Supplementary Information

IRS4, a novel modulator of BMP/Smad and Akt signalling during early muscle differentiation

Gina Dörpholz, Arunima Murgai, Jerome Jatzlau, Daniel Horbelt, Mohammad Poorgholi Belverdi, Christina Heroven, Isabelle Schreiber, Gisela Wendel, Karen Ruschke; Sigmar Stricker, and Petra Knaus
Figure S1

(a) Identification of IRS4 as a BMPRII interacting protein. IRS4 specific peptides were identified by mass spectrometry upon precipitation of HA-BMPRII-LF from HA-BMPRII stably transfected HEK293T cell lysates both in the absence (peptides marked in yellow) and presence of BMP2 (peptides underlined in red).

(b) IRS4 and IRS2 are capable of interacting with BMPRII-LF. Transfected HEK293T cells were subjected to immunoprecipitation using an α-myc antibody; precipitates and TCL were analysed by Western blotting using indicated antibodies. Incubation with beads only served as IP control.

Supplementary Figure S1: IRS4 interacts with BMPRII
(a) Identification of IRS4 as a BMPRII interacting protein. IRS4 specific peptides were identified by mass spectrometry upon precipitation of HA-BMPRII-LF from HA-BMPRII stably transfected HEK293T cell lysates both in the absence (peptides marked in yellow) and presence of BMP2 (peptides underlined in red). (b) IRS4 and IRS2 are capable of interacting with BMPRII-LF. Transfected HEK293T cells were subjected to immunoprecipitation using an α-myc antibody; precipitates and TCL were analysed by Western blotting using indicated antibodies. Incubation with beads only served as IP control.
Supplementary Figure S2: Impact of IRS4 on Smad proteins and on transcriptional activity of Smads

(a) Expression of IRS4. HEK293T cells and C2C12 cells were transfected with IRS4-myc or β-galactosidase as control; lysates were subjected to Western blotting using indicated antibodies. The expected molecular weight of either overexpressed mouse IRS4 or endogenous protein are indicated by arrows. IRS4 Santa Cruz antibody detects particularly human IRS4, IRS4 Bioss and Origene antibodies detect solely the mouse or human protein, respectively.

(b) The IRS4-induced decrease of Smad1 protein level is ligand-independent. Transfected C2C12 cells were stimulated with 10 nM BMP2 for 30 min and subjected to flow cytometry analysis. IRS4 transfected cells were gated on single cells (blue curve) and the endogenous Smad1 level was quantified. Solid lines depict unstimulated and dotted lines BMP2 stimulated samples. Bar chart shows MFI values relative to β-galactosidase w/o ligand.

(c) IRS4 does not affect Smad5 protein levels. Transfected C2C12 cells were gated on myc-positive single cells (blue curve) via flow cytometry and the endogenous Smad5 level was analysed. Bar chart depicts MFI values of myc-positive cells compared to those of all cells (grey curve) derived from 2 independent experiments.

(d) IRS4 does not affect FLAG-Smad5 levels. Transfected C2C12 cells were incubated with 5 µg/ml cycloheximide for 6 h; ethanol
was applied as vehicle control. Lysate were subjected to Western blotting using indicated antibodies. Quantification depicts total FLAG-Smad5 levels (see red indication on the blot) normalised to β-actin relative to β-galactosidase. (e) Impact of IRS proteins on transcriptional activity of BMP Smads. C2C12 cells transfected with BRE-luc, RL-TK and IRS1-4-myc or β-galactosidase were stimulated with 5 nM BMP2 for 6 h (a) or 24 h (b), respectively. Bar chart depicts means ± SD of RLU from triplicate measurements relative to β-galactosidase. (g) IRS4 reduces BMP2-induced ID1 protein expression. Transfected C2C12 cells were stimulated with 5 nM BMP2 for 6 h. Lysates were subjected to Western blotting using indicated antibodies. (h) C2C12 cells transfected with BRE-luc, RL-TK and IRS4-myc, truncations thereof or β-galactosidase were stimulated with 5 nM BMP2 for 6 h. Bar chart depicts means ± SD of RLU from triplicate measurements relative to β-galactosidase. RLU = relative luciferase units (BRE-luc/RLTK-luc)

