GRAF1 Deficiency Leads to Defective Brown Adipose Tissue Differentiation and Thermogenic Response

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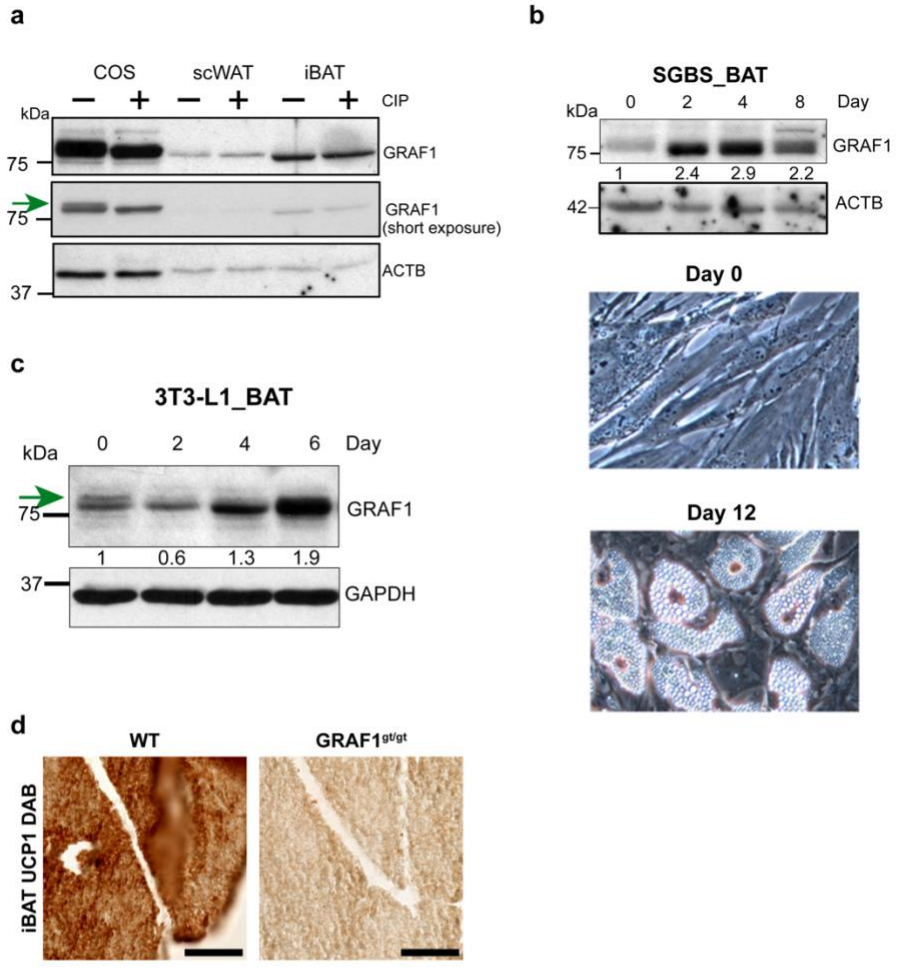

a
COS
scWAT
iBAT
− + − + − + CIP
kDa
75
GRAF1
75
GRAF1
(short exposure)
37
ACTB
b
SGBS_BAT
kDa
0 2 4 8 Day
75
GRAF1
1 2.4 2.9 2.2
42
ACTB
Day 0
Day 12
c
3T3-L1_BAT
kDa
0 2 4 6 Day
75
GRAF1
1 0.6 1.3 1.9
37
GAPDH
d
WT
GRAF1gt/gt
iBAT UCP1 DAB

