**Nicastrin Deficiency Induces Tyrosinase-Dependent Depigmentation and Skin Inflammation**

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Skin depigmentation diseases, such as vitiligo, are pigmentation disorders that often destroy melanocytes. However, their pathological mechanisms remain unclear, and therefore, promising treatments or prevention has been lacking. Here, we demonstrate that a zebrafish insertion mutant showing a significant reduction of *nicastrin* transcript possesses melanosome maturation defect, Tyrosinase-dependent mitochondrial swelling, and melanophore cell death. The depigmentation phenotypes are proven to be a result of γ-secretase inactivation. Furthermore, live imaging demonstrates that macrophages are recruited to and can phagocytose melanophore debris. Thus, we characterize a potential zebrafish depigmentation disease model, a *nicastrin*mutant, which can be used for further treatment or drug development of diseases related to skin depigmentation and/or inflammation.

**INTRODUCTION**

The variability among human traits, such as skin color, is a popular research topic. Differences in the melanin-producing activity of melanocytes, which are pigment cells located near the basement membrane of skin (Drochmans, 1960), not only affect coloring (Lerner and McGuire, 1961), but also contribute to UV resistance and cancer risk (Cesarini, 1988). Compared with skin-color variation or albinism, acquired hypopigmentation, depigmentation, vitiligo, or pigment loss is a cell-death-mediated melanocyte loss (Lerner and Nordlund, 1978; Picardo et al., 2015) accompanied by immune cell infiltration, such as macrophages (Le Poole et al., 1996). The abnormal pigment loss can lead to white macules, patches on skin, gray hair, or even retinal pigment epithelium (RPE) hypopigmentation, RPE degeneration, and visual impairment (Albert et al., 1983; Sparrow et al., 2010). Although the disease has a prevalence around 0.5–2% of the world population, its pathomechanism remains elusive (Picardo et al., 2015).

Several genes, including *TYR* and genes related to autoimmune diseases, such as *IL2RA* and *MHC*, were found to be associated with the occurrence of vitiligo in previous genome-wide association studies (Jin et al., 2010; Jin et al., 2012). Consistently, macrophages were observed in inflammatory vitiligo sites accompanied by melanocyte removal (Le Poole et al., 1996). Furthermore, defects in mitochondrial genomic content (Karvonen et al., 1999) and environmental risk factors, such as hydroquinone monobenzyl ether (Hariharan et al., 2010), were shown to increase the prevalence of vitiligo and to induce depigmentation, respectively. Adding to the complexity of the situation, the treatment of γ-secretase inhibitor was reported to induce hair-color changes in a previous clinical trial (Fleisher et al., 2008) and pigment loss in animal models (Hamada et al., 2014; Kumano et al., 2008; Moriyama et al., 2006); however, a clear pathological investigation has not been reported.

γ-secretase is a multiprotein complex formed by Presenilin, Pen2, Aph1 and Nicastrin (Chen et al., 2003; Semeels et al., 2005). The deficiency of Nicastrin has been shown to compromise the transportation and stability of γ-secretase (Zhang et al., 2005), and therefore diminish the activity of γ-secretase. Nonetheless, there are controversial studies showing that γ-secretase substrates could still be processed without Nicastrin (Hu et al., 2015; Zhao et al., 2010), suggesting that the requirement of Nicastrin could vary from cells to cells. So far, altered expression, mutations or polymorphisms in *nicastrin* have been associated with the occurrence of inflammatory acne inversa (Wang et al., 2010), life-long cognitive ability (Deary et al., 2005), and neurodegeneration (Tabuchi et al., 2009; Zhong et al., 2009).

Here we show that nicastrin deficiency in the zebrafish embryos leads to curled-up tail, depigmentation with ruptured melanosomes, swollen mitochondria, necrotic-like nuclei, and non-apoptotic death of melanophores, the fish counterparts of melanocytes. Blocking Tyrosinase by inhibitors or mutants prevented melanophore death in the
nicastrinhi1384 mutants. In addition, the treatment of γ-secretase inhibitor mimicked the phenotypes of nicastrin-deficient mutants and also led to a Tyrosinase-dependent reduction in mitochondrial cristae surface and depigmentation. Furthermore, the damaged melanosomes were phagocyted and retained in the recruited macrophages or nearby melanophores. In summary, the nicastrin deficiency in insertional nicastrinhi1384 mutants compromises γ-secretase activity and leads to melanosome defects, Tyrosinase-dependent mitochondrial swelling and depigmentation, which, in turn, recruits macrophages to phagocytose damaged melanosomes.

RESULTS
nicastrin deficiency leads to curled-up tail and depigmentation
To study the function of nicastrin, the zebrafish nicastrinhi1384 line (Amsterdam et al., 2004) was employed. The inserted GT2.0 sequence was located in the intron 1 of the nicastrin gene (Supplementary Figure S1a); it led to a significant downregulation of nicastrin expression, as revealed by reverse transcription-PCR and whole mount in situ hybridization (Supplementary Figure S1b and c), manifesting that nicastrinhi1384 homozygotes are nicastrin-deficient mutants. A quarter of the offspring showed hypopigmentation at 48 hours postfertilization (hpf) and one-eighth had a curled-up tail phenotype, in addition to hypopigmentation at 76 hpf (Supplementary Figure S2a–e). The mutants exhibited a progressive pigment loss, which can be easily observed in regions including the eyes and epidermis around the tectum (Figure 1). Phenotypes of embryos were correlated with their genotypes (Supplementary Figure S2f). Morpholino knockdown and mRNA overexpression were then used to mimic and rescue the phenotypes, respectively. The nicastrin splicing morpholino partially suppressed the expression of nicastrin and, therefore, only mimicked the hypopigmentation in the tectum and induced a melanophore stripe pattern defect on the dorsal midline (Supplementary Figure S3), which has been reported in presenilin1 and presenilin2 morphants (Nornes et al., 2009). In contrast, nicastrin overexpression rescued pigment loss temporarily and prevented tails from curling up in mutants (Supplementary Figure S4) but had no effect in siblings (not shown).

