# Supplementary Information

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Supplementary Experimental Procedures

Cell culture and experimental treatments

F9 embryonal carcinoma cells were cultured DMEM with 15% fetal calf serum, 12% CO2, 37°C. MEFs immortalized with SV40 T and t antigens using plasmid 830Tt (iMEFs; Östling et al., 2007), murine neuroblastoma cells Neuro2A (N2A), and HeLa cells were cultured in DMEM with 10% fetal calf serum, 5% CO2, 37°C. Cells were exposed to EtOH or heat for 15 min to 24 h. EtOH was added directly to the culture medium. EtOH concentrations were determined as described in the paper. Cells were subjected to HS at 42°C in a water bath, or, alternatively into a cell incubator for long kinetics (from 3 to 24 h).

N2A cells in 24-well plates were transfected with Hsp70 promoter-Firely Luciferase and Renilla Luciferase expression vectors (ratio 1:5), using Lipofectamine 2000 (Invitrogen). After 24 hrs, the transfectants were exposed to EtOH (0.2 or 0.5%) or HS (42°C) treatments for the indicated times. Luciferase assays were performed with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions and firefly luciferase activity value was normalized to Renilla activity value. N2A cells, at 70% confluency, were cotransfected (ratio 1:1) with expression plasmids encoding Myc-epitope-tagged HSF1 (Holmberg et al., 2001) and green fluorescent protein (GFP)-fused expression plasmid SUMO-1 (Poukka et al., 2000) using Lipofectamine 2000 (Invitrogen) in Opti-MEM medium (Invitrogen) with a total of 24 µg DNA per 60 cm² dish. The green fluorescent protein (GFP)-fused expression plasmids SUMO-1 WT (kindly provided by Hetti Poukka, University of Helsinki, Helsinki, Finland), as well as the Myc epitope-tagged human HSF1 expression plasmid, have been described by Holmberg et al. (2001). HDAC inhibitors were added 3 hours before and during exposure to HS or EtOH (trichostatin (TSA), 1µM; valproic acid (VPA) 1 mM; nicotinamide (NAM) 10 mM).
For rescue experiments in iMEFs, Hsf2α and β cDNA, cloned from E16.5 mouse brain, were inserted into vector PCEMM_CTAP (Euroscarf P30536) with CMV-driven expression of insert and GFP used as an indirect reporter (IRES). Retrovirus production and iMEFs transduction was performed as described (Sandrin & Cosset, 2006).

**In situ hybridization**

Radioactive labelled probe for HSF1 or HSF2 corresponded to full-length cDNA (Sarge et al, 1991; Goodson et al, 1995). Radioactive ISH was performed as described (Wilkinson, 1992).

**Wound Healing Assay**

Independent clones of immortalized MEFs (iMEFs isolated after immortalization of MEFs by SV40 T and t) from \( Hsf2^{+/-} \) and \( Hsf2^{-/-} \; (\text{Östling et al, 2007}) \) or from the \( Hsf2^{+/-} \) knockout mice (1 \( Hsf2^{+/-} \) and 1 \( Hsf2^{-/-} \), immortalized according to the same protocol) were cultivated in DMEM high glucose pyruvate glutamax (Invitrogen) complemented with 10% FBS and antibiotics. Three to four independent experiments for each clone were performed. Subconfluent cultures were wounded by about 16 linear scratches (0.1 cm wide) per 10cm² dish, and subsequently treated with 1% EtOH. After 24h, control and EtOH-treated cells were stained with Coomassie blue and rinsed twice by PBS. Between 50 and 100 pictures per culture dish (the totality of scratch length) were taken by a Leica DFC420 C camera and binocular Leica S6D (magnification 16x). Either cell numbers that have migrated in the scratched zone, or the surface covered by these cells was calculated for each clone with Image J software. The same results were obtained with both methods.

Cell proliferation after 24 hrs was determined after Coomassie blue staining and by cell counting using the same method. No significant differences in proliferation were observed between untreated and EtOH treated samples.
**Immunocytochemistry**

HeLa cells were fixed with ice-cold acetone for 8-10 min. at -20°C and then submitted to immunocytochemistry with rabbit polyclonal anti-HSF1 (1/500; rabbit polyclonal Abcam) or mouse monoclonal anti-SUMO-1 (1/500; Zymed). Secondary antibodies: anti-rabbit Alexa488 and anti-mouse-Texas red. Pictures were taken on Zeiss, magnification 63x to 100x.

**Western blot analysis**

Tissue extracts and whole-cell extracts were prepared as described by Mosser et al (1988) or Loones et al (2000). 30–40 μg of proteins per sample was used for 9% SDS-PAGE. Anti-HSF1 monoclonal antibody was used at 1:2500 dilution (Ab4, Abcam ab81279 or ThermoFisher AB4-RT629); anti-HSF2 antibody at 1:2500 (3E2, Abcam); anti-SUMO-1 (GNP1, 33-2400, Invitrogen) was used at 1:400 dilution for tissue extracts; 1:1500 for whole cell extracts; anti-GFP (Santa Cruz, sc-3996) was used at 1:1500; anti-actin at 1:5000 (Ab20272, Abcam), anti-HSC70 at 1:5000 (SPA-815, Stressgen). In supplementary Fig S1B, HSF2 was detected using polyclonal antibody SFI57 (Östling et al., 2007; 1:2000 dilution) and HSF1 with rabbit anti-HSF1 (Santa Cruz sc-9144; 1:2000 dilution). Hyperphosphorylation of HSF1 was checked on 7% SDS-PAGE. Calf intestinal phosphatase (Biolabs) was used for 1 h at 37°C, according to the manufacturer recommendations.

