***Drosophila* caspase transduces Shaggy/GSK-3 β kinase activity in neural precursor development**

**Hirotaka Kanuka^{1,2}, Erina Kuranaga^{1,2},
Kiwamu Takemoto^{1,2}, Tetsuo Hiratou²,
Hideyuki Okano^{3,4} and Masayuki Miura^{1,2,*}**

¹Department of Genetics, Graduate School of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan, ²Laboratory for Cell Recovery Mechanisms, RIKEN Brain Science Institute, Wako, Saitama, Japan, ³Department of Physiology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan and ⁴Core Research for Evolutional Science and Technology (CREST), Shinjuku-ku, Tokyo, Japan

Caspases are well known for their role in the execution of apoptotic programs, in which they cleave specific target proteins, leading to the elimination of cells, and for their role in cytokine maturation. In this study, we identified a novel substrate, which, through cleavage by caspases, can regulate *Drosophila* neural precursor development. Shaggy (Sgg)46 protein, an isoform encoded by the *sgg* gene and essential for the negative regulation of Wingless signaling, is cleaved by the Dark-dependent caspase. This cleavage converts it to an active kinase, which contributes to the formation of neural precursor (sensory organ precursor (SOP)) cells. Our evidence suggests that caspase regulation of the wingless pathway is not associated with apoptotic cell death. These results imply a novel role for caspases in modulating cell signaling pathways through substrate cleavage in neural precursor development.

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Introduction

Caspases, key mediators in cell-killing pathways, are a class of cysteine proteases that includes mammalian interleukin-1 β -converting enzyme (ICE) and the *ced-3* gene of *Caenorhabditis elegans* (*C. elegans*) (Miura and Yuan, 1996). A functional apoptotic program requires multiple caspases, each contributing to a different phase of apoptosis. During this program, activated caspases cleave a variety of substrates, including other caspases, which ultimately leads to morphological changes that indicate apoptosis. The requirement for 'killer caspases' in this process has been demonstrated through a variety of experimental approaches. Since several members of the caspase family process each other, resulting in the effective amplification of cell death signals (Thornberry and Lazebnik, 1998), studies on the

activation mechanisms of these caspases and identification of caspase substrates are crucial for understanding the cell death machinery. However, although many caspase substrates have been identified, the biological functions of most of these substrates remain largely unknown. The identification of caspase targets and determination of the effects of caspase-mediated cleavage on their function will be critical to understanding the mechanisms of apoptosis as well as other downstream events.

Recently, evidence for nonapoptotic functions of caspases in cellular processes has been accumulating. Caspase activity is required in some cells for the regulation of survival pathways, cell motility, receptor internalization, and may regulate cell-cycle progression and proliferation (Algeciras-Schimmich *et al*, 2002). In particular, recent studies implicate effector caspases in mouse lens development, highlighting a potential role for the caspase cleavage of α -spectrin (Lee *et al*, 2001), and caspase activation is required for the terminal differentiation of human epidermal keratinocytes that are necessary for the loss of the nucleus (Weil *et al*, 1999). Moreover, a previous report implicated caspase-8 as a negative regulator of erythroid development through its processing of GATA-1 (De Maria *et al*, 1999), and a more recent study demonstrated that caspases are required for terminal erythroid differentiation through the cleavage of lamin B and acinus (Zermati *et al*, 2001). In *Drosophila*, caspase activity is required for the individualization of sperm differentiation (Arama *et al*, 2003; Huh *et al*, 2004b), and provided a model where apoptotic cells activate signaling cascades for compensatory proliferation (Huh *et al*, 2004a; Perez-Garijo *et al*, 2004; Ryoo *et al*, 2004), but the precise mechanisms remain to be elucidated. Recently, a cell death-independent role for *Drosophila* caspases in Rac-mediated cell motility was reported (Geisbrecht and Montell, 2004), and loss of the *Drosophila* caspase DRONC leads to an unappreciated role in the maintenance of adult tissues including the eye, wing, and midgut (Chew *et al*, 2004; Daish *et al*, 2004). These findings point to the involvement of caspases in regulating cell death, survival, and differentiation.

In *Drosophila*, seven caspases (DCP-1, drICE, Dredd, DRONC, Decay, DAMM, and STRICA) have been identified and shown to be required for cell death induced by various stimuli (Hay *et al*, 2004). These caspases are mediated, in part, by an Apaf-1 homolog, Dark (Dapaf-1/HAC-1), and two Bcl-2 family proteins (Drob-1/Debcl/dBorg-1/dBok and Buffy/dBorg-2) (Baehrecke, 2002). In addition, caspase-dependent cell death can be induced by the *Drosophila* killer proteins Reaper and Hid, and strongly inhibited by the overexpression of caspase inhibitory proteins, p35, DIAP1, and DIAP2 (McCall and Steller, 1997). The *Drosophila* caspases have distinct specificities for substrate recognition (Hawkins *et al*, 2000; Song *et al*, 2000), which could potentially be used to modulate nonapoptotic signaling pathways during development. In this study, we identified a novel substrate that, through cleavage by caspases, can be involved in the regula-

*Corresponding author. Department of Genetics, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel.: +81 3 5841 4860; Fax: +81 3 5841 4867; E-mail: miura@mol.f.u-tokyo.ac.jp

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tion of *Drosophila* neural precursor development. Here we reveal that the Shaggy (Sgg)46 protein, an isoform encoded by the *sgg* gene and essential for the negative regulation of Wingless signaling, is converted by caspase cleavage into an active kinase, which then might contribute to the formation of neural precursor (sensory organ precursor (SOP)) cells.

