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HP1BP3 expression determines maternal behavior and offspring survival

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Maternal care is an indispensable behavioral component necessary for survival and reproductive success in mammals, and postpartum maternal behavior is mediated by an incompletely understood complex interplay of signals including effects of epigenetic regulation. We approached this issue using our recently established mice with targeted deletion of heterochromatin protein 1 binding protein 3 (HP1BP3), which we found to be a novel epigenetic repressor with critical roles in postnatal growth. Here, we report a dramatic reduction in the survival of pups born to *Hp1bp3*$^{-/-}$ deficient mouse dams, which could be rescued by co-fostering with wild-type dams. *Hp1bp3*$^{-/-}$ females failed to retrieve both their own pups and foster pups in a pup retrieval test, and showed reduced anxiety-like behavior in the open-field and elevated-plus-maze tests. In contrast, *Hp1bp3*$^{-/-}$ females showed no deficits in behaviors often associated with impaired maternal care, including social behavior, depression, motor coordination and olfactory capability; and maintained unchanged anxiety-associated hallmarks such as cholinergic status and brain miRNA profiles. Collectively, our results suggest a novel role for HP1BP3 in regulating maternal and anxiety-related behavior in mice and call for exploring ways to manipulate this epigenetic process.

Keywords: Anxiety, chromatin, epigenetics, histone, HP1BP3, knockout mouse, maternal behavior, microRNA, social, survival

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With the evolution of live birth synchronized with lactation, maternal behavior toward young became essential for species survival in mammals. It increases the probability that offspring will reach maturity and is essential for the survival of mammalian newborns (Wang & Storm 2010). As rodents are born deaf, blind and immobile, maternal behaviors including nest building, gathering pups together in the nest, and keeping them warm are critical for survival. Maternal behavior and care is of utmost importance for humans as well, and across a broad range of disciplines, there is a growing consensus on the importance of early childhood in influencing the trajectory of mental health later in life (Stewart-Brown & Schrader-Mcmillan 2011).

A new mother displays a dramatic change in behavior as soon as the babies are born: she immediately cares for the young and defends them. The expression of these essential components of maternal behavior is the culmination of changes controlled by pregnancy hormones affecting the activity of neural circuitry in late pregnancy. Recently, major progress has been made in our understanding of the organization and molecular mechanisms of the neuroendocrine networks that govern mammalian parturition, lactation and maternal behavior. These changes involve a complex interplay between hormonal signaling (Bridges & Mann 1994; Insel *et al.* 1997; Martyn *et al.* 2012), gene expression patterns (Driessen *et al.* 2014; Eisinger *et al.* 2013; Mileva-Seitz *et al.* 2016) and epigenetic regulation (Stolzenberg *et al.* 2012, 2014) in numerous brain regions.

Genetic mouse models have been instrumental in uncovering genes and pathways important for mediating maternal behavior, and knockout mice have confirmed roles for serotonergic signaling (Alenina *et al.* 2009; Angoa-Perez *et al.* 2014b; Lerch-Haner *et al.* 2008), and olfaction (Wang & Storm 2010; Weiss *et al.* 2011) among others. Knockout mice have also been invaluable in defining the roles of pregnancy hormones in inducing maternal behavior, and mouse models with impaired oxytocin (Pedersen *et al.* 2006; Takayanagi *et al.* 2005) or prolactin (Lucas *et al.* 1998; Martyn *et al.* 2012) function showed dramatic impairments in their maternal care and responsiveness. Finally, mouse models have highlighted the importance of epigenetic regulation for maternal behavior (Stolzenberg & Champagne 2016). Thus, deletion of the neuron-expressed epigenetically imprinted genes *Peg3* (Champagne *et al.* 2009; Li *et al.* 1999) and *Mest* (Lefebvre *et al.* 1998), as well as the epigenetic silencing complex component *Mbd2* (Hendrich *et al.* 2001), all led to impaired

maternal behavior. In this regard, we have recently characterized a novel histone variant called HP1BP3, and found that it is a transcriptional repressor that interacts with the epigenetic repressor HP1 and is highly expressed in the brain (Garfinkel *et al.* 2015b). Deletion of HP1BP3 in a knockout (KO) mouse model had a profound effect, and only 40% of *Hp1bp3*$^{-/-}$ pups born to heterozygous parents survived to adulthood. Furthermore, surviving mice were proportionate dwarfs, and their reduced size was linked to altered expression of endocrine insulin like growth factor 1 (IGF-1) pathway components (Garfinkel *et al.* 2015a). We now find that HP1BP3 deficient dams show impaired maternal behavior and anxiety reactions, leading to reduced litter survival. HP1BP3 is highly conserved between mice and humans, and epigenetic regulation of the *HP1BP3* gene itself was recently implicated in human postpartum depression (PPD; Guintivano *et al.* 2013; Osborne *et al.* 2015), indicating that this gene may play a conserved role in maternal behavior. Based on these considerations, we used HP1BP3 deficient mice for studying the role of *HP1BP3* in maternal behavior and anxiety.

Materials and methods

Animals

Hp1bp3$^{tm1a(EUCOMM)Wtsi}$ mice with a C57BL/6N genetic background were acquired from the European Conditional Mouse Mutagenesis Program (EUCOMM) and have been described previously (Garfinkel *et al.* 2015a). In these mice, a FlipROSAβGeo cassette (Schnutgen *et al.* 2005) was inserted into intron 7 of the *Hp1bp3* gene, leading to the production of a truncated transcript. The genotype of all animals was assessed using a PCR-based genotyping protocol. At 21 days of age, tails were clipped, the tissue was digested and DNA was extracted and purified. PCR was performed on 1–2 μg of DNA using the primer triplet a, b and c. The knockout specific primer pair a/b produces a product of 150 bp and the wild-type (WT) specific primer pair a/c produces a product of 650 bp. The primer sequences used were: a-TCGTGGTATCGTTATGCGCC; b-GAAAGGTGAGTCTGTCCCCG; c-CCCTTCTGCAACACAGCATC. The mice were maintained under a schedule of 12 h light, 12 h dark with food and water *ad libitum,* and were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols had the approval of the Institutional Committee on Animal Care and Use at The Alexander Silverman Institute of Life Sciences, The Hebrew University of Jerusalem. Mice were weaned at the age of 21 days.

