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# The basic helix-loop-helix transcription factor Hand1 regulates mouse development as a homodimer



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## ABSTRACT

Hand1 is a basic helix-loop-helix transcription factor that is essential for development of the placenta, yolk sac and heart during mouse development. While Hand1 is essential for trophoblast giant cell (TGC) differentiation, its potential heterodimer partners are not co-expressed in TGCs. To test the hypothesis that Hand1 functions as homodimer, we generated knock-in mice in which the Hand1 gene was altered to encode a tethered homodimer (TH). Some *Hand1*<sup>TH/-</sup> conceptuses in which the only form of Hand1 is Hand1<sup>TH</sup> are viable and fertile, indicating that homodimer Hand1 is sufficient for mouse survival. ~2/3 of *Hand1*<sup>TH/-</sup> and all *Hand1*<sup>TH/TH</sup> mice died *in utero* and displayed severe placental defects and variable cardiac and cranial–facial abnormalities, indicating a dosage-dependent effect of *Hand1*<sup>TH</sup>. Meanwhile, expression of the Hand1<sup>TH</sup> protein did not have negative effects on viability or fertility in all *Hand1*<sup>TH/+</sup> mice. These data imply that Hand1 homodimer plays a dominant role during development and its expression dosage is critical for survival, whereas Hand1 heterodimers can be either dispensable or play a regulatory role to modulate the activity of Hand1 homodimer *in vivo*.

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## Introduction

The basic helix-loop-helix (bHLH) transcription factor genes encode two highly conserved and functionally distinct domains; a basic DNA binding domain and a HLH dimerization domain that make up a region of approximately 60 amino-acids (Jones, 2004). The basic DNA binding domain binds to DNA at a consensus hexanucleotide sequence known as the E box, while the HLH domain mediates the dimerization with different HLH partners, which together contribute to the transcription specificity *in vivo* (Massari and Murre, 2000). In general, the tissue-specific class II bHLH transcription factors function by forming heterodimers with the ubiquitously expressed class I bHLH transcription factors, the E proteins (Massari and Murre, 2000). During development, many tissue specific basic helix-loop-helix (bHLH) transcription factors, such as MyoD, Twist, Ascl and Hand, play critical roles in specification of various cell lineages (Castanon et al., 2001;

Firulli et al., 1998; Megeney and Rudnicki, 1995; Philogene et al., 2012; Riley et al., 1998; Tanaka et al., 1997). Basic HLH factors can either form homodimer or heterodimers, and dimerization partner choice becomes a key factor in regulating the activities of tissue specific bHLH transcription factors (Jones, 2004; Massari and Murre, 2000). The function of specific dimer complexes has been directly approached by using a ‘tethered’ strategy through linking two monomer partner sequences with a flexible linker sequence such as to study the role of MyoD:E47 in inducing myogenesis (Neuhold and Wold, 1993) in nonmyogenic cells *in vitro* and the role of tethered Twist homodimers and heterodimer in somatic myogenesis when over-expressed in mesoderm of *Drosophila in vivo* (Castanon et al., 2001).

Hand1 is a tissue specific bHLH factor that is expressed in placenta, extra-embryonic membranes, heart and many neural crest derivatives during murine embryogenesis (Cross et al., 1995; Cserjesi et al., 1995). Studies of *Hand1* knockout models (Firulli et al., 1998; Maska et al., 2010; Morikawa and Cserjesi, 2004; Riley et al., 1998, 2000; Scott et al., 2000) indicate that Hand1 plays an essential role during various developing processes including trophoblast giant cells (TGC) differentiation, yolk sac vasculature and heart morphogenesis and lateral mesoderm development. Hand1 is a class II tissue specific bHLH transcription factor and was first cloned

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independently by several groups by virtue of its ability to interact the E-protein E47 (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995). *In vitro* biochemical studies have shown that Hand1 can heterodimerize with class I E-factors (Itf2, Alf1) and other closely related class II factors such as Hand2 (Firulli et al., 2000; Scott et al., 2000), but can also form homodimers (Firulli et al., 2000; Scott et al., 2000). Hand1 heterodimerization versus homodimerization can be regulated through different mechanisms such as the phosphorylation status of Hand1 (Firulli et al., 2003) and by tertiary interaction of Hand1 with the non-bHLH factor FHL2 (Hill and Riley, 2004). We have previously found that none of the various putative Hand1 heterodimer partners (Alf1, Itf2, and Ascl2) are expressed in the TGC layer of the placenta (Scott et al., 2000). Moreover, we have found that co-transfection of an E-protein expression vector inhibited the ability of Hand1 to promote differentiation of Rcho-1 trophoblast cells to the TGC fate and to transactivate a TGC-specific promoter (*Pr13d2* gene) (Scott and Cross, unpublished). Based on this evidence, we hypothesized that Hand1 may function as a homodimer *in vivo* to regulate TGC differentiation. To test this hypothesis, we generated a knock-in allele that produces a tethered homodimer (TH) form of Hand1 and generated mice that express either only tethered homodimer (*Hand1<sup>TH/-</sup>* and *Hand1<sup>TH/TH</sup>*) or both the monomer and tethered homodimer of Hand1 (*Hand1<sup>TH/+</sup>*). We find that while some *Hand1<sup>TH/-</sup>* mice are viable and fertile, there is a dosage-dependent effect of *Hand1<sup>TH</sup>* and overabundant *Hand1<sup>TH</sup>* leads to placental defects and embryonic lethality. Meanwhile, presence of the *Hand1<sup>TH</sup>* did not have negative effects on viability or fertility in all *Hand1<sup>TH/+</sup>* mice.

## Material and methods

### Plasmids

Site-directed mutagenesis was carried out according to manufacturer's instructions using the Altered Sites *in vitro* mutagenesis kit (Promega). pCMV-FLAG Hand1 $\Delta$ b, containing a RRR to GSG substitution in the basic domain, was constructed using site-directed mutagenesis of pCMV-FLAG Hand1 (Scott et al., 2000) (AS oligo 5'-aat gct ctc tgt gcc gga tcc ctc ctt ctt ggg tcc-3'). For construction of tethered expression cassettes, EcoRI sites were first introduced by site directed mutagenesis 5' to the Hand1 (AS oligo 5'-gtt cat gtt gga GAA TTC cct gcc ctg tgc tg-3') and Itf2 (S oligo 5'-ctt ggt ttg tgt GAA TTC tcc aac atg cat cac caa cag-3') initiation methionine sequences (shown in bold). In a similar manner, PstI sites were introduced immediately 3' to the same Hand1 (AS oligo 5'-ggc ggc gtt ggc ctC TGC AGa ctg gtt tag ctc-3') and Itf2 (S oligo 5'-atg gga cag tCT GCA Ggt cca agt tgc-3') coding regions, thereby removing the STOP codons (shown in bold). A PstI site was also introduced 5' of a second Hand1 cDNA (AS oligo 5'-gct gcc cac gag gtt caC TGC AGa gag gct cct gcc ctg-3'). EcoRI/PstI (both containing Hand1 and Itf2) and PstI/XhoI (containing Hand1) fragments from these SDM reactions were ligated together in pBluescript SK+ and digested with PstI. Annealed oligos with PstI overhangs, encoding a (GGGS)<sub>n</sub> linker (S 5'-ggg ggt tcc gcc ggg ggt tct gga ggt ggg agc ggc gga ggg tcc gcc gga gga act gca-3', AS 5'-gtt cct ccg ccg gac cct ccg ccg ctc cca cct cca gaa ccc ccg ccg gaa ccc cct gca-3') were then ligated into the PstI site. Clones where the oligos had incorporated in the correct orientation were verified by sequencing. Finally, EcoRI/XhoI fragments of these were ligated into EcoRI/XhoI-digested pCMV-FLAG-Hand1 to yield pCMV-FLAGHand1:Hand1 and pCMV-FLAGItf2:Hand1. For the construction of pCMV-FLAG Hand1:Hand1  $\Delta$ b the same approach was used. The mammalian expression vectors pCMV-Ascl2 and p $\beta$ Actin-LacZ (Cross et al., 1995) and pCMV-Itf2 (Chiaramello et al., 1995) have been previously described.

### Trophoblast cell culture and transfection

Rcho-1 cells were cultured in RPMI-1640 medium (Sigma) supplemented with 20% fetal calf serum (Hyclone), 50  $\mu$ M  $\beta$ -mercaptoethanol, and 1 mM Na-pyruvate, as previously described (Cross et al., 1995). For TGC differentiation assays, Rcho-1 stem cells were transfected using Lipofectamine PLUS (Gibco BRL) 5 h after plating to coverslips. In initial experiments, 250 ng of p $\beta$ Actin-LacZ and 375 ng of expression vector were added per 35 mm well, with empty expression vector (pcDNA3) added to a 1.0  $\mu$ g total. Cells were fixed 48 h post-transfection in 4% paraformaldehyde and permeabilized with methanol. Following incubation with mouse anti-FLAG (1/200 dilution, IBI) and rabbit anti- $\beta$ -galactosidase (1/400 dilution, Cappel) primary antibodies, and anti-mouse-FITC and anti-rabbit-TRITC (Sigma, 1/50 dilution) secondary antibodies, cells were stained with bisbenzimidazole (Sigma) and examined by fluorescent microscopy. In subsequent experiments, cells were transfected with 250 ng pCMV-IRES:EGFP (Clontech) and 375 ng of the indicated expression vectors (to 1.0  $\mu$ g total with pcDNA3). Cells were lightly fixed 48 h post-transfection in 4% paraformaldehyde and stained with bisbenzimidazole. Giant cell differentiation was scored as the percent of TRITC- or GFP-positive cells that had the enlarged nuclei characteristic of TGCs (Cross et al., 1995). Percent TGC differentiation values represent the mean  $\pm$  SE for 25 fields examined for each treatment group using a 40 $\times$  objective, and were similar in 2–3 separate experiments.