Results

Dark-dependent caspase activation is involved in macrochaete formation

Loss of maternal and zygotic Dark (Dapaf-1/HAC-1) function in *Drosophila* results in decreased caspase activity and reduced apoptosis in the embryonic and larval brain (Kanuka *et al*, 1999a; Rodriguez *et al*, 1999; Zhou *et al*, 1999). Macrochaetes are external sensory organs located on the notum and are typical structures of the *Drosophila* peripheral nervous system (PNS). Four successive asymmetric cell divisions from a common precursor cell called the SOP generate a sensory bristle consisting of four different non-neuronal support cells and one neuron (Figure 1A). As shown in Figure 1B, four large macrochaetes are observed on the wild-type scutellum. However, extra macrochaetes often appeared on the scutellum of *dark* mutant flies (48%) (Figure 1C; Kanuka *et al*, 1999a; Rodriguez *et al*, 1999). To determine if the appearance of these extra macrochaetes was caspase-dependent, the caspase inhibitory protein p35 was expressed in the scutellum (GAL4/UAS system; Brand and Perrimon, 1993). Indeed, the inhibition of caspases in the scutellum by *scabrous*-GAL4 (*sca*-GAL4) resulted in the formation of ectopic macrochaetes similar to those observed in the *dark* mutants (Figure 1D and E; Kanuka *et al*, 1999a). This phenotype was not limited to p35 expression, given that both DIAP1 and DIAP2 expression also resulted in additional macrochaetes (Table I). Interestingly, blockage of the downstream Dark target, DRONC, either through the expression of a dominant-negative form (DRONC DN) or ectopic expression of an inverted repeat (IR) dsRNA against *DRONC* (which abolishes protein expression, data not shown), induced the same phenotype (Table I). These results concerning DRONC function are consistent with a previous report that mild but not complete, loss of function of *dronc* also causes extra macrochaete formation (Chew *et al*, 2004). The more severe phenotype we observed likely stems from overexpression of DRONC DN, which may inhibit caspase function more effectively than in the partial *dronc* mutant. In contrast, the ectopic expression of p35 and DRONC DN specifically in SOP cells, through the use of *neur*-GAL4, did not result in extra macrochaetes (Figure 1F–I, Table I). Taken together, the Dark/DRONC-dependent caspase activation pathway appears to be involved in regulating the macrochaete number before SOP cell formation.

Dark-dependent caspase activation negatively regulates SOP cell formation

We next examined whether extra macrochaetes are actually produced from extra SOP cells. Initially during the formation of SOP cells, a group of cells called a proneural cluster, characterized by the expression of the proneural genes *achaete* and *scute*, forms at the future site of the macrochaetes in the wing disc (Jan and Jan 1994). These proneural clusters also express the *scabrous* gene (Figure 1A and F), followed by

the expression of *achaete* and *senseless*, in one or a few cells singled out from the proneural cluster (Figure 1A and 2A; Nolo *et al*, 2000). We performed immunostaining using antibodies against Achaete and Senseless on wing discs from the third-instar larval stage. In wild-type larvae, two sets of SOP cells were seen in scutellum region of one wing disc (Figure 2A). However, in both *dark* mutants and DRONC DN-expressing flies, we observed one extra SOP cell expressing Achaete and Senseless (Figure 2B and C). This result indicates that caspase activation is involved in the control of SOP cell formation in the scutellum area.

As shown in Figures 1A and 2D, one SOP cell will produce a set of one neuron and one thecogen cell in the pupal notum. These SOP daughter lineage cells can be characterized by specific marker proteins, Elav (neuron) and Prospero (thecogen) (Jan and Jan, 1998). To examine whether the extra SOP cells induced by caspase inhibition at the larval stage have the potential to develop into a complete set of extra sensory organs, the scutellum region of the pupal notum (24–30 h after pupal formation (APF)) was subjected to immunostaining. Indeed, we observed extra sets of both Elav- and Prospero-positive cells in *dark* mutants and DRONC DN-expressing flies (Figure 2F and G). These observations suggest that caspase activity negatively mediates the formation of scutellum SOP cells.

Caspase activation does not correlate with cell death in SOP cell development

To identify the exact moment at which caspase activity regulates macrochaete formation, we transiently expressed p35 using heat shock. Consistent with the SOP cell-specific expression of p35 and DRONC DN, no significant increase in macrochaete number was seen as a result of caspase inhibition during SOP cell formation (i.e. late third-instar, 6–12 h APF) or after it (24–30 h APF) (Figure 3A). At the stage prior to the appearance SOP cells, however, the transient expression of p35 had a strong effect on the production of extra bristles (Figure 3A). These data strongly support the idea that the inhibition of caspase activity leads to the formation of extra SOP cells rather than affecting differentiation at a later stage.

As caspases are well-known executioners of apoptosis and cell death, which lead to the elimination of cells (Thornberry and Lazebnik, 1998), we investigated whether the increased number of SOP cells resulting from reduced caspase function was related to the inhibition of cell death. At first, we examined whether caspases are actually activated in the *scabrous*-expressing proneural clusters that contain SOP cells. We previously reported the development of an indicator molecule for caspase activation (SCAT3) that uses fluorescence resonance energy transfer (FRET) between two types of fluorescent protein (ECFP and Venus), linked by a peptide containing the caspase-3 cleavage sequence, DEVD (Takemoto *et al*, 2003). Using this probe, we can monitor caspase activation at the single-cell level by observing the changes in the Venus/ECFP ratio. After performing confocal imaging of caspase activity in *sca*-GAL4 + UAS-SCAT3 flies, a decrease in FRET (i.e. activation of caspase) was observed in *scabrous*-expressing proneural clusters in wild-type wing discs (Figure 3B). We also confirmed that this change of ratio was clearly recovered by coexpressing DRONC DN (Figure 3C), indicating that *scabrous*-positive cells actually

