p38 MAPKs regulate the expression of genes in the dopamine synthesis pathway through phosphorylation of NR4A nuclear receptors

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Summary

In *Drosophila*, the melanization reaction is an important defense mechanism against injury and invasion of microorganisms. *Drosophila* tyrosine hydroxylase (TH, also known as Pale) and dopa decarboxylase (Ddc), key enzymes in the dopamine synthesis pathway, underlie the melanin synthesis by providing the melanin precursors dopa and dopamine, respectively. It has been shown that expression of *Drosophila TH* and *Ddc* is induced in various physiological and pathological conditions, including bacterial challenge; however, the mechanism involved has not been fully elucidated. Here, we show that ectopic activation of p38 MAPK induces *TH* and *Ddc* expression, leading to upregulation of melanization in the *Drosophila* cuticle. This p38-dependent melanization was attenuated by knockdown of *TH* and *Ddc*, as well as by that of *Drosophila HR38*, a member of the NR4A family of nuclear receptors. In mammalian cells, p38 phosphorylated mammalian NR4As and *Drosophila* HR38 and potentiated these NR4As to transactivate a promoter containing NR4A-binding elements, with this transactivation being, at least in part, dependent on the phosphorylation. This suggests an evolutionarily conserved role for p38 MAPKs in the regulation of NR4As. Thus, p38-regulated gene induction through NR4As appears to function in the dopamine synthesis pathway and may be involved in immune and stress responses.

Key words: p38 MAPK, NR4A nuclear receptor, Tyrosine hydroxylase, Stress response

Introduction

Melanin is a widespread pigment found in the skin, hair and eyes of animals, protecting them from harmful environmental stimuli. In arthropods, the melanization reaction is also an important defense mechanism against injury and invasion of a wide variety of microorganisms (Cerenius and Soderhall, 2004; Nappi and Vass, 1993; Sugumaran, 2002). Rapid deposition of melanin at the wound site prevents the loss of hemolymph and leads to killing of the invading microorganisms by toxic intermediates, such as reactive oxygen species, that are produced during the melanin biosynthesis. In *Drosophila*, tyrosine-derived catecholamines, such as dopa and dopamine, serve as the precursors of melanin, which constitutes the black and brown pigments in the cuticle and wing (Wittkopp et al., 2003). *Drosophila* tyrosine hydroxylase (TH, also known as Pale) catalyses the oxidation of tyrosine to dopa, which is the first and rate-limiting step in dopamine biosynthesis. Dopa decarboxylase (Ddc) converts dopa into dopamine. Some fractions of dopa and dopamine are oxidized by phenoloxidase (PO) in the hemolymph and converted into quinones, which are then polymerized non-enzymatically to form dopa- and dopamine-melanin, respectively.

Mutations in the *pale* locus, which encodes TH, have been shown to result in unpigmentated embryos that are unable to hatch (Jürgens et al., 1984; Neckameyer and White, 1993). It has also been shown that *TH,* together with *Ddc*, is required for melanin synthesis in the cuticle of adult flies, and expression of both genes is sufficient to cause ectopic melanin patterns in the wing (True et al., 1999). A microarray analysis of *Drosophila* genes revealed that *TH* and *Ddc* were upregulated in the flies after septic injury with a mixture of *Escherichia coli* and *Micrococcus luteus* (De Gregorio et al., 2001). In addition, in the moth *Manduca sexta*, the corresponding tyrosine-hydroxylase-encoding gene was upregulated in response to inoculation with *M. luteus* (Gorman et al., 2007), suggesting the possible

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involvement of *DTH* and *Ddc* in immune-associated melanization. Despite these lines of evidence, however, the regulatory mechanism of *TH* and *Ddc* gene expression in the context of melanization has been elusive.

p38 MAPKs are preferentially activated in response to a wide variety of physical, chemical and biological stress stimuli, and thereafter phosphorylate various substrates, such as transcription factors and other protein kinases, regulating cellular immune and stress responses (Kyriakis and Avruch, 2001; Widmann et al., 1999; Zarubin and Han, 2005). Activation of p38 is regulated by two classes of protein kinases, MAPK kinases (MAP2Ks) and MAP2K kinases (MAP3Ks). MAP3Ks phosphorylate and thereby activate MAP2Ks, and activated MAP2Ks in turn phosphorylate and activate MAPKs. In *Drosophila*, three p38-MAPK-encoding genes, *p38a* (also known as *Mpk2*), *p38b* and *p38c*, have been identified (Davis et al., 2008; Han et al., 1998a; Han et al., 1998b). As with mammalian p38s, *Drosophila* p38a and p38b have been shown to be activated by immune stimulation and various stressors, and flies lacking the *p38a* or *p38b* gene are susceptible to some environmental stressors (Craig et al., 2004; Cully et al., 2010; Han et al., 1998b). Recently, it has been shown that the *p38a* and *p38b* double-mutant flies are viable in near-sterile condition but hypersensitive to microbial infection (Chen et al., 2010). By contrast, p38c appears to be an atypical p38 MAPK, because it has mutations in the TGY motif, which is required for activating phosphorylation by MAP2Ks (Davis et al., 2008).

Recent studies have shed light on the tissue- and cell-type-specific roles of *Drosophila* p38 family members in innate immune responses. Oral ingestion of microbial components induces activation of *Drosophila* p38, and p38 in turn transcriptionally induces *Dual oxidase* (*Duox*) in the midgut of flies and contributes to the survival of flies (Ha et al., 2009). In hemocytes, p38b-mediated 'phagocytic encapsulation' of bacteria increases the infection tolerance of flies injected with *Salmonella typhimurium* (Shinzawa et al., 2009). Importantly, Hodgetts and colleagues have demonstrated that p38c is required for septic-injury-dependent *Ddc* expression in epidermal tissues (Davis et al., 2008). These findings suggest that the *Drosophila* p38 MAPKs have a physiological role in innate immunity, as has been shown with p38 in other species, such as mammals and *Caenorhabditis elegans* (Kim et al., 2002; Matsuzawa et al., 2005).

In the present study, we found that constitutive activation of *Drosophila* p38 in the dorsal midline of flies induced black pigmentation, which was a consequence of upregulated melanization. In this p38-induced melanization, *Drosophila* TH, Ddc and the nuclear receptor HR38 were found to play a crucial role. *Drosophila* HR38 belongs to the NR4A subfamily of nuclear receptors that function as transcription factors and has been shown to play pivotal roles in adult cuticle formation and atypical ecdysteroid signaling in flies (Baker et al., 2003; Kozlova et al., 2009; Kozlova et al., 1998). We found that p38 phosphorylated mammalian NR4As and *Drosophila* HR38 and potentiated these NR4As to transactivate the rat *TH* promoter, with this transactivation being, at least in part, mediated by the phosphorylation. This suggests an evolutionarily conserved role for p38 in the regulation of NR4As. We thus propose that p38-regulated gene induction through NR4A nuclear receptors serves as a regulatory mechanism for the dopamine biosynthesis pathway in the immune and stress responses in *Drosophila* and mammals.

Results

Ectopic expression of ASK1ΔN induces black pigmentation in the fly thorax

To explore target genes downstream of *Drosophila* p38, we took advantage of the MAP3K *Drosophila* ASK1 (also known as Pk92B) as a potential p38-activating MAP3K (Kuranaga et al., 2002). We constructed an expression vector for *Drosophila* ASK1ΔN, lacking the ASK1 N-terminal regulatory domain, which is similar in its primary structure to the mammalian ASK1ΔN that has been shown to act in a constitutively active manner (Mizumura et al., 2006; Takeda et al., 2000). In *Drosophila* S2 cells, p38a was strongly activated by coexpression with ASK1ΔN compared with that by wild-type (WT) ASK1, as determined by an antibody against phosphorylated p38 that specifically recognizes the dual-phosphorylation of Thr and Tyr residues in the well-conserved TGY motif in the activation loop of p38 (Fig. 1A; supplementary material Fig. S1A). We also found that p38b was activated by coexpression with ASK1ΔN, although we could not evaluate the activation state of p38c because of the lack of the TGY motif (supplementary material Fig. S1A,B). Although ASK1ΔN also activated *Drosophila* JNK, which is a further stress-activated MAPK, the extent of JNK activation by ASK1ΔN was similar to that by ASK1WT (Fig. 1B). These results suggest that ASK1ΔN acts as a constitutively active mutant selectively for the p38 pathway.

