Chapter 2

Materials and Methods
# MATERIALS

**Chemicals**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
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<tr>
<td>Acetic acid (glacial)</td>
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<td>Agarose (Type II)</td>
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<td>Sisco Chemicals, India</td>
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<td>Glycogen - 20 mg/ ml</td>
<td>Roche Applied Science, USA</td>
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Hematoxylin Merck Specialities (GR grade), India
Hydrochloric acid Merck Specialities (GR grade), India
Hydroxyquinoline Qualigens Fine Chemicals (ExcelaR grade), India
Isoamyl alcohol Thomas Baker, India.
Paraffin wax (60-62°C) Merck Specialities (GR grade), India
Phenol Merck Specialities (GR grade), India
Picric acid saturated Qualigens Fine Chemicals (ExcelaR grade), India
Potassium aluminium sulphate SD Fine Chemicals (AR grade), India
Potassium chloride Merck Specialities (GR grade), India
Proteinase K Bangalore Genei, India
Primers TCGA, India and Sigma- Aldrich, India.
SDS Amersham Biosciences, Sweden
Sodium acetate Merck Specialities (GR grade), India
Sodium hydroxide Merck Specialities (GR grade), India
Tris Merck Specialities (GR grade), India
Water- PCR clean Biosolve BV, Netherlands and Sigma- Aldrich, USA
Xylene Merck Specialities (GR grade), India

**Enzymes/ Markers/ Kits**

100 bp DNA ladder (100-1517 bp) New England Biolabs, USA
DNeasy® Blood & Tissue Kit Qiagen, USA
Taq DNA polymerase and 10X buffer SibEnzymes, Russia
**Buffer/ Reagents/ Solutions**

**2% (w/v) Agarose gel – 70 ml**

2% (w/v) agarose was dissolved by boiling in 70 ml of 0.5X TBE. The volume was made up to 70 ml by adding MQ water. 0.4 µg/ml ethidium bromide (v/v) was added after the solution cools to ~50°C and swirled to mix and poured on the gel tray to mould.

**Bouin’s fixative – 125 ml**

75 ml saturated picric acid

5 ml glacial acetic acid

25 ml formaldehyde 40%

**6X Gel loading buffer**

0.25% (w/v) bromophenol blue

30% (v/v) glycerol in autoclaved MQ water

**DPX for histology**

The DPX used for mounting was prepared by mixing DPX & xylene in 4:1 ratio.

**0.5 M EDTA (pH 8)**

The solution was prepared in autoclaved MQ water. The pH was adjusted to 8 with 5 M sodium hydroxide and the solution was stirred vigorously to achieve complete dissolution of the EDTA and sterilized by autoclaving.

**Ehrlich’s Hematoxylin**

Solution I - 1 gm hematoxylin powder was dissolved in 5 ml glacial acetic acid, 50 ml ethanol and 50 ml glycerol.

Solution II - 5 gm potassium aluminum sulphate was dissolved in 50 ml distilled water by boiling to prepare the saturated solution.

Solutions I and II were mixed and the mixture was exposed to sunlight for ripening in a glass flask plugged with cotton. Ripening was complete in 2-3 mo.
**Tris-Cl equilibrated Phenol**

Phenol was equilibrated to pH > 7.8 with 0.5 M and 0.1 M Tris-Cl pH 8.0, for use in DNA isolation (DNA partitions into organic phase at acidic pH) (Sambrook & Russell, 2001). The phenol stored at -20°C was warmed to ambient temperature and melted at 68°C. Hydroxyquinoline which is an antioxidant, a weak metal ions chelator as well as provides yellow colour to the phenol, was added to a final concentration of 0.1%. An equal volume of 0.5 M Tris-Cl pH 8.0 was added to the liquefied phenol and mixed on a magnetic stirrer for 15 min in an amber bottle. The phenol was allowed to stand for phase separation and the aqueous phase was aspirated with a glass pipette. Following this, the phenol was equilibrated repeatedly with 0.1 M Tris-Cl pH 8.0 as described above, until the pH was > 7.8 (checked periodically with pH paper). The equilibrated phenol was then layered with 0.1 M Tris-Cl pH 8.0 containing 0.2% mercaptoethanol and stored in amber bottle at 4°C for few mo.

