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# MGRN1-dependent pigment-type switching requires its ubiquitination activity but not its interaction with TSG101 or NEDD4

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## Summary

Mice lacking the E3 ubiquitin ligase mahogunin ring finger-1 (MGRN1) have a pleiotropic phenotype that includes spongiform neurodegeneration, embryonic patterning defects, and dark fur due to a defect in pigment-type switching. The only MGRN1 ubiquitination target identified to date is tumor susceptibility gene 101 (TSG101), a component of the endosomal trafficking machinery. Here, we show that MGRN1 also interacts with but does not ubiquitinate NEDD4, a HECT-domain ubiquitin ligase involved in endosomal trafficking. Using transgenesis in mice, we demonstrate that pigment-type switching likely requires MGRN1's ubiquitin ligase activity but not its ability to bind TSG101 or NEDD4. This indicates that MGRN1-dependent ubiquitination of an as-yet unidentified target protein is required for agouti-mediated melanocortin signaling.

### Keywords

Mahogunin Ring Finger-1 (MGRN1); pigment-type switching; agouti; melanocortin signaling; TSG101; NEDD4

Melanocortin receptors are a family of five G-protein coupled receptors that regulate diverse physiological processes (Eves and Haycock, 2010). Signaling through the melanocortin 1 receptor (MC1R) is required for the production of black/brown pigment (eumelanin). In most mammals, transient expression of the MC1R inverse agonist, agouti signaling protein (ASIP), triggers a switch in pigment production from eumelanin to yellow/red pheomelanin. ASIP signaling requires the type-I transmembrane protein attractin (ATRN) and the RING-domain E3 ubiquitin ligase mahogunin ring finger-1 (MGRN1) (reviewed by Walker and Gunn, 2010). ATRN facilitates ASIP binding to MC1R (He et al., 2001), while the role of MGRN1 remains unclear. MGRN1 has been reported to associate with MC1R, MC2R and MC4R. Although MC1R itself does not appear to be ubiquitinated, at least in a heterologous cell system (Perez-Oliva et al., 2009), two studies suggest MGRN1 may regulate melanocortin signaling by ubiquitinating a protein that binds melanocortin receptors

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(Cooray et al., 2011; Perez-Oliva et al., 2009). Alternatively, MGRN1 may regulate melanocortin signaling indirectly through an effect on endosomal trafficking. MGRN1 contains a “late viral domain” motif of the P[S/T]AP family (PSAP at amino acids 384-387 in isoform I) that mediates its association with its only identified ubiquitination target to date, TSG101 (Jiao et al., 2009b; Kim et al., 2007). TSG101 is a component of the endosomal complex required for transport-I (ESCRT-I) that mediates sorting of ubiquitinated transmembrane proteins into multivesicular bodies, a key step in their trafficking to the lysosome for degradation. MGRN1-mediated ubiquitination of TSG101 regulates its ESCRT function, with loss of MGRN1 disrupting TSG101-dependent trafficking. This has led to speculation that loss of MGRN1 disrupts the trafficking of activated melanocortin receptors, leading to prolonged signaling and/or increased recycling to the plasma membrane (Overton and Leibel, 2011).

MGRN1 also contains a PPXY “late viral domain” (PPGY at amino acids 361-364 of isoform I). As PPXY motifs interact with WW domains in NEDD4-family ubiquitin ligases (Ingham et al., 2005), we tested whether MGRN1 interacts with ITCH or NEDD4 using co-immunoprecipitation (co-IP) of lysates from transiently transfected and untransfected HEK293T cells (see Supplementary Materials for Methods). While GFP-tagged MGRN1 (MGRN1-GFP) did not associate with Myc-tagged ITCH (Figure 1A), it did co-IP with HA-tagged and endogenous NEDD4, as did endogenous MGRN1 (Figure 1B–C and data not shown). To test whether the PPGY motif mediates the ability of MGRN1 to bind NEDD4, co-IP studies were repeated using MGRN1-GFP expression constructs in which both proline residues or both prolines and the tyrosine residue of the PPGY motif were mutated to alanines (MGRN1$^{AAGY}$-GFP and MGRN1$^{AAGA}$-GFP, respectively). Mutating both prolines greatly reduced the association of NEDD4 with MGRN1, while also mutating the tyrosine abolished the interaction (Figure 1D). The cellular distribution of the PPGY mutants did not differ from wild-type MGRN1-GFP in HEK293T cells (Figure 1E–G), and there was no obvious difference in the pattern of NEDD4 expression between wild-type and *Mgrn1* null melanocytes (Figure 1H–I). These data indicate that interaction between MGRN1 and NEDD4 does not determine their subcellular localization.