**Nuclear/cytosolic fractionation**

Nuclear/cytosolic fractionation was done following a modified protocol from Mendéz and Stillman (2000). Three E16.5 mouse cortices were resuspended at 4°C in 100μL fresh Buffer A (10mM Hepes pH7.9; 10mM KCl; 0.34M sucrose, 10%glycerol, 1 mM DTT, 1 mM PMSF,
0.03% NP40, 0.15mM spermine, 0.15mM Spermidine, 1x phosphatases and protease inhibitors cocktails (Roche)). Extracts were incubated for 5 min on ice. Nuclei were collected in pellet 1 (P1) by low-speed centrifugation (4 min, 1,300 × g, 4°C). The supernatant (S1) was further clarified by high-speed centrifugation (15 min, 20,000 × g, 4°C) to remove cell debris and insoluble aggregates and constitute the cytosolic extract. Nuclei were washed once in buffer A, resuspended in 125µL extraction buffer (10mM Hepes pH7.9; 0.4M NaCl; 0.1mM EGTA, 5%glycerol, 0.5 mM DTT, 0.5mM mM PMSF, 1x phosphatase and protease inhibitors cocktails (Roche)) and were then submitted to 2–4 rapid freeze-thaw cycles, as described (Mezger et al., 1989). High-speed centrifugation (30 min, 20,000 × g, 4°C) clarified supernatant (S2) that constitute the soluble nuclei extract used for western blot. The following antibodies were used: anti- Phospho-Ser^{326}-HSF1 (ADI-SPA-902, Enzo Life Sciences; 1:1000); anti-Histone H3 (Abcam ab1791; 1:1500).

**Immunoprecipitation**

HSF was immunoprecipitated from 300 µg of total extracts from embryonic cortices or cell extracts with 5 µL of anti-HSF antibodies (1 µg). Conversely, immunoprecipitation was performed with 5 µL (1 µg) of anti-acetyl-Lysine (Millipore, AB3879) or of anti-SUMO-1 (GNP1, 33-2400, Invitrogen) and blotted against anti-HSF1 (1:2000). The pull-down was performed using Agarose beads coupled to protein G according to the manufacturer’s instructions (Roche). For HSP70 or HSP90 detection, mouse mAbs (SPA820 P conjugate) were purchased from and used as described (StressGen). In Fig 3D, anti-HSF1 monoclonal antibody (Ab81279) was used at a 1:2500 dilution. Anti-SUMO-1 was used at 1:400.

**In vitro EGS cross-linking**

EGS (Ethylene glycol disuccinate bis (sulfo-N-succinimidyl) ester sodium salt (1; Sigma...
Aldrich, 39104), was added from 1 mM to 10 mM (for cells) and up to 20 mM (for fetal cortices) final concentrations, followed by incubation at 22°C for 30 min. After quenching of the cross-linking reactions with an excess of glycine (75 mM final concentration), samples were resolved by electrophoresis through a 9% or 7% SDS-polyacrylamide gel and analysed by immunoblotting with antibodies specific to HSF1 (1:2500; rat monoclonal, Abcam ab81279) or HSF2 (1:2500, 3E2, rat monoclonal, Abcam ab44824).

Transfection and SiRNA

SiRNAs was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Functional, non-targeting control siRNA (Dharmacon) was used as negative control (D-001810). The targeted sequences of the Hsf2 gene were: #1: 5’GAGGGGAGUACAACUGCAUU3’; #2: 5’CAGCAGGACUGAAGGUUUAUU3’. For each well, 500 pmol RNAi duplex in 250 µl Opti-MEM (Invitrogen, 31985), and 5 µl of lipofectamine in 250 µl Opti-MEM media were combined and mixed gently. After 15 min at room temperature, the RNAi mixture was added to each well containing cells. Medium was changed 6 h after transfection, and cells were incubated for an additional 24 h prior to the experiments.
**Antibodies used in immunohistochemistry**

The following antibodies and dilution used for immunohistochemistry and further detected using avidin-biotin Vector kits, using Diaminobenzidine as a chromogen.

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<tr>
<th>Antibody</th>
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<tr>
<td>HSF1</td>
<td>Neomarker rt-629</td>
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<tr>
<td>HSF1</td>
<td>Abcam ab47369</td>
<td>1:100</td>
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<tr>
<td>HSF2</td>
<td>Abcam ab44824</td>
<td>1:10</td>
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**Bioinformatic web sites**

Transfac (http://www.gene.regulation.com)

Genomatix (http://www.genomatix.de)

iHOP (http://www.ihop-net.org)

ENSEMBL (http://ensembl.org)

These web sites were used for the search of HSE in HSF2 target genes or primer design.