Notably, melanin-containing debris, a hallmark of melanophore death (Hamada et al., 2014; Parichy and Turner, 2003), was observed on the tectum (Figure 1b and c) and later around the eyes (Figures 1a and 3a) in nicastrinhi1384 mutants. The expression of dct, a melanophore-specific marker (Kelsh et al., 2000), and pnp4a, an iridophore-specific marker (Curran et al., 2010), was found progressively downregulated from 76 hpf in all regions and around the eyes, respectively (Supplementary Figure S5). The RPE was also found gradually degenerated and accompanied with photoreceptor loss after 3 days postfertilization (dpf) (Supplementary Figure S6). The findings prove that melanophore loss in nicastrinhi1384 homozygotes is a result of cell death rather than a change in cell fate or gene expression. In summary, these results demonstrate that nicastrin deficiency leads to curled-up tail and depigmentation in nicastrinhi1384 mutants.

nicastrin deficiency leads to melanosome maturation defect, mitochondrial swelling, and non-apoptotic melanophore cell death
Depigmentation has been extensively studied in zebrafish mutants, such as pmel17 (Schonthaler et al., 2005), tm7 (Ardùni and Henion, 2004; also known as dalmatian in Cornell et al., 2004; and touchdown in Kelsh et al., 1996), V-ATPase (Krauss et al., 2013), vacuolar protein sorting (vps)11 (Clancy et al., 2013), and ttyp1A (Krauss et al., 2014). Similar to the previously mentioned mutants, nicastrinhi1384 mutants also developed hypopigmentation before degeneration (Figure 1 and Supplementary Figure S2b), suggesting a correlation between melanin synthesis or deposition defect and depigmentation. Transmission electron microscopy was applied to investigate the melanosomes. We chose to analyze the melanosomes of the RPE because of its easy accessibility to examine a vast number of melanosomes for statistical reasons. At 24 hpf, wild-type (WT) melanosomes contained multi-vesicular endosome-like structures (Supplementary Figure S7a and b). The empty vesicles were gradually filled (Supplementary Figure S7c and e–g), and melanosomes developed into mature ovoid or ellipsoidal

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**Figure 1. nicastrinhi1384 mutants show a gradually depigmented phenotype.**
(a) The black pigments in the eyes do not show a dramatic difference between WT embryos and nicastrinhi1384 mutants at 52 hpf. Later, the black pigment gradually disappears from the eyes of (76 hpf to 124 hpf) nicastrinhi1384 mutants but not WT counterparts. (b) A progressive decrease of pigments is also observed on the epidermis around the tectum in (52 hpf to 124 hpf) nicastrinhi1384 homozygotes when compared with WT embryos. (c) Cell debris (red arrowheads) of melanophores can be spotted in the enlarged image from 100 hpf nicastrinhi1384 mutant in b (red square). hpf, hours postfertilization; WT, wild-type. Bar = 100 μm.
found dramatically reduced (swelling) in the melanophores of 

\textit{nicastrin}^{hi1384} homozygotes (Figure 2a). In addition, 

abundant necrotic-like melanophores, in contrast to melanophores with normal nucleus in the WT (Figure 2b), were spotted in \textit{nicastrin}^{hi1384} mutants at 4 dpf with many ruptured melanosomes (Figure 2b). Melanophores of different damages—including ruptured melanosomes, swelling and/or ruptured mitochondria (Günther et al., 2011; Hamabe et al., 2005; Niquet et al., 2003), crenellated nuclei (Abraham et al., 2007), nuclear chromatin clumping (Balvan et al., 2015), ruptured plasma membrane (Murakami et al., 2015), electron-lucent cytoplasm (Sonakowska et al., 2016), and chromatin condensation (Trump et al., 1997)—depicted with transmission electron microscopy were arranged in a tentative temporal sequence, which suggests a non-apoptotic, programmed necrosis (Supplementary Figure S8). Consistently, no apoptotic melanophores were observed in \textit{nicastrin}^{hi1384} mutants (Supplementary Figure S9), and the pigmentation loss could not be rescued or prevented by a broad-spectrum caspase inhibitor, zVAD-fmk (not shown). These observations support the idea that the toxic substances that leak from the improperly developed melanosomes impair the structure and functions of mitochondria (e.g., ATP production [Paumard et al., 2002] and cytochrome C release [Zhang et al., 2008]) and subsequently lead to necrosis.

**Depigmentation in \textit{nicastrin}^{hi1384} homozygotes is Tyrosinase-dependent**

Tyrosinase downstream metabolites, such as eumelanin, in the melanosome, have been demonstrated to induce DNA double-strand breaks in cell culture (Hill and Hill, 1987) and indicated to cause melanocyte death in animal models (Johnson and Jackson, 1992; Krauss et al., 2014; McNeill et al., 2007). To validate whether the necrosis is induced by the Tyrosinase downstream products, phenylthiourea (PTU), a common Tyrosinase inhibitor (Hall and Orlow, 2005) was used. When the activity of Tyrosinase was suppressed, beginning at 22 hpf to prevent the formation of eumelanin and its precursors (Figure S10a), \textit{dct} expressing cells were easily observed in the RPE at 3 dpf in all the treated \textit{nicastrin}^{hi1384} offspring, whereas about a quarter of the non-treated ones showed weak or nearly no \textit{dct} expression (Supplementary Figure S10b). Similar results were found for \textit{pnp4a} expression (Supplementary Figure S10c). These results demonstrate that after PTU treatment melanophores in the mutants survive and keep their melanin-generating ability by expressing \textit{dct} (also known as TRP-2 or TYRP2). To further validate whether the Tyrosinase inhibitor could block the progression of depigmentation in \textit{nicastrin}^{hi1384} mutants even after pigment loss has begun, homozygotes were treated with PTU after 48 hpf to block the formation of eumelanin and its precursors so as to reduce the accumulated cell damage in the melanophores. The depigmentation was prevented; both melanophores and iridophores persisted after the treatment in the RPE (Figure 3a) and also on the tectum epidermis (Supplementary Figure S10d). Consistently, after PTU treatment, the ratio of swollen mitochondria was significantly decreased in \textit{nicastrin}^{hi1384} mutants (Figure 3c). Quantitatively, in the non-treated group, around 39% of the
melanophores showed normal nuclei, whereas in the PTU-treated group > 94% of the melanophores showed normal nuclei (Figure 3b and c). These results demonstrate that the PTU treatment prevents mitochondrial swelling and melanophore necrosis in nicastrin mutants. Notably, no significant difference in the ratios of normal mitochondria and nuclei in PTU-treated nicastrin mutants by 22 hpf and 48 hpf was found (P = 0.26 and 0.29, respectively).

In addition, the later PTU treatment suggested that reducing the accumulated damages within a tolerable range could prevent melanophore cell death. To test this, different doses of PTU were used to suppress melanin production; the results showed that the lower the melanin production rate, the fewer melanophores die in nicastrin mutants (Supplementary Figure S11), which intriguingly echoed the association of blue and green eyes and tanning ability with vitiligo prevalence in previous clinical observations (Dunlap et al., 2017; Jin et al., 2010; Jin et al., 2012).