Both wild-type and catalytically inactive MGRN1 (C278A/C281A mutant, MGRN1$^{AVVA}$-GFP) interacted with NEDD4 (Figure 2A–C). Surprisingly, heating lysates prior to IP did not always disrupt the interaction, especially with MGRN1$^{AVVA}$-GFP (Figure 2B, top panel). As ubiquitination-defective ligases often bind more of (or more tightly to) their targets (Itahana et al., 2007; Jiao et al., 2009b), we investigated whether MGRN1 ubiquitinates NEDD4. In HEK293T cells, NEDD4 was detected as 2-3 bands ~130 kDa (Figure 2B–C). Although the relative abundance of NEDD4 did not change in cells over-expressing MGRN1-GFP or MGRN1$^{AVVA}$-GFP (Figure 2C, second panel), the smallest isoform preferentially immunoprecipitated from cells over-expressing MGRN1, especially MGRN1$^{AVVA}$-GFP (Figure 2B–C). No ubiquitinated NEDD4 was detected (Figure 2B, middle panel, and Figure 2C, two bottom panels) and no obvious changes in NEDD4 levels were observed when proteasomal degradation was inhibited by treating cells with MG132 (Figure 2C). Furthermore, neither the molecular weight nor levels of NEDD4 differed in *Mgrn1* null melanocytes (from *Mgrn1*$^{md\text{-}nc/md\text{-}nc}$ mice) relative to control melanocytes

(Figure 2D). Together, these results indicate that MGRN1 does not ubiquitinate NEDD4 or target it for proteasomal degradation.

The predominant ubiquitinated protein the studies described above was MGRN1-GFP (Figure 2B and C). A single band was observed, not a ladder, and its molecular weight (~105 kDa) was consistent with MGRN1 (~70 kDa) + GFP (~27 kDa) + 1 ubiquitin (~9 kDa). The same band was detected in cells co-transfected with HA-$Ub^{K0}$ (which contains no lysines, preventing it's incorporation into polyubiquitin chains), confirming MGRN1-GFP is mono-ubiquitinated (Figure 2E). This is unlikely to reflect auto-ubiquitination since highly over-expressed catalytically inactive $MGRN1^{AVVA}$-GFP was also modified (Figure 2C and E). Nor does MGRN1-GFP appear to be a direct ubiquitination target of NEDD4 since NEDD4-interaction-deficient $MGRN1^{AAGA}$-GFP was also mono-ubiquitinated (Figure 2E). Over-expression of wild-type or catalytically inactive MGRN1-GFP or HA-NEDD4 did not affect the other protein's molecular weight or levels (Figure 2F), indicating that MGRN1 and NEDD4 do not ubiquitinate or promote the degradation of one another. NEDD4 may target itself for degradation since over-expression of wild-type HA-NEDD4 led to reduced levels of full-length NEDD4 and high levels of smaller, ubiquitinated bands that likely represent NEDD4 degradation products (Figures 2F and S1).

