Hypopigmentation and Maternal-Zygotic Embryonic Lethality Caused by a Hypomorphic Mbtps1 Mutation in Mice

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ABSTRACT The site 1 protease, encoded by Mbtps1, mediates the initial cleavage of site 2 protease substrates, including sterol regulatory element binding proteins and CREB/ATF transcription factors. We demonstrate that a hypomorphic mutation of Mbtps1 called woodrat (wrt) caused hypocholesterolemia, as well as progressive hypopigmentation of the coat, that appears to be mechanistically unrelated. Hypopigmentation was rescued by transgenic expression of wild-type Mbtps1, and reciprocal grafting studies showed that normal pigmentation depended upon both cell-intrinsic or paracrine factors, as well as factors that act systemically, both of which are lacking in wrt homozygotes. Mbtps1 exhibited a maternal-zygotic effect characterized by fully penetrant embryonic lethality of maternal-zygotic wrt mutant offspring and partial embryonic lethality (~40%) of zygotic wrt mutant offspring. Mbtps1 is one of two maternal-zygotic effect genes identified in mammals to date. It functions nonredundantly in pigmentation and embryogenesis.

KEYWORDS pigmentation coat color site 1 protease cholesterol maternal-zygotic effect lethality

The site 1 protease (SIP), a transmembrane serine protease also known as subtilisin-kexin-isoenzyme 1, operates within the Golgi apparatus, where it performs the initial extracytoplasmic cleavage of site 2 protease (S2P) substrates during regulated intramembrane proteolysis, a process by which transmembrane proteins are cleaved within a membrane-spanning helix to release cytosolic domains (Ehrmann and Clausen 2004). SIP cleaves the sterol regulatory element binding proteins (SREBPs) to regulate cholesterol homeostasis; CREB/ATF transcription factors (Ye et al. 2000), CREBH (Zhang et al. 2006), CREB4 (Stirling and O’ hare 2006), OASIS (Murakami et al. 2006), and Luman (Raggo et al. 2002) in response to endoplasmic reticulum (ER) stress; and the surface glycoprotein precursors of lymphocytic choriomeningitis virus, Lassa fever virus, and Crimean-Congo hemorrhagic fever virus (Beyer et al. 2003; Kunz et al. 2003; Lenz et al. 2001; Vincent et al. 2003).

In the mouse, targeted disruption of the SIP-encoding gene Mbtps1 prevented normal epiblast formation and subsequent implantation of the embryo (Mitchell et al. 2001), resulting in lethality at an early developmental stage (Yang et al. 2001). Liver-specific knockout of Mbtps1 yielded viable mice in which blood cholesterol and triglyceride levels and the expression of genes involved in fatty acid and cholesterol synthesis and low-density lipoprotein (LDL) clearance were affected (Yang et al. 2001). Cartilage-specific Mbtps1-deficient mice died shortly after birth, exhibiting severe chondrodysplasia caused by the retention of type 2 collagen in the ER (Patra et al. 2007).

We recently generated a viable hypomorphic allele of Mbtps1, called woodrat, with the substitution Y496C in the extracellular domain. Homozygosity for the woodrat mutation caused hypersensitivity to dextran sodium sulfate–induced colitis by impairing the unfolded protein response in intestinal epithelial cells (Brandl et al. 2009) and conferred resistance to lymphocytic choriomeningitis virus through an...
effect on dendritic cells (Popkin et al. 2011). We demonstrate here that cell-intrinsic and -extrinsic functions of S1P are required for normal coat color. In addition, the \textit{wrt} mutation of \textit{Mbtps1} causes maternal-zygotic effect embryonic lethality.

**MATERIALS AND METHODS**

**Mice**

Mice were maintained in specific pathogen-free conditions in The Scripps Research Institute Vivarium under the supervision of the Department of Animal Resources. Animals were fed a normal (Teklad #7012; Harlan) or a 5% cholesterol-enriched diet (TD00337; Harlan). All experimental procedures were conducted in accordance with institutional guidelines for animal care and use. C57BL/6j mice were purchased from The Jackson Laboratories. \textit{Tyr\textsuperscript{-}\textit{ghost}} mutant mice were produced and maintained in the Beutler lab. The \textit{Mbtps1\textsuperscript{wrt/wrt}} strain was generated by N-ethyl-N-nitrosourea mutagenesis of C57BL/6j mice (Brandl et al. 2009) and maintained by breeding \textit{Mbtps1\textsuperscript{wrt/wrt}} males to \textit{Mbtps1\textsuperscript{wrt/+}} females. The \textit{Tyr\textsuperscript{-}\textit{ghost}} and \textit{Mbtps1\textsuperscript{wrt}} strains are described at http://mutagenetix.utsouthwestern.edu.