**5 mg/ml Ethidium Bromide**

The solution was prepared in autoclaved MQ water. Dissolved the ethidium bromide by vortexing vigorously to ensure complete dissolution and stored at 4°C covered with aluminum foil.

**Eosin solution**

0.01% (w/v) eosin powder in 95% ethanol.

**Lysis Buffer**

50 mM Tris-Cl (pH 8.0)

5 mM EDTA

0.5% SDS

The volume was made up with autoclaved MQ water.

**Mayer’s albumin** prepared by stirring equal volume of egg albumin and glycerol. A pinch of crystalline phenol was added after mixing. It was stored at -20°C.
**Phosphate buffer saline – A (pH 7.2 - 7.39) – 500 ml**

5 g NaCl,

0.125 g KCl

0.72 g Na$_2$HPO$_4$

0.125 g KH$_2$PO$_4$

The solution was prepared in autoclaved MQ water and sterilized by autoclaving.

**10 mg/ml Proteinase K**

The solution was prepared in autoclaved MQ water and stored in -20°C.

**10% (w/v) SDS**

The SDS was dissolved in autoclaved MQ water by heating to 50-60°C and final volume was made up as the solution cooled to room temperature. The solution was not sterilized and was stored at room temperature.

**Seasoned paraffin wax**

For seasoning, the paraffin wax was melted at ~70°C and allowed to solidify thrice to remove all the air bubbles. The molten paraffin was filtered through Whatmann filter paper 1 to remove all the suspended particles and used for histology.

**3 M Sodium acetate (pH 5.2)**

The solution was prepared in autoclaved MQ water and pH was adjusted with glacial acetic acid. The solution was sterilized by autoclaving.

**5 M Sodium hydroxide**

The solution was prepared in cold autoclaved MQ water to prevent heating of the solution. Final volume was made up after the solution cooled to room temperature.

**10X Taq Buffer**

600 mM Tris-Cl (pH8.5)

15 mM MgCl$_2$

250 mM KCl

100 mM 2-mercaptoethanol

1% Triton X-100

1X Taq buffer was used for PCR.
**5X TBE Stock solution (pH ~8.3) – 500 ml**

27 g Tris buffer

13.75 g Boric acid

0.5 M EDTA- 10 ml

The solution was prepared in autoclaved MQ water. The stock solution was diluted to 0.5x TBE in MQ water for use.

**TE (pH 8)**

100 mM Tris-Cl (pH 8)

10 mM EDTA (pH 8)

The volume was made up with autoclaved MQ water.

**1 M Tris-Cl (pH 8)**

The Tris base was dissolved in autoclaved MQ water and the pH was adjusted to 8 with concentrated HCl. The solution was cooled to room temperature before making the final adjustments to the pH. The solution was sterilized by autoclaving.
METHODS

1. Establishment of the Hmox1 mouse colony

- Animal Husbandry protocols

To determine the role of Hmox1 in rodent embryogenesis, the first step was to establish the Hmox1 gene-targeted mouse colony in our animal house. We were aware that genetically modified mice have to be housed in a stable aseptic environment. In the absence of infrastructure for housing gene-targeted and transgenic mice in our Animal house, a room was prepared for installation of an IVCS (Ventilator Model No. V3, Citizen Industries, India). Ambient temperature of 21-24°C and relative humidity of 40-70% was maintained with air conditioners, oil filled heater (in winter) and humidifier. A 14 h light/10 h dark cycle was maintained in the animal room with an automatic timer. Breeding pairs which were heterozygous for the Hmox1 gene were obtained from the “Small Animal Facility” of NII and the mouse colony was established in the animal room. Wild type progeny obtained from the Hmox1 breeding pairs were also set for mating to determine the normal breeding pattern of this gene-targeted mouse line. Temperature and relative humidity was monitored daily and all precautions were taken to minimize noise, vibrations, dust and odour in the animal room.