To doubly confirm the results, the tyrosinaseck112b mutant was employed (Park et al., 2016). This mutant, containing an 18-base pair insertion in the tyrosinase gene, showed significantly decreased Tyrosinase activity at 28.5 °C, which led to a temperature-sensitive albino phenotype (Supplementary Figure S12). The reduction of dct staining in nicastrinhi1384 homozygotes was entirely averted in the nicastrinhi1384; tyrosinaseck112b double homozygotes (Supplementary Figure S13), establishing that the depigmentation in nicastrinhi1384 mutants depends on their Tyrosinase activity.

Tyrosinase-dependent pigment loss in nicastrinhi1384 mutants is due to decreased γ-secretase activity

Previously, nicastrin was reported to be not essential for γ-secretase to process one of its substrates, Notch (Hu et al., 2015; Zhao et al., 2010). To validate whether the depigmentation in the nicastrin-deficient zebrafish is a result of γ-secretase inactivation, WT embryos were treated with a
γ-secretase inhibitor, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (Geling et al., 2002). The WT embryos treated with DAPT beginning at 4 somite stage (ss) showed immature melanosomes and swollen mitochondria (Figure 4b, left). Necrotic-like melanophores with ruptured melanosomes and mitochondrial cristae reduction (left one; green asterisk) in melanophores with normal nucleus (left one; blue arrow) are also observed. (c) The depigmentation phenotype is also observed in DAPT-treated 4 dpf WT (DAPT), which could be rescued by PTU co-treatment (DAPT+PTU). (d) The ratio of mitochondria with normal cristae density and the ratio of melanophores with normal nuclei are significantly reduced in the DAPT-treated group (DAPT+). (e) The phenomenon is also prevented by PTU co-treatment (DAPT+/PTU+). DAPT-: treated with DMSO by 4 ss; DAPT+: treated with DAPT by 4 ss; PTU-: treated with E3 egg medium by 2 dpf; PTU+: treated with PTU by 2 dpf. DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; ss, somite stage; dpf, days postfertilization; PTU, phenylthiourea; SD, standard deviation; TEM, transmission electron microscopy; WT, wild-type.

The results support the hypothesis that nicastrin deficiency leads to decreased γ-secretase activity and then gives rise to Tyrosinase-dependent mitochondrial swelling and depigmentation.

**Innate immune response was likely activated by necrotic-like melanophores**

Cell necrosis and mitochondrial stress were previously indicated to generate damage-associated molecular patterns to trigger innate immune response and subsequently activate adaptive immune system in vitiligo (Richmond et al., 2013). Additionally, nicastrin was found associated with an inflammatory skin disease, acne inversa (Wang et al., 2010). To examine if the innate immune system was activated in nicastrinhi1384 mutants, a macrophage reporter line, Tg(mpeg1: m-Cherry)dp23 (Ellert et al., 2011), was used. The macrophages were recruited to the location of melanophores and phagocytosed the damaged ones (Figure 5a and Supplementary

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**Figure 4.** Treatment of γ-secretase inhibitor mimics the phenotypes of nicastrinhi1384 mutants, which can be prevented by a Tyrosinase inhibitor. (a) The scheme of DAPT or DAPT+PTU–co-treatment. (b) TEM images of DAPT-treated WT. Bar = 500 nm. (c) Nomarski observations of control, DAPT-treated and PTU+DAPT–co-treated embryos. Bar= 100 μm. (d) Quantitation (mean +/- SD) of mitochondrion status and nucleus status of melanophores is shown. (b) After DAPT treatment, WT larvae show immature melanosomes (blue arrowheads) and mitochondrial cristae reduction (left one; green asterisk) in melanophores with normal nucleus (left one; red arrow). (b) The ruptured melanosomes (right one; red arrowheads) with necrotic-like nucleus (right one; red arrow) and swollen mitochondria (green asterisk) are also observed. (c) The depigmentation phenotype is also observed in DAPT-treated 4 dpf WT (DAPT), which could be rescued by PTU co-treatment (DAPT+PTU). (d) The ratio of mitochondria with normal cristae density and the ratio of melanophores with normal nuclei are significantly reduced in the DAPT-treated group (DAPT+). (e) The phenomenon is also prevented by PTU co-treatment (DAPT+/PTU+). DAPT-: treated with DMSO by 4 ss; DAPT+: treated with DAPT by 4 ss; PTU-: treated with E3 egg medium by 2 dpf; PTU+: treated with PTU by 2 dpf. DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; ss, somite stage; dpf, days postfertilization; PTU, phenylthiourea; SD, standard deviation; TEM, transmission electron microscopy; WT, wild-type.
Videos S1 and S2). It has also been suggested that melanocyte itself, in addition to macrophages, is a member of the innate immune system (Le Poole et al., 1993). The phagocytosis of damaged melanophores by nearby melanophores was also observed in nicastrinhi1384 mutants (Figure 5b). A similar phenomenon was also observed with transmission electron microscopy (Supplementary Figure S15). These results imply that the melanophore damage can activate an innate immune response in nicastrinhi1384 mutants.

DISCUSSION

Our findings show an unreported function of nicastrin in promoting melanosome maturation and maintaining the existence of melanophores through regulating γ-secretase activity. The depigmentation can be easily restrained by using Tyrosinase inhibitors or tyrosinase mutants (Figure 5b). A similar phenomenon was also observed with transmission electron microscopy (Supplementary Figure S15). These results imply that the melanophore damage can activate an innate immune response in nicastrinhi1384 mutants.

Mutations in different γ-secretase subunits lead to depigmentation

It has been demonstrated that the mutations in γ-secretase subunits, such as Presenilin 1 and Presenilin 2 (Haas and De Strooper, 1999; Selkoe and Hardy, 2016), prompt Alzheimer’s disease through modulating the processing of amyloid protein precursor and trafficking of amyloid-β (Haas and Selkoe, 1998; Sannerud et al., 2016). In addition, γ-secretase is known to regulate various developmental processes, such as somite formation, through the modulation of Notch processing (Rida et al., 2004) by releasing its intracellular domain (Jorissen and De Strooper, 2010). Recently, enzymatic components (presenilin proteins) of γ-secretase were shown to play a role in pigmentation (Jiang et al., 2018; Wang et al., 2006). Similar to amyloid protein precursor, melanin synthesis enzymes, Tyrosinase, Tyrp1 and Tyrp2 (also known as Dct) are type I membrane proteins and subject to the regulation of γ-secretase-dependent trafficking (Wang et al., 2006). Therefore, it is conceivable that zebrafish Nicastrin, similar to mouse Presenilin, regulates the trafficking of Tyrosinase through cleaving Tyrosinase C-terminal fragment, which contains a melanosomal targeting signal (Calvo et al., 1999). Although we could not demonstrate the localization of Tyrosinase in nicastrin mutants, because of the lack of zebrafish Tyrosinase antibodies, we showed that nicastrin mutants similarly exhibit a melanosome developmental defect. This finding suggests an intriguing regulation among the activity of Tyrosinase, Tyrosinase C-terminal fragments and Tyrosinase localization in relation to depigmentation, which remains to be investigated.