For phenotypic rescue experiments, a BAC (CH29-17A18) containing the wild-type allele of \textit{Mbtps1} derived from the NOD/LtJ strain was injected into the male pronucleus of fertilized oocytes from matings of C57BL/6j females and \textit{Mbtps1\textsuperscript{wrt/wrt}} males. The expression of the transgenic wild-type \textit{Mbtps1} allele in mice was confirmed by detection via polymerase chain reaction (PCR) of a GATA simple sequence length polymorphism present in the BAC and informative for C57BL/6j vs. NOD/LtJ strains using the following primers: 5\textsuperscript{-}CCAGCGGTTAATGGCATCTGAAATG-3\textsuperscript{-} and 5\textsuperscript{-}ATTGTCTAAGCTGGGTGGCAGAG-3\textsuperscript{-}.

**Real-time PCR analysis**

RNA from skin was isolated using the Trizol reagent (Invitrogen). DNase-treated RNA underwent randomly primed cDNA synthesis and real-time PCR analysis. SYBR Green-based real-time PCR was performed using the DyNAamo SYBR Green qPCR Kit (Finnzymes). \textit{Mif}t-specific primers were obtained from QIAGEN, and signals were normalized to \textit{\textbeta}-actin. Normalized data were used to quantitate relative levels of \textit{Mif}t using \\DeltaACt analysis.

**Skin grafts**

Recipient mice were anesthetized and the flank hair shaved with electronic clippers. A graft bed was prepared on the lateral thoracic region under aseptic conditions. The graft bed was prepared by carefully removing the epidermis and dermis to the level of the panniculus carnosus without disturbing the vascular bed. Donor thoracic skin was prepared in the same manner, i.e., removing the epidermis and dermis and placing in a sterile Petri dish wetted with phosphate-buffered saline. The donor skin was then placed into the recipient vascular bed. Donor thoracic skin was removed from a given cross, unless otherwise specified. The statistical significance of all other differences was determined by a two-tailed Student’s \textit{t}-test. Differences with a \textit{P} value <0.05 were considered statistically significant. All error bars represent SD.

**RESULTS**

**S1P is required for normal coat color**

The recessive \textit{woodrat} \textit{(wrt)} mutation was isolated by positional cloning based on a hypopigmentation phenotype (Brandl et al. 2009). Mild uniform hypopigmentation was first observed in \textit{Mbtps1\textsuperscript{wrt/wrt}} and \textit{Mbtps1\textsuperscript{+/wrt}} progeny from a given cross, unless otherwise specified. The statistical significance of all other differences was determined by a two-tailed Student’s \textit{t}-test. Differences with a \textit{P} value <0.05 were considered statistically significant. All error bars represent SD.

Four mice per genotype were fasted for 4 hr before blood collection from the retro-orbital sinus. Serum concentrations of total cholesterol and triglycerides were determined enzymatically on a Cobas Mira Plus autoanalyzer using the cholesterol R1 and triglycerides reagent methods, respectively (Roche Diagnostics). Colorimetric changes were measured at 500 nm. Lipoprotein-associated cholesterol was separated using the SPIFE 3000 agarose electrophoresis system (Helena Labs, Beaumont, TX) (Contois et al. 1999) and verified by fast-protein liquid chromatography with a Superose 6HR column and in-line post column analysis as described previously (Kieft et al. 1991).
which carried the transgene. Genomic DNA from a \( \text{Mbtps1}^{\text{wrt/wrt}} \) mouse was sequenced extensively over the region included within the BAC, so that 92.4% of all nucleotides comprising coding regions and splice junctions (de\( \text{f}\)ined as 10 bp at either end of each intron) were examined on at least one strand to a phred score of 30 or greater. No mutations other than the \( \text{Mbtps1}^{\text{wrt}} \) allele were detected.