Maintenance of these mice involved sterilizing the IVCS cage assemblies, bedding, potable water and water bottles routinely. Paddy husk was used as bedding material and shredded paper was provided in cages as enrichment and nesting material. Water and pellet feed was made available to the mice *ab-libitum*. Pellet feed was sterilized by gamma-irradiation (15 KGY, Shriram Institute For Industrial Research, Delhi) while all other material was sterilized by autoclaving. The soiled cages were changed once a week. In addition, precautions were taken before entering the animal room and handling the mice to prevent the accidental carriage of infectious agents and murine pathogens into the facility. Laboratory coats, face masks, gloves, head and shoe covers were worn before handling the mice. Special care was taken while handling the mice during cage changing especially in case of pregnant females,
newly born pups and lactating mothers to avoid physical stress. Handling of the mice during biopsy collection and vaginal examination was also done very gently and carefully to avoid any kind of stress or injury.

Accurate record of each breeding pair and details of genotype of their progeny was maintained. Handling, husbandry and disposal of the mice were carried out according to CPCSEA guidelines and all experimental protocols were approved by the Department of Zoology Institutional Animal Ethics Committee.

• Genotyping of the progeny mice

Tagging and tail biopsy collection

As significant prenatal lethality is observed in Hmox1 gene-targeted mice, we maintained this mutation in non-sibling mating of heterozygous mice. Routine genotyping of progeny is an integral part of maintaining this mouse colony. Genotyping of mice was done by multiplex PCR using genomic DNA isolated from tail biopsies.

At approximately 6 weeks of age, the progeny of Hmox1 mice from heterozygous breeding pairs were ear tagged with numbered metallic small animal ear tags from National Band & Tag Company, USA and at the same time 1 cm tail biopsies were collected. The mouse ear that was to be tagged, metal tag and the applicator were disinfected with 70% ethanol to prevent infection after ear tagging. Tail biopsies were snipped off with surgical blades and frozen at -70°C till further use. Fresh surgical blades were used for each tail biopsy to prevent cross contamination.

Genomic DNA isolation and quantitation

The frozen tail biopsies were thawed rapidly in a 37°C water bath to bring them to ambient temperature and 500 µl Lysis Buffer containing 400 µg/ml proteinase K was added to each tube. These were incubated for 18 h at 55°C in a shaker incubator (Neolab Instruments) set at 150 rpm. After incubation the lysates were allowed to cool to ambient temperature and extracted with phenol: chloroform: isoamyl alcohol at ambient temperature as follows. To each tube 250 µl each of Tris-
equilibrated phenol and choloform : isoamyl alcohol mixture (24:1) was added and mixed gently on a rotor wheel (Neolab Instruments) for 15 min. Phase separation was done by centrifugation at 13,000 rpm for 5 min in a microcentrifuge (MiniSpin, Eppendorf, Germany) and 500 µl of aqueous phase was collected in 2 ml tubes without disturbing the interface. The phenol : chlo roform : isoamyl alcohol extraction step was repeated and 400 µl of aqueous phase was collected. An equal volume of chloroform : isoamyl alcohol mixture (24:1) was added to the aqueous phase, mixed on rotor wheel for 10 min, centrifuged at 13,000 rpm for 5 min and 250 µl of aqueous phase was collected in 1.5 ml tubes. To this 1/10 volume of 3 M sodium acetate (pH 5.2) was added and DNA was precipitated with two volumes of ice cold absolute ethanol. The DNA was pelleted by centrifugation at 13,000 rpm for 3 min at ambient temperature and left overnight at 4°C in 800 µl of 70% ethanol to remove any excess salt. The following day the tubes were warmed to ambient temperature, vortexed gently to release the DNA pellet and centrifuged at 13,000 rpm for 5 min. This washing step was repeated, the DNA pellet was air dried and left overnight at ambient temperature for dissolving in 50 µl of TE buffer. The samples were stored at 4°C till the genomic PCR was completed and then transferred to -20°C for long term storage.

For spectrophotometric quantitation an aliquot of the genomic DNA was diluted 1:100 in TE buffer and the absorbance was measured at 260 and 280 nm in a double beam spectrophotometer (Cecil CE 7200). DNA concentration was determined from the absorbance at 260 nm.

The \( A_{260}/A_{280} \) ratio of the tail biopsy samples ranged from 1.84 to 1.96.