### Generation of *Hand1<sup>TH</sup>* knock-in mice

For cloning purpose, a BglII site was introduced 5' to the Hand1 transcription initiation site by PCR, as previous described (Riley et al., 2000). Targeting vectors consisted of a 6.0 kb KpnI/BglII 5' arm (upstream of transcription start site) and a 2.6 kb Sall/BglII 3' arm (part of intron 1, exon 2, and sequence 3' to exon 2) in a pUC8 vector backbone containing HSV-*thymidine* kinase cassette. BglII/Sall fragments containing tethered homodimer Hand1 sequences were ligated between the two vector arms. For the tethered homodimer Hand1, this fragment was constructed as described above, except that the second *Hand1* monomer consisted of genomic sequences. A PGK-*puro<sup>res</sup>* cassette flanked by LoxP sites was subsequently inserted into the Sall site found in intron 1. Constructs were linearized via digestion with KpnI and transfected into the *Hand1<sup>+/-</sup>* ES cell line 14A3 (Riley et al., 2000) via electroporation. Following positive–negative selection using gangcyclovir and puromycin (1.5  $\mu$ g/mL), ES cell clones were isolated and screened for proper homologous recombination following Southern blotting as previously described (Joyner, 1993). The genotyping results are shown by PCR. Chimeras were generated via aggregation of targeted *Hand1<sup>TH(puro)/+</sup>* ES cells and 8-cell stage wildtype embryos, as previously described (Joyner, 1993). One male chimera (8D11) transmitted the *Hand1<sup>TH(puro)</sup>* allele through the germline, and was outbred to CD1 females. Progeny were crossed with CAGGS-nlsCre mice (a gift from A. Nagy), and pups were genotyped via PCR for excision of the PGK-*puro<sup>res</sup>* cassette. *Hand1<sup>TH/+</sup>* mice were obtained and further bred with *Hand1<sup>+/-</sup>* mice, the genotype of the progeny was determined by PCR. Experiments were done in accordance with the guidelines of the Canadian Council on Animal Care and the University of Calgary Animal Welfare Committee (Protocol No. M01025).

### Histological analysis of placental tissues

Placentas and embryos were dissected and harvested from timed matings and fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS). Following fixation, embryos were

rinsed in PBS then dehydrated through a graded ethanol series and embedded in paraffin wax. Histological sections were cut and stained with hematoxylin and eosin.

RNA *in situ* hybridization on whole mount conceptuses and sections was carried out as previously described (Scott et al., 2000). *Hand1*<sup>TH/+</sup> X *Hand1*<sup>TH/-</sup> offspring were dissected out of the uterus at E8.5 and E10.5 (E0.5 is defined as noon of the day on which vaginal plugging was detected). *In situ* probes used include: *Pr13d2/Pl1* (Prolactin family 3, subfamily d, member 2 / placental lactogen-1), *Tpbpa*, *Pr13b1/Pl2* (Prolactin family 3, subfamily b, member 1 / placental lactogen-2), *Ascl2* and *Gcm1*. Digoxigenin-labeled probes were prepared by using digoxigenin labeling mix (Boehringer Mannheim) and detected by using an anti-digoxigenin-alkaline phosphatase conjugate (Boehringer Mannheim).

Paraffin sections were dewaxed and rehydrated, and stained with antibodies against laminin (1:300; Sigma L9393) and phospho-histone H3 (1:300; Upstate 06-570). Immunostaining sections were counterstained with nuclear marker hematoxylin.

#### Characterization of Hand1 antibody

Anti-mouse Hand1 antibody was obtained from Santa Cruz Biotech (C-19; Cat. No. sc-9413). The specificity of the Hand1 C19 antibody was shown by Western blotting of extracts from wild-type, differentiated trophoblast stem cells showing a major band at the expected size of 25 kDa. Extracts generated from *Hand1*<sup>-/-</sup> cells (Hemberger et al., 2004) were used as negative controls and showed no band at 25 kDa. Some initial experiments were also performed using a Hand1 antibody from Abcam (Cat. No. ab11846) as reported previously by others (Martindill et al., 2007). However we found that this antibody reacted with multiple bands on Western blots and produced signals both on westerns and immunofluorescent nuclear staining of trophoblast cells even in the *Hand1*<sup>-/-</sup> mutant background (data not shown).

#### Western blotting

Placenta tissues were obtained from the E8.5 and E10.5 *Hand1*<sup>TH</sup> conceptuses. To prepare protein extracts, placenta tissues were homogenized in RIPA lysis buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) with 1 mM PMSF, 1 mM sodium orthovanadate, and 10 µg/mL proteinase inhibitor cocktail (Roche) freshly added (Aubin et al., 2004). About 20 µg of all protein extract from each E8.5 placenta or 40 µg protein extract from each E10.5 placenta was loaded in each lane of the gel. Western blot was performed by standard methods using antibodies against Hand1 (1:300; Santa Cruz sc-9413), β-actin (1:1000; Santa Cruz). Results were analyzed by densitometry using ImageJ 1.34s software as described (<http://rsb.info.nih.gov/ij/>).

#### In vitro transcription/translation and immunoprecipitation

*In vitro* transcription/translation was performed with the Promega TNT rabbit reticulocyte lysate kit and plasmids pT7-Flag-Hand1, pT7-Flag-TH Hand1, pHis-Alf1 and pT7-c-jun according to the manufacturer's instructions. Proteins were labeled via the addition of [<sup>35</sup>S]methionine to the reaction. Proteins were either phosphorylated by treatment with protein kinase A (New England Biolabs) in 10 × PKA reaction buffer (NEB) with the addition of ATP, HALT™ phosphatase inhibitor cocktail, and 10 mM okadaic acid for 30 min at 30 °C; or dephosphorylated by addition of EDTA plus Shrimp alkaline phosphatase in Alk Phos. buffer, also for 30 min at 30 °C. Proteins were made in separate reactions and then mixed for 30 min at 4 °C in buffers specific to the phosphorylation state of the proteins (150 mM NaCl, 50 mM Tris-Cl pH 7.5, 1% Triton X-100,

1 × HALT™ phosphatase inhibitor cocktail, 10 µM okadaic acid, 25 µM MgCl<sub>2</sub> for the phosphorylated set; same salt/Tris/Triton but addition of 1 mM EDTA for the dephosphorylated set), in the presence of anti-FLAG M2 affinity gel, followed by an overnight incubation at 4 °C on a rotating wheel. Flag beads were washed four times with buffers specific to maintaining the phosphorylated state (see above), resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, boiled, and subjected to SDS-PAGE. Proteins were transferred from gels to PVDF membrane and were incubated in Enhance Reagent (NEN), allowing the detection of labeled proteins by fluorography.

#### Ectoplacental cone culture and Hand1 immunofluorescence staining

The chorion and ectoplacental cone were dissected at E8.5 from *Hand1*<sup>TH/-</sup> X *Hand1*<sup>TH/-</sup> crosses in 1 × PBS. Explants were placed in 0.125% trypsin/EDTA (Gibco) in PBS for 10 min at 37 °C. Dispersed cells were then cultured on gelatin coated coverslips in fibroblast-conditioned TS cell medium with bFGF and heparin for 3 days at 37 °C in 95% humidity, 5% CO<sub>2</sub>, with daily medium change. Yolk sacs were removed during dissection for genotyping. Coverslips were fixed in 2% paraformaldehyde for 20 min on ice, permeabilized with 0.25% Triton X-100 in PBS for 10 min and blocked in 5% donkey serum in PBS. Cells were then incubated in 1:100 goat anti-mouse Hand1 antibody (C-19; Santa Cruz sc-9413) overnight at 4 °C and incubated in 1:500 Cy3 conjugated donkey anti-goat IgG (Jackson Immuno-Research 705-166-147). For immunofluorescent quantitative analysis, all samples were treated with the same staining procedures without knowledge of genotypes.

#### Microscopic equipment and software

Leica DMR light microscope (for light and immunofluorescence images), Leica MZ95 dissection microscope (for dissected embryo/placenta images) with Photometrics Coolsnap of camera and RS Image 1.0.1 imaging software program were used to obtain micrographs. For immunofluorescent quantitative analysis, all images were taken under the same conditions with fixed exposure time. Minimal image processing was done using Adobe Photoshop 7.0 and figures were constructed using Canvas 9.0.2.