**Primers for ChIP and RT-qPCR experiments**

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<tr>
<th>ChIP primers</th>
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<td>5’-TATGTCTGACCCAGAGGAGAGA-3’</td>
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<tr>
<td>Cyclophilin</td>
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<td>5’-TCAGCGTCTGTCGACCCTGACC-3’</td>
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<tr>
<td>Hsp70.1</td>
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<td>5’-TCAGCGTCTGTCGACCCTGACC-3’</td>
</tr>
<tr>
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<td>5’-CCGCACAGGACACTGAAGGC-3’</td>
</tr>
<tr>
<td>Dlk1</td>
<td>5’-CCACACACAGGGCCTGGTCC-3’</td>
<td>5’-TCACATTATCCAGAGAGGTCG-3’</td>
</tr>
<tr>
<td>Dcx</td>
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<td>CGCAACAGAAATTGCAGAGTAG</td>
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<td>5’-TTA CCC GCA GTC AGG CAG GC-3’</td>
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<td>Myo10</td>
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<td>5’-GAGGGAGGGTTAGAAGAAAA-3’</td>
</tr>
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<td>5’-TTGCTCTCCAGGGATCTGT-3’</td>
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<td>RT-qPCR primers</td>
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<td>Reverse</td>
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<td>NudC</td>
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<td>5’-GCCTAGTCTCAGTGAGGCTT-3’</td>
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<td>5’-GCCGGCTGCAATTGTCGCCGC-3’</td>
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<tr>
<td>Cdk5rap2</td>
<td>5’-CACCATATGCTGGGCTTGAT-3’</td>
<td>5’-GCTCAAGGCGCTTCAGTGGTT-3’</td>
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<tr>
<td>Myo10</td>
<td>5’-CTTTGAGGAGGAAACTGGAAG-3’</td>
<td>5’-CGTGGCAAGTACCTAGTGGATG-3’</td>
</tr>
<tr>
<td>Map1</td>
<td>5’-CGAAGATCGCCACACACTCC-3’</td>
<td>5’-TGACCTGGAGGAGATCCTATG-3’</td>
</tr>
<tr>
<td>Mark2</td>
<td>5’-GGGACCAGCAGAATCTCTACCC-3’</td>
<td>5’-GCTGAAAGTAGCTGCCAGAG-3’</td>
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<td>Dyn1h1</td>
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<td>5’-GGGAGCTGAGGAGATCAATT-3’</td>
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<td>Tubb2b</td>
<td>5’-CAAGATGTCCGACCCACTTCA-3’</td>
<td>5’-GCCGAACATAGCAGTGAAAC-3’</td>
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</table>

**Supplemental References**


KIF5C and KIF2A cause malformations of cortical development and microcephaly. Nat Genet 45: 639-647


Legends to Supplementary Figures

Figure S1. Modes of chronic prenatal alcohol exposure, HSF1 and HSF2 expression profiles in the developing brain, and induction of HSF1 DNA-binding activity and persistence of HSF2 DNA-binding activity in the fetal cortex exposed to alcohol (related to Fig 1).

(A) **Effect of different modes of chronic fetal alcohol exposure on BrdU incorporation:** feeding with alcohol-containing food (CAI), daily force-feeding (gavage, GAV), or twice-daily chronic intraperitoneal injections (CIP). (Left) Quantification of BrdU-labelled cells at E16.5, per 0.05 mm² (n = 5 different section on 5 different animals; upper panel; p-values: CAI, p= 0.0049; GAV, p = 0.0011; CIP = 0.0099) or per 100 cells (lower panel; p-values: CAI, p= 0.0010; GAV, p = 0.0531; CIP, p = 0.0153). A decrease in BrdU incorporation upon FAE was observed in the germinal proliferative zones (gz). *: p ≤ 0.05; **: p ≤ 0.01. Data information: Differences were considered statistically significant when P values were <0.05. When Anova was found significative (p<0.05) we performed the Student’s *t*-test with Bonferroni adjustment. Data are presented as mean ± s.e.m. (Right) Representative images corresponding to the left panels (scale bars, 10 μM).

(B and C) **HSF1 is ubiquitously expressed in the developing brain.** (B) Upper and middle panel: WB analysis of whole-cell protein extracts from fetal mouse telencephala (E13) or cortices (E15-E18) at different stages, with anti-HSF1 or anti-HSF2 antibodies. For each stage, equal loading (40 μg) was verified by antibodies against actin. Tel, telencephalon; Fl, forelimb; Hl, hindlimb; N2A, Neuro2A neuroblastoma cells (positive control). Lower panel: major HSF2 isoforms α and β are indicated (Fiorenza et al, 1995; Goodson et al, 1995); F9 embryonal carcinoma cells (positive control; Rallu et al, 1997).

(C) (Left) **In situ hybridization analysis of Hsf1 and Hsf2 mRNA levels (white signal) in parasagittal sections of the mesencephalon at E14.5.** vz, ventricular zone; ven, ventricle.
(Right) Immunohistochemical detection of HSF1 or HSF2 in the cortex at E16.5. cp, cortical plate (brown signal). Arrowhead points to outer layers.

(D) Chronic EtOH exposure by gavage or twice-daily intraperitoneal injections activates **HSF1 and keeps HSF2 active in the fetal cortex.** Gel-shift assays of HSF1 and HSF2 DNA-binding activities using supershifting with HSF1 (α1) or HSF2 antibodies (α2) in E16.5 cortices after gavage (GAV), or twice-daily intraperitoneal injections (CIP). The specific supershifting induced by the addition of anti-HSF1 (α1) or anti-HSF2 antibodies (α2) is illustrated by black and white arrowheads, respectively. HSF-HSE, CHBA, NS, free: as in Figure 1B. The specific supershift induced by the addition of anti-HSF1 (α1) or anti-HSF2 antibodies (α2), was illustrated by red and blue bars, respectively (no antibody: black bars), as in Fig 4.

**Supplemental information relative to Figure S1** (related to Fig 1).

HSF1 is expressed in the neuroepithelium of various species (reviewed in Abane & Mezger, 2010). We observed that HSF1 was expressed at each stage of corticogenesis, as was HSF2 (Fig S1B). As previously described (Rallu et al, 1997; Kallio et al, 2002; Chang et al, 2006), HSF2 expression was restricted to the proliferative zones of the brain (FigS1 C, left panel), including the cortex, and was also expressed in the upper layers of the developing cortical plate from E15.5, (cp; FigS1 C, right panel; arrow). In contrast, Hsf1 mRNA and protein were ubiquitously expressed in the proliferative and non-proliferative zones of the developing brain, including the cortex (Fig S1C).
Figure S2. HSF1 nuclear localization increases after CAI.