**Genomic PCR**

The genomic PCR was based on the protocol used in Prof. Anupam Agarwal’s laboratory (Table 2). The multiplex PCR involved two sets of primers; one specific for the WT allele producing an amplicon of 146 bp and the other specific for the KO allele producing an amplicon of 283 bp. Their protocol was adapted in our laboratory to make it more cost effective by substituting the HotStar Taq DNA polymerase with SibEnzyme Taq DNA polymerase. The reaction volume of 50 µl contained 2 µl template DNA, 0.2 µM of WT primers, 0.4 µM of KO primers, 160 µM of dNTP mix, 2.5 U of Taq DNA polymerase and 0.69 M betaine (wherever required). The reaction
Table 2: PCR genotyping protocol (Personal communication; Prof. Anupam Agarwal, University of Alabama, Birmingham, Alabama, USA)

Two primer sets were used for multiplex PCR.

Wild type allele

1. HO-1 WT 5'-3' Forward – GTA CAC TGA CTG TGG GTG GGG GAG
2. HO-1 WT 5'-3' Reverse - AGG GCC GAG TAG ATA TGG TAC
3. Amplicon - 146 bp

Knockout allele

1. HO-1 KO 5'-3' Forward - GCT TGG GTG GAG AGG CTA TTC
2. HO-1 KO 5'-3' Reverse - CAA GGT GAG ATG ACA GGA GAT C
3. Amplicon - 283 bp.
Table : 2

Each 50 µl reaction contains the following components -

<table>
<thead>
<tr>
<th>Component (Concentration)</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR clean ddH₂O</td>
<td>37.5 µl</td>
<td>-</td>
</tr>
<tr>
<td>10X Taq Buffer</td>
<td>5 µl</td>
<td>1X</td>
</tr>
<tr>
<td>dNTP mix (8 mM, 2 mM each)</td>
<td>1 µl</td>
<td>160 µM</td>
</tr>
<tr>
<td>HO-1 WT Forward (10 pm/ µl stock)</td>
<td>1 µl</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>HO-1 WT Reverse (10 pm/ µl stock)</td>
<td>1 µl</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>HO-1 KO Forward (20 pm/ µl stock)</td>
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<td>0.4 µM</td>
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<tr>
<td>HO-1 KO Reverse (20 pm/ µl stock)</td>
<td>1 µl</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>Qiagen HotStar Taq DNA Polymerase (5 U/ µl)</td>
<td>0.5 µl</td>
<td>2.5 U</td>
</tr>
<tr>
<td>Template DNA (100 ng/ µl)</td>
<td>2 µl</td>
<td>200 ng</td>
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Thermal cycling conditions –

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>(with heated lid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>5 min</td>
<td>repeat 35 cycles</td>
</tr>
<tr>
<td>95°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>54°C</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>72°C</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>4°C</td>
<td>Hold</td>
<td></td>
</tr>
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</table>
mix was prepared for n + 1 reactions as shown in Table 3 and aliquoted into precooled 0.2 ml PCR tubes, in the PCR workstation. To each tube 2 µl of 1:10 dilution of tail DNA was added and the reaction was carried out in a thermal cycler (Applied Biosystems 2720, USA) using thermal cycling conditions mentioned in Table 2. Handling and addition of the DNA was done in a fume hood, away from the PCR workstation, to avoid accidental DNA contamination. The work areas and pipettes used for PCR were UV irradiated before and after use to prevent carryover contamination.

Following the PCR 10 µl of each reaction, mixed with 2 µl of 6X gel loading buffer was electrophoresed in 2% (w/v) agarose gel containing 0.4 µg/ ml ethidium bromide. Electrophoresis was carried out in 0.5X TBE buffer at 5 V/cm till the BPB dye front migrated ~9.8 cm from the front of the well. The gel was viewed and imaged in a gel documentation system (ChemiDoc XRS, Bio-Rad Gel documentation system or AlphaImager EC, Alpha Innotech). Gel pictures were printed in a thermal printer (Digital Graphic Printer UP-D890) on high gloss thermal paper from Sony (Type V UPP-110HG).

Densitometric analysis of gel pictures

Quantity One software provided with Bio Rad gel documentation system was used for imaging and analyzing the agarose gels. Densitometric analysis of the gel pictures was done using quantitation protocol optimized in our laboratory (Sonika Sharma, Ph.D. Thesis). After background subtraction each band was converted to its Trace Quantity (intensity x mm) which is the area under its intensity profile curve.