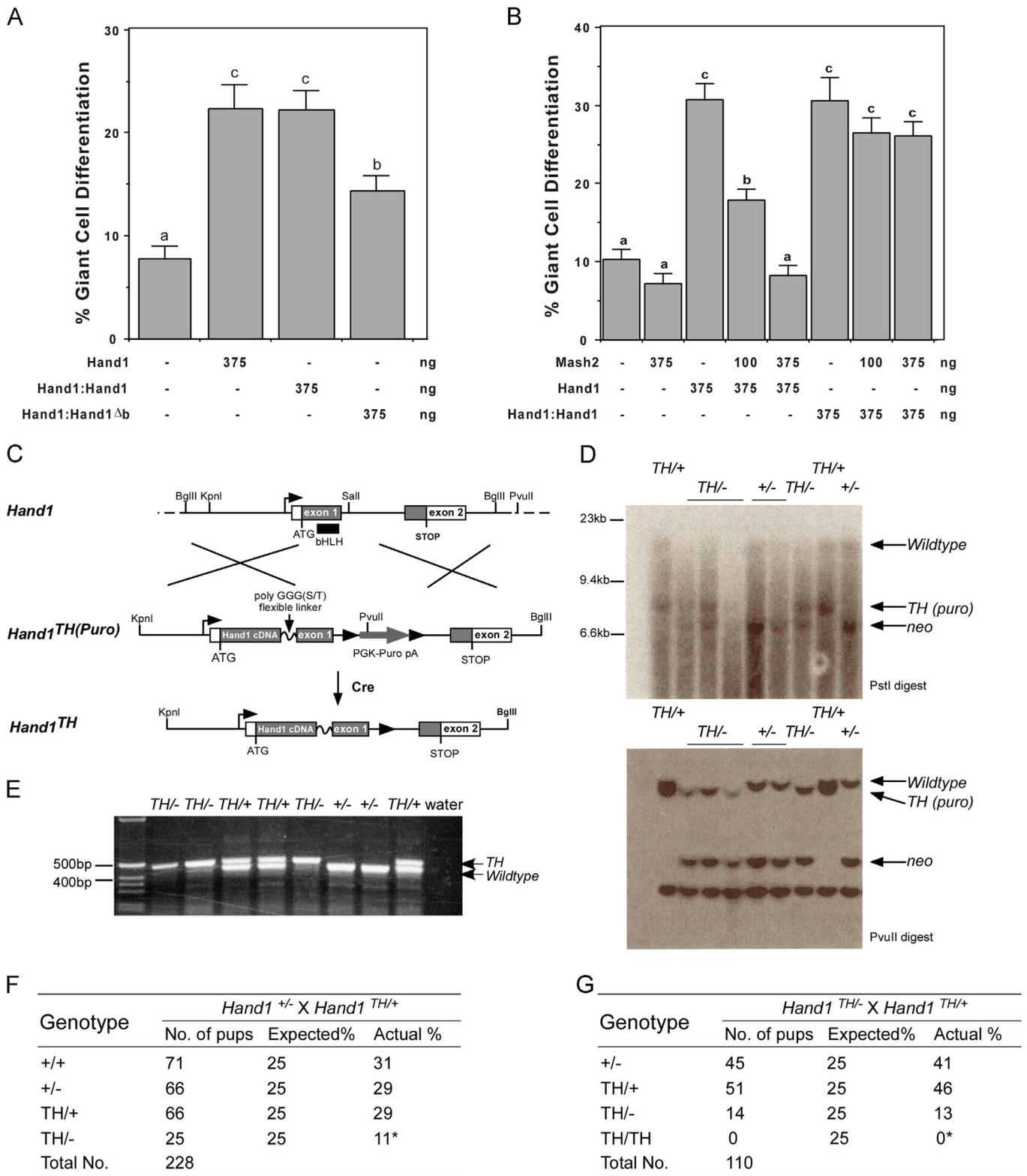
#### Quantitative and statistical analysis

Quantitative analysis on Hand1 staining of ectoplacental cone cultures was conducted through measurement of the integrated density, mean gray value and area of individual cell using ImageJ. At least 500 cells from EPC culture of each conceptuses were measured. Statistical analyses were performed by a Student's *t* test or two way ANOVA. Data are shown as the mean ± standard error of the mean. A *P* value of < 0.05 was considered to be statistically significant.

## Results

#### Tethered homodimer Hand1 (*Hand1*<sup>TH</sup>) promotes TGC differentiation *in vitro*

In order to assess the ability of specific Hand1 dimer complexes to promote TGC differentiation, a tethered homodimer construct was transfected into Rcho-1 trophoblast cells. Strikingly, the *Hand1*<sup>TH</sup> (*Hand1*: *Hand1*) promoted TGC differentiation to a similar extent as monomer (wildtype) *Hand1* (Fig. 1A). To test if the ability of *Hand1*<sup>TH</sup> to promote TGC differentiation was also dependent on binding to DNA as wildtype *Hand1*, point mutations



**Fig. 1.** Introduction of tethered Hand1 homodimers (*Hand1*<sup>TH</sup>) *in vitro* and *in vivo*. (A) Promotion of TGC differentiation by Hand1<sup>TH</sup>. The Hand1<sup>TH</sup> (Hand1:Hand1) promoted TGC differentiation to a similar extent as wildtype monomer Hand1 in transfected Rcho-1 cells. Introduction of point mutation that inhibits DNA-binding activity in other bHLH proteins into the Hand1 basic domain (Hand1 $\Delta$ b) of Hand1<sup>TH</sup> also impairs its ability to promote TGC differentiation. Different superscripts indicate statistically significant differences ( $P < 0.05$ ). (B) Promotion of TGC differentiation by the tethered Hand1 homodimer is resistant to inhibition by Ascl2. Ascl2 inhibits the ability of wildtype Hand1 to promote TGC formation in transfected Rcho-1 cells in a concentration-dependent fashion. However, Ascl2 cannot inhibit the ability of Hand1<sup>TH</sup> (Hand1:Hand1) to promote TGC differentiation. Different superscripts indicate statistically significant differences ( $P < 0.05$ ). (C) Targeting ES cells to derive *Hand1*<sup>TH</sup> ‘knock-in’ alleles. The *Hand1*<sup>TH(puro)</sup> targeting construct is shown in the middle to replace part of the two exons and the intron in the endogenous Hand1 locus. Later on, Cre recombinase was introduced into the ES cell line to excise the Puro cassette. (D) Genotyping of targeted clones by Southern blotting. Genomic DNA was digested with PstI (top) or PvuII (bottom) and probed using 5’ and 3’ probes, respectively, as described in material and methods. neo, *Hand1*<sup>neo</sup> null allele. (E) *Hand1*<sup>TH</sup> knock-in pups were genotyped by PCR. (F) Breeding of *Hand1*<sup>+/-</sup> with *Hand1*<sup>TH/+</sup> knock-in mice. \* Chi square analysis,  $P < 0.05$ . (G) Breeding *Hand1*<sup>TH/+</sup> with *Hand1*<sup>TH/-</sup> knock-in mice. \* Chi square analysis,  $P < 0.05$ .

that inhibit DNA-binding activity in other bHLH proteins were introduced to one of the two Hand1 basic domains in the Hand1<sup>TH</sup> construct (Hand1:Hand1Δb). The ability to promote TGC differentiation by Hand1:Hand1Δb was significantly lower than that of wildtype Hand1 and the Hand1<sup>TH</sup> (Fig. 1A). Initial experiments in which immunostaining for the N-terminal FLAG epitope was carried out indicated that these constructs were expressed both in a comparable percentage of cells and at quantitatively similar levels (data not shown). Therefore, the decreased activity of Hand1:Hand1Δb was not due to decreased levels of this protein being present. As two functional basic domains are required for binding of a bHLH dimer to DNA (Voronova and Baltimore, 1990), these data suggest that the Hand1 complex that promoted TGC differentiation in these assays is a Hand1 homodimer.

To further examine if Hand1 activity in TGC differentiation assays can be ascribed to homodimer complexes, co-transfection experiments were performed in which increasing amounts of *Ascl2* expression construct were added. *Ascl2* inhibits the ability of Hand1 to promote TGC formation in transfected Rcho-1 cells in a concentration-dependent fashion (Scott et al., 2000). Strikingly, *Ascl2* did not significantly inhibit the activity of the Hand1<sup>TH</sup> (Fig. 1B). This held true even when higher concentrations of *Ascl2* expression construct, which fully abolish wild-type Hand1 activity, were added (Fig. 1B). It appears that tethering two Hand1 monomers together, and thus driving Hand1 homodimer formation, circumvents the inhibitory activity of *Ascl2*.

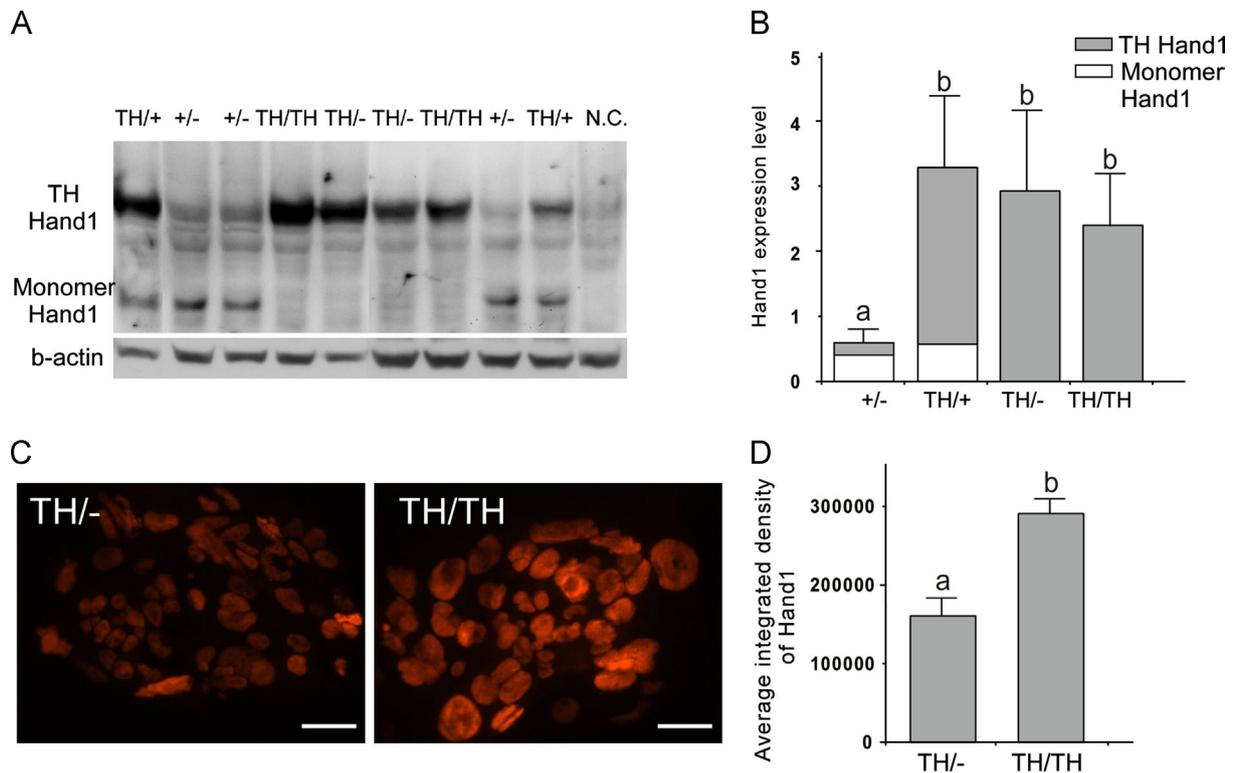
#### Hand1<sup>TH</sup> is sufficient for mouse survival

