Liver-specific expression of the agouti gene in transgenic mice promotes liver carcinogenesis in the absence of obesity and diabetes

Alexander I. Kuklin  
*Oak Ridge National Laboratory*

Randall L. Mynatt  
*Oak Ridge National Laboratory*

Mitchell L. Klebig  
*Oak Ridge National Laboratory*

Laura L. Kiefer  
*Glaxo Wellcome*

William O. Wilkison  
*Glaxo Wellcome*

*See next page for additional authors*

Follow this and additional works at: [http://trace.tennessee.edu/utk_biocpubs](http://trace.tennessee.edu/utk_biocpubs)

Part of the [Molecular Biology Commons](http://trace.tennessee.edu/utk_biocpubs)

**Recommended Citation**  
Molecular Cancer 2004, 3:17 doi:10.1186/1476-4598-3-17
Liver-specific expression of the agouti gene in transgenic mice promotes liver carcinogenesis in the absence of obesity and diabetes

Alexander I Kuklin†1,3, Randall L Mynatt†1,4, Mitchell L Klebig1,5, Laura L Kiefer2,6, William O Wilkison2,7, Richard P Woychik1,8 and Edward J Michaud*1

Address: 1Life Sciences Division, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831, USA, 2Glaxo Wellcome, 5 Moore Drive, Research Triangle Park, NC 27709, USA, 3Transgenomic, Inc., 12325 Emmet Street, Omaha, NE 68164, USA, 4Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA 70808, USA, 5Department of Biochemistry and Cellular & Molecular Biology, The University of Tennessee, Knoxville, TN 37996, USA, 6Paradigm Genetics, 108 Alexander Drive, Research Triangle Park, NC 27709, USA, 7GlaxoSmithKline, Inc., 5 Moore Drive, Research Triangle Park, NC 27709, USA and 8The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609, USA

Email: Alexander I Kuklin - akuklin@transgenomic.com; Randall L Mynatt - mynattrl@pbrc.edu; Mitchell L Klebig - uvi@ornl.gov; Laura L Kiefer - lkiefer@paragen.com; William O Wilkison - william.o.wilkison@gsk.com; Richard P Woychik - rick@jax.org; Edward J Michaud* - michaudejjii@ornl.gov

* Corresponding author    †Equal contributors

Published: 02 June 2004
Received: 02 April 2004
Accepted: 02 June 2004

Molecular Cancer 2004, 3:17

This article is available from: http://www.molecular-cancer.com/content/3/1/17

© 2004 Kuklin et al; licensee BioMed Central Ltd. This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL.

Abstract

Background: The agouti protein is a paracrine factor that is normally present in the skin of many species of mammals. Agouti regulates the switch between black and yellow hair pigmentation by signalling through the melanocortin 1 receptor (Mc1r) on melanocytes. Lethal yellow (Ay) and viable yellow (Avy) are dominant regulatory mutations in the mouse agouti gene that cause the wild-type protein to be produced at abnormally high levels throughout the body. Mice harboring these mutations exhibit a pleiotropic syndrome characterized by yellow coat color, obesity, hyperglycemia, hyperinsulinemia, and increased susceptibility to hyperplasia and carcinogenesis in numerous tissues, including the liver. The goal of this research was to determine if ectopic expression of the agouti gene in the liver alone is sufficient to recapitulate any aspect of this syndrome. For this purpose, we generated lines of transgenic mice expressing high levels of agouti in the liver under the regulatory control of the albumin promoter. Expression levels of the agouti transgene in the liver were quantified by Northern blot analysis. Functional agouti protein in the liver of transgenic mice was assayed by its ability to inhibit binding of the α-melanocyte stimulating hormone (αMSH) to the Mc1r. Body weight, plasma insulin and blood glucose levels were analyzed in control and transgenic mice. Control and transgenic male mice were given a single intraperitoneal injection (10 mg/kg) of the hepatocellular carcinogen, diethylnitrosamine (DEN), at 15 days of age. Mice were euthanized at 36 or 40 weeks after DEN injection and the number of tumors per liver and total liver weights were recorded.

Results: The albumin-agouti transgene was expressed at high levels in the livers of mice and produced a functional agouti protein. Albumin-agouti transgenic mice had normal body weights and normal levels of blood glucose and plasma insulin, but responded to chemical initiation of the liver with an increased number of liver tumors compared to non-transgenic control mice.

Conclusions: The data demonstrate that liver-specific expression of the agouti gene is not sufficient to induce obesity or diabetes, but, in the absence of these factors, agouti continues to promote hepatocellular carcinogenesis.
Background
The wild-type agouti coat color exhibited by many mammals consists of individual hairs that are black with a sub-terminal band of yellow [1]. The mouse agouti gene product is a secreted paracrine factor that regulates the alternate production of black and yellow pigments produced by hair-bulb melanocytes [2-4]. Binding of αMSH to the Mc1r on the surface of hair-bulb melanocytes results in the production of black pigment that is deposited in the growing hair. The agouti gene is transiently expressed in the skin during the mid-portion of the hair growth cycle. At this time, the agouti protein binds to the Mc1r, thereby excluding αMSH binding and causing a switch from black to yellow pigment production by melanocytes, which results in the appearance of the sub-terminal yellow band in the otherwise black hair [5-10].

