Dissection of the pathway required for generation of vitamin A and for *Drosophila* phototransduction

Tao Wang, Yuchen Jiao, and Craig Montell

Department of Biological Chemistry, Department of Neuroscience, and Center for Sensory Biology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

Dietary carotenoids are precursors for the production of retinoids, which participate in many essential processes, including the formation of the photopigment rhodopsin. Despite the importance of conversion of carotenoids to vitamin A (all-trans-retinol), many questions remain concerning the mechanisms that promote this process, including the uptake of carotenoids. We use the *Drosophila* visual system as a genetic model to study retinoid formation from β-carotene. In a screen for mutations that affect the biosynthesis of rhodopsin, we identified a class B scavenger receptor, SANTA MARIA. We demonstrate that SANTA MARIA functions upstream of vitamin A formation in neurons and glia, which are outside of the retina. The protein is coexpressed and functionally coupled with the β, β-carotene-15, 15′-monooxygenase, NINAB, which converts β-carotene to all-trans-retinal. Another class B scavenger receptor, NINAD, functions upstream of SANTA MARIA in the uptake of carotenoids, enabling us to propose a pathway involving multiple extraretinal cell types and proteins essential for the formation of rhodopsin.

Introduction

Retinoids (vitamin A and its derivatives) are critical for processes ranging from the immune response to neuronal plasticity, development, visual pigment generation, cell proliferation, and other essential physiological processes (for review see Lane and Bailey, 2005; Travis et al., 2007). In animals, all retinoids must be acquired from the diet either as preformed vitamin A (all-trans-retinol) or must be formed from the provitamin A precursor, carotenoids. The dietary carotenoids are synthesized in plants, certain fungi, and bacteria, and, to become biologically active, must first be absorbed and then delivered to the site in the body where they are converted to vitamin A (for review see von Lintig et al., 2005).

The β, β-carotene-15, 15′ monooxygenase (BCO) is the key enzyme in vitamin A formation, which catalyzes the centric cleavage of β-carotene to yield retinaldehyde (all-trans-retinal; von Lintig and Vogt, 2000; Kiefer et al., 2001; Paik et al., 2001; Redmond et al., 2001; Fig. 1 A). However, until relatively recently, the identities of these enzymes in vertebrates and invertebrates were not known. In *Drosophila*, BCO is encoded by *ninaB* (*neither inactivation nor afterpotential B*), and mutations in this gene disrupt retinoid production and phototransduction as a result of elimination of rhodopsin (von Lintig et al., 2001). As carotenoids are highly lipophilic molecules, specific proteins must exist to transport them to specialized target tissues and to absorb the provitamin A into cells.

It has been suggested that class B scavenger receptors may play important roles in the cellular uptake of carotenoids (for review see von Lintig et al., 2005). In *Drosophila*, mutations in the *ninaD* gene (Johnson and Pak, 1986), which encodes a membrane protein homologous to the mammalian class B type I scavenger receptor (SR-BI; Acton et al., 1994), result in a defect in the uptake of carotenoids and synthesis of retinoids (Kiefer et al., 2002). SR-BI plays critical roles in cholesterol and high-density lipoprotein metabolism and in maintaining plasma cholesterol levels (Acton et al., 1996). SR-BI also mediates cellular uptake of free cholesterol (Acton et al., 1996), triglycerides (Stangl et al., 1999), phospholipids (Thuahnai et al., 2001), and vitamin E (Goti et al., 2001). Moreover, SR-BI is expressed in the human intestine (Hauser et al., 1998; Levy et al., 2004), where it is proposed to mediate absorption of dietary β-carotene (Reboul et al., 2005; van Bennekum et al., 2005). The combination of these studies suggests that class B scavenger receptors may function as carotenoid receptors. Although the molecular mechanism through which class B scavenger

Correspondence to Craig Montell: cmontell@jhmi.edu

Abbreviations used in this paper: BCO, β, β-carotene-15, 15′ monooxygenase; EMS, ethyl methanesulfonate; ERG, electroretinogram; *nina, neither inactivation nor afterpotential*; *norpA, no receptor potential A*; PDA, prolonged depolarization afterpotential; *pinta, PDA is not apparent*; *santa maria, scavenger receptor acting in neural tissue and majority of rhodopsin is absent*; *UAS, upstream activator sequence.*

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receptors mediate absorption of carotenoids is not known, it might involve binding of carotenoid containing lipoproteins or micelles via the extracellular domain, separating the two transmembrane segments (Tao et al., 1996; Gu et al., 2000), followed by uptake of carotenoids through a process independent of endocytosis (Acton et al., 1996).

The photopigment, rhodopsin, consists of a seven-transmembrane protein, opsin, and a chromophore (3-hydroxy-11-cis retinal and 11-cis retinal in *Drosophila* and mammals, respectively), which is formed through metabolism of vitamin A (Montell, 1999; Travis et al., 2007). In *Drosophila*, light results in a cis- to trans-isomerization of the chromophore, and this transformation represents the only light-driven step during phototransduction. The all-trans-retinol is converted to 11-cis-retinal in pigment cells in a light-dependent, rather than an enzyme-dependent, manner (Wang and Montell, 2005), whereas the pathway leading from dietary carotenoids to all-trans-retinal takes place outside of retina tissues (Gu et al., 2004). Deprivation of vitamin A, either by depletion of dietary retinoids or as a result of mutations in the vitamin A pathway causes reductions in rhodopsin levels and defects in vision.

In contrast to mammals, in *Drosophila*, retinoids are not required for viability but appear to be required exclusively in the retina (Harris et al., 1977). As such, *Drosophila* represents a highly tractable animal model to study the metabolism of vitamin A in vivo. The *ninaB* gene encodes a BCO, which functions outside the retina for conversion of carotenoids to all-trans-retinal (Stephenson et al., 1983; von Lintig et al., 2001; Kiefer et al., 2002). Thus, a key question concerns the identity of the scavenger receptor that is functionally coupled to NINAB for the uptake of carotenoids. It has been suggested that the class B scavenger receptor, NINAD, is the protein that functions in concert with NINAB (Fig. 1 A; Gu et al., 2004). However, *ninaD* expression is enriched in bodies, whereas *ninaB* expression is reported to be enriched in heads (von Lintig et al., 2001; Kiefer et al., 2002), which questions how the two differentially expressed gene products are coupled.

In the present study, we describe the isolation of the *santa maria* (*scavenger receptor acting in neural tissue and majority of rhodopsin is absent*) locus, which encodes a new member of class B scavenger receptor family. Mutation of *santa maria* profoundly affected the visual response and production of rhodopsin, both of which were restored by providing all-trans-retinal to the diet. We found that *santa maria* functioned downstream of *ninaD*, in a step required for the conversion of carotenoids to vitamin A. The *santa maria* gene functioned outside of the retina and appeared to display a similar expression pattern as *ninaB* in fly heads. We provide evidence that *santa maria* and *ninaB* function in the same cells in vivo. Based on these results, we propose that the class B scavenger receptor, SANTA MARIA, is functionally coupled with the BCO enzyme, NINAB, in the conversion of carotenoids to retinaldehyde. In contrast to NINAB and SANTA MARIA, we show that the other class B scavenger receptor, NINAD functions in the uptake of carotenoids primarily in the midgut. Combined with our previous demonstration that the retinoid binding protein, PINTA (PDA [prolonged depolarization afterpotential] is not apparent), functions in the retinal pigment cells in the final step in the generation of the chromophore, we propose a pathway involving the NINAB, NINAD, PINTA, and SANTA MARIA proteins acting in multiple cell types in the conversion of carotenoids to the rhodopsin chromophore.