In order to test the role of Hand1<sup>TH</sup> *in vivo*, Hand1<sup>TH</sup> knock-in mice were generated (Fig. 1C, D and E). Hand1<sup>TH/+</sup> knock-in mice

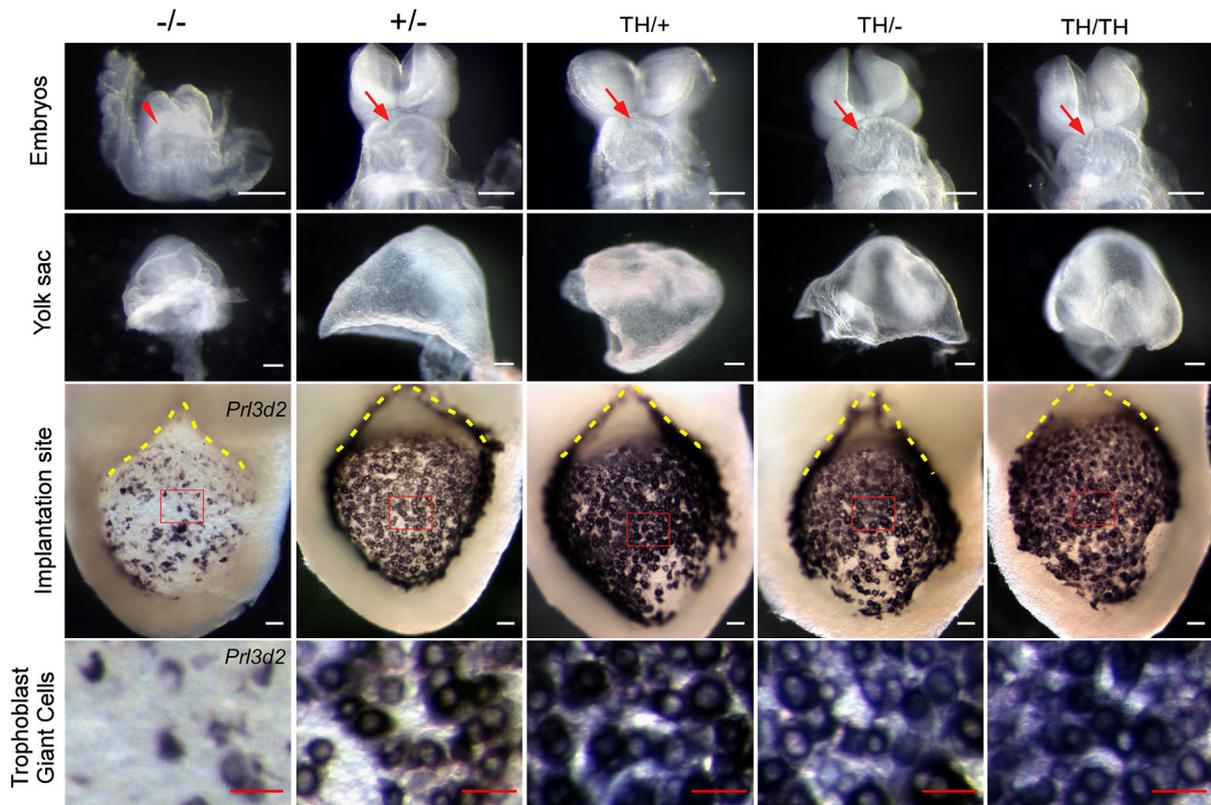
were born at the expected Mendelian ratio and appeared to be normal and fertile (Fig. 1F). Among the offspring of Hand1<sup>+/-</sup> and Hand1<sup>TH/+</sup> crosses, Hand1<sup>TH/-</sup> mice were born and they are fully viable and fertile. However, their frequency was reduced to ~1/3 of expected Mendelian ratio (Fig. 1F). The Hand1<sup>TH/-</sup> adult mice were apparently normal and phenotypes that might be indications of lateral mesoderm derivatives, neural crest derivatives, cranial facial or cardiac defects were not observed. These phenotypes include weight difference, food intake changes, cranial facial phenotypes and cyanosis (data not shown).

In order to determine if the survival and rescue of the Hand1<sup>TH/-</sup> mice was possibly due to cleavage of Hand1<sup>TH</sup> protein into monomer Hand1 proteins, Western blotting was performed on tissue lysates from placental tissues. No monomer protein band was detected in samples from Hand1<sup>TH/-</sup> or Hand1<sup>TH/TH</sup> conceptuses at either E10.5 or E8.5 (Fig. 2A and Supplementary Fig. 1A), indicating that the survival of Hand1<sup>TH/-</sup> mice is due to function of the Hand1<sup>TH</sup> protein. Moreover, to examine if the survival of Hand1<sup>TH/-</sup> was likely due to intermolecular interaction and formation of intermolecular heterodimers, we studied the interaction of either monomer or TH Hand1 with E factor Alf1 that are *in vitro* transcribed/translated. Under either phosphorylation or dephosphorylation status after treatment with protein kinase A or Alkaline phosphatase respectively, the binding of Alf1 to TH Hand1 is minimal and dramatically reduced than monomer Hand1 ( $P < 0.001$ ; Supplementary Fig. 2), indicating that the TH Hand1 prefers to form stable intra-molecular homodimers rather than inter-molecular dimers.

Hand1 null (Hand1<sup>-/-</sup>) mutants die by E8.5 and have unlooped hearts, 'blistered' yolk sacs, smaller implantation sites, and fewer and under-differentiated TGCs surrounding the implantation site



**Fig. 2.** Hand1<sup>TH</sup> protein expression *in vivo*. (A) Hand1<sup>TH</sup> protein is stably formed *in vivo*. Western blot on placenta protein lysates from Hand1<sup>TH</sup> conceptuses at E10.5 stained with Hand1 antibody. N.C.: negative control (lysates from uterus smooth muscle tissue without Hand1 expression). (B) Quantitative analysis of Western blot results of Hand1 protein expression in E10.5 placenta protein lysates shows expression of Hand1<sup>TH</sup> protein is significantly higher than Hand1 monomer proteins *in vivo*. Different superscripts indicate statistically significant differences ( $P < 0.05$ ). (C) Immunofluorescence staining of Hand1 protein in E8.5 Hand1<sup>TH/-</sup> and Hand1<sup>TH/TH</sup> EPC cells followed by three days of culture. Scale bars: white, 50  $\mu$ m. (D) Quantitative analysis of Hand1 immunofluorescence staining results of E8.5 EPC cultured cells demonstrated that Hand1<sup>TH</sup> protein expression level is significantly higher in Hand1<sup>TH/TH</sup> trophoblast cells than in Hand1<sup>TH/-</sup> cells, suggesting a dosage dependent effect.



**Fig. 3.** Gross observation of *Hand1*<sup>TH</sup> conceptuses at E8.5. Heart is unlooped in *Hand1*<sup>-/-</sup> embryos (red arrow head) at E8.5. In contrast, heart is looped in *Hand1*<sup>+/-</sup> and all *Hand1*<sup>TH</sup> embryos (red arrows). E8.5 yolk sac appears to be 'blistered' in *Hand1*<sup>-/-</sup> conceptuses. Yolk sac appears to be smooth in *Hand1*<sup>+/-</sup> and all *Hand1*<sup>TH</sup> conceptuses. E8.5 implantation site is outlined by yellow dashed line. Implantation site size in *Hand1*<sup>-/-</sup> conceptus appears to be much smaller than in other genotype. TGCs are stained by *Pr3d2*/*PI1* probe in conceptuses through whole mount *in situ*. TGCs size and number in E8.5 *Hand1*<sup>-/-</sup> conceptuses appear to be smaller and significantly reduced ( $P < 0.05$ , data not shown). TGCs size and number in E8.5 *Hand1*<sup>TH</sup> conceptuses appear to be not significantly different as in *Hand1*<sup>+/-</sup> conceptuses ( $P > 0.05$ , data not shown). Scale bars: white, 200  $\mu$ m; red, 100  $\mu$ m.

(Fig. 3) (Firulli et al., 1998; Morikawa and Cserjesi, 2004; Riley et al., 1998; Scott et al., 2000). *Hand1*<sup>TH/+</sup> mice were bred to *Hand1*<sup>TH/-</sup> mice in order to generate mice with either one or two copies of *Hand1*<sup>TH</sup>. All *Hand1*<sup>TH/-</sup> and *Hand1*<sup>TH/TH</sup> conceptuses appeared to be normal at E8.5 (Fig. 3), indicating that the heart, yolk sac and TGCs defects caused by absence of monomer *Hand1* could be rescued by *Hand1*<sup>TH</sup>. Therefore, *Hand1* may function as homodimer in various tissues during development.