We next established a *Drosophila* transgenic strain harboring *UAS-DASK1ΔN* in which cDNA encoding ASK1ΔN was inserted downstream of the UAS binding sequence for the transcription factor GAL4. We then performed crosses of this strain with various 'driver' strains that express GAL4 through ubiquitous or tissue-specific promoters. When the *pannier* (*pnr*)-*GAL4* strain, in which GAL4 is expressed in a longitudinal dorsal domain extending along the thoracic and abdominal segments (Calleja et al., 2000), was crossed with the *UAS-DASK1ΔN* strain, the progeny (*pnr>ASK1ΔN*) exhibited black pigmentation in the thorax cuticle, which corresponded to the *pnr-GAL4* expression region in the thorax; no such pigmentation was observed in the *pnr-GAL4* flies without the *UAS-DASK1ΔN* transgene (Fig. 1C,D). To confirm that this pigmentation was induced by overexpressed ASK1ΔN, we generated transgenic flies expressing the inverted repeat (IR) RNA for *ASK1*, which specifically inhibits *ASK1* expression in a GAL4-dependent manner (*UAS-DASK1-IR*). The pigmentation in *pnr>ASK1ΔN* was suppressed by coexpression with *ASK1-IR*, excluding the possibility that this phenotype was caused by an artifact of the expression system (Fig. 1E).

Intriguingly, the pigmentation in *pnr>ASK1ΔN* was confined to the thorax, although *pnr-GAL4* is expressed in the dorsal midline both of the thorax and abdomen (Fig. 1C,D). This raised the possibility that the dorsal midline of the thorax is a preferential region where ASK1ΔN expression induces visible pigmentation. To test this possibility, we examined whether and where ubiquitous expression of ASK1ΔN induces pigmentation using a heat-shock-driven GAL4 (*hs*)*>ASK1ΔN* flies to ubiquitously express ASK1ΔN upon heat shock. These flies, which were subjected to a 1-hour heat shock at 37℃ during their pupal stage, showed the pigmentation in the lower medial region of the thorax cuticle, together with a defect in wing expansion

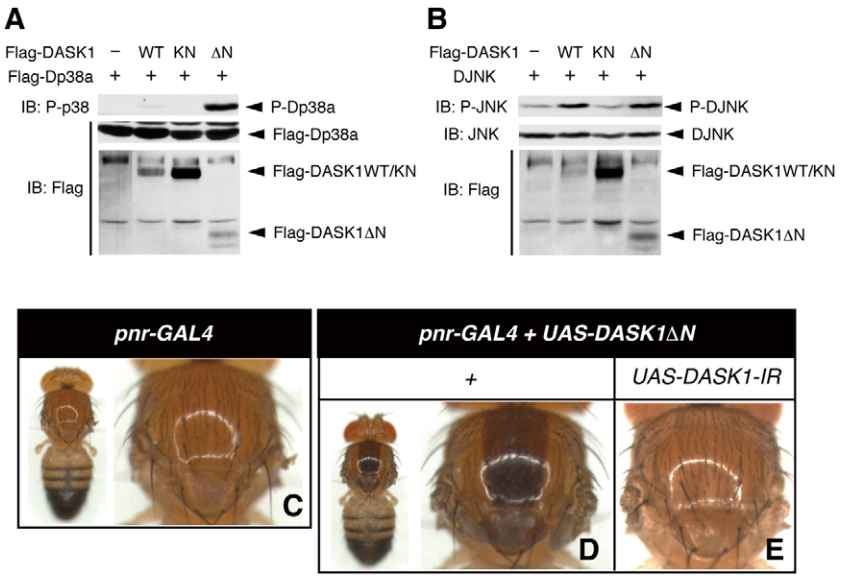

Fig. 1. Ectopic expression of ASK1ΔN induces black pigmentation in flies. (**A**) Activation of *Drosophila* p38 (Dp38) by *Drosophila* ASK1 (DASK1) in S2 cells. S2 cells were transiently transfected with pUAST-Flag-p38a alone or together with either pUAST-Flag-ASK1WT, KN or ΔN (WT, wild type; KN, kinase-negative) driven by pWAGAL4. Cell lysates were subjected to immunoblotting (IB). (**B**) Activation of *Drosophila* JNK (DJNK) by *Drosophila* ASK1 in S2 cells. S2 cells were transiently transfected with pUAST-JNK alone or together with either pUAST-Flag-ASK1WT, KN or ΔN driven by pWAGAL4. Cell lysates were subjected to immunoblotting. (**C**,**D**) ASK1ΔN-induced pigmentation in the thorax cuticle of *pnr>ASK1ΔN* fly. Whole flies (wings and legs were removed for observation) and magnified thoraxes of *pnr-GAL4/+* (C) and *UAS-DASK1ΔN/+*; *pnr-GAL4/+* (D) are shown. (**E**) ASK1ΔN-induced pigmentation is abolished by coexpression with ASK1-IR. Thoraxes of *UAS-DASK1ΔN/UAS-DASK1-IR*; *pnr-GAL4/+* are shown.

(Fig. 2A,B). By contrast, ASK1ΔN expression in immune-related tissues, such as fat body and hemocytes, did not induce visible pigmentation (data not shown). These results suggested that ASK1ΔN induces pigmentation preferentially in the dorsal midline of the thorax and that tissue-specific factor(s) and/or mechanism(s) are required for ectopic pigmentation.

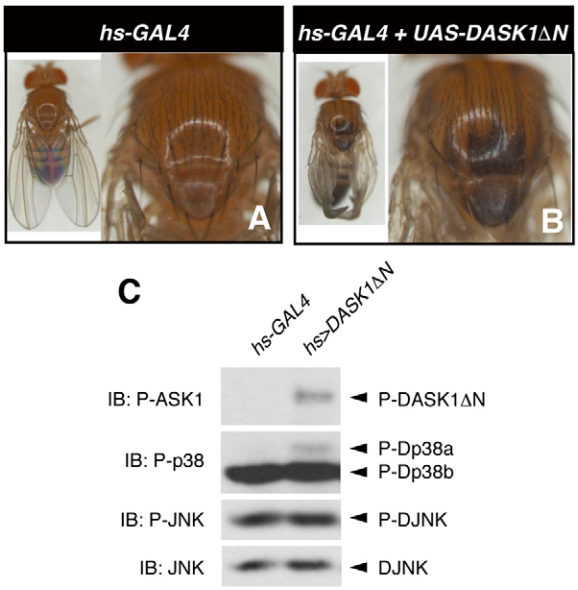

Fig. 2. *hs-GAL4*-dependent *Drosophila* ASK1ΔN expression induces black pigmentation in the thorax cuticle. (**A**,**B**) The *hs-GAL4/+* (A) and *UAS-DASK1Δ /+*; *hs-GAL4/+* (B) flies were subjected to a 1-hour heat shock at 37℃ during their pupal stage and were then maintained at 25℃ until they became adults. Whole flies and their magnified thoraxes are shown. (**C**) *hs-GAL4*-dependent ASK1ΔN expression induces *Drosophila* p38 activation. Adult males of *hs-GAL4/+* and *UAS-DASK1ΔN/hs-GAL4* were subjected to a 1-hour heat shock at 37℃ and then maintained at 25℃ for 5 hours. Flies were lysed and subjected to immunoblotting (IB).

ASK1ΔN-dependent pigmentation is mediated by the *Drosophila* p38 pathway

To evaluate the contribution of p38 in ASK1ΔN-dependent pigmentation, we examined whether expression of ASK1ΔN also selectively activates p38 in vivo (Fig. 2C). Heat-shock-dependent expression of ASK1ΔN in the *hs>ASK1ΔN* flies induced p38 activation (Fig. 2C, the upper and weaker phosphorylated p38 band in the blot), although the basal activity of p38 (the lower stronger band) was high even in the absence of ASK1ΔN expression, probably owing to the heat shock used for induction of *GAL4*; an analysis using the *p38a*-null and *p38b*-null flies suggested that the upper and lower phosphorylated p38 bands corresponded to the activated forms of p38a and p38b, respectively (supplementary material Fig. S1C). By contrast, ASK1ΔN expression did not induce JNK activation, indicating that ASK1ΔN expression also selectively activates p38 in vivo.

We next examined the requirement of the *Drosophila* p38 pathway for ASK1ΔN-induced pigmentation. Expression of dominant-negative p38a (p38a-DN) (Adachi-Yamada et al., 1999) or IR RNA-mediated knockdown of *p38a* or *p38b* in the *pnr>ASK1ΔN* flies abolished the pigmentation (Fig. 3A–C,J), whereas knockdown of *p38c* only had a marginal effect (Fig. 3D). The pigmentation was also suppressed by knockdown of a *Drosophila* MAP2K gene in the p38 pathway, *licorne* (*lic*) (also known as *Mkk3*) (Han et al., 1998b) (Fig. 3E), strongly suggesting that the p38 pathway is required for the ASK1ΔN-dependent pigmentation.