Figure 1 Dark-dependent caspase activation is involved in macrochaete formation. (A) Macrochaetes, extra sensory organs on the notum, are typical structures of the *Drosophila* PNS. Four successive asymmetric cell divisions from a common precursor cell called the sensory organ precursor (SOP) generate a sensory bristle comprised of four different non-neuronal support cells and one neuron. (B–D) Caspase inhibition caused increased numbers of extra macrochaetes in the scutellum. Scanning electron microscopic images of the macrochaetes in the scutellum of the adult notum are shown. Each arrowhead indicates extra macrochaetes. The numbers indicate the percentages of the population of flies that contained extra macrochaetes in the scutellum. The indicated genotypes are as follows: wild type (*w¹¹¹⁸*, B), dark mutant (*dark^{P1041}/dark^{P1041}*, C), *sca>p35* (*sca-GAL4/+;UAS-p35/+*, D), and *dpp>p35* (*dpp-GAL4/UAS-p35*, E). (F–I) Proneural clusters including SOP cells in the third-instar larval wing disc. Proneural clusters are visualized by Scabrous expression (green; GFP) (F), and SOP cells are marked by Neuralized (magenta; *A101-lacZ* enhancer trap line) (H and I). A merged picture is shown in (G). The genotype is *sca-GAL4/UAS-GFP;A101-lacZ/+*.

Table I Caspase activation is involved in macrochaete formation

Genotype	Affected cells	Ratio of phenotype (total N)
<i>w¹¹¹⁸</i>	All types of cells	4.0% (255)
<i>dark^{P1041}</i>		48.0% (189)
<i>sca-GAL4 + UAS-lacZ</i>		10.9% (228)
<i>sca-GAL4 + UAS-p35</i>	<i>sca</i> + cell cluster (including SOP)	39.2% (197)
<i>sca-GAL4 + UAS-DRONC DN</i>		55.2% (232)
<i>sca-GAL4 + UAS-DIAP1</i>		60.8% (194)
<i>sca-GAL4 + UAS-DIAP2</i>		40.4% (183)
<i>sca-GAL4 + UAS-lacZ IR</i>		4.6% (205)
<i>sca-GAL4 + UAS-DRONC IR</i>		47.3% (138)
<i>neur-GAL4 + UAS-lacZ</i>		4.7% (148)
<i>neur-GAL4 + UAS-p35</i>		5.1% (117)
<i>neur-GAL4 + UAS-DRONC DN</i>		1.5% (131)

Summary of phenotypes pertaining to increased macrochaete numbers. Flies were crossed and kept on standard *Drosophila* medium at 25°C. After exclusion, the number of adult flies that exhibited at least one extra macrochaete in the scutellum were counted and ratios (%) were estimated. *neur-GAL4* flies express GAL4 protein specifically in SOP cells, while *sca-GAL4* flies express GAL4 protein in proneural clusters that include SOP cells. *UAS-lacZ* and *UAS-lacZ IR* were used as negative controls.

have a DEVDase-like caspase activity that can be inhibited by the dominant-negative form of DRONC.

A large amount of naturally occurring cell death is observed in the wing disc of second- and third-instar larvae (Milan *et al*, 1997; Adachi-Yamada *et al*, 1999). This cell death is programmed to eliminate excess cells produced by a high level of proliferation or differentiation (Adachi-Yamada *et al*, 1999; Moreno *et al*, 2002). Consistent with this, we also observed massive cell death by TUNEL staining in the third-instar larval wing disc (Figure 3D and E), allowing for the possibility that the extra SOP cells normally disappear via cell death in wild-type flies. Surprisingly, whereas containing an amount of caspase activity (Figure 3B and C), careful examination of wing discs at early and late larval stages ($n=21$ and $n=28$, respectively), in which caspase inhibition appeared to be effective (Figure 3C), showed no dead cells in the *scabrous*-expressing proneural clusters in wild-type flies (Figure 3D and E). However, since TUNEL labeling is a transient event, it is difficult to rule out the possibility of inhibition of death, even in the case that these are no TUNEL-positive cells in *scabrous* cluster. The mechanism to produce extra macrochaete by inhibition of caspase could arise through several possible mechanisms, and we also prefer the mechanism that the cell fate change could be induced by caspase inhibition. These results suggest that the increased number of SOP cells seen in the caspase-inhibited wing discs was not a result of the artificial survival of cells that otherwise should have undergone apoptotic cell death, but rather was due to inhibition of an apoptosis-independent caspase activity.

Genetic identification of Sgg as a component of caspase-regulated macrochaete development

To identify possible downstream targets of this apoptosis-independent caspase activity, we performed a dominant-modifier screen in which we monitored macrochaete numbers. The ectopic expression of DRONC DN using *sca-GAL4*

gave rise to a dose-dependent increase in the number of macrochaetes (Table I and data not shown). In our modifier screen, we identified 29 suppressors and three enhancers covering large chromosomal regions. We secondarily screened and identified small-deficiency lines, and subsequently tested the ability of available pre-existing mutants located in these regions to reproduce the phenotype exhibited by the *sca-GAL4 + UAS-DRONC DN* flies. As a result, components of Notch signaling (*numb*, *Su(H)*, *Delta*, and *neur*) and the Wingless pathway (*wingless* and *shaggy*) were identified (Table II and data not shown). Both of these signaling pathways are important for macrochaete development, especially in the regulation of positional patterning and number (Simpson *et al*, 1999). Thus, we further analyzed the Wingless pathway in relation to caspase-mediated SOP cell formation.