Recessive mutations in the agouti gene affect only the coat color of mice, causing either a partial or complete loss of yellow pigment in the hair [11,12]. The dominant agouti mutations, lethal yellow (Ay) and viable yellow (A^v), affect coat color by causing an increase in the amount of yellow pigment in the hair. Additionally, these dominant mutations cause mice to develop type II diabetes (peripheral insulin resistance, pancreatic islet hyper trophy and hyperplasia, hyperinsulinemia, and hyperglycemia), obesity (hyperphagia and increased adipose mass), increased somatic growth (increased fat-free dry mass and slightly longer bones), and increased susceptibility to hyperplasia and carcinogenesis in numerous tissues [reviewed in refs. [13-22]]. This syndrome is manifested in lethal yellow and viable yellow mice because they carry regulatory mutations in the agouti gene that cause the normal protein to be produced at abnormally high levels throughout the body [23-26].

In addition to its normal role of regulating pigmentation through Mc1r, agouti can also antagonize αMSH binding to other melanocortin receptor family members [5,27-31]. The ability of agouti to antagonize binding of αMSH to the Mc4r is of particular relevance, as Mc4r is expressed in the adipose tissue also appears to contribute to the obesity syndrome. Transgenic mice with adipocyte-specific agouti expression were shown to have significantly increased fat mass compared to control mice, which was accompanied by an increase in the protein levels of three transcription factors (Pparg, peroxisome proliferator-activated receptor gamma; Stat1, signal transducer and activator of transcription 1; and Stat3) in their adipose tissue [36]. These three transcription factors were also upregulated in mature 3T3-L1 adipocytes in culture following treatment with recombinant agouti protein [36]. Additionally, recombinant agouti protein causes an increase in fatty acid synthase expression and activity, and the accumulation of triglycerides in cultured adipocytes [37]. Together, these results suggest that the obesity-related factors of the dominant agouti syndrome are mediated by agouti expression in both the brain and peripheral tissue(s).

Dominant mutations in the agouti gene also cause an increase in the susceptibility to hyperplasia and carcinogenesis in the liver [38-45], skin [46,47], lung [44,48-50], mammary gland [38,39,51-54], and urinary bladder [55]. It is likely that agouti-mediated antagonism of melanocortin receptors is mainly responsible for the obesity and diabetes of lethal yellow and viable yellow mice, but it is not known if melanocortin receptors are involved in their increased susceptibility to cancer. Whereas the obesity-related factors may contribute to the increased predisposition to carcinogenesis, there is some evidence to support the hypothesis that ectopic expression of the agouti gene per se may promote carcinogenesis in the liver and lung, even in the absence of hyperinsulinemia and obesity [44].

The liver is a primary site of insulin-mediated glucose disposal and lipogenesis in the mouse. Based on this fact, and on the previous reports of increased susceptibility to hepatic carcinogenesis in dominant agouti mutant mice, we were interested in determining if agouti expression in the liver alone would be sufficient to induce any of the phenotypes observed in lethal yellow or viable yellow mice. For this purpose, we generated lines of transgenic mice in which the wild-type murine agouti cDNA was expressed only in the liver at levels similar to or greater than those observed in lethal yellow or viable yellow mice. Transgenic and control mice were compared with respect to body weights, blood glucose levels, plasma insulin levels, and tumorigenic responses to chemical initiation in the liver.

Results
The albumin promoter directs expression of the wild-type agouti cDNA to the liver in transgenic mice
The albumin promoter was used to direct liver-specific expression [56] of the wild-type murine agouti cDNA in transgenic mice. Three lines of transgenic mice were established and two of these lines were characterized in detail: FVB/N-Tg(Alb1-a)86R and FVB/N-Tg(Alb1-a)83R (hereafter referred to as alb-agouti 86 and alb-agouti 83, respectively). As expected, agouti expression was detected only in the liver of the transgenic mice after an agouti cDNA probe
was hybridized to Northern blots containing ~2.5 µg of poly (A)+ RNA from adult muscle, liver, small intestine, brain and white adipose tissue. The expression level of agouti in the liver of an alb-agouti 86 mouse is compared to that of a BAPa20 mouse (FVB/N-TgN(BAPa)20Rpw) in Figure 1. BAPa20 is a line of transgenic mice in which the wild-type agouti cDNA is under the regulatory control of the human β-actin promoter and enhancer. It was previously demonstrated that BAPa20 mice express the agouti gene in a ubiquitous manner and, consequently, become hyperinsulinemic and obese [57]. As seen in Figure 1, alb-agouti 86 mice express the agouti cDNA in the liver at a higher level than do BAPa20 mice.

The expression levels of agouti in the livers of alb-agouti 86 and 83 transgenic mice was next estimated by densitometry and compared to the levels of agouti expressed in the livers of BAPa20, lethal yellow (Ay/a) and viable yellow (Avy/a) mice (Fig. 2). Viable yellow mice have coat colors ranging from completely yellow (y) to mottled yellow and agouti (m) to pseudoagouti (p), and the amount of yellow pigment in the coat is correlated with the level of agouti expression throughout the body and the severity of the obesity, diabetes, and neoplasia that they display [16]. The alb-agouti 86 mice express agouti in the liver at ~13.7 times the level in lethal yellow liver, and at a level that is also substantially greater than in BAPa20 liver and the livers of all three phenotypic classes of viable yellow mice. In contrast, the alb-agouti 83 mice express agouti in the liver at ~0.8 times the level in lethal yellow liver, at approximately one third the level in BAPa20 liver, but still at a greater level than in the livers of all three classes of viable yellow mice. Thus, under the hypothesis that expression of agouti in the liver alone is sufficient to induce the obesity
and diabetes of viable yellow mice, both lines of alb-agouti transgenic mice express the wild-type agouti cDNA in the liver at levels that should be adequate to induce these effects.