Knockdown of another MAP2K gene, *Mkk4*, attenuated the ASK1ΔN-dependent pigmentation (Fig. 3F). Because mammalian MKK4 is known to mediate both the JNK and p38 pathways, this result suggested that the ASK1ΔN-dependent pigmentation might be attributed to basal JNK activity, in addition to ASK1ΔN-induced p38 activation. However, it was difficult to assess the requirement of the *Drosophila* JNK pathway for the ASK1ΔN-induced pigmentation; the loss of JNK activity, accomplished by knockdown of JNK or overexpression of a dominant-negative form of JNK or the JNK phosphatase Puckered, in the *pnr-GAL4*-expressing region,

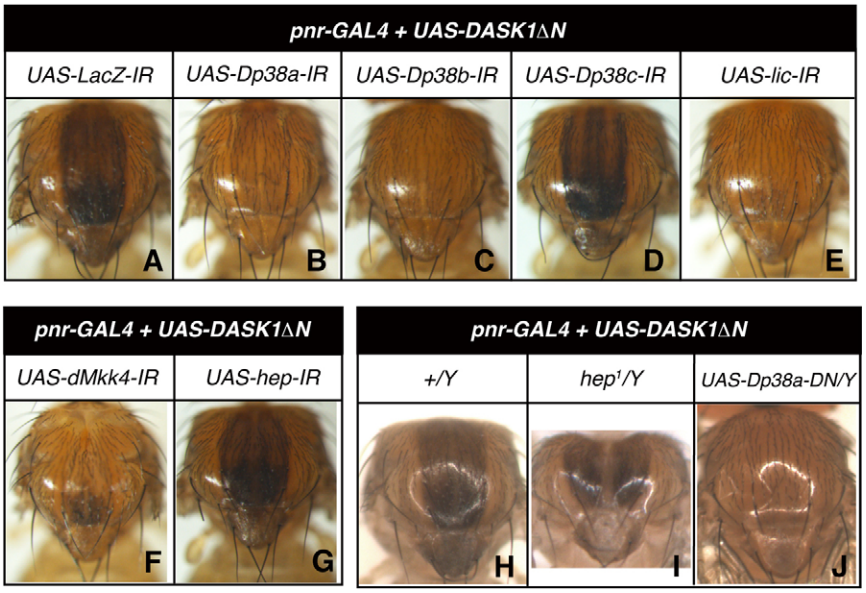

Fig. 3. *Drosophila* ASK1ΔN-dependent pigmentation is mediated by the *Drosophila* p38 pathway. Thoraxes of female flies with the following genotypes are shown: *UAS-DASK1ΔN /UAS-LacZ-IR*; *pnr-GAL4/+* (**A**), *UAS-DASK1ΔN/UAS-Dp38a-IR*; *pnr-GAL4/+* (**B**), *UAS-DASK1ΔN/+*; *pnr-GAL4/UAS-Dp38b-IR* (**C**), *UAS-DASK1ΔN/UAS-Dp38c-IR*; *pnr-GAL4/+* (**D**), *UAS-DASK1ΔN/UAS-lic-IR*; *pnr-GAL4/+* (**E**), *UAS-DASK1ΔN/+*; *pnr-GAL4/dMkk4-IR* (**F**), *UAS-DASK1ΔN/+*; *pnr-GAL4/UAS-hep-IR* (**G**), *+/Y*; *UAS-DASK1ΔN/+*; *pnr-GAL4/+* (**H**), hep^1*/Y*; *UAS-DASK1ΔN/+*; *pnr-GAL4/+* (**I**), and *UAS-Dp38a-DN/Y*; *UAS-DASK1ΔN/+*; *pnr-GAL4/+* (**J**).

mostly resulted in lethality before eclosion, probably owing to defects in dorsal or thorax closure (Riesgo-Escovar et al., 1996; Sluss et al., 1996; Zeitlinger and Bohmann, 1999). Among the strains we tested, the *pnr>ASK1ΔN* flies in a hemizygous background for hep^1, a hypomorphic allele of the gene encoding a MAP2K of the JNK pathway (Glise et al., 1995), and those crossed with the *UAS-hep-IR* (*4353R-2*) line avoided lethality but retained the ASK1ΔN-dependent pigmentation (Fig. 3G,I). The contribution of JNK to ASK1ΔN-dependent pigmentation might thus be relatively low compared with that of p38, although these results do not completely exclude the possibility that basal JNK activity plays some roles.

p38-dependent pigmentation is caused by melanization

Black and brown pigments in *Drosophila* cuticle are known to comprise dopa and dopamine melanin (Wittkopp et al., 2003). Fig. 4A shows the current model of the melanin biosynthesis pathway in *Drosophila* (True, 2003; Wittkopp et al., 2002). Tyrosine is converted into dopa by TH, and dopa is converted into dopamine by Ddc. Some fractions of dopa and dopamine are converted into dopa and dopamine melanin, respectively, by means of the PO system. Dopamine is also catalyzed into *N*-β-alanyl dopamine (NBAD) by Ebony, and NBAD is subsequently converted into yellowish NBAD sclerotin. The melanization pattern in the thorax cuticle appears to be determined by the balance of genes involved in the melanin synthesis pathway including *TH*, *Ddc* and *ebony*, all of which have been reported to be highly expressed in the epidermal cells of adult thorax (Gibert et al., 2007; Wittkopp et al., 2002). Consistent with the previous finding that overexpression of Ebony suppressed melanin accumulation, probably owing to a reduction in the levels of dopa and dopamine (Wittkopp et al., 2002), we found that coexpression of Ebony with ASK1ΔN abolished ectopic black pigmentation (Fig. 4B,C). Moreover, we found that, in a hemizygous background for a *yellow* (y^1) mutation, in which flies exhibit a yellowish body color compared with WT flies, pigmentation in the *ASK1ΔN*-expressing region, particularly in the scutellum (the one-fourth to one-third caudomedial portion in each photo), was brown rather than black (Fig. 4D,E). Because *yellow* is known to be required for the production of black melanin (Walter et al., 1991; Wittkopp et al., 2002), *yellow*-dependent production of dopa melanin appears to be required for black pigmentation in *pnr>ASK1ΔN* flies. These results suggested that upregulation of dopa and dopamine melanin was involved in *Drosophila*-p38-dependent pigmentation.

TH and Ddc are required for p38-dependent melanization

Given that the dopa and dopamine levels are regulated by TH and Ddc, we assessed whether TH and Ddc are involved in the p38-dependent melanization. Expression of IR RNA for *TH* or *Ddc*,

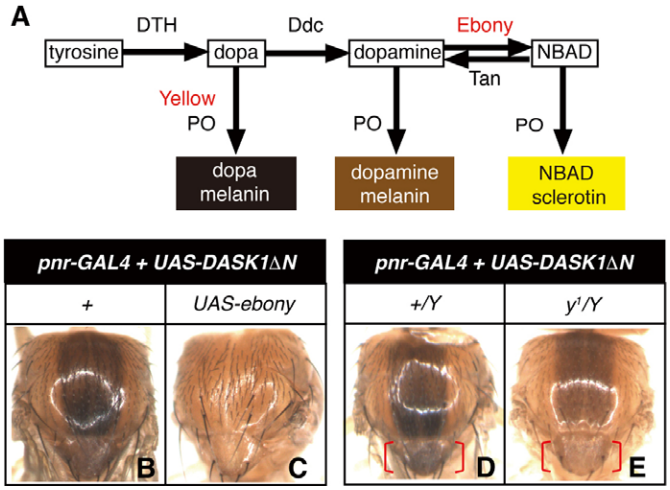

Fig. 4. *Drosophila* p38-dependent pigmentation is caused by melanization. (**A**) A current model of the *Drosophila* melanin biosynthesis pathway. (**B**,**C**) *Drosophila* ASK1ΔN-induced pigmentation is abolished by coexpression with Ebony. Thoraxes of *UAS-DASK1ΔN/+*; *pnr-GAL4/+* (B) and *UAS-DASK1ΔN/UAS-ebony*; *pnr-GAL4/+* (C) are shown. (**D**,**E**) ASK1ΔN-induced pigmentation is switched from black to brown in a hemizygous background for *yellow* mutation. The scutellum is enclosed by the brackets. Thoraxes of *+/Y*; *UAS-DASK1ΔN/+*; *pnr-GAL4/+* (D) and y^1*/Y*; *UAS-DASK1ΔN/+*; *pnr-GAL4/+* (E) are shown.

which targets all known splicing variants of each gene (Friggi-Grelin et al., 2003; Shen et al., 1993), attenuated basal pigmentation in the thorax of *pnr-GAL4* flies, indicating the requirement of TH and Ddc for basal pigmentation (Fig. 5A,B). Knockdown of either gene also abolished ASK1ΔN-induced pigmentation, suggesting that TH and Ddc were essential for p38-dependent melanization (Fig. 5C–E).