In cells that receive Wingless signals, Armadillo (Arm)/ β -catenin accumulates and translocates to the nucleus to activate Wingless-responsive genes. This signal-induced accumulation of Arm/ β -catenin results from an interruption of its normal turnover mediated by phosphorylation and subsequent ubiquitination-proteasomal degradation (Wodarz and Nusse 1998). The kinase Sgg, the *Drosophila* GSK-3 β ortholog, is an antagonist of Wingless signal transduction and has been shown to be critical for this phosphorylation of Arm/ β -catenin (Dierick and Bejsovec 1999). In our modifier screen, we found a regulatory mutant of Wingless (*wg^{SP-1}*) that was able to suppress the increase in extra macrochaetes, while a completely null mutant of Sgg (*sgg^{D127}*) enhanced the phenotype (Table II). This result genetically suggests that endogenous caspase activity antagonizes Wingless-dependent macrochaete formation. In agreement with this, loss of Sgg function (achieved by an *sgg^{D127} -/-* mitotic clone) resulted in extra macrochaetes and microchaetes in the dorsal part and scutellum of the adult notum (Figure 4A–D), where Wingless is highly expressed (Figure 4E). Similarly, a previous study reported that *sgg^{D127} -/-* adult flies produced by transient rescue of the *sgg* gene also exhibited severe hyperplasia of macrochaetes in the same region (Ruel *et al*, 1993a). In support of these data, *sgg -/-* cells in the wing discs significantly expressed both Senseless and Achaete, marker proteins for SOP cells (Figure 4F), indicating that these extra macrochaetes are mainly produced by ectopic differentiated SOP cells. In contrast, the ectopic expression of Sgg10, one of the major isoforms of the *sgg* gene transcripts, caused a clear loss of macrochaetes in the scutellum (Figure 4H, compared with 4G) and loss of SOP cells in the wing disc (Figure 6G). This phenotype depends on Sgg kinase activity because a kinase-negative form of Sgg10 (Sgg10 A81T) did not affect macrochaete formation (Figure 4I). The same phenotypes were reported using another GAL4 driver (*sc(455.2)-GAL4*) (Bourouis, 2002). Taken together, these data indicate that Sgg kinase activity negatively regulates SOP cell and macrochaete development.

Shaggy kinase isoform is a substrate for caspase

We next investigated how caspases are involved in the regulation of Sgg kinase activity. Some typical caspase families recognize and cleave motifs corresponding to DEVD (Asp-Glu-Val-Asp). Interestingly, Sgg46, one of three major isoforms of Sgg (Ruel *et al*, 1993b), contains two such motifs, suggesting it may be a direct target for caspase cleavage

Figure 2 Dark-dependent caspase activation negatively regulates SOP cell formation. (A–C) Caspase inhibition induces the formation of extra SOP cells. The SOP cells in the scutellum of the third-instar larval wing disc are marked by Senseless (red, A–C) and Achaete (blue, A'–C'). Merged pictures are shown in (A''–C''). Each arrowhead indicates extra SOP cells. The indicated genotypes are as follows: wild type (w^{1118} , A–A''), dark mutant ($dark^{P1041}/dark^{P1041}$, B–B''), and *sca-GAL4 + UAS-DRONC DN* (*sca-GAL4/+; UAS-DRONC DN/+*, C–C''). (D–G) The extra SOP cells can produce daughter cells. The daughter lineage cells in the scutellum of the pupal notum (24–30 h APF) marked by Elav (neuron marker, magenta) and Prospero (theogen marker, green). The scheme of the wild-type notum is shown in (D). Each arrowhead indicates an extra set of neuron/theogen cells. The indicated genotypes are as follows: wild type (w^{1118} , E), dark mutant ($dark^{P1041}/dark^{P1041}$, F), and *sca-GAL4 + UAS-DRONC DN* (*sca-GAL4/+; UAS-DRONC DN/+*, G).

(Figure 5A). Both of the caspase-cleavage DEVD domains are found in the N-terminal portion of Sgg46, before the kinase domain that is shared with Sgg10. To examine whether Sgg46

can be processed by caspases, Myc-tagged Sgg46 was expressed along with various caspases in *Drosophila* S2 cells (Figure 5B). As a positive control, staurosporine, which is

Figure 3 Caspase activation does not correlate with cell death in SOP cell development. (A) Caspases affect macrochaete development at the stage before the beginning of SOP cell formation. The scheme of the development of SOP cells and their daughter lineage is shown. The larvae or pupa (*hs-GAL4 UAS-35/+*) were collected during the indicated time points and heat-shocked at 37°C for 1 h. After they eclosed, the number of adult flies that exhibited at least one extra macrochaete in the scutellum was counted, and the ratio (%) is shown. (B, C) Caspase activity was observed in cells that expressed Scabrous. Wing discs from third-instar larvae were examined by *in situ* confocal imaging of caspase activity using FRET-based probe, SCAT3. Ratio images of SCAT3-expressing cells are shown in (B, C). The indicated genotypes are as follows: wild type (*sca-GAL4 UAS-SCAT3/+*, B) and *sca-GAL4 + UAS-DRONC DN* (*sca-GAL4 UAS-SCAT3/+; UAS-DRONC-DN/+*, C). (D, E) There were no dead cells among those that expressed Scabrous. Wing discs from early- (D) and late- (E) stage third-instar larvae (*sca-GAL4/UAS-GFP*) were examined by TUNEL staining (apoptotic cells, magenta, D, E) and Scabrous expression (proneural clusters, green, D', E'). Merged pictures are shown in (D'', E''). Each arrowhead indicates the position of Scabrous-expressing clusters.

Table II Sgg as a component of caspase-regulated macrochaete formation

	Genotype	Ratio of phenotype (total N)
<i>sca-GAL4 + UAS-DRONC DN x</i>	<i>w¹¹¹⁸</i>	18.3% (139)
	<i>wg^{SP-1}</i>	2.8% (210)
	<i>sgg^{D127}</i> (null for all Sgg isoforms)	30.1% (196)

The genetic interaction of DRONC DN-expressing flies with Wingless pathway components. *sca-GAL4/CyO;UAS-DRONC DN/TM3, Sb* flies were crossed to flies with the indicated genotype. The number of F1 progeny adult flies exhibiting at least one extra macrochaete in the scutellum was counted and a ratio (%) estimated.

known to induce cell death via caspase activation in S2 cells, was used (Kanuka *et al*, 1999b). A single fragment (≤ 39 kDa), which was likely processed from full-length Sgg46 protein, was observed specifically in drICE- and DRONC-expressing cells, in addition to staurosporine-treated cells (Figure 5B). We did not detect this fragment when a putative noncleavable form of Sgg46, Sgg46 D300G, was expressed with drICE, indicating that caspases are responsible for cleaving Sgg46 at Asp³⁰⁰ (Figure 5B).

