild type mice. Scale bar: 20 μm.

C) Representative apoptotic cardiomyocyte nucleus (arrowed) in heart section subjected to TUNEL assay and stained for α-actinin (green) and DAPI (blue). Scale bar: 10 μm.

Figure S2 IQGAP1-null and wild type mice did not differ for expression of matrix genes, profibrotic cytokines and angiogenic factors.

Real Time PCR was performed on wild type and IQGAP1-null mice in basal conditions (Sham) and after 1 and 12 weeks of AB for the following genes: A) collagen I α1; B) collagen III α1, C) fibronectin, D) Tumor Growth Factor β1 (TGF-β1), E) Connective Tissue Growth Factor (CTGF), F) Matrix Metallo Proteinase-9 (MMP-9), G) Hypoxia Inducible Factor 1α (HIF-1 α) and H) Vascular endothelial growth factor (VEGF). I) Renin activity measured on plasma form wild type

and IQGAP1-null sham operated and after 1 week AB.

(*n*=6 hearts/group) *p<0.05; **p<0.01; ***p<0.001

Figure S3 IQGAP1-null and wild type cardiac fibroblasts showed similar proliferation rate and matrix deposition.

A) Cardiac fibroblasts growth curve measured by crystal violet staining (*n*=3 independent experiments). Real Time PCR was performed on wild type and IQGAP1-null cardiac fibroblasts (*n*=3 independent experiments) untreated (NT) or treated for 36 hours with 10 ng/mL TGF-β for the following genes: B) collagen I α1; C) collagen III α1, D) fibronectin, E) Tumor Growth Factor β1 (TGF-β1). F). Immunofluorescence for fibronectin (green) and actin (red) on wild type and IQGAP1-null cardiac fibroblasts untreated (NT) or treated for 48 hours with 10 ng/mL TGF-β.

*p<0.05; **p<0.01

Figure S4 IQGAP1-null and wild type showed similar phosphorylation of JNK1/2/3 and p38 in response to AB.

Heart protein extracts from wild type mice sham-operated (S) or subjected to aortic banding (AB) for the indicated times were analyzed by Western blotting for phosphorylated and total JNK1/2/3 and p38 (upper panel). Densitometric quantification of Western blot bands, expressed as phospho/total protein ratio (n=4 hearts/group) (lower panels).

*p<0.05

Figure S5 Absence of MEK1/2 and AKT phosphorylation in response to 12 week AB in both wild type and IQGAP1-null mice.

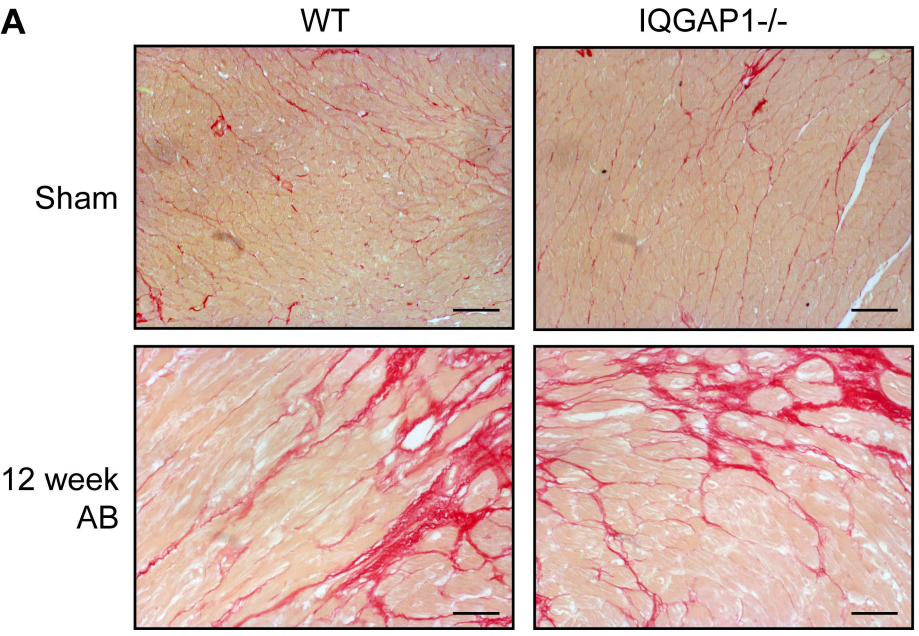
Heart protein extracts from wild type mice sham-operated (Sham) or subjected to aortic banding for 12 weeks (12w AB), were analyzed by Western blotting for phosphorylated and total MEK1/2, ERK1/2 and AKT. Graphs shows densitometric quantification of Western blot bands, expressed as