Figure S1. GRAF1 expression is elevated following BAT differentiation. **a**. Lysates from COS cells expressing GRAF1, as well as lysates from adult scWAT and iBAT, were treated with calf intestinal alkaline phosphatase (CIP) to determine GRAF1 phosphorylation status. COS cells transfected with GRAF1 for 20 hours were further exposed to Carbonyl Cyanide Chlorophenylhydrazone (CCCP) to induce GRAF1 phosphorylation, serving as a positive control. Notably, CIP treatment eliminated the GRAF1 band shift in COS cell lysates, while exerting no impact on the band distinctions between scWAT and iBAT. **b**. GRAF1 levels in SGBS at indicated time points following treatment of brown adipocyte differentiation medium was assessed by Western blotting **(top panel).** Representative microscopy images of SGBS prior to and following treatment of brown adipocyte differentiation medium for 12 days **(bottom panel)**. Note accumulation of various small lipid droplets and characteristic brown phenotype observed at Day 12. **c**. GRAF1 levels in mouse 3T3-L1 adipocytes treated with BAT-induction cocktail was assessed by Western blotting. **d.** Representative UCP1 DAB stain of iBAT isolated from cold exposed 2–3-month-old GRAF1$^{gt/gt}$ and WT mice. scale bar: 100μm. Quantitative analysis of images using Fiji revealed a significant reduction in % positivity of UCP1 staining (61.0% +/- 25.2% for WT (n=3) and 2.8% +/- 1.4% for GRAF1$^{gt/gt}$ (n=4); $p<0.05$). Green arrows in **a** and **c** indicate the presence of additional GRAF1 band. Densitometric values of GRAF1 in **b** and **c** were measured and presented below each corresponding GRAF1 band.

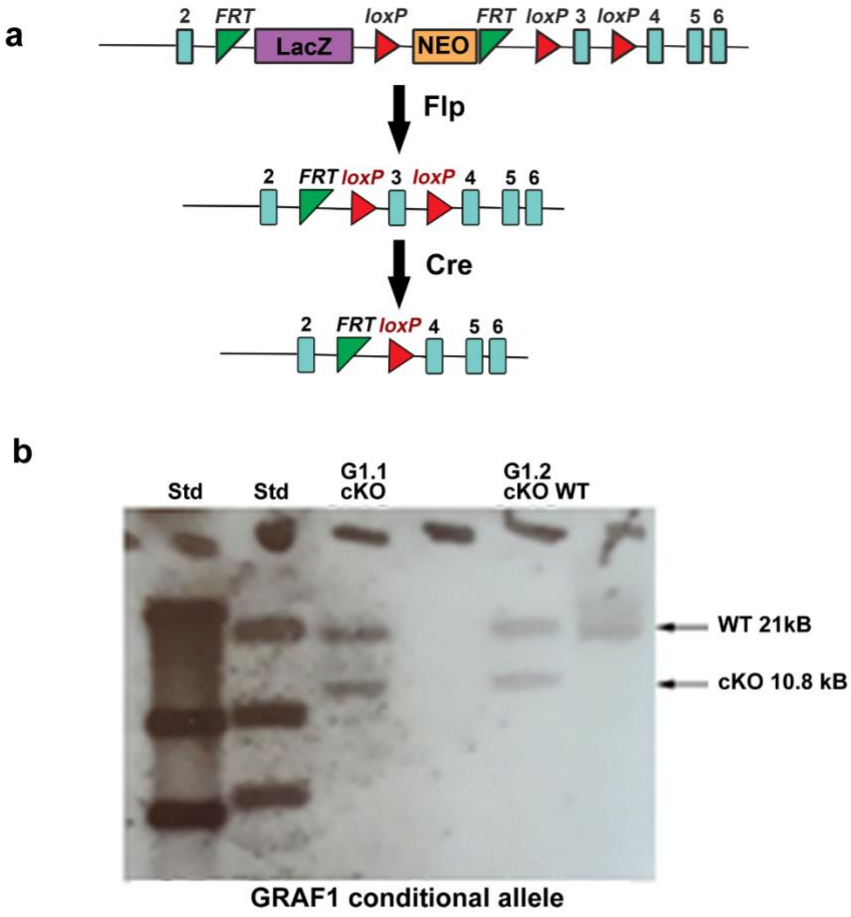

a
2
FRT
LacZ
loxP
NEO
FRT
loxP
3
loxP
4
5
6
Flp
2
FRT
loxP
3
loxP
4
5
6
Cre
2
FRT
loxP
4
5
6
b
Std
Std
G1.1
cKO
G1.2
cKO
WT
WT 21kB
cKO 10.8 kB
GRAF1 conditional allele
c
GRAF1/HPRT1
1.5
1.0
0.5
0.0
*
**
siCon+V
siCon+Y
SiG1+V
SiG1+Y