A recent report showing the role of p38c in the induction of *Ddc* in the epidermis of bacteria-injected flies motivated us to examine whether the ASK1ΔN-dependent p38 activation induced expression of *TH* and *Ddc* in flies (Davis et al., 2008). Although we initially examined the *pnr>ASK1ΔN*-dependent increase in the mRNA expression of *TH* and *Ddc* in adult flies, we failed to detect it, probably owing to high basal expression (Gibert et al., 2007). We therefore examined the *hs>ASK1ΔN* pupae and their control *hs>GFP* pupae 36–48 hours after their puparium formation, when *TH* and *Ddc* expression is reportedly decreased (Davis et al., 2007a). RNA was extracted from the pupae maintained at 25℃, in which only leaky expression of *GFP* or *ASK1ΔN* from the *hs* promoter was induced, avoiding the effect caused by heat-shock-dependent activation of p38. Quantitative real-time PCR (qRT-PCR) revealed that mRNA expression of *TH* and *Ddc* was induced in the *ASK1ΔN*-expressing pupae, compared with that in the control pupae (Fig. 5F), suggesting that *Drosophila* p38 activation induced the transcription of *TH* and *Ddc*.

HR38 is required for p38-dependent melanization

In mammals, the NR4A subfamily of nuclear receptors consists of three transcription factors: NR4A1 (also known as Nur77 and NGFI-B), NR4A2 (also known as Nurr1), and NR4A3 (also known as NOR-1) (Maxwell and Muscat, 2006). NR4A2 has been shown to regulate expression of the genes encoding tyrosine hydroxylase and aromatic L-amino acid decarboxylase (*AADC*), a mammalian ortholog of *Ddc*, and to play crucial roles in the development of dopaminergic neurons (Jankovic et al., 2005; Perlmann and Wallen-Mackénzie, 2004). NR4As bind to the NBRE (NGFI-B response element), through which NR4A2 regulates the mammalian *TH* promoter (Maxwell and Muscat, 2006). HR38 is the single *Drosophila* ortholog of the mammalian NR4A family and has been shown to bind to the NBRE (Baker et al., 2003; Fisk and Thummel, 1995). Consistent with this, it has recently been reported that HR38 binds to the NBRE-like sequence upstream of the *Ddc* gene and that ectopic expression of HR38 influences expression of *Ddc* in a tissue-dependent manner (Davis et al., 2007b). Furthermore, we found that four NBRE-like sequences existed in the *Drosophila TH* gene promoter (–2748 bp, –2318 bp, –2148 bp, and –459 bp from the transcription start site). These findings suggest that HR38 is involved in p38-dependent transcriptional control of *Drosophila TH* and *Ddc*.

To evaluate this possibility, we knocked down *HR38* by *pnr-GAL4*-dependent expression of IR RNA for *HR38*. Because of the lethality of the *pnr>DHR38-IR* flies at a conventional temperature of 25℃, we maintained the flies at 18℃ to reduce GAL4-dependent expression of *HR38-IR* and thus avoided the lethality (Fig. 5G). ASK1ΔN-induced melanization at 18℃, which was less obvious than that at 25℃, probably owing to the reduced GAL4 activity, was inhibited by coexpression of *HR38-IR* (Fig. 5H,I), suggesting that HR38 was required for the p38-dependent melanization. Taken together with the result that expression of *HR38* did not change upon *ASK1ΔN* expression (Fig. 5F), *Drosophila* p38 appears to induce *TH* and *Ddc* through post-translational activation of HR38.

Mammalian p38 activation potentiates NR4As to transactivate the rat *TH* promoter

Because the structure of the NR4A family factors including *Drosophila* HR38 is highly conserved among species (Baker et al., 2003; Wang et al., 2003), we expected that p38 regulates gene expression through NR4As by a conserved mechanism. We therefore evaluated the transactivation function

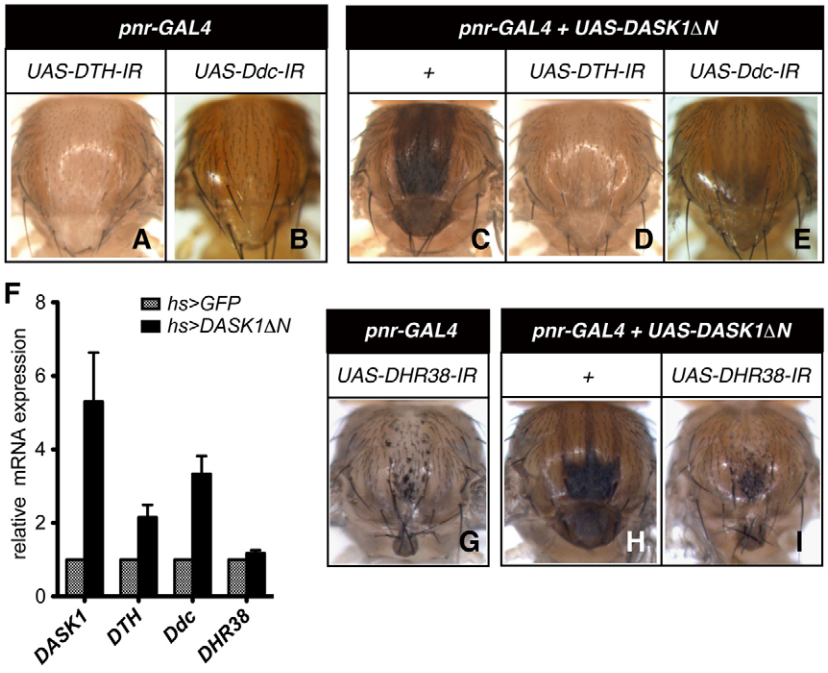

Fig. 5. *Drosophila* TH, Ddc, and HR38 are required for *Drosophila* p38-dependent melanization. (**A**–**E**) *Drosophila TH* and *Ddc* are required for ASK1ΔN-induced melanization. Thoraxes of *UAS-DTH-IR/+*; *pnr-GAL4/+* (A), *UAS-Ddc-IR/+*; *pnr-GAL4/+* (B), *UAS-DASK1ΔN/+*; *pnr-GAL4/+* (C), *UAS-DASK1ΔN/UAS-DTH-IR*; *pnr-GAL4/+* (D), *UAS-Ddc-IR/+*; *UAS-DASK1ΔN/+*; *pnr-GAL4/+* (E) are shown. (**F**) mRNA expression of *Drosophila TH* (*DTH*) and *Ddc* is induced by *Drosophila* ASK1ΔN (DASK1ΔN) expression. Total RNA isolated from pupae of *UAS-GFP^{S65T}/+*; *hs-GAL4/+ (hs>GFP)* and *UAS-DASK1ΔN/+*; *hs-GAL4/+ (hs>DASK1ΔN)* at 36–48 hours after puparium formation was subjected to qRT-PCR analysis for the indicated genes. Results shown are the means ± s.e.m. for five independent RNA preparations from 7–10 pupae each. (**G**–**I**) HR38 is required for ASK1ΔN-induced melanization. Thoraxes of *UAS-DHR38-IR/+*; *pnr-GAL4/+* (G), *UAS-DASK1ΔN/+*; *pnr-GAL4/+* (H), and *UAS-DASK1ΔN/UAS-DHR38-IR*; *pnr-GAL4/+* (I) are shown. Flies were crossed and maintained at 18℃ to avoid the lethality.

of NR4As by coexpressing the reporter gene *TH/–9kb*, which contained the rat *TH* promoter (–9 kb to +15 bp) fused to the luciferase gene (Iwawaki et al., 2000), in rat PC12 cells and measuring the relative luciferase activity. Although expression of mammalian ASK1ΔN or either of the NR4As activated the promoter to some extent, coexpression of ASK1ΔN and either of NR4As synergistically activated the promoter (Fig. 6A). The reporter gene *TH/NBRE1* containing three copies of NBRE, but not the control gene *TH/NBRE1-mt* containing the same copies of mutated NBRE sequences, was activated only when either of NR4As was coexpressed with ASK1ΔN (Fig. 6B), indicating that mammalian ASK1ΔN potentiated NR4As to transactivate the rat *TH* promoter through the NBREs. Importantly, synergistic activation of the *TH/–9kb* promoter by coexpression of ASK1ΔN and NR4A2 was dose-dependently suppressed by treatment with the p38 inhibitor SB203580 but not with the JNK inhibitor SP600125 (Fig. 6C), suggesting that p38 activation was required for the ASK1ΔN-induced transactivation functions of NR4As. Moreover, coexpression of NR4A2 with the constitutively active MKK6 (also known as MAP2K6) mutant MKK6DD, which is a MAP2K that selectively activates p38, induced synergistic activation of the *TH/–9kb* promoter (Fig. 6D), suggesting that activation of p38 is sufficient to potentiate NR4As to transactivate the *TH* promoter.