Presenilin 2 has been shown to colocalize with PMEL (Sannerud et al., 2016), a melanosome component, which suggests that γ-secretase is located in melanosomes. The downregulation of nicastrin compromises the function of γ-secretase and then disturbs the development of melanosomes. After that, the melanosome defect leads to the release of melanosomes toxic contents, such as eumelanin and its precursors, which harm organelles, such as mitochondria. The severe damage triggers the necrosis of melanosomes and leads to the release of DAMP, which subsequently recruit phagocytes, such as macrophages, to phagocyte the damaged melanophores and present their antigens. DAMP, damage-associated molecular pattern.

Figure 6. Model of depigmentation and immune activation in nicastrinhi1384 mutants. Nicastrin, a member of γ-secretase, is required to maintain melanosome. The downregulation of nicastrin compromises the function of γ-secretase and then disturbs the development of melanosomes. After that, the melanosome defect leads to the release of melanosomes toxic contents, such as eumelanin and its precursors, which harm organelles, such as mitochondria. The severe damage triggers the necrosis of melanosomes and leads to the release of DAMP, which subsequently recruit phagocytes, such as macrophages, to phagocyte the damaged melanophores and present their antigens. DAMP, damage-associated molecular pattern.
**Mechanisms of melanophore cell death are diverse**

Melanophore death was found in several zebrafish models accompanied by various melanosome defects, including immature, abnormally-shaped and/or ruptured melanosomes, such as mutants of *nicastrin* (this study), *tpm7* (McNeill et al., 2007), *vps11* (Clancey et al., 2013; Thomas et al., 2011), V-ATPase (Nuckels et al., 2009), and *tyrplA* (Krauss et al., 2014). Moreover, in human patients, immature melanosomes have been observed at active vitiligo sites, whereas mature ones have been seen at stable vitiligo sites (Xiong et al., 2017). All these studies suggest that melanosome maturation defect is a predisposition leading to acquired pigment loss in human patients.

The mechanisms of zebrafish melanophore death have been studied in some detail for several genes, including *kit*, *vps11*, *tpm7*, and *tyrplA*. In *kit* mutants, melanophores underwent apoptotic death with DNA fragmentation (Parichy et al., 1999). Furthermore, treatment of zVAD-fmk prevented depigmentation, demonstrating that *kit* melanophage death is caspase-dependent (McNeill et al., 2007). In *vps11* mutants, melanophores, necrotic-like phenotypes have been observed: lacking caspase-dependent activity, increased cathepsin activity, swollen organelles, and plasma membrane disruption (Clancey et al., 2013). Treatment with bafilomycin A1 (autophagy and vacuolar-type H⁺-ATPase inhibitor, Yoshimori et al., 1991) restored melanophore morphology and number in *vps11* mutants. Consistently, immunoblotting illustrated a rise of LC3B II, an autophagosome marker, in *vps11* mutants (Clancey et al., 2013). Bafilomycin A1 also has been shown to increase the pH value and enhance Tyrosinase activity in *vps11* mutants, as well as in *vps39* and *slc45α2* (*oca4*) mutants (Beiril et al., 2014; Dooley et al., 2013). Thus, bafilomycin A1 restores pigmentogenesis through different mechanisms. However, bafilomycin A1 treatment of *nicastrin* mutant led to an acceleration of pigment loss but no pigment restoration (not shown).

Similar to *nicastrin* mutants, *tpm7* mutants showed a necrotic-like melanophore phenotype, and their melanophage death did not depend on zVAD-fmk-sensitive Caspases but Tyrosinase activity (McNeill et al., 2007; this study). However, melanophage death in the *nicastrin* mutant is unlikely due to the Mg²⁺/Ca²⁺ ion channel sensitivity defect, because the pigment loss was not precluded by supplementation with a high concentration of magnesium or calcium ions (not shown) as in *tpm7* mutants (Elizondo et al., 2005; McNeill et al., 2007). Whereas zebrafish *tyrplA* mutation induced the pigment loss through the melanin synthesis pathway, which was rescued by Tyrosinase inhibitor and tyrosinase mutant (Krauss et al., 2014), the death of cutaneous melanocytes in mouse *Tyrp1* (*TRP-1*) mutants was also found to be Tyrosinase-dependent (Johnson and Jackson, 1992). Both have an Arg to Cys change adjacent to the N-terminal EGF-like domain of Tyrp1, which might inhibit its functions in the melanosome membrane, eventually leading to melanosome disruption and leakage of toxic melanin precursors into the cytoplasm (Johnson and Jackson, 1992). These observations exemplify that melanophore death induced by various mechanisms has a common downstream executor, Tyrosinase, that generates toxic metabolites and induces non-apoptotic cell death.

**Damaged melanophores in *nicastrin* mutants may activate innate immune response**

In necrosis, unlike in apoptosis, cells lose their plasma membrane integrity and release damage-associated molecular patterns to activate macrophages, immune cells that play a critical role in regulating host innate immunity, tissue repair, and amplification of inflammatory response (Poon et al., 2010; Sanz et al., 2014). The deficiency of *nicastrin* transcript led to massive melanophore necrosis accompanied by the recruitment of macrophages and phagocytosis of damaged melanophores. These observations demonstrate that inflammatory response is activated around the necrotic-like melanophores and suggest the contribution of *nicastrin* to acne inversa, a human inflammatory skin disorder (Wang et al., 2010). In addition, macrophages can present antigens found in the target cells and activate an adaptive immune response (Poon et al., 2010). Although the T-cell response in the mutants was not investigated, the occurrence of an autoimmune response against melanophores, shown in chemical-induced acquired pigment loss (Iokura et al., 2015; van den Boom et al., 2011), was not excluded. Furthermore, the T cells were detected from 3 dpf around thymic lobes in zebrafish (Langenau et al., 2004), which echoes the observation of a relative fast pigment loss after 3 dpf (Figure 1), and a frequent accumulation of black pigment around thymic lobes (Supplementary Figure S11, *nicastrin* mutants with little or no PTU). Therefore, whether the damaged melanophore in *nicastrin* mutants activates adaptive immune response that synergistically facilitates the death of melanophores is worth further investigation.