Animal breeding and cross-fostering

Hp1bp3$^{-/-}$ and *Hp1bp3*$^{+/+}$ female mice were bred with stud males of opposite genotypes (*Hp1bp3*$^{+/+}$ and *Hp1bp3*$^{-/-}$, respectively) and vaginal plugs were assessed daily. Pregnant females were isolated 4 days prior to parturition. Following birth, the total number of pups per litter was counted, and the percentage of pups excluded from the nest was scored. Litters were then observed on a daily basis for 1 week. A separate set of experiments was carried out in which a surrogate experienced non-lactating *Hp1bp3*$^{+/+}$ mother was placed in the home-cage of a pregnant *Hp1bp3*$^{-/-}$ female. Following parturition, the number of surviving pups from the *Hp1bp3*$^{-/-}$ mother was documented.

Tissue and blood collection

Hp1bp3$^{-/-}$ and *Hp1bp3*$^{+/+}$ female mice aged 2–6 months were bred as described above. Prior to parturition, mice were housed in pairs of one *Hp1bp3*$^{-/-}$ and one *Hp1bp3*$^{+/+}$ to allow survival of the pups born to *Hp1bp3*$^{-/-}$ females. The number of pups was held constant at 5 pups per dam. Mice were killed by CO_2 asphyxiation and cervical dislocation 48 h postpartum. Blood collected by cardiac puncture was allowed to clot in blood collection tubes (BD), then centrifuged (6000 × *g*, 5 min at 4°C), and serum was separated and stored at −80°C until analysis by ELISA. Inguinal mammary glands were next surgically removed and fixed in 4% phosphate-buffered paraformaldehyde (pH = 7.2) for 24 h. Samples were embedded in paraffin, and 4 μm sections were placed on slides and stained with Hematoxylin and eosin for histological analysis.

Serum hormone measurements

Serum concentrations of Prolactin and Oxytocin were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits from Abcam (Cambridge, UK) and Enzo Life sciences (Farmingdale, NY, USA), respectively, according to the manufacturer's instructions. Results were detected using a Synergy H1 Multi-Mode Reader (Biotek, Winooski, VT, USA). Serum concentrations of progesterone and estrogen were measured using commercially available Chemiluminescent Microparticle Immunoassay (CMIA) kits from Abbot (Longford, Ireland).

Behavioral phenotype testing

All testing was performed in an isolated behavior room at 23–24°C. Animals were brought to the testing room 2 h before testing began, to acclimate them to the environment. All behavior testing occurred in the second half of the light part of the light/dark cycle. All behaviors were videotaped and scored later by a single trained observer blind to genotype. Each behavioral test was separated by at least 24 h and tests were performed in the order presented. Heterozygotes were not included for purposes of controlling subject number. This allowed each test to be completed in 1 day and during the same part of the light/dark cycle, in an effort to reduce variability. All mice used in these studies were aged 2–6 months, and were the products of heterozygous pairings in order to control for the possible confound of parental genotype.

Maternal behavior test

Two days following parturition, postpartum female mice were separated from their pups and placed one at a time in a clean plastic chamber (26 × 42 cm) with standard wood chip bedding and a cotton nestlet in one corner and allowed 15 min of free exploration. Four of their pups were consecutively placed at the opposite end of the cage, with 30–40 second intervals. The experiment was terminated when all pups were retrieved or after 2 min. The latency to approach and the latency to retrieve the pups were scored manually from the videotape. In order to control for the possible confounds of parental genotype, a separate set of experiments was done using *Hp1bp3*$^{+/+}$ foster pups instead of the mother's own. In both sets of experiments, the females were postpartum mothers.

Sucrose preference test

Mice were group housed by genotype, five per cage and given access to two bottles, one containing water and the other with a 2% sucrose solution. After 72 h of habituation to the two bottles, daily intake was measured each morning for 4 days by weighing the bottles. Bottles were rotated daily to eliminate location bias.

Wire hang

The wire hang test was performed as described before (Aartsma-Rus & van Putten 2014). Mice were placed on a wire cage lid, and the lid was inverted and positioned 25 cm above soft bedding. The latency to falling was recorded, and 10 min was set as the maximum test time. Mice were allowed three trials, and the maximum duration was chosen for analysis.

Rotarod

Motor co-ordination and balance were tested using a commercially available accelerating rotarod apparatus (Model 7650, Ugo Basile,

Napoli, Italy). Mice were placed on the rotating cylinder (3 cm in diameter) and confined to a section approximately 6.0 cm long by gray plastic dividers. The rotational speed of the cylinder was increased from 5 to 40 r.p.m. over a 5-min period. Latency at which mice fell off the rotating cylinder was measured. Each mouse was given three trials.

Olfactory habituation

The olfactory habituation–dishabituation test was performed as described previously (Yang & Crawley 2009). Briefly, mice were placed in a clean cage, and allowed to acclimate for 1 h. Odors were prepared by diluting natural banana and almond extracts 1 : 100 in distilled water, and by collecting urine from unfamiliar mature females. Cotton tipped wooden applicators were dipped in the odor solutions, and presented to the mice. Each odor was presented three consecutive times for 2 min each, with an inter-trial interval of 1 min. The time spent sniffing the applicator was recorded for each trial.

Social exploration and social habituation

Free range social exploration was performed in the subject's home-cage. A previously unknown pre-pubertal female was inserted for 3 min of free interaction investigation, and investigation times of the subject mouse were evaluated by an observer. Chambered social preference experiments were conducted in a white Perspex box ($37 \times 22 \times 35\ cm^3$). For stimuli mice presentation, two triangulated chambers were placed in two opposite corners (12 cm isosceles, 35 cm high). To enable exploration, slits facing the center of the box were grooved. Social exploration paradigm was started after 20 min of habituation to the setup. Then, a stimulus mouse (3–4 weeks old juvenile male) and an object (Pen) were inserted into the chambers for 5 min. Social habituation-dishabituation paradigm experiments were started after 15 min of habituation to the white Perspex box (without chambers). Then a novel stimulus mouse was inserted for 3 min of free interaction investigation. Three more similar trials with inter-trial intervals of 15 min were conducted using the same stimulus mouse. Finally, an additional trial was performed with a different novel stimulus mouse. In both paradigms, investigation times of the subject mouse were evaluated by an observer.

Open field

The open field apparatus consisted of a Plexiglass box with 30 cm high walls measuring $60 \times 60\ cm^2$. The subject was placed in one corner of the arena, and its location was recorded for 5 min. The critical measure was the time spent in the inner area of the arena (>5 cm away from any wall).

Elevated plus maze

The elevated plus maze apparatus consisted of two open arms and two closed arms each measuring 35×6.25 cm and elevated 50 cm above the ground. The subject was placed in the center of the apparatus facing the open arm and its location was recorded for 5 min. An arm entry was defined as the mouse having all four paws into the arm. The plus maze was wiped clean between trials with a 10% alcohol solution.