p38 directly phosphorylates NR4As

Because *Drosophila* p38 activation did not upregulate *HR38* expression (Fig. 5F), we sought to examine whether p38 regulates NR4As by protein modification such as phosphorylation. To this end, we used a bacterially expressed GST-tagged N-terminal portion of NR4A2 (GST–NR4A2-NT) as a substrate of p38 in an in vitro kinase assay. This portion of NR4A2 includes most of consensus phosphorylation sites targeted by p38 (Ser-Pro or Thr-Pro; indicated as red arrowheads in Fig. 7D). Whereas p38α (also known as MAPK14) and p38β (also known as MAPK11) phosphorylated GST–NR4A2-NT only faintly, both of them were activated by coexpression with ASK1WT, but not with kinase-negative (KN) form of ASK1, and effectively phosphorylated GST–NR4A2-NT (Fig. 7A). Because ASK1WT alone did not phosphorylate NR4A2-NT (data not shown), p38 MAPKs activated by ASK1 appear to directly phosphorylate NR4A2.

To assess whether p38 MAPKs also phosphorylated NR4As within cells, we treated, or left untreated, lysates of NR4A2-expressing cells with λ-phosphatase (λPPase) and subjected them to immunoblotting (Fig. 7B). PPase-treated NR4A2 migrated slightly faster than untreated NR4A2 (Fig. 7B, lanes 1 and 2), suggesting that NR4A2 was basally phosphorylated to some extent. Whereas coexpression of p38β did not affect the migration of NR4A2 probably due to the inactive states of p38β as determined using an antibody against phosphorylated p38 (Fig. 7B, lanes 3 and 4), coexpression of p38β and ASK1ΔN markedly retarded the migration of NR4A2 in a manner sensitive to λPPase (Fig. 7B, lanes 5 and 6). We also found that, in HEK-293 cells coexpressing p38β and ASK1ΔN, HR38 and NR4A2 exhibited similarly retarded migrations, both of which were sensitive to SB203580 (Fig. 7C). These results suggested that p38 phosphorylated NR4As within cells.

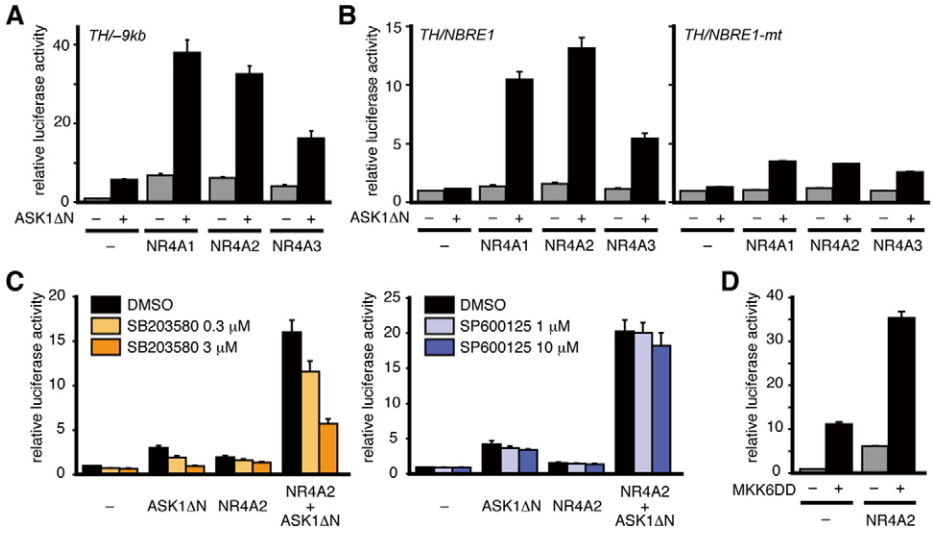

Fig. 6. Mammalian p38 activation potentiates NR4As to transactivate the rat *TH* promoter. (**A**) Mammalian ASK1ΔN induces the transactivation function of NR4As. The rat *TH/–9kb* reporter gene and one of the NR4As, in the presence or absence of ASK1ΔN, were transiently expressed in PC12 cells. The luciferase activity was measured and converted into a fold increase relative to that of the cells transfected with an empty vector (first column). Results shown are representative of three independent experiments and are the means ± s.e.m. for triplicate determinations. (**B**) ASK1ΔN-induced transactivation functions of NR4As are mediated largely through NBREs. The *TH/NBRE1* or *TH/NBRE1-mt* reporter gene and either of NR4As, in the presence or absence of ASK1ΔN, were transiently expressed in PC12 cells. The luciferase activity was measured and shown as in A. (**C**) Mammalian p38 but not JNK is required for the ASK1ΔN-induced transactivation functions of NR4A2. NR4A2 and/or ASK1ΔN together with the *TH/–9kb* reporter gene were transiently expressed in PC12 cells. At 4 hours after the transfection, the indicated concentrations of SB203580 or SP600125 were added to the culture medium. After a further 20 hours, the luciferase activity was measured and indicated as a fold increase relative to that of control DMSO-treated cells transfected with an empty vector (black columns). Results shown are representative of three independent experiments and are the means ± s.e.m. for triplicate determinations. (**D**) Mammalian p38 is sufficient to activate NR4A2. NR4A2 and/or a constitutively active mutant of MKK6 (MKK6DD) together with the *TH/–9kb* reporter gene were transiently expressed in PC12 cells. The luciferase activity was measured and shown as in A.