**Tyrosinase inhibitors could be a preventive anti-vitiligo drug**

Vitiligo has been widely believed to be an auto-inflammatory disease, because it was associated with immune-related genetic loci (Jin et al., 2010; Jin et al., 2012), carried lesion-infiltrating natural killer cells (Yu et al., 2012), and had a positive responsive case reported in immune suppression therapy (Bayer and Chiu, 2017). However, only a small proportion of patients showed response to the treatment (Rodrigues et al., 2017). This study provides an alternative strategy to avert the occurrence of vitiligo: topical...
pre-administration of Tyrosinase inhibitors. Although there is currently no report using Tyrosinase inhibitors as a drug to alleviate pigment loss, several clinical observations and animal model studies suggest that such a preventive treatment could be effective. First, melanosome defects have been found in clinical samples (Xiong et al., 2017) and the previously mentioned animal models. Second, melanin synthesis is associated with vitiligo (Jin et al., 2010; Jin et al., 2012). Third, vitiligo correlates with tanning ability (Dunlap et al., 2017). Fourth, melanocyte toxic factors are Tyrosinase substrates (van den Boorn et al., 2011; Tokura et al., 2015). Fifth, Tyrosinase inhibitors have shown promising results in different pigment loss disease models (Johnson and Jackson, 1992; Krauss et al., 2014; McNeill et al., 2007; this study). All these data indicate the potential of Tyrosinase inhibitors for vitiligo prevention.

Additionally, among Tyrosinase inhibitors only PTU has been shown to prevent depigmentation in animal models. Zebrafish is a great animal model for performing drug screening because of its high-throughput efficiency (MacRae and Peterson, 2015) and similarity to mammals (Howe et al., 2013). Therefore, the nicastrin\(^{\text{hi1384}}\) line can be applied to identifying non-melanotoxic Tyrosinase inhibitors.

**MATERIALS AND METHODS**

**Zebrafish**

Fish were raised and maintained according to the standard operating protocol of the Zebrafish Core Facility of the National Health Research Institutes (NHRI), Taiwan (You et al., 2016). Laid eggs were collected within 30 minutes after mating started, sorted into 50–60 eggs per 9 cm petri-dish with E3 medium (5 mM NaCl [Amresco, Solon, OH), 0.17 mM KCl [Amresco], 0.33 mM CaCl\(_2\) [I.T. Baker, Hsinchu, Taiwan], 0.33 mM MgSO\(_4\) [I.T. Baker]) and raised at 28.5 °C unless otherwise specified. Embryos and larvae were staged as previously described (Kimmel et al., 1995). Experimental procedures on fish embryos were approved by NHRI’s Institutional Animal Care and Use Committee (NHRI-IACUC-103122-Æ and NHRI-IACUC-107094-A) and carried out in accordance with the approved guidelines.

**Imaging**

Embryos or larvae mounted with 4% methyl cellulose (Sigma, Darmstadt, Germany) or 2% low melting agarose (Zymeset, Taipei, Taiwan) was imaged with a AxioVision A1 (Zeiss, Oberkochen, Germany) or Nikon SMZ1500 (Nikon, Tokyo, Japan) or Andor Dragonfly confocal imaging platform (Oxford Instruments, Abingdon, United Kingdom). Images of melanophores and iridophores were captured with transmitting light and incident light, respectively.

**Transmission electron microscope image**

Samples fixed with the fixation buffer, containing 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), 1% glutaraldehyde (Electron Microscopy Sciences) and 0.1% Tween 20 (Sigma) in phosphate buffered saline (Protech, Taipei, Taiwan), were sequentially treated with 4% osmium-tetroxide (Electron Microscopy Sciences), 1.5% potassium ferrocyanide (Sigma), and uranyl acetate (Electron Microscopy Sciences); then they were gradually dehydrated with ethanol (Merck, Darmstadt, Germany) and infiltrated with EMBed-812 embedding kit (Electron Microscopy Sciences). The ultrathin sectioning and micrographs were performed with the assistance of the Image Core, Institute of Molecular Biology, Academia Sinica, Taiwan.

**Drug treatments**

The activity of Tyrosinase was inhibited by 0.2 mM PTU (Sigma) in E3 egg medium, which was refreshed daily, from 22 hpf or 48 hpf to the desired stages (Figures 3 and S10).

A final concentration of 100 μM (unless stated otherwise) γ-secretase inhibitor IX/DAPT (Calbiochem, Darmstadt, Germany) was freshly made from 10 mM γ-secretase inhibitor/DMSO (Sigma) stock solution with E3 egg medium or PTU. Although stock was gradually added into the desired solution with constant vortex, unavoidable precipitation appeared gradually. To reduce variability, the solution was refreshed twice a day. For Supplementary Figure S14, precipitation was not observed at the applied concentrations, the solution was refreshed once a day.

**Statistics**

Mitochondria in 10 different melanophores per sample with three biological replicates were counted and grouped into normal or swollen mitochondria as mentioned. The nuclei of melanophores on the RPE per sample with three biological replicates were counted and grouped into live nuclei or necrotic-like nuclei as mentioned. The counted number of mitochondria and nuclei of each sample were then used to determine a ratio (group number/total number). The average, standard deviation of the ratios and two-tailed Student \(t\) tests were calculated with Excel.

**Data availability statement**

Datasets related to this article can be found at https://doi.org/10.17632/6y6b8mr4nv.1, an open-source online data repository hosted at Mendeley Data.

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**CONFLICT OF INTEREST**

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: CHH and YYJ; Formal Analysis: CHH; Funding Acquisition: YYJ; Investigation: CHH and GGL; Resources: YYJ; Supervision: YYJ; Visualization: CHH; Writing - Original Draft Preparation: CHH, GGL and YYJ; Writing - Review and Editing: CHH and YYJ.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2019.07.702.

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SUPPLEMENTARY MATERIAL AND METHODS

Genomic PCR and reverse transcription—PCR
Genomic DNA was extracted with 50 mM NaOH and 1 M Tris-HCl (pH 8.0). To genotype nicastrin<sup>hi13384</sup> mutants, PCR was carried out with nicastrin exon 1-1 primer (5'-GGGTCCACGCTTCACATTACAGC-3') and nicastrin intron 1-2 primer (5'-CGTGTCTGGTTTTCAGACGCGC-3'), GT 2.0 virus (5'-CGCAAACCTACAGGTGGGGTC-3'), and One PCR mix (MB203-0100, GeneDireX, Taiwan).

