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REVIEW

A Study on the Knob Formation Mechanism in the *Knobbed* Mutant (*K*) of the Silkworm, *Bombyx mori*

Sachiko SHIMURA*

Invertebrate Gene Function Research Unit, National Institute of Agrobiological Sciences (NIAS) (Tsukuba, Ibaraki 305–8634, Japan)

Abstract

The *Knobbed* mutant of the silkworm, *Bombyx mori*, is a dermal mutant characterized as having paired knobs in specific regions of larval segments. To clarify the knob formation mechanisms, the epidermal cell proliferation and morphology of the knobbed region during the fourth instar were investigated. Mitosis of the epidermis in the non-knobbed region was mainly observed to occur at 48 hours (h) after ecdysis. Conversely, numerous mitotic cells were observed in the knobbed region not only at 48 h but also 24h after ecdysis. Mitotic cells were also observed at 72 h after ecdysis. According to observation using the transmission electron microscope, epidermal cells at the knobbed region were considerably longer and more slender than those of the non-knobbed region. The unusual shape of the epidermal cells in the knobbed region appeared attributable to abnormally prolonged mitotic activity. In this paper, based on these morphological observations, the process of knob formation was discussed.

Discipline: Biotechnology
Additional key words: epidermal cell, immunohistochemistry, proliferation

Introduction

The *Knobbed* mutant is a dermal mutant with characteristic knobs (protuberances), which form at specific dorsal regions of several segments (Fig. 1, 2a, b)[1]. It originated from the "Ryukaku" which was retaken from China in 1897[16]. The *Knobbed* (*K*) gene dominates, and the locus is located at the position of 25.4 on the genetic map of the eleventh linkage group[5].

The knobs formed dorsal regions, which correspond to larval marking regions - for example the lunar marking (Fig. 1, 2a, b). Accordingly, there are reports having analyzed the relationship between the larval markings and knob formation[2,4,33,43]. The knobs are formed on the extra-marking expressed by *Multi-lunar* (*L*), *multi-star* (*ms*) and *E* pseudoallele[3,43]. In contrast, the knobs are reduced when *Knobbed* is combined with *Striped* (p^S), *od* translucent (*od*), *Slow-growing* (*Slg*) and *Splashed* (*Spl*)[2,3,33,43].

Ashino (1940)[4] first suggested that the knob formation was due to excessive proliferation of the epidermal cells during the molting stages. Nagashima *et al*. (1959)[32] observed histological changes in the knobbed region of the integument during the molting stage, and reported that markedly increased cell division occurred in the knobbed region and also that the epidermal cells became narrower than normal cells. However, numerous questions needed to be resolved, for example: How the knobs were formed during larval growth? Did the epidermis in the knobbed region consist of stratified cells as previously reported[8]? If the epidermis was formed by stratified cells, is cuticle also secreted by underlying epidermal cells? Although the normal epidermal cells of *B. mori* are not subject to mitosis in the molting stage of larval instars[22,23], is it true that epidermis in the knobbed region proliferate in the molting stage as previous reported[4,32]? In addition, the *Knobbed* gene is also known to increase some commercial characteristics, such as cocoon weight[34,44,48]. Therefore, elucidating the mechanisms of knob formation might also help clarify how commercial

Present address:
Insect Mimetics Research Unit, NIAS (Tsukuba, Ibaraki 305–8634, Japan)
*Corresponding author: e-mail sshimura@affrc.go.jp
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characteristics can be improved.

Thus, to clarify the cause of the knobbed formation, I examined the mitotic activity of epidermal cells of the *Knobbed* mutant by whole-mount immunohistochemical staining; using an antibody specific for M-phase nuclei[38,39]. In addition, the morphological characteristics of the integument in knobbed regions were observed using light and electron microscopy[40]. In this paper, I review these papers and discuss the knob formation process.