Next, we isolated an S2 cell line that stably expresses the cell death activator *reaper* under the control of the heat-shock promoter. Expression of *reaper* remarkably induced the endogenous caspase activity (DEVDase) 60 min after heat-shock treatment (Figure 5C) and the subsequent processing of drICE into the active form (Figure 5D). Correlated with this caspase activation, two small Myc-tagged fragments and a large fragment recognized by an antibody against the cleaved form of Sgg46 (Asp³⁰⁰, Figure 5E) were observed (Figure 5F and G). These fragments could not be detected in S2 cells expressing Sgg46 D300G (upper band in Figure 5F and large band in 5G) and Sgg46 D235G (lower band of Figure 5F, data not shown), suggesting that Sgg46 can be cleaved by endogenous caspases at Asp²³⁵ and Asp³⁰⁰.

Caspase-dependent cleavage alters Sgg46 function in SOP cell development

Previous genetic studies of *sgg* mutant flies showed the interesting observation that some phenotypes of *sgg* mutants can be rescued by the expression of *sgg10* or *sgg39* (the other *sgg* isoform similar to *sgg10*), but not *sgg46* (Ruel *et al*, 1993a,b), suggesting that Sgg46 might be an inactive form. The Sgg10 kinase phosphorylates the Arm protein and induces its degradation (Yanagawa *et al*, 1997; Ruel *et al*, 1999). We tested various forms of Sgg protein for this activity (Figure 6A). As previously reported, expression of Sgg10 induced Arm phosphorylation and degradation in a kinase-dependent manner (Figure 6B, myc-Sgg10 and myc-Sgg10 K83R, a kinase-negative form). In contrast, full-length Sgg46 did not produce the same effects on the Arm protein (Figure 6B, myc-Sgg46). Interestingly, expression of a putative cleaved form of Sgg46, containing the kinase domain (myc-Sgg46 Δ N235 and myc-Sgg46 Δ N300), led to Arm phosphorylation and degradation in a manner similar to that of Sgg10 (Figure 6B). These results suggest that full-length Sgg46 is an inactive form that can be converted into an active kinase via caspase-dependent cleavage.

Figure 4 Identification of Shaggy as a component of caspase-regulated macrochaete development. (A-E) Loss of *sgg* function induces extra macrochaetes in the Wingless-expressing region. The FLP/FRT-mediated mosaic analysis was used for making an *sgg* -/- clone. Extra macrochaetes (black arrowheads, A-C) were frequently seen in the specific region of the notum with Wingless expression (E). Blue arrowhead in (D) shows extra microchaetes. (F) Loss of *sgg* function induced extra SOP cells. After producing the *sgg* -/- clone, third-instar larval wing discs were examined for Senseless (red, F) and Achaete (blue, F'') expression. *sgg* -/- clones (arrowhead) lack GFP (green, F'). Merged pictures are shown in (F''). (G-I) The ectopic expression of Sgg induced a loss of macrochaetes that was dependent on its kinase activity. Asterisks indicate the position of missing macrochaetes in the scutellum (H). Sgg10 A81T was used as a kinase-negative form. The indicated genotypes are as follows: wild type (*w¹¹¹⁸*, G), *sgg10* (*sca-GAL4/+;UAS-sgg10/+*, H), and *sgg10 A81T* (*sca-GAL4/+;UAS-sgg10 A81T/+*, I).

Figure 5 A Shaggy kinase isoform is a substrate for caspases. (A) Sgg46 contains two putative cleavage sites recognized by caspase. The *sgg* gene encodes two major types of proteins, Sgg10 and Sgg46. Sgg46 has a large N-terminal domain compared with Sgg10. These Sgg proteins commonly contain both a protein kinase catalytic domain. Note that the two caspase-cleavage motifs (DEVD²³⁵ and DEVD³⁰⁰) in Sgg46 are located before the kinase domain. (B) *Drosophila* caspase induces cleavage of Sgg46. (Left panel) S2 cells were cotransfected with pUAST-myc-sgg46 (800 ng) and pWAGAL4 (200 ng) and the indicated construct (pUAST-lacZ, -HA-drICE, -HA-DCP-1, -HA-Dredd, or -HA-DRONC) (200 ng). At 12 h after the transfection, staurosporine (1 μ M) was added to the medium. (Right panel) S2 cells were transfected with the indicated plasmid (pUAST-myc-sgg46, -sgg46 D300G, -sgg46 D235G, or -sgg46 D235G/D300G) (800 ng) along with pWAGAL4 (200 ng) and pUAST-HA-drICE (200 ng). In both cases, 24 h after transfection, the cells were collected and subjected to immunoblotting using an anti-myc antibody. The D235 fragment of sgg46 ran off the end of this gel. (C, D) Expression of Reaper induces a rapid activation of caspases, including drICE. Caspase activities (DEVDase) in control S2 cells (squares) and S2 cells stably transfected with pCasper-*hs-reaper* (diamonds) were measured at the indicated time points (min) after heat-shock treatment at 37°C for 1 h (C). The processing of drICE was also detected by using antibodies specific for the drICE active form (D, arrowhead). (E) The antibody-specific scheme for the cleavage sites of Sgg46 at Asp³⁰⁰. (F, G) Endogenous active caspases evoked by Reaper can cleave Sgg46. S2 cells that were stably transfected with pCasper-*hs-reaper* were cotransfected with the indicated plasmid (pUAST-myc-sgg46 or pUAST-sgg46 D300G) (800 ng) and pWAGAL4 (200 ng) into stably transfected. At 18 h after the transfection, the cells were heat-shocked at 37°C for 1 h. The cells were then collected and subjected to immunoblotting using anti-myc and anti-active Sgg46 antibodies.