**alb-agouti transgenic mice produce functional agouti protein in the liver**

Recombinant murine agouti protein inhibits the binding of \[^{125}\text{I}\]-NDP-\(\alpha\)MSH to the Mc1r in murine melanoma B16F10 cells. This assay was used as previously described \[58,59\] to determine if the alb-agouti transgene produces a functional agouti protein in the liver of the transgenic mice. Prior to the assay, an S Sepharose chromatography procedure was used to enrich for agouti protein in liver homogenates from alb-agouti 86 transgenic mice. Liver homogenates from non-transgenic control mice and liver homogenates from non-transgenic control mice spiked with recombinant agouti protein were treated in the same manner. Relative inhibition of \[^{125}\text{I}\]-NDP-\(\alpha\)MSH binding to the Mc1r for each of the three samples is displayed in Figure 3. The alb-agouti 86 sample inhibited \[^{125}\text{I}\]-NDP-\(\alpha\)MSH binding in the assay almost as effectively as the control sample spiked with recombinant agouti protein, whereas the control sample from non-transgenic mice did not significantly affect \(\alpha\)MSH binding.

**alb-agouti transgenic mice have normal body weight, and normal levels of plasma insulin and blood glucose**

To determine if expression of the agouti gene in the liver alone is sufficient to induce obesity and diabetes, the body weight, and levels of blood glucose and plasma insulin were analyzed in the alb-agouti transgenic mice and compared to those of non-transgenic siblings. There were no significant differences \((p > 0.05)\) in body weight between transgenic and control mice fed a diet containing 11% fat by weight (Fig. 4). Levels of plasma insulin and blood glucose did not differ significantly \((p > 0.05)\) between the alb-agouti transgenic mice and the non-transgenic controls, whereas both insulin and glucose levels were significantly elevated in BAPa20 mice (positive controls) as expected (Table 1). Therefore, these data indicate that expression of the agouti gene in the liver alone is not sufficient to induce obesity or diabetes.

**alb-agouti transgenic mice respond to chemical initiation of the liver with an increased number of tumors per liver**

To determine if the presence of agouti in the livers of alb-agouti 83 and 86 transgenic mice promotes liver carcinogenesis in the absence of obesity and diabetes, a single intraperitoneal injection of the liver carcinogen, DEN (10 mg/kg body weight), was administered to transgenic and control male mice at 15 days of age. Body weights of all injected mice were recorded from 4–42 weeks of age, and there were no significant differences \((p > 0.05)\) between either of the transgenic lines and their non-transgenic littermates at any time point (data not shown). Three mice from each of the transgenic and control groups were euthanized at various times after DEN injection, and it was determined that 36 and 40 weeks post-injection were the most appropriate times for sampling the mice, because tumors at those ages were macroscopically visible and had not yet coalesced (data not shown). Therefore, separate groups of transgenic and control mice \((n = 18–20\) per group) were euthanized at 36 weeks or 40 weeks post-injection, and their tumor numbers and liver weights were recorded (Fig. 5).

At 36 weeks after DEN injection, the number of tumors per liver was 2.3 ± 0.4 (mean ± one standard error of the
of tumors per liver for the transgenic mice was positively correlated with the level of agouti expression in the liver. Whereas transgenic mice had more tumors per liver than did the control mice, the size distribution of tumors was similar in transgenics and controls (data not shown). The fact that alb-agouti 86 mice have normal body weights and levels of blood glucose and plasma insulin, but responded to DEN with significantly more liver tumors than did the non-transgenic mice demonstrates a more direct effect of agouti in the promotion of liver carcinogenesis.

Total liver weight was also recorded at 36 and 40 weeks after DEN injection. Both lines of transgenic mice had greater average liver weights than did the control mice (Fig 5B). The differences in liver weights between alb-agouti 86 mice and controls were significant ($p < 0.05$) at both 36 weeks (1.95 g ± 0.06 vs. 1.63 g ± 0.07) and 40 weeks (1.97 g ± 0.08 vs. 1.67 g ± 0.03) after DEN injection, whereas the differences between alb-agouti 83 mice and controls were not significant ($p > 0.05$). Unlike the livers from obese lethal yellow and viable yellow mice, where visual examination reveals an excessive amount of fat deposition in the liver (E. J. M., personal observations), the livers from lean alb-agouti 83 and 86 mice did not appear to be different from control livers in terms of fat content (data not shown). This suggests that the heavier livers in DEN injected alb-agouti transgenic mice resulted from agouti protein-induced hyperplasia and/or greater tumor burdens, not of an increased triglyceride content of the liver.