Determination of circulation AChE and BChE activity

Blood collected by cardiac puncture from age matched 2–6 month old female mice was allowed to clot in BD, then centrifuged (6000*g*, 5 min at 4°C), and serum was separated. The acetylcholine hydrolyzing activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were measured by the Ellman's assay (Ellman *et al.* 1961) as described (Arbel *et al.* 2014). Briefly, samples were diluted 1 : 10 with 0.2 M phosphate buffer pH 7.4. Ellman's reagent (dithionitrobenzoate, DTNB) at a final concentration of 1 mM with and without the BChE-specific inhibitor Tetraisopropyl pyrophosphoramide (iso-OMPA) was added to each sample, the mixture was incubated at RT for 20 min under darkness. Then, acetylthiocholine iodide was added and absorbance of the corresponding plate wells at 405 nm was monitored with a Synergy H1 Multi-Mode Reader (Biotek, Winooski, VT, USA). at 15 time points with 2 min intervals. AChE and BChE activities were calculated based on the concentration of the resultant 5-thio-2-nitrobenzoate anion ($\epsilon_{405} = 13.6\ M^{-1}\ cm^{-1}$); taking into account the average optical density (OD) increment per minute.

RNA-seq library preparation and sequencing

Total RNA was isolated from the prefrontal cortexes of three *Hp1bp3*$^{+/+}$ and three *Hp1bp3*$^{-/-}$ 8 week old non-stressed female mice using the miRNeasy kit (Qiagen, Hilden, Germany). RNA quality and quantity assessments were performed on Agilent Nano Chip on the bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The RNAseq library was then built using NEBNext Small RNA Library Prep set (NEB, Ipswich, MA, USA) from 300 ng total RNA. Size selection was performed on a 4% agarose gel. Library quality was assessed using the Agilent 4200 TapeStation system (Agilent, Santa Clara, CA, USA). Pooled libraries were sequenced to yield an average of 18 million 2×30 bp paired-end reads per sample using the Illumina NextSeq 500 with 150v2 reagent kit Illumina, San Diego, CA, USA. Normalization and differential expression of micro-RNA was assessed using Deseq2 software (Love *et al.* 2014).

Data and statistical analysis

All results were expressed as mean ± SEM. *P*-values were calculated by two tailed, unpaired Student's *t*-tests. For sequencing results, *P*-values acquired by Student's *t*-tests were corrected with False Discovery Rate (FDR).

Results

Reduced survival of pups born to Hp1bp3$^{-/-}$ females

In order to assess the impact of HP1BP3 deficiency on fertility, we crossed *Hp1bp3*$^{-/-}$ males with *Hp1bp3*$^{+/+}$ females, and *Hp1bp3*$^{-/-}$ females with *Hp1bp3*$^{+/+}$ males. Both male and female *Hp1bp3*$^{-/-}$ mice were fertile, although we observed a sex specific effect on the number of pups observed the morning following parturition, which was significantly smaller when the dam was an *Hp1bp3*$^{-/-}$ female [Fig. 1a; $t(15) = 3.5$, $P < 0.005$]. This could be due either to reduced litter size *in-utero*, or early postnatal mortality and cannibalization of the pups. The latter option is lent support by the striking observation that while nearly 100% of the observed pups born to WT dams were alive after 7 days, only 20–30% of the pups observed with *Hp1bp3*$^{-/-}$ females survived beyond postpartum day 2 [Fig. 1b; $t(6) = 4.6$, $P < 0.005$]. Since all pups in this experiment shared the same heterozygous *Hp1bp3*$^{-/+}$ genotype, it seemed unlikely that this lethality was due to their genetic makeup, implying a maternal cause. One of the most basic components of postnatal maternal care is providing nourishment through nursing. HP1BP3 deficient mice are inherently small (Garfinkel *et al.* 2015a), and we now find that the wet weight of mammary glands from lactating *Hp1bp3*$^{-/-}$ dams is greatly reduced relative to their WT littermates [Fig. 1c; $t(10) = 5.06$, $P < 0.001$]. While the reduced size of the mammary tissue may impact milk quantity, the actual existence of milk in primiparous lactating *Hp1bp3*$^{-/-}$ dams was confirmed by palpitation around the nipple. Furthermore, histological analysis of the mammary tissues of these mice showed no change in the density of terminally differentiated glandular profiles compared to WT mice (Fig. 1d), suggesting that the neonatal lethality is not due to gross impairments in mammary gland

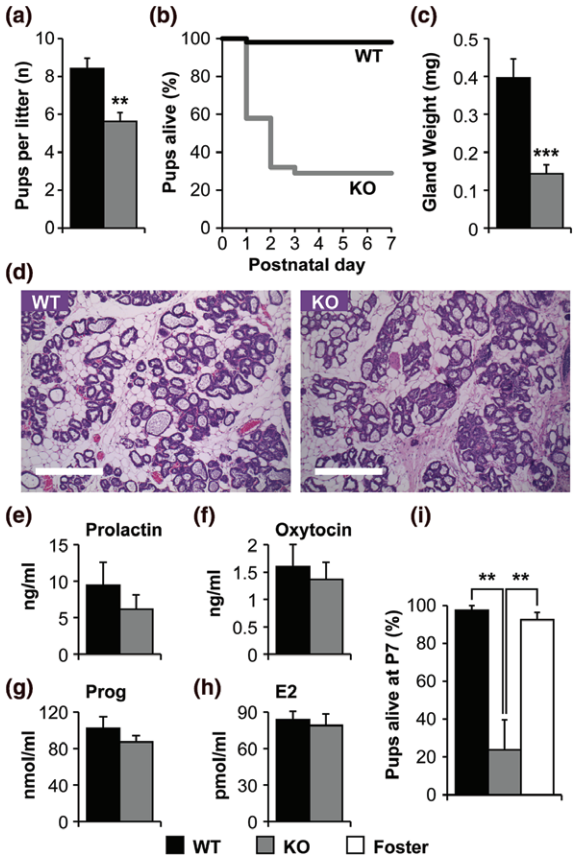

Figure 1: Reduced fertility with intact nursing in *Hp1bp3*$^{-/-}$ females. (a) Pups from litters born to *Hp1bp3*$^{+/+}$ (WT, black bar, 7 litters) or *Hp1bp3*$^{-/-}$ (KO, gray bar, 11 litters) dams were counted the morning following parturition. (b) The pups were then observed daily and their numbers recorded. The original number observed (KO = 62, WT = 59) was set as 100%, and the percentage of live pups remaining each day is presented. (c) Wet weight of inguinal mammary glands from WT ($n = 6$, black bar) and KO ($n = 8$, grey bar) dams that were 48 h into their first lactational period. (d) Histology of mammary tissue in WT and KO dams that were 48 h into their first lactational period. Data are representative of $n = 3$ mice per genotype. Scale bar, 300 μm. Levels of prolactin (e), oxytocin (f), progesterone (g) and estrogen (h) were measured in the serum of WT ($n = 7$, black bar) and KO (n = 8, grey bar) dams that were 48 h into their first lactational period. (i) The percentage of pups still alive at postnatal day 7 is shown for litters born to and raised by WT dams (black bar), KO (gray bar) dams or KO dams housed with experienced WT mothers (white bar, 5 litters). Data are mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with WT controls.