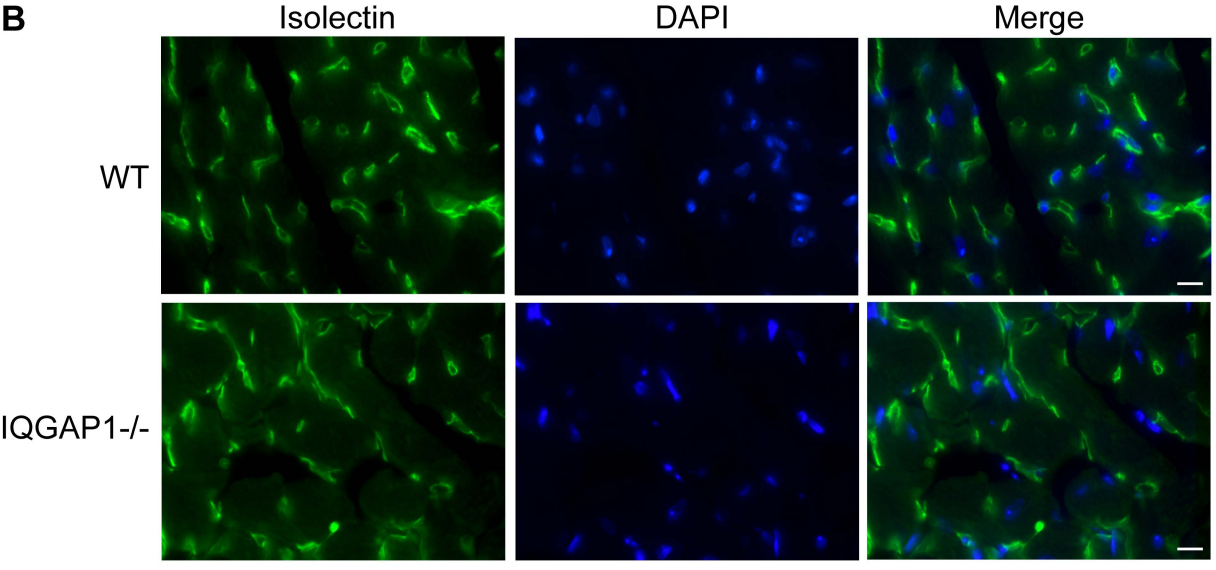
phospho/total protein ratio (n=4 hearts/group).

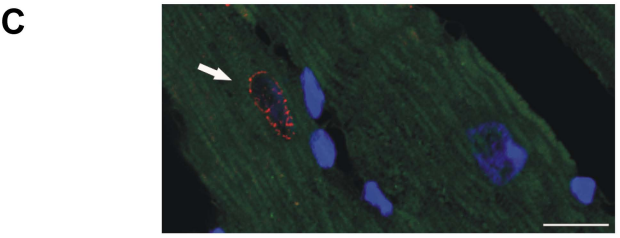
Supplementary References

1. Tarone G, Amedeo MR, Di Renzo MF, Comoglio P. Monoclonal antibodies to the collagen binding domain of human plasma fibronectin. *Exp Cell Biol* 1984;**52**:225-236.
2. Brancaccio M, Fratta L, Notte A, Hirsch E, Poulet R, Guazzone S*, et al.* Melusin, a muscle-specific integrin beta1-interacting protein, is required to prevent cardiac failure in response to chronic pressure overload. *Nat Med* 2003;**9**:68-75.
3. Patrucco E, Notte A, Barberis L, Selvetella G, Maffei A, Brancaccio M*, et al.* PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. *Cell* 2004;**118**:375-387.