**Discussion**

The current investigations were undertaken to determine if agouti expression in the liver of transgenic mice could recapitulate any aspects of the dominant pleiotropic syndrome (i.e., obesity, hyperglycemia, hyperinsulinemia, and/or liver cancer) observed in lethal yellow and viable yellow mice. To address this question, lines of transgenic mice were generated in which the wild-type agouti cDNA was ectopically expressed only in the liver under the regulatory control of the albumin promoter. Different lines of transgenic mice were shown to express the agouti gene in the liver at levels that were similar to or greater than the levels detected in the livers of mice that express agouti ubiquitously and exhibit the pleiotropic syndrome (lethal yellow, viable yellow, and BAPa20 mice).

Although the liver is a key lipogenic tissue in the mouse and a major site of glucose disposal (conversion to glycogen), the finding that body weight, blood glucose and plasma insulin did not differ significantly between the alb-agouti transgenic and control mice demonstrates that agouti expression in the liver alone is insufficient to induce obesity, hyperglycemia or hyperinsulinemia. However, expression of agouti in the livers of the transgenic mice did cause an increase in the susceptibility to DEN-induced

---

**Figure 4**

Growth rates of alb-agouti transgenic mice and wild-type littermate controls. Body weights of alb-agouti 86 (A) and alb-agouti 83 (B) mice were recorded once every four weeks from 4–36 weeks of age and compared to littermate control mice. Shown are the means ± one standard error of the mean (for many of the means, the standard errors are too small to see the error bars). Sample sizes range from 6–22 for each mean. The weights of transgenic and control mice did not differ significantly ($p > 0.05$) at any time.
liver carcino genesis. The total tumor burden in the livers of the exposed mice was estimated by considering both the numbers of tumors visible on the surface of the livers and the total weights of the livers. Thus, whereas the alb-agouti 86 transgenic mice had significantly more liver tumors than control mice at 40, but not 36, weeks after injection, the transgenic livers were significantly heavier than the control livers at both time points (Fig. 5). Taken together, these results indicate that the agouti protein stimulated a significant increase in liver hyperplasia and/or tumor burden of the alb-agouti 86 transgenic mice at the earlier time point (36 weeks) as well.

The present observation that agouti has a primary role in promoting liver carcinogenesis, independent of obesity and diabetes, is in agreement with previous findings on viable yellow mice [44]. Viable yellow mice express the agouti gene in a ubiquitous manner, but exhibit a wide range in the level of expression of the gene and the associated phenotypes. At one end of the spectrum are individuals with very high levels of ubiquitous agouti expression; these mice have completely yellow coats, and they are obese and hyperinsulinemic. At the other end of the spectrum are mice with very low levels of ubiquitous agouti expression; these mice have coat colors that appear almost normal (called pseudoagouti), and they have normal body weights and levels of circulating insulin. Mice in the middle of the spectrum exhibit a moderate level of ubiquitous agouti expression, have coats that are a patchwork of yellow and agouti hairs (i.e., mottled), and are likely to become obese and hyperinsulinemic. Wolff and colleagues [44] fed viable yellow mice and control mice a diet supplemented with lindane (γ-hexachlorocyclohexane) for 24 months, then examined the mice for the development of tumors. They found that both the yellow mice (obese and hyperinsulinemic) and the pseudoagouti mice (lean and normoinsulinemic) had a higher prevalence of chemically initiated liver and lung tumors (i.e., a greater number of mice with tumors) than did control mice. Tumor prevalence, however, was highest in the yellow mice. Thus, a key discovery of these experiments, and of particular relevance to this study, was that a very low level of ubiquitous agouti expression did not cause obesity or hyperinsulinemia, but was sufficient to promote carcinogenesis in some tissues. The higher prevalence of liver tumors in pseudoagouti mice than in control mice suggested that the tumor promoting effect of agouti was a direct consequence of agouti expression in the liver. However, because pseudoagouti mice express the agouti gene in a ubiquitous manner, albeit at a low level, it remained a possibility that this low-level ubiquitous expression of agouti may have elicited some other physiological response that was in turn responsible for the tumor phenotype. The fact that a low level of constitutive agouti expression in the skin of pseudoagouti mice is sufficient to cause a subtle alteration in coat color [60] lends credence to this possibility. The alb-agouti transgenic mice presented here have agouti expressed only in the liver and, although they are lean and normoinsulinemic, are predisposed to an increased number of liver tumors. In fact, the number of tumors per liver in the transgenic mice was correlated with their level of agouti expression in the liver. These results demonstrate that agouti expression in the liver is sufficient to promote liver carcinogenesis, independent of any other apparent agouti-mediated physiological effects.

The molecular mechanism underpinning the role of the agouti protein in promoting liver carcinogenesis is currently unknown. Whether agouti promotes carcinogenesis by antagonizing a melanocortin receptor in the liver, or by acting in a melanocortin-independent manner remains to be determined. In this regard, it is interesting to note that the only melanocortin receptor currently known to be expressed in a widespread manner, including in the liver, is Mc5r [61-63], but agouti protein appears to have little to no effect on antagonizing the binding of αMSH to Mc5r [5,64]. Whether agouti antagonizes the interaction of a different ligand with the Mc5r is not known. The data presented here set the stage for future studies aimed at elucidating the mechanism of action of the agouti gene in the promotion of hepatocellular hyperplasia and neoplasia.