(A) Representative images of subcellular localization of HSF1 detected by immunohistochemistry. Lateral cortex from control (CTR) and CAI fetuses at E16.5 (magnification 4×100x; Scale bars, 10 µM).

(B) Quantification of the number of HSF1-positive nuclei before or after CAI (**, p< 0.01).

(C) Representative immunoblot analysis of nuclear-cytoplasmic subcellular distribution of Phospho-Ser326-HSF1 after fractionation of control (CTR) and E16.5 cortices exposed to CAI, and the corresponding quantification (n=2).

Supplemental information relative to Figure S2 (related to Fig 1).

The number of neurons showing nuclear HSF1 labelling increases significantly in cortices treated with CAI (FigS2A and B). To verify that HSF1 translocated to the nucleus after CAI was in an active form, we performed nuclear-cytoplasmic fractionation of E16.5 cortices and checked for the presence of HSF1 phosphorylated on serine 326 (conserved between human and mouse species) using an antibody against PhosphoSer326-HSF1. An increase in the signal corresponding to PhosphoSer326-HSF1 was observed in the nucleus after CAI, but not in the cytoplasm (although an increase in total HSF1 protein levels was observed in the cytoplasm). Some “leaking” of nuclear material was observed in the cytoplasmic fraction as often occurs with tissues, however, despite this, an enrichment of the active PhosphoSer326-HSF1 form was observed in the nucleus (and not in the cytoplasm). We therefore concluded that the nuclei of cortical neurons are enriched in an active form of HSF1 after CAI, in line with Hashimoto-Torii et al, 2014.
Figure S3. (A) Schematic representation of HSE sites in mouse p35, Dcx, Dclk1, Chl1, MapT, Myo10, and Mark2 genes. The position of the HSEs, which was determined using Genomatix software and conserved between at least 3 mammalian species (humans, mice and rats or humans, cows and mice) and that were found to be bound by HSF2 in our ChIP experiments (p35, Dcx, Dclk1, Chl1 Fig 2A and see also Fig S8C) or in ChIPSeq experiments (Myo10, MapT, and Mark2; Mendillo et al, 2012; Vihervaara et al, 2013) and whose expression was affected by fetal alcohol exposure in an HSF2-dependent manner (Fig 6C), are schematically indicated. For p35, where GC boxes and the HSE have already been described in the literature, they are indicated as grey boxes as in Chang et al., 2006). For the other genes, HSEs are illustrated by grey ellipses. For Dcx, the HSE was positioned at -160 nucleotides from the beginning of the 5’-UTR in a region known to be important for Dcx expression (Karl et al, 2005). In the case of Dclk1, the HSE was positioned according to the ATG (+171 nucleotides downstream). In Myo10, the HSE was positioned at +814 nucleotides from the transcription start, for Mark2, the first intron at +8536 nucleotides from the beginning of the 5’-UTR. The sequence of the HSE (in red) in p35 and Chl1 allows for the binding of only one HSF trimer.

Figure S4. CAI results in a decrease in p35 protein levels, in line with reduction in p35 mRNA levels in fetal cortices.

(Left) Representative immunoblot of p35 protein expression in cortices in Hsf2+/− mice before (CTR) or after CAI, and comparison with the levels of p35 in Hsf2+/− control cortices. (Right) quantification (n= 2 experiments).
Figure S5. Alcohol exposure activates HSF1 and HSF2 iMEFs. (Related to Fig 3).

(A and B) Ethanol exposure induces HSF1 and HSF2 DNA-binding activity in iMEFs. Gel-shift analyses of HSF1 and HSF2 DNA-binding activities in immortalized Hsf2+/+ iMEFs, using supershifting with anti-HSF1 (α1; black arrowhead) or -HSF2 antibodies (α2; white arrowhead) after ex vivo exposure to 1% EtOH for 3 hours (A; 1-2% is the optimal EtOH concentration for HSF1 induction in iMEFs) or after a 1-hour HS at 42°C (B). HSF-HSE, CHBA, NS, free: as in Fig 1.

(C) ChIP analysis of the recruitment of HSF1 and HSF2 by the Hsp70 promoter in control iMEFs (CTR), after HS (42°C) or 1% EtOH exposure (35 min). Cyclo: the Cyclophilin B gene that does not contain HSE was used as a negative control. Hsf2-/- iMEFs were used as a negative control for in vivo HSF2 binding.

(D and E) EtOH induces persistent HSF1 DNA-binding activity ex vivo. (D) Gel-shift assays of the kinetics of induction of HSF DNA-binding activity upon ex vivo treatment of Hsf2-/- iMEFs by either a 41°C HS or 2% EtOH. HSF-HSE, CHBA, NS, free, α1: as in Fig 1.

(E) Quantification of the intensity of the HSF1-HSE complex in two additional independent kinetic experiments.

(F) Induction of Hsp70 by ethanol exposure in iMEFs. (Left) Quantitative RT-PCR analysis of Hsp70 mRNA levels upon exposure to different EtOH concentrations (upper panel) or HS at 41°C for 0.5 or 1.0 hour, without or with 1.0 hour of recovery (1.0+1.0 R) at 37°C (lower panel). n= 6. *: p < 0.001, **: p= 0.00189, and ***: p= 0.0013. The increase in Hsp70 mRNA levels with 0.2 and 0.5% EtOH was not statistically significant (upper panel) in the case of iMEFs. Data information: Differences were considered statistically significant when P values were <0.05, using unpaired two-way Student's t-test. Data are presented as mean ± s.e.m. (Right) Western blot analysis of Hsp70 accumulation after EtOH exposure at
different concentrations (1 hour; upper panel) or HS stress (1 hour at 42°C) and 3 h of recovery (lower panel). Tub: tubulin.