Development of *Knobbed* mutant

First, I observed the morphology of the *Knobbed* mutant from the first instar larva to the adult moth. While the knob shapes were not clearly apparent in the first instar larva (Fig. 1a), in the second instar, tiny but distinct knob-shaped bumps were observed on the third thoracic segment and the second and fifth abdominal segments (Fig. 1b). These bumps increased in size and were prominent in the third instar larvae (Fig. 1c). After entering the fourth instar, the knobs increased further as the larva grew. At this stage, these knobs also emerged on the dorsal surface of the second thoracic segment and at the base of the caudal horn on the eighth abdominal segment (Fig. 1d and e). In the pupal stage, knobs formed on the corresponding regions (Fig. 1f). Adult moths also developed knobs, although only two pairs were visible on the second and fifth abdominal segments (Fig. 1g).

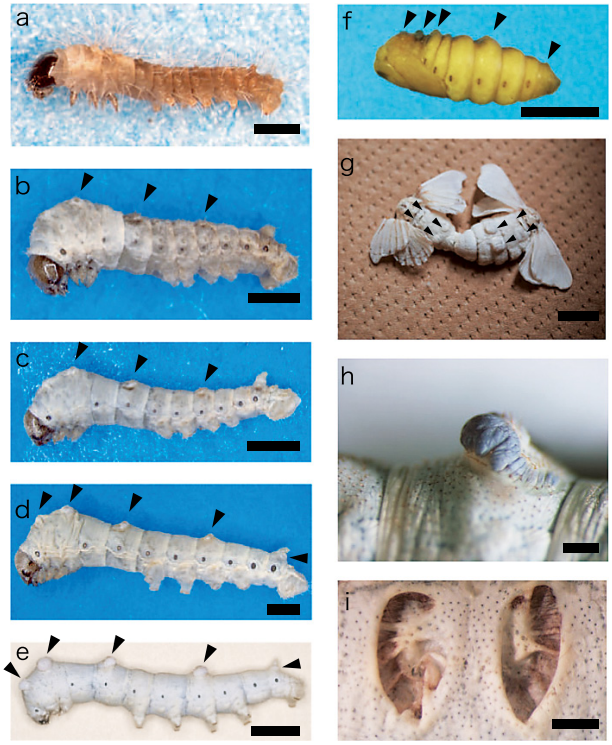

Fig. 1. Photographs of the *Knobbed* mutant at various developmental stages from the first instar larva to the adult

a-e: First, second, third, fourth and fifth instar larvae, respectively. f: pupa, g: adult moth. h: lateral view of a knob from the fifth instar day 0 larva. i: internal view of the knobbed regions of the fifth instar day 0 larva. Arrowheads show the position of the knobs. Scale bars represent 1 mm (b), 2 mm (c and d), 1 cm (e-g), and 0.5mm (a, h and i). (From Shimura *et al.*, 2009b)

The regions of the knobbed integument protruded conspicuously from the body, giving the surface of the larvae in these areas a rough, wrinkled appearance (Fig. 1h). The knobs themselves were hollow (Fig. 1i) and distributed with fat bodies and tracheae similarly to the normal epidermis.

Cell proliferation of the knobbed regions during the fourth instar

Next, to investigate changes in mitotic activity in the epidermis during the fourth instar, I observed mitotic activity in the epidermis of the knobbed region by whole mount immunohistochemistry using an antibody for the M-phase nucleus that was highly specific for the phosphorylated form of the amino-terminus of histone H3 (PH3)[15]. Anti-PH3 was marked with Alexa 488 (shown in green), while the nucleus was marked with propidium iodide as counter stain (shown in red). The knobbed region of the second abdominal segment was excised together with the surrounding unknobbed regions (Fig. 2a, b and c). As controls, the corresponding region of the third abdominal segment of the mutant larva was used (Fig. 2a, b and i).

At 0 h after the third ecdysis, no mitotic figure was detected in the epidermis (Fig. 2d:nuclei show red). However, at 24 h, numerous mitotic cells were detected in the knobbed regions, but few mitotic cells in the surrounding non-knobbed regions (Fig. 2e: mitotic cells show green). At 48 h, mitotic figures were observed in both knobbed and non-knobbed regions (Fig. 2f). Even at 72 h, at the beginning of the molting stage, a few but clear mitotic figures were detected (Fig. 2g), while during the molting stage (96 h), no mitotic cells were observed (Fig. 2h). These results suggest that mitoses occur in the epidermis of the knobbed regions during most of the intermolt/feeding stage, but that this mitotic activity ceases soon after entering the molting stage.