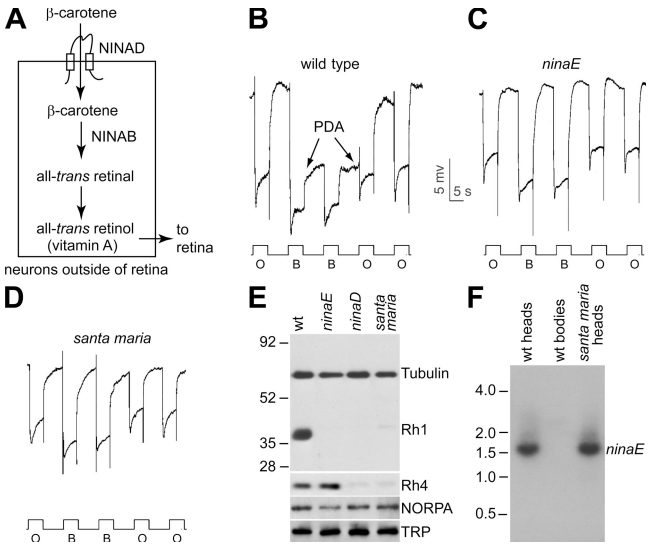

Figure 1. **Rhodopsin biosynthesis defects in *santa maria*[1].** (A) Previously proposed model (Gu et al., 2004) suggesting that NINAB and NINAD are functionally coupled in extra-retinal neurons. (B–D) ERG paradigm that elicits PDA in wild-type but not in mutants that disrupt production of the major rhodopsin (Rh1). Flies (~2 d after eclosion) were dark adapted for 1 min and subsequently exposed to five 5-s pulses of orange light (O) or blue light (B) interspersed by 7 s as indicated. A PDA is induced in wild type by blue light, as indicated by the arrows in B, and terminated by orange light. All the flies analyzed were in a w^{1118} background. (B) Wild type (w^{1118}), (C) $ninaE^{P334}$, (D) *santa maria*[1]. (E) Rh1 and Rh4 were reduced in *santa maria*[1] flies. The Western blot, which contained extracts prepared from the heads (~2 d after eclosion) of wild-type, $ninaE^{P334}$, $ninaD^{P246}$, and *santa maria*[1] flies (all in a w^{1118} background), were probed with anti-Rh1 and anti-tubulin antibodies. Molecular mass markers (kD) are indicated to the left. The same blot was reprobed with anti-Rh4 and anti-NORPA antibodies. The same samples were probed on a separate blot with anti–transient receptor potential (TRP) antibodies. (F) The *ninaE* mRNA levels were not affected in *santa maria*[1] flies. The Northern blot, which contained 2 μg of total RNA in each lane, was probed with *ninaE* DNA probe. Single-stranded RNA markers are indicated to the left.

Results

A mutant defective in the generation of rhodopsin

To identify new genes in *Drosophila* that functioned in the generation of rhodopsin and other aspects of phototransduction, we conducted a screen of chromosome 2 for homozygous viable mutations that caused a defect in electroretinogram (ERG) recordings (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200610081/DC1). ERGs are extracellular recordings that measure the summed retinal response to light. In *Drosophila*, the chromophore stays bound to the light-activated metarhodopsin, and a second photon of light is required for the reconversion of the metarhodopsin to the inactive rhodopsin (Pak, 1979; Montell, 1999). The major rhodopsin (Rh1) responds to either orange or blue light, whereas metarhodopsin responds effectively to orange light only. As a consequence,

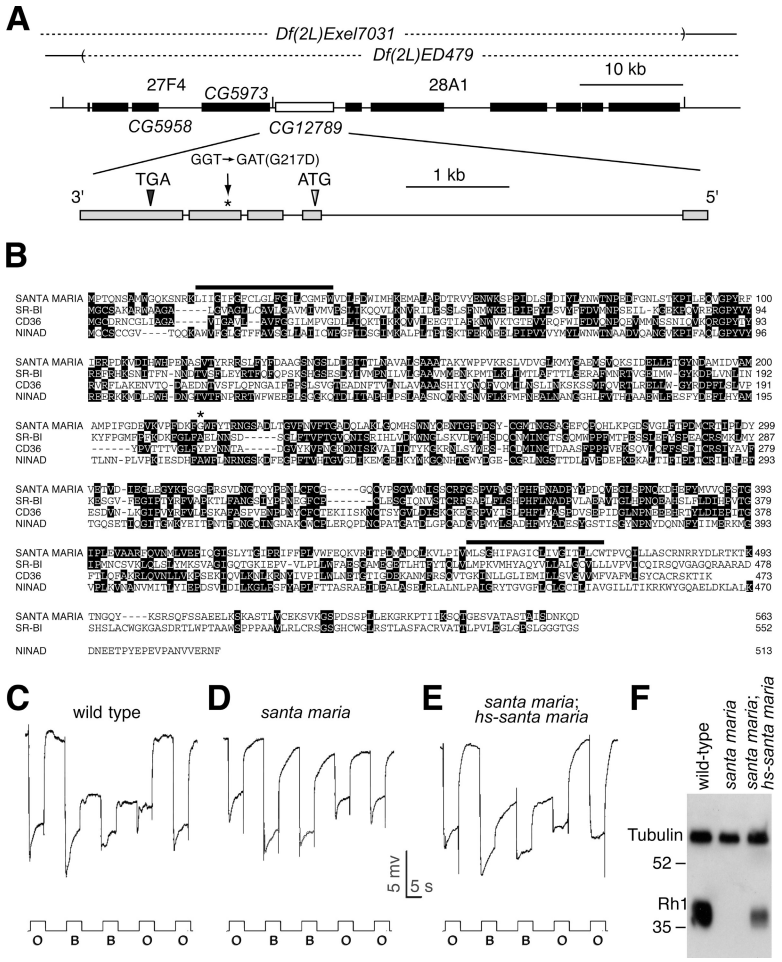

Figure 2. **Identification of the *santa maria* gene.** (A) Mapping of the *santa maria* mutation. The *santa maria*^{1} gene was localized to 27F4-28A1, based on the failure of the following deficiencies to complement the *santa maria* phenotype: *Df(2L)ED479* and *Df(2L)Exel7031*. *CG12789* is indicated by the open box, and the other nine genes in this interval are indicated by the black boxes. The intron–exon structure of *CG12789* is indicated below. The position of the mutation in *CG12789* (GGT to GAT), which changes glycine 217 to an aspartic acid, is indicated. This mutation was identified by comparing the sequences in *santa maria*^{1} and the original line used in the screen. (B) Alignment of the SANTA MARIA amino acid sequence with human SR-BI, human CD36, and NINAD. Identical residues, which are found in at least two proteins, are enclosed in black boxes. The running tallies of amino acids are indicated to the right. The two predicted transmembrane segments in SANTA MARIA are indicated by the black lines above the sequences. The position of the G217D change is indicated by the asterisk. (C–E) Rescue of the PDA phenotype in *santa maria*^{1} by expression of a *santa maria* transgene under control of the *heat shock protein 70* promoter (*hs–santa maria*). The ERG paradigm using orange (O) and blue light (B) is as described in Fig. 1 B. (C) PDA in wild type (*w*^{1118}). (D) Absence of a PDA in *santa maria*^{1} flies. (E) A PDA was restored in *santa maria*^{1}*;hs–santa maria/+* flies. (F) Rh1 expression increased in *santa maria*^{1} flies upon expression of the *hs–santa maria* transgene (*santa maria*^{1}*;hs–santa maria/+*). We applied a 2-h heat shock to the flies immediately after eclosion. Head extracts were prepared from flies ~2 d after eclosion and probed with anti-Rh1 and anti-tubulin antibodies.

blue light causes stable activation of metarhodopsin, resulting in a PDA (Fig. 1 B). The PDA requires a molar excess of the active form of the metarhodopsin over the available arrestin, which is required to arrest the activity of the metarhodopsin (Dolph et al., 1993). Thus, when the Rh1 level is decreased, as occurs upon mutation of the structural gene for the Rh1 opsin (*ninaE*), a PDA is not produced (Fig. 1 C; O'Tousa et al., 1985; Zuker et al., 1985).

One of mutant lines isolated in the ERG screen displayed a PDA-defective ERG phenotype, similar to that observed in *ninaE* (*ninaE*^{P322}) flies (Fig. 1 D). Mutations in three second-chromosomal genes, *ninaA*, *ninaC*, and *ninaD*, are known to reduce or eliminate the PDA (Matsumoto et al., 1987; Montell and Rubin, 1988; Schneuwly et al., 1989; Colley et al., 1991; Kiefer et al., 2002). The new mutation complemented *ninaA*, *ninaC*, or *ninaD* (unpublished data). Therefore, this mutation disrupted a new gene required for the generation of the PDA, which we refer to as *santa maria*.

As the PDA phenotype is usually due to a reduction in the level of Rh1, we checked the Rh1 concentration and found that it was severely reduced in the *santa maria*^{1} mutant, as was the case in *ninaE*^{P334} and *ninaD*^{P246} flies (Fig. 1 E). We also checked *ninaE* (*rh1*) mRNA expression in *santa maria*^{1} using Northern blots and found that it was not reduced compared with wild type (Fig. 1 F). Thus, the reduction in Rh1 protein was not due to disruption in expression or stability of the *ninaE* mRNA.

In addition to Rh1, there are four minor rhodopsins (Rh3–6) expressed in the retina (Montell, 1999). These minor opsins are spatially localized in nonoverlapping subsets of the smaller R7 and R8 cells (Montell et al., 1987; Zuker et al., 1987; Chou et al., 1996; Huber et al., 1997; Papatsenko et al., 1997). To address whether the *santa maria*^{1} mutation reduced the expression of an opsin other than Rh1, we checked the protein levels of Rh4 and found that the concentration of this protein was also diminished (Fig. 1 E). The levels of other photoreceptor proteins, such as the eye-enriched PLC (NORPA [no receptor potential A]) and the transient receptor potential channel, did not change (Fig. 1 E). Therefore, the *santa maria*^{1} mutation caused a reduction in the concentration of rhodopsins but did not result in a general defect in the expression of photoreceptor cell proteins.