Although the functional importance of the MGRN1-NEDD4 interaction is unclear, the association of MGRN1 with two proteins involved in endosomal trafficking suggested MGRN1 might mediate pigment-production by regulating MC1R down-regulation via endosomal trafficking. We used transgenesis in mice to test whether the interaction of MGRN1 with TSG101 or NEDD4 is required for normal pigment-type switching *in vivo*. As transgenic expression of wild-type *Mgrn1* isoform I under control of the human β-actin promoter rescued all aspects of the $Mgrn1^{md\text{-}nc/md\text{-}nc}$ (null) mutant phenotype (Jiao et al., 2009a), we generated $Mgrn1^{md\text{-}nc/md\text{-}nc}$ mice carrying TSG101-interaction-deficient $Mgrn1^{ASAA}$ and NEDD4-interaction-deficient $Mgrn1^{AAGY}$ and $Mgrn1^{AAGA}$ mutant isoform I transgenes. Although some $Mgrn1^{AAGY}$ bound NEDD4 (Figure 1F), both $Mgrn1^{AAGY}$ and $Mgrn1^{AAGA}$ transgenes were generated because the tyrosine is part of a putative sorting signal and mutating it might disrupt more than the NEDD4 interaction. Two $Mgrn1^{ASAA}$ (TSG101-interaction-deficient) transgene founders were obtained. One line completely rescued the coat color defect of $Mgrn1^{md\text{-}nc/md\text{-}nc}$ mice (Figure 3B and Table 1), while the other only showed partial rescue when homozygous (Figure 3C–D and Table 1). For NEDD4-interaction-deficient transgenes, one $Mgrn1^{AAGA}$ and three $Mgrn1^{AAGY}$ founders were obtained. One $Mgrn1^{AAGY}$ transgene completely rescued the $Mgrn1^{md\text{-}nc/md\text{-}nc}$ pigmentation defect (Figure 3E) (Table 1). Transgenic animals from the "non-rescuing" line were mostly black but typically had small patches of agouti-banded hairs. The third founder did not breed. The $Mgrn1^{AAGA}$ transgene completely rescued the pigmentation phenotype of $Mgrn1^{md\text{-}nc/md\text{-}nc}$ mice (Figure 3F) (Table 1). The putative tyrosine-based sorting signal must not be active and/or essential for MGRN1-dependent pigmentation since $Mgrn1^{AAGY}$ and $Mgrn1^{AAGA}$ transgenes rescued the $Mgrn1^{md\text{-}nc/md\text{-}nc}$ coat color defect. The normal pigmentation phenotype of mice only expressing mutant forms of *Mgrn1* indicates that the role of MGRN1 in pigment-type switching is independent of its interaction with and/or regulation of TSG101 or NEDD4.

MGRN1 has been reported to bind MC1R and decrease it's signaling to cAMP with no effect on receptor ubiquitination, levels, localization or down-regulation (Perez-Oliva et al., 2009). MGRN1 and Gαs competed for MC1R binding, with catalytically inactive MGRN1 displacing Gαs as efficiently as wild-type MGRN1. To test whether MGRN1 ubiquitin ligase activity is required for pigment-type switching, we generated catalytically inactive *Mgrn1*$^{AVVA}$ mutant transgenic mice using the scheme described above. In two independent lines, the coat color of *Mgrn1*$^{md\text{-}nc/md\text{-}nc}$ mice expressing the *Mgrn1*$^{AVVA}$ transgene was indistinguishable from that of non-transgenic *Mgrn1*$^{md\text{-}nc/md\text{-}nc}$ mice (Figure 3G–H and Table 1). Quantitative RT-PCR was performed to compare *Mgrn1* expression in the skin of mice expressing only catalytically inactive *Mgrn1* to *Mgrn1*$^{md\text{-}nc/+}$ mice (which have normal, agouti coat color) and *Mgrn1*$^{md\text{-}nc/md\text{-}nc}$ mice carrying the TSG101-interation deficient *Mgrn1*$^{ASAA}$ transgene that rescued the *Mgrn1* mutant pigmentation defect. Although skin *Mgrn1* expression was not as high in the *Mgrn1*$^{AVVA}$ transgenic lines as in the *Mgrn1*$^{ASAA}$ line, it was significantly higher than in *Mgrn1*$^{md\text{-}nc/+}$ animals (Table 2). This suggests that failure of the *Mgrn1*$^{AVVA}$ transgenes to rescue the pigmentation defect reflects a requirement for MGRN1 ubiquitin ligase activity in pigment-type switching rather than insufficient expression of the transgene.