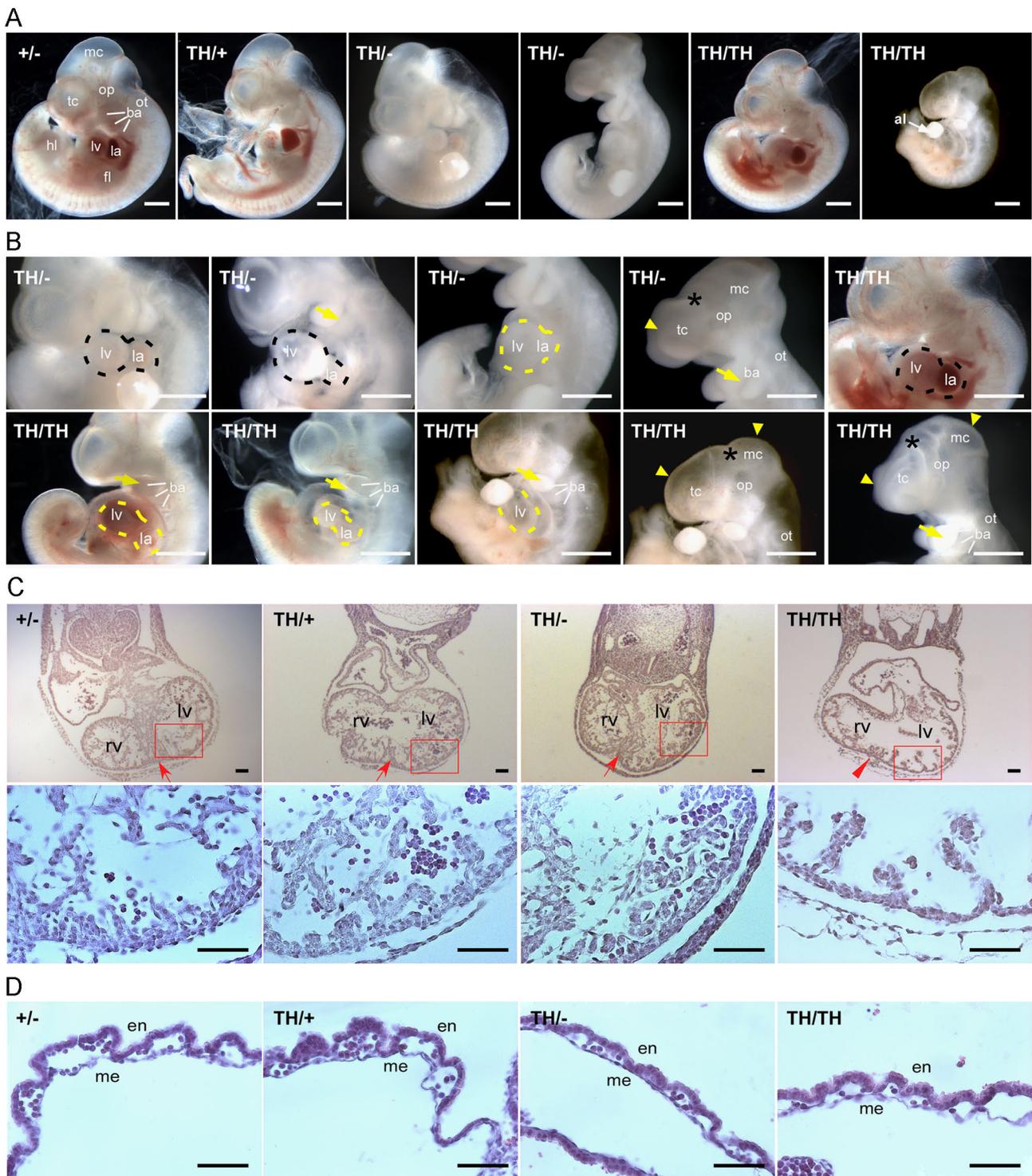
#### The *Hand1*<sup>TH</sup> protein has dosage-dependent effects *in vivo*

Whereas at least some of the *Hand1*<sup>TH/-</sup> pups were viable, none of *Hand1*<sup>TH/TH</sup> pups were detected at birth (Fig. 1G). Embryos were then dissected at different embryonic stages (E8.5, 10.5, 12.5, 14.5) and examined for signs of embryonic and extra-embryonic defects. *Hand1*<sup>+/-</sup> and *Hand1*<sup>TH/+</sup> embryos were normal at each stage studied (Supplementary Table 1). In contrast, ~2/3 of *Hand1*<sup>TH/-</sup> embryos died between stage E10.5 and 14.5, and all *Hand1*<sup>TH/TH</sup> embryos died between E10.5 and 12.5 (Supplementary Table 1).

The fact that at least some of the *Hand1*<sup>TH/-</sup> embryos survived and yet all of the *Hand1*<sup>TH/TH</sup> embryos died suggested that overdose of the *Hand1*<sup>TH</sup> protein was deleterious. Western blotting of E10.5 placental tissue lysates showed that, relative to *Hand1* monomer, the *Hand1*<sup>TH</sup> protein expression level was significantly higher *in vivo* (Fig. 2A and B). Even accounting for the fact that each *Hand1* molecule in the *Hand1*<sup>TH</sup> protein contains two monomers worth, the *Hand1*<sup>TH</sup> was expressed at least two-fold higher than expected. Western results also demonstrated that *Hand1*<sup>TH</sup> protein overdose is not evident at E8.5 (Supplementary Fig. 1). To further determine if *Hand1*<sup>TH</sup> protein expression is

correlated with genetic dosage, quantitative analysis was then conducted (Fig. 2C and D). Because the tissue composition of *Hand1* positive cells such as TGCs within *Hand1*<sup>TH/TH</sup> placenta at E10.5 is dramatically different from that of other *Hand1*<sup>TH</sup> genotype and the dosage effect of *Hand1*<sup>TH</sup> is not evident yet at E8.5, western on whole placenta lysates from E10.5 or E8.5 are not capable of addressing this question. Thus, we performed immunofluorescent staining on E8.5 ectoplacental cone cells after three days of culture in conditioned TS cell medium, quantified the integrated fluorescent intensity of each trophoblast cell (at least 500 cells were quantified for each sample) and calculated the average integrated intensity of individual trophoblast cell. All images were taken under the same conditions with fixed exposure time to ensure the fluorescent intensity is correlated to *Hand1* protein expression. Quantitative results demonstrated that *Hand1*<sup>TH/TH</sup> trophoblast cells express significantly higher level of dimer protein than *Hand1*<sup>TH/-</sup> cells, indicating a strong association between the *Hand1*<sup>TH</sup> protein level and its genetic dosage (Fig. 2C and D).

In a previous study using rat Rcho-1 cells as a model, sequestration of *Hand1* in the nucleolus and subsequent release during differentiation was suggested to act as a molecular switch to determine trophoblast cell fate (Martindill et al., 2007). To examine if altered protein sub-cellular localization could also be a likely mechanism of *Hand1*<sup>TH</sup> lethal effects, *Hand1* immunofluorescent staining was performed. *Hand1* protein stains diffusely in the nuclei and its sub-cellular localization did not differ among EPC cells culture from *Hand1*<sup>TH/+</sup>, *Hand1*<sup>TH/-</sup>, *Hand1*<sup>TH/TH</sup> and *Hand1*<sup>+/-</sup> samples (Supplementary Fig. 3). Good specificity of *Hand1* staining has been validated using mouse *Hand1*<sup>+/+</sup> and *Hand1*<sup>-/-</sup>



**Fig. 4.** Gross and histological observation of *Hand1<sup>TH</sup>* conceptuses at E10.5. (A) Phenotypic analysis of *Hand1* homodimer embryos at E10.5 shows *Hand1<sup>TH/+</sup>* mice embryos appear to be apparently normal, whereas some *Hand1<sup>TH/-</sup>* and *TH/TH* embryos appears to be grossly underdeveloped. mc, Metencephalon; tc, telecephalon; op, optic vesicle; ot, otic pit; ba, bronchial arches; la, left atrium; left ventricle; fl, forelimb; hl, hindlimb; al, allantois. White arrow, unattached allantois with a ball shape in one of the *Hand1<sup>TH/TH</sup>* embryos. (B) *Hand1<sup>TH/-</sup>* and *TH/TH* embryos appear to have variable cranial-facial and heart defects. Stars, abnormal head sub-division; yellow arrow head, less developed telecephalon and metencephalon; yellow arrow, less developed brachial arches; black dashed line, outline of the normal heart left ventricle and atrium morphology; yellow dashed line, outline of the less developed ventricle and atrium in some *Hand1<sup>TH/-</sup>* and *TH/TH* embryos. (C) H&E staining of sections shows heart histology at E10.5. The ventricle wall in the *Hand1<sup>TH/TH</sup>* embryo is apparently thinner and less trabeculated. Arrowhead indicates no left and right ventricle septum formed in the *Hand1<sup>TH/TH</sup>* embryo. Arrows indicate left and right ventricle septum formed. (D) H&E staining of sections shows yolk sac histology at E10.5 is not significantly different between *Hand1<sup>TH/-</sup>* and *Hand1<sup>TH/+</sup>* embryos. me, Mesoderm; en, endoderm. Scale bars: white, 500  $\mu$ m; black, 100  $\mu$ m.

trophoblast stem cell lines Rs26 and H5-1 (Supplementary Fig. 4). It is worth noting here that in contrast to what was published (Martindill et al., 2007), alteration of *Hand1* sub-cellular localization was not observed in trophoblast cells during differentiation (this study).