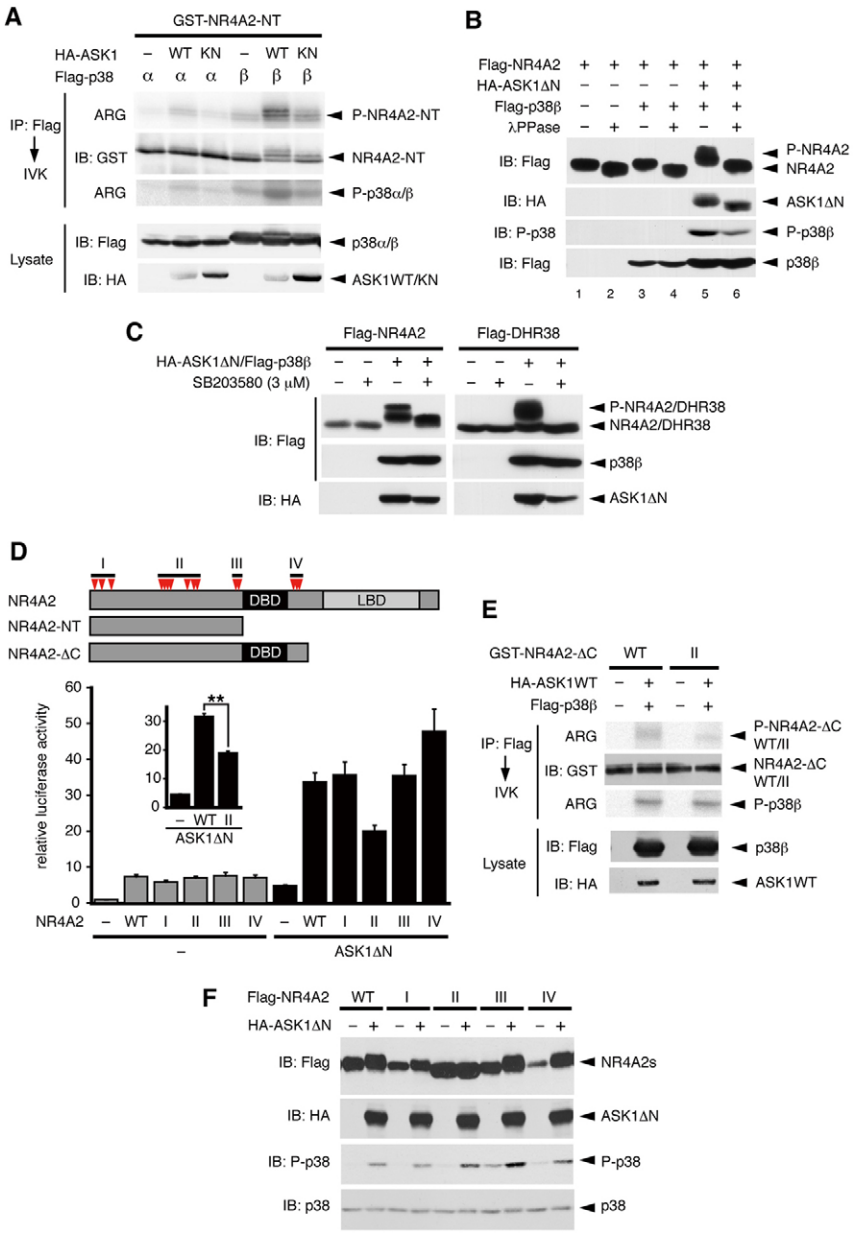

Fig. 7. Mammalian p38 potentiates NR4As, at least in part, by phosphorylation. (**A**) p38α and p38β phosphorylate NR4A2 in vitro. The indicated tagged proteins (WT, wild type; KN, kinase-negative) were transiently expressed in HEK-293 cells and were immunoprecipitated (IP) with anti-Flag antibody. The immune complex was incubated with recombinant GST–NR4A2-NT (1–261 amino acids) in a kinase buffer in vitro. Samples were subjected to SDS-PAGE followed by autoradiographing (ARG) and immunoblotting (IB) for the indicated tags. The proteins detected are shown on the right-hand side (P- indicates the phosphorylated form). (**B**) p38 phosphorylates NR4A2 in HEK-293 cells. The indicated expression plasmids were transiently transfected in HEK-293 cells. Cell lysates were divided into two aliquots: one was untreated and the other was treated with λPPase. Samples were subjected to immunoblotting. (**C**) p38 phosphorylates *Drosophila* HR38 (DHR38) in HEK-293 cells. The indicated expression plasmids were transiently transfected in HEK-293 cells. At 4 hours after the transfection, 3 μM of SB203580 was added to the culture medium. After a further 24 hours, cell lysates were subjected to immunoblotting. (**D**) ASK1ΔN-induced transactivation activity is reduced in the cluster II mutant of NR4A2. The diagram at the top shows the structural features of full-length, N-terminal 261 amino acids (NT) and N-terminal 369 amino acid (ΔC) forms of NR4A2, the latter two of which were used as GST fusion proteins in Fig. 7A and E, respectively. A total of 15 potential phosphorylation sites targeted by p38 (Ser-Pro or Thr-Pro; indicated as red arrowheads) in both human and mouse NR4A2 and the four clusters of the potential phosphorylation sites that we designated I, II, III and IV are also shown. The rat *TH/–9kb* reporter gene and either of the four mutants, in which all the potential Ser and Thr residues in each cluster were replaced with Ala (columns I, II, III or IV), in the presence or absence of ASK1ΔN, were transiently expressed in PC12 cells. The luciferase activity was measured and indicated as a fold increase relative to that of cells transfected with an empty vector (–, first column). Results shown are representative of two independent experiments and are the means ± s.e.m. for triplicate determinations. The inset shows that the difference in the extent of ASK1ΔN-induced transactivation activity between WT NR4A2 and the cluster II mutant was statistically significant (**$P<0.01$, using a Student *t*-test). Results shown are the means ± s.e.m. for six independent experiments performed in duplicate or triplicate determinations. (**E**) NR4A2 cluster II includes p38β phosphorylation site(s). In vitro kinase (IVK) assays were performed as in Fig. 7A using recombinant GST–NR4A2-ΔC (WT; all the potential phosphorylation sites are preserved) and its cluster II alanine mutant (II) as substrates. (**F**) p38 phosphorylates NR4A2 cluster II in HEK-293 cells. The indicated expression plasmids were transiently transfected in HEK-293 cells. After 24 hours, cell lysates were subjected to immunoblotting.

p38 MAPKs potentiate NR4As, at least in part, by phosphorylation

We next examined the involvement of p38-dependent phosphorylation of NR4As in their transactivation functions. A total of 15 potential (consensus) phosphorylation sites targeted by p38 (Ser-Pro or Thr-Pro) exist in both human and mouse NR4A2 as depicted in the diagram in Fig. 7D. Although we initially tested the NR4A2 mutants carrying single replacements of all potential sites with Ala residues, the ASK1ΔN-induced of none of the mutants had significantly different transactivation functions from that of WT NR4A2 (data not shown). We therefore examined the effect of combinational Ala mutations on NR4A2 activity. The 15 potential phosphorylation sites are distributed to four clusters that we designated I (Ser11, Ser16 and Thr36), II (Ser126, Thr129, Thr132, Ser140, Thr168, Ser18 and Thr185), III (Ser250 and Ser256) and IV (Ser351, Ser356 and Ser359). We generated four mutants, in which all potential Ser and Thr residues in each cluster were replaced with Ala. Each of these mutants activated the *TH/–9kb* promoter to a similar extent to that of WT NR4A2 (Fig. 7D, left six columns), indicating that these cluster mutants maintained their basal transactivation functions. Among the four mutants, however, ASK1ΔN-dependent synergistic activation of the *TH/–9kb* promoter by the cluster II mutant was partially, but significantly, reduced compared with that by WT (Fig. 7D, right six columns, and *inset*), suggesting that phosphorylation of multiple Ser and Thr residues in cluster II was required for p38-dependent activation of NR4A2.

To examine whether cluster II actually includes p38 phosphorylation site(s), we generated a GST-tagged NR4A2-ΔC protein that lacked C-terminal ligand-binding domain (LBD) and either contained all the phosphorylation site clusters (NR4A2-ΔC WT) or had the Ala replacement mutations in cluster II (NR4A2-ΔC II) (Fig. 7D). In vitro kinase assays using these proteins as substrates revealed that NR4A2-ΔC II was less phosphorylated by p38β than NR4A2-ΔC WT (Fig. 7E). Moreover, the retarded migration of NR4A2 coexpressed with ASK1ΔN was only not observed in the cluster II mutant (Fig. 7F). These results suggest that p38 activation potentiated NR4As, at least in part, by direct phosphorylation of cluster II in NR4As to transactivate the *TH* promoter.

Discussion

Here, we found that ectopic expression of *Drosophila* ASK1ΔN induced p38-dependent black pigmentation, which was caused by upregulated melanization. We also found that *Drosophila* p38 activation induced *TH* and *Ddc* expression and that these genes were essential for the p38-dependent melanization. Taking into account the diverse functions of p38 in immune and stress responses in a wide variety of organisms, regulation of basal and/or stimulation-induced *Drosophila TH* and *Ddc* expression might be an important device for p38 to exert its protective activity against harmful environmental stimuli in *Drosophila*.

Several lines of evidence have revealed that expression of *Drosophila TH* and *Ddc* is regulated at the transcription level in various physiological and pathological conditions. In the developmental process, these genes are transcriptionally upregulated before eclosion and play a role in the pigmentation of the adult cuticle (Davis et al., 2007a). Upon wounding, by pricking with a sterile needle, *Drosophila TH* and *Ddc* expression is induced in the epidermal cells near the wound site and contributes to repair the integument (Mace et al., 2005; Pearson et al., 2009). Septic injury with microbes is another setting in which regulation of *Drosophila TH* and *Ddc* expression is involved, as described in the introduction. Importantly, *Drosophila p38c* has been shown to be selectively required for *E.-coli*- and *S.-aureus*-induced *Ddc* expression in the epidermis (Davis et al., 2008). In our genetic interaction studies, we found that *Drosophila p38a* and *p38b*, rather than *p38c*, were required for ASK1ΔN-induced melanization (Fig. 3). Thus, not only *Drosophila* p38c but also p38a and p38b might regulate *TH* and *Ddc* expression in a manner dependent on cell types and/or cellular context.

We have shown, using the *hs* driver strain, that the dorsal midline of the thorax is the preferential region where expression of *Drosophila* ASK1ΔN induces visible melanization (Fig. 2). One plausible explanation for this regional selectivity is that *Drosophila* p38 activated by ASK1ΔN induces *TH* and *Ddc* expression efficiently in cells in the corresponding region of the thorax. Alternatively, it is possible that, although *Drosophila* p38 induces *TH* and *Ddc* in a wide range of cells, the upregulated *TH* and *Ddc* contribute to visible melanization only in the dorsal midline of the thorax. In the latter case, *Drosophila* p38-dependent regulation of *TH* and *Ddc* might have roles, such as in immune and stress responses, that are independent from melanization in tissues other than the thorax cuticle.