The *santa maria* gene encodes a class B scavenger receptor

To identify the gene responsible for the *santa maria* phenotype, we mapped the site of the mutation to the 27F4 to 28A2 region (Fig. 2 A; see Materials and methods), which included 10 known or predicted genes (http://flybase.bio.indiana.edu) spanning the region between *CG5261* and *CG6630*. Among these 10 genes, the predicted amino acid sequences of three genes suggested

that they were excellent candidates for encoding SANTA MARIA. Two of them (*CG5958* and *CG5973*) encode putative retinoid binding proteins, and the third (*CG12789*) encodes a homologue of class B scavenger receptors. The predicted CG12789 protein shares 33% identity with the human SR-BI (hSR-BI; Calvo and Vega, 1993; Acton et al., 1994); 26% identity with mouse CD36, the founding member of this family (Endemann et al., 1993); and 30% identity with the *Drosophila* scavenger receptor NINAD, which also functions in rhodopsin biosynthesis (Johnson and Pak, 1986; Kiefer et al., 2002; Fig. 2 B). Class B scavenger receptor family are suggested to consist of two transmembrane domains and cytoplasmic N- and C-termini (Tao et al., 1996).

To find out which of the three candidates was the *santa maria* gene, we introduced transgenes encoding *CG5958*, *CG5973*, and *CG12789* into *santa maria*^{1} flies. *CG12789*, expressed under the control of the *heat-shock protein 70* promoter (*hs-CG12789*), restored a wild-type PDA (Fig. 2, C–E) and increased the level of the Rh1 protein in the mutant flies (*santa maria*^{1}*;hs–santa maria/+*; Fig. 2 F). The level of rhodopsin in these flies was lower than in wild-type, possibly because of the relative weakness of the *heat-shock protein 70* promoter in some cell types. In contrast, neither the *CG5958* nor the *CG5973* transgenes rescued the PDA defect or increased Rh1 levels in the *santa maria*^{1} flies (unpublished data). Therefore, *CG12789*, which encodes a predicted class B scavenger receptor, is the *santa maria* gene.

santa maria functions outside of the retina

Some gene products that are essential for production or transport of the chromophore function in the retina, whereas others play roles outside the retina. The two proteins required in the retina are the retinoid binding protein, PINTA, which functions in pigment cells for chromophore synthesis (Wang and Montell, 2005), and a oxidoreductase, NINAG, which is required in the compound eye for chromophore synthesis (Sarfare et al., 2005). In contrast, two gene products that have been reported to operate outside of the retina for carotenoid metabolism are *ninaD*, which encodes a class B scavenger receptor (Pak, 1979; Kiefer et al., 2002), and *ninaB*, which encodes a BCO (Pak, 1979; von Lintig and Vogt, 2000; Kiefer et al., 2001). These findings raise the question as to the tissue and cellular requirements for *santa maria*.

To determine whether *santa maria* was required in the compound eye, we used two approaches. First, we generated mosaic flies using a mitotic recombination approach that leads to the generation of fully homozygous mutant eyes in otherwise heterozygous animals (Stowers and Schwarz, 1999). We found that the mosaic flies expressed normal levels of Rh1 (Fig. 3 A), indicating that *santa maria* was not required in the compound eye. Second, we tested for rescue of the *santa maria*^{1} phenotype, after expressing wild-type *santa maria* in the retina, using the *GAL4/UAS* (*upstream activator sequence*) system (Brand and Perrimon, 1993). This approach results in expression of genes that are linked 3′ to the *UAS*, to occur specifically under the control of the GAL4 transcription factor. Therefore, we generated *UAS–santa maria* transgenic flies, and introduced

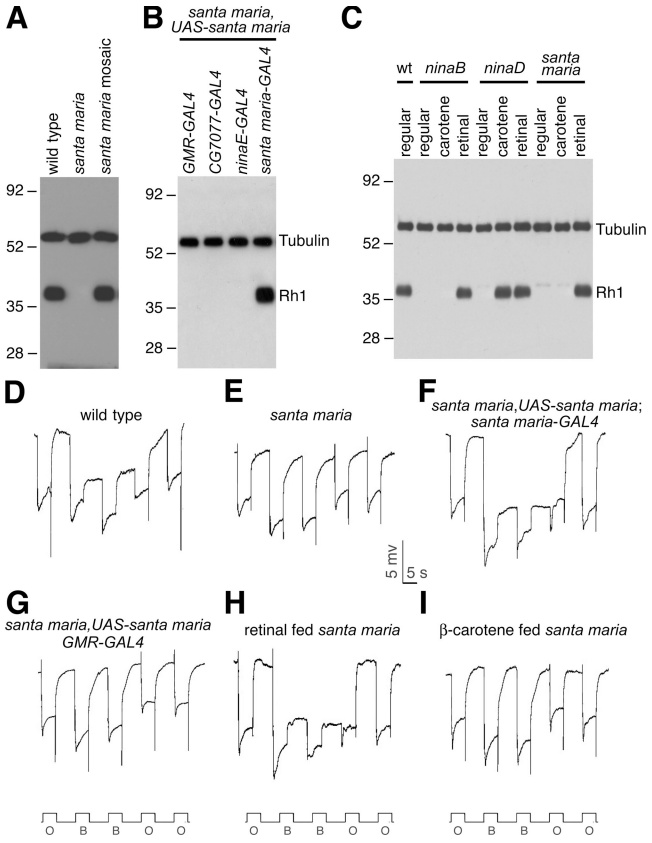

Figure 3. ***santa maria* is required for carotenoid metabolism outside of the retina.** (A) *santa maria* functions outside of the compound eye for production of Rh1. The level of the Rh1 protein was normal in *santa maria*^{1} mosaic eyes: *santa maria*^{1} *FRT/*P[*GMR-hid*]*G1* P[*neoFRT*]*40 l(2)CL-L;* P[*GAL4-ey.H*]*SS5* P[*UAS-FLP1.D*]*JD2*. The samples were prepared from flies ∼2 d after eclosion, and the blot was probed with ant-Rh1 and anti-tubulin antibodies. (B) Expression of *santa maria* in different sets of retinal cells using the *GAL4/UAS* system (Brand and Perrimon, 1993) did not restore Rh1 levels. Expression of *UAS–santa maria* was driven ubiquitously in the eye using the *GMR-GAL4* (Freeman, 1996) or specifically in pigment or photoreceptor cells using the *CG7077-GAL4* (not depicted) or the *ninaE-GAL4*, respectively. To examine the level of Rh1, we prepared head extracts from flies ∼2 d after eclosion, and a Western blot was probed with anti-Rh1 and anti-tubulin antibodies. (C) Normal Rh1 levels were restored in *santa maria*^{1} flies by feeding all-trans-retinal but not by feeding with β-carotene. The *ninaB*^{P315}, *ninaD*^{P246}, and *santa maria*^{1} flies were fed either 0.2 mM all-trans-retinal or 0.2 mM β-carotene. To perform the Western blot, we prepared extracts from flies ∼4 d after eclosion and probed the filter with anti-Rh1 and anti-tubulin antibodies. (D–I) ERGs using a series of orange (O) and blue (B) light stimuli as de scribed in Fig. 1 B (see event markers at the bottom of G–I). All the flies were in a *w*^{1118} background. Flies were fed normal food unless indicated otherwise. (D) wild-type; (E) *santa maria*^{1}; (F) *santa maria*^{1}*,UAS–santa maria;santa maria–GAL4/+*; (G) *santa maria*^{1}*,UAS–santa maria;GMR-GAL4/+*; (H) all-trans-retinal fed *santa maria*^{1}; (I) β-carotene fed *santa maria*^{1}.

GAL4 transgenes into these flies that direct expression of *santa maria* in different retinal cells. Normal Rh1 levels or a wild-type PDA were not restored in *santa maria*^{1} upon expression of *UAS–santa maria* throughout the eye (*GMR-GAL4*) or exclusively in pigment cells (*CG7077-GAL4*) or photoreceptor cells (*ninaE-GAL4*; Fig. 3, B and D–G; and Fig. S2, A–C, available at http://www.jcb.org/cgi/content/full/jcb.200610081/DC1). The lack of rescue was not due to a problem with the *UAS–santa maria* transgene, as the *santa maria*^{1} phenotype was reversed in flies containing a *santa maria–GAL4* in combination with the