For genotyping and sequencing tyrosinase<sup>kl22b</sup>, the primers applied to the T7E1 assay in a previous study were used (Park et al., 2016).

The total RNA, extracted with RNAzol (R4533, Sigma), was used to generate cDNA with SuperScript III FirstStrand (18080-051, Invitrogen, Waltham, MA) with oligo dT or random hexamer.

PCR-based chromosome walk
The chromosome walk followed the instructions given in a previously published protocol (Tan et al., 2005). The genomic sequence was initially amplified with nicastrin exon 1-3 primer (5'-AAAAAACACTCTCCATG-3') and sitefinder primer 1 (5'-CAGCAGGCTCTATCAACACACCTCCTGCA CAGCCTCTCAAGGGGCGCCGNNNNNNNGC-3'). A nested amplification was then used to generate next amplicons with nicastrin exon 1-2 primer (5'-GGTGATCTGTGAATAGG-3') and with sitefinder primer 2 (5'-CAGCAGGCTCTATCAACACACCTCCTGCA CAGCCTCTCAAGGGGCGCCGNNNNNNNGC-3'). A nested amplification was then used to generate next amplicons with nicastrin exon 1-2 primer (5'-GGTGATCTGTGAATAGG-3') and with sitefinder primer 2 (5'-CAGCAGGCTCTATCAACACACCTCCTGCA CAGCCTCTCAAGGGGCGCCGNNNNNNNGC-3'). A nested amplification was then used to generate next amplicons.

In vitro Tyrosinase activity assay
The Tyrosinase activity assay was modified from a previous publication (Uchida et al., 2014). Briefly, twenty-five 30-hpf embryos were deyolked (Link et al., 2006) and homogenized in 30 μl of 100 mM potassium phosphate buffer with 0.1% TritonX-100 (PBT)(pH 6.8) with a sonicator. The program for sonication was 10 seconds sonicator on and 20 seconds off, with 12 repeats. After the lysate was centrifuged, 20 μl of 500 μg/ml protein lysate and PBT was added with 50 μl of 50 mM PBT and 20 μl of 1.22 mg/ml L-DOPA in 50 mM PBT. The L-DOPA containing mixtures were incubated at 28.5 °C for 13 hours. The images of mixtures were captured by HTC Desire 12 and their optical density was measured at 450 nm with Nanodrop (ThermoFisher Scientific).

For Supplementary Figure S2e, the phenotypes of offspring from eight pairs nicastrin<sup>hi13384</sup> carriers were calculated and converted into a ratio (number of specified phenotype/total number). The means and standard errors of each group were then calculated. The means (small square), standard errors (large square), 95% percentile (+), and the maximum and minimum values (×) were drawn with Qtiplot (http://www.qtiplot.com/index.html).

For Supplementary Figures S10b, S10c, and S13, the number of embryos in each group characterized was calculated and used to form a ratio (group embryo number/total embryo number).

Whole mount in situ hybridization
To generate whole mount in situ hybridization templates for probes, cDNA generated from in-house AB embryos with SuperScript III FirstStrand (18080-051, Invitrogen) was amplified with dcr primer set (5'-GTTTTGGATGCATGTACCTG-3' and 5'-CAGGTTGTGTAGATTGGAC-3') or pnp4a primer set (5'-TTTTCACACAAACACATGC-3' and 5'-GCCACCCTCTGATCGACAT-3') and cloned into pGem-T Easy (A1360, Promega, Madison, WI). Next, MEGAscript (ThermoFisher Scientific, Waltham, MA) and DIG RNA Labeling Mix (11277073910, Roche) were used to generate labeling probes. The whole mount in situ hybridization procedure followed the instructions in previously published protocols (Hsu et al., 2015; This and This, 2008).

nicastrin knockdown and mRNA rescue experiments
A total of 8 ng nicastrin exon 1-intron 1 (E1I1) morpholino (5'-CGATAATAGTACTCAGATGTG-3') was used to mimic the phenotype of nicastrin<sup>hi13384</sup> mutants. Reverse transcription—PCR and sequencing were then used to confirm its knockdown efficiency (Supplementary Figure S3c).

For the rescue experiment, 200 pg zebrafish nicastrin mRNA generated from pCS2+nicastrin was used to inject nicastrin<sup>hi13384</sup> mutant embryos at the one-cell stage.

Sectioning and hematoxylin & eosin staining
Samples were collected at stages as indicated, fixed with 4% paraformaldehyde, rehydrated with phosphate buffered saline with 0.1% Tween20, mounted in 10% low melting agarose, and gradually dehydrated to 75% ethanol. The samples were then processed with the assistance of the Pathology Core Laboratory of the National Health Research Institutes, Taiwan. The images of sections were taken with a Zeiss AxiosVision A1.

Supplementary References


Supplementary Figure S1. nicastrin^{h1384} has a GT2.0 virus insertion in the intron 1 region, which leads to a significant downregulation of nicastrin expression. Results of (a) nicastrin^{h1384} PCR-based chromosome walk, (b) RT-PCR, and (c) nicastrin WISH are shown. (a) The sequencing result is illustrated at the top and the sequence of GT 2.0 virus on the bottom. The sequence fragments of the GT 2.0 virus are found in the intron 1 of nicastrin gene. (b) The transcript of mutant’s nicastrin, from exon 1 to exon 3, is unable to be detected in the mutant cDNA generated with oligo dT (T) or random hexamer (R) primers, whereas its expression can be detected in the WT. The expression of a positive control, foxc1a, is detected in both WT and nicastrin^{h1384} mutants. (c) Additionally, the expression of nicastrin is examined by WISH, and results show that it is severely downregulated in nicastrin^{h1384} mutants at 4 dpf. dpf, days postfertilization; RT-PCR, reverse transcription—PCR; WISH, whole mount in situ hybridization; WT, wild-type.