To investigate further how *Drosophila* p38 regulates *TH* and *Ddc* expression, we focused on HR38, a member of the NR4A nuclear receptor family, and found that it was also required for *Drosophila* p38-dependent melanization (Fig. 5). NR4A nuclear receptors are classified as a subgroup of orphan nuclear factors. However, NR4As have recently been found to function independently from ligand binding (Baker et al., 2003; Wang et al., 2003) and, instead, are regulated by their expression levels and/or post-transcriptional regulation. At least in *Drosophila* p38-dependent melanization, HR38 appeared to be regulated post-transcriptionally rather than transcriptionally, because we found that *Drosophila* ASK1ΔN expression did not induce mRNA expression of HR38 (Fig. 5F). Consistent with this, we found that mammalian p38 MAPKs directly phosphorylated mammalian NR4As (Fig. 7), consistent with the previous findings that several other kinases, including Akt, ERK, JNK and RSK, phosphorylated NR4As (Kolluri et al., 2003; Masuyama et al., 2001; Slagsvold et al., 2002; Wingate et al., 2006). Among the NR4A2 mutants we generated, in which potential phosphorylation sites targeted by p38 were replaced with Ala residues, only the cluster II mutant failed to be efficiently activated by mammalian p38 (Fig. 7D), suggesting that phosphorylation of multiple Ser and Thr residues in the cluster II is required for p38-induced activation of NR4A2. The partial reduction of both p38-dependent phosphorylation and activation of the cluster II mutant also suggests that a combination of phosphorylation of other clusters with that of cluster II is required (Fig. 7D,E). Comparison of amino acid sequences of NR4As (Fig. 8) reveals that most of potential phosphorylation sites targeted by p38 in each protein are in the relatively unconserved regions, not in the highly conserved DNA-binding and ligand-binding domains, and that the relative locations of these sites within a molecule vary among NR4As with the exception of the serine residue corresponding to Ser181 of NR4A2. This information supports the idea that a combination of phosphorylation of multiple residues, rather than phosphorylation of one crucial residue, might be a common

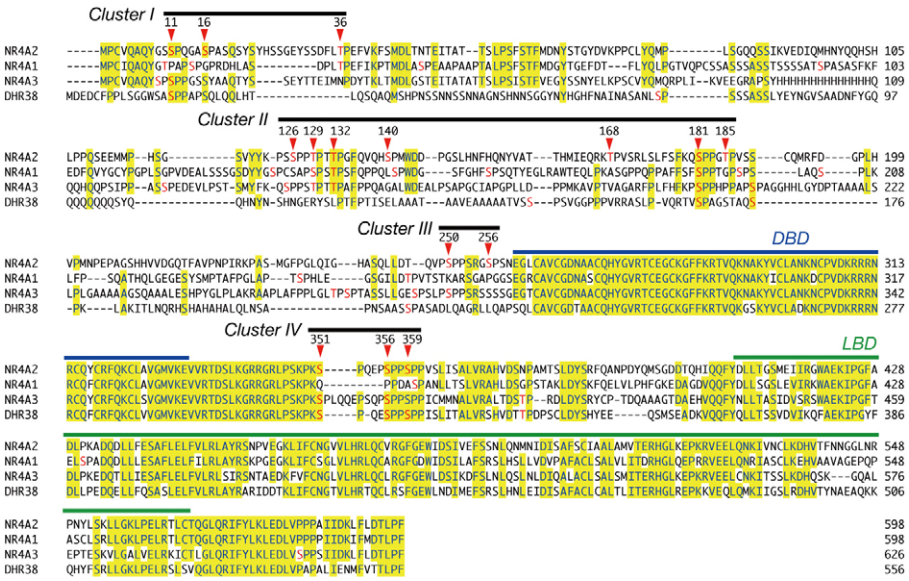

Fig. 8. ClustalW alignment of the amino acid sequences of NR4A nuclear receptors (human NR4A2, NR4A1 and NR4A3 and *Drosophila* HR38). Residues identical among three or four proteins are shown on a yellow background. Potential phosphorylation sites targeted by p38 (Ser-Pro or Thr-Pro) are shown in red letters. The 15 potential phosphorylation sites in NR4A2 are indicated as red arrowheads with their amino acid positions given. The four clusters of the potential phosphorylation sites that we designated I, II, III and IV are indicated with the black lines. The DNA-binding domain (DBD) and ligand-binding domain (LBD) are indicated as blue and green lines, respectively.

transactivation mechanism of NR4As induced by p38 MAPKs. Nevertheless, other mechanisms (e.g. p38-dependent induction and/or recruitment of certain coactivators of NR4As) might facilitate the phosphorylation-induced transactivation functions of NR4As.

In mammals, NR4A2 has been shown to regulate the expression of genes such as *TH* and *AADC* that are important in the synthesis and storage of dopamine in central dopaminergic neurons (Jankovic et al., 2005; Perlmann and Wallen-Mackénzie, 2004). As one of our approaches to elucidate the role of p38 and NR4Rs in mammals, we examined dopamine levels in brains from ASK1-deficient adult mice, in which basal p38 activity is reduced compared with those from WT mice (Takeda et al., 2004). No significant difference in dopamine levels between WT and ASK1-deficient whole brains was detected (data not shown). However, dopamine levels are increased, at least in the striatum and prefrontal cortex, in ASK1-deficient mice (Kumakura et al., 2010), suggesting that the basal activity of p38 is not involved in the upregulation of dopamine levels, at least in the healthy mouse brain. Recently, it has been shown that genetic mutations of *NR4A2* are associated with Parkinson's disease, a neurodegenerative disorder primarily affecting dopaminergic neurons (Le et al., 2003). Given that various potential roles of p38 in the regulation of the nervous system have been proposed (Takeda and Ichijo, 2002), p38 might participate in the pathophysiological regulation of dopaminergic neurons through NR4A2.

In correlation with the possible involvement of *Drosophila* p38-dependent induction of *Drosophila TH* and *Ddc* in immune responses in flies as discussed above, mammalian NR4As have also been shown to be induced by inflammatory stimuli and to regulate inflammatory gene expression (Pei et al., 2005; Pei et al., 2006). Further analyses of the regulatory mechanisms of NR4As by p38 both in *Drosophila* and mammals will shed light on the precise roles of p38–NR4As axis in immune responses. Moreover, we expect that our model of p38-dependent melanization in *Drosophila* will provide a useful tool to genetically search for signaling components that are crucially involved in the p38-dependent regulation of immune and stress responses.

Materials and Methods

Expression plasmids

pUAST-ASK1 wild type (WT) was described previously (Kuranaga et al., 2002). cDNA encoding *Drosophila* ASK1ΔN that lacked N-terminal 558 amino acids was generated by PCR and subcloned into pUAST (pUAST-ASK1ΔN). ASK1 cDNAs of WT, kinase-negative [KN; Lys618 was substituted by Met as described previously (Kuranaga et al., 2002)], and ΔN were subcloned into pUAST with an N-terminal Flag tag. *Drosophila* p38a, p38b, p38c and HR38 cDNAs were cloned by reverse transcript (RT)-PCR from total RNA isolated from *Drosophila* embryos or adult flies and subcloned into pUAST and pcDNA3 (Invitrogen) with an N-terminal Flag tag, respectively. pUAST-JNK was obtained from Makoto Nakamura (Matsuyama University, Japan). Head-to-head inverted repeat constructs, pUAST-ASK1-IR, pUAST-TH-IR and pUAST-HR38-IR, were generated by inserting cDNA fragments of *Drosophila* ASK1 [nucleotides (nt) 1759–2257], TH (nt 244–720 of variant I) and HR38 (nt 1–139), respectively, into pUAST-R57 (a gift from Ryu Ueda, National Institute of Genetics, Mishima, Japan). A driver plasmid pWAGAL4 that expresses GAL4 under the control of the *actin5C* promoter was a gift from Yasushi Hiromi (National Institute of Genetics, Mishima, Japan) pcDNA3 expressing HA–ASK1ΔN, HA–ASK1WT, HA–ASK1KN, Flag–mouse-p38α and Flag–mouse-p38β were described previously (Mizumura et al., 2006; Nishitoh et al., 1998; Saitoh et al., 1998). pcDNA3-Flag-NR4A2 was generated by subcloning mouse NR4A2 cDNA from pCMX-Nurr1 (Iwawaki et al., 2000). cDNA encoding the N-terminal 261 and 369 amino acids of NR4A2 (NR4A2-NT and NR4A2-ΔC, respectively) were generated by PCR and subcloned into pGEX4T-1 (GE Healthcare). cDNAs encoding mouse NR4A1 and NR4A3 were cloned by RT-PCR from total RNA isolated from RAW264.7 cells and subcloned into pcDNA3-Flag. A constitutively active mutant of human MKK6 (MKK6DD; Ser208 and Thr211 were both replaced with Asp) and a series of Nurr1 mutants, in which Ser and/or Thr were replaced with Ala, were obtained using the QuikChange site-directed mutagenesis kit (Stratagene) and subcloned into pcDNA3, with a C-terminal HA tag, and pcDNA3-Flag, respectively. pTH/–9kb, pTH/NBRE1, and pTH/NBRE1-mt were as described previously (Iwawaki et al., 2000). pGL4.70 [hRluc] was purchased from Promega.