**Figure S3**

![Graphs](image)

**Supplementary Figure S3**: Expression of IRS4 and myogenic markers during mouse limb development  
(a-d) IRS4 is expressed during mouse limb development. RNA from mouse limbs of indicated embryonic developmental stages was isolated, reverse-transcribed and subjected to gene expression analysis via qRT-PCR. Bar charts summarise triplicate measurements and depict MNE ± SEM representative for 2 independent experiments. Data corresponds to these combined in Fig 5a.
Supplementary Figure S4: Characterisation of BMP signalling in immortalised human myoblasts

(a) RNA from cultured human myoblasts was isolated, reverse-transcribed and used for gene expression analysis via qRT-PCR. Bar chart depicts means ± SEM from triplicate measurements. (b) Human myoblasts were stimulated with 10 nM BMP2 for indicated times; lysates were subjected to Western blotting using indicated antibodies. (c) Human myoblasts were differentiated with or without 10 nM BMP2 for indicated times; gene expression was analysed via qRT-PCR. Bar chart depicts MNE ± SEM from triplicate measurements. (d) Concomitantly, images were acquired at indicated times. (e) IRS4 is expressed in human myoblasts. IRS4 expression was verified via flow cytometry using a specific antibody (black line; control IgG: grey shaded curve). (f) siRNA-mediated knockdown of IRS4. Knockdown efficiency was analysed via qRT PCR (left) and Western blotting (right) (g) Control samples for the In situ proximity ligation assay (PLA) of IRS4 and BMPRII or Smad1. Human myoblasts
were subjected to *in situ* PLA (green signal) to visualize the endogenous association of IRS4 with BMPRII or Smad1; nuclei and the actin cytoskeleton were stained using DAPI and Phalloidin594. The association of Smad1 with Smad4 served as positive control.

**Figure S5**

**a**

![Images showing staining results](image)

**b**

![Images showing staining results](image)
Supplementary Figure S5: Targeted IRS4 knockdown decreases myogenic differentiation of primary foetal myoblasts and primary postnatal satellite cells

(a) (b) IRS4 knockdown affects myogenesis in primary mouse cells. Primary mouse myoblasts isolated from E18.5 limb muscles (a) or primary mouse satellite cells isolated from P7 limb muscles (b) were transfected with siRNA targeting either nonspecific sequences (scr si) or mouse IRS4 (mIRS4 si) and differentiated for three (a) or two (b) days, respectively. Myosin heavy chain (MHC) and/or the actin cytoskeleton were stained using a specific antibody or Phalloidin594; nuclei were stained using DAPI. Images were quantified using ImageJ. Bar charts depict means ± SD of at least 16 images per condition.