Supplementary Figure S2. nicastrin^{h1384} mutants show hypopigmentation and curled-up tail phenotypes. Lateral views of (a and c) WT-like and (b and d) mutant-like nicastrin^{h1384} offspring are shown. (e) Ratio of each phenotype* in the whole population (n = 433). (f) Each corresponding genotype is analyzed by a tested PCR mixture having mutant-specific and WT-specific PCR primers. The mutant-like nicastrin^{h1384} show (b) hypopigmentation or weak-pigmentation at 52 hpf and some of them show (d) curled-up tail phenotype at 76 hpf. (e) The ratio of HY is around 25% of the total population, whereas the CU has a ratio around 12.5%. Noticeably, curled-up tail can be found only in the hypopigmented larvae. (f) By using genotyping, all those larvae showing HY or HY plus CU are nicastrin^{h1384} homozygotes. The PCR-amplified fragment is 1148 bp for the WT allele and 304 bp for the mutant allele. bp, base pair; hpf, hours postfertilization; CU: curled-up tail; CUO: curled-up tail only; HY, hypopigmentation; HY plus CU, hypopigmentation and curled-up tail; HYO, hypopigmentation only; *WT, wild-type like.
Supplementary Figure S3. Injection of nicastrin splicing morpholino (MO) partially mimics the phenotypes of nicastrin^{hi1384} mutants. Dorsal views of (a) head and (b) trunk are shown with head toward the left. WT larvae injected with 8 ng E111 nicastrin morpholino show hypopigmentation instead of depigmentation observed in nicastrin^{hi1384} homozygotes. Unlike WTs dorsal midline melanophores, which show a parallel and symmetric distribution, the melanophores in nicastrin^{hi1384} mutants and nicastrin morphants show an asymmetric and uneven distribution. (c) PCR-amplification of nicastrin cDNA in WT and morphants (triplicates). The RT-PCR of nicastrin transcript shows only a partial reduction of WT nicastrin transcript (blue arrowhead) in morphants with an additional alternative splicing product induced by morpholino (red arrowhead). (d) The image illustrates the splicing junction in WT (1) and the alternative splicing junction in nicastrin E111 morphants (2). (e) The alternatively spliced mRNA in E111 morphants has an additional 167 bp intronic sequence (in black), which is predicted to have an in-frame stop codon in the transcript. (f) The predicted products of normal nicastrin mRNA and the alternatively spliced nicastrin mRNA induced by injecting E111 MO. The latter is predicted to encode an early truncated nicastrin protein.

Supplementary Figure S4. The curled-up tail and depigmentation phenotypes in nicastrin^{hi1384} mutants are prevented and temporarily averted by nicastrin mRNA injection, respectively. (a) The progression of depigmentation in the eyes of nicastrin^{hi1384} mutants with nicastrin mRNA rescue are shown. The depigmentation was temporarily prevented with 200 pg nicastrin mRNA injection at 3 dpf. However, pigment loss can still be observed in mRNA-injected nicastrin^{hi1384} mutants at 6 dpf. (b) Quantitation of curled-up tail ratio in Con and Res. The semi-penetrated curled-up tail phenotype is prevented in nicastrin^{hi1384} mutants injected with 200 pg nicastrin mRNA. Con, nicastrin^{hi1384} mutants; dpf, days postfertilization; Res, rescued nicastrin^{hi1384} homozygotes.
Supplementary Figure S5. Melanophores and iridophores are gradually decreased in nicastrin<sup>h1384</sup> homozygotes. WISH of (a) dct, which marks melanophores, and (b) pnp<sup>-</sup>4a, an iridophore-specific marker, are shown. Melanophores are gradually reduced in all regions of nicastrin<sup>h1384</sup> homozygotes, whereas iridophores are only gradually lost in the eyes of nicastrin mutants. hpf, hours postfertilization; WISH, whole mount in situ hybridization.
Supplementary Figure S6. The retinal pigment epithelium is lost in nicastrin<sup>h1384</sup> homozygotes. The RPE and ONL layers can be observed both in WT embryos and nicastrin<sup>h1384</sup> mutants at 2 dpf with no significant difference. Gaps on the RPE are observed at 3 dpf in the eyes of nicastrin<sup>h1384</sup> mutants. A significant loss of RPE is subsequently found beginning at 4 dpf, and photoreceptor degeneration appeared at 7 dpf. dpf, days postfertilization; GCL, ganglion cell layer; INL, inner nuclear layer (amacrine cells and horizontal cells); ONL, outer nuclear layer (rod and cone cells); RPE, retinal pigment epithelium (melanophores); WT, wild-type. Bar = 25 μm.
Supplementary Figure S7. The maturation process of WT RPE melanosomes. TEM images of the cytoplasm of (a) 24 hpf and (d) 48 hpf melanophores are shown. The enlarged images of (b) and (c) and (e), (f), and (g) are shown from respectively marked areas in (a) and (d). (a) and (b) Small multi-vesicular endosome-like melanosomes can be found in the cytoplasm of 24 hpf melanophores, with many hole-like structure within (red arrowheads). (a) and (c) The melanosomes become larger and filled with dark substances. After 2 dpf, ellipsoidal melanosomes (red arrow in (d), ovoid melanosomes (d and g; green arrows in (d)) and hole-filling melanosomes (e and f) can be observed in the melanophores (d). dpf, days postfertilization; hpf, hours postfertilization; RPE, retinal pigment epithelium; TEM, transmission electron microscopy; WT, wild-type. Bar in (a) and (d) = 500 nm; bar in (b), (e), (f), and (g) = 100 nm.
Supplementary Figure S8. The possible progression of melanophore cell death in nicastrin<sup>hi34</sup> homozygotes. Stage I: melanophores show ruptured melanosomes (red arrowhead), moderately inflated mitochondria (green asterisk, Figures 1b and 2gi in Niquet et al., [2003]) and smooth nuclear envelope. Bar = 1 μm. Stage II: melanophores display ruptured melanosomes (red arrowheads), severely inflated mitochondria (green asterisks, Figure 2d in Günther et al., [2011]) and irregular nuclear envelope (N). Bar = 2 μm. Stage III: melanophores show ruptured melanosomes (red arrowheads), severely inflated mitochondria (green asterisks) and crenellated nucleus (N, Figure 4f in Abraham et al., [2007]). Bar = 1 μm. Stage IV: melanophores exhibit ruptured melanosomes (red arrowheads), mitochondria (green asterisks), membrane rupture (yellow arrowheads, Figure 2c in Hamabe et al., [2005]), and early necrotic-like nucleus (N): fuzzy nuclear envelope with clumped chromatin (Figure 4c in Balvan et al., [2015]). Bar = 1 μm. Stage V: melanophores show ruptured melanosomes (red arrowheads), plasma membrane rupture (green arrowhead, Figure 2f in Murakami et al., [2015]), and late necrotic-like nucleus (NL): entirely condensed chromatin (Figure 4b in Trump et al., [1997]) and electron-lucent cytoplasm (Figure 6 in Sonakowska et al., [2016]). Bar = 1 μm. N, nucleus; NL, late necrotic-like nucleus.
Supplementary Figure S9. No apoptotic melanophores are detected in nicastrin<sup>hi1384</sup> mutants. Anti-active Caspase-3 antibody (BD Biosciences, 559565; Jette et al., 2008) was used to detect the presence of apoptotic melanophores in nicastrin<sup>hi1384</sup> mutants. The apoptotic cells in the positive control, udu<sup>ho24</sup> mutants (Lim et al., 2009), show Caspase-3 activation with condensed DAPI staining (thick arrowheads; inserts are four times enlarged images). In contrast, no apoptotic-like activated Caspase-3 staining (no colocalized condensed DAPI staining) is observed in both WTs and nicastrin<sup>hi1384</sup> mutants. Although there is one suspected activated Caspase-3 fluorescent cell observed in the WT’s melanophore (arrow), the cell’s DAPI staining is not condensed (see enlarged image within), suggesting a non-apoptotic Caspase-3 activation in the WT’s melanophore (Kavanagh et al., 2014; Nakajima and Kuranaga, 2017). In addition, the colocalization of DAPI and activated Caspase-3 in the melanophore also indicates that the presence of melanin does not obstruct the fluorescence observation in the melanophores. The numbers (1, 2, and 3) indicate triplicates of respective embryos. Bar = 50 µm. DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; hpf, hours postfertilization; WISH, whole mount in situ hybridization.
Supplementary Figure S10. The loss of melanophores or iridophores in the eyes and tectum epidermis is prevented by Tyrosinase inhibitor treatment. (a) The treatment scheme of PTU in b and c. Different levels of (b) dct and (c) pnp4a WISH and their statistics in treated nicastrinhi1384 offspring. Around 20–30% of the nicastrinhi1384 offspring show weak dct expression or nearly non-dct expression. In contrast, a similar population is not observed in the PTU-treated group. Less than 10% of the PTU-treated group's offspring show normal-like dct expression; and nearly 90% of its offspring have normal dct expression. (c) Similarly, only normal-like pnp4a expressing larvae can be found in the PTU-treated group. The larvae expressing nearly non-pnp4a in eyes are observed only in the control nicastrinhi1384 offspring. (d) The protective effect of Tyrosinase inhibitor on tectum epidermis. The pre-selected nicastrinhi1384 homozygotes are treated with E3 medium or PTU at 48 hpf and fixed at 76 hpf. The homozygotes cultured in PTU show fewer necrotic-like melanophores (red arrowhead in E3) and show a larger area covered by melanophores (statistical data on the right), including both the necrotic-like ones and well-expanded ones, in three biological replicates. Although only one of the three pairs showed a significant difference in a two-tailed t test (likely because of the low sample number), the grouping two-tailed t test showed an even smaller P value between the E3-treated group and the PTU-treated group than that of individual pairs. Con, control; hpf, hours postfertilization; PTU, phenylthiourea; WISH, whole mount in situ hybridization.
Supplementary Figure S11. Low melanin production averts depigmentation in nicastrin<sup>hi1384</sup> mutants. WT (left panel) and nicastrin<sup>hi1384</sup> mutants (right panel) treated with different doses of PTU from 22 hpf and 48 hpf, respectively. When WT embryos were treated with different concentrations of PTU, embryos showed different melanin synthesis capability at 52 hpf (left panel). The higher concentration the PTU, the lower the melanin synthesis activity (sequentially from left top to left bottom). When nicastrin<sup>hi1384</sup> mutants were treated with PTU after 48 hpf, the higher concentration the PTU, the less severe the depigmentation (sequentially from right top to right bottom). hpf, hours postfertilization; PTU, phenylthiourea; WT, wild-type.
Supplementary Figure S12.  Tyrosinase<sup>ck112b</sup> homozygote is a temperature-sensitive albino mutant with extremely low Tyrosinase activity at 28.5 °C. (a) The sequence of tyrosinase<sup>ck112b</sup> homozygotes. The ck112b allele has a 2 bp deletion and a 20 bp insertion in the tyrosinase gene. The sequence “CC” is replaced with “AGACTCCACAGACTGGGA”, leading to an in-frame 18 bp insertion in the tyrosinase gene. (b) The genotyping results of tyrosinase<sup>ck112b</sup> heterozygotes. By using genomic amplification, the carriers of tyrosinase<sup>ck112b</sup> show an easily identified extra amplified fragment on gel. Its homozygous larvae show (c) a temperature-sensitive albino phenotype at 28.5 °C and (d) and (e) a significant decrease of Tyrosinase activity at 28.5 °C. Reference: sequence from database; WT: sequence from sibling; tyrosinase<sup>ck112b</sup> mutant: sequence from ck112b allele. bp, base pair.

