

Cytochrome b_5 null mouse: a new model for studying inherited skin disorders and the role of unsaturated fatty acids in normal homeostasis

Robert D. Finn · Lesley A. McLaughlin ·
Catherine Hughes · Chengli Song ·
Colin J. Henderson · C. Roland Wolf

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Abstract Microsomal cytochrome b_5 is a ubiquitous, 15.2 kDa haemoprotein implicated in a number of cellular processes such as fatty acid desaturation, drug metabolism, steroid hormone biosynthesis and methaemoglobin reduction. As a consequence of these functions this protein has been considered essential for life. Most of the ascribed functions of cytochrome b_5 , however, stem from in vitro studies and for this reason we have carried out a germline deletion of this enzyme. We have unexpectedly found that cytochrome b_5 null mice were viable and fertile, with pups being born at expected Mendelian ratios. However, a number of

intriguing phenotypes were identified, including altered drug metabolism, methaemoglobinemia and disrupted steroid hormone homeostasis. In addition to these previously identified roles for this protein, cytochrome b_5 null mice displayed skin defects closely resembling those observed in autosomal recessive congenital ichthyosis and retardation of neonatal development, indicating that this protein, possibly as a consequence of its role in the de novo biosynthesis of unsaturated fatty acids, plays a central role in skin development and neonatal nutrition. Results from fatty acid profile analysis of several tissues suggest that cytochrome b_5 plays a role controlling saturated/unsaturated homeostasis. These data demonstrate that regional concentrations of unsaturated fatty acids are controlled by endogenous metabolic pathways and not by diet alone.

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Present Address:

R. D. Finn
School of Applied Sciences, Ellison Building,
Northumbria University, Newcastle NE1 8ST, UK

L. A. McLaughlin · C. Hughes · C. J. Henderson ·
C. Roland Wolf (✉)
Cancer Research UK Molecular Pharmacology Unit,
Biomedical Research Institute, Level 5, Ninewells
Hospital & Medical School, Dundee DD1 9SY, UK
e-mail: c.r.wolf@dundee.ac.uk

C. Song
School of Medical Instrument and Food Engineering,
University of Shanghai for Science and Technology,
200093 Shanghai, China

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Introduction

For more than 40 years microsomal cytochrome b_5 has been proposed to modulate the activity of cytochrome P450 enzymes, including those involved in endogenous processes such as cholesterol and steroid hormone synthesis/breakdown, as well as the metabolism of exogenous xenobiotics, chemical

toxins and drugs (Akhtar et al. 2005; Yamazaki et al. 2002; Borthiry et al. 2007; Kurian et al. 2006). The modulation of P450 activity by cytochrome b_5 is reported to be both substrate- and P450-specific, with evidence of both stimulation and inhibition of substrate metabolism (Yamazaki et al. 2002; Schenkman and Jansson 2003). Cytochrome b_5 has also been described as a facilitator in the metabolism of endogenous substrates such as the desaturation of fatty acids (Jeffcoat et al. 1977) and methaemoglobin reduction to haemoglobin (Jaffe 1981). Importantly, for many of these pathways, cytochrome b_5 has been deemed obligatory and therefore by implication possibly essential for life. However, as all the evidence to date is drawn exclusively from in vitro studies, the in vivo roles of this enzyme have thus remained speculative.

In order to address the in vivo function of microsomal cytochrome b_5 in drug metabolism, we have recently generated a mouse model (hepatic b_5 null (HBN)) in which microsomal cytochrome b_5 was conditionally deleted in the liver (Finn et al. 2008). HBN mice had significantly reduced in vitro rates of NADPH- and NADH-dependent metabolism of a range of model substrates and probe drugs; furthermore, significant changes in drug pharmacokinetics were also found, demonstrating that cytochrome b_5 does indeed play a significant role in the in vivo disposition of drugs.

A conditional approach was taken in these studies because of the anticipation that complete deletion of microsomal cytochrome b_5 would be embryonic lethal. However, as the functions of this protein have been based on in vitro data, we also carried out germ line deletion (b_5 complete null (BCN)). Remarkably, cytochrome b_5 null animals were viable, fertile and produced grossly normal pups at expected Mendelian ratios. However, cytochrome b_5 null mice exhibited a number of unexpected and extraordinary phenotypes, identifying new roles for this protein in skin lipid homeostasis and neonatal development.

Materials and methods

Chemicals

Unless stated, all reagents were purchased from Sigma–Aldrich (Poole, UK).

Cytochrome b_5 complete null (BCN) mice

Homozygous cytochrome b_5 complete knockout mice (BCN, $Cytb_5^{-/-}$) and control (wild-type, $Cytb_5^{+/+}$) mice were generated as previously described (McLaughlin et al. 2010).

All mice were maintained on a standard chow (RM1—Special Diet Services, Essex, UK) under standard animal house conditions, with free access to food and water, and 12 h light/12 h dark cycle. All animal work was carried out in accordance with the Animal Scientific Procedures Act (1986) and after local ethical review.

Tissue histology

Skin sections were removed post mortem from the backs of 10 week old male wild-type and BCN mice ($n = 3$ for each genotype) and fixed flat on Whatman 3MM paper (GE Healthcare, Amersham, UK) in formal saline (Gurr® BDH, VWR International, Lut-terworth, UK) before being sectioned and stained with hematoxylin and eosin. Measurements were taken of the epidermis, dermis and hair shaft thickness using an Olympus IX50 microscope (Olympus UK Ltd., Wat-ford, UK) with Zeiss Axio Vision version 4.5 software (Carl Zeiss Ltd., Welwyn Garden City, UK).