**Supplemental information relative to Figure S5** (related to Fig 3).

We analyzed the HSE-binding activities of HSFs in iMEFs upon EtOH exposure: iMEFs displayed an increase in HSF DNA-binding activity to heat shock elements (HSE) in gel-shift assays, with the contribution of HSF1 as well as HSF2, as indicated by the supershifting of the whole HSF-HSE complex by either anti-HSF1 or HSF2-antibodies (Fig S5A; black and white arrowheads, respectively). In contrast, heat-shocked iMEFs typically showed only HSF1 activation, as expected in gel-shift assays (Fig S5B; black and white arrowheads, respectively). By ChIP, we observed that the *in vivo* binding of HSF2 to the *Hsp70* promoter was increased in iMEFs treated by EtOH as it is upon HS (Fig S5C; see also Östling et al, 2007; Ahlskog et al, 2010). In addition, HSF1 DNA-binding activity is persistently induced by alcohol exposure (Fig S5D and E).

HSF1 and HSF2 binding at the *Hsp70* promoter region was followed by a 2- and 4-fold increase in *Hsp70* mRNA levels, in response to 1 or 2% EtOH exposure, respectively (Fig S5F, left panel). The elevation in *Hsp70* mRNA and protein levels was not as high as for HS, in line with the lowest DNA-binding activity of HSF observed in EMSA, compared with HS (Fig S5A and B).

These experiments led us to conclude that HSF1 and HSF2 are both activated in terms of DNA-binding in response to *ex vivo* alcohol exposure, and influence the expression of classic *Hsp* target genes.
**Figure S6. Persistent HSF1 activation and post-translational modifications.**

A) Gel-shift assays from N2A cells exposed to heat shock (42°C; 0.5 h) or EtOH (0.5%, which we have determined is the optimal dose of EtOH to provoke HSF1 activation in N2A cells) for 5 or 7 h.

(B) N2A cells were transfected with an *Hsp70-Luciferase* reporter construct. After 24 hours, transfected cells were exposed to EtOH (0.2 or 0.5%) or HS (42°C) for the indicated times. Values are indicated as the ratio of luciferase activity present in stressed samples versus control samples. The data represent means ± SD from 12 independent experiments performed for each time point and each condition.

(C) Quantitative RT-PCR analysis of *Hsp25*, *Hsp70*, and *Hsp90* mRNA levels in N2A cells exposed to 0.5% EtOH for 1 or 2 hours. (n=3; asterisk, p<0.001). P-values for *Hsp25*, p = 0.000567 for 1 hour and p = 0.000467 for 2 hours; for *Hsp40*, p = 0.000912 for 1 hour and p = 0.000601 for 2 hours; for *Hsp70*, p = 0.000334 for 1 hour and p = 0.000167 for 2 hours; for Hsp90, p = 0.000587 for 1 hour and p = 0.000321 for 2 hours. Data information: Differences were considered statistically significant when *P* values were <0.05, using unpaired two-way Student's *t*-test. Data are presented as mean ± s.e.m.

(D) Representative immunoblots (left) and corresponding quantification (n= 2 experiments), showing increased levels of HDAC1 and SIRT1 (but not of HDAC2) in N2A cells exposed to EtOH, that might at least transiently explain the lack of HSF1 acetylation upon EtOH exposure.

(E) (Left) Control for HSF1 hyperphosphorylation in iMEFs: calf intestinal phosphatase (Calf Int. Phos.) abrogates the HS-induced HSF1 migration shift characteristic of HSF1 hyperphosphorylation in WB. (Right) Immunoblotting of HSF1 phosphoSer303/307 (Phos-Ser303/307): iMEFs (HS at 43°C for 15 min or 2% EtOH for 0.5 h).
(F) (Upper panel) Immunoblotting of HSF1 phosphoSer303/307 (Phos-Ser303/307). N2A extracts as in (Fig 3G). Immunoblot analysis of the level of Ser303/307 phosphorylation of HSF1. (Lower panel) Corresponding quantification of the ratio of HSF1 phosphorylation at Ser303/307 in stress versus control situations. Loading control: β-actin.

**Figure S7. EtOH induces delayed colocalization of HSF1 and SUMO-1 in nuclear stress bodies, compared with HS** (related to Fig 3).

Immunocytochemical detection of HSF1 and SUMO-1. HeLa cells were untreated (C), heat-shocked at 42°C or treated by 0.5% EtOH, and/or allowed to recover (after a 2h stress) at 37°C for the indicated times. White arrows point to nuclear stress bodies (nSBs), in which HSF1 and SUMO-1 are colocalized. Magnification: 63x.

**Supplemental information relative to Figure S7.** (related to Fig 3).

We confirmed the specific profile of HSF1 sumoylation upon EtOH exposure, by taking advantage of the propensity of human cells to accumulate HSF1 in nuclear stress granules or bodies (nSBs) upon stress. nSBs typically appear as brightly stained irregular subnuclear structures in immunocytochemistry (Cotto et al 1997; Jolly et al 1999; Sarge et al 1993). HSF1 and SUMO-1 are prominently colocalized in nSBs, although only transiently even during prolonged HS (Hietakangas et al, 2003). Accordingly, we found that the distribution of HSF1 was initially diffuse, and did not overlap with that of SUMO-1 in control HeLa cells, but accumulated in nSBs and was colocalized with SUMO-1 upon a 1-h or 2-h HS. This HSF1 localization in nSBs started to disappear at 4 h and was absent during recovery. In contrast, upon EtOH exposure, no nSB could be observed at 1 h (although the diffuse HSF1 pattern was slightly different from what was observed in control cells). nSBs with HSF1 and SUMO-1 colocalization were observed from 2 h of EtOH exposure and persisted both at 4 h,
and after a 3-h recovery period. Similar delayed and persistent HSF1 and SUMO-1 colocalization was obtained with 0.2% or 2% EtOH (data not shown).