development and function. To further test this notion, we assessed the levels of hormones related to lactation and maternal responsiveness. Circulating levels of oxytocin, prolactin, progesterone and estrogen were measured in the serum of lactating WT and *Hp1bp3*$^{-/-}$ females 48 h postpartum. None of these hormones were significantly different between *Hp1bp3*$^{-/-}$ and WT females (Fig. 1e–h; $P \geq 0.3$ for all). Finally, pregnant *Hp1bp3*$^{-/-}$ females were placed in a cage with non-lactating experienced *Hp1bp3*$^{+/+}$ mothers 4 days prior to parturition. The pups in these co-fostered litters showed survival similar to WT litters, in contrast to the litters of lone *Hp1bp3*$^{-/-}$ females [Fig. 1i; $t(7) = -4.2$, $P < 0.005$]. Collectively, these observations indicated that *Hp1bp3*$^{-/-}$ females can provide the nourishment necessary for early postnatal survival, and it is likely a behavioral characteristic of the postpartum HP1BP3 deficient mother that is responsible for the poor survival rate of their pups.

Hp1bp3$^{-/-}$ *females show abnormal maternal care*

To explore the underlying cause of the poor postnatal survival, we closely observed litters the morning following parturition. As expected, in litters of control females, all pups were huddled in the nest (Fig. 2a). In contrast, we found that in *Hp1bp3*$^{-/-}$ litters nearly half of the pups were scattered outside the nest, and they appeared neglected (Outcast pups, Fig. 2a–c; $P < 0.005$), easily distinguishable by sticking to the cotton nest and woodchip. These observations suggested that HP1BP3 deficient females exhibit impaired maternal nurturing behavior, and we further tested this notion using the pup-retrieval assay. To this end, *Hp1bp3*$^{-/-}$ and *Hp1bp3*$^{+/+}$ females were crossed as before, and 48 h postpartum the dams were separated from their pups, which were then returned to them one at a time at 30 second intervals over the course of 2 min. We observed a striking difference in the behavior of the mothers following contact with the pups. *Hp1bp3*$^{+/+}$ mothers behaved as expected, grabbing the pups and rapidly retrieving them to the cotton nest. In contrast, *Hp1bp3*$^{-/-}$ mothers took at least fivefold longer, often failing completely to retrieve the pups [Fig. 2d–f; $t(39) = 3.2$, $P < 0.001$]. The impaired retrieval was not due to failure of the mothers to detect the pups, because latency to approach and sniff the pups was similar to that of WT mothers (Initial contact, Fig. 2g; $P > 0.05$). In order to verify that the lack of retrieval is not due to any intrinsic characteristic of pups born to the *Hp1bp3*$^{-/-}$ females, we repeated the test using *Hp1bp3*$^{+/+}$ foster pups. Similar results were also observed under these circumstances, with no difference between genotypes in the latency to approach the WT pups (Fig. 2i; $P > 0.05$), and a nearly complete failure of *Hp1bp3*$^{-/-}$ females to retrieve the pups [Fig. 2h; $t(62) = 8.8$, $P < 0.001$]. To test if maternal behavior improves over time, we repeated the test 2 months following parturition. Here too *Hp1bp3*$^{-/-}$ females failed to retrieve the pups in spite of normal retrieval by *Hp1bp3*$^{+/+}$ mothers (not shown), indicating that the effect is long term. The reduced survival of *Hp1bp3*$^{-/-}$ pups was hence attributed to impaired maternal behavior of *Hp1bp3*$^{-/-}$ females.

Depression, fatigue and anosmia do not impair the maternal care of Hp1bp3$^{-/-}$ *females*

Since *Hp1bp3*$^{-/-}$ mice are smaller than their littermates (Garfinkel *et al.* 2015b), and in order to rule out a role for motor weakness or impaired motor coordination of the mother in the impaired retrieval, we performed wire hang and rotarod tests, respectively (Fig. 3a,b). Clearly, the *Hp1bp3*$^{-/-}$ females