Two independent transgenic founders, both female heterozygotes, were crossed to male \( \text{Mbtps1}^{\text{wrt/wrt}} \) mice and the heterozygous progeny were crossed to the parental \( \text{Mbtps1}^{\text{wrt/wrt}} \) strain to achieve homozygosity for the \( \text{wrt} \) allele in a fraction of the offspring, 50% of which would be expected to inherit the transgene. Nine of them showed the hypopigmentation phenotype, all were homozygous for the \( \text{wrt} \) allele and none carried a transgene (\( P = 0.00195; \) exact binomial probability). Nineteen offspring carried a transgene, derived from one founder or the other, and all showed a wild-type coat color (\( P = 0.00000191; \) exact binomial probability). Combining these probabilities by Fisher’s method, the null hypothesis that the transgene does not affect the woodrat phenotype is rejected (\( \chi^2 \) on 4 df = 38.8; \( P < 0.0001 \)).

\( \text{Mitf} \) is a key transcriptional regulator of melanocyte development, and several mutant alleles of \( \text{Mitf} \), including \( \text{mi-enu5, mi-bcc2} \) (Hansdottir et al. 2004), and \( \text{mi-vit} \) (Boissy et al. 1987; Lerner 1986), cause hypopigmentation similar in various aspects to that observed in \( \text{wrt} \) homozygotes. However, we found no significant difference between \( \text{Mitf} \) mRNA expression in the skin of wild-type vs. \( \text{Mbtps1}^{\text{wrt/wrt}} \) mice (Figure 1C). Consistent with this finding, melanocytes of normal morphological appearance were present in the hair follicles of \( \text{Mbtps1}^{\text{wrt/wrt}} \) mice; however, the functional status of those melanocytes was not evaluated. These findings suggest that the hypopigmentation of \( \text{Mbtps1}^{\text{wrt/wrt}} \) mice is not caused by aberrant melanocyte development.

To determine whether the \( \text{wrt} \) pigmentation phenotype resulted from a systemic metabolic defect or from a defect localized to the skin, syngeneic skin transplants were performed (Figure 1D). Shaved dorsal skin from \( \text{Mbtps1}^{\text{wrt/wrt}} \) or C57BL/6J mice was grafted onto C57BL/6J or \( \text{Mbtps1}^{\text{wrt/wrt}} \) mice, respectively. In a wild-type C57BL/6J animal, \( \text{Mbtps1}^{\text{wrt/wrt}} \) grafts retained their hypopigmented phenotype, whereas C57BL/6J skin transplanted onto a \( \text{Mbtps1}^{\text{wrt/wrt}} \) individual acquired a hypopigmented phenotype. Control C57BL/6J skin grafts maintained their black color when transplanted onto a \( \text{Tyr}^{\text{ghost}} \) homozygous mutant mouse, and reciprocal transplantation of skin from the \( \text{Tyr}^{\text{ghost}} \) homozygous mutant onto wild-type recipients remained white (Figure 1E). These results indicate that normal pigmentation depends upon two independent processes, one systemic and one cell-autonomous or paracrine, both of which are disrupted by homozygosity for the \( \text{Mbtps1}^{\text{wrt}} \) mutation.

**Maternal-zygotic effect lethality caused by the woodrat mutation**

Homozygosity for the \( \text{wrt} \) allele biases against survival in utero. Of 87 progeny in 23 litters born from crosses of \( \text{Mbtps1}^{\text{wrt/wrt}} \) males to \( \text{Mbtps1}^{\text{wrt+}} \) females, in which an equal number of homozygous and
heterozygous progeny would be expected, 60 phenotypically normal and 27 phenotypically affected mice were produced ($P = 0.0004$). When fetuses from this cross were examined at 11 (n = 7 embryos, 1 litter), 14 (n = 6 embryos, 1 litter), 17 (n = 8 embryos, 1 litter), and 20 days of gestation (n = 6 embryos, 1 litter), 100% were found alive (Table 1), suggesting that death of homozygotes occurred before embryonic day 11. In independent crosses of heterozygotes, among 18 litters, 90 phenotypically normal and 17 phenotypically affected mice were produced ($P = 0.0295$). All litters were carefully counted on the day of birth, and no postnatal deaths were observed before genotyping at 19 days of age.