Thermal imaging

Thermal imaging experiments were performed in a closed room at a constant temperature of 20°C with tolerance of 1°C and lighting kept to a minimum. This arrangement ensured temperature variations that may adversely affect the performance of the thermal camera were negligible. Temperature measurements were obtained using a high-performance thermal camera (Cedip Infrared Systems, France) containing a 320 × 240 pixel focal plane array operating in the mid-infrared range (3–5 μm) with a thermal sensitivity of 0.02°C and spatial resolution of 0.2 mm, positioned 50 cm above the subject.

Body temperature measurements of 3 week old male wild-type and BCN pups, ($n = 3$ for each genotype) were taken at the same time of the day with a 2-day interval over a period of 6 weeks to allow temperature changes to be related to growth. In order to determine skin temperature, a small area (20 mm diameter) on the back of each mouse was shaved,

immediately before temperature measurements were taken. Average and maximum temperatures of the shaved and haired areas were calculated using the specialized software associated with the camera (Song et al. 2007).

Mouse milking

Nursing females (first litter) were milked 10 days postpartum. Briefly, mice were given an i.p. injection of oxytocin (100 μ l, 5 U/ml) to simulate milk production. After 20 min, treated mice were anesthetized by inhalation of isoflurane and milk production facilitated by gentle squeezing of the nipples. The milk released was collected by pipette and samples were stored at -70°C prior to analysis.

Lipid analysis

Detailed fatty acid profile analysis was performed on milk, plasma, skin and liver by the Nutrition Analytical Service, University of Stirling, UK, according to the following method. Total lipids were extracted from skin and liver by homogenising in 20 volumes of chloroform/methanol (2:1 v/v) in an Ultra Turrax tissue disrupter (Fisher Scientific, Loughborough, UK). Total lipids were prepared from all samples according to the Folch method and non-lipid impurities were removed by washing with 0.88% (w/v) KCl (Folch et al. 1957). The weight of lipids was determined gravimetrically after evaporation of the solvent and overnight desiccation under vacuum. Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification of total lipids according to the method of Christie (Christie 2003). Extraction and purification of FAME was performed as described by Ghioni et al. (1996). FAME were separated and quantified by gas-liquid chromatography using a Thermo Fisher Trace GC 2000 (Thermo Fisher, Hemel Hempstead, UK) equipped with a fused silica capillary column (ZB wax, 30 m \times 0.32 mm i.d.; Phenomenex, Macclesfield, UK) with hydrogen as carrier gas and using on-column injection. The temperature gradient was from 50 to 150°C at $40^{\circ}\text{C}/\text{min}$ and then to 195°C at $1.5^{\circ}\text{C}/\text{min}$ and finally to 220°C at $2^{\circ}\text{C}/\text{min}$. Individual methyl esters were identified by comparison with known standards and by reference to published data. Data were collected and processed using the Chromcard for Windows (version 2.00)

computer package (Thermoquest Italia S.p.A., Milan, Italy).

Statistical analysis

Data sets were analyzed for statistical significance using an Unpaired student *t*-test (www.graphpad.com/quickcalcs/t-test1.cfm).

Results and discussion

Global deletion of microsomal cytochrome *b*₅ is not lethal

To establish the *in vivo* role(s) of microsomal cytochrome *b*₅ we generated a mouse germline deletion of exons 2–5 of the gene. Mice lacking microsomal cytochrome *b*₅, were unexpectedly viable, born at expected Mendelian ratios and exhibited no gross anatomical abnormalities. Cytochrome *b*₅ complete null (BCN) mice of both sexes were fertile, indicating that cytochrome *b*₅ is not necessary for sexual maturation, demonstrating that cytochrome *b*₅ does not play an obligatory role in testosterone biosynthesis (Akhtar et al. 2005). However, analysis of testicular testosterone levels revealed that they were approximately 40% lower in BCN males and this was accompanied by the almost complete loss of the *in vitro* activity of Cyp17, a P450 essential for testosterone biosynthesis (McLaughlin et al. 2010). In addition to its role in transferring electrons to P450s involved in the metabolism of steroids, cytochrome *b*₅ can also act as an electron donor to P450s involved in the metabolism of drugs and foreign compounds. In agreement with our previous findings in the conditional hepatic knockout model, BCN mice also had a marked reduction in *in vivo* P450 drug metabolism confirming the pivotal role this protein may play in drug metabolism *in vivo* (Finn et al. 2008; McLaughlin et al. 2010).

Cytochrome *b*₅ is thought to play a key role in the reduction of methaemoglobin to haemoglobin (Jaffe 1981) and in agreement with this role, 10 week old male BCN mice had significantly higher levels of circulating methaemoglobin (tenfold) than their wild-type counterparts (Fig. S1). Methaemoglobinemia in humans is clinically defined as having methaemoglobin levels greater than 1%, with homozygous methaemoglobin reductase-deficient patients possessing

levels greater than 10% (Percy et al. 2005). By this definition, BCN mice are methaemoglobinemic, however none of the other physiological phenotypes associated with methaemoglobinemia due to disruption of cytochrome *b₅* or cytochrome *b₅* reductase in humans were observed (Hegesh et al. 1986; Giordano et al. 1994; Manabe et al. 1996). These data suggest that although cytochrome *b₅* is part of the pathway of methaemoglobin reduction, the known alternative pathways such as the glucose/glucose-6-phosphate dehydrogenase are capable of partially compensating for loss of this protein and maintaining methaemoglobinemia at asymptomatic levels (Jaffe 1981). BCN mice may, however, be more susceptible to acquired methaemoglobinemia under conditions of metabolic stress or to chemicals known to induce methaemoglobinemia (Molthrop et al. 1994; Mary and Bhupalam 2000).

