**Supplement**

**Material and Methods**

*Generation of a targeting vector for bag-1 gene knockout*

A mouse 129/Sv genomic library was screened using a mouse cDNA encoding the short form of *bag-1* as a probe. A positive phage containing an approximately 15 kb insert was identified (phage no1). A 14-kb *Hpa*I - *Not*I fragment from this phage was subcloned into Bluescript KS cut with *Eco*RV plus *Not*I and partially sequenced. This fragment was found to contain all seven coding exons of *bag-1* (Fig. 1a). To generate the 3´arm of the targeting vector, a 6 kb *Bam*HI fragment, located 3´of exon 2 was made blunt and subcloned into the unique *Xho*I site of the plasmid pPNT, and the resulting clone is designated p1988. To generate the 5´arm of the targeting vector, a 1,084 bp fragment located 5´to the start codon(s) in the first exon of *bag-1* was amplified via PCR using primers P5 (5´-GCG CGG TAC CAG CCG GGA AAT CTT TTT GTC) and P6 (5´-GCG CGG ATC CCT TTC CAC CTC TCC CAG AGC TC). The PCR was performed with HF Taq polymerase (Clontech). The primers had *Kpn*I and *Bam*HI sites at their ends to allow their ligation into the plasmid p1988 after cutting with *Kpn*I plus *Bam*HI, and the resulting clone is designated p1992. The final construct is shown in Fig. 1a.

The targeting vector (25 µg) was linearized by digestion with *Not*I and electroporated into E14 embryonic stem cells that were selected in G418 (400 µg/ml) and gancyclovir (2 µM). The G418-gancyclovir-resistant ES colonies were picked and subjected to PCR analysis using primers P1 (5´-GTT GTC ACC AGC CTT ACC TTA GC) and P2 (5´-GAT TCG CAG CGC ATC GCC TT). Positive ES clones were subjected to Southern analysis using a 5´probe of 400 bp external to the targeting construct. Three positive ES clones were injected into C57Bl/6 blastocysts and

transferred into pseudopregnant female recipients (Charles River). The resulting chimaeras were bread with C57Bl/6 females. Germline transmission was screened by coat colour and confirmed by the same Southern analysis.

*Genotyping of bag-1 mutant mice*

Heterozygous *bag-1* mice were genotyped by Southern blot analysis as described in Fig. 1. Alternatively, genotyping was performed by a PCR assay. The wild-type allele was detected with primers P1 and P4. The binding site for P3 (5´-ACT TCT AGT CAA CTC CCT GCT GCT) was deleted in the targeted allele. The targeted allele was detected with P1 and P2.

*Cultures of neural stem cells and primary embryonic fibroblasts*

The forebrain was dissected from 11.5 and 12.5 day old mouse embryos and transferred to 100 µl HBSS. After treatment with trypsin (Worthington; 0.05 %, 10 min), cell suspensions were generated by trituration. The trypsin was inactivated with trypsin inhibitor from egg yolk sack (Sigma; 0.05 %) and the cells were plated on 75 ml culture dishes (Greiner) in 5 ml Neurobasal medium (Invitrogen) containing Glutamax (1:100), B27 Supplement (Invitrogen) and basic fibroblast growth factor (bFGF) and Epidermal growth factor (EGF; Cell Concepts) at final concentrations of 10 ng/ml each. The cells were grown at $37^0$C in a 5 % $CO_2$ humidified atmosphere. Medium was changed every second day. The cells from the supernatant were centrifuged at 400g, triturated and cultured in fresh medium. Cells were passaged at least 6 times before they were taken for the first experiment. At this time, the cultures contained exclusively neurospheres. For the analysis of the cells they were centrifuged and plated on poly-DL-ornithine and laminin coated glass coverslips in the experiments shown in Fig. 4, and without laminin in the experiments shown in

Supplementary Fig. 2. The cultures were diluted so that a maximum of 6 colonies in total should grow on one coverslip. The cells were grown for 24 hours in the same medium and subsequently subjected to immunhistochemical analyses. Transfection of neurospheres with the RNAi oligonucleotides was performed using the nucleofactor transfection system (Amaxa) and double-stranded oligonucleotides with U6 promotor cassette was prepared using the Clontech PCR system. The oligonucleotide sequences used were: Bag-1RNAi-s: 5´-GGGCAACTAGCCAAATGTC-3' 5/6 Bag-1RNA1-as: 5´-GACATTTGGCTAGTTGCCC-3', scrambled-s: 5´-GGCGCAAGATACCATATGC-3´ and scambled-as: 5´-GCATATGGTATCTTGCGCC-3´. PCRs were performed according to the manufacturer`s instructions. PCR products for Bag-1RNAi-s and Bag-1RNAi-as as well as scrambled-s and scrambled-as were mixed in a 1:1 molar ratio and transfected to the neural stem cells. Independent transfections were performed using the pEGFP-C1 plasmid (Clontech). They revealed transfection efficacy in the range of 35-50%. Knock down of Bag-1 isoforms were evaluated by Western blot and immunohistochemical analysis and quantification of the respective band intensities for Bag-1.