We also observed that no offspring were born from crosses of Mbtps1wrt/wrt males and Mbtps1wrt/wrt females; these female mice were surveyed daily from day 18 to day 24 after observation of the corroboratory plug. Mbtps1wrt/wrt females crossed to Mbtps1wrt/+ males or Mbtps1wrt/+ females gave birth to heterozygous pups, but in the former cross, never to homozygous pups; litters were counted on the day of birth and deaths among pups were not observed before genotyping at postnatal day 19. However, as already noted, Mbtps1wrt/+ females crossed to Mbtps1wrt/wrt males produced both heterozygous and homozygous offspring. Thus, the wrt allele causes maternal-zygotic effect lethality in that homozygous females can only give birth to offspring lacking wild type Mbtps1.

To determine the stage at which development of homozygous maternal-zygotic mutants fails, embryos from Mbtps1wrt/+ females crossed to Mbtps1wrt/+ males were examined after various gestation periods. Mbtps1wrt/+ females crossed to Mbtps1wrt/+ males appeared pregnant 8 days postcoitum and left and right uterine horns were highly vascularized and displayed rounded bulges (Figure 2A). However, no fetal bodies were found within their uteri at 8, 11, or 14 days of gestation (Figure 2B and Table 1). Thus, death and resorption of maternal-zygotic mutant embryos (homozygous wrt embryos derived from homozygous wrt mothers) occur before the eighth day of gestation.

### Low cholesterol levels in wrt homozygotes are not responsible for hypopigmentation

Because S1P has nonredundant functions in both cholesterol and triglyceride homeostasis through cleavage of SREBP (Sakai et al. 1998), we measured the levels of cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein-C (LDL-C)/very low-density lipoprotein-C (VLDL-C) in Mbtps1wrt/wrt mice fasted for 4 hr (Figure 3A). Serum concentrations of cholesterol and lipoproteins were significantly reduced in homozygous mutant mice as compared to wild-type controls. Triglyceride levels were not affected by the wrt mutation. To determine whether a high-cholesterol diet could rescue the low levels of serum cholesterol, HDL-C, and LDL-C/VLDL-C, as well as the pigmentation defect in wrt homozygotes, mice were fed a high-cholesterol diet and observed for 1 week. The high-cholesterol diet increased the concentrations of total cholesterol and LDL-C/VLDL-C in the serum of Mbtps1wrt/wrt mice relative to levels in Mbtps1wrt/+ mice fed a regular diet (Figure 3A). However, the high-cholesterol diet did not change the abnormal coat pigmentation of Mbtps1wrt/wrt mice.

There are three isoforms of SREBP, SREBP2 preferentially up-regulates the expression of genes involved in cholesterol synthesis, whereas SREBP1c selectively induces lipogenic genes without affecting cholesterol synthesis genes (Eberle et al. 2004), and SREBP1a leads to accumulation of both cholesterol and triglycerides. We hypothesized that the disproportionate effect of the woodrat mutation on serum cholesterol vs. triglyceride concentration might reflect substrate specificity, i.e., more efficient regulated intramembrane proteolysis processing of SREBP1c relative to SREBP2 in Mbtps1wrt/wrt mice. Consistent with this hypothesis, we observed that whereas the SREBP1 precursor was equally processed into its 68 kD active form in hepatocytes from Mbtps1wrt/wrt, Mbtps1wrt/+, and wild-type mice, a reduced amount of precursor SREBP2 was processed into its cleaved, active form in Mbtps1wrt/wrt compared with Mbtps1wrt/+ or wild-type hepatocytes (Figure 3B).

### DISCUSSION

In previous studies, we established that a gene trap allele of Mbtps1 was lethal both in the homozygous state and in trans with Mbtps1wrt (Brandl et al. 2009). However, although hypopigmentation was the phenotype used to map the Mbtps1wrt mutation, no firm conclusion could be drawn concerning cause and effect. Using transgenesis, we have now adduced strong evidence that the previously reported coat color anomaly of the wrt strain does, in fact, result from the hypomorphic wrt mutation of Mbtps1. In a compound heterozygous cross, Mbtps1wrt/wrt mice that were also transgenic for the wild-type Mbtps1 locus never displayed hypopigmentation and all mice that did display hypopigmentation were of the Mbtps1wrt/wrt genotype, and lacked the transgene.