Together these findings confirm *in vivo* the numerous functions assigned to microsomal cytochrome *b₅* through *in vitro* studies carried over the past four decades.

Deletion of microsomal cytochrome *b₅* results in the development of ichthyosis

Ten days after birth hair growth had commenced in wild-type mice but in contrast BCN pups remained hairless and exhibited a generalized scaling of the skin and hyperkeratosis on the tail which by 4 weeks of age resulted in development of constrictive bands, referred to as ‘ringtail’, a condition observed in rats kept in dry, low humidity environments (Fig. 1a, b). At 4 weeks, the hair coat of BCN mice had developed a “static” appearance and was noticeably thinner than in age-matched controls (Fig. 1c). By 10 weeks of age coat condition had improved further however, the difference in thickness compared to wild-type mice could still be clearly seen on the ventral surface (Fig. 1d). Microscopic examination of hair types from BCN and wild-type mice found no significant difference in the absolute numbers or proportion of each hair type between the two genotypes (data not shown). These data therefore suggest that deletion of microsomal cytochrome *b₅* delays hair follicle development.

Histological examination of dorsal skin sections from 10 week old male wild-type (Fig. 1e, g) and BCN (Fig. 1f, h) mice identified pronounced keratinization of the stratified squamous epithelium in the

latter and concomitant significant thickening of the epidermis— $13.2 \mu\text{m} \pm 0.6$ versus $10.2 \mu\text{m} \pm 0.3$ (Fig. 1, compare e & f). Furthermore, hair shaft width was significantly finer in BCN mice than in wild-type animals ($12.7 \mu\text{m} \pm 0.09$ vs. $14.7 \mu\text{m} \pm 0.3$; Fig. 1i). Similar increases in epidermal thickness and keratinization have been reported on disruption of the 12R-lipoxygenase, *ALOX12B* locus, in mice, an enzyme involved in an alternative pathway of arachidonic acid metabolism in the skin, and suggests that cytochrome *b₅* may also contribute to this pathway of eicosanoid biosynthesis (Juanes et al. 2009). The skin phenotypes observed in BCN mice are highly similar to those observed in the human diseases classed as autosomal recessive congenital ichthyosis (ARCI) (Akiyama and Shimizu 2008).

A static hair phenotype has also been observed in stearoyl-CoA desaturase (*SCD1*) null mice (Miyazaki et al. 2001). However, in *SCD1* null mice, the effect is associated with atrophy of the sebaceous glands, as a result of lipid depletion, whereas in BCN mice the sebaceous glands were slightly enlarged (Fig. 1g, h). Also, the characteristic phenotypes associated with ichthyosis have not been reported for *SCD1* null mice. These data therefore suggest a role for cytochrome *b₅* in lipid and eicosanoid metabolism in skin, disruption of which leads to the development of ichthyosis. Ichthyosis was not reported in the methaemoglobinemia patient identified by Hegesh et al. who expressed a splice variant of cytochrome *b₅*; however this is most likely due to the presence of residual cytochrome *b₅* expression/activity and not complete loss as in BCN mice (Hegesh et al. 1986; Giordano et al. 1994).

Cytochrome *b₅* is essential for skin lipid homeostasis but its deletion does not disrupt epidermal barrier function

In light of the above data, we carried out fatty acid analysis of the skin. Significant changes in the levels of most saturated, monounsaturated and branched-chain fatty acid species, many of which are known to have emollient and moisturizing properties e.g. behenic and erucic acid, were observed in the skin of 10 week old male BCN mice (Table S1). In addition, significant changes in the levels of α -linolenic acid (decreased by 35%) and eicosadienoic acid (increased by 40%) were found (Table S1).