**Conclusions**

In summary, we have demonstrated that liver-specific expression of the agouti gene in transgenic mice was insuf-

---

### Table 1: Circulating glucose and insulin concentrations in transgenic and control mice

| Line                | Blood glucose (mg/dl) | Plasma insulin (µU/ml) |
|---------------------|-----------------------|------------------------|
|                     | controls              | transgenics            | controls | transgenics |
| alb-agouti 83       | 116 ± 8 (5)           | 115 ± 7 (5)            | 32 ± 3 (10) | 28 ± 3 (6) |
| alb-agouti 86       | 115 ± 12 (5)          | 112 ± 7 (5)            | 26 ± 3 (6)  | 32 ± 3 (11) |
| BAPa20              | 122 ± 12 (5)          | 241 ± 37 (8)*          | 31 ± 6 (5)  | 197 ± 33 (9)* |

aData are presented as the mean ± one standard error of the mean. Numbers in parentheses indicate sample sizes. Asterisks denote significant differences between transgenic and control mice (p < 0.05).
sufficient to alter body weight, blood glucose level, or plasma insulin level, but it did promote DEN-initiated liver carcinogenesis. Because alb-agouti transgenic mice developed more liver tumors than did the control mice, the expression of the agouti gene in the liver alone, in the absence of obesity and diabetes, is sufficient to promote the development of liver tumors in mice. These data suggest that the increased susceptibility of lethal yellow and viable yellow mice to carcinogenesis in a variety of tissues is mediated, at least in part, by the tumor promoting effect of agouti expression in the target tissue, rather than being just a secondary consequence of the obesity-related factors.

Methods
Agouti expression construct
An agouti expression construct (plasmid AlbPE-a) containing the murine albumin promoter and enhancer, the wild-type murine agouti cDNA, and the simian virus 40 (SV40) polyadenylation sequences was generated as follows. A Clal fragment containing the agouti cDNA and SV40 polyadenylation sequences was isolated from plasmid clone BAPa [57]. The Clal ends were filled in with Klenow and SalI linkers were ligated to the fragment [65]. The SalI fragment was then cloned into the SalI site of plasmid NB0.3 alb [56], downstream of the albumin promoter and enhancer to generate the plasmid AlbPE-a. This expression construct was verified by DNA sequencing. The AlbPE-a plasmid was digested with SacI and KpnI to excise the 3.5-kb expression cassette from vector sequences for microinjection into fertilized mouse eggs.

Transgenic mice
The pronuclei of fertilized eggs from a random-bred closed-colony stock of FVB/N mice were microinjected with the AlbPE-a expression cassette, along with a tyrosinase minigene expression cassette [66] (at a total DNA concentration of 3 ng/µl in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5), to generate lines of transgenic mice as described [67].

The tyrosinase expression cassette [66] produces pigmentation in the hair of albino FVB/N mice. Cointegration of the AlbPE-a and tyrosinase expression cassettes permits visual identification of the alb-agouti transgenic mice by their coat color. Genomic DNA was obtained by tail biopsy and all mice were genotyped for inheritance of the AlbPE-a transgene by probing Southern blots of BamHI-digested DNA with an agouti cDNA probe, as described [2]. Transgenic founder mice were mated to wild-type FVB/N mice to establish independent transgenic lines, and mice were maintained hemizygous for the transgene by mating transgene carriers to FVB/N mice and genotyping the offspring by Southern blot analysis. For those lines in which the AlbPE-a transgene was shown to cosegregate with coat color, the mice were thereafter genotyped by coat color with confirmation by Southern blotting a few mice at each generation. All experiments involving mice in this study were conducted under approved Institutional Animal Care and Use Committee protocols.
Northern blot analysis
Isolation of total RNA and poly (A)+ RNA, preparation of Northern blots, radiolabeling of hybridization probes, and hybridization conditions were as described [68]. In order to quantify levels of agouti expression in the livers of various strains of mice (Fig. 2), a Northern blot containing ~2.5 µg of poly (A)+ liver RNA per lane was first hybridized with an agouti cDNA probe. The hybridization signals were detected with a FUJIX BAS 1000 phosphorimager and quantified with MacBAS software (Fuji Medical Systems). The blot was then stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (Gapd) probe and the Gapd transcript levels were quantified as described above to control for the amount of RNA loaded for each sample. For each mRNA sample, the level of agouti expression was reported as the ratio of agouti over Gapd mRNA signals. The level of agouti mRNA expression in lethal yellow mice was assigned the value of 1.0, and the expression levels in viable yellow mice and transgenic mice were normalized relative to this value.

Assay for functional agouti protein
Non-transgenic control mice and transgenic mice were euthanized by cervical dislocation and the livers were quickly excised, washed in ice-cold phosphate buffered saline, pH 7.4 (PBS), homogenized, and stored at -80°C. Three livers from each genotype were pooled prior to homogenization. Liver homogenates were enriched for agouti protein as follows. Liver homogenates (2 ml of 20 mg/ml protein) from non-transgenic control mice (negative control), from non-transgenic control mice spiked with 33 nM recombinant agouti protein (positive control), and from alb-agouti 86 mice, each containing 10 mM phosphoramidon, were incubated on a Nutator (4°C for 1 hr) with 2 ml of S Sepharose cation exchange resin equilibrated in PBS. The resin was washed with 0.5 M NaCl in 20 mM HEPES, pH 7.5 and eluted with 1.0 M NaCl in 20 mM HEPES, pH 7.5. The eluants were desalted on a PD-10 column (Pharmacia) equilibrated in PBS.