Figure 6 Caspase-dependent cleavage alters Sgg46 function in SOP cell development. **(A)** Scheme of the variants of Myc-tagged Sgg proteins used in this study. **(B)** The cleaved form of Sgg46, but not full-length form, acts as an active kinase in Arm phosphorylation and degradation. The indicated plasmids (*pUAST-lacZ*, *-myc-sgg10*, *-myc-sgg46*, *-myc-sgg10 K83R*, *-myc-sgg46 Δ N235*, and *-sgg46 Δ N300*) (600 ng) were transfected with *pWAGAL4* (200 ng) and *pUAST-Arm* (200 ng) into S2 cells. At 24 h after transfection, the cells were collected and subjected to immunoblotting using anti-myc, anti-Arm, and anti-phosphorylated Arm antibodies. Asterisk indicates a nonspecific band. **(C–F)** Ectopic expression of the cleaved form of Sgg46, but not the full-length form, induces a loss of macrochaetes. Asterisks indicate the position of the lost macrochaetes in the scutellum (D–F). The indicated genotypes are as follows: wild type (*w¹¹¹⁸*, C), *sgg10* (*sca-GAL4/+;UAS-myc-sgg10/+*, D), *sgg46* (*sca-GAL4/+;UAS-myc-sgg46/+*, E), and *sgg46 Δ N300* (*sca-GAL4/+;UAS-myc-sgg46 Δ N300/+*, F). **(G–I)** Ectopic expression of the cleaved form of Sgg46, but not the full-length form, induces a loss of SOP cells without cell death. Third-instar larval wing discs with the indicated genotypes shown in (D–F) were examined for cell death (TUNEL, green) and Senseless expression (red). The ectopic expression of Myc-tagged Sgg proteins by *sca-GAL4* was also confirmed (blue).

We next examined whether these findings were applicable to macrochaete and SOP cell development *in vivo* by using transgenic flies expressing Sgg proteins. As shown above, the ectopic expression of Sgg10 by *sca-GAL4* caused the loss of macrochaetes and SOP cells (Figure 6D, compared with 6C). No apoptotic cells in the myc-Sgg10 protein-expressing region of the wing disc could be detected, indicating that this disappearance did not result from the death of SOP cells (Figure 6G). Consistent with the immunoblotting results, full-length Sgg46 did not influence macrochaete and SOP cell formation (Figure 6E and H), whereas the cleaved form of Sgg46 (Sgg46 Δ N300) worked in a manner similar to that of Sgg10 (Figure 6F and I). After crossing *sca-GAL4 + UAS-DRONC DN* to *UAS-sgg10*, most F1 progeny showed a clear loss of macrochaetes in the scutellum (data not shown), indicating that Sgg kinase activation might be downstream of caspases. These observations suggest that the processing of Sgg46 by caspases leads to the formation of an active kinase that can negatively regulate SOP cell development.

Finally, we investigated Sgg46 contributes significantly to macrochaete and SOP cell formation *in vivo*. The ectopic expression of Sgg46 D235G/D300G by *sca-GAL4* significantly induced extra macrochaetes and SOP cells (Figure 7A–C). Since Sgg46 D235G/D300G could not be cleaved by caspases (Figure 5B, F and G), this noncleaved Sgg46 might act as dominant-negative form against endogenous Sgg function. Furthermore, an ectopic knockdown of Sgg protein expression by dsRNA-expressing constructs (Figure 7D) revealed that the specific reduction of the Sgg46 protein induced extra macrochaetes (Figure 7E–G). However, inhibition of Sgg46 is less effective at producing extra macrochaetes than inhibiting Dark or DRONC, suggesting that modulation of Sgg kinase activity may not be the only mechanism contributing to SOP formation. It still remains to be examined whether or not Sgg46 is actually cleaved and converted into an active form in proneural clusters, and will require further examination *in vivo*. Based on our findings that loss of Sgg function or inhibition of caspase activity resulted in extra macrochaetes mainly in the scutellum of the adult notum (pSC and aSC), where Wingless is highly expressed (Figure 4A–E), and that caspases are activated in *scabrous*-expressing cluster (Figure 3B and C), we could consider that *scabrous*-expressing SOP cells that will produce pSC and aSC macrochaetes are located in specific region, where precise formation of each set of macrochaetes might require both (1) Wg expression (to increase bristle) and (2) caspase activation (to decrease bristle). Thus, it appears that Dark-dependent caspase signaling mediates the total Sgg kinase activity by processing Sgg46 into an active form, thereby negatively regulating Wingless-sensitive macrochaete development (Figure 7H).

Discussion

Signal transduction mediated by the caspase family

Although the contribution of caspases to development through apoptosis has been well documented, caspase downstream targets and potential nonapoptotic roles have remained elusive. In this study, we observed that the Sgg46 protein, an isoform encoded by the *sgg* gene and essential for the negative regulation of Wingless signaling, could be cleaved by caspases and thereby converted into an active kinase. This cleaved form of Sgg46 was then able to

contribute to the formation of neural precursor (SOP) cells (Figure 7H). However, whereas a plethora of homologous Sgg/GSK-3 β proteins in many species including yeast and mammals have been identified, we could not find any isoforms similar to Sgg46, even in the mosquito *Anopheles* genome (data not shown). This indicates that the additional Sgg46 domain attached to the conserved kinase domain of Sgg/GSK-3 β , which is able to be processed by caspases, might be an evolutionarily unique phenomenon in *Drosophila*.