For preparation of primary embryonic fibroblasts, the embryos were decapitated and the intestinal organs removed. The embryo bodies were washed three times in PBS and trypsinized in 0.1 % Trypsin/EDTA (Invitrogen) for 10 min at $37^0$C. Each single embryo body was passed through a 21 gauche needle and cells transferred to individual culture dishes. The plates were filled with DMEM medium containing 10 % FCS and 1 % non essential amino acids (NEAA, Invitrogen). Cells were passaged 3 times, then put on individual 10 cm cell culture plates and grown under low serum conditions (0.5 % FCS) for 24 h prior to stimulation with IGF-1 for the indicated time points.

*Westernblot techniques and antibodies*

Blots were blocked at room temperature for 1 hour with 5 % skim milk, in Tris buffered saline (TBS), pH 7.5 containing 0.1 % polyvinylpyrrolidone (PVP) and 0.01 % Tween-20. The blots were then incubated overnight at 4ºC with antibodies diluted according to the manufacturer´s instructions (Phospho-Ser112-Bad, Phospho-Ser136-Bad, Phospho-Ser155-Bad, Bad, FKHR, Phospho-Ser473-Akt, Phospho-Thr308-Akt, Akt, each 1:1000 (New England Biolabs/Cell Signaling; rabbit-anti-Bag-1, 1:400, from SantaCruz). Immunoreactive bands were detected with a horseradish-peroxidase-conjugated secondary antibody (260 ng per ml goat anti-rabbit, Biotrend). The membrane was then reacted with ECL reagent (Amersham) and exposed to X-ray film. Films were scanned with an INTAS Duo-Store image analysis system (INTAS).

*Immunofluorescence techniques for cell cultures and tissue sections*

Vibratome stections and cell cultures derived from 11.5 and 12.5-day-old embryos were fixed with 4 % paraformaldehyde, and either 14 µm paraffin sections were prepared or 200 µm Vibratome (TPI) sections. All staining procedures were performed at room temperature. Sections were rinsed 3 times with TGT buffer (40 mM Tris-Cl pH 7.8, 0.7 % NaCl, 0.04 % $KH_2PO_4$, 0.15 % $Na_2PO_4$, 0.1 % gelatine), and blocked with 10 % goat serum in TGT buffer. Primary antibodies used were: $p75^{NTR}$ monoclonal antibody (Chemicon), Islet 1/2 (Clone 39.4D5, DSHB, Iowa), nestin polyclonal antibodies (kind gift from R. McKay, NIH, Bethesda, ML), doublecortin (Chemicon), Pax6 (R&D Systems). The sections were again washed three times and incubated with Cy2 or Cy3-coupled secondary IgGs. Alternatively the

sections were incubated with DAPI and Propidium iodide. After 30 min the sections were washed three times with TGT buffer, mounted with PBS/50 % glycerol (v/v) and observed under a Leica confocal microscope (TCS, Leica). Numbers of overlapping pixels were quantified for P-Akt, B-Raf and Cytochrome-c-oxidase immunoreactivity in cultured motoneurons using the Leica confocal picture analysis software. Pixels were measured at maximal resolution of the confocal microscope.

Cells were fixed with 4 % paraformaldehyde in phosphate buffered saline (PBS). The cells were washed with Tris buffered saline (TBS; 25 mM Tris-Cl, pH 7.4, 0.8 % NaCl, 0.2 % KCl), 10 % goat serum and 0.05 % Tween-20. Cells were immunostained for 24 h at 4°C with rabbit anti-B-Raf (1:1000; and Cytochrome-c-oxidase; Molecular Probes), mouse anti neurofilament-M (Sigma), Doublecortin (Chemicon), Pax6 (R&D Systems) or Bag-1(SantaCruz) or rabbit anti nestin. Controls were performed in the absence of the first antibody. Cells were washed 3 times with TBS containing 10 % goat serum, and were incubated for 30 min with 2 µg/ml Cy-3 coupled goat anti-rabbit antibodies. Cells were washed again three times with TBS and 10 % goat serum, then covered with Mowiol in 50 % glycerol/PBS (v/v) and observed under a Leica confocal microscope. Serial vibratome sections from lumbar spinal cord of 4 *bag-1*$^{-/-}$ and 3 control littermates were stained against p75$^{NTR}$ and labelled cells in the ventrolateral region were counted under the confocal microscope. Counts were performed blindly before the result of genotyping was revealed to the investigator.

*Caspase-3 immunofluorescence in tissue sections of embryonic mice*

Embryos or tissue samples were fixed overnight in fresh buffered 4% paraformaldehyde and 7 µm paraffin serial sections were prepared. Deparaffinized sections were microwaved in 10 mM sodium citrate buffer (pH 5.5), washed in PBS.

Subsequently the slides were incubated in peroxidase blocking solution (1.5% $H_2O_2$ in PBS), washed with PBS and pre-incubated in blocking solution (5% goat serum in PBS). Primary antibody against Caspase-3 (Cell Signalling) was diluted 1:100 in blocking solution and applied overnight at $4^0$C. After subsequent processing with biotin conjugated goat anti-rabbit Ig (Dako) in blocking solution for 2 hours, antigen-antibody complexes were detected using fluorescent antibodies conjugated with Texas Red (Vector) according to the manufacturer´s instructions.