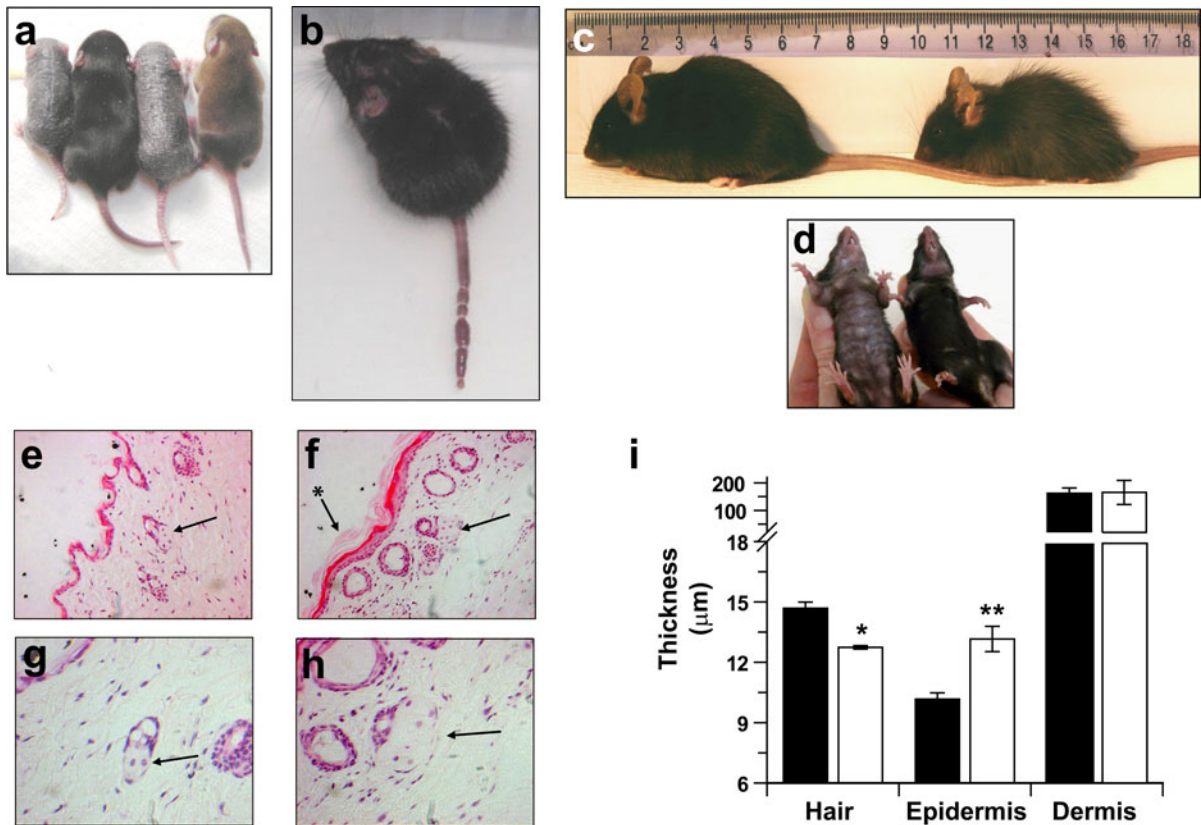


Fig. 1 Characterization of skin related phenotypes from cytochrome *b*₅ null mice. **a** 10 day old wild-type and BCN pups illustrating the scaled appearance of BCN pups. **b** A 4 week old BCN mouse demonstrating the “ringtailed” phenotype. **c** Age-matched (4 weeks) wild-type (*left*) and BCN mice demonstrating the differences in coat appearance/condition and the smaller size of the BCN mice. **d** Ventral view of 10 week old male BCN (*left*) and wild-type mice demonstrating marked thinning of the hair. **e, g** Hematoxylin and eosin staining of skin from 10 week old male wild-type mice, magnification 20× & 40×, respectively. **f, h** Hematoxylin and eosin staining of skin

from 10 week old male BCN mice, magnification 20× & 40×, respectively. *Arrows* indicate the sebaceous glands (**e–h**) and the keratinization of the stratified squamous epithelium (**f**—*asterisk*ed arrow) in BCN mice. **i** Measurements of hair shaft width, epidermal and dermal thicknesses in 10 week old male BCN and wild-type mice. Values represent the mean ± SD of 50 measurements from each mouse (*n* = 3). *Black* and *white bars* represent wild-type and BCN mice respectively. * *P* ≤ 0.05, ** *P* ≤ 0.005, *** *P* ≤ 0.001 comparing wild-type and BCN mice

Calculation of desaturation indices (i.e. the ratio of unsaturated to saturated fatty acids), as a measure of SCD activity (Attie et al. 2002), for both palmitic and stearic acid showed a 50 and 54% reduction, respectively, in BCN compared to wild-type mice (Fig. 2a). This may account for some of the similarities observed between BCN and SCD1 null mice. The skin phenotype of SCD1 null mice was ascribed to the disruption of the epidermal lipid barrier, due to loss of ω-hydroxylated very long chain fatty acids and the abolishment of lymphoid enhancer-binding factor 1 (*lef1*) expression, a transcription factor essential for correct development of the hair follicle

and the sebaceous gland; however, in agreement with the morphological differences of the sebaceous gland between BCN and SCD1 null mice, *lef1* expression levels are not significantly changed in 10 week old male BCN mice (data not shown) (Binczek et al. 2007; DasGupta and Fuchs 1999). Members of the CYP4 family function in lipid metabolism as ω-hydroxylases, acting on long-chain fatty acids and Loughran et al. have showed that cytochrome *b*₅ is essential for the in vitro ω-hydroxylation of arachidonic acid (Hardwick 2008; Kalsotra and Strobel 2006; Loughran et al. 2001). The skin phenotypes observed in BCN mice may therefore result from a