2. Experimental mice

Criteria for the experimental mice were based on the comprehensive breeding data of the Hmox1 mouse colony. Adult healthy virgin females between 2-6 mo and males between 2-8 mo of age were used for the timed matings. These were progeny obtained from younger breeding pairs (maternal age 3-9 mo). All these criteria were set to exclude effects of age- related reproductive irregularities. Only the males with
Each 50 µl reaction contains the following components –

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<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>H₂O (Molecular biology grade)</td>
<td>30.8 µl</td>
<td>-</td>
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<tr>
<td>SibEnzymes 10X Taq Buffer</td>
<td>5 µl</td>
<td>1X</td>
</tr>
<tr>
<td>SibEnzymes dNTP mix (10 mM, 2.5 mM each)</td>
<td>0.8 µl</td>
<td>160 µM</td>
</tr>
<tr>
<td>HO-1 WT Forward (10 pm/ µl stock)</td>
<td>1 µl</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>HO-1 WT Reverse (10 pm/ µl stock)</td>
<td>1 µl</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>HO-1 KO Forward (20 pm/ µl stock)</td>
<td>1 µl</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>HO-1 KO Reverse (20 pm/ µl stock)</td>
<td>1 µl</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>SibEnzymes Taq DNA Polymerase (5 U/ µl)</td>
<td>0.5 µl</td>
<td>2.5 U</td>
</tr>
<tr>
<td>Template DNA (1:10 dilution)</td>
<td>2 µl</td>
<td>~ 70 ng</td>
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Thermal cycling conditions –

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<td></td>
</tr>
<tr>
<td>4°C</td>
<td>Hold</td>
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</tbody>
</table>

Table 3: Adapted PCR genotyping protocol. Modifications made have been highlighted in grey.
proven fertility (studs) were used for experiments to avoid pseudopregnancy on account of male infertility.

3. Sample collection

- Timed matings

Non-sibling timed matings were set in 1:1 ratio of males and females of the same genotype. These mice were chosen from the same generation with a maximum permissible age difference of 2 mo. The matings were set in the evening in conventional autoclaved polypropylene cages with steel grid covers. Feed pellets and water was provided ab-libitum. The next morning the vagina of the female was examined for seminal plug deposition as a mark of copulation. The day of plug detection was considered as 0.5 dpc (Nagy et al., 2003). The plug positive female was separated from the male and dissected at the required gestational age only if it appeared pregnant.

- Harvesting of mouse embryos

The pregnant female was euthanized humanely by cervical dislocation on the day of dissection to harvest embryos of a particular gestational age. The abdomen of the euthanized mouse was sprayed generously with 70% ethanol to avoid contamination with mouse hairs during dissection. Next the abdomen was cut open and the coils of intestine were pushed away gently. The two horns of the pregnant uterus were located as shown in Figure 3A and the blood vessels, extraneous tissues were cut and removed as shown in Figure 3B. The uteri were cut with scissors across the cervix and ovaries to free them from the female’s body as shown in Figure 3B and gently placed in a 10 cm glass petridish containing PBS – A at ambient temperature. Each conceptus was separated out by cutting transversely between the implantations as shown in Figure 3C and placed in a 5 cm petridish for further dissection. The muscle layer of the uterus was cut away with fine scissors without damaging the extraembryonic tissues. For embryos of 12.5 dpc to 14.5 dpc the junction of Reichert’s membrane and the placenta was carefully nipped off (Figure 4A) to expose the visceral yolk sac enclosing the embryo attached to the placenta.
**Figure 3**: Dissection of the pregnant female mouse. **A**: The gut was displaced and removed, revealing the pregnant uteri on the floor of the body cavity. **B**: Mesometrium and blood vessels attached to the uterus were removed gently by holding the uterus with forceps as shown. Both the uteri were finally removed out of the female’s body by cutting across the cervix. **C**: After the uterus was placed in a petridish, each conceptus was separated by cutting transversely between the implantation sites.
Adapted from (Nagy et al., 2003).
Figure 3