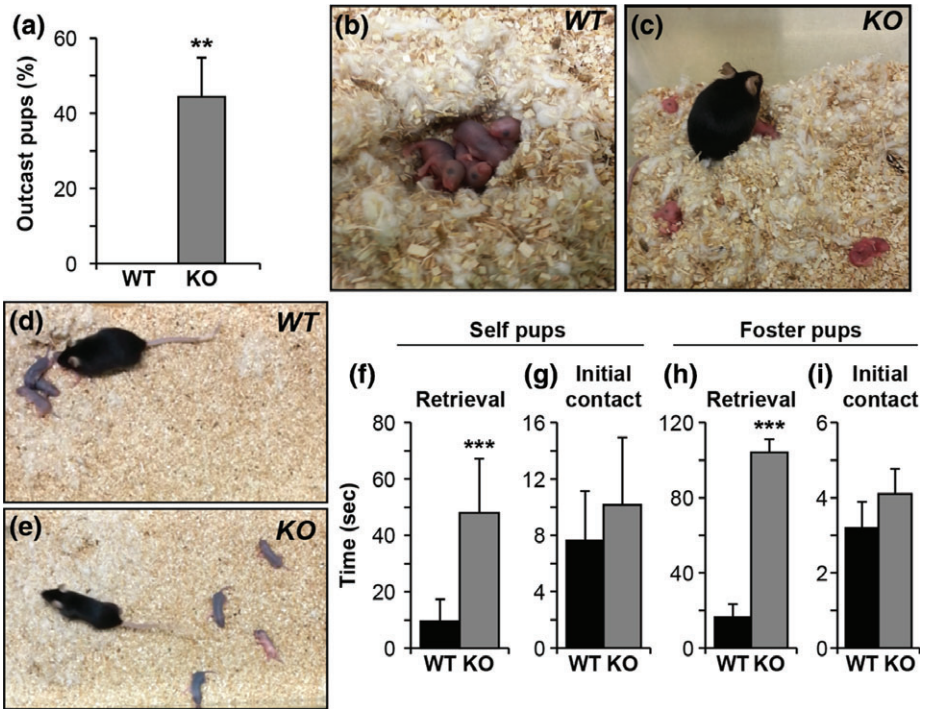

Figure 2: Altered maternal behavior of *Hp1bp3*$^{-/-}$ females. (a–c) Litters were observed on the morning following parturition for *Hp1bp3*$^{+/+}$ (WT, black bars, $n = 10$) and *Hp1bp3*$^{-/-}$ (KO, grey bars, $n = 10$) dams and (a) the percentage of pups not gathered in the nest was noted. (d–i) Pup retrieval experiment. WT ($n = 10$) and KO ($n = 10$) postpartum dams were introduced into a new cage with a cotton nest, and four of their own 2 day old pups (f, g) or four unfamiliar WT foster pups were presented to them at 30-second intervals. The time to initial contact for self (g) and foster (i) pups, and the time to complete retrieval to the cotton nest for self (f) and foster (h) pups were measured. Pups not collected after 2 min were given a time value of 120 seconds. A representative end of experiment is shown for WT (d) and KO (e) dams. Data are mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with +/+ controls.

performed normally, and showed no signs of impairment compared to WT females ($P > 0.05$). Another possible cause for reduced maternal care could be PPD, and a potential connection was recently described between HP1BP3 and PPD in humans (Guintivano *et al.* 2013). We therefore submitted *Hp1bp3*$^{-/-}$ and WT females to a sucrose preference test. Yet, *Hp1bp3*$^{-/-}$ females showed a preference for sucrose similar to that of WT controls (Fig. 3c; $P > 0.05$), suggesting that the observed phenotype is not directly related to depression. Maternal care is also highly dependent on olfactory cues, and anosmia can lead to impaired maternal care. We therefore tested the ability of *Hp1bp3*$^{-/-}$ females to recognize odors using a habituation-dishabituation paradigm. *Hp1bp3*$^{-/-}$ females habituated to the odorants presented to them, and showed no difficulty in recognizing the different odors, indicating that anosmia is also probably not a major underlying cause for the observed behavior (Fig. 3d). These findings excluded fatigue, depression and anosmia as causes for the impaired maternal behavior of *Hp1bp3*$^{-/-}$ females.

Hp1bp3$^{-/-}$ *females show small social behavior differences*

A number of reports have described impaired maternal care in association with altered social behavior (Bond *et al.* 2003; Girirajan & Elsea 2009; Won *et al.* 2012). Therefore, we next examined the social behavior of HP1BP3 deficient females. Social interest was tested using both free range and chambered paradigms. For the free range test, a previously unknown stimulus of a pre-pubertal female mouse was introduced into the subject home cage, and social interactions were scored over a period of 3 min. We did not observe any lack of social interest in the examined *Hp1bp3*$^{-/-}$ females. In fact, the latter showed a marked increase in the time they spent performing olfactory investigation of the stimulus female [Fig. 4a, $t(16) = -5.3$, $P < 0.001$]. Similar results were obtained when the subject mice were presented with two chambers, one containing a stimulus pre-pubertal male mouse, and the other one empty. Both *Hp1bp3*$^{-/-}$ and *Hp1bp3*$^{+/+}$ females spent a significantly greater proportion of their time next to the chamber containing the stimulus mouse than the empty chamber (Fig. 4b, $P < 0.001$). As in the free range test, here too the effect was greater for HP1BP3 deficient females ($P < 0.05$). Social recognition was assayed using a habituation-dishabituation test. Social habituation of HP1BP3 deficient and WT females was measured as a difference in olfactory investigation of the same previously unknown pre-pubertal male during each of four successive 3 min trials (ITI = 15 min). Both WT and *Hp1bp3*$^{-/-}$

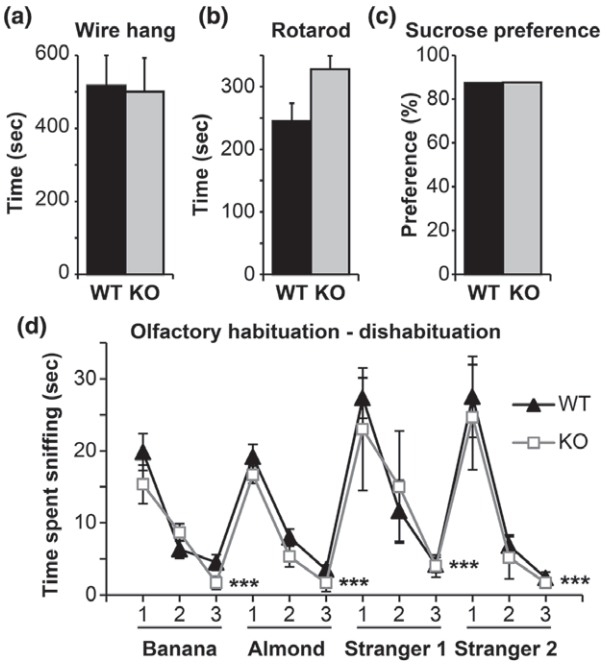

Figure 3: *Hp1bp3*$^{-/-}$ mice are not motor-impaired, anhedonic or anosmic. *Hp1bp3*$^{+/+}$ (WT, $n = 10$, black bar) and *Hp1bp3*$^{-/-}$ (KO, $n = 10$, grey bar) females were tested for motor deficiencies on the wire hang test (a), and the rotarod test (b), and latency to fall is shown for both. (c) WT ($n = 10$, black bar) and KO ($n = 10$, grey bar) females were subjected to a sucrose preference test. Sucrose preference was measured as the fraction of 2% sucrose out of total liquid ingested over a period of 72 h. (d) Olfactory habituation was measured as a difference in olfactory investigation of four different scents (Banana, Almond, stranger urine 1 and stranger urine 2), each during three successive 2 min trials (ITI = 3 min). The first trial for each consecutive scent functions as a dishabituation trial for the previous scent. WT (black triangles, $n = 6$) and KO (white squares, $n = 6$) mice are shown. Data are mean ± SEM. ***$P < 0.001$ significant decrease between each trial as compared to the first trial.

females showed a significant decrease in social olfactory investigation upon subsequent presentations of the same male in trials 3 [$t(7) = 5.5$, $P < 0.001$ and $t(9) = 5.2$, $P < 0.001$] and 4 [$t(7) = 7$, $P < 0.001$ and $t(8) = 5.9$, $P < 0.001$] as compared to trial 1 (Fig. 4c). The olfactory investigation decrease in trials 3 and 4 was not due to a general decrease in olfactory investigation because presentation of a novel male during trial 5 resulted in a similar amount of investigation as trial 1 with the original female (Fig. 4c). We conclude that *Hp1bp3*$^{-/-}$ females present limited differences in social behavior.