The presence of functional agouti protein in the livers of the transgenic mice was assayed by measuring the ability of enriched liver homogenates to inhibit the binding of α-MSH to the Mc1r. Murine melanoma B16F10 cells were cultured and used in the [125I]-NDP-α-MSH binding assay as previously described [58,59]. B16F10 cells were incubated for 2 hr at room temperature with 0.1 nM [125I]-NDP-α-MSH plus increasing amounts of each of the enriched samples described above. Cells were washed twice with ice-cold PBS to remove free ligand before the addition of 125 µl of scintillation cocktail. Bound radioactive ligand was measured using a Wallac 1650 Microbeta counter.

Body weight, plasma insulin, and blood glucose analyses
Transgenic and control mice were fed a diet containing 11% fat by weight (Rodent laboratory diet 5015, PMI Feeds), weaned at three weeks of age, and weighed every four weeks from 4–36 weeks of age. Blood samples were collected by retro-orbital sinus puncture from anesthetized, nonfasted, 40–50 week old male mice between 9–11 a.m. Plasma insulin levels were measured in duplicate by radioimmunoassay according to the manufacturer’s recommendations (ICN Biomedicals) with porcine insulin as a standard. Glucose concentrations were measured with the One-Touch glucose determination system (Johnson & Johnson).

Mice used in the carcinogenesis studies were fed a diet containing 4.5% fat by weight (Rodent laboratory diet 5001, PMI Feeds), weaned at three weeks of age, and weighed every two weeks from 4–42 weeks of age.

Liver tumor analysis
Diethylnitrosamine (DEN) was purchased from Sigma Chemical Co. A single intraperitoneal injection of DEN (10 mg/kg) was administered to fifteen-day-old transgenic and control male mice. Groups of animals were weighed, euthanized by cervical dislocation at 36 or 40 weeks after injection, and necropsied. The livers were removed, weighed, and examined for visible lesions, which were counted and measured (diameter).

Statistical analyses
All statistical analyses were performed with the JMP computer software package (SAS Institute Inc.).

List of abbreviations
alb, albumin promoter; alb-agouti mice, transgenic mice expressing the wild-type agouti cDNA in the liver under the regulatory control of the albumin promoter; α-MSH, α-melanocyte stimulating hormone; A9, viable yellow allele of the agouti gene; A5, lethal yellow allele of the agouti gene; DEN, diethylnitrosamine; Gapd, glyceraldehyde-3-phosphate dehydrogenase; Mc1r, melanocortin 1 receptor; Mc4r, melanocortin 4 receptor; Pparg, peroxisome proliferator activated receptor alpha; Stat1, signal transducer and activator of transcription 1; Stat3, signal transducer and activator of transcription 3; SV40, simian virus 40.

Authors’ contributions
All authors participated in the study design. MLK and RPW generated the transgene construct and the transgenic mice. AIK, MLK, and RLM performed the Northern blot analyses. RLM, LLK, and WOW performed the assay for functional agouti protein. MLK and RLM weighed mice on the 11%-fat-by-weight diet. RLM performed the plasma insulin and blood glucose measurements. AIK and EJM...
weighed mice on the 4%-fat-by-weight diet and performed the tumorigenesis studies. EJM drafted the manuscript, and all authors provided comments, critique and suggestions for its improvement. All authors read and approved the final manuscript.

Acknowledgements

We thank Dr. Frederick F. Becker for valuable discussions on DEN-induced liver carcinogenesis experiments. We thank Drs. D. K. Johnson, E. M. Rinchik, L. B. Russell, and Y. Wang for critically reading the manuscript. Funding to generate and characterize the lines of transgenic mice was provided by Glaxo Wellcome (to RPW). Additional funding to perform the tumorigenesis experiments was provided by the Office of Health and Environmental Research, U.S. Department of Energy, under contract DE-AC05-960R22464 with Lockheed Martin Energy Systems, Inc. (to RPW); and by the Office of Biological and Environmental Research, U.S. Department of Energy, under contract DE-AC03-00OR22725 with UT-Battelle, LLC (to EJM).

References

1. Slivers WK: The agouti and extension series of alleles, umbrous, and sable. In The Coat Colors of Mice: A Model for Mammalian Gene Action and Interaction New York: Springer-Verlag, 1979:6-44.

2. Bultman SJ, Michaud EJ, Woychik RP: Molecular characterization of the mouse agouti locus. Cell 1992, 71:1195-1204.

3. Miller MW, Duhl DMJ, Vrieling H, Cordes SP, Ollmann MM, Winkels BM, Barsh GS: Cloning of the mouse agouti gene predicts a secreted protein ubiquitously expressed in mice carrying the lethal yellow mutation. Genes Dev 1993, 7:454-467.

4. Matsunaga N, Virador V, Santis C, Vieira WD, Furumura M, Matsunaga N, Kobayashi N, Hearing VJ: In situ localization of agouti signal protein in murine skin using immunohistochemistry with an ASP-specific antibody. Biochem Biophys Res Commun 2000, 270:186-172.