Figure S8. Alcohol induces the formation of HSF1-HSF2 heterocomplexes with specific migration signatures in gel-shift assays in F9 cells (related to Fig 4 and 6C (C) for the part).

(A) Gel-shift assays after EtOH exposure (0.5%) or HS at 43°C in F9 embryonal carcinoma cells. In these cells, as in the developing cortex, HSF2 displays high constitutive DNA-binding activity, but HSF1 does not (Rallu et al, 1997). Black, red, and blue bars as in Fig 4.

(B) Corresponding quantification of the intensity and supershifting of HSF-HSE complexes in stressed F9.

(C) ChIP analysis of the binding of HSF1 and HSF2 to the HSEs of the Nde1 and Chl1 genes in CTR and CAI situations at E16.5.

(Upper panels) Quantification of the occupancy of HSE by HSF1 or HSF2 using ChIP and quantitative PCR (ratio of the ChIP signal versus input signal) on Nde1 and Gapdh (left) and Chl1 and Cyclophilin B genes in E16.5 fetal cortices from control dams (C) or those subjected to CAI (CAI); for Nde1 and Gapdh, n=6 independent litters for CAI; n=3 independent litters for controls; for Chl1 and Cyclophilin B, n=2 independent litters for C and n=4 for CAI. Hatched and solid bars: ChIP with anti-HSF2 and anti-HSF1, respectively. Color code for gene families: Nde1 (orange), Chl1 (dark gray). P-values for Hsp70: p = 0.001482 for HSF1 enrichment in CAI compared to C, and p = 0.000686 for HSF2; for Nde1 (CTR/CAI: for HSF1 p = 0.00291. For Chl1: no significant enrichment for HSF1 (p = 0.09675); significant reduction in enrichment for HSF2 in CAI compared to C (p = 0.04123). *** p≤0.001, ** for p≤0.01; * for p≤0.05. as in Fig 2A. For Chl1: (p=0.09675). No enrichment of HSF2 or HSF1 for Gapdh (p = 0.176985 for HSF1 and p =0.195512 for HSF2) and Cyclo
(p= 0.133646 for HSF1 and p=0.152153 for HSF2). Data information: Differences were considered statistically significant when P values were <0.05, using unpaired two-way Student's t-test. Data are presented as mean ± s.e.m.

(Lower panel) *Nde1* HSE is located within the gene body at +14077 nucleotides from the ATG. This HSE can be occupied by only one trimer, which, altogether with our biochemical analyses, is in favor of binding of both HSF1 and HSF2 as heterotrimers upon CAI.

**Supplemental information relative to Figure S8.** (related to Fig 4 and 6C).

*Nde1* is a gene that is not notably affected by alcohol or by HSF2 (see supplementary Fig 11C). However, it is bound by HSF2 in control conditions and by HSF1 and HSF2 upon CAI. This HSE can by occupied by only one trimer, which, altogether with our biochemical analyses, is in favor of binding of both HSF1 and HSF2 as heterotrimers upon CAI. We therefore used this gene as a model for in vivo binding of HSF1 and HSF2 heterotrimers (see working hypothesis, Figure 4A).

**Figure S9. Alcohol induces HSF1 and HSF2 oligomerization** (related to Fig 4)

(A) WB analysis of the oligomeric state of HSF1 and HSF2 after chemical crosslinking with EGS in N2A whole cell extracts (9% SDS PAGE).

(B) Same as in (A) but run on a 7% SDS PAGE to allow better observation of trimeric forms.

(C) Same as in (B) but with E17.5 cortical extracts from control or CAI fetuses.

Arrow: position of HSF1 or HSF2 monomers. Arrowhead: position of HSF1 or HSF2 trimers. Mono-, di- and trimers are indicated by gray ellipses. Asterisk: band typically observed for HSF1 and HSF2 in E10.5 to E17.5 cortical extracts, possibly derived from alternative splicing, transcription starts, or post-translational modifications. In cortices, both HSF1 and HSF2 were present in complexes of similar sizes, which seemed to extend towards even
higher molecular weights ($\geq 250$ kDa). In addition, their intensity was enhanced after CAI at 10\(\mu\)M EGS, likely corresponding to trimers or higher order oligomers, which may correspond to interactions between heterotrimers as demonstrated for Drosophila HSF (Clos et al, 1990; Xiao et al, 1991), or between heterotrimers and other proteins. Note that discrete bands were detected in uncrosslinked samples due to difficulties in EGS quenching and, possibly, to the diffusion of the remaining EGS in the 7% gel. Higher concentrations of EGS were necessary to crosslink HSF complexes from embryonic cortices (20 \(\mu\)M).