Figure S2. Development of GRAF1 conditional knockout mouse. **a**. Schematic showing use of "knock-out first" targeted ES cells to generate Cre-dependent GRAF1 conditional knockouts. **b.** Southern blot analysis confirmed deletion of GRAF1 exon 3 in embryonic stem cells. **c**. qRT-PCR analysis illustrating the expression levels of GRAF1 in SGBS cells. The cells were transfected with specified siRNA for 24 hours and subsequently treated with either Vehicle(V) or the ROCK inhibitor Y27632(Y) during the brown adipocyte differentiation. n=3/group. siG1 refers to siGRAF1. Data (**c**) are represented as mean ± SEM, ns, not significant; *P < 0.05; **P < 0.01 by two-way ANOVA with post-hoc Fisher's LSD test.

Supplement Table 1: primers for real time PCR

mouse target gene		
GRAF1	5'-TGGAAGGGTACCTGTACGTG-3'	5'-ATCCCGTTGGTAGGTACAGT-3'
UCP1	5'-AGGATTGGCCTCTACGACTCA-3'	5'-CAATGAACACTGCCACACCTC-3'
PPARγ	5'-GTGAGACCAACAGCCTGACG-3'	5'-AGTGGTTCACCGCTTCTTTCA-3'
ND5	5'-AGCATTCGGAAGCATCCTTG-3'	5'-TTGTGAGGACTGGAATGCTG-3'
HPRT1	5'-GCTTGCTGGTGAAAAGGACCTCTCGAAG-3'	5'-CCCTGAAGTACTCATTATAGTCAAGGGCAT-3'
Elovl3	5'-TCCGCGTTCTCATGTAGGTCT-3'	5'-GGACCTGATGCAACCCTATGA-3'
Adiponectin	5'-ATGGCAGAGATGGCACTCCT-3'	5'-CCCTTCAGCTCCTGTCATTCC-3'
Cox7a	5'-CAGCGTCATGGTCAGTCTGT-3'	5'-AGAAAACCGTGTGGCAGAGA-3'
Cpt1a	5'-GGACTCCGCTCGCTCATT-3'	5'-GAGATCGATGCCATCAGGGG-3'
Cpt1b	5'-GGTCCCATAAGAAACAAGACCTCC-3'	5'-CAGAAAGTACCTCAGCCAGGAAAG-3'
FGF1	5'-CCGAAGGGCTTTTATACGGCT-3'	5'-CAGTTCTTCTCCGCATGCTTC-3'
FGF21	5'-AGATCAGGGAGGATGGAAC-3'	5'-TCAAAGTGAGGCGATCCATA-3'
CS	5'-CTCCTGTTGCAGCTGTAGCTCT-3'	5'-AAATTCGTGGAAGAAGCACTGG-3'
TBP	5’-TATGACCCCTATCACTCCTG-3’	5’-TTCTTCACTCTTGGCTCCTGT-3’
human target gene		
GRAF1	5'-CACTTCCGAGAGACGCTCAA-3'	5'-CTTCCGCTTCGCTGAAGACA-3'
HPRT1	5’-GGCCATCACATTGTAGCCCT-3’	5’-GTCCCCTGTTGACTGGTCATT-3’
UCP-1	5'-AATCAGCTCCGCCTCTCTCA-3'	5'-TCTTGCTTCCTAAACTAGGTGCT-3'