Figure S1







Tunel-positive cardiomyocyte nucleus in IQGAP1-/- heart at 12 week AB

Figure S2

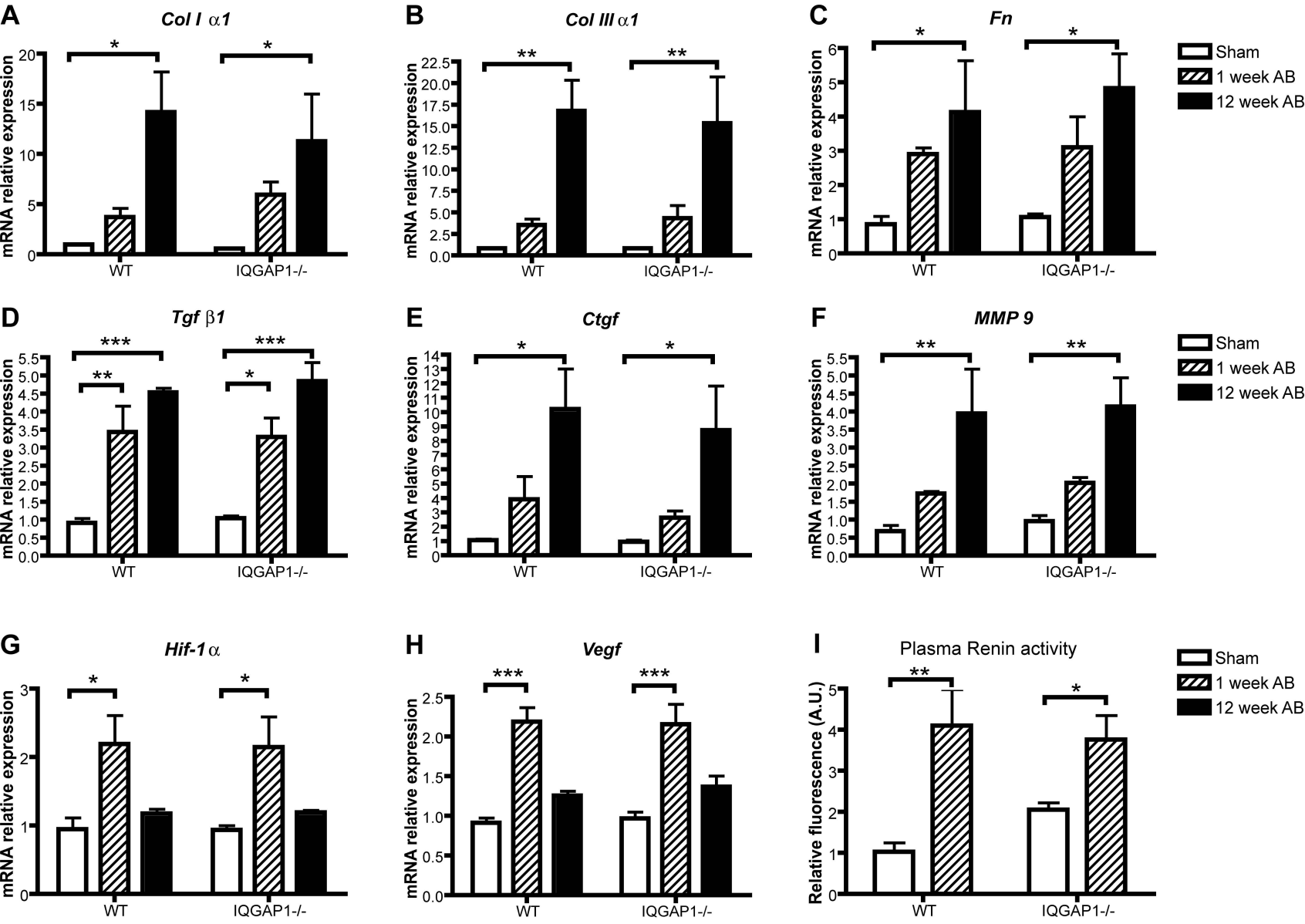

A
Col I α1
mRNA relative expression
WT
IQGAP1-/-
B
Col III α1
C
Fn
D
Tgf β1
E
Ctgf
F
MMP 9
G
Hif-1α
H
Vegf
I
Plasma Renin activity
Relative fluorescence (A.U.)
Sham
1 week AB
12 week AB