To determine whether such prolonged mitotic activity is specific to the knobbed region, I compared this change in mitotic activity with those occurring in corresponding regions of the third abdominal segment without any knob. The sites selected for observation were chosen based on the location of the bristles, which can be used as

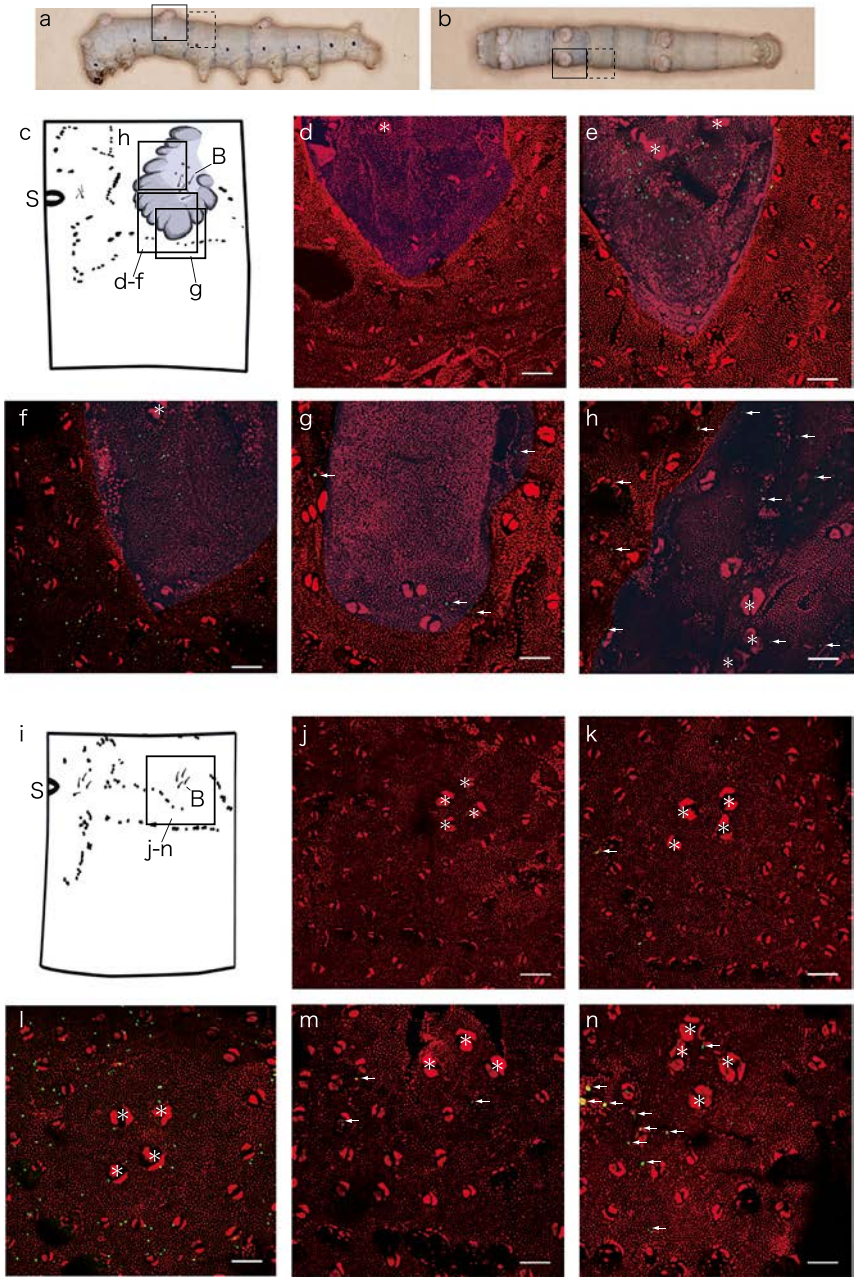

Fig. 2. Detection of mitotic cells in the epidermis of the *Knobbed* mutant during the 4th instar

Photographs show the lateral and dorsal views (a) and (b) of the larva of the *Knobbed* mutant. The area surrounded by a solid line on the second abdominal segment, on which a knob exists, was used for this study, while that surrounded by dotted line on the third abdominal segment, which is the normal epidermis corresponding to the knobbed region on the second abdominal segment, was used for the control. Regions (c) and (i) show drawings of the left half of the second and third abdominal segments, respectively. A swollen knob exists in the area of the larval marking, indicated by the blue shaded area. Black dots indicate the sites of muscle attachment, while B shows bristles used as location markers. Boxes labeled (d) – (h) and (j) – (n) indicate regions observed by confocal laser scanning microscopy.