Hp1bp3$^{-/-}$ *mice show reduced Anxiety-like behavior*

Impaired maternal behavior is sometimes associated with altered anxiety-like behavior (Bielsky *et al.* 2004; Mosienko *et al.* 2012). To investigate this aspect, we assessed the response of *Hp1bp3*$^{-/-}$ mice to open field and elevated plus maze tests. Interestingly, female *Hp1bp3*$^{-/-}$ mice demonstrated significantly less anxiety-like behaviors in both tests. Thus, *Hp1bp3*$^{-/-}$ females spent significantly more time in the inner area of the open field arena [$t(12) = -2.18$, $P < 0.05$] and entered it more frequently [$t(9) = -2.4$, $P < 0.05$] (Fig. 5a–c) than did their WT littermates. There were no genotype differences in the overall distance traveled (Fig. 5d). As for the elevated plus maze tests, female *Hp1bp3*$^{-/-}$ mice entered the open arms [Fig. 5g, $t(10) = 5.6$, $P < 0.001$] and spent more time in them than did their WT littermates [Fig. 5f, $t(9) = 4.25$, $P < 0.005$]. Here too there were no significant genotype differences in overall activity in the elevated plus maze as measured by the total distance traveled (Fig. 5h). Altered anxiety is often accompanied by modified capacity of circulation cholinesterases to hydrolyze acetylcholine (Shenhar-Tsarfaty *et al.* 2015; Soreq 2015), and in human post-partum mothers it is reflected by elevated serum cholinesterase activities (Grisaru *et al.* 2006). However, biochemical measurements of circulation cholinesterases, named collectively 'cholinergic status' demonstrated unmodified cholinergic status in *Hp1bp3*$^{-/-}$ mice (Fig. 6a,b). The reduced anxiety reactions of these mice were hence indicated to associate with other mechanism(s) of action.

Hp1bp3$^{-/-}$ *mice show unchanged brain microRNA profiles*

Impaired anxiety is often associated with altered brain microRNA(miR) profiles (Leung & Sharp 2010; Meerson *et al.* 2010). To find if this was the case for the *Hp1bp3*$^{-/-}$ mice, we performed miR-deep sequencing analysis of prefrontal cortex samples from both *Hp1bp3*$^{-/-}$ and *Hp1bp3*$^{+/+}$ mice. An average of 18 million reads were obtained per sample, and these were aligned to miRbase (version 21). Surprisingly, no significant changes were detected between *Hp1bp3*$^{-/-}$ and *Hp1bp3*$^{+/+}$ mice in any of the sequenced microRNAs (Fig. 6c, Table S1, Supporting Information). This lack of change spanned all levels of abundance, and we observed none of those changes in the expression of miRs that were previously implicated in mediating stress responses in the prefrontal cortex (Fig. 6d; Meerson *et al.* 2010; Rinaldi *et al.* 2010; Shaltiel *et al.* 2013; Uchida *et al.* 2010). We therefore conclude that the altered behavior of *Hp1bp3*$^{-/-}$ mice was not due to modified brain microRNAs.

Discussion

In this work we observed dramatically reduced survival rates in litters born to *Hp1bp3*$^{-/-}$ females. Since the females were crossed with males of the opposite genotype (i.e. *Hp1bp3*$^{-/-}$ female with *Hp1bp3*$^{+/+}$ male and vice versa), all of the pups had the same *Hp1bp3*$^{+/-}$ genotype, and we therefore surmised that the effect must be due to the maternal HP1BP3 deficiency. Supporting this notion, litters born to *Hp1bp3*$^{-/-}$ females showed restored viability in co-fostering experiments, indicating that maternal HP1BP3 deficiency does not impact development *in-utero*. Rather, it is the postnatal care practiced by *Hp1bp3*$^{-/-}$ females which is not sufficient to support pup survival.

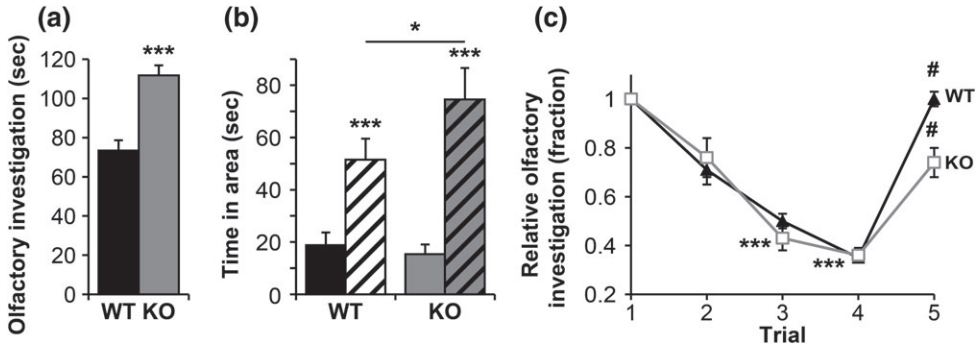

Figure 4: Small changes in social behavior of *Hp1bp3*$^{-/-}$ mice. (a) *Hp1bp3*$^{+/+}$ (WT, $n=6$, black bar) and *Hp1bp3*$^{-/-}$ (KO, $n=6$, grey bar) females were tested for social exploration in a free range test. A previously unknown stimulus pre-pubertal female mouse was introduced, and social interactions were scored over a period of 3 min. (b) WT ($n=7$) and KO ($n=7$) females were tested in a chambered test for social preference. Time spent near the non-social (plain bars) and social (striped bars) are presented for each genotype. (c) Social habituation of WT (black triangles) and KO (white squares) mice was measured as a difference in olfactory investigation of the same pre-pubertal female during each of four successive 3 min trials (ITI = 15 min). A fifth dishabituation trial depicts the response to a novel stimulus mouse in a 3 min pairing 15 min after the fourth trial. Data are mean ± SEM. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared with WT controls for a, b, and between each trial as compared to the first trial for c. $\#P<0.001$ between the fourth trial and the dishabituation trial for c.