In general, all data indicate that the accelerated phenotype of *Hand1<sup>TH/TH</sup>* compared to *Hand1<sup>TH/-</sup>* appeared to be correlated with increased level of *Hand1* protein within the nucleus and has little to do with altered protein subcellular localization.

**Table 1**  
Detailed phenotypes of conceptuses derived from *Hand1*<sup>TH/+</sup> X *Hand1*<sup>TH/-</sup> crosses at E10.5.

	Genotype			
	+/-	TH/+	TH/-	TH/TH
Gross morphology				
Somite no.	36.07 ± 3.17	32.54 ± 6.45	32.00 ± 3.57	30.83 ± 1.94 *
Crown rump length#	1568 ± 49.28	1409 ± 86.44	1346 ± 69.77	1144 ± 65.29 *
Abnormal heart	0/23	2/19	7/17	10/19
Abnormal face	0/23	2/19	4/17	4/19
Pale yolk sac	0/23	0/19	1/17	11/19
Allantois not attached to placenta	0/23	1/19	1/17	7/19
Normal	23/23	17/19	9/17	4/19
Placental histology				
Allantois not attached	0/6	0/6	0/7	4/12
Allantois attached but shallow labyrinth	0/6	0/6	1/7	8/12
Reduced TGC	0/6	0/6	1/7	12/12
Normal	6/6	6/6	6/7	0/12

\*  $P < 0.05$  by *t* test.

### Characterization of fetal and placental defects in *Hand1*<sup>TH</sup> conceptuses and association with genetic dosage

Whereas development of all *Hand*<sup>TH</sup> mice was grossly normal at E8.5 (Fig. 3), observations at later stages indicated that some *Hand1*<sup>TH/-</sup> and all *Hand1*<sup>TH/TH</sup> conceptuses developed several abnormalities. At E10.5, there were variable heart and craniofacial defects present in *Hand1*<sup>TH/-</sup> and *Hand1*<sup>TH/TH</sup> embryos (Fig. 4A, B and Table 1). About 50% of the *Hand1*<sup>TH/TH</sup> conceptuses showed abnormal heart morphologies (Table 1). Gross and histological observations of the heart showed underdeveloped ventricles and hypoplasia of both ventricular walls in some E10.5 embryos at (Fig. 4C and Table 1). About 60% of *Hand1*<sup>TH/TH</sup> conceptuses had a pale yolk sac (Table 1). Unlike *Hand1* null mutants (Morikawa and Cserjesi, 2004; Riley et al., 1998), histological sections of the *Hand1*<sup>TH/TH</sup> yolk sac did not show any morphological abnormalities of endoderm or mesoderm in the yolk sac vasculature (Fig. 4D), suggesting that the pale yolk sac was secondary. In the placentas of *Hand1*<sup>TH</sup> knock-in mice, about 30% of the *Hand1*<sup>TH/TH</sup> embryos failed to have chorioallantoic attachment (Fig. 5A and Table 1). H&E staining, laminin immunohistochemistry (as a marker of the basement membrane in fetal blood vessels in the placenta) and *Gcm1* gene expression (as a marker of trophoblast cells in the branched villi) demonstrated that almost all of the rest (about 70%) of *Hand1*<sup>TH/TH</sup> placentas had limited branching morphogenesis of the labyrinth (Fig. 5A and Table 1). Almost all *Hand1*<sup>TH/TH</sup> placentas had a strikingly smaller labyrinth/implantation site by E10.5. *Hand1*<sup>TH/-</sup> conceptuses with one copy of *Hand1*<sup>TH</sup> have shown similar phenotypes as *Hand1*<sup>TH/TH</sup> with two copy of *Hand1*<sup>TH</sup> but with milder phenotype and much lower penetrance (Figs. 4 and 5 and Table 1).

### Characterization of trophoblast lineage development in *Hand1*<sup>TH</sup> conceptuses

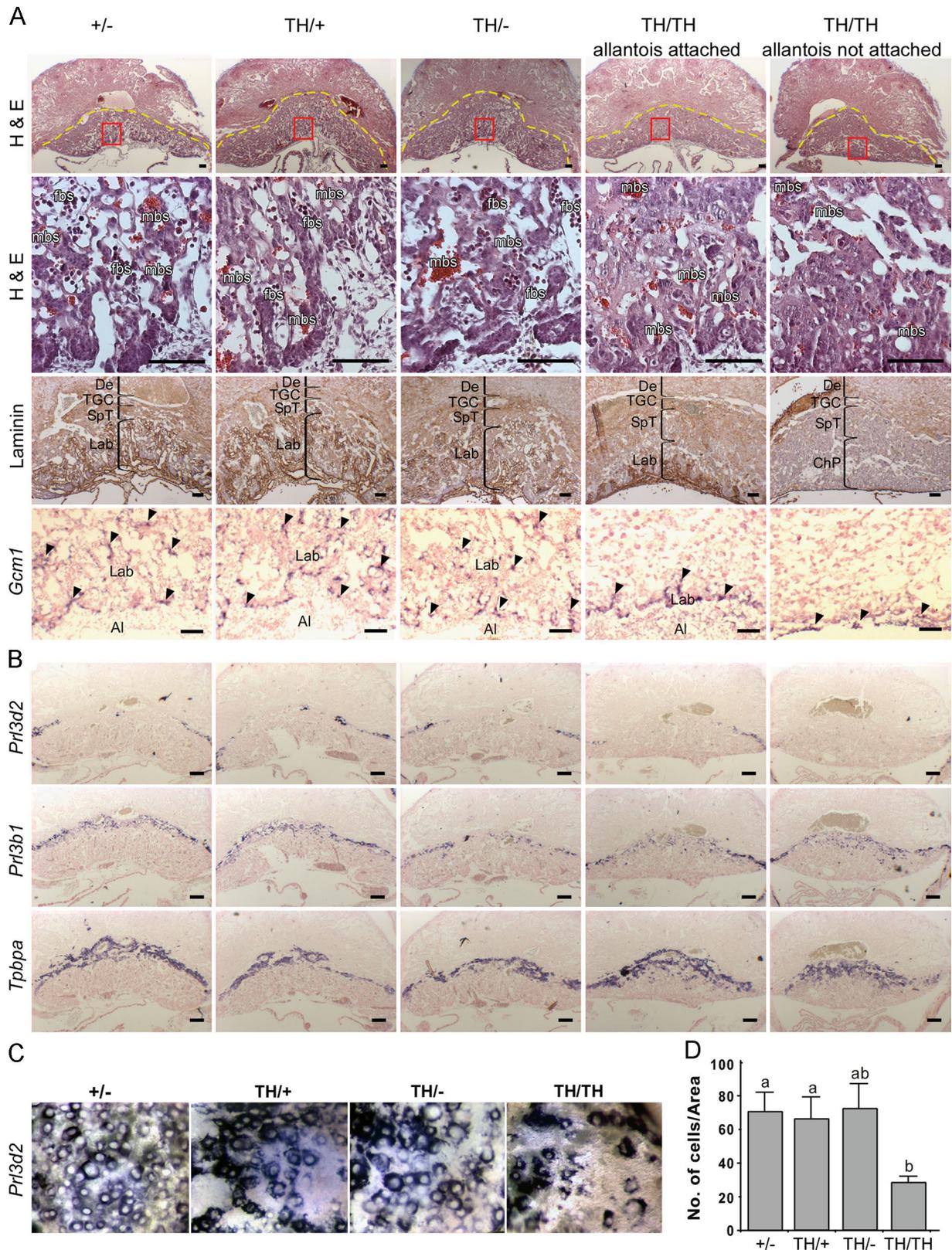
In searching for reasons why overdose of *Hand1*<sup>TH</sup> leads to abnormal development and lethality we investigate further into trophoblast lineage development, because *Hand1*<sup>TH</sup> conceptuses have prominent placenta abnormalities with highest penetrance and trophoblast has been a best established system for study of *Hand1* function. Characterization of the spongiotrophoblast and TGC layers in *Hand1*<sup>TH</sup> conceptuses at E10.5 showed that the spongiotrophoblast layer (stained by *Tpbpa*) was not significantly altered, while all *Hand1*<sup>TH/TH</sup> placentas had reduced *Prl3d2/Pl1* and *Pr13b1/Pl2* positive cells (Fig. 5B–D), indicating a reduction in several sub-types of TGCs which were well characterized by these

markers previously (Simmons et al., 2007). It is worth noting here that unlike what was observed in the *Hand1*<sup>-/-</sup> mutants in which TGC shows smaller nuclear size and very faint *Pr13d2/Pl1* staining, nuclear size and *Pr13d2/Pl1* staining intensity of the mural TGCs were not significantly different in *Hand1*<sup>TH/+</sup>, *Hand1*<sup>TH/-</sup> and *Hand1*<sup>TH/TH</sup> placentas ( $P > 0.05$ ; Fig. 5C and D and data not shown), indicating a different kind of TGCs defect (neomorph).