Regions (d) – (h) show confocal images of knobbed and surrounding un-knobbed regions of the second abdominal segment during the 4th instar (d: 0h, e: 24 h, f: 48 h, g: 72 h, h: 96 h after 3rd ecdysis). (j) – (n) show confocal images of the corresponding region of the third abdominal larval segment from the knobbed mutant larva during the 4th instar (j: 0, k: 24 h, l: 48 h, m: 72 h, n: 96 h after 3rd ecdysis). The knobbed area is indicated by a blue shaded area (d-h). The mitotic cells are labeled with an anti-phosphorylated histone H3 antibody (shown in green) and the nuclei are labeled with propidium iodide (shown in red) as a counter-stain. Asterisks indicate bristles used as location markers to determine the sampling site. Arrows indicate mitotic cells in attached hemocytes and fat bodies. Fat bodies and hemocyte cells are identified using Z-axis observation. Scale bars represent 100 µm. (Modified from Shimura *et al*., 2009b)

markers since they are ever-present at the corresponding sites (Fig. 2i). At 24h, relatively few mitotic figures were detected (Fig. 2k), although many were detected at 48 h (Fig. 2l). No mitotic cell was detected at other stages such as 0, 72 and 96 h after the 3rd ecdysis (Fig. 2j, m, and n). This result of the third abdominal segment was the same as in previous reports[22,23]. In both segments, hemocytes and fatbody cells were also shown in mitosis (Fig. 2g, h, k, m and n; cells indicated by arrows).

These differences in the relative occurrence of mitotic cells suggest that mitoses occur in the epidermal cells of knobbed areas for a prolonged period during the intermolt/feeding stage, and that these mitoses result in knob formation by increasing the number of epidermal cells. Next, to clarify the process of knob formation at cellular level, the morphological characteristics of the integument in knobbed regions were compared with the integument of normal non-knobbed regions in 4th instar larvae by light and electron microscopy.

Light microscopy

The histological changes in the integument from the knobbed region were compared with changes in the normal, non-knobbed region by light microscopy. Staging of the molting larva was done by the spiracle index (SI) of Kiguchi and Agui (1981)[24]. It was clear that the thickness of the cuticle and epidermal layer in the normal non-knobbed region increased during the intermolt/feeding stage (Fig. 3a-d). In the control non-knobbed region, the epidermal layer consisted of simple cuboidal cells, while the basal plasma membrane and the attached basement membrane were flat and smooth throughout the 4th instar (Fig. 3a-e). At the mid-molting stage (SI=D2), when the exo-cuticle was being secreted (Fig. 3e), the epidermal layer thickened, and the apical plasma membrane of epidermis appeared to partially extend upward, probably to form folds of the newly formed integument as suggested by Ito (1951)[19]. In contrast, the epidermal cells of the knobbed region was remarkably taller and more slender than the cells in the equivalent area of the non-knobbed region during the intermolt/feeding stage (Fig. 3f-j), which conformed well to the observations of Nagashima *et al*. (1959)[32]. Interestingly, the epidermal cells of the knobbed region were relatively irregular in size resulting in the basement membrane becoming distorted during the intermolt/feeding stage, especially on day 2 (Fig. 3h). However, at the spiracle apolysis stage (SI=B), the basement membrane became flat (Fig. 3i). Thereafter, at the mid-molting stage (SI=D2), the epidermal layer was considerably wrinkled, with the basement membrane becoming partially detached from the basal plasma membrane (Fig. 3j), probably due to the increased number of epidermal cells and the growth of each cell. These detachments might be related to the absorption of substances from the hemolymph necessary for active cell growth and cuticle secretion during the molting stage[36].