Supplementary Materials and Methods

Supplementary Table S1: Primers for cloning

| Primer name          | sequence 5’-3’                                      |
|----------------------|-----------------------------------------------------|
| mIRS1 fwd            | CCACCATGCCGAGCCCTCCCGG                               |
| mIRS2 fwd            | CCACCATGCTAGCGGCACCC                                 |
| rIRS3 fwd            | CCACCATGAGCTGAGGAGG                                 |
| mIRS4 fwd            | CCACCATGAGCTGAGGAGG                                 |
| Myc c-term rev       | ATGAAGATGAGATGAGAG                                  |
| rIRS3 rev            | TAGCTAGCCAGCATCAAGCTTCTCCAAGCAGAGATGCCCTTTTAC       |
| mIRS4 myc rev        | AAGCTTATTGAGAAGATGCTTTAG                            |
| mIRS4 E201stop fwd   | TCGGCCGCCTCATCCTCTCATAAGCGACGACGCCCTCCCTTTAG        |
| mIRS4 E201 myc stop  | AAGCTTATTGAGAAGATGCTTTAG                            |
| mIRS4 Y337stop fwd   | GCCTGTGAGAGAGAGATGAGTTTTTCTCCCGAGAGGCTTTTAG        |
| mIRS4 Y337myc stop   | GCCTGTGAGAGAGAGATGAGTTTTTCTCCCGAGAGGCTTTTAG        |
| mIRS4 P604stop fwd   | AAGCAGAGCAGAAACACTTGTCGCCACCCCCACCCCTTTAG          |
| mIRS4 P604 myc stop  | GCCTGTGAGAGAGAGATGAGTTTTTCTCCCGAGAGGCTTTTAG        |
| mIRS4 P908stop fwd   | AAGCAGAGCAGAAACACTTGTCGCCACCCCCACCCCTTTAG          |
| mIRS4 P906 myc rev   | AAGCAGAGCAGAAACACTTGTCGCCACCCCCACCCCTTTAG          |
| mIRS4 P1175stop fwd  | AAGCAGAGCAGAAACACTTGTCGCCACCCCCACCCCTTTAG          |
| mIRS4 A1174 myc rev  | CGGCGCGCAGCGCTGAGGAGCAGACGAGCTTTTCTCCCGAGAGGCTTTTAG |
| mIRS4 E1189stop fwd  | TCGTGGGACAGCAATGACGAGCAGACGAGCTTTTCTCCCGAGAGGCTTTTAG |
| mIRS4 E1189myc rev   | TCGTGGGACAGCAATGACGAGCAGACGAGCTTTTCTCCCGAGAGGCTTTTAG |
| mIRS4 dPH fwd        | CCACCATGAGAGCAAGGCGG                                 |
| mIRS4 dPH PTB fwd    | CCACCATGAGAGCAAGGCGG                                 |
### Supplementary Table S2: Primers for qRT-PCR

| primer      | species | sequence 5’-3’                          | accession number    |
|-------------|---------|-----------------------------------------|---------------------|
| IRS4        | Human   | F TCTTGCTGACAGTGCCATTC                  | ENST0000372129      |
|             |         | R CTTGTGGGCGTCTCTTC                    | NM_003604.2         |
| ID1         | Human   | F GCTGCTCTACGACATGAACG                 | NM_002165           |
|             |         | R CCCACTGAAGGCTCTCTGATG                |                     |
| HPRT        | Human   | F CTTGTGGACCTGCTGGATT                 | NM_000194           |
| MyoG        | Human   | F CAGGGGTCGCCAGCGAATGC                | ENST0000024165      |
|             |         | R ATCTGTAGGGCTAGCCCGTGGA              | NM_002479.5         |
| ACVRI       | Human   | F AAGCCTGGGAGCATTGTAAC                | NM_001105           |
|             |         | R TCACCTGGCTACTCAGGAGA                |                     |
| BMPRIa      | Human   | F CATCTTGGAGGAGTCTAAGAA               | NM_004329           |
| BMPRIb      | Human   | F CTGCCATAAGTGAGAAGCAAAC              | NM_001203           |
| ActRIIA/ACVR2A | Human | F CCTGACAGCTTTGCACTACTCATTT        | NM_001616           |
| ActRIIB/ACVR2B | Human | F TGAAGCAGAAGAAGCCTAGATCTGGAAGGAA   | NM_001106           |
| BMPRII      | Human   | F CATGGAGAAGCAGCTAGCTGCTGTA          | NM_001204           |
| IRS4        | Mouse   | F GTCTTGGAGAACCCCAAGAGAG            | NM_010572.2         |
|             |         | R TGGTTTTGGCGATGGAAAAA                | NM_010495           |
| ID1         | Mouse   | F CTTCTTGGAGGACAGAGGAA               | NM_011039.2         |
| Pax7_1      | Mouse   | F CTGGATGAGGGCTAGCTTGA               | NM_011039           |
| Pax7_2      | Mouse   | F CCGTGGTTTCTCATGTTGTTG              | NM_011039           |
| Myf5        | Mouse   | F CTGTCTGCTCCGCGTCTGAGAC            | NM_008656.5         |
| MyoG_1      | Mouse   | F CCAAGGTCCTCTGCCTGCTGATG               | NM_031189.2         |
| MyoG_2      | Mouse   | F CTACAGCCTTTGCTACAGCTC              | NM_031189           |
| MyoD        | Mouse   | F AAGCACTACAGGGCCAGTCA                | NM_010866           |
| GAPDH       | Mouse   | F AACTTGGCAACTATATGTTGGAAAGG         | NM_008084           |
| HPRT_1      | Mouse   | F TTGTGATAGCTAGCGCTTGTAGG              | NM_013556           |
| HPRT_2      | Mouse   | F TATGCGAGGATATTGAGAAAA               | NM_013556           |