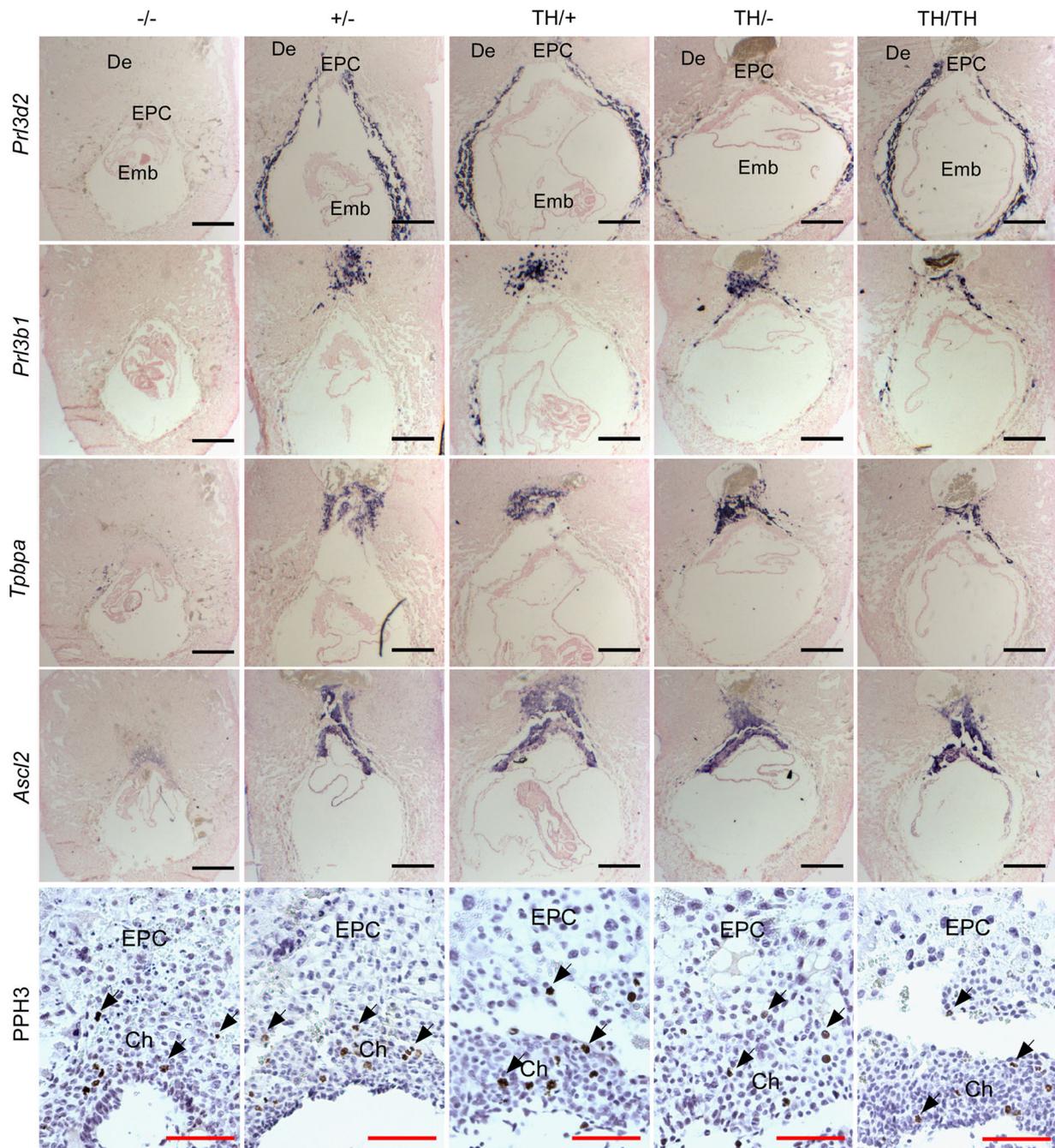
To test if reduced TGCs at E10.5 are due to a TGCs premature differentiation caused by *Hand1*<sup>TH</sup>, we performed various analyses at E8.5 and E9.5. At E8.5, H&E and phospho-histone 3 staining did not show significant changes in the chorion (trophoblast stem cell pool) or ectoplacental cone (TGC progenitor pool) for either total cell numbers or percentage of cells undergoing mitosis ( $P < 0.05$ ; Fig. 6 and Supplementary Fig. 5). Staining of TGCs by *Pr13d2/Pl1* probe and TGC progenitors spongiotrophoblast by *Tpbpa* and *Ascl2* probes, showed no apparent change (Fig. 6). Staining of *Pr13b1/Pl2*, which is only expressed in parietal TGCs lining the implantation site after E9.5 (Simmons et al., 2008), was not prematurely present or increased in *Hand1*<sup>TH/TH</sup> mice at E8.5 (Fig. 6). Staining of trophoblast lineage at E9.5 demonstrated apparent decrease of *Pr13d2/Pl1*, *Pr13b1/Pl2* and *Pr12c/Plf* staining without apparent change of spongiotrophoblast markers *Tpbpa* and *Ascl2* in *Hand1*<sup>TH/TH</sup> mice (data not shown). In short, these data indicate a neomorphic phenotype in trophoblast cells and suggest no premature differentiation of TGC caused by *Hand1*<sup>TH</sup>.

## Discussion

*Hand1* was first identified by virtue of its ability to interact with E proteins (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995), but it was more recently shown to form homodimers as well (Firulli et al., 2000; Scott et al., 2000). The tethered dimer approach has been used to study the activity of various MyoD dimers in cultured cells (Neuhold and Wold, 1993) and Twist dimers in *Drosophila* (Castanon et al., 2001; Wong et al., 2008). Using a similar approach we generated a construct encoding a tethered dimer form of *Hand1* that was stable in cells and show evidence of a constitutively homodimerized form. Using a gene knock-in approach, we have developed the first tethered dimer system in a mouse model to address the biological significance of the alternative dimer forms of *Hand1* and we have made three main conclusions. First, the *Hand1* homodimer is sufficient for mouse survival and plays a dominant role in development, specifically in trophoblast development. Second, the level of *Hand1* homodimer expressed *in vivo* is critical and overdose leads



**Fig. 5.** Characterization of placenta and trophoblast lineage in *Hand1<sup>TH</sup>* implantation sites at E10.5. (A) Characterization of labyrinth development in *Hand1<sup>TH</sup>* conceptuses at E10.5. E10.5 placenta H&E staining low magnification and high magnification showed chorion-allantois attachment fail to occur in some *Hand1<sup>TH/TH</sup>* conceptuses, and labyrinth is not normally developed in *Hand1<sup>TH/TH</sup>* conceptuses as shown by strikingly reduced fetal blood spaces. mbs, Maternal blood space; fbs, fetal blood space. E10.5 placenta laminin antibody staining for basement membrane showed limited branching morphogenesis in *Hand1<sup>TH/TH</sup>* conceptuses. De, decidua; TGC, trophoblast giant cells; SpT, spongiotrophoblast; Lab, labyrinth; ChP, chorionic plate. E10.5 placenta *in situ* hybridization staining for labyrinth syncytiotrophoblast using *Gcm1* probe consistently showed limited branching morphogenesis in *Hand1<sup>TH/TH</sup>* conceptuses. Scale bars: black, 100  $\mu$ m. (B) Characterization of spongiotrophoblast and giant cell layers development at E10.5. E10.5 placenta *in situ* hybridization using *Prl3d2/P11*, *Prl2c/P1f*, *Prl3b1/P12* and *Tpbpa* probes showed *Prl3d2/P11*, *Prl2c/P1f* and *Prl3b1/P12* positive TGCs are reduced in *Hand1<sup>TH/TH</sup>* knock-in conceptuses, while spongiotrophoblast development is not significantly changed in the *Hand1<sup>TH</sup>* placentas. Scale bars: black, 200  $\mu$ m. (C) E10.5 placenta whole mount *in situ* hybridization using *Prl3d2/P11* probe shows that TGC nuclear size and *Prl3d2/P11* staining intensity are not reduced in *Hand1<sup>TH</sup>* knock-in conceptuses. (D) Quantitative analysis showed that TGC density (TGC cell number per area) is significantly reduced in *Hand1<sup>TH/TH</sup>* conceptuses at E10.5. Different superscripts indicate statistically significant differences ( $P < 0.05$ ).



**Fig. 6.** Characterize of the spongiotrophoblast and TGCs development in *Hand1<sup>TH</sup>* knock-in conceptuses at E8.5. E8.5 implantation sites *in situ* hybridization using *Prl3d2/Pl1*, *Prl3b1/Pl2*, *Tpbpa* and *Ascl2* probes. At E8.5, the expression pattern and intensity of these probes in *Hand1<sup>TH</sup>* implantation sites is not apparently different comparing to the *Hand1<sup>-/-</sup>* controls. Immuno-staining of anti-phosphohistone 3 (PPH3) antibody showed that PPH3 positive mitotic cells (arrows) percentage in *Hand1<sup>TH</sup>* implantation sites are not significantly different from the *Hand1<sup>+/-</sup>* controls ( $P > 0.05$ , [Supplementary Fig. 3](#)). De, deciduas; EPC, ectoplacental cone; Emb, embryo; Ch, chorion. Scale bars: black, 400  $\mu\text{m}$ ; red, 100  $\mu\text{m}$ .

to embryonic phenotypes and lethality. Third, *Hand1* heterodimers are either dispensable for embryonic development or play a regulatory role to counterbalance the activity of the *Hand1* homodimer *in vivo*.

*Hand1* tethered homodimer is sufficient for mouse survival and plays a dominant role during development

The most striking finding in our studies is that a significant fraction (about one-third) of *Hand1<sup>TH/-</sup>* mice were viable and fertile. In addition, all embryonic and extra-embryonic phenotypes in *Hand1* null conceptuses at E8.5 were rescued by the *Hand1<sup>TH</sup>* allele.