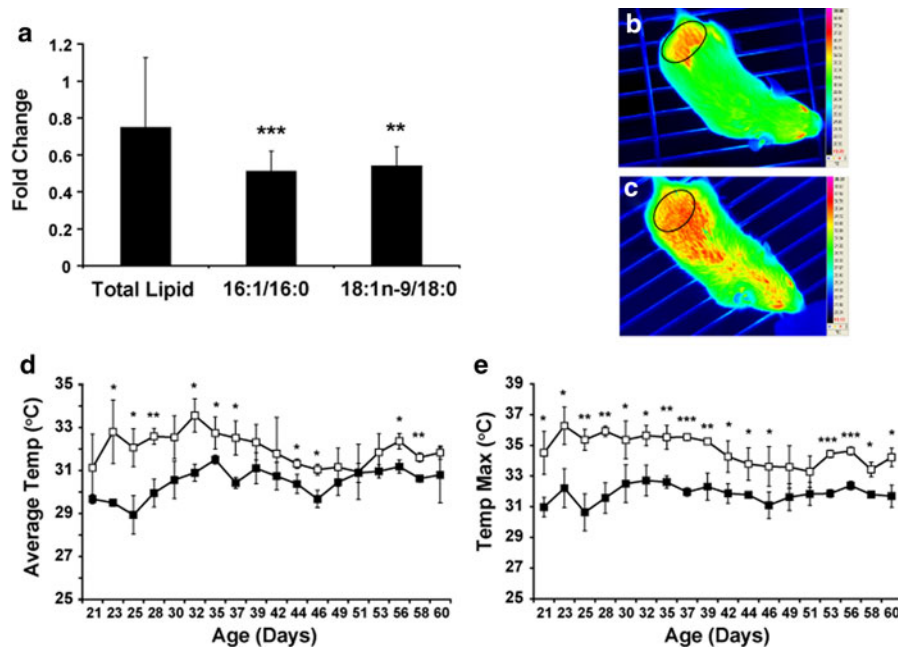


Fig. 2 Characterization of skin lipid and temperature changes in cytochrome *b*₅ null mice. **a** Total lipid content and desaturation indices determined from skin fatty acid profile analysis carried out on 10 week old male wild-type and BCN mice. Values represent the mean \pm SD, where $n = 3$ for wild-type mice and $n = 6$ for BCN mice. **b** and **c** Thermal imaging of 4 week old male wild-type and BCN mice respectively. Temperature measurements were recorded from in 3 week old

wild-type and BCN mice for a period of 6 weeks as outlined in the “Materials and methods”. **d** Average temperature and **e** maximum temperature of the haired coat area plotted against age. Values represent the mean \pm SD, where $n = 3$. *Black* and *white squares* represent wild-type and BCN mice respectively. * $P \leq 0.05$, ** $P \leq 0.005$, *** $P \leq 0.001$ comparing wild-type and BCN mice

combination of a change in unsaturated fatty acid biosynthesis and a reduction in the ω -hydroxylation of long-chain fatty acids. In support of this possibility, Lefèvre et al. have identified mutations in the gene *FLJ39501*, a member of the CYP4F subfamily, associated with lamellar ichthyosis (Lefèvre et al. 2006).

Approximately 30% of ARCI cases cannot be explained by genetic changes in known ichthyosis loci; the data presented here suggest that polymorphisms in the cytochrome *b*₅ pathway represent novel candidate genes for study. The striking similarities between the BCN phenotype and the human disease suggest that the BCN mouse could potentially be a valuable genetic model for developing new treatments for this disease and suggest new approaches for therapeutic intervention.

The loss of the epidermal lipid barrier in the skin of SCD1 null mice has been associated with perturbations in temperature control (Binczek et al. 2007), we therefore used thermal imaging to assess whether

the changes in skin fatty acids observed in BCN mice also resulted in disruption of this barrier. A small patch of back hair was shaved on 3 week old male BCN and wild-type mice to allow skin surface temperature measurements to be recorded together with similar measurements from haired regions over a 6 week period (Fig. 2b, c). In shaved skin areas, despite significantly higher measurements at two (out of 18) time-points, no overall difference between BCN and wild-type mice in either average or maximum temperatures was detected over the 6 week period (Fig. S2). In contrast, BCN mice had consistently significantly higher average and maximum temperatures in the haired skin areas compared to wild-type mice over the entire 6 week period, however from 5 to 6 weeks of age the differences in both average and maximum temperatures between genotypes decreased and the trend was for these measurement to approach those recorded for wild-type mice (Fig. 2d, e). These data indicated that the heat losses observed in BCN mice are primarily due

differences in hair coverage rather than changes in epidermal barrier permeability and confirm the visual observations that coat thickness improved with age in BCN mice.

Disruption of mammary gland cytochrome *b*₅ activity identifies roles in the determination of milk composition and neonatal development

As indicated by the thermal imaging data, the changes in coat condition, possibly associated with the changes in skin lipid composition, improved as

the mice mature, however coat condition did start to deteriorate again with age and especially in nursing females. In nursing females, this led to hair loss with skin reddening, particularly under the chin, around the eyes and snout, with the birth of each subsequent litter (Fig. 3a, b). Post-weaning, the dams' coat condition recovered and the skin inflammation subsided. Associated with these effects, pups from successive BCN litters were progressively smaller (unlike wild-type) suggesting the possibility that the dams' milk was nutritionally deficient (Fig. 3c, d). This hypothesis was supported by the finding that the