Supplementary Figure S13. Depigmentation in nicastrin<sup>n1384</sup> mutants is prevented in the tyrosinase mutant background. (a) The expression of a melanophore marker, dct, and (b) the statistics in WT, nicastrin<sup>n1384</sup> mutants, and nicastrin<sup>n1384</sup>; tyrosinase<sup>ck112b</sup> double mutants at 4 dpf. nicastrin<sup>n1384</sup> homozygotes show a significant reduction in dct expression, whereas dct is normally expressed in both WT and nicastrin<sup>n1384</sup>; tyrosinase<sup>ck112b</sup> double mutants. All embryos are phenotyped. nicastrin<sup>n1384</sup> mutants show curled-up tail; tyrosinase<sup>ck112b</sup> mutants show albinism; double mutants show both phenotypes. dpf, days postfertilization; WT, wild-type.
Supplementary Figure S14. DAPT treatment facilitates the pigment loss in *nicastrin<sup>h1384</sup>* mutants. (a) The 3 dpf WTs show acquired pigment loss when treated with 25 μM DAPT, but not with 5 μM DAPT or DMSO. In contrast, the depigmentation in *nicastrin<sup>h1384</sup>* mutants treated with 5 μM DAPT progress faster than those treated with DMSO, suggesting a synergistic effect of *nicastrin* deficiency and inhibition of γ-secretase activity. All *nicastrin<sup>h1384</sup>* mutants are genotyped. (b) Non-genotyped offspring from heterozygous *nicastrin<sup>h1384</sup>* carriers show severe depigmentation when treated with 25 μM DAPT. DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; dpf, days postfertilization; WT, wild-type.

Supplementary Figure S15. Damaged melanophores are phagocytosed. The melanophores show mitochondrial swelling (green asterisks), leaky melanosome (red arrowhead) with a relative intact nucleus was found phagocytosed by a neighboring cell in a 3 dpf *nicastrin<sup>h1384</sup>* homozygote (left panel). The necrotic-like melanophore with diluted cytoplasm and damaged melanosomes was phagocytosed in a 4 dpf *nicastrin<sup>h1384</sup>* homozygote (right panel). The purple double-headed arrow indicates the cell membranes of the phagocyte and the phagocytosed melanophore. dpf, days postfertilization; N, nucleus. Bar = 2 μm.