Convincing biochemical and phenotypic evidence shows that the survival of some *Hand1<sup>TH</sup>* mice and the rescue of early defects are actually due to function of tethered homodimer *Hand1* rather than any possible *Hand1* heterodimers formed. First, to rule out the possibility that the *Hand1<sup>TH</sup>* protein was simply cleaved back into monomer which forms heterodimers *in vivo*, we performed Western blot analysis and found no evidence of such cleavages. To rule out another possibility of intermolecular heterodimers formation, we performed co-immunoprecipitation analysis and showed that *in vitro* intermolecular heterodimers formation is minimal comparing to monomers. Our *in vitro* co-transfection study demonstrates that *Hand1<sup>TH</sup>* is stably formed to drive TGC differentiation and resistant to *Ascl2* inhibition. *In vitro* studies

from other groups also support the idea that the tethered bHLH proteins preferentially form intramolecular dimers and rarely interact with other bHLH monomers or dimers to form intermolecular dimers or tetramers (Bakiri et al., 2002; Lemerrier et al., 1998; Neuhold and Wold, 1993). Second, if Hand1 heterodimers form through Hand1<sup>TH</sup> cleavages or intermolecular heterodimer formation and that is the reason for survival and rescue in Hand1<sup>TH</sup> mice, the phenotype of Hand1<sup>TH</sup> mice should be hypomorphic. However, evidence showed that the phenotype of TH Hand1 mice are neomorphic. A hypomorph model generated by Firulli et al. (2010) demonstrates that Hand1 with a expression 30–40% of that in wildtype (+/+) mice showing similar phenotypes as in Hand1 null mice such as unattached and large allantois, thickened yolk sac, caudal defects and die between E10.5 and E12.5. Whereas, in the Hand1<sup>TH/-</sup> mice, all null defects at E8.5 has been rescued, the primary phenotype at E10.5 or E12.5 is smaller labyrinth and pale yolk sac, with almost all the allantoises attached to chorions and thickened yolk sac has never been observed at either gross or histological level. Moreover, the TGCs phenotype in Hand1<sup>TH</sup> mice is strikingly different as in the Hand1 null mutant. All these data support a neomorphic phenotype of Hand1<sup>TH</sup> allele. Lastly, to assess dosage effects we have generated mice carrying two Hand1<sup>TH</sup> alleles. We reasoned that if there was Hand1<sup>TH</sup> protein partial cleavage or intermolecular heterodimers formation, and either was the explanation for incomplete rescue in Hand1<sup>TH/-</sup> mice, then Hand1<sup>TH/TH</sup> mice should have an even higher rate of rescue. However, the opposites are true.

These data indicate that the homodimer form of Hand1 is sufficient for mouse survival, suggesting a dominant role of Hand1 homodimer. This contradicts that conventional wisdom in the bHLH transcription factor field that class II factors such as Hand1 primarily functions by forming heterodimers with E proteins to play major roles during normal development. However, like our study there are accumulating evidence suggesting bHLH factor homodimers could play dominant roles during development (Castanon et al., 2001; Philogene et al., 2012). A recent study in *Caenorhabditis elegans* with similar strategies as our study by expressing tethered bHLH factor HLH-8/HLH-8 homodimers in *hlh-8* null animals suggested that it is HLH-8/HLH-8 homodimers but not HLH-8/HLH-2 (HLH-2 is Daughterless/E factor ortholog) heterodimer that rescued M patterning and enteric development (Philogene et al., 2012). All suggest a newer paradigm for understanding bHLH transcription factor control of development.

While a dominant role of Hand1<sup>TH</sup> in placenta/trophoblast development has been clearly indicated by our *in vitro* experiments and *in vivo* rescue and neomorphic evidence, identifying its function in particular embryonic tissue can be complicated. Hand1 plays an essential role during development of multiple embryonic tissues such as in heart morphogenesis or lateral mesoderm development, the role of which seems to be required for survival (Maska et al., 2010; McFadden et al., 2005). Thus, survival of Hand1<sup>TH/-</sup> could indicate play a dominant role of Hand1<sup>TH</sup> in these embryonic tissues. However, there are two additional possibilities we cannot rule out with present model. First, Hand1<sup>TH</sup> may be dispensable for development of particular embryonic tissues. This possibility has been indicated by a Hand1 X Wnt1Cre conditional knockout model, in which a dispensable role of Hand1 in synthetic neural development was identified (Vincentz et al., 2012). Second, Hand1 homodimer is dispensable whereas Hand1 heterodimer is required for development of particular embryonic tissue, in which case particular embryonic defects may be present in viable Hand1<sup>TH/-</sup> mice but are compatible with life. This possibility has been indicated by a Hand1 X Tlx2Cre conditional knockout model in which mutant mice displayed histological phenotypes of thinner and disorganized enteric smooth muscle layers. Not all of the mice developed apparent and severe phenotype such as hernia

and about one fourth of them survive to adulthood (Maska et al., 2010). Further identification of the role of Hand1 homodimer in various embryonic tissues awaits new models in which Hand1<sup>TH</sup> expression is introduced to a conditional knock-out background and awaits better characterization of histological phenotypes and biological or molecular read-outs of Hand1 transcription in each particular tissue.

#### *Dosage effect of Hand1 tethered homodimer in regulating mouse development*

While Hand1 tethered homodimers are sufficient for mouse survival, it was also clear that the level of Hand1 homodimer is critical since overabundant Hand1<sup>TH</sup> causes embryonic lethality. The lethality is not due to expression of Hand1<sup>TH</sup> protein *per se* since Hand1<sup>TH/+</sup> mice are perfectly normal. That the lethality is due to overdose of Hand1 is implied by the exacerbated phenotype of Hand1<sup>TH/TH</sup> compared to Hand1<sup>TH/-</sup> conceptuses and supported by quantitative analysis of Hand1<sup>TH</sup> expression.

The mechanism by which Hand1<sup>TH</sup> protein is significantly higher expressed than Hand1 monomers by E10.5 is unclear, however, can be speculated at the mRNA level and protein level. The Hand1 gene promoter region contains several potential binding sites for Hand1 itself. However, based on expression of lacZ (Firulli et al., 1998) and luciferase (Riley et al., 2000) reporter genes knocked into the Hand1 locus, there is no evidence of transcriptional auto-regulation of Hand1. The alternative explanation is that forced dimerization of the Hand1 protein makes it more stable. Various studies have shown that tethered dimers usually have dramatically (usually ~10 fold or more) increased affinity for specific DNA binding sequences (Asahara et al., 1999; Robinson and Sauer, 1996; Sieber and Allemann, 1998; Zhou, 2001). Binding to DNA can inhibit the degradation by the ubiquitin pathway of bHLH proteins (Abu Hatoum et al., 1998). Therefore, it is likely that the Hand1<sup>TH</sup> protein becomes more stable and less susceptible to degradation at post-translational level and leads to accumulated high expression levels in the cell.

To understand why overdose of Hand1<sup>TH</sup> leads to developmental abnormalities, we focused on study of trophoblast cell development, the phenotypes of which has the highest penetrance and are indicated as the primary reason for embryonic lethality. Although the number of parietal TGCs lining the implantation site was reduced, we found that there is no compelling evidence that TGC precursor cell number was significantly affected or TGCs precociously differentiated. This result first seems strange as it is not consistent with the *in vitro* results that Hand1<sup>TH</sup> can over-ride the activity of Ascl2 to maintain TGCs precursor properties. However, after we carefully characterized endogenous expression pattern of Hand1 and Ascl2 *in vivo*, we observed Hand1 and Ascl2 expressions are mutually exclusive in most ectoplacental cells/TGCs precursors at E8.5 and E9.5 (unpublished data provided by Simmons). Therefore, this discrepancy could be explained by the endogenous temporal/spatial expression patterns of Hand1 and Ascl2, which precludes Hand1<sup>TH</sup> from overriding function of Ascl2 in TGCs lineage *in vivo*.

#### *Role of Hand1 monomers or heterodimers in vivo*

The role of Hand1 monomers/heterodimers is very intriguing as indicated by our model. On one hand, 1/3 of Hand1<sup>TH/-</sup> mice are fully viable indicating that Hand1 monomers/heterodimers are dispensable for survival. While a dispensable role of Hand1 has been reported in sympathetic nerve differentiation during embryogenesis, this is not surprising (Vincentz et al., 2012). On the other hand, survival of all Hand<sup>TH/+</sup> mice demonstrate that the presence of Hand1 monomers /heterodimers can rescue the embryonic

lethality caused by Hand1<sup>TH</sup> overdose, indicating an important regulatory role of Hand1 heterodimers. A possible model is that Hand1 heterodimers functionally counterbalance the dominant effect of Hand1 homodimers in regulating gene expression. A comparable mechanism has actually been suggested in *Drosophila* for the Twist bHLH transcription factor. Twist forms homodimers to activate myogenic genes and direct somatic myogenesis, whereas Twist heterodimers with the E protein Daughterless represses these somatic myogenic genes (Castanon et al., 2001). The repression activity is dependent on the repression domain of Daughterless which is sensitive to certain tissue context and development stages (Wong et al., 2008). In addition to repressive activity on the same set of genes, Twist heterodimers might directly compete with homodimers for common binding factors to antagonize its activity (Wong et al., 2008). A direct test of this hypothesis for Hand1 is difficult because firstly, the bona fide transcriptional targets of Hand1 are unknown, which prevents us from examining DNA binding ability of Hand1 heterodimer or homodimer to its putative targeting DNA binding sites, and secondly the Hand1 antibody available for Western blot is not good enough for immunoprecipitation preventing us from testing its endogenous dimerization partners.

## Conclusions

Tissue specific bHLH transcription factors are dedicatedly regulated at the level of dimerization during development. The role of Hand1 homodimers and heterodimers based on our model is complex and has been well summarized (Supplementary Table 2). It is clear in our model that cells require the dominant function of Hand1 homodimer and are very sensitive to their levels; their effect can be modulated and counterbalanced by heterodimers.

## Acknowledgement

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2013.07.025>.

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