Electron microscopic observation

The ultrastructure of the integument was compared between knobbed and normal non-knobbed regions on days 0 and 3, which is the end of the intermolt/feeding stage in the 4th instar. The epidermal cells in the knobbed region were considerably taller and more slender than equivalent cells in the non-knobbed region on day 3 (compare Fig. 4b and 4d). As for the fine structure of the epidermis, the basement membrane of the knobbed region appeared slightly thinner than that of the normal region (Fig. 4). Besides, the intercellular spaces were more clearly observed in the epidermis of the knobbed region (Fig. 4a, b).

These characteristics seem unrelated to larval marking formation and/or pigmentation. Histological studies of the epidermis of *B. mori* have been performed by many researchers[2,19,20,30,31,42]. However, nobody reported any difference in the morphology of epidermis between marking and non-marking regions, although a conspicuous difference in epidermal pigment granules was observed[20]. I observed the morphology of larval integument of the Eri silkworm, *Samia ricini*[37], but could not detect any difference in the shape of epidermal cells between marking and non-marking regions (data not shown). Therefore, the cellar characteristics mentioned above are considered specific to the knobbed region.

Process of knob formation

Collectively, it might therefore be possible to consider the process of knob formation as follows. First, (1) In the penultimate 4th instar larvae of the normal strain, mitoses occur in the anterior half of the segment (where the knobs are formed) only during the mid-intermolt/feeding stage and not the molting stage[39]. However, in the knobbed regions, active mitoses occur in the epidermal cells for most of the intermolt/feeding stage (Fig. 2)[38]. (2) This extraordinary long mitotic activity results in an increase in the number of epidermal cells, which makes the epidermal cells irregular, taller and more slender. (3) After mitosis ceases at the beginning of the molting stage[38], each of these highly dense epidermal cells grows and expands, which may account for the undulating characteristics of the epidermis and also how the basement membrane becomes partially detached from the basal plasma mem-