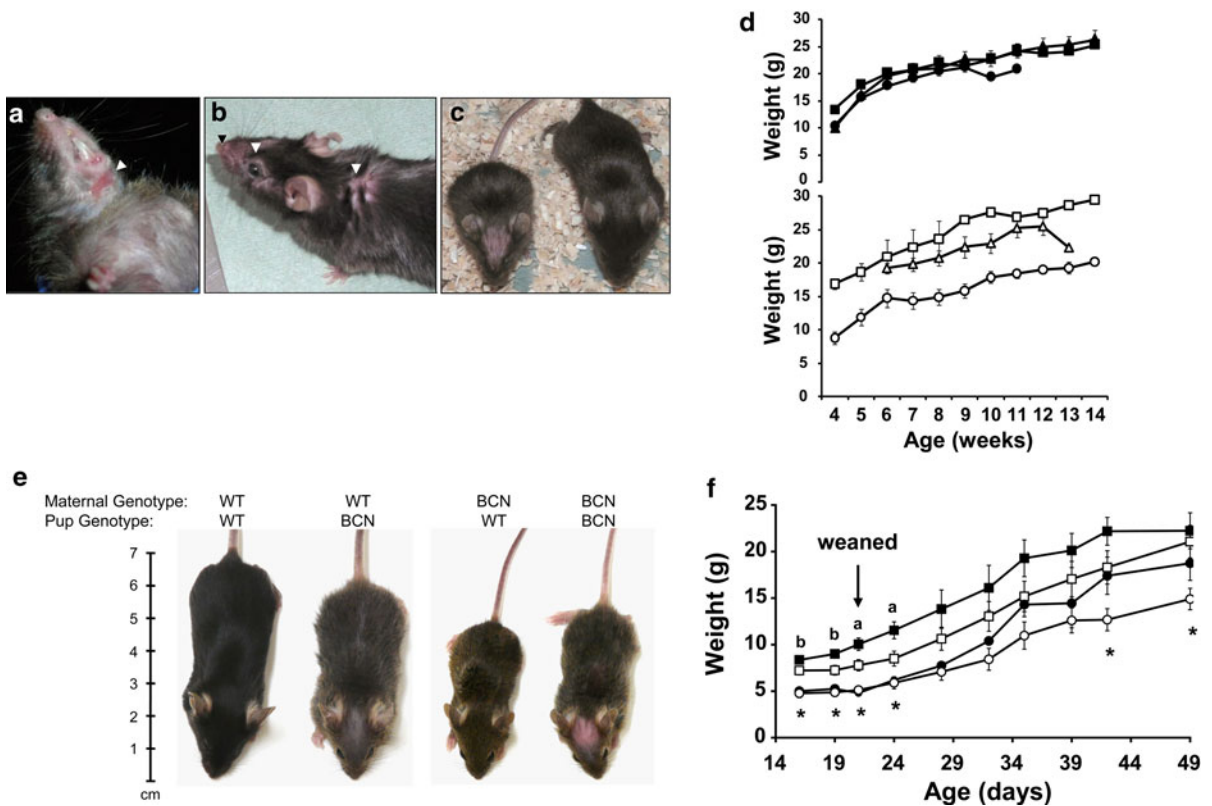


Fig. 3 Cytochrome *b*₅ null mice nurturing phenotype. **a** and **b** Progressive loss of coat condition in BCN females with successive litters. *Arrows* indicate the development of dry, red and inflamed skin under the chin (**a**); dry skin around eyes and muzzle, and poor coat condition accompanied by hair loss (**b**). **c** Age-matched (23 days old) BCN (*left*) and wild-type weanlings from the third litter of the relevant homozygous cross, showing the marked difference in size. **d** Time course of average litter weights for sequential litters from wild-type (*upper panel*) and BCN (*lower panel*). *Black squares, triangles and circles* represent wild-type litters 1, 2 and 3, respectively, while *white squares, triangles and circles* represent BCN litters 1, 2 and 3, respectively. Values represent the mean ± SEM.

e Fostering of 3 day old wild-type pups (third litter) to a BCN mother and BCN pups (second litter) to a wild-type mother indicates growth retardation is due to nutrient deficiencies. **f** Growth curves for wild-type pups left with their wild-type mother (*black squares*), BCN pups fostered to the wild-type mother (*white squares*), wild-type pups fostered to a BCN mother (*black circles*) and BCN pups left with their BCN mother (*white circles*). Values represent the mean ± SEM. of *n* = 4–6. * *P* ≤ 0.05, comparing BCN pups fostered to a wild-type mother and BCN pups left with their BCN mother. **a** *P* ≤ 0.05 and **b** *P* ≤ 0.005, comparing wild-type pups fostered to a BCN mother and wild-type pups left with their wild-type mother

fostering of BCN pups to a wild-type mother significantly reduced the growth retardation. In contrast, when wild-type pups were nursed by a BCN female a striking reduction in growth was observed (Fig. 3e, f).

The possibility that BCN milk is deficient in essential nutrients was investigated by comparing the fat content of milk taken 10 days postpartum from nursing wild-type and BCN females (both nursing their first litter). BCN milk contained a significantly higher level of total lipid; the predominant change being an increase in total saturated fatty acid levels; consistent with the role of cytochrome *b*₅ in fatty acid desaturation (Fig. 4a, Table S2). However, a small increase in n-6 polyunsaturated fatty acids was also observed. Monounsaturated and n-3 polyunsaturated fatty acid content was unchanged. The increase in saturated fat was associated with an increase in lauric, myristic, palmitic and arachidic acid (increased by 72, 75, 56 and 67% respectively) and the increase in n-6 polyunsaturated fats was due specifically to a 32% ($P = 0.021$) increase in linoleic acid (Fig. 4b). In addition, the level of α -linolenic acid was significantly increased in BCN milk although total n-3 polyunsaturated fatty acid levels were unchanged (Fig. 4b).