Even if the Sgg46 isoform is unique, similar forms of caspase-dependent regulation may occur in other signaling pathways. The tumor suppressor APC (adenomatous polyposis coli) is implicated in the Wntless/Wnt signaling pathway that is involved in early embryonic development and tumorigenesis in vertebrates. Browne *et al* (1998) and Webb *et al* (1999) reported that mammalian APC is processed to a 90-kDa amino-terminal fragment by caspase-3. This cleavage at the DNID site of APC may be biologically relevant, given that the 90-kDa fragment consists of a sequence that is highly conserved in the human, rat, mouse, *Xenopus*, and *Drosophila* APC (Webb *et al*, 1999). Further circumstantial evidence for caspase-mediated regulation of the Wnt pathway comes from the observation that caspases also induce the cleavage of Arm/ β -catenin in mammals, a protein known to accumulate in cells depleted of functional APC. This regulation appears to link cell–cell signaling to changes in transcription and cell movement (Brancolini *et al*, 1997). Although the precise functions of the cleaved APC and Arm/ β -catenin products remain unknown, these findings strongly suggest a conservation of the caspase-dependent regulation of Wntless/Wnt signaling between *Drosophila* and mammalian systems. Considering the number of caspases present in the different systems (three in *C. elegans*, seven in *Drosophila*, and 14 in mammals) (Baehrecke, 2002), a broad and manifold evolution of caspase families may allow for a diversity of specific substrates with various nonapoptotic roles.

Caspase-dependent modification of kinase activity

The constitutive activation of kinases can lead to catastrophic effects in a cell. Here, we clearly showed that full-length Sgg46 is an inactive form that can be converted into an active kinase via caspase-dependent cleavage. While some substrates are functionally inactivated upon caspase-mediated cleavage, other proteins and enzymes can be activated, mostly via the removal of an inhibitory or regulatory domain. The physiological consequence of this gain-of-function cleavage in apoptosis remains unclear. Several members of the PKC family and MAP kinase pathway are constitutively activated by the separation of N-terminal regulatory from C-terminal catalytic domains (e.g. p21-activated kinase PAK2 and ROCK-1), and this activation is important for cytoskeletal reorganization and plasma membrane blebbing (Rudel and Bokoch 1997; Coleman *et al*, 2001; Sebbagh *et al*, 2001). Interestingly, a DEVD motif-specific caspase that cleaves MEKK-1 is specifically activated when cells lose matrix contact and this cleavage is required for the MEKK-1 kinase activity (Cardone *et al*, 1997). In another example, MST1, mammalian STE20-like kinase 1, is cleaved and activated by caspases during apoptosis (Graves *et al*, 1998; Lee *et al*, 1998; Ura *et al*, 2001). This raises the possibility that the N-terminal portion of Sgg46 might inhibit its own kinase domain, there-

Figure 7 Crosstalk between caspase and Wingless signaling by modulation of effector molecules. (A, B) Ectopic expression of caspase-insensitive Sgg46 protein induces an increase in extra SOP cells and macrochaetes. The indicated genotypes are as follows: *lacZ* (*sca-GAL4/UAS-lacZ*, A) and *sgg46 D235G/D300G* (*sca-GAL4/UAS-myc-sgg46 D235G/D300G*, B). The numbers indicate the percentages of the population of flies that contained extra macrochaetes in the scutellum, and the arrowhead indicates an extra macrochaete. (C) The third-instar larval wing disc of (B) was examined for apoptotic cells (TUNEL, green) and Senseless expression (magenta). (D) RNAi-mediated knockdown of Sgg protein expression. dsRNA, expressed as an extended hairpin-loop RNA by the UAS vector, was used to knock down Sgg protein expression in cultured cells and flies. (Left panel) S2 cells were transfected with the indicated plasmid (*pUAST-lacZ*, *-sgg10 IR* or *-sgg46 IR*) (400 ng) and *pWAGAL4* (200 ng), *pUAST-HA-CARD* (100 ng), *pUAST-myc-sgg10* (200 ng), or *pUAST-myc-sgg46* (200 ng). At 24 h after the transfection, the cells were collected and subjected to immunoblotting using an anti-myc and anti-HA antibody. (Right panel) Adult flies with the indicated genotypes (*UAS-lacZ* (*UAS-lacZ IR/+; hs-GAL4/+*), *UAS-sgg10 IR* (*UAS-sgg10 IR/hs-GAL4*), and *UAS-sgg46 IR* (*UAS-sgg46 IR/hs-GAL4*)) were heat-shocked at 37°C for a total of 1 h each day for 5 days. At 5 h after the last heat shock, the whole flies were subjected to immunoblotting using an anti-Sgg and anti- β -tubulin antibody. (E–G) The specific knockdown of Sgg46 function by RNAi induced an increase in the number of extra macrochaetes. The indicated genotypes are as follows: *lacZ IR* (*sca-GAL4/+; UAS-lacZ IR/+*, E), *sgg10 IR* (*sca-GAL4/+; UAS-sgg10 IR/+*, F), and *sgg46 IR* (*sca-GAL4/+; UAS-sgg46 IR/+*, G). The numbers indicate the percentage of the population of flies that contained extra macrochaetes in the scutellum, and the arrowheads indicate extra macrochaetes. (H) The scheme shows a pathway predicted from this study: caspases antagonize Wingless signaling by regulating Sgg kinase in neural precursor development.

by imposing an additional form of regulation on its kinase activity.