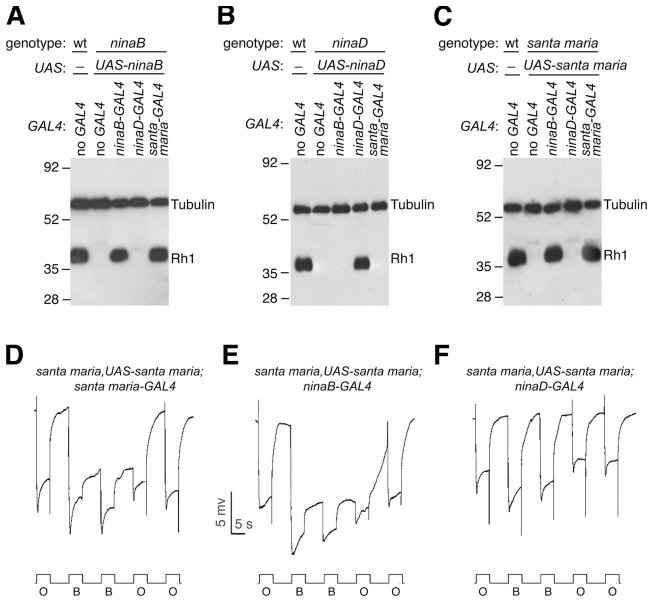

Figure 4. ***santa maria* but not *ninaD* appear to be functionally coupled with *ninaB*.** (A–C) Western blots containing head extracts prepared from flies ~2 d after eclosion were probed with anti-Rh1 and anti-tubulin antibodies. (A) Rh1 protein levels in *ninaB*^{P315} flies after expression of *UAS-ninaB* under control of *ninaB-GAL4*, *ninaD-GAL4*, or *santa maria–GAL4*. (B) Rh1 protein levels in *ninaD*^{P246} flies after expression of *UAS-ninaD* under the control of *ninaB-GAL4*, *ninaD-GAL4*, or *santa maria–GAL4*. (C) Rh1 protein levels in *santa maria*^{1} flies after expression of *UAS–santa maria* under control of *ninaB-GAL4*, *ninaD-GAL4*, or *santa maria–GAL4*. (D–F) ERG paradigm to test for a PDA, as described in Fig. 1 B. All flies were in a *w*^{1118} background. (D) *santa maria*^{1}*,UAS–santa maria;santa maria–GAL4;* (E) *santa maria*^{1}*,UAS–santa maria;ninaB-GAL4;* (F) *santa maria*^{1}*, UAS–santa maria;ninaD-GAL4*.

UAS–santa maria (Fig. 3, B and F). These results demonstrate that *santa maria* is required outside the retina for biosynthesis of rhodopsin.

Dietary all-trans-retinal but not β-carotene rescues the *santa maria*^{1} phenotype

Neither flies nor mammals can synthesize β-carotene but must obtain this vitamin A/chromophore precursor from the diet. Both *ninaB* and *ninaD*, whose activities are required outside of the retina for rhodopsin biogenesis, function in the pathway from β-carotene to all-trans-retinal because both mutant phenotypes are rescued by supplementation of the food with all- trans-retinal (Stephenson et al., 1983; Gu et al., 2004; Fig. 3 C and Fig. S2, D, E, G, and H). In contrast, PINTA and NINAG are required in the compound eyes and function subsequent to the generation of all-trans-retinal, as supplementation with all- trans-retinal does not restore Rh1 to wild-type levels in these mutants (Wang and Montell, 2005; Ahmad et al., 2006). Because *santa maria* acts outside of the compound eyes, it may also function in a step necessary for the conversion of β-carotene to vitamin A. Therefore, we checked whether the *santa maria*^{1} phenotype could be rescued by addition of all-trans-retinal to the diet. As was the case with *ninaB* and *ninaD*, we found that supplementation of the food with all-trans-retinal (0.2 mM) restored the Rh1 levels and the PDA in *santa maria*^{1} flies (Fig. 3, C and H).

Table I. ***santa maria* functions together with *ninaB* in neural cells**

Mutant	GAL4	UAS	Rh1 levels
ninaB^{P315}	No	No	–
ninaB^{P315}	*ninaB-GAL4*	*UAS-ninaB*	+
ninaB^{P315}	*ninaD-GAL4*	*UAS-ninaB*	–
ninaB^{P315}	*santa maria–GAL4*	*UAS-ninaB*	+
ninaB^{P315}	*repo-GAL4*	*UAS-ninaB*	+
ninaB^{P315}	*elav-GAL4*	*UAS-ninaB*	+
ninaD^{P246}	No	No	–
ninaD^{P246}	*ninaB-GAL4*	*UAS-ninaD*	–
ninaD^{P246}	*ninaD-GAL4*	*UAS-ninaD*	+
ninaD^{P246}	*santa maria–GAL4*	*UAS-ninaD*	–
ninaD^{P246}	*repo-GAL4*	*UAS-ninaD*	–
ninaD^{P246}	*elav-GAL4*	*UAS-ninaD*	+
ninaD^{P246}	*drm-GAL4*	*UAS-ninaD*	+
ninaD^{P246}	*ninaD-GAL4*	*UAS–santa maria*	–
ninaD^{P246}	*ninaD-GAL4*	*UAS–SR-BI*	–
santa maria^{1}	No	No	–
santa maria^{1}	*ninaB-GAL4*	*UAS–santa maria*	+
santa maria^{1}	*ninaD-GAL4*	*UAS–santa maria*	–
santa maria^{1}	*santa maria–GAL4*	*UAS–santa maria*	+
santa maria^{1}	*repo-GAL4*	*UAS–santa maria*	+
santa maria^{1}	*elav-GAL4*	*UAS–santa maria*	+
santa maria^{1}	*drm-GAL4*	*UAS–santa maria*	–
santa maria^{1}	*santa maria–D-GAL4*	*UAS-ninaD*	–
santa maria^{1}	*santa maria–D-GAL4*	*UAS–SR-BI*	–

The summary of Rh1 levels is based on the data in Figs. 3–7. Plus signs indicate wild-type Rh1 (rhodopsin) levels, and minus signs indicate reduced Rh1 levels.

Both *ninaB* and *ninaD* function in the generation of retinoids from carotenoids; however, only the *ninaD*^{P246} phenotype, not the *ninaB*^{P315} phenotype, is rescued by high doses of β-carotene (Fig. 3 C and Fig. S2, F and I; Stephenson et al., 1983; Gu et al., 2004). NINAB is an essential enzyme necessary for all-trans-retinal production, whereas NINAD is a scavenger receptor, which promotes the uptake of carotenoids. This latter function can be bypassed by large concentrations of dietary carotenoids. Based on these data, it has been proposed that *ninaB* functions downstream of *ninaD* (Gu et al., 2004). To test whether the *santa maria*^{1} phenotype was rescued by carotenoids, we fed the mutant flies 0.2 mM β-carotene. We found that addition of β-carotene did not rescue the *santa maria*^{1} phenotype (Fig. 3 I).

SANTA MARIA and NINAB appear to be functionally coupled

NINAB is a BCO, which converts β-carotene to all-trans-retinal; therefore, NINAB would need to be coexpressed with a β-carotene receptor, to promote influx of β-carotene into the cells. The two class B scavenger receptors, SANTA MARIA and NINAD, are candidate proteins that could be functionally coexpressed with NINAB, and serve this role. To address whether NINAD or SANTA MARIA function in the same cells as NINAB, we used the *GAL4/UAS* system (Brand and Perrimon, 1993). To conduct these experiments, we generated *ninaB-GAL4*, *ninaD-GAL4*, and *santa maria–GAL4* transgenic flies (see Materials and methods) and introduced them into the *ninaB*^{P315}, *ninaD*^{P246}, and *santa maria*^{1} mutant backgrounds,