Under normal circumstances the level of fat contained in milk is tightly regulated but the composition can change depending on the maternal diet and circulating levels of individual fatty acids (Innis 2007; Rudolph et al. 2007). Genetic disruption of lactogenesis or lipogenesis, for example through the inactivation of the α -lactalbumin gene or of the activity of the mammary Akt pathway, has however been shown to increase the fat content of milk resulting in increased viscosity (Stinnakre et al. 1994;

Schwertfeger et al. 2003). This has been suggested in both these situations to prevent pups obtaining sufficient qualities of milk to support normal growth and thus leading to growth retardation. Indeed, milk from BCN mice appeared to have increased viscosity which would be consistent with the increase in saturated fat content and could possibly lead to an inability of BCN pups to obtain sufficient nutrition to support normal growth. Cytochrome *b*₅ may therefore be an important determinant in regulating the consistency of milk by virtue of its role in controlling fatty acid desaturation in the mammary gland.

However, we believe that the underlying reason for the reduced growth is due to a metabolic deficiency rather than a nurturing issue as the phenotype was progressive in subsequent generations. In addition to dietary intake, polyunsaturated fatty acids such as arachidonic, docosahexaenoic and eicosapentaenoic acid can be synthesised from linoleic and α -linolenic acid via the actions of $\Delta 5$ and $\Delta 6$ desaturases in maternal tissues. These desaturases have been shown to be expressed in lactating mammary glands of rats and are active in the conversion of linoleic acid to the γ -linolenic and arachidonic acid (Rodriguez-Cruz et al. 2006). Additionally, the in vitro findings of Guillou et al. have shown that exogenously added cytochrome *b*₅ is capable of enhancing $\Delta 6$ desaturase activity (Guillou et al. 2004). The loss of desaturase activity could explain the increase in linoleic acid in the milk; humans genetic variations in these gene loci have been associated with altered polyunsaturated fatty acid levels in milk (Xie and Innis 2008). It is therefore possible that the changes in the levels of linoleic and α -linolenic acid in BCN milk are responsible for the growth retardation observed in BCN pups.

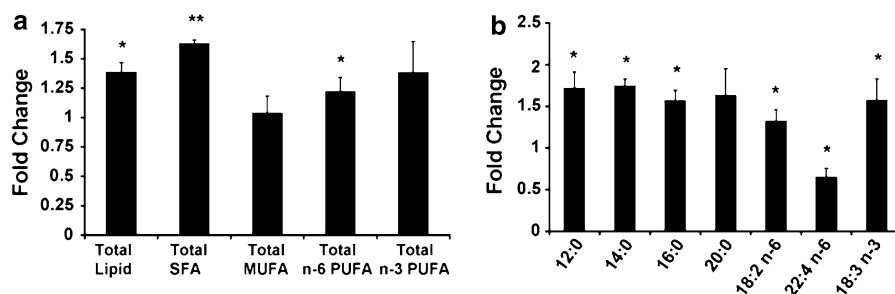


Fig. 4 Milk fatty acid profile analysis from wild-type and cytochrome *b*₅ null mice. Fatty acid profile analysis was carried out on milk from wild-type and BCN females nursing their first litters 10 days postpartum. **a** Total and **b** individual fatty acid

levels. Values are presented as fold change relative to wild-type levels and represent the mean \pm SD, where $n = 3$. * $P \leq 0.05$, ** $P \leq 0.005$ and *** $P \leq 0.001$ comparing wild-type and BCN mice

In support of this, studies in rodents have shown that excess levels of these fatty acids result in postnatal growth restriction (Bongiovanni et al. 2007; Church et al. 2008). The animals used in this study were on a normal diet, nutritionally balanced for saturated and unsaturated fatty acids. It is therefore intriguing that in spite of this, defects in endogenous unsaturated fatty acid biosynthesis caused the above phenotypes. The progressive degradation in the health of dams, suggests that an equilibrium exists between circulating fatty acids and breast milk, but that this is not sufficient to reverse the nutritional deficiency.

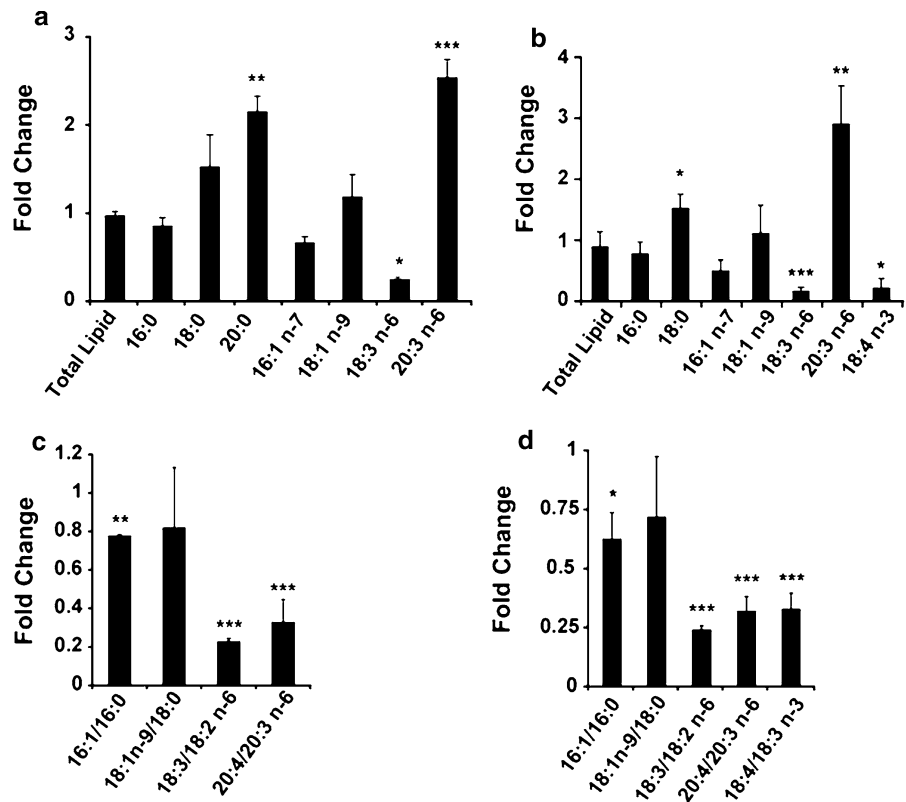
Disruption of microsomal cytochrome *b*₅ provides evidence for a central role in mono and polyunsaturated fatty acid homeostasis