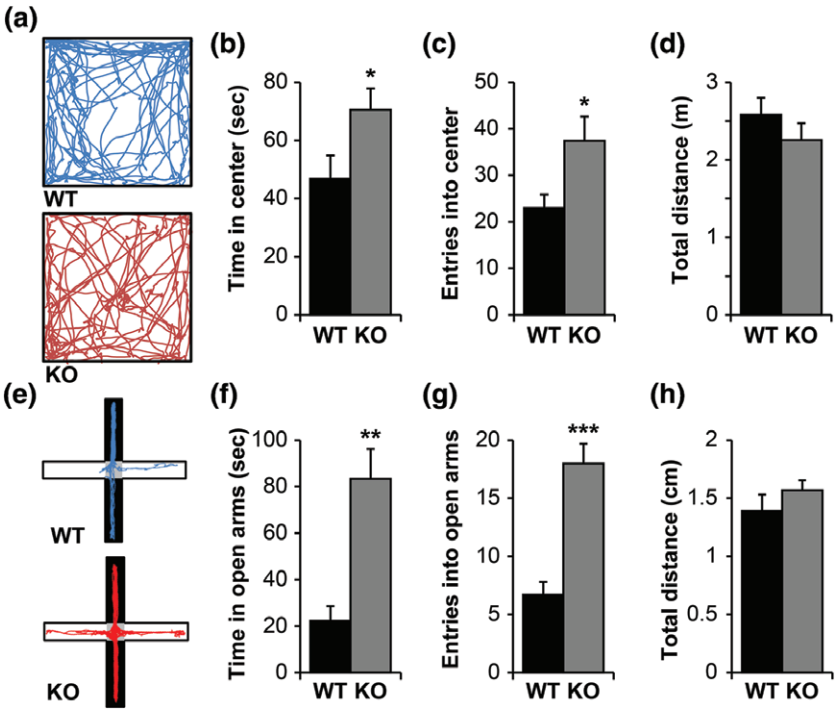

Figure 5: *Hp1bp3*$^{-/-}$ mice have reduced anxiety-related behavior. (a–d) Behavior in the open field test was measured for *Hp1bp3*$^{+/+}$ (WT, $n=6$, black bar) and *Hp1bp3*$^{-/-}$ (KO, $n=6$, grey bar). KO animals spent significantly more time in the center area of the open field arena (b), and entered the center area more frequently than their WT littermates (c), in spite of traveling similar distances (d). (e–h) The same mice were tested with the elevated plus maze. KO animals spent significantly more time in the open arms of the elevated plus maze (f), and entered the open arms more frequently than their WT littermates (g). There was no difference in the total distance traveled (h). Data are mean ± SEM. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared with +/+ controls.

Several independent experimental strategies were used in an effort to approach the underlying cause for the impaired maternal behavior of *Hp1bp3*$^{-/-}$ females. Our previous observation of altered levels of systemic IGF-1 and IGF binding proteins in *Hp1bp3*$^{-/-}$ mice (Garfinkel *et al.* 2015a) raises the possibility that HP1BP3 may regulate other systemic hormones as well. Hormones play a major role in inducing and mediating maternal care; in this regard, prolactin (Bridges & Mann 1994; Lucas *et al.* 1998; Martyn *et al.* 2012) and oxytocin (Bielsky *et al.* 2004; Insel *et al.* 1997; Pedersen *et al.* 2006) have long been known to be crucial for both the induction of lactation, as well as maternal responsiveness and behavior. Upstream of these hormones, postpartum changes in the levels of the sex steroid hormones estrogen and progesterone have also been shown to be necessary for the onset of maternal behavior (Champagne *et al.* 2001; Siegel & Rosenblatt 1975; Siegel & Rosenblatt 1978). We therefore assessed the levels of circulating

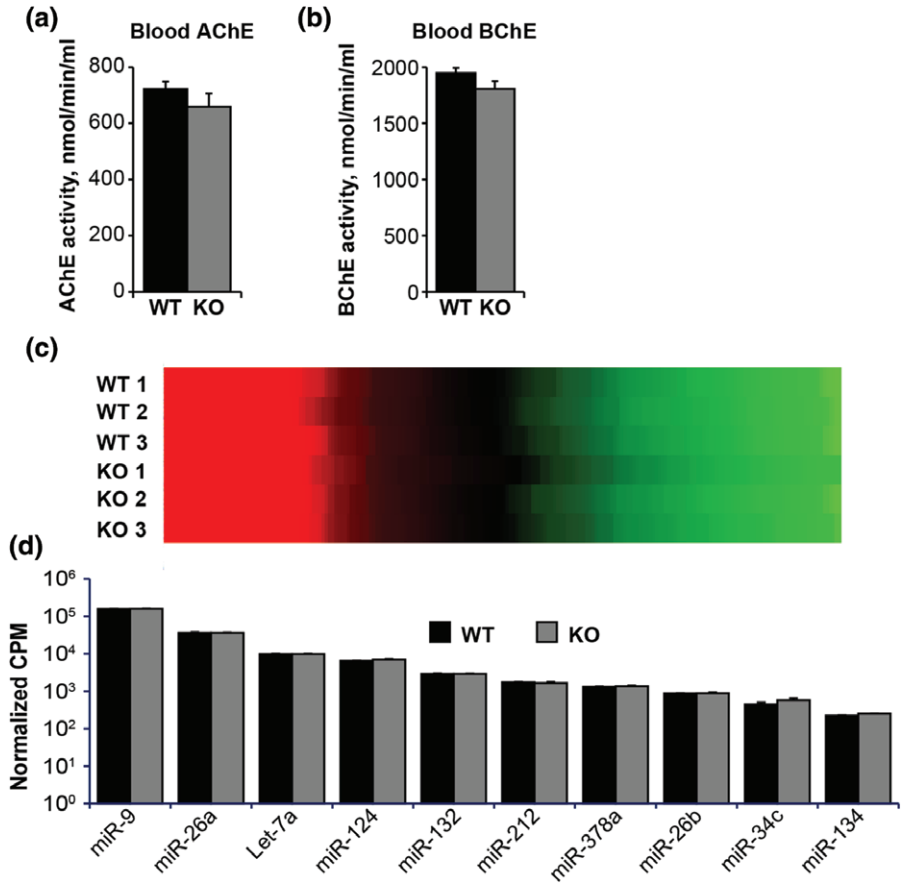

Figure 6: Unchanged blood cholinergic activity and prefrontal cortex microRNA profile in *Hp1bp3*$^{-/-}$ mice. (a) AChE and (b) BChE activity were assessed in blood collected from *Hp1bp3*$^{+/+}$ (WT, $n=9$, black bar) and *Hp1bp3*$^{-/-}$ (KO, $n=9$, grey bar) mice. (c) A heatmap showing results of deep sequencing of microRNAs from prefrontal cortex of WT ($n=3$) and KO ($n=3$) mice. Depicted are relative levels of all microRNAs (red–high, green–low) with normalized count per million (cpm) higher than 100. (d) Expression levels of 10 abundant miRs implicated in stress responses.

prolactin, oxytocin, progesterone and estrogen and found that *Hp1bp3*$^{-/-}$ females had normal levels of all hormones 48 h postpartum, suggesting that the impaired behavior is probably not directly related to alterations in the circulation of these hormones. It should be noted, however, that levels of circulating hormones do not always perfectly mirror the situation in the brain (Jones *et al.* 1983). Also, we cannot rule out altered expression of receptors within specific brain regions, and future work will assess changes in oxytocin and prolactin signaling in the brain.