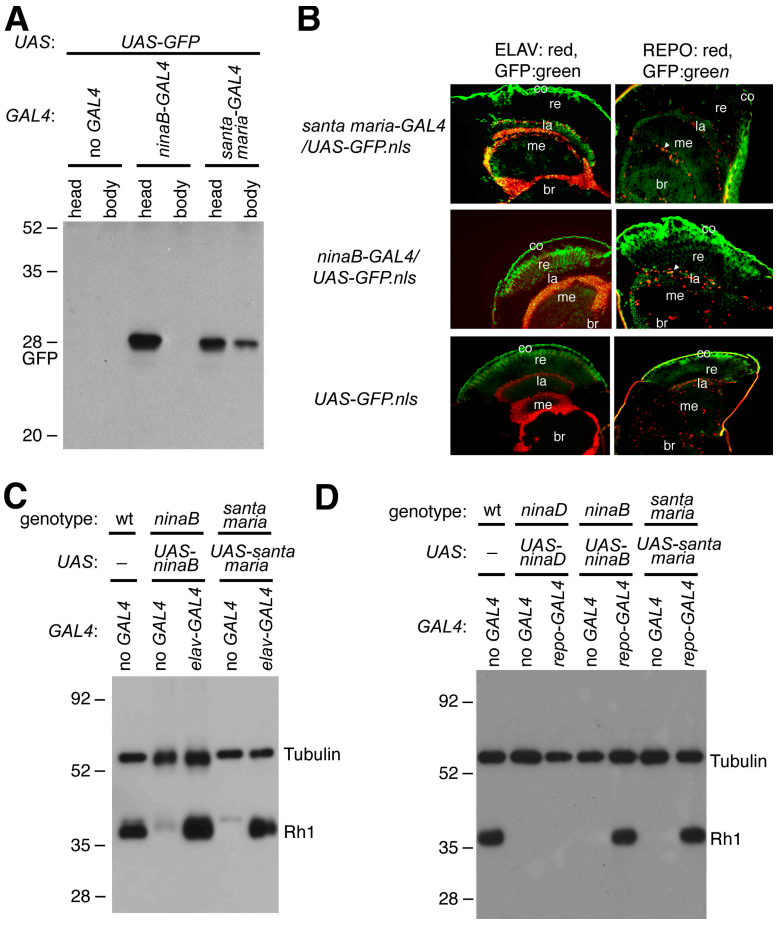

Figure 5. ***ninaB* and *santa maria* were expressed and functioned in neuronal and glia cells.** (A) Examination of *ninaB* and *santa maria* expression in heads and bodies using a *UAS-GFP* reporter. Extracts, which were prepared from the heads and bodies of flies containing *UAS-GFP* and either the *ninaB-GAL4* or the *santa maria–GAL4*, were fractionated by SDS-PAGE, and a Western blot was probed with anti-GFP antibodies. The same membrane was stained with Ponceau S to compare total proteins in each lane (not depicted). (B) Testing for colocalization of the *santa maria–* and *ninaB*-GFP reporters with neuronal and glial cell markers (anti-ELAV and anti-REPO, respectively) in adult heads. Because both REPO and ELAV are nuclear proteins, we used a GFP with a nuclear localization signal (GFP.nls). To conduct the experiments, sections of adult heads were prepared from *ninaB-GAL4/UAS-GFP.nls* and *santa maria–GAL4/UAS-GFP.nls* flies and stained with rabbit anti-GFP (green) and either rat anti-ELAV (red) or mouse anti-REPO (red). br, brain; co, cornea; la, lamina; me, medulla; re, retina. The arrowheads indicate one example in each set, in which there is overlap between the green (anti-GFP) and red (anti-ELAV or anti-REPO) signals. The labeling of the cornea is nonspecific because of autofluorescence. Similar patterns of anti-GFP staining were observed with different *santa maria–GAL4* and *ninaB-GAL4* lines, in combination with *UAS-GFP.nls* (not depicted). (C) Western blot showing that expression of *UAS-ninaB* or *UAS–santa maria* in neurons, using the *elav-GAL4*, restores Rh1 expression in the $ninaB^{P315}$ and $santa\ maria^{1}$, respectively. Head extracts were prepared from flies ~2 d after eclosion and probed with anti-Rh1 and anti-tubulin antibodies. (D) Western blot demonstrating that expression of *UAS-ninaB* or *UAS–santa maria* in glia, under control of the *repo-GAL4*, increases Rh1 expression in $ninaB^{P315}$ and $santa\ maria^{1}$. The blot was performed as indicated in C.

along with *UAS-ninaB*, *UAS-ninaD*, and *UAS–santa maria*. Each of these *GAL4* and *UAS* lines was effective because the $ninaB^{P315}$, $ninaD^{P246}$, or $santa\ maria^{1}$ phenotype was rescued by the *GAL4/UAS* transgenes corresponding to the same genes (Fig. 4 and Table I).

We found that the $ninaB^{P315}$ and $santa\ maria^{1}$ phenotypes were rescued by expression of one gene under the control of the other *GAL4* line. Specifically, expression of *UAS-ninaB* using the *santa maria–GAL4* restored Rh1 levels in $ninaB^{P315}$ flies (Fig. 4 A and Table I), whereas expression of *UAS–santa maria* under control of the *ninaB-GAL4* rescued the $santa\ maria^{1}$ phenotype (Fig. 4, C and E; and Table I). Indistinguishable results were obtained using two independent *santa maria–GAL4* and three *ninaB-GAL4* lines (unpublished data). These data indicate that *santa maria* is functionally coexpressed with *ninaB* in the same cells.

In contrast to these results, the NINAB BCO did not function together with the other scavenger receptor, NINAD, as previously proposed (Gu et al., 2004). Expression of *UAS-ninaB* or *UAS–santa maria* under control of either of two *ninaD-GAL4* lines did not reduce the severity of the $ninaB^{P315}$ and $santa\ maria^{1}$ phenotypes, respectively (Fig. 4, A, C, and F; Table I; and not depicted). To determine the sensitivity of this analysis, we conducted a dilution experiment and found that we could detect 2% the wild-type levels of Rh1 (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200610081/DC1). Because the levels of Rh1 produced in $santa\ maria^{1}$;*UAS–santa maria* flies, either in the presence or absence of *ninaD-GAL4*, were both at the threshold for detection (≤2% wild-type levels; Fig. S3), we conclude that if there was any rescue with the *ninaD-GAL4*, it was ≤2%. Furthermore, within the resolution of our analysis, expression of *UAS-ninaD* using the *ninaB-GAL4* or *santa maria–GAL4* did not increase Rh1 levels in $ninaD^{P246}$ flies (Fig. 4 B and Table I). These results were not due to ineffectiveness of the *ninaD-GAL4* or the *UAS-ninaD*, as the $ninaD^{P246}$ phenotype was rescued by cointroduction of these transgenes into the mutant flies (Fig. 4 B). Thus, *ninaD* was not functionally coexpressed with *ninaB*, consistent with the proposal that SANTA MARIA is the critical scavenger receptor operating in combination with NINAB.

ninaB and *santa maria* were both required in neurons and glia

To find out which cell types express *ninaB* and *santa maria*, we first tested whether expression of these genes was enriched in bodies or heads, using a *GFP* reporter. We prepared extracts from the heads and bodies of *ninaB-GAL4/UAS-GFP* and *santa maria–GAL4/UAS-GFP* flies and probed Western blots with anti-GFP antibodies. In *ninaB-GAL4/UAS-GFP* flies, GFP was detected exclusively in the heads, whereas in *santa maria–GAL4/UAS-GFP* flies, the GFP was found in both heads and bodies (Fig. 5 A and not depicted). Because *ninaB* and *santa*

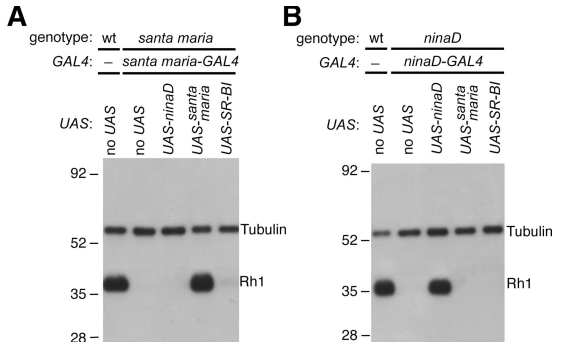

Figure 6. ***ninaD* and *santa maria* cannot substitute for each other.** (A) Expression of neither *ninaD* nor *SR-BI* restored Rh1 expression in *santa maria*^{1} flies. The indicated *UAS* transgenes were expressed in *santa maria*^{1}*;santa maria–GAL4* flies. The Western blot, processed as indicated in Fig. 5 C, was probed with anti-Rh1 and anti-tubulin antibodies. (B) The reduced Rh1 expression in *ninaD*^{P246} flies was not rescued by expression of either *santa maria* or *SR-BI*. The indicated *UAS* transgenes were expressed in *ninaD*^{P246}*;ninaD-GAL4* flies. The Western blot, processed as indicated in Fig. 5 C, was probed with anti-Rh1 and anti-tubulin antibodies.

maria appear to be functionally coexpressed, the two gene products may collaborate primarily in fly heads for the generation of all-trans-retinal.

To address the cell types in adult heads expressing reporters under control of the *ninaB* and *santa maria* enhancer/promoters, we performed double-labeling experiments. We stained head sections obtained from *ninaB-GAL4/UAS-GFP.nls* and *santa maria–GAL4/UAS-GFP.nls* flies with anti-GFP antibodies, in combination with glial (anti-REPO) or neuronal markers (anti-ELAV). A GFP with a nuclear localization signal was used (GFP.nls), as REPO (Campbell et al., 1994; Xiong et al., 1994) and ELAV (Bier et al., 1988; Robinow and White, 1988) are both nuclear proteins. We found that the GFP colocalized with ELAV and, to a lesser extent, with REPO (Fig. 5 B), which indicated that *ninaB* and *santa maria* were expressed in both neuronal cells and glia cells. The presumptive expression patterns for *santa maria* and *ninaB*, which were detected using the *GAL4*/*UAS* system, appeared to reflect the patterns obtained by direct enhancer/promoter-reporter fusions, as we observed similar patterns of expression after staining the flies with anti-GAL4 and anti-GFP (unpublished data).

To investigate the cell type in which *ninaB* and *santa maria* appear to function, we tested for rescue of the mutant phenotypes using the *GAL4*/*UAS* approach. We found that expression of *UAS-ninaB* or *UAS–santa maria* in neurons or glia, using the *elav-GAL4* or the *repo-GAL4*, respectively, restored Rh1 levels in both *ninaB*^{P315} and *santa maria*^{1} flies (Fig. 5, C and D; and Table I). However, the mutant phenotypes were not rescued by introduction of either *UAS-ninaB* or *UAS–santa maria* in combination with *GAL4* drivers that were expressed in other cell types and tissues, such as muscle cells, salivary glands, and fat bodies (unpublished data). These results suggest that *ninaB* and *santa maria* both function in neurons and glia cells.