Cellular differentiation via caspase activation

Little is known about the proteins cleaved by caspases during the differentiation process, and only a limited number of distinct substrates have been identified. For instance, in erythroblasts, PARP, lamin B, and acinus are cleaved, while GATA-1, a transcription factor essential for erythrocyte

formation, and ICAD, remains intact. Interestingly, MST1 kinase was identified as a crucial caspase-3 effector in myoblast differentiation (Fernando *et al*, 2002): the cleaved and activated MST1 enhances the activity of downstream MAP kinases to promote skeletal muscle differentiation. In this report, we observed caspase activation in clusters of neural precursor cells that was able to induce slight but significant changes in SOP cell differentiation through the cleavage of Sgg46 rather than by cell death. It is still unclear why

Dark-dependent caspase activation in the *scabrous*-expressing regions of wing discs does not induce cell death. Similar observations of caspase-dependent cellular differentiation have been made, for example, in the lens, keratinocytes, erythrocytes, and sperm (De Maria *et al*, 1999; Weil *et al*, 1999; Lee *et al*, 2001; Arama *et al*, 2003; Huh *et al*, 2004b). In these cases, caspase activation does not lead to any apoptotic or cell death-related events. Furthermore, differentiation-related caspase activation must be tightly regulated to prevent cells from dying through apoptosis. During cellular differentiation, caspase activation is apparently very limited, transient, or localized. For instance, during megakaryocyte differentiation, the limited caspase activation is confined to dot-like structures (De Botton *et al*, 2002). When senescent megakaryocytes die, however, the site of caspase activation changes from a localized to a diffused and greatly enlarged cytosolic area. Thus, restricting or lowering the levels of caspase activation in specific cellular spaces might enable Sgg46 processing without concurrent cell death.

Conclusion

We showed that the caspase family antagonizes the Wingless pathway during macrochaete development via Sgg in a kinase-dependent manner. This process is achieved through the caspase-dependent cleavage of Sgg46, which converts it to an active kinase. Our study clearly indicates an apoptosis-independent mechanism in which caspase activation modulates a signaling pathway via substrate cleavage. Such caspase cleavage of intracellular target proteins may strongly depend on the cellular context, including the differentiation status. Clearly, much remains to be learned about the potential dual role of caspases in apoptosis and cellular differentiation. Characterization of the molecules that regulate this limited caspase activation and the relevant substrates will provide exciting new insights into processes that, beyond cell death, might link caspase cleavage to important nonapoptotic biological processes.

Materials and methods

Fly stocks

Flies were raised on standard *Drosophila* medium at 25°C. The fly strains used in this study were described in Supplementary data. The ectopic expression of various kinds of Sgg proteins and sgg dsRNA in the fly was achieved using the GAL4/UAS system. All *pUAST* constructs were injected into *w¹¹¹⁸*; *Dr/TMS*, *Sb P[ry+]*, $\Delta 2-3$ embryos to produce transgenic flies as described (Kanuka *et al*, 1999b). At least three independent transformed lines were obtained for each transgenic construct. *Drosophila* crosses were carried out by standard procedures at 25°C.

Histology, immunohistochemistry, and TUNEL

Flies were prepared for scanning electron microscopy as described (Kanuka *et al*, 1999b). For light microscopic images of the adult notum, flies were anesthetized and examined with a Nikon SMZ1000 microscope (Nikon) equipped with an AxioCam digital camera (Carl Zeiss). Immunohistochemistry for wing discs and pupal notum were described previously (Tsuneizumi *et al*, 1997; Sato *et al*, 1999). TUNEL labeling of the wing discs was carried out as

described (Hay *et al*, 1994) with some modifications; in the secondary antibody incubation step, the wing discs were also incubated with Cy2- or Cy3-conjugated streptavidin (Jackson). All fluorescently labeled samples were examined with an LSM510 confocal microscope (Carl Zeiss). The antibodies used for immunostaining in this study were described in Supplementary data.

Confocal imaging analysis of caspase activity in vivo

Confocal imaging analysis of caspase activation was performed based on our previous report with several modifications (Takemoto *et al*, 2003). Briefly, wing discs dissected from third-instar larvae were prefixed in 4% PFA at 4°C for 5 min, and then fixed in fresh 4% PFA at room temperature for 20 min. After several washes by PBS, confocal FRET images were acquired on the Aquacosmos/Ashura system (Hamamatsu Photonics) using an IX-71 equipped with a UPlanApo 10 $\times$ 0.40 NA objective (Olympus), a spinning disk-type confocal unit (CSU10, Yokogawa, Tokyo), a diode-pumped solid-state laser (430 nm) (Melles Griot), and a three CCD (charge-coupled device) color camera (C7780-22; Hamamatsu Photonics). Image acquisition and analysis were performed using Aquacosmos/Ashura software (Hamamatsu Photonics).

Cell culture, transfections, immunoblotting, and caspase activity

Drosophila S2 cells were cultured as described previously (Kanuka *et al*, 1999a). To generate stable transformants of the *reaper* expression plasmid, S2 cells were transfected with *pCasepeR-hs-reaper* and *pAct5cp-Neomycin*, and stable S2 cell lines were selected and established as described previously (Kondo *et al*, 1997). For the detection of proteins, S2 cells were cultured in six-well plates (5×10^5 cells/well) and transfected using CellFectin (GIBCO) with various amounts of the indicated plasmids plus *pWAGAL4*, which expresses the GAL4 protein under the control of the *actin5C* promoter. After transfection, the cells were lysed in SDS sample buffer. All samples were separated by 10% SDS-PAGE and subjected to immunoblotting. The antibodies used for immunoblotting in this study were described in Supplementary data. The signals were visualized using ECL plus (Amersham). Specific antibodies that recognize cleaved forms of Sgg46 and drICE were raised in rabbits using a synthetic peptide corresponding to the N-terminus of Sgg46 (AKPKNR) and the C-terminus of drICE (SQTETD) conjugated with keyhole limpet hemocyanin as the immunogen. These specific antibodies were purified by sequential protein affinity purification and used for immunoblotting (1:100 dilution). The assay for caspase activity in S2 cells was performed as described (Kanuka *et al*, 1999a).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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