Failure of catalytically inactive MGRN1 to confer normal pigment-type switching indicates that displacement of Gαs from MC1R by MGRN1 is not sufficient for normal pigment production. *Mgrn1* null mutant mice expressing a wild-type *Mgrn1* transgene under control of a melanocyte-specific promoter had agouti-banded hairs (He, 2003), suggesting MGRN1-dependent ubiquitination of an as-yet unknown target in melanocytes is required for normal pigment-type switching. It is tempting to speculate that it is the same ubiquitinated protein that co-precipitates with MC1R from HEK293T cells (Perez-Oliva et al., 2009). Identifying this protein will provide new insights into the regulation of melanocortin signaling and pigment-type switching.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Significance

Melanocortin receptors regulate numerous important physiological functions, including pigmentation, body weight, sexual behavior and exocrine function. It has generally been assumed that MGRN1 mediates the trafficking and/or lysosomal degradation of melanocortin receptors via it's only identified ubiquitination target to date, TSG101, but the fact that MGRN1 associates with MC1R, MC2R and MC4R suggests it may have a direct effect on these receptors and regulate their signaling independent of TSG101-mediated trafficking. Our data show that the ubiquitin ligase activity of MGRN1, but not its association with TSG101 or NEDD4, is required for normal pigment-type switching.

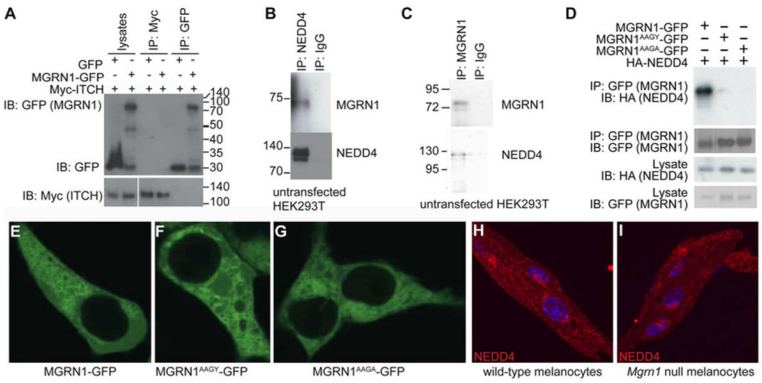


**Figure 1.**

Interaction between MGRN1 and NEDD4-family proteins. (A) MGRN1 does not interact with ITCH. Lysates from HEK293T cells transfected with GFP or MGRN1-GFP and Myc-ITCH plasmids were subjected to IP using antibodies against Myc or GFP. Immunoblotting (IB) indicated no association of Myc-ITCH with GFP or MGRN1-GFP. (B–C) MGRN1 interacts with NEDD4, as demonstrated by IP of endogenous NEDD4 (C) or MGRN1 (D) from untransfected HEK293T cells followed by IB for each protein. (D) The PPGY motif in MGRN1 mediates its association with NEDD4. Lysates from HEK293T cells transfected with indicated plasmids were subjected to IP for GFP. IB for HA (NEDD4) demonstrated reduced association of NEDD4 with MGRN1$^{AAGY}$-GFP and no association with MGRN1$^{AAGA}$-GFP. (E–I) Interaction between MGRN1 and NEDD4 does not regulate their cellular localization. HEK293T cells expressing wild-type (E) or NEDD4-interaction-deficient (F–G) MGRN1-GFP imaged by confocal microscopy. In all cases, the GFP signal was predominantly observed on cytoplasmic membranes. Immunofluorescence for endogenous NEDD4 revealed a similar pattern of staining in wild-type (H) and *Mgrn1*$^{md\text{-}nc/md\text{-}nc}$ (I) melanocytes: in addition to a strong perinuclear signal, NEDD4 (red) was detected on the cell surface and cytoplasmic membranes. DAPI-stained nuclei are shown in blue.