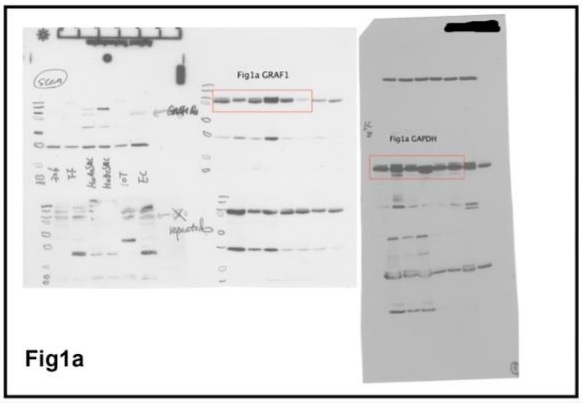


Fig1a

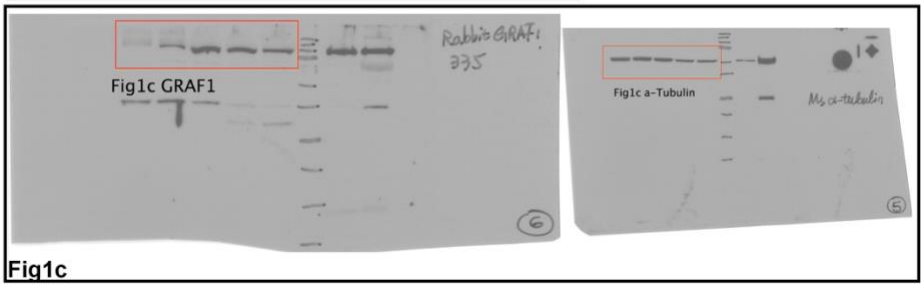


Fig1c

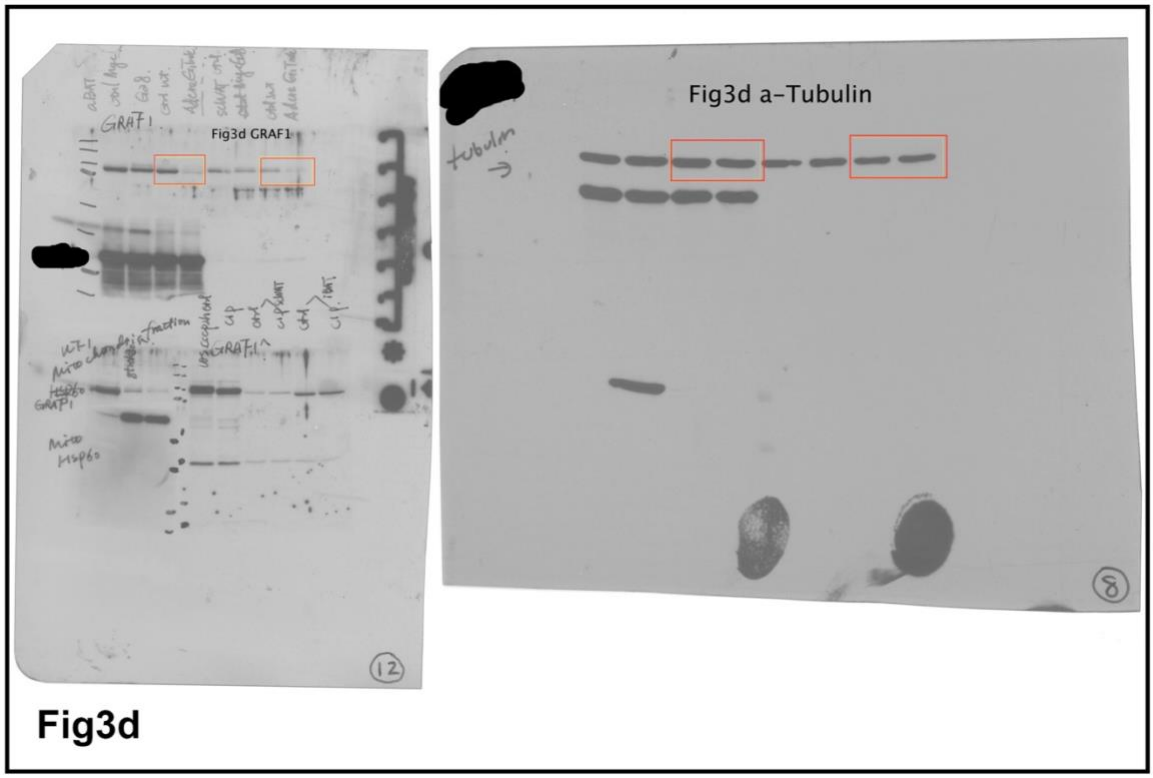


Fig3d

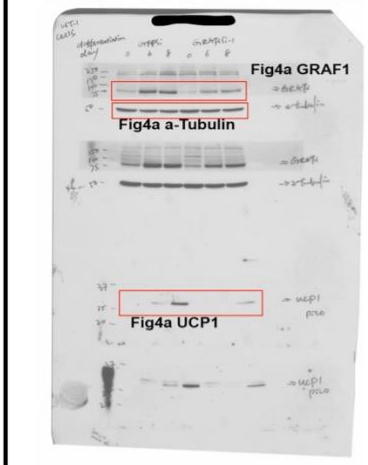


Fig4a GRAF1
Fig4a a-Tubulin
Fig4a UCP1