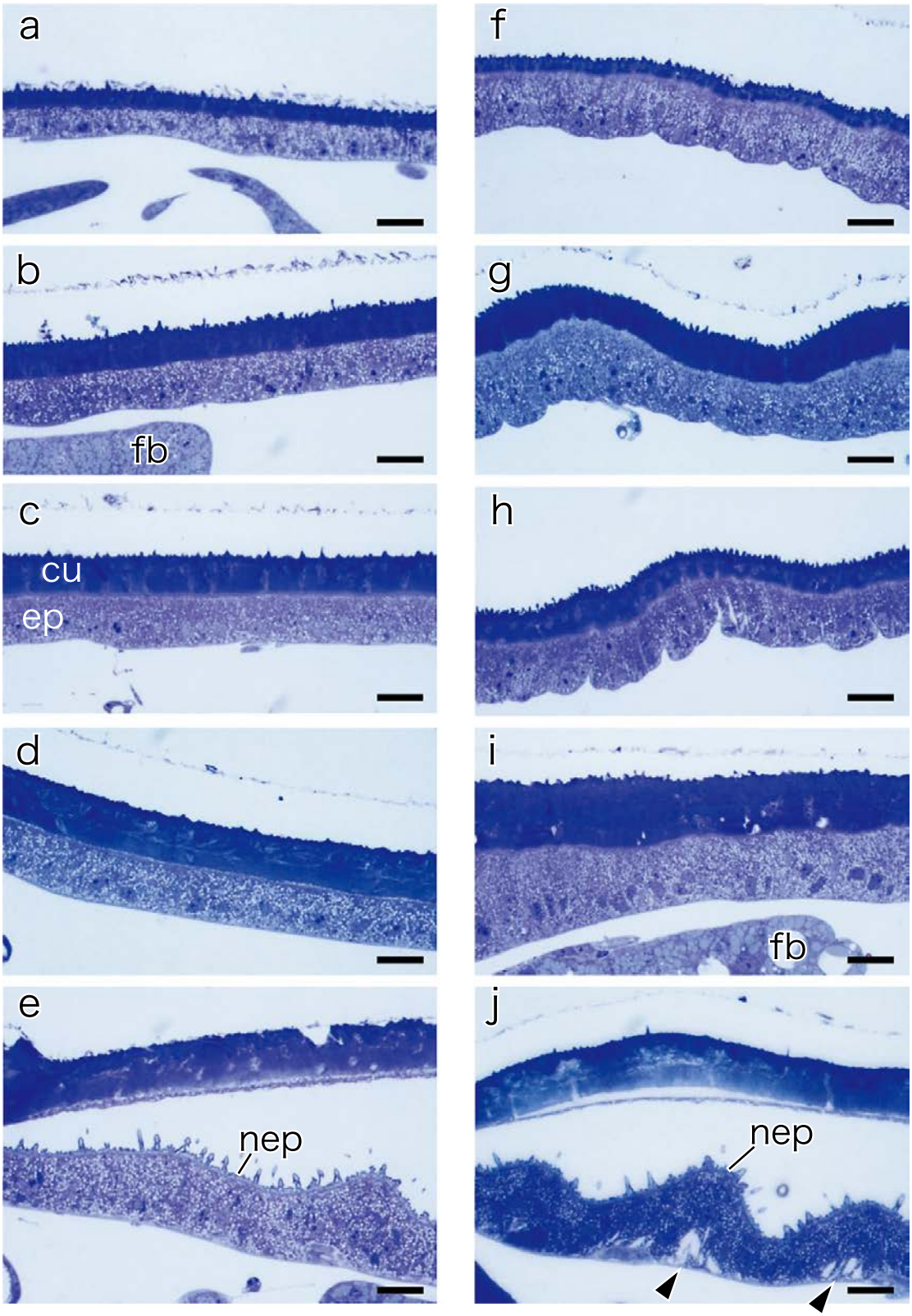

Fig. 3. Light micrographs of integument from normal non-knobbed regions (a-e) and knobbed regions (f-j) in 4th instar larvae

Micrographs a and f: day 0, b and g: day 1, c and h: day 2, d and i: just at the beginning of the molting stage, e and j: mid-molting stage. cu: cuticle, ep: epidermis, nep: new epicuticle and papillae, fb: fat body. Black arrowheads show where the basement membrane has become detached from the basal plasma membrane. Scale bar = 20 μm. (From Shimura *et al.*, 2010)