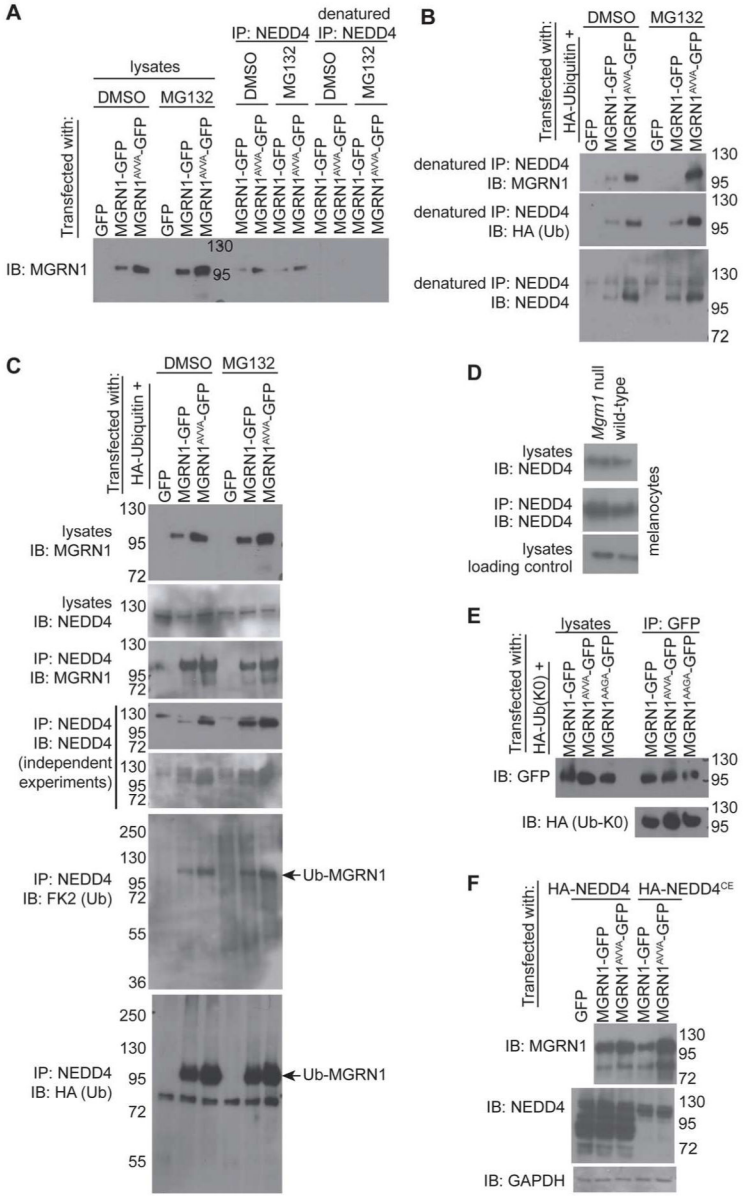


**Figure 2.**

MGRN1 and NEDD4 do not regulate one another. (A–C) HEK293T cells expressing indicated plasmids were treated with 10 μM MG132 (proteasome inhibitor) or DMSO (control), lysed, and subjected to IP for NEDD4 then IB as indicated. (A–B) NEDD4 associated with wild-type and catalytically inactive (AVVA mutant) MGRN1-GFP (A), sometimes even following heat denaturation (B). Following IP for NEDD4, IB for ubiquitinated proteins detected MGRN1-GFP but not NEDD4 (B–C). (D) *Mgrn1* null mutant melanocytes express normal levels of NEDD4. (E) NEDD4 does not directly mono-ubiquitinate MGRN1-GFP. Lysates from HEK293T cells expressing indicated plasmids were heat denatured and subjected to IP for GFP. IB for GFP (MGRN1) and HA ($Ub^{K0}$) detected the same band, even in samples expressing NEDD4-interaction-deficient $MGRN1^{AAGA}$-GFP. (F) MGRN1 and NEDD4 do not target one another for degradation.