The finding that wild-type Rh1 levels are restored if *ninaB* and *santa maria* are expressed in either neurons or glia raises the possibility that the specific cell type expressing these two genes is not critical, as long as the two gene products are coordinately expressed. Therefore, we coexpressed *ninaB* and *santa maria* in the retinal pigment cells, which express the retinoid binding protein PINTA and normally function in the final step in the production of the chromophore—conversion of all-trans-retinol to 11-cis-retinal (Wang and Montell, 2005). To conduct these experiments, we introduced into *ninaB*^{P315} or *santa maria*^{1} flies a pigment cell *GAL4* (*CG7077-GAL4*) together with both *UAS-ninaB* and *UAS–santa maria*. However, in neither mutant was the reduced Rh1 level increased (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200610081/DC1). These results suggest that there are one or more additional components that are required for the *ninaB*- and *santa maria*–dependent conversion of carotenoids to vitamin A. Alternatively, we cannot exclude the possibility that there is a problem in the transport of carotenoids to the retina.

NINAD and SANTA MARIA have unique functions

Because NINAD and SANTA MARIA share considerable amino acid homology (30% identity) and both are required for the generation of retinoids from carotenoids, the two scavenger receptors might have the same molecular functions. To address whether NINAD and SANTA MARIA can functionally substitute for each other, we tested whether *UAS–santa maria* could rescue the *ninaD*^{P246} phenotype if it was expressed in those cells that normally express *ninaD*. In addition, we performed the reciprocal experiment by expressing the *UAS-ninaD* transgene under control of the *santa maria–GAL4* in *santa maria*^{1} flies. However, Rh1 levels were not restored either in *santa maria*^{1} flies containing the *santa maria– GAL4*/*UAS-ninaD* transgenes or in *ninaD*^{P246} flies containing the *ninaD-GAL4*/*UAS–santa maria* transgenes (Fig. 6).

A mammalian homologue of NINAD and SANTA MARIA, SR-BI, has been suggested to function in the uptake of a variety of lipids, including β-carotene (van Bennekum et al., 2005). Therefore, we considered the possibility that SR-BI may have the same molecular function as either NINAD or SANTA MARIA. To test this proposal, we introduced a *UAS–SR-BI* transgene and expressed *SR-BI* under control of the *ninaD-GAL4* or the *santa maria–GAL4* in *ninaD*^{P246} or *santa maria*^{1} flies, respectively. However, expression of *SR-BI* did not rescue either the *santa maria*^{1} or *ninaD*^{P246} phenotypes (Fig. 6, A and B; Table I; and Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200610081/DC1).

NINAD was expressed and required in the midgut

Because the class B scavenger receptor, NINAD, is not functionally coexpressed with NINAB, it would appear that *ninaD* is required in cells distinct from those in which *ninaB* and *santa maria* function. The expression of *ninaD* has been detected in the midgut primordia in embryos (Kiefer et al., 2002), raising the possibility that NINAD may function in the midgut for absorption of carotenoids into animals. To test whether *ninaD* is expressed in the midgut, we used a GFP reporter. We found that GFP fluorescence in *ninaD-GAL4*/*UAS-GFP* flies was detected almost exclusively in the midgut (Fig. 7 A). To address whether

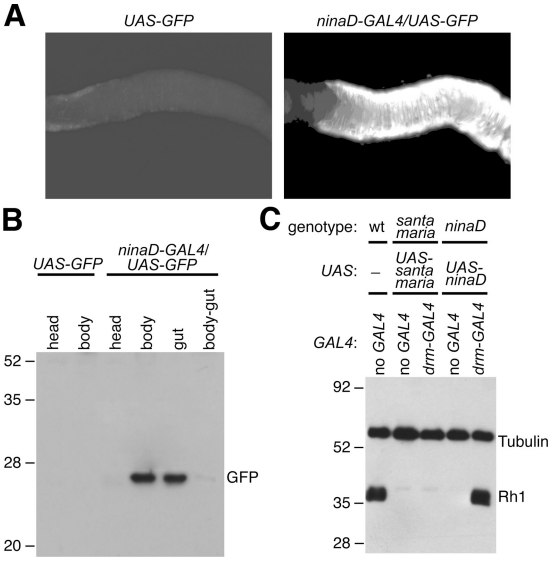

Figure 7. ***ninaD* was expressed and functioned in the gut.** (A) GFP fluorescence in the midgut of *ninaD-GAL4/UAS-GFP* flies but not in flies containing only the *UAS-GFP* transgene. (B) Expression of *ninaD* was enriched in the gut. Extracts were prepared from the heads, bodies, guts, and bodies without guts of *ninaD-GAL4/UAS-GFP* flies and from the heads and bodies of *UAS-GFP* flies. The total proteins in each lane was detected by staining with Ponceau S (not depicted). The Ponceau S staining in the gut lane was lighter than in the other lanes because the same number of bodies and guts (rather than the same mass) was loaded into each lane. (C) Expression of *UAS-ninaD* using a midgut *GAL4* line (*drm-GAL4*) rescued the $ninaD^{P246}$ phenotype. The Western blot, which contained head extracts prepared from flies ~2 d after eclosion, was probed with anti-Rh1 and anti-tubulin antibodies.

ninaD expression was enriched in gut, we prepared extracts from *ninaD-GAL4/UAS-GFP* fly heads, bodies, dissected guts, and bodies without guts and probed a Western blot with anti-GFP antibodies. To aid in the comparison, the extracts were prepared in a constant volume consisting of the same actual numbers of dissected guts and bodies, rather than the same total mass. GFP was detected in bodies and dissected guts, but not in heads (Fig. 7 B). Moreover, the GFP signal was dramatically reduced in bodies after removal of the guts. The results further supported the conclusion that *ninaD* was expressed primarily in the midgut.

To address whether *ninaD* functioned in the midgut, we directed *UAS-ninaD* expression under control of *drm-GAL4*, which drives expression in the midgut, as well as in the small intestines and Malpighian tubules (Green et al., 2002). We found that expression of *ninaD* under the control of *drm-GAL4* fully restored the Rh1 levels in $ninaD^{P246}$ flies, whereas expression of *santa maria* using the *drm-GAL4* did not increase Rh1 levels in $santa\ maria^{1}$ flies (Fig. 7 C and Table I). Based on these results, we propose that the NINAD functions in absorption of carotenoids in the midgut.

Discussion

In *Drosophila*, a reduction in rhodopsin levels results from mutations affecting either the synthesis or transport of the opsin or chromophore subunits (Harris et al., 1977; Stephenson et al., 1983). As such, genetic screens for mutations that affect rhodopsin levels provide an excellent opportunity to identify and characterize the roles of gene products required for production of the opsin, vitamin A, and the chromophore. Several genes required for rhodopsin biosynthesis have been previously reported, including those that are essential for steps involved in the synthesis or transport of the opsin (O'Tousa et al., 1985; Zuker et al., 1985; Colley et al., 1991; Rosenbaum et al., 2006), all-trans-retinal from β-carotene (von Lintig et al., 2001; Kiefer et al., 2002), and the chromophore from vitamin A (Sarfare et al., 2005; Wang and Montell, 2005). However, there remained many questions concerning the cellular sites for the various steps in vitamin A/chromophore synthesis, the nature of the proteins that participate in the uptake, transport, and synthesis of the intermediates, and the identities of receptors and enzymes that functioned coordinately in the same cells.

In animals, ranging from flies to humans, dietary β-carotene is the substrate for production of vitamin A, and the vitamin A is subsequently converted into the chromophore. The critical step in the conversion of β-carotene to vitamin A is the centric cleavage by BCO, which in *Drosophila* is encoded by the *ninaB* gene (von Lintig et al., 2001). The key question concerns the identity of the receptor protein that operates in concert with NINAB and is necessary for the uptake of carotenoids in the *ninaB* expressing cells. It has been suggested that the class B scavenger receptor encoded by *ninaD* serves this function (Gu et al., 2004). However, we have found that the *ninaD* phenotype was not rescued by expression of wild-type *ninaD* in *ninaB* expressing cells. Moreover, we found that *ninaB* was expressed primarily in the heads, whereas *ninaD* was only detected in the bodies.

As *ninaD* does not operate in concert with *ninaB*, there would appear to be another receptor that serves this function. In the current work, we identify a new class B scavenger receptor, SANTA MARIA, and provide evidence that it is functionally coupled to the NINAB BCO. In support of these conclusions, we found that SANTA MARIA is homologous to known class B scavenger receptors and mutations in *santa maria* disrupt the biogenesis of rhodopsin. Moreover, both *ninaB* and *santa maria* are expressed in fly heads and function in neurons and glia. Most important, expression of *ninaB* under control of the *santa maria* promoter rescued the *ninaB* phenotype and expression of *santa maria* using the *ninaB* promoter rescued the *santa maria* phenotype. Therefore, we suggest that carotenoids are taken up from circulation by SANTA MARIA, thereby providing the substrate for processing of carotenoids to all-trans-retinal by the NINAB BCO.