Antibodies and reagents

Mouse monoclonal antibody to Flag tag (M2) and rat monoclonal antibody to the HA tag (clone 3F10) were purchased from Sigma and Roche, respectively. Phosphorylation-specific antibodies for JNK (Thr183 and Tyr185) and p38 (Thr180 and Tyr182) MAPKs were purchased from Cell Signaling. Anti-JNK rabbit polyclonal antibody (JNK1-FL) and anti-p38 goat polyclonal antibody were purchased from Santa Cruz Biotechnology. Anti-GST goat polyclonal antibody was purchased from GE Healthcare. Phosphorylation-specific antibodies to Thr838 of human ASK1 was used for monitoring *Drosophila* ASK1 activity as previously described (Kuranaga et al., 2002). The p38 inhibitor SB203580 and the JNK inhibitor SP600125 were purchased from Calbiochem.

Fly stocks and generation of transgenic flies

Driver strains and *hs-GAL4* (Bloomington *Drosophila* Stock Center) were used for the ectopic expression of *Drosophila* ASK1WT and ASK1ΔN. *UAS-Dp38a-DN*

(Adachi-Yamada et al., 1999), *UAS-ebony* (Wittkopp et al., 2002), *UAS-GFP^{S65T}*, and *UAS-LacZ-IR* (Kennerdell and Carthew, 2000) strains and *hep^{1}* (Glise et al., 1995), and *y^{1}w^{1}* (Wittkopp et al., 2002) mutant strains were used for genetic interaction assays. Other IR strains, *UAS-Dp38a-IR* (5475R-2), *UAS-Dp38b-IR* (7393R-1), *UAS-hep-IR* (CG4153R-2), *UAS-dMKK4-IR* (9738R-1), and *UAS-Ddc-IR* (10697R-1) were provided by NIG-FLY stock center, and *UAS-lic-IR* (20166) and *UAS-Dp38c-IR* (105173) were provided by Vienna *Drosophila* RNAi Center. Mutant strains of *p38a* (Crag et al., 2004) and *p38b* (Shinzawa et al., 2009) were used for characterization of phosphorylated *Drosophila* p38 bands (see supplementary material Fig. S1). For generation of transgenic flies harboring *UAS-DASK1ΔN*, *UAS-DASK1-IR*, *UAS-DTH-IR*, and *UAS-DHR38-IR*, each pUAST plasmid was injected into *w^{1118}*; *Dr/TMS, Sb P[ry^{+}, Δ2–3]* embryos as described previously (Kuranaga et al., 2002). We carried out crosses between *Drosophila* strains at 25℃ by standard procedures.

Cell culture and transfection

S2 cells were cultured in Schneider's *Drosophila* medium (Gibco) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin G and 5 mg/ml Bacto Pepton (BD Difco) at 26℃. PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 10% heat-inactivated horse serum and 100 units/ml penicillin G under a 5% CO_2 atmosphere at 37℃. HEK-293 cells were cultured in DMEM containing 10% FBS, 4.5 mg/ml glucose and 100 units/ml penicillin G under a 5% CO_2 atmosphere at 37℃. Transfection of expression plasmids into S2 cells, PC12 cells and HEK293 cells was performed with Cellfectin (Invitrogen), Lipofectamine 2000 (Invitrogen) and FuGENE6 (Roche), respectively, according to the manufacturers' instructions. We differentially used PC12 cells and HEK-293 cells for the NBRE-dependent promoter assays and the transfection-based phosphorylation assays, respectively; the former assays have been shown to depend upon cell types (Iwawaki et al., 2000) and worked well in PC12 cells, but not in HEK-293 cells, whereas the latter assays require a high transfection efficiency, which was achieved in HEK-293 cells, but not in PC12 cells.

Immunoblotting analysis

Cells or flies were lysed with a lysis buffer [1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 μg/ml aprotinin]. In Fig. 7F, another lysis buffer [1% Triton X-100, 1% deoxycholate, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 1 mM PMSF, 5 μg/ml Leupeptin and PhosSTOP (Roche)] was used. Cell or fly extracts were clarified by centrifugation, and the supernatants were resolved by SDS-PAGE and electroblotted onto PVDF membranes. After blocking with 5% skimmed milk powder in TBS-T (50 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.05% Tween 20), the membranes were probed with antibodies. The antibody–antigen complexes were detected using the ECL system (GE Healthcare).

qRT-PCR analysis

UAS-GFP^{S65T}/+; *hs-GAL4/+* and *UAS-DASK1ΔN/+*; *hs-GAL4/+* pupae were maintained at 25℃, at which only leaky expression from the heat-shock promoter was induced. Total RNA was isolated at 36–48 hours after puparium formation using TRIzol (Invitrogen) and reverse transcribed with the QuantiTect Reverse Transcript Kit (QIAGEN). Quantitative PCR was performed with Power SYBR Green PCR Master Mix using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The following oligonucleotides were used: *rp49* forward, 5′-CGGATCGATATGCTAAGCTGT-3′; *rp49* reverse, 5′-GCGCTTGTTCGATCCGTA-3′; *ASK1* forward, 5′-CCCCCTCCATAATATCACTCAC-3′; *ASK1* reverse, 5′-AACCCCTTTATTCTCCCTCTTAA-3′; *TH* forward, 5′-TTCGGAGGCGGCATTG-3′; *TH* reverse, 5′-ACAGCCGACCAAGAACGATT-3′; *Ddc* forward, 5′-TCTGGAGAATATACGCGAAAGG-3′; *Ddc* reverse, 5′-CACTTCTCCGGCTTCTCG-3′; *HR38* forward, 5′-GCGTTCTGTGATCAGGGTTAGG-3′; *HR38* reverse, 5′-GCACAACCTGGAGATCGACAT-3′. The expression value of each gene was normalized to that of the control *rp49* gene and represented as relative mRNA expression by setting the value of *hs>GFP* as 1.

Luciferase reporter assays

PC12 cells seeded in 12-well plates were transiently transfected with expression and reporter plasmids using Lipofectamine 2000 (Invitrogen). Each transfection included 0.2 μg of pGL4.70 [hRluc] (Promega) for normalization of transfection efficiency and 0.3 μg of a reporter plasmid. The total amount of plasmid DNA was kept at 1 μg/well by supplementation with empty pcDNA3. At 24 hours after the transfection, cell extracts were analyzed for firefly luciferase and *Renilla* activities using the dual luciferase kit (Promega).

In vitro kinase assays

GST–NR4A2-NT, GST–NR4A2-ΔC WT and GST–NR4A2-ΔC II were prepared as described previously (Saitoh et al., 1998). At 24 hours after the transfection, HEK-293 cells were lysed with a lysis buffer (1% Triton X-100, 1% deoxycholate, 12 mM β-glycerophosphate, 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and 5 μg/ml aprotinin). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with anti-Flag antibody gel (M2 gel, Sigma). The immune complex was incubated in a kinase buffer containing 50 mM Tris-HCl pH 8.0, 20 mM $MgCl_2$, 1 mM DTT, 100 μM ATP and 0.3 μCi of [γ-^{32}P]ATP together with GST–NR4A2-NT at 25℃ for 20 minutes. Kinase reactions were stopped by adding SDS sample buffer and samples were subjected to SDS-PAGE. Phosphorylation of substrate proteins was analyzed by storage phosphor screen autoradiography using the STORM imaging system 840 (GE Healthcare).

Phosphatase treatment

Cell lysate was treated with 2 units/μl λ protein phosphatase (λPPase, New England Biolabs) in a buffer containing 50 mM Hepes pH 7.5, 5 mM DTT, 0.1 mM EDTA, 0.01% Brij-35 and 2 mM $MnCl_2$ at 30℃ for 30 minutes. Reactions were stopped by adding SDS sample buffer.

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