More than 300 gene products are known to affect mouse pigmentation (Montoliu et al. 2011), including proteins that regulate melanocyte proliferation and development (e.g., Mitf, Kit, Edn1), melanosome biogenesis (e.g., Oca2, Scl45a2, BLOC-1 complex), melanosome transport (e.g., Rab27a, melanophilin, myosin Va), and melanogenesis (e.g., Tyr, Dct, Mc1r). Most classical coat color genes encode factors produced in the skin and/or hair follicle that signal locally to melanocytes or within them (Hirobe 2011; Slominski et al. 2004). In general, less is
known of factors that circulate through the bloodstream and exert systemic control over pigmentation. One well-studied example is α-melanocyte-signaling hormone, which is produced in humans by melanocytes and keratinocytes (Chakraborty et al. 1996; Rousseau et al. 2007; Wakamatsu et al. 1997) and in mice and humans by the pituitary gland (Hirobe et al. 2004; Krude et al. 1998; Pears et al. 1992), through cleavage of the precursor protein proopiomelanocortin. α-melanocyte-signaling hormone from either tissue source in humans, and from the pituitary gland via the bloodstream in mice, promotes the production of eumelanin by melanocytes. Other systemic factors influencing pigmentation include steroid hormones (e.g., estrogen, progesterone, androgen), fatty acids (e.g., linoleic acid, palmitic acid), and iron (Hirobe 2011).

Using reciprocal immunologically compatible skin grafts, we showed that normal pigmentation of the fur depends upon two processes, one cell-autonomous or paracrine and one systemic, both of which are disrupted by homozygosity for the wt mutation. The effects of Mbtps1 activity on cells or tissues at remote locations may be interpreted in several ways. First, it has been noted that both an ER/Golgi membrane-anchored and a shed, soluble form of SIP exist, and the soluble form might conceivably exert a systemic effect on pigmentation. Second, it may be imagined that specific metabolites, dependent upon the enzymatic activity of SIP cleavage products, might be needed for this process. Although we showed that serum cholesterol levels were reduced in Mbtps1wt/wt mice, cholesterol does not seem to be the crucial metabolite needed for normal pigmentation, since a high cholesterol diet did not normalize the coat pigmentation of Mbtps1wt/wt mice.

In breeding experiments, maternal-zygotic Mbtps1wt/mutant offspring (homozygotes derived from homozygous mutant mothers) displayed fully penetrant embryonic lethality, whereas zygotic mutant offspring (homozygotes derived from heterozygous mothers) displayed partial (~40%) embryonic lethality, and heterozygous mutant offspring of homozygous mothers were fully viable. These findings demonstrate a maternal-zygotic effect of Mbtps1, to our knowledge the second mammalian gene to which such an effect has been ascribed. Mouse Zfp57 was the first identified mammalian maternal-zygotic effect gene and was found to participate in the maintenance of genomic DNA methylation imprints without which mouse embryos died in midgestation (Li et al. 2008). ZFP57, together with its cofactor KAP1, recruits DNA methyltransferases to a methylated hexanucleotide within numerous imprinting control regions (Quenneville et al. 2011; Zuo et al. 2011). A role for SIP in genomic imprinting remains to be tested.

Experiments in which Drosophila was used support an evolutionarily conserved requirement for the function of the S1P/S2P module during embryonic development. Analogously to wt mutant mice, ds2p homozygous mutant fli embryos from heterozygous mothers emerged at a normal frequency, whereas less than 50% of the expected number of homozygous offspring derived from homozygous mothers survived (Matthews et al. 2009). The survival rate of homozygous offspring of homozygous female flies was not reported in this study, leaving open the possibility that ds2p may function as a maternal-zygotic effect gene. However, in contrast to the nonredundant function of SIP in mice, the caspase drICE can partially compensate for dS2P deficiency in Drosophila, thus permitting a significant proportion of homozygous fli embryos derived from homozygous mothers to survive to adulthood (Amarnah et al. 2009). Notably, maternal effect embryonic lethality of homozygous mutant flies was completely rescued by supplementation of the embryo culture medium with fatty acids (Matthews et al. 2009). Fatty acids and/or cholesterol may likewise be critically lacking during development of Mbtps1wt/wt concepti in utero.

We found that cholesterol and lipoproteins were preferentially reduced relative to triglycerides in serum from Mbtps1wt/wt mice, an effect that we attribute to a more severe impairment of SREBP2 processing as compared with SREBP1 processing. Y496, the residue mutated in SIP, may provide a contact critical for interaction with SREBP2.

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