In support of the role of cytochrome *b*₅ in overall fatty acid homeostasis, we found that in 16 week old male BCN mice the plasma level of γ -linolenic acid was significantly decreased (by 75%) and that of dihomo- γ -linolenic acid increased (by 153%) (Fig. 5a, Table S3). Additionally, in these mice, hepatic levels of these fatty acids followed a similar

pattern with γ -linolenic acid decreased by 84% and dihomo- γ -linolenic acid increased by 195% (Fig. 5b, Table S4). Furthermore, hepatic levels of octadecatetraenoic acid were decreased by 80% and eicosatetraenoic acid increased from undetectable levels to 0.03 ± 0.01 mg/g tissue (Fig. 5b). These fatty acids correspond to the substrates and products of various reactions catalysed by the Δ 5 and Δ 6 desaturases (Napier et al. 2003), and the differences observed in the desaturation indices for these fatty acids provides evidence for cytochrome *b*₅ as a modulator of the activity of these enzymes (Fig. 5c, d). In view of the effects ascribed to dietary ω -3 and ω -6 fatty acids on human health, it is important to understand the role of the endogenous biosynthetic pathways on circulating levels of these unsaturated fatty acids.

Monounsaturated fatty acids can be obtained either from the diet or by de novo synthesis involving a cytochrome *b*₅-dependent pathway (Hackett and Strittmatter 1984; Schenkman and Jansson 2003). We therefore determined saturated and monounsaturated fatty acid levels in plasma and liver from 10 and 16 week old male BCN mice (Tables S3, S4). At 16 weeks of age profound differences in both plasma

Fig. 5 Hepatic and plasma fatty acid profile analysis in wild-type and cytochrome *b*₅ null mice. Fatty acid profile analysis was carried out on plasma and livers from 16 week old wild-type and BCN mice as described in the “Materials and methods”. **a** Plasma fatty acid analysis, **b** liver fatty acid analysis, **c** plasma desaturation indices and **d** liver desaturation indices. Values are presented as fold change relative to wild-type levels and represent the mean \pm SD, where $n = 3$. * $P \leq 0.05$, ** $P \leq 0.005$ and *** $P \leq 0.001$ comparing wild-type and BCN mice



and hepatic levels of stearic (increasing by 50 and 47%, respectively) and palmitoleic acid (decreasing by 34 and 52%, respectively) were observed (Fig. 5a, b). Plasma and hepatic levels of palmitic and oleic acid were effectively unchanged and only the hepatic level of palmitoleic acid was significantly changed at 10 weeks of age (Table S4). Determination of desaturation indices for both palmitic and stearic acid revealed decreases for both fatty acids in BCN mice; however, only the desaturation index for palmitic acid was significantly changed (Fig. 5c, d). These data demonstrate that under normal dietary conditions cytochrome *b*₅-dependent pathways affect circulating mono and polyunsaturated fatty acid concentrations and their activity will therefore influence the beneficial effects of these compounds. Interestingly, Zhu et al. have recently identified a cytochrome *b*₅/*b*₅ reductase fusion protein, NAD(P)H cytochrome *b*₅ oxidoreductase (Ncb5OR), located in the lumen of the endoplasmic reticulum, which when deleted in mice results in marked decreases in hepatic levels of palmitoleic and oleic acid, suggesting a role for this protein in electron transport to SCD1 (Zhu et al. 2004; Larade et al. 2008).

In summary, we have generated a novel genetic model that identifies the numerous and varied in vivo roles of cytochrome *b*₅ and now allows these pathways to be studied in detail. Using this model we have identified that cytochrome *b*₅ plays a key role in a wide range of metabolic processes of central importance to both nutrition, drug metabolism and a range of human diseases and further demonstrate the importance of de novo unsaturated fatty acid biosynthesis in human health (McLaughlin et al. 2010). With the exception of the changes in hepatic lipid metabolism, none of the other phenotypic changes observed in BCN mice are seen in the conditional hepatic knockout model (Finn et al. 2008). This finding indicates that these phenotypic changes are most likely due to the specific absence of cytochrome *b*₅ in the particular tissue and not to the systematic disruption of cytochrome *b*₅ function, a fact that would also explain, with the exception of plasma and hepatic fatty acid changes which mimic each other, the differences in fatty acid profiles obtained from different tissues. These studies also demonstrate that certain endogenous pathways of fatty acid homeostasis cannot be substituted by dietary means. The relationship between de novo biosynthesis and dietary intake of fatty acids and how dietary intervention

may influence the pathogenesis of human disease will be a fascinating area of further study.

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