**Supplementary figures**

**Supplementary Figure 1**

**Activated caspase 3 immunoreactivity is increased in E10.5 and E11.5 *bag-1*$^{-/-}$ embryonic forebrain.**

(a) Antibodies against activated caspase 3 were used to detect cells in which proapoptotic signalling pathways are activated.

(b) Quantification of caspase 3 immunoreactive cells revealed increased numbers in *bag-1*$^{-/-}$ embryonic forebrain compared to *bag-1*$^{+/+}$ at E10.5 (see also table 1) and E11.5 (shown here and in table 1).

**Supplementary Figure 2**

**Enhanced apoptosis of differentiating neurons from *bag-1*$^{-/-}$ Pax6-positive neural stem cells.**

(a) Quantitative analysis of Pax6-positive cells in cultures of differentiating *bag-1*$^{+/+}$ and *bag-1*$^{-/-}$ neural stem cells after 2 days on Poly-L-Lysine.

(b) Quantitative analysis of Doublecortin (Dc)-positive cells in cultures of differentiating *bag-1*$^{+/+}$ and *bag-1*$^{-/-}$ neural stem cells after 2 days on Poly-L-Lysine.

(c) Quantification of cells with condensed nuclei in *bag-1*$^{-/-}$ and control cultures of neural stem cells.

(d) Images of *bag-1*$^{+/+}$ and *bag-1*$^{-/-}$ cultures of neural stem cells after 2 days under differentiating conditions on Poly-L-Lysine. Arrows point to condensed nuclei with Pax6 immunoreactivity. Scale bar: 10 µm.

(e) The number of originally plated neural stem cells (mean ± sd) in *bag-1*$^{-/-}$ cultures is significantly reduced after 48 h under differentiating conditions. Statistical analysis was carried out by student´s t-test: ***$P$<0.001 or **$P$<0.01.

**Supplementary Figure 3**

**Knock down of Bag-1 by RNAi results in loss of Bag-1 immunohistochemistry.**

Transfection of scrambled RNAi resulted in no detectable changes in Bag-1 immunohistochemistry, while transfection of the RNAi construct showed an almost complete loss of Bag-1 immunoreactivity in a portion of the cultured cells. Arrows point to cells that do not express Bag-1, arrowheads to condensed nuclei. Scale bar: 20 µm.

**Supplementary Figure 4**

**Enhanced apoptosis of differentiating neurons in the forebrain of *bag-1*$^{-/-}$ *mice*.**

(a) Quantitative analysis of apoptosis in Pax6-positive cells in the forebrain of E11.5 *bag-1*$^{+/+}$ and *bag-1*$^{-/-}$ mice.

(b) Quantitative analysis of apoptosis in the Doublecortin-positive (Dc) cell population in the forebrain of E11.5 *bag-1*$^{+/+}$ and *bag-1*$^{-/-}$ mice. Error bars represent ± SD.

(c) Immunohistochemical detection of Pax6, Doublecortin (Dc) and Bag-1 in the brain of *bag-1*$^{+/+}$ and *bag-1*$^{-/-}$ embryos (E11.5). DAPI staining was used as a nuclear marker to identify condensed or fragmented nuclei. Arrow heads indicate apoptotic cells. Bag-1 is expressed in Pax6-positive cells in *bag-1*$^{+/+}$ embryos. Note that the thickness of the neuroepithelium is reduced in *bag-1*$^{-/-}$ brain at E11.5. Scale bars from left to right: 80 µm, 80 µm, 80µm and 100 µm.

**Supplementary Figure 5**

Model for the interaction of a complex formed by Bag-1, Hsp70, B-Raf and Akt and its substrate Bad in wild-type (a) and disturbance of this process in *bag-1*$^{-/-}$ mice (b).

**Supplementary Table 1**

Number of apoptotic cells in spinal cord and brain sections of *bag-1*$^{+/+}$ and *bag-1*$^{-/-}$. * indicate significant differences measured by students t-test: ****P*<0.001.

| | apoptotic cells / section (n = 60) | |
|---|---|---|
| marker | *bag-1*$^{+/+}$ | *bag-1*$^{-/-}$ |
| Pax6, spinal cord, E11.5 | 1.23 ± 0.45 | 6.85 ± 0.77*** |
| Dc, spinal cord, E11.5 | 0.25 ± 0.14 | 0.38 ± 0.16 |
| Pax6, brain, E11.5 | 1.23 ± 0.45 | 6.85 ± 0.77*** |
| Dc, brain, E11.5 | 0.25 ± 0.14 | 0.38 ± 0.16 |
| Activated Caspase 3, brain E10.5 | 2.85 ± 0.72 | 10.69 ± 1.42*** |
| Activated Caspase 3, brain E11.5 | 1.23 ± 0.46 | 6.85 ± 0.77*** |