5. Lu D, Willard D, Patel IR, Kadhswell S, Overtol L, Kost T, Luther M, Chen W, Woychik RP, Wilkison WO, Cone RD: Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. Nature 1994, 371:799-802.

6. Blanchard SG, Harris CO, Itoopo OR, Nichols JS, Parks DJ, Truesdale AT, Wilkison WO: Agouti antagonism of melanocortin binding and action in the B16F10 murine melanoma cell line. Biochemnistry 1995, 34:10406-10411.

7. Siegrist W, Drozdz R, Coti R, Willard DH, Wilkison WO, Eberle AN: Interactions of alpha-melanotropin and agouti on B16 melanoma cells: evidence for inverse agonism of agouti. J Recept Signal Transduct Res 1997, 17:75-90.

8. Ollmann MM, Lamoreux ML, Wilson BD, Barsh GS: Interaction of Agouti protein with the melanocortin 1 receptor in vitro and in vivo. Genes Dev 1998, 12:316-330.

9. Eberle AN, Bodj I, Oroz G, Suli-Vargha H, Jaggv V, Zumsneg U: Agouti: agonist and agonist activities of the mouse agouti protein. Fragment (91-131) at the melanocortin-1 receptor. J Recept Signal Transduct Res 2001, 21:25-45.

10. Adel-Palek ZA, Scott MC, Furumura M, Lamoreux ML, Ollmann M, Barsh GS, Hearing VJ: The melanocortin 1 receptor is the principal mediator of the effects of agouti signaling protein on mammalian melanocytes. J Cell Sci 2001, 114:1019-1024.

11. Husted CM, Perry WL, Siracusa LD, Raspberry C, Cobb L, Cattanach BM, Kovaltchuk R, Copeland NG, Jenkins NA: Molecular genetic characterization of six recessive viable alleles of the mouse agouti locus. Genetics 1995, 140:255-265.

12. Miltenberger R, Wakamatsu K, Ito S, Woychik RP, Russell LB, Michaud EJ: Molecular and phenotypic analysis of 25 recessive, homozygous-viable alleles at the mouse agouti locus. Genetics 2002, 160:659-674.

13. Wolff GL, Roberts DW, Galbraith DB: Prenatal determination of obesity, tumor susceptibility, and coat color pattern in viable yellow (Avy) mice. The yellow mouse syndrome. J Hered 1986, 77:151-159.

14. Wolff GL: Body weight and cancer. Am J Clin Nutr 1987, 45:168-180.

15. Jackson IJ: Colour-coded switches. Nature 1993, 362:587-588.

16. Yen TT, Gill AM, Frigeri LG, Barsh GS: Wolff GL: Obesity, diabetes, and neoplasia in yellow Ay/- mice: ectopic expression of the agouti gene. FASEB J 1994, 8:479-480.

17. Perry WL, Copeland NG, Jenkins NA: The molecular basis for dominant yellow agouti coat color mutations. Bioessays 1994, 16:705-707.

18. Delesalle J, Argenso AC, Siracusa LD: Mechanisms for the pleiotropic effects of the agouti gene. Proc Natl Acad Sci USA 1995, 92:4721-4724.

19. Kleiberg ML, Wilkinson JE, Woychik RP: Molecular analysis of the mouse agouti gene and the role of dominant agouti- locus mutations in obesity and insulin resistance. In Molecular and Genetic Aspects of Obesity. Pennington Center Nutrition Series Volume 5. 1st edition. Edited by: Bray GA, Ryan DH. Baton Rouge: Louisiana State University Press; 1996:120-160.

20. Michaud EJ, Mynatt RL, Miltenberger R, Kleiberg ML, Wilkinson JE, Zemel MB, Woychik RP: Role of the agouti gene in obesity. J Endocrinol 1997, 155:207-209.

21. Wolff GL, Roberts DW, Mountjoy KG: Physiological consequences of ectopic agouti gene expression: the yellow obese mouse syndrome. Physiol Genomics 1999, 1:151-163.

22. Wolff GL: Regulation of agouti expression in vivo: a historical perspective. Pigment Cell Res 2003, 16:2-15.

23. Michaud EJ, Bultman SJ, Stubbs LJ, Woychik RP: The embryonic lethality of homozygous lethal yellow mice (Ay/Ay) is associated with the disruption of a novel RNA-binding protein. Genes Dev 1993, 7:1201-1213.

24. Michaud EJ, Bultman SJ, Kleiberg ML, van Vught MJ, Stubbs LJ, Russell LB, Woychik RP: A molecular model for the genetic and phenotypic characteristics of the mouse yellow (Ay) mutation. Proc Natl Acad Sci USA 1994, 91:2562-2566.

25. Duhl DMJ, Stevens ME, Vrieling H, Saxton PJ, Miller MW, Epstein CJ, Barsh GS: Pleiotropic effects of the mouse yellow (Ay) mutation explained by deletion of a maternaly expressed gene and the simultaneous production of agouti fusion proteins. Development 1994, 120:465-470.

26. Duhl DM, Vrieling H, Miller KA, Wolff GL, Barsh GS: Neomorphic agouti mutations in obese yellow mice. Nat Genet 1994, 8:59-65.