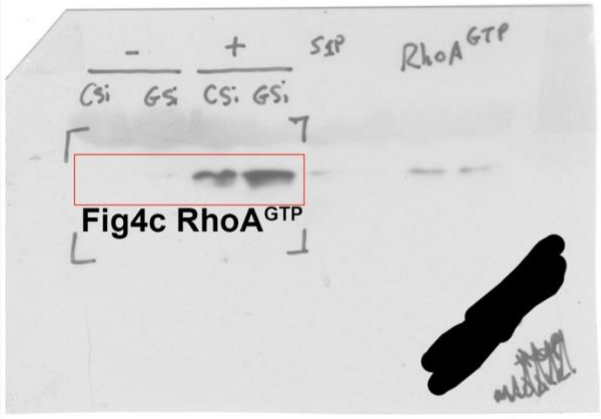


Fig4c RhoA GTP

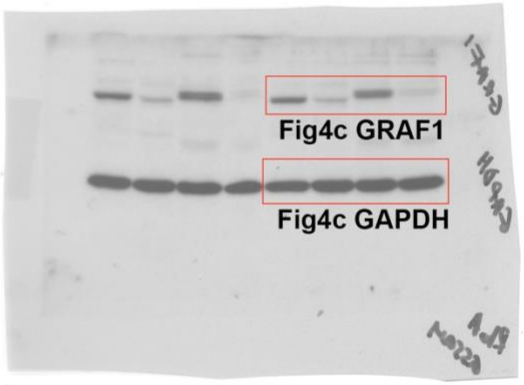


Fig4c GRAF1
Fig4c GAPDH

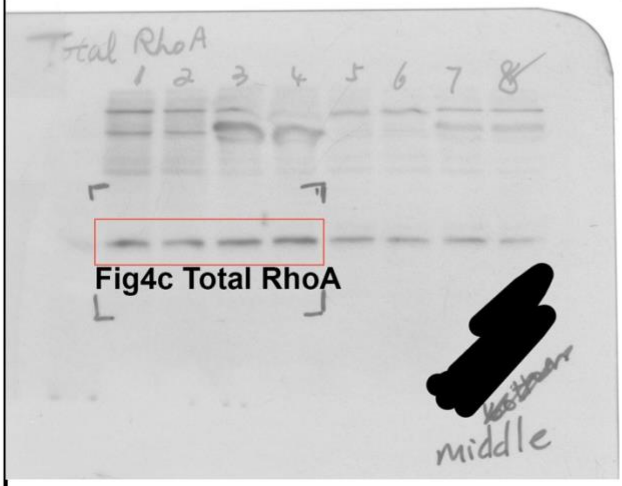
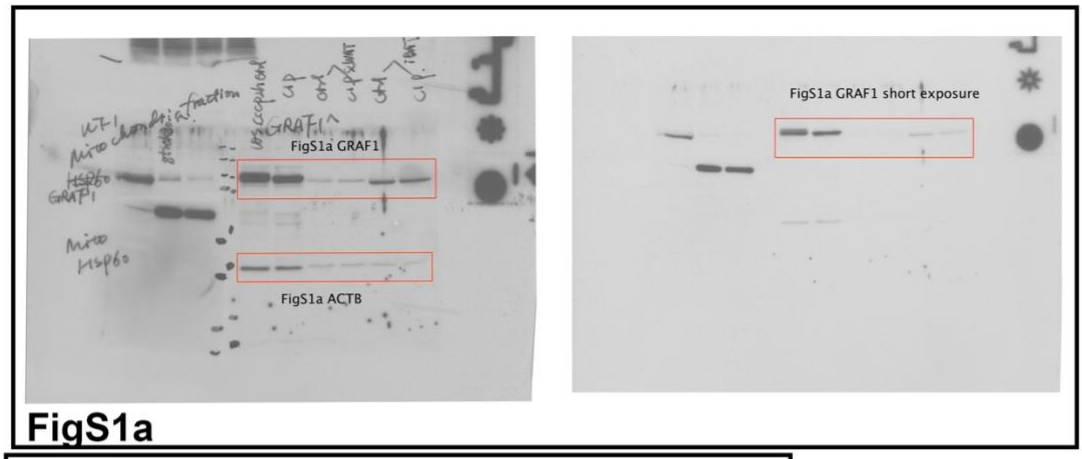
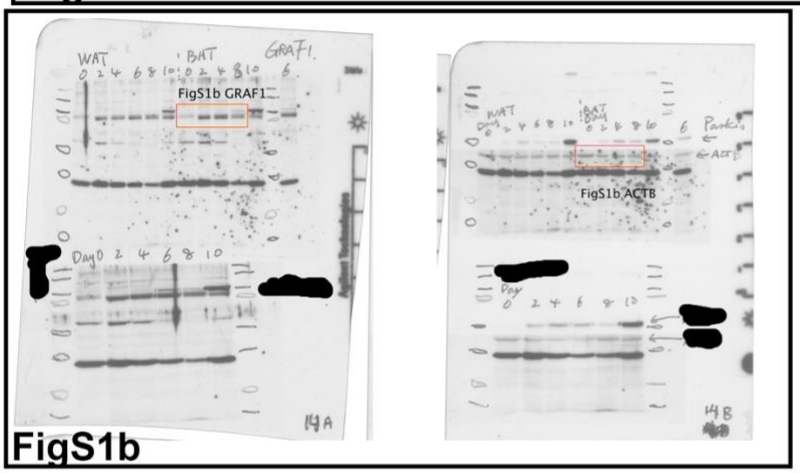