We suggest that the mammalian class B scavenger receptors SR-BI and CD36 may also function in all-trans-retinal production, through coupling with a BCO. SR-BI and CD36 are expressed in the liver (Fluiter and van Berkel, 1997; Ji et al., 1999) and in the intestines (Hauser et al., 1998; Levy et al., 2004) and appear to function in mediating absorption of β-carotene (Reboul et al., 2005; van Bennekum et al., 2005). In mammals, *BCO* mRNA is also detected in the small intestine and liver (Kiefer et al., 2001; Paik et al., 2001; Redmond et al., 2001), raising the possibility that one of these scavenger receptors

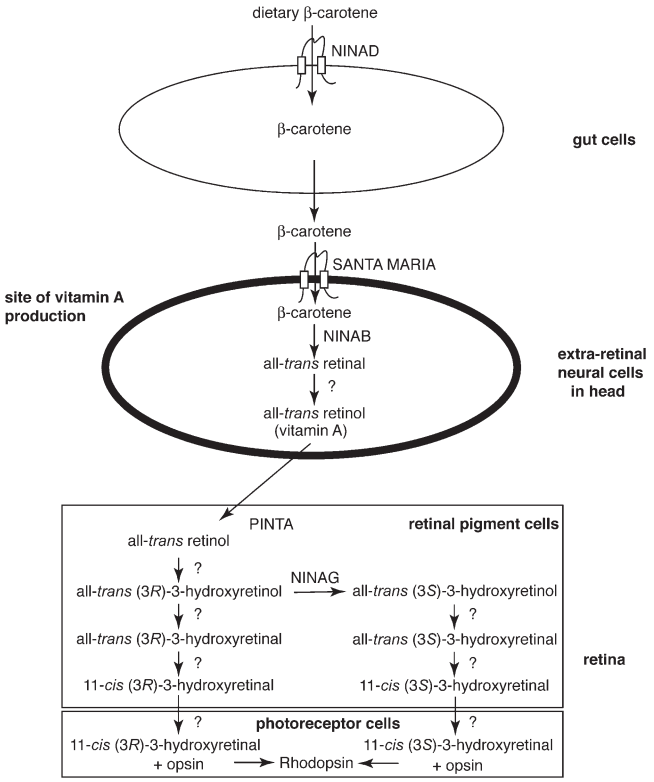

Figure 8. **Model of the pathway for production of vitamin A and the chromophore.** Absorption of β-carotene into the gut cells is dependent on the NINAD scavenger receptor. The β-carotene is subsequently taken up into extra-retinal neurons and glia in the head via the SANTA MARIA scavenger receptor and cleaved by the NINAB BCO. Vitamin A is subsequently transported to the retinal pigment cells, where it is converted to the chromophore, through a process dependent on the PINTA retinoid binding protein (Wang and Montell, 2005) and the NINAG oxidoreductase (Ahmad et al., 2006). The chromophore is finally transported to the photoreceptor cells and binds to the opsin, resulting in the generation of rhodopsin. The proteins required for the indicated steps have not been identified (question marks).

is functional coupled with BCO in the conversion of β-carotene to all-trans-retinal. BCO is highly expressed in the retinal pigment epithelium of both human and monkey eyes (Yan et al., 2001; Bhatti et al., 2003), suggesting that the biosynthesis of all-trans-retinal from carotenoids may occur in RPE cells, which are well known to function in the generation of the chromophore from all-trans-retinol (for review see Travis et al., 2007). Interestingly, CD36 also appears to be expressed in RPE cells, suggesting that it may participate in the metabolism of β-carotene to all-trans-retinal in RPE cells. However, the association of a specific mammalian scavenger receptor and a BCO has not been described.

In principle, the conversion of carotenoids to all-trans-retinal could be a relatively simple process, which occurs in one cell type (e.g., cells in the gut) exclusively through the activity of a coupled scavenger receptor and a BCO. Although NINAB and SANTA MARIA function together for the centric cleavage of β-carotene, our data suggest that the production of all-trans-retinal from β-carotene is more complicated than previously envisioned. NINAD is another class B scavenger receptor required for the generation of all-trans-retinal; yet, NINAD and SANTA MARIA cannot substitute for each other. The *ninaD* gene is expressed primarily in the gut, and expression of *ninaD* using a *GAL4* driver that directs expression in the gut rescues the *ninaD* phenotype. Surprisingly, *ninaD* function can also be rescued by expression specifically in neurons (Gu et al., 2004; unpublished data), demonstrating that expression of *ninaD* either in the midgut or specifically in neurons rescues *ninaD* function. Furthermore, *ninaB* and *santa maria* are expressed and function in glia and neurons distinct from those in which *ninaD* functions.

The data from the current work enables the formulation of the pathway critical for the conversion of carotenoids to production of all-trans-retinal, which is subsequently metabolized into the chromophore (Fig. 8). The pathway begins with the uptake of dietary carotenoids into the gut, through a process that involves the NINAD scavenger receptor. The β-carotene does not appear to be metabolized in the gut or in the *ninaD* expressing neurons. Rather, the SANTA MARIA scavenger receptor and the NINAB BCO function coordinately in neurons and glia, for the uptake and centric cleavage of β-carotene. The all-trans-retinal is then metabolized into vitamin A and transferred to the retinal pigment cells, where it is converted into the chromophore, through a process involving the PINTA retinoid binding protein (Wang and Montell, 2005). The NINAG oxidoreductase also participates in the production of the chromophore, in a step subsequent to the formation of vitamin A (Sarfare et al., 2005; Ahmad et al., 2006), although it remains to be determined whether it functions in the retinal pigment cells or in photoreceptor cells.

The proposed pathway is not yet complete. Because NINAD appears to operate upstream of NINAB/SANTA MARIA, there may be additional proteins that facilitate the uptake of carotenoids into the gut and in the transport to the neurons and glia that express NINAB/SANTA MARIA. In addition, there may be yet-to-be-identified dehydrogenases, as well as other proteins that participate in the uptake of the chromophore into photoreceptor cells. Given the evolutionary conservation of the known components that are required for vitamin A/chromophore production, these yet-to-be-identified *Drosophila* proteins are also likely to participate in the carotenoid metabolic pathway in mammals.

Materials and methods

Ethyl methanesulfonate (EMS) mutagenesis

The *santa maria*^{1} mutant was isolated by performing EMS mutagenesis and screening for second-chromosome mutations affecting the PDA. To perform the screen (Fig. S1), we mutagenized an isogenized *cn,bw* stock with EMS as we have described recently (Wang et al., 2005). The mutagenized flies were mated to DTS91, *Sco*/CyO flies, and the homozygous viable F3 progeny were screened by performing ERGs, using blue and orange light, similar to the paradigm previously described (Pak, 1995).

Fly stocks

The Bloomington Stock Center was the source for the second-chromosome deficiency kit and the following stocks: *Df(2L)ED479*, *Df(2L)Exel7031*, *y w*; *P[GMR-hid]G1 P[neoFRT]40 l(2)CL-L*/CyO; *P[GAL4-ey.H]SS5 P[UAS-FLP1.D]JD2*, *GMR-GAL4*, *drm-GAL4*, *UAS-EGFP*, *UAS-GFP.nls*, P{*Sgs3-GAL4.PD*}3, P{*Lsp2-GAL4.H*}TP1, P{*w*[+*mW.hs*]=*Switch1*}106, P{*GawB*}*EDTP*^{DJ694}, P{*GawB*}*DJ667*, *elav-GAL4*, and *repo-GAL4*. W. Pak (Purdue University, West Lafayette, IN) provided *ninaE*^{P332}, *ninaD*^{P246}, and

ninaB^{P315}; J. O'Tousa (University of Notre Dame, Notre Dame, IN) provided *UAS-ninaB* and *UAS-ninaD* flies; and C. Desplan (New York University, New York, NY) supplied the *ninaE-GAL4*.