Figure S3

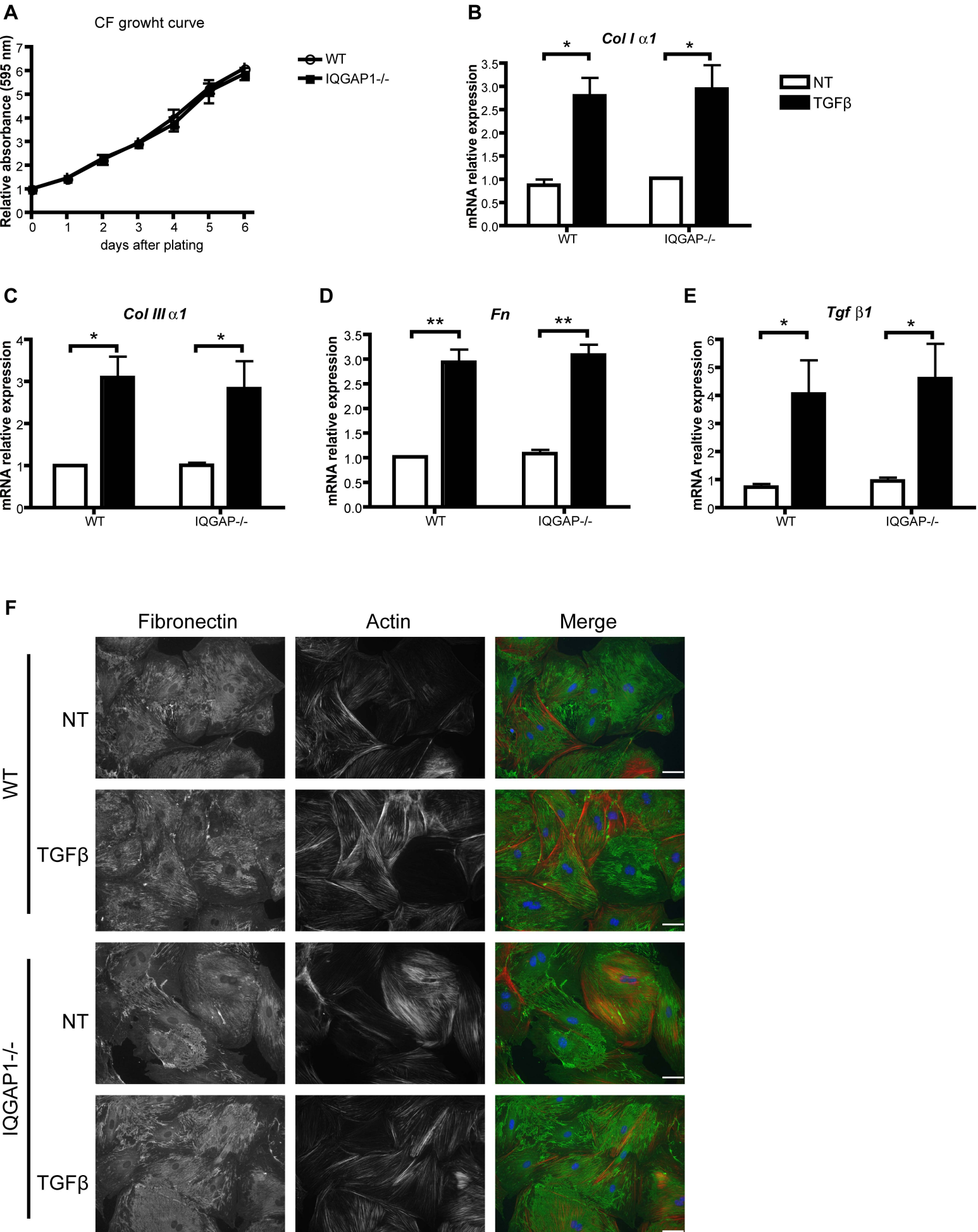

A
CF growht curve
Relative absorbance (595 nm)
days after plating
WT
IQGAP1-/-
B
Col I α1
mRNA relative expression
NT
TGFβ
WT
IQGAP-/-
C
Col III α1
D
Fn
E
Tgf β1
mRNA realtive expression
F
Fibronectin
Actin
Merge
WT
IQGAP1-/-
NT
TGFβ