**Supplemental information relative to Figure S9.** (related to Fig 4).

**HSF2 and HSF1 both participate in trimeric complexes**

Since fetal alcohol exposure in embryonic cortices led to both persistent HSF1 and HSF2 binding activities, we asked whether these two molecules co-existed as homo- or heterotrimers. The binding of both HSF1 and HSF2 to the \textit{Nde1} gene, which has an HSE site that cannot accept more than a single trimer (Fig S8C and 4A) was suggestive of the induction of heterotrimer formation upon fetal alcohol exposure. It is known that the activation of either HSF1 or HSF2 is accompanied by homotrimerization (Sarge et al, 1993; Sistonen et al, 1994; Sandqvist et al, 2009). We thus analyzed the oligomeric status of the EtOH-resistant/induced HSF2 and EtOH-induced HSF1 by chemical crosslinking with EGS, followed by WB analysis. In contrast to HS, which did not notably modify HSF2 oligomerization, EtOH exposure led to increased accumulation of HSF2 in dimeric and trimeric forms (>170 kDa and < 250kDa; Supporting Information Fig S9A). Similar results were obtained for HSF1, although in this case, the effect of EtOH was not as strong as that of HS (Supporting Information Fig S9A), in line with the intensities of HSF-DNA complexes in gel-shift assays (Fig 4B). Trimers clearly appeared as the major oligomeric species in low acrylamide percentage gels, upon HS or EtOH treatments (Supporting Information Fig S9B). Importantly,
the presence of HSF1 and HSF2 in alcohol-induced trimers was also observed in fetal cortices from dams treated with CAI (Supporting Information Fig S9C). These trimers could be homotrimers containing only HSF1 or HSF2 or, conversely, heterotrimer.

Figure S10. HSF2 is not necessary for HSF1 activation in iMEFs and protects iMEFs from migration defects in response to alcohol (related to Fig 5 and 6).

(A) In contrast to its essential role in fetal cortices, HSF2 is not necessary for HSF1 activation in iMEFs. Gel-shift assays of $Hsf2^{+/+}$ iMEF extracts (from $Hsf2^{tm1Mmr}$ knockout mice), showing that HSF1 activity can be observed and induced by 1.0% EtOH in the absence of HSF2, in contrast to what is observed in $Hsf2^{-/-}$ fetal cortices. HS: 1 hour at 42°C. Note that, as in WT iMEFs, HSF1 activity induced by EtOH is lower than with HS.

(B) The lack of HSF2 enhances alcohol-induced migration defects in iMEFs.

(Left) Representative wound healing assays upon 1% EtOH exposure for 2 $Hsf2^{tm1Mmr}$ iMEF clones ($Hsf2^{+/+}$ and $Hsf2^{-/-}$; Kallio et al, 2002) before and after 24 h of migration. (Right) Quantification of the number of cells that migrated into the wound (Ratio between values from EtOH-exposed and control cells), for 3 independent clones of each genotype from $Hsf2^{tm1Mmr}$ or one from the $Hsf2^{tm1Ijb}$ knockout model (McMillan et al, 2002); average of the values for $Hsf2^{tm1Mmr}$ clones. Hatched and dotted bars: $Hsf2^{+/+}$ and $Hsf2^{-/-}$, respectively.

(C) Reintroducing HSF2 in $Hsf2^{-/-}$ iMEFs reinstates alcohol-induced migration defects.

Rescue experiment and quantification as in (B). (Left) Ratio between values from EtOH-exposed and control cells. Stable HSF2 α or β expression in an $Hsf2^{tm1Mmr/tm1Mmr}$ clone diminishes or abolishes EtOH-induced migration defects, respectively. The dotted line indicates a ratio equal to one. (Right) Representative immunoblot of HSF2 α or β expression levels in $Hsf2^{tm1Mmr/tm1Mmr}$ iMEFs stably expressing HSF2 α or β.

(D) Western blot analysis of the HSF1 expression levels in the developing cortex
**compared to other organs** Tel, telencephalon; FL, forelimbs; HL, hindlimbs. F9 embryonal carcinoma cells were loaded as positive control. Loading control: non-stress inducible Heat shock cognate, Hcs70. **HSF1 expression levels are lower in the developing cortex compared with other organs** (illustrated here at E13.5). In contrast, we previously observed that HSF2 is expressed at high levels in the developing cortex compared with other organs (see Rallu et al, 1997), This means that the HSF1:HSF2 ratio is lower in the developing cortex compared with fore- or hind-limbs.

**Supplemental information related to Figure S10.** (related to Fig 5 and 6).

**HSF2 is not required for HSF1 activation by alcohol exposure in iMEFS**

We first examined the response of *Hsf2*<sup>-/-</sup> iMEFs to ethanol. In contrast to what was seen in the fetal cortex and in N2A cells, the absence of HSF2 did not compromise the activation of HSF1 upon ethanol exposure in iMEFs.

**Loss of HSF2 increases the severity of EtOH-induced migration defects in iMEFs**

We compared the migration of *Hsf2*<sup>+/+</sup> and *Hsf2*<sup>-/-</sup> mouse embryonic fibroblasts (iMEFs) in wound-healing assays, after exposure to EtOH. We analyzed three independent *Hsf2*<sup>+/+</sup> iMEF clones (Östling et al, 2007) and three independent *Hsf2*<sup>-/-</sup> clones from the *Hsf2*<sup>tm1Mmr</sup> knockout (Kallio et al, 2002); and one of each genotype from the *Hsf2*<sup>tm1Ijb</sup> knockout (McMillan et al, 2002). The migration of *Hsf2*<sup>-/-</sup> iMEFs was markedly and reproducibly impaired in the presence of EtOH, whereas the migration of *Hsf2*<sup>+/+</sup> clones was only slightly affected by EtOH (Fig S11C). HSF2 is expressed as two major isoforms, HSF2<sub>α</sub> and β, which seem to display differential effects on *Hsp* genes (See Fig S1B; Fiorenza et al, 1995; Goodson et al, 1995; He et al, 2003; Leppä et al, 1997). Rescue experiments in one *Hsf2*<sup>-/-</sup> iMEF clone from the *Hsf2*<sup>tm1Mmr</sup> knockout showed that the overexpression of HSF2<sub>α</sub> or HSF2<sub>β</sub>, respectively, partially or totally abolished the impairment of *Hsf2*<sup>-/-</sup> iMEFs (Fig S11C). These results
suggested that the migration defects induced by alcohol in iMEFs are limited in the presence of HSF2, but exacerbated by its absence, in contrast to what happens in the fetal cortex upon FAE.