Over-expression of wild-type or catalytically inactive MGRN1-GFP did not affect NEDD4 molecular weight or levels, nor did over-expression of wild-type or catalytically inactive (C744E mutant) HA-NEDD4 affect MGRN1-GFP expression.

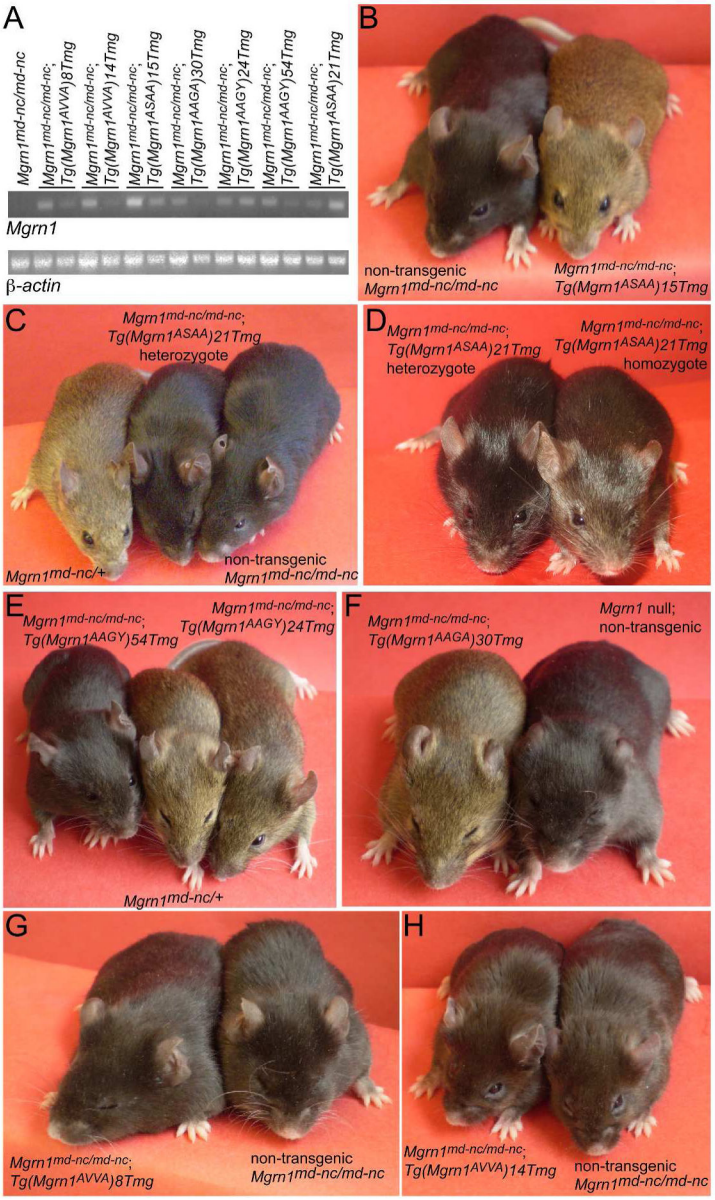


**Figure 3.**

Normal pigment-type switching requires MGRN1's ubiquitin ligase activity but not its association with TSG101 or NEDD4. (A) RT-PCR products demonstrating expression of mutant *Mgrn1* transgenes in skin. (B) TSG101-interaction-deficient $Mgrn1^{ASAA}$ transgenic line 15 showed rescue of the $Mgrn1^{md\text{-}nc/md\text{-}nc}$ coat color defect. (C–D) The coat color of $Mgrn1^{md\text{-}nc/md\text{-}nc}$ mice heterozygous for $Mgrn1^{ASAA}$ line 21 transgene was similar to non-transgenic *Mgrn1* mutants (C), but presumed transgene homozygotes produced some pheomelanin and were dark agouti (D). (E–F) $Mgrn1^{md\text{-}nc/md\text{-}nc}$ mice carrying NEDD4-interaction-deficient $Mgrn1^{AAGY}$ (E) and $Mgrn1^{AAGA}$ (F) transgenes had either normal agouti coat color ($Mgrn1^{AAGY}$ line 24 and $Mgrn1^{AAGY}$ line 30) or were mostly black with small patches of agouti hairs ($Mgrn1^{AAGY}$ line 54; note agouti hairs on face of animal shown). (G–H) The coat color of mice expressing only catalytically inactive *Mgrn1*