**Target sequences of used siRNAs**

All siRNA oligonucleotides (ON-TARGET plus) were obtained from Thermo Scientific/Dharmacon.

siRNA hIRS4 (hIRS4 si) target sequence: GGG-AAG-AUU-CAG-ACU-UUA-U
siRNA mIRS4 (mIRS4 si) SMART pool, target sequences: UCA-AAC-AGG-UCA-CAA-AGC-CAGUCU-U, GAA-CUC-AAG-UAA-AGC-CCA-A, CAA-AGA-AGC-CAA-AGAGGU-A

Non-targeting siRNA#1 (scr si) target sequence: UGG-UUU-ACA-UGU-CGA-CUA-A

**Antibodies**

The following antibodies were used for Western blotting: β-actin (#A5441, Sigma-Aldrich), GAPDH (#2118, Cell Signaling), ID1 (sc-488, Santa Cruz), Caveolin-3 (sc-5310, Santa Cruz), myogenin (sc-13137), myosin [skeletal, fast] (#M4276, Sigma-Aldrich), total Smad1 (#6944, Cell Signaling), total Smad5 (#12167-1-1-AP, Proteintech), p-Smad1/5/8 (#9511, Cell Signaling), p-Akt Ser473 (#4060S, Cell Signaling), p-GSK3β Ser9 (#9336, Cell Signaling), p-p70S6K Thr421/Ser424 (#9204, Cell Signaling), p-p38 pTGpY (#V1211, Promega), IRS4 (sc-28830, Santa Cruz; bs-0187R, Bioss; #TA303856, Origene), p-Tyrosine-1000 (#8954, Cell Signaling), BMPRII (#612292, BD Biosciences), FLAG® Tag M2 (#F3165, Sigma Aldrich), HA Tag (#H3663, Sigma-Aldrich) and myc Tag (#2276, Cell Signaling) followed by HRP-conjugated secondary antibodies goat-α-mouse/rabbit (#115-035-068/#111-035-046, Dianova).

**Isolation of primary foetal mouse myoblasts and cytospin**

For the isolation of primary myoblasts, limbs derived from E18.5 foetuses were used. First, the muscle tissue was dissected from the skin and bones and homogenised. Homogenates were transferred into Hank’s buffered Salt Solution (HBSS; Biochrom AG/Thermo Scientific) supplemented with 2 mM MgSO₄ and 2 mM CaCl₂. Then, 50 μl 15% collagenase (Sigma-Aldrich) was added and incubated at 37°C and 1000 rpm for 40 min with rigorous vortexing in between. Each sample was supplemented with growth medium (DMEM (inc. 4.5 g/l glucose) BioWhittaker Cambrex), pipetted through a 40 μM cell strainer into 15 ml tubes and centrifuged at room temperature at 2000 rpm for 5 min. The cell pellet was resuspended in growth medium, added onto poly-D-lysine (1 mg/ml; Millipore) coated glass cover slips, incubated for 1 h at
room temperature and subjected to cytospin-preparation at 200 rpm for 5 min followed by fixation and immunofluorescence staining. For RNA isolation, cDNA synthesis and semi-quantitative PCR analysis, pure primary myoblast population was used.