**Figure S11.** (A) Neuronal positioning defects induced by ETOH are less pronounced in *Hsf2*<sup>-/-</sup> (KO) compared with *Hsf2*<sup>+/+</sup> (WT) fetuses (related to Fig 6). Non-normalized (raw) data of the number of BrdU-labelled cells in fetal cortices from pregnant dams, non-exposed (CTR) or chronically intoxicated with food containing EtOH *ad libitum* (CAI) per 0.05 mm<sup>2</sup>. Superficial layers, I-III; deep layers, IV-VI (n = 3 litters per condition). P-values for *Hsf2*<sup>+/+</sup>: layers I-III, *p* = 0.0281; layers IV-VI, *p* = 0.0061.

(B) Quantitative RT-PCR analysis of mRNAs for *Dclk2, Nde1, Ndell, NudC, Lis1, p39, Cdk5rap2, Rtnn, Dis1, Dynclh1, Tubb2b*. Ratio of levels in chronically intoxicated (CAI) versus control embryonic cortices (CTR) A cut-off at 20% is indicated by dashed lines (see below). The graph represents the mean value of the ratio between each sample value and each control value. P values were calculated by comparing ratios obtained for the control (CTR) condition versus the CAI one. For *Dclk2, Nde1, Ndell, Lis1, p39, Cdk5rap2*, n=5 cortices from individual embryos WT CTR; n=7 KO CTR; n=6 WT CAI; n=4 KO CAI. For *Disc1, Dyn, NudC, Tubb2*, n=3 for each conditions for the following genes.

For P-values, see Table S1. *** *p*< 0.0001; ** *p*< 0.001; * *p*<0.01. A ratio of 1 is indicated by the black line. P-values (see Table S1 for details): Same colour code as in Fig 1C and D, 2A, and 6B. Note that Dclk2 and Rtnn display an intermediate behaviour, compared to affected genes (Fig 6C) and non-affected genes: the ratio CAI/CTR is slightly below 20% for Dclk2 and Rtnn, but, respectively, only slightly or not dependent on HSF2.

Data information: Differences were considered statistically significant when *P* values were <0.05, using unpaired two-way Student's *t*-test. Data are presented as mean ± s.e.m.
Table S1. Mean, s.e.m and p-values for RT-qPCR experiments in Figure 6 and Supplemental Figure S11 B. Data information: Differences were considered statistically significant when P values were <0.05, using unpaired two-way Student's t-test. Data are presented as mean ± s.e.m.
El Fatimy Supporting Information Figure S1

A

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Bar graphs showing the number of BrdU-positive cells per mg of tissue for VZ and SVZ regions. CTR, CAI, GAV, CIP indicate different modes of fetal alcohol exposure. Error bars indicate SEM.

B

Western blots of HSF1 and HSF2 expression at different stages (E13, E15, E16, E17, E18, and N2A) and between groups (F9, E9, E15, E17). WB HSF1 and WB actin indicate Western blots for HSF1 and actin, respectively.

C

Immunohistochemistry showing HSF1 and HSF2 expression at E14.5 and E16.5.

D

Electrophoretic gel showing DNA binding activity with different antibodies (α1, α2) for CTR and GAV, and CIP and CTR groups. HSE-HSF indicates HSE (heat shock element) binding to HSF (heat shock factor). CHBA, NS, and free indicate different bands on the gel.
El Fatimy Supporting Information Figure S2

A

100x

CTR

CAI

B

HSF1-positive nuclei/100 cells

CTR  CAI

**

C

Nucleus

CTR  CAI

PhSer326  ns

Total HSF1

Actin

Histone H3

Nucleus

Signal intensity/actin

CTR  CAI  CTR  CAI

PhSer326-HSF1  HSF1

Cytoplasm

CTR  CAI

PhSer326  ns

Total HSF1

Actin

Histone H3

Cytoplasm

Signal intensity/actin

CTR  CAI  CTR  CAI

PhSer326-HSF1  HSF1
**El Fatimy Supporting Information Figure S6**

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**B**

Hsp70-Luc activity in N2A

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**C**

N2A Hsp mRNAs

**D**

- **WB HDAC1**: CTR, 30', 3 h
- **WB actin**: CTR, 30', 3 h

- **WB HDAC2**: CTR, 30', 3 h

- **WB SIRT1**: CTR, 30', 3 h

**E**

- **Calf Int. Phos**: C, HS, ElOH
- **WB: HSF1**: C, HS, ElOH
- **WB: Phos-Ser303/307**: C, HS, ElOH

**F**

- **HSF1-Myc GFP-SUMO-1**: C, HS, ElOH
- **HSF1-SUMO-1**: C, HS, ElOH
- **WB: Phos-Ser303/307**: C, HS, ElOH
**Supporting Information Figure S11**

A. 

BrdU positive cells/0.05 mm²

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B. 

RT-qPCR

**E17.5 cortices**

WT KO

- Dcx family
- Nde1 family
- Lis1 (Nde1/Nde1 interactor)
- Cdk5 regulators

CAI/CTR

- Dclk2
- Nde1
- Nde1
- NudC
- Lis1
- p39
- Cdk5rap2
- Rtn
- Disc1
- Dyn1h1
- Tubb2b

Significance levels:

- * p < 0.05
- ** p < 0.01
### Table S1. Mean, sem and p-values for RT-qPCR experiments in Figure 6 and S11

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