Figure S4

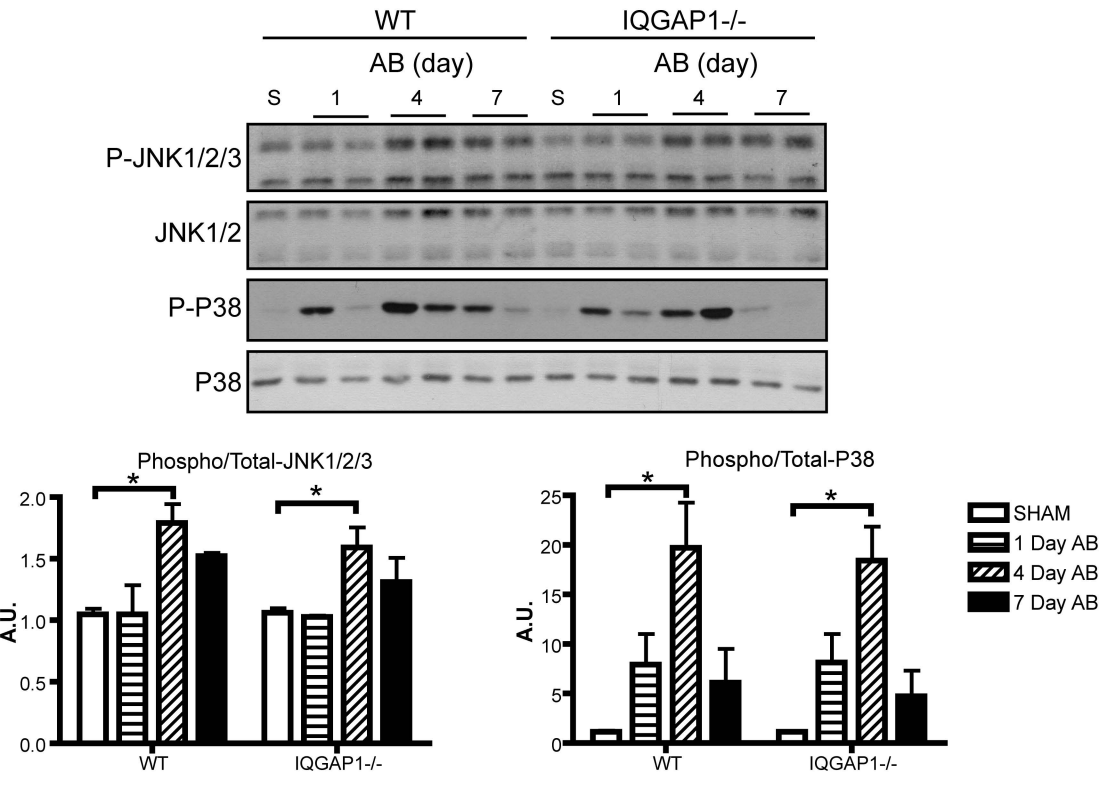
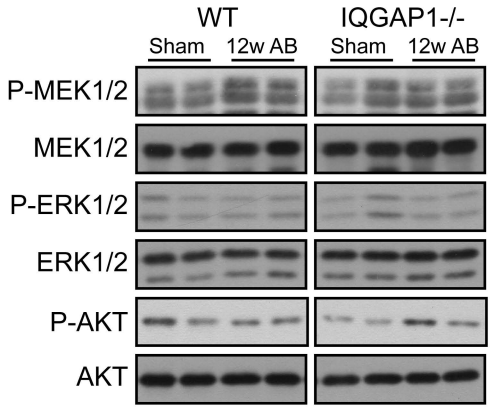
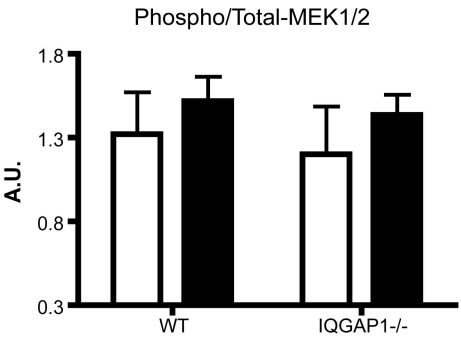
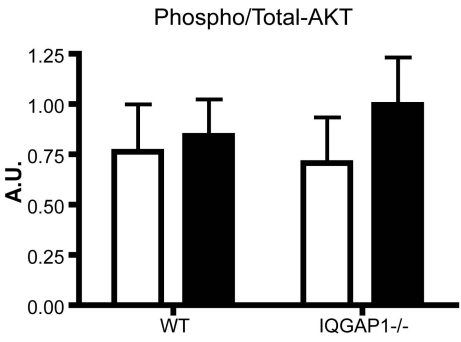
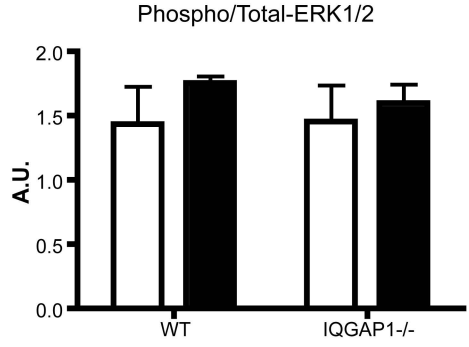

WT
IQGAP1-/-
AB (day)
AB (day)
S
1
4
7
S
1
4
7
P-JNK1/2/3
JNK1/2
P-P38
P38
Phospho/Total-JNK1/2/3
A.U.
2.0
1.5
1.0
0.5
0.0
*
*
WT
IQGAP1-/-
Phospho/Total-P38
A.U.
25
20
15
10
5
0
*
*
WT
IQGAP1-/-
SHAM
1 Day AB
4 Day AB
7 Day AB

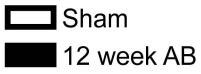
Figure S5


WT
IQGAP1-/-
Sham
12w AB
Sham
12w AB
P-MEK1/2
MEK1/2
P-ERK1/2
ERK1/2
P-AKT
AKT


Phospho/Total-MEK1/2
A.U.
1.8
1.3
0.8
0.3
WT
IQGAP1-/-


Phospho/Total-AKT
A.U.
1.25
1.00
0.75
0.50
0.25
0.00
WT
IQGAP1-/-


Phospho/Total-ERK1/2
A.U.
2.0
1.5
1.0
0.5
0.0
WT
IQGAP1-/-


Sham
12 week AB