The fly stocks generated were as follows: Fig. 2, *santa maria*^{1}; *hs–santa maria/+*; Fig. 3, (1) *P[GMR-hid]G1 P[neoFRT]40 l(2)CL-L/ P[neoFRT]40 santa maria*^{1}*;P[GAL4-ey.H]SS5 P[UAS-FLP1.D]JD2*, (2) *santa maria*^{1}*,UAS–santa maria;GMR-GAL4/+*, (3) *CG7077* (pigment cell)*-GAL4;santa maria*^{1}*,UAS–santa maria*, (4) *santa maria*^{1}*,UAS–santa maria/santa maria*^{1}*,ninaE-GAL4*, (5) *santa maria*^{1}*,UAS–santa maria;santa maria–GAL4/+*; Fig. 4, (1) *ninaB-GAL4/+;ninaB*^{P315}*,UAS-ninaB*, (2) *ninaD-GAL4/+;ninaB*^{P315}*,UAS-ninaB*, (3) *santa maria–GAL4/ +;ninaB*^{P315}*,UAS-ninaB*, (4) *ninaD*^{P246}*,UAS-ninaD;ninaB-GAL4/+*, (5) *ninaD*^{P246}*,UAS-ninaD;ninaD-GAL4/+*, (6) *ninaD*^{P246}*,UAS-ninaD;santa maria–GAL4/+*, (7) *santa maria*^{1}*,UAS–santa maria;ninaB-GAL4/+*, (8) *santa maria*^{1}*,UAS–santa maria;ninaD–GAL4/+*, (9) *santa maria*^{1}*,UAS–santa maria;santa maria–GAL4/+*; Fig. 5, (1) *ninaB-GAL4/UAS-GFP*, (2) *santa maria–GAL4/UAS-GFP*, (3) *ninaB-GAL4/UAS-GFP.nls*, (4) *santa maria–GAL4/UAS-GFP.nls*, (5) *elav-GAL4;ninaB*^{P315}*,UAS-ninaB*, (6) *elav-GAL4;santa maria*^{1}*,UAS–santa maria*, (7) *ninaD*^{P246}*,UAS-ninaD;repo-GAL4/+*, (8) *ninaB*^{P315}*,UAS-ninaB/ninaB*^{P315}*,repo-GAL4*, (9) *santa maria*^{1}*,UAS–santa maria;repo-GAL4/+*; Fig. 6, (1) *santa maria*^{1}*;santa maria–GAL4/UAS-ninaD*, (2) *santa maria*^{1}*;santa maria–GAL4/UAS–santa maria*, (3) *santa maria*^{1}*;santa maria–GAL4 /UAS-SR-BI*, (4) *ninaD*^{P246}*;ninaD-GAL4/UAS-ninaD*, (5) *ninaD*^{P246}*;ninaD-GAL4/UAS–santa maria*, (6) *ninaD*^{P246}*;ninaD-GAL4/UAS-SR-BI*; Fig. 7, (1) *ninaD-GAL4/UAS-EGFP*, (2) *santa maria*^{1}*,UAS–santa maria;drm-GAL4/+*, (3) *ninaD*^{P246}*,UAS-ninaD;drm-GAL4/+*.

Deficiency mapping the *santa maria* mutation

The *santa maria*^{1} mutation was crossed to the fly stocks that comprised the second-chromosome deficiency kit. The mutation was uncovered by *Dl(2l)XE-3801*, which deleted 27E2 to 28D1, but not by other deficiency lines in the kit. To map the *santa maria* locus further, we used smaller deficiencies in the region and found that the mutation was uncovered by *Df(2L)ED479* (27F4 to 28B1) and *Df(2L)Exel7031* (27F3 to 28A1; Fig. 2 A). Based on these data, we localized the mutation responsible for the *santa maria* phenotype to 27F4 to 28A1. This interval is ~30 kb and included 10 genes spanning from *CG5261* to *CG6630* (Fig. 2 A).

ERG recordings

ERG recordings were performed as previously described (Wes et al., 1999). In brief, two glass microelectrodes filled with Ringer's solution were inserted into small drops of electrode cream placed on the surfaces of the compound eye and the thorax. A Newport light projector (model 765) was used for stimulation. The ERGs were amplified with an electrometer (IE-210; Warner) and recorded with an A/D converter (MacLab/4s) and the Chart v3.4/s program (A/D Instruments). Five 5-s light pulses (orange, blue, blue, orange, and orange) were given to each fly, and the interval time between each pulse was 7 s. All recordings were performed at room temperature.

Generation of transgenic flies

To express the *santa maria* cDNA under the control of the *heat shock protein 70* (*hs*) promoter and the *UAS* promoter, the cDNA (EST clone RH67675; Drosophila Genomics Resource Center) was subcloned between the NotI and XbaI sites of the pCaspeR-*hs* vector (Thummel and Pirrotta, 1992) and same site of the pUAST vector (Brand and Perrimon, 1993), respectively. To express SR-BI in flies, the human cDNA (EST clone 6384348; Invitrogen) were subcloned between the NotI and XbaI sites of pUAST.

To express GAL4 under control of the *ninaB*, *ninaD*, and *santa maria* transcriptional control regions, we subcloned the following ~2.0-kb genomic DNA fragments between the KpnI and BamHI sites of the GAL4 vector, pGATB (Brand and Perrimon, 1993): (1) *ninaB*, −1790 to +98 base pairs 5′ to the transcription starting site; (2) *ninaD*, −2021 to +13 base pairs 5′ to the transcription starting site; and (3) *santa maria*, −2030 to +30 base pairs 5′ to the transcription starting site. The promoter-*GAL4* fragments were excised from pGATB using KpnI and NotI and introduced between the same sites of pCaspeR4 (Thummel and Pirrotta, 1992). The constructs were injected into of *w*^{1118} embryos, and transformants were identified on the basis of eye pigmentation.

Western blots

To perform Western blots, fly heads, bodies, and dissected guts were homogenized in SDS sample buffer with a Pellet Pestle (Kimble/Kontes). The proteins were fractionated by SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore) in Tris-glycine buffer. The blots were probed with mouse anti-tubulin primary antibodies (1:2,000 dilution; Developmental Studies Hybridoma Bank), mouse anti-Rh1 antibodies (1:2,000 dilution; Developmental Studies Hybridoma Bank), rabbit anti-Rh4 antibodies (1:1,000 dilution), rabbit anti-NORPA antibodies (1:2,000 dilution; Wang et al., 2005), rabbit anti-GFP antibodies (1:1,000; Santa Cruz Biotechnology, Inc.), or rabbit anti–SR-BI (1:1,000 dilution; Novus Biologicals) and, subsequently, with anti-rabbit or -mouse IgG peroxidase conjugate (Sigma-Aldrich). The signals were detected using ECL reagents (GE Healthcare).

Northern blots

Total RNAs were prepared using Trizol reagent (Invitrogen), and the RNA samples (2 μg each) were fractionated on 3% formaldehyde and 1.2% agarose gels. The RNAs were transferred to nitrocellulose membranes and allowed to hybridize with ^{32}P-labeled probes, which were prepared using random primers and a *ninaE* PCR product (nucleotides 300–900 of the *ninaE* cDNA) as the template. The hybridization was performed at 65°C in 7% SDS, 2 mM EDTA, and 0.5 M Na_2HPO_4, pH 7.2, and the membranes were washed at 65°C in 0.5× SSC and 0.1% SDS.

Immunolocalizations

We prepared coronal sections (8 μm) from adult fly heads, which were frozen in OCT medium (Tissue-Tek). The primary antibodies consisted of rabbit anti-GFP antibodies (1:50 dilution; Santa Cruz Biotechnology, Inc.) combined with either mouse anti-REPO antibody (1:20 dilution; Developmental Studies Hybridoma Bank) or rat anti-ELAV antibodies (1:100 dilution; Developmental Studies Hybridoma Bank). To facilitate detection of the primary antibody staining, we used the following secondary antibodies (1:200 dilution; Invitrogen): anti-rabbit labeled with Alexa Fluor 488 (for detection of anti-GFP), anti-mouse Alexa Fluor 568 (for detection of anti-REPO), or anti-rat Alexa Fluor 568 (for detection of anti-ELAV). The sections were examined using a microscope (Axioplan; Carl Zeiss MicroImaging, Inc.) with Plan-Apochromat 20× objectives, and images were acquired with a camera (SensiCam; Cooke) and IPLab 3.6.5 software. The images were transferred into Photoshop 7.0 (Adobe) to assemble the figures.

Guts were dissected from flies ~1 d after eclosion and rinsed with 1× PBS buffer. The GFP fluorescence was examined using an Axioplan microscope with a Plan-Apochromat 20× objective, and images were acquired with a SensiCam camera and IPLab 3.6.5 software. The images were transferred into Photoshop 7.0 to assemble the figures.

Online supplemental material

Fig. S1 shows the genetic scheme to identify mutations on the second chromosome that disrupt the ERG. Fig. S2 shows that *santa maria*, *ninaB*, and *ninaD* function outside of retina for carotenoid metabolism. Fig. S3 shows that the Rh1 protein level in *santa maria*^{1} flies does not increase as a result of expression of *UAS–santa maria* under control of the *ninaD-GAL4*. Fig. S4 shows that coexpression of *ninaB* and *santa maria* in the retinal pigment cells of *ninaB*^{P315} or *santa maria*^{1} flies does not restore Rh1 levels. Fig. S5 shows that SR-BI is expressed in the *ninaD-GAL4/UAS–SR-BI* and *santa maria–GAL4/UAS–SR-BI* flies. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200610081/DC1.

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