Total RhoA
1 2 3 4 5 6 7 8
Fig4c Total RhoA
middle

Fig4 a and c





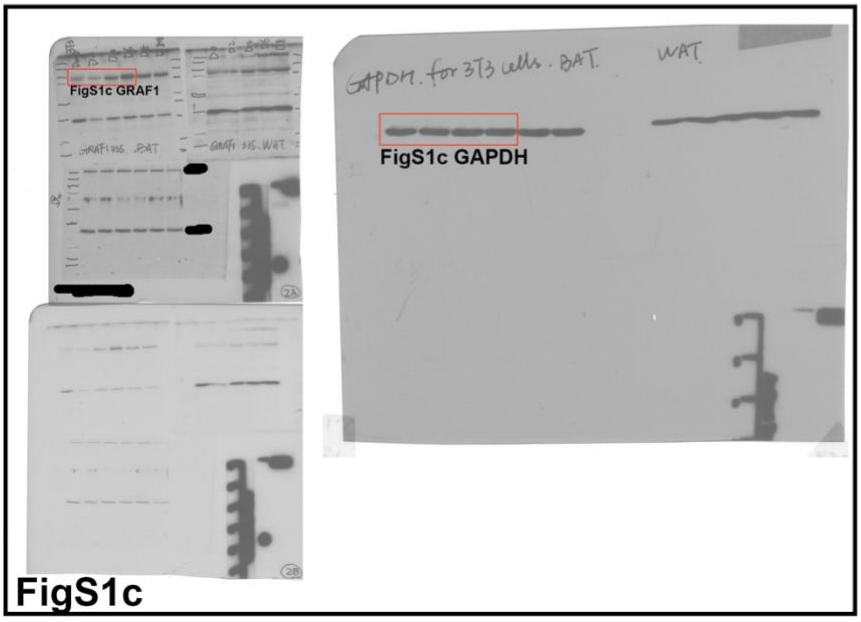


Fig S3 The original western blot membranes. The cropped versions presented in the article are highlighted in red frame.