27. Kim JH, Itoopo OR, Bunke K, Truesdale AT, Willard DH, Nichols JS, Blanchard SG, Mynatt RL, Woychik RP, Michaud EJ, Wilkison WO, Moustaid N: Agouti regulation of melanocortin action. J Physiol 1997, 527:379-384.

28. Yang YK, Ollmann MM, Wilson BD, Dickinson C, Yamada T, Barsh GS, Gantz I: Effects of recombinant agouti-signaling protein on the melanocortin action. Mol Endocrinol 1997, 11:274-280.

29. Mountjoy KG, Willard DH, Wilkison WO: Agouti antagonism of melanocortin-4 receptor: greater effect with desacetyl-alpha-melanocyte-stimulating hormone (MSH) than with alpha-MSH. Endocrinology 1999, 140:2167-2172.

30. Mountjoy KG, Kong PL, Taylor JA, Willard DH, Wilkison WO: Melanocortin receptor-mediated mobilization of intracellular free calcium in HEK293 cells. Physiol Genomics 2001, 5:11-19.

31. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkeimer LR, Gu W, Kesterson RA, Boston BA, Cone RD, Smith FJ, Campfield LA, Bunting F: Agouti regulation of the melanocortin-4 receptor results in obesity in mice. Cell 1997, 88:131-141.

32. Yeo GS, Farooqi IS, Aminian S, Halsall DJ, Stanhope RG, R’Orrilly S: A frameshift mutation in MC4R associated with dominantly inherited human obesity. Nat Genet 1998, 20:111-112.

33. Vaisse C, Clement K, Guy-Grand B, Froguel P: A frameshift mutation in human MC4R is associated with a dominant form of obesity. Nat Genet 1998, 20:113-114.

34. Butler AA, Cone RD: The melanocortin receptors: lessons from knockout models. Neuropeptides 2002, 36:77-84.

35. Mynatt RL, Stevens JM: Agouti regulates adipocyte transcription factors. Am J Physiol Cell Physiol 2001, 280:C954-C961.

36. Jones BH, Kim JH, Zemel MB, Woychik RP, Michaud EJ, Wilkison WO, Moustaid N: Upregulation of adipocyte metabolism by
agouti protein: possible paracrine actions in yellow mouse obesity. Am J Physiol 1996, 270:E192-E196.
38. Heston WE, Vlahakis G: Influence of the A\(^\prime\) gene on mammary-gland tumors, hepatomas, and normal growth in mice. J Natl Cancer Inst 1968, 40:1161-1166.
39. Wolff GL: Differential growth of hepatoma-susceptible liver induced by genome × genome interaction. Cancer Res 1970, 30:1722-1725.
40. Wolff GL, Pitot HC: Variation of hepatic malic enzyme capacity with hepatoma susceptibility in mice of different genotypes. Cancer Res 1972, 32:1861-1863.
41. Wolff GL, Morrissey RL, Chen JJ: Amplified response to pheno-barbital promotion of hepatotumorgenesis in obese yellow A\(^\prime\)A (C3H × VY) F-1 hybrid mice. Carcinogenesis 1986, 7:1855-1859.
42. Hansen LA, Malarkey DE, Wilkinson JE, Rosenberg M, Woychik RP, Tenen DM: Effect of the viable-yellow (A\(^\prime\)) agouti allele on skin tumorigenesis and humoral hypercalcemia in v-H-ras transgenic TG.AC mice. Carcinogenesis 1998, 19:1837-1845.
43. Heston WE: Relationship between the lethal yellow (A\(^\prime\)) gene of the mouse and susceptibility to induced pulmonary tumors. J Natl Cancer Inst 1947, 7:463-465.
44. Hansen LA, Malarkey DE, Wilkinson JE, Rosenberg M, Woychik RP, Tenen DM: Effect of the viable-yellow (A\(^\prime\)) agouti allele on skin tumorigenesis and humoral hypercalcemia in v-H-ras transgenic TG.AC mice. Carcinogenesis 1998, 19:1837-1845.
45. Heston WE, Heron MG: Relationship between the lethal yellow (A\(^\prime\)) gene of the mouse and susceptibility to induced pulmonary tumors. J Natl Cancer Inst 1947, 7:463-465.
46. Heston WE, Heron MG: Relationship between the lethal yellow (A\(^\prime\)) gene of the mouse and susceptibility to induced pulmonary tumors. J Natl Cancer Inst 1947, 7:463-465.
47. Heston WE: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
48. Heston WE, Smith GH: Strain C3H-A-vv/fb mice: ninety percent incidence of mammary tumors transmitted by either parent. Science 1970, 170:185-187.
49. Heston WE, Vlahakis G: Elimination of the effect of the A\(^\prime\) gene on pulmonary tumors in mice by alteration of its effect on normal growth. J Natl Cancer Inst 1961, 27:1189-1196.
50. Heston WE, Vlahakis G: Elimination of the effect of the A\(^\prime\) gene on pulmonary tumors in mice by alteration of its effect on normal growth. J Natl Cancer Inst 1961, 27:1189-1196.
51. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
52. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
53. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
54. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
55. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
56. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
57. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
58. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
59. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
60. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
61. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
62. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
63. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
64. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
65. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
66. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
67. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
68. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
69. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
70. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
71. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
72. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.
73. Little CC: The relationship of coat color to the spontaneous incidence of mammary tumors in mice. J Exper Med 1934, 59:229-250.