A

Coils of gut pushed out of the way
Kidney, fat pad, ovary, and oviduct
Pregnant uterus
Fat pad (larger in older mice)
Bladder

B

Pull up with forceps
Use tips of forceps or scissors to tear mesometrium
Mesometrium with blood vessels
Cut cervix with scissors
Pull uterus out to side

C

Uterus dissected into medium in the dish
Muscular wall of uterus contracts
Cut mesometrium
Remnants of mesometrium
Decidual tissue
via umbilical cord blood vessels (Figure 4B). In embryos of the later stages this step is not required as the Reichert’s membrane becomes very thin and begins to degenerate. The yolk sac was cut and separated very carefully from the base of the placenta on the embryonic side so that there was no contamination with maternal tissue. The umbilical cord blood vessels were cut carefully to separate the embryo and the placenta as shown in Figure 4C. The embryo was removed from the yolk sac (Figure 4D), examined in a stereozoom microscope (Nikon SMZ800 with Fiber epi-illumination) and photographed using a digital camera (Nikon Coolpix 8400) attached to the microscope. The crown-rump lengths of the embryos at gestational ages 9.5 dpc to 12.5 dpc were measured with the reticle micrometer lens (Nikon) attached to the eye piece of the stereozoom microscope. For gestational age 13.5 dpc to 18.5 dpc embryos the crown -rump length was measured with ImageJ software (ImageJ 1.40g version, USA). The embryos (12.5 dpc to 18.5 dpc) and placentas (11.5 dpc to 18.5 dpc) were weighed and fixed in Bouin’s fixative for 6-22 h for histological analysis. The time of fixation was based on crown-rump length of the embryo and weight of the placenta (Kaufmann, 1998). Fixed tissues were washed and stored in 70% ethanol till further analysis.

The 9.5 dpc to 11.5 dpc embryos were dissected out carefully, photographed and frozen at -70°C. The weight of 10.5 dpc and 11.5 dpc embryos were noted before freezing. All the glassware and dissection instruments were sterilized by autoclaving. For dissection of embryos from HET females, all the dissection instruments were cleaned and washed with autoclaved MQ water followed by PBS-A in between the embryos, to prevent cross-contamination.

The dissected yolk sacs were collected in precooled 2 ml tubes and frozen at -70°C till further processing for determining the genotype of the embryos. At 9.5 dpc and 10.5 dpc, dissecting the yolk sac without maternal tissue contamination was very difficult. In addition the embryos were too small in size to be handled for histology and therefore those harvested from HET females were used for genotyping.

All harvested embryos were staged according to Theiler’s Staging Criteria to determine the developmental stage. Theiler’s Staging Criteria are based on the typical external characteristic features observed during mouse embryo development (Theiler, 1972).
**Figure 4**: Diagrammatic representation of dissection of 13.5 dpc conceptus to recover the embryos as well as the extraembryonic tissues - the placenta and yolk sac. **A**: The muscle layer of the uterus surrounding each conceptus was removed quickly to release it with intact Reichert’s membrane. **B**: Embryo enclosed by yolk sac attached to placenta via umbilical cord blood vessels was exposed after removal of Reichert’s membrane. **C & D**: The yolk sac and the umbilical cord blood vessels were cut from the base of placenta carefully to separate the embryo, placenta and the yolk sac. Adapted from (Nagy *et al.*, 2003).
Figure 4

Conceptus removed from the uterus

A
Embryo enclosed in Reichert’s membrane

B
Embryo after removal of Reichert’s membrane

C
Umbilical cord blood vessels →

Placenta removed

D
Yolk sac →

Embryo covered by amnion
4. Genotyping of mouse embryos

The genomic DNA isolation from 10.5 dpc and 11.5 dpc embryos and yolk sacs was done as described for the tail biopsy DNA. For 10.5 dpc embryos, 10 µg of glycogen was added to the aqueous phase before precipitating the genomic DNA with sodium acetate and ethanol. Glycogen acts as an inert carrier for precipitating DNA when concentration is expected to be low (Tracy, 1981; Helms et al., 1985).