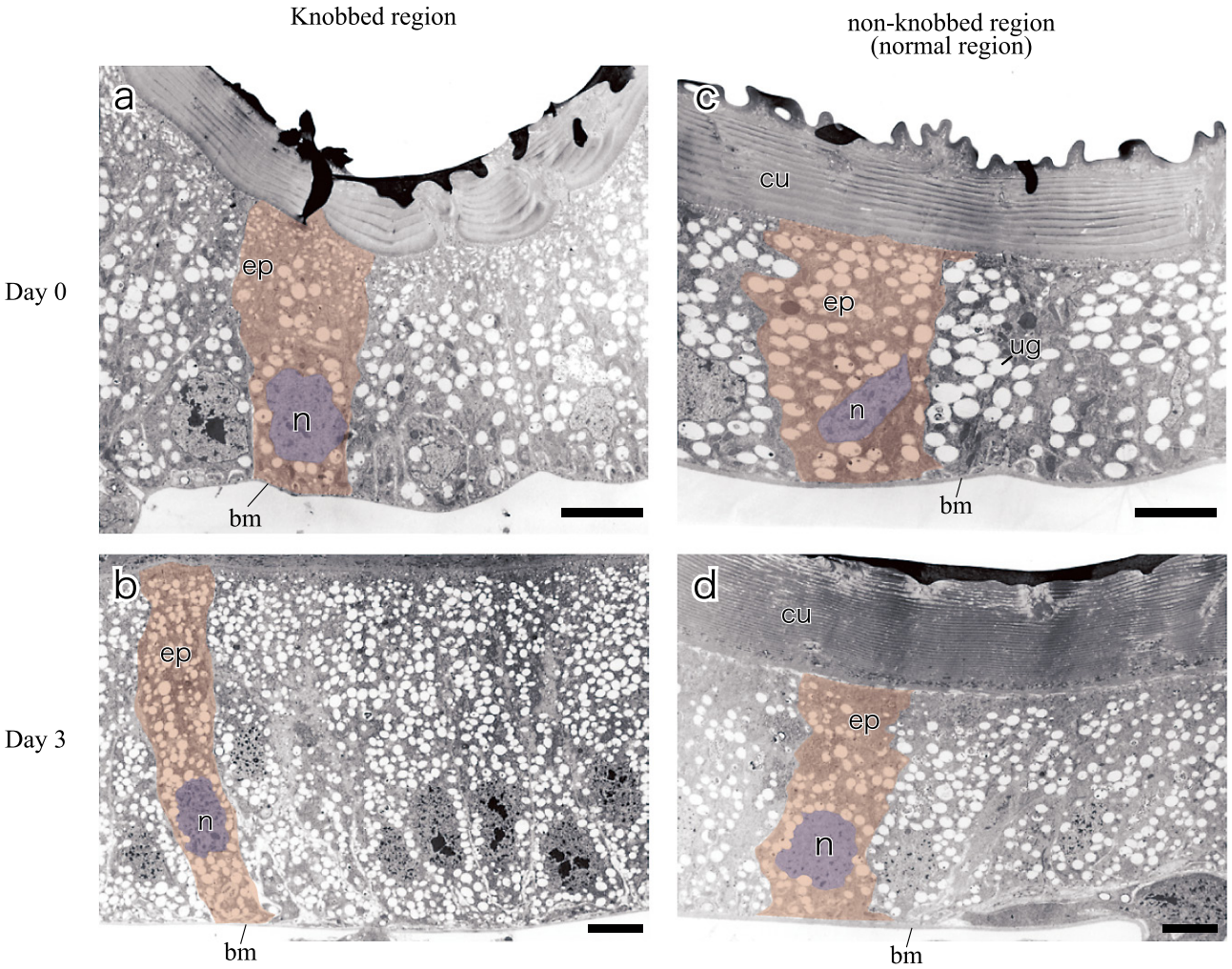

Fig. 4. Electron micrographs of knobbed regions (a, b) and normal non-knobbed regions (c, d) in fourth instar larvae
The single epidermal cell and nucleus are indicated by red and blue shading, respectively. (a) and (c) show photographs at Day 0, (b) and (d) are photographs at Day 3 after the 3rd ecdysis (beginning of molting stage). cu: cuticle, ep: epidermis, n: nucleus; ug: uric acid granules. Bm: basement membrane. Scale bars = 5 µm. (Modified from Shimura *et al*., 2010)

brane at the mid-molting stage. (5) This is followed by the development of conspicuous outgrowths of new integument, which form the large, swollen knobs at ecdysis into the 5th instar.

Perspective

By positional cloning using *Bombyx* genome information, the genes responsible for certain mutants, such as those of larval body color, have been identified[7,9,10,17,18,26,29]. Unfortunately, although a knobbed area was mapped at position 25.4 on chromosome 11[5], the responsible gene has not been identified.

Recently, the *Wingless/Wnt-1* gene, which encodes a member of the Wnt family of secreted signal proteins that control proliferation and differentiation during development[6,41], has been identified by positional cloning as a candidate gene responsible for the *Multi-lunar* (*L*)[46]. The multi-lunar markings appearing on the sub-dorsal line of the larva are controlled by a dominant gene *L* located on chromosome 4[12]. When the knobbed larva is crossed with the *Multi-Lunar* larva, knobs form on the multi-lunar markings[3,43]. This result raises the possibility of the gene responsible for *knobbed* phenotype working in the downstream of the Wnt-1 signaling pathway.

TEM observation showed that the basement membrane was slightly thinner in the *Knobbed* region than in the normal region. The basement membrane contains a number of proteoglycans, such as perlecan, laminin and heparan sulfate proteoglycans (HSPGs), which play critical roles in regulating cell signaling and migration[25,27,28,35,45]. For example, HSPGs play essential roles in regulating the signaling activities of Wnt in *Drosophila*[11,13,14,21,47]. The difference in thickness of the basement

membrane might also be an important factor behind excessive cell proliferation. Although the molecular mechanisms of knob formation remains unclear, I believe this research will provide better insight.

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