Next, we assessed a panel of behavioral patterns that have previously been associated with altered maternal care. Primarily, maternal care depends on a functional main olfactory system (Belluscio *et al.* 1998; Gandelman *et al.* 1971; Wang & Storm 2010; Weiss *et al.* 2011), yet we found no evidence of anosmia in *Hp1bp3*$^{-/-}$ females. Also, social interest and recognition, which have been shown to correlate with maternal behavior in mouse models (Takayanagi *et al.* 2005; Won *et al.* 2012), were both intact in these mice. Another behavioral state with a major impact on maternal care is depression, and post-partum depressive behavior is associated with reduced maternal care in both humans (Field 2010) and mice (Maguire & Mody 2008). Interestingly, and apparently consistent with our own notion in mice, it was recently reported that the hyper-methylation status of the *HP1BP3* gene promoter in blood DNA from pregnant women was an excellent antenatal predictor of PPD (Guintivano *et al.* 2013; Osborne *et al.* 2015). One potential cause for post-partum anxiety is altered cholinergic status (Grisaru *et al.* 2006); however, we excluded that cause by demonstrating that *Hp1bp3*$^{-/-}$ mice sustained unchanged circulation cholinesterase activities as compared to normal controls. Furthermore, we did not observe evidence for depressive behavior in the *Hp1bp3*$^{-/-}$ female mice, that did not differ from WT littermates in the sucrose preference test. Yet, it should be noted that depression does not always manifest in mice as expected, a relevant example being mice with targeted deletion of serotonin production in the brain. These mice show impaired maternal care (Angoa-Perez *et al.* 2014b), but no signs of depression (Angoa-Perez *et al.* 2014a), even though selective serotonin reuptake inhibitors (SSRIs) are the most widely used pharmacotherapy for treating PPD (De Crescenzo *et al.* 2014). Thus, our results do not rule out a connection between the impaired maternal care observed in *Hp1bp3*$^{-/-}$ mice and PPD in humans, but exclude cholinergic imbalance as a cause for such symptoms.

Impaired maternal behavior in knockout mouse models is often associated with altered anxiety-like behavior. However, the direction of change is not consistent, with some cases showing increased (Sadakata *et al.* 2012; Won *et al.* 2012) and others presenting reduced anxiety-like behavior (Bielsky *et al.* 2004; Martel *et al.* 2008; Mosienko *et al.* 2012; Satoh

et al. 2011). In fact, recent work assessing the correlation between anxiety-like and maternal behaviors in rodents found no direct connection between the two (Curley *et al.* 2012). Therefore, it is difficult to say if the reduced anxiety-like behavior in HP1BP3 deficient mice plays an etiological role in the impaired maternal behavior, or, is merely associated with it.

The neural circuitry responsible for activating and sustaining maternal behavior is complex and involves multiple brain regions, including among others the olfactory bulbs, the amygdala, the medial preoptic area (mPOA), the ventral bed nucleus of stria terminalis (vBNST), the ventral tagmental area (VTA) and the nucleus acumbens (NAcc). When anxiety-like behavior is considered, the frontal cortex plays a major role (Bishop *et al.* 2004; Davidson 2002), and the family of brain-expressed microRNAs are held responsible by many (Meerson *et al.* 2010; Rinaldi *et al.* 2010; Uchida *et al.* 2010). However, unbiased profiling of frontal cortex miRNAs from *Hp1bp3*$^{-/-}$ mice compared to controls by RNA-sequencing showed no significant differences, excluding this cause as well. Additionally, recent work has shown extensive changes in protein-coding transcripts in several of the above brain regions in response to parturition in mice (Driessen *et al.* 2014; Eisinger *et al.* 2013; Zhao *et al.* 2014). Considering the role of HP1BP3 as a regulator of transcription, it is conceivable that HP1BP3 may be necessary for reaching the correct maternal postpartum transcriptional signature. It is interesting in this regard to note that two of the most significantly changed genes in the maternal mPOA were Igf1 and Igfbp5 (Driessen *et al.* 2014), both previously shown by us to be targets of HP1BP3 mediated regulation (Garfinkel *et al.* 2015a, 2015b). Since HP1BP3 is widely expressed in the brain (Allen brain atlas and data not shown), it is difficult to predict which brain regions are directly affected by HP1BP3 deficiency, and future work will aim to map the location of HP1BP3 action. An interesting step in this direction is the recent description of a role for HP1BP3 in hippocampus-dependent learning and memory (Neuner *et al.* 2016).

A fascinating aspect of the establishment and maintenance of maternal behavior is the role of epigenetic regulators. Within the nucleus, transcription factor action at DNA sequences is ultimately regulated by chromatin structure, and chromatin modifying proteins interact to determine the local accessibility of DNA. Tight chromatin configurations block access of transcriptional machinery to transcription start sites, resulting in gene silencing. In this regard, chromatin remodeling has been shown to facilitate both hormone and non-hormone dependent maternal behavior, suggesting that this is a shared mechanistic pathway accounting for maternal responsiveness in lactating and non-lactating females (Stolzenberg *et al.* 2012, 2014). Also, epigenetic regulators of long-term anxiety reactions have been shown (Sailaja *et al.* 2012). Relevant to this rationale are a number of studies demonstrating that HP1BP3 interacts and colocalizes with heterochromatin protein 1 (HP1) (Garfinkel *et al.* 2015b; Hayashihara *et al.* 2009), a key factor involved in mediating epigenetic gene silencing (Cheutin *et al.* 2003; Haldar *et al.* 2011; Lachner *et al.* 2001). This suggests the intriguing hypothesis that HP1BP3 could play a role in epigenetic priming of the brain for maternal behavior, which is functionally disturbed in the absence of this protein.

In conclusion, our results suggest a novel role for HP1BP3 in regulating maternal and anxiety-related behavior in mice and call for exploring ways to manipulate this epigenetic process.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1: Small RNA sequencing results. All miRNAs with normalized counts of at least 10 cpm are shown.