(*Mgrn1*$^{AVVA}$ transgenic *Mgrn1*$^{md\text{-}nc/md\text{-}nc}$ mutants) was indistinguishable from that of non-transgenic *Mgrn1*$^{md\text{-}nc/md\text{-}nc}$ mice in two independent lines. The pigmentation of *Mgrn1*$^{md\text{-}nc/+}$ mice carrying the transgene was normal (not shown).

## Table 1

Coat color effect of mutant *Mgrn1* transgenes.

| Genotype | # mice observed[a] | # agouti | # black |
|---|---|---|---|
| *Tg(Mgrn1$^{ASAA}$)15; Mgrn1$^{md\text{-}nc/md\text{-}nc}$* | >50 | >50 | 0 |
| *Tg(Mgrn1$^{ASAA}$)21; Mgrn1$^{md\text{-}nc/md\text{-}nc}$* | 45 | 0 | 45[b] |
| *Tg(Mgrn1$^{AAGY}$)24; Mgrn1$^{md\text{-}nc/md\text{-}nc}$* | >30 | >30 | 0 |
| *Tg(Mgrn1$^{AAGY}$)54; Mgrn1$^{md\text{-}nc/md\text{-}nc}$* | 46 | 0 | 46[c] |
| *Tg(Mgrn1$^{AAGA}$)30; Mgrn1$^{md\text{-}nc/md\text{-}nc}$* | >35 | >35 | 0 |
| *Tg(Mgrn1$^{AVVA}$)8; Mgrn1$^{md\text{-}nc/md\text{-}nc}$* | 55 | 0 | 55 |
| *Tg(Mgrn1$^{AVVA}$)14; Mgrn1$^{md\text{-}nc/md\text{-}nc}$* | 41 | 0 | 41 |

[a] For transgenes that rescued coat color, pups from matings where both parents were *Mgrn1$^{md\text{-}nc/md\text{-}nc}$* were genotyped by phenotype (agouti vs. black); # mice genotyped by PCR is provided, preceded by > to indicate additional (ungenotyped) transgenic mice were observed.

[b] Most matings between two ASAA line 21 transgene positive parents produced pups with dark agouti fur (presumed to be homozygous for the transgene), as shown in Figure 3D. Pups from one such mating also had dilute coat color.

[c] Most of these mice had small patches of agouti-banded hairs.

## Table 2

Relative *Mgrn1* expression in *Mgrn1*$^{AVVA}$ transgenic mice.

| Genotype | *Mgrn1* relative quantification value[a] (range) |
|---|---|
| *Mgrn1*$^{md\text{-}nc/+}$ | 1.0 (0.5 – 2.1) |
| *Tg(Mgrn1*$^{ASAA}$*)15; Mgrn1*$^{md\text{-}nc/md\text{-}nc}$ | 35.2 (33.4 – 37.2)[b] |
| *Tg(Mgrn1*$^{AVVA}$*)8; Mgrn1*$^{md\text{-}nc/md\text{-}nc}$ | 13.9 (10.8 – 17.8)[b] |
| *Tg(Mgrn1*$^{AVVA}$*)14; Mgrn1*$^{md\text{-}nc/md\text{-}nc}$ | 13.1 (7.6 – 22.8)[b] |

[a] *Mgrn1* expression normalized against *Gpi*, with *Mgrn1* used as baseline.

[b] Significantly higher expression than *Mgrn1*$^{md\text{-}nc/+}$ ($p < 0.03$).