DNA was isolated from 9.5 dpc embryos with DNeasy® Blood & Tissue Kit, Qiagen, USA as per manufacturer’s instructions. The frozen embryos were quickly thawed in a water bath at 37°C and 180 µl Buffer ATL plus 20 µl proteinase K were added to the tubes. This was followed by incubation at 56°C for 18 h in a shaker incubator set at 150 rpm. After incubation the lysates were allowed to cool to ambient temperature and DNA was purified using the Spin-Column protocol provided in the DNeasy® Blood & Tissue handbook. The DNA was eluted from the DNeasy Mini spin column using 100 µl of Buffer AE. DNA quality and concentration was estimated on Nanodrop spectrophotometer (ND-1000, Nanodrop Technologies, USA)

All the reagents used were at ambient temperature unless specified. Precautions were taken to prevent cross-contamination between the samples.

DNA isolated from embryos (9.5 dpc to 11.5 dpc) and yolk sacs (12.5 dpc to 18.5 dpc) were used for PCR genotyping. The genomic PCR protocol described for genotyping the Hmox1 mice was used except that betaine was not required. A 1:10 dilution of yolk sac or embryo DNA used as template in these reactions gave a lot of non-specific amplification. Therefore 2 µl of stock DNA was used as template to obtain a clear genotype of the embryos.

5. Histology of placenta

Placenta samples that were stored in 70% ethanol were dehydrated by passing through different grades of ethanol as follows – 80% ethanol for 30 min, 90% for 30
min, 96% (I) for 60 min, 96% (II) for 30 min, 100% (I) for 60 min and 100% (II) for 30 min. These were cleared in xylene for 45 min followed by three changes in paraffin wax at 55°C (45 min each). For making blocks, the placentas were placed in glycerol smeared cavity blocks containing molten paraffin wax and were allowed to cool overnight. While embedding the placenta in the paraffin wax and making the blocks air bubbles were avoided.

The wax was trimmed to obtain a rectangular block which was mounted on a wooden chuck. The chuck was then fixed on to the sample holder of the microtome (Spencer, Model No- 820, AO Instruments Company, USA). Radial sections of 8 µm thickness were cut serially using a disposable microtome blade (Dura-edge, USA) and any loss of sections in between was noted. Wax ribbons containing placenta sections were floated on the 20% ethanol placed on Mayer’s albumin coated glass slides to ease the stretching process. These wax ribbons were stretched in a water bath maintained at ~55°C and dried at 40°C overnight.

For staining the sections, the wax was first removed by passing through xylene (two changes for 5 min each). Following this, the sections were rehydrated in descending ethanol grades (5 min each), starting with 100% ethanol (two changes) followed by 90%, 70%, 50%, 30% ethanol and distilled water. The sections were stained in Ehrlich’s Hematoxylin for 10-15 min till nuclei of the cells were stained and were differentiated by dipping in tap water and ammonia water (0.05% v/v). Next these were dehydrated by passing through ascending grades of ethanol (5 min each) starting with distilled water, followed by 30%, 50%, 70% and 90% ethanol and stained with eosin solution for 2 sec. After this sections were dehydrated in 90% ethanol followed by two changes in 100% ethanol, before clearing in xylene. The cleared sections were mounted with DPX and dried overnight at ambient temperature.

Mid radial sections of the placenta were examined and photographed in the stereozoom microscope (Nikon SMZ800) at 2X magnification to capture the tissue organization. For finer details, sections were photographed at 10X magnification in an inverted microscope (Nikon Eclipse TS100) with compatible Nikon digital camera.
6. Statistical analysis

SigmaPlot 12.0 software was used for statistical analysis of data obtained from four groups that included WT, HET and KO samples from heterozygous matings and samples from wild type matings. Dot density graphs of crown-rump length of embryos, weights of placentas and embryos from four groups of samples were plotted and tested for normal distribution. If they passed the Normality test (Shapiro-Wilk Test) and Equal Variance test, they were analysed for differences using the one-way analysis of variance (ANOVA). These data sets were further checked for pairwise differences within the groups by Tukey’s test which is based on the comparison of the group means.

The data sets which were not distributed normally were tested for differences by comparison of the medians, using Kruskal-Wallis One Way Analysis of Variance on Ranks. The pairwise differences within the groups were further checked by non parametric test- Dunn’s Method which is based on comparisons of the medians.

For all the tests, statistical significance was determined at $p = 0.05$. 