**Isolation of primary postnatal mouse satellite cells**

Forelimb and hindlimb muscles harvested from day 7 postnatal mice (P7) were minced and digested using 20 µl collagenase A (100 µg/µl; Sigma-Aldrich) in DMEM in a shaking water bath at 37°C and 1400 rpm for 30 min followed by addition of 2 U/ml dispase (Sigma-Aldrich) for another 30 min. Next, the sample was passed through a 20G syringe followed by a 70 µm cell strainer to obtain a single cell suspension.

For the isolation of satellite cells via fluorescence-activated cell sorting (FACS), fluorescently labelled antibodies as listed below were added to the single cell suspension and incubated for 1 h on ice. The cells were washed three times in HBSS by centrifugation at 4°C 300 g for 5 min and subsequently resuspended in 500 µl HBSS containing 5% BSA. Cells were sorted via FACS-based exclusion to deplete cells expressing surface markers CD45, CD31, Ly-6A/E-Sca1 and Ter119 and positive selection for α-7 integrin using a BD FACSARia II (BD Biosciences).

**Supplementary Table S3: Antibodies used for FACS-based sorting of primary mouse satellite cells**

| Antigen          | Fluorophore | Used amount | provider           | Ordering number |
|------------------|-------------|-------------|--------------------|----------------|
| CD45             | APC         | 10 µl       | Life Technologies  | #17-0451-83    |
| CD31             | APC         | 10 µl       | Life Technologies  | #17-0311-82    |
| Ly-6A/E-Sca1     | APC/Cy7     | 10 µl       | Biolegend          | #108126        |
| Ter119           | APC         | 10 µl       | Life Technologies  | #17-5921-83    |
| α-7 integrin     | PE          | 5 µl        | AbLab              | #53-0010-05     |

**Cultivation and transfection of primary mouse myoblasts and satellite cells**

Sorted satellite cells were either seeded on matrigel coated (Corning® Matrigel® Basement Membrane Matrix Growth Factor Reduced) 10-mm cover slips for immunofluorescence stainings or 24-well plates for RNA isolation, cDNA synthesis and subsequent semi-
quantitative PCR analysis. Cells were cultured in DMEM containing 20% FCS, 10% horse
serum, 2 mM L-glutamine and penicillin (100 units/ml)/streptomycin (10 µg/ml) at 37°C and 5%
CO₂; the medium was exchanged every other day.
Differentiation was induced using DMEM supplemented with 5% horse serum, 2 mM
L-glutamine and penicillin (100 units/ml)/streptomycin (10 µg/ml).
For siRNA-mediated knockdown of mouse IRS4 in primary myoblasts or satellite cells,
Lipofectamine RNAiMAX (Invitrogen) was used according to manufacturer’s instructions; as
control non-targeting siRNA was used. In short, cells were transfected with 25-75 nM siRNA
and differentiation was induced 48 h post-transfection.

**Semi-quantitative PCR analysis**
RNA isolated from freshly isolated, pure primary myoblasts or isolated and cultured primary
satellite cells was subjected to cDNA synthesis and amplified by PCR using 2 µl cDNA
template, 2 µl dNTPs (1.25 mM; Fermentas), 1 µl of each specific primer (10 µM, see
Supplementary Table S2) and 0.5 µl Taq polymerase (5-10 U/µl; homemade) in a final reaction
volume of 20 µl. The amplification reaction consisted of an initial denaturation step at 95°C for
10 min followed by 30 cycles of 15 sec at 95°C and 60 sec at 60°C. PCR products were
separated on 1.5% agarose gels and visualised via ethidium bromide staining.