

# Caspase-mediated changes in histone H1 in early apoptosis: prolonged caspase activation in developing olfactory sensory neurons

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Programmed cell death or apoptosis is required for the patterning and development of multicellular organisms. However, apoptosis is a difficult process to measure because the dead cells are rapidly degraded by their neighbors within a few hours. The post-caspase activation events that determine whether a cell will undergo apoptosis remain elusive. Here we report that apoptosis-specific nuclear events that occur before DNA fragmentation can be distinguished by monitoring the histone H1 status. In both mammals and *Drosophila*, dying cells failed to be immunolabeled with an anti-H1 monoclonal antibody, AE-4. Real-time imaging of caspase activation and H1 dynamics in mammalian neural cells revealed that H1 changed its location in the nucleus after caspase activation. In addition, the timing of this re-localization was largely dependent on the apoptotic stimulus used. From the staining patterns of AE-4 and anti-active caspase-3 antibodies, cells undergoing the transition from caspase activation to the apoptotic H1 change could be identified as H1-positive caspase-activated cells, providing a novel criterion for early apoptosis and making it possible to characterize caspase-activated cells in tissues. On the basis of these staining patterns, we found that many olfactory sensory neurons in the developing mouse olfactory epithelium showed sustained caspase activity without the H1 change, suggesting a unique caspase function in these neurons.

*Cell Death and Differentiation* (2008) 15, 1429–1439; doi:10.1038/cdd.2008.71; published online 16 May 2008

During animal development, the precise, regional execution of programmed cell death (PCD) is required for the proper patterning and sculpting of the embryonic primordium. Classic examples include digit formation in the vertebrate limb bud and excess neuron removal in the developing nervous system.<sup>1,2</sup>

The most abundant form of PCD in developing animals is apoptosis, typically characterized by nuclear events such as pyknotic chromatin body formation and DNA fragmentation. The mechanisms of apoptosis have been extensively investigated. A family of cysteine proteases known as caspases has conserved roles in a proteolytic cascade, in which the caspases cleave one another and key intracellular proteins to kill the cell in a controlled manner.<sup>3</sup>

Caspases are activated early in apoptosis, and their activation has been considered a principal indicator of apoptosis. However, caspases also appear to function as regulatory molecules in various events associated with cell-fate determination, including dendritic pruning, sperm differentiation, and border-cell migration in *Drosophila*.<sup>4,5</sup> To evaluate the physiological roles of caspases *in vivo*, it is important to identify common molecular changes occurring after caspase activation that indicate whether caspase-activated cells are dying or not.

Chromatin changes are observed in cells undergoing apoptosis. The linker histone H1 (H1) inhabits an integral part of the chromatin structure that is thought to include a recognition site for caspase-activated DNase, which attacks the DNA late in apoptosis.<sup>6–8</sup> H1 undergoes alterations during apoptosis *in vitro*, including phosphorylation and poly(ADP)-ribosylation.<sup>9–11</sup> Once thought of as a static, non-participating structural element, H1 is now known to be a dynamic component of the machinery responsible for chromosomal events. Thus, H1 might be altered in the apoptotic process, making the chromosomal DNA vulnerable to cleavage.

Here, we found that a commercially available anti-H1 antibody (clone AE-4) could be used to distinguish early changes in the apoptotic process *in vivo*. The AE-4 anti-H1 antibody failed to recognize specifically H1 in cells dying from caspase-dependent apoptosis, in both cultured cells and tissue sections. The loss of AE-4 immunoreactivity correlated with a caspase-mediated early apoptotic change in the localization of H1 and preceded the DNA fragmentation identified by TdT-mediated dUTP-biotin nick end labeling (TUNEL). Live imaging revealed that the time between caspase activation and H1 localization change was variable

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**Keywords:** apoptosis; caspase; neuron

**Abbreviations:** AC-3, active caspase-3; CHX, cycloheximide; H1, linker histone H1; PCA, perchloric acid; PCD, programmed cell death; OSN, olfactory sensory neuron; STS, staurosporine; SVZ, subventricular zone; TUNEL, TdT-mediated dUTP-biotin nick end labeling

Received 25.1.08; revised 27.3.08; accepted 14.4.08; Edited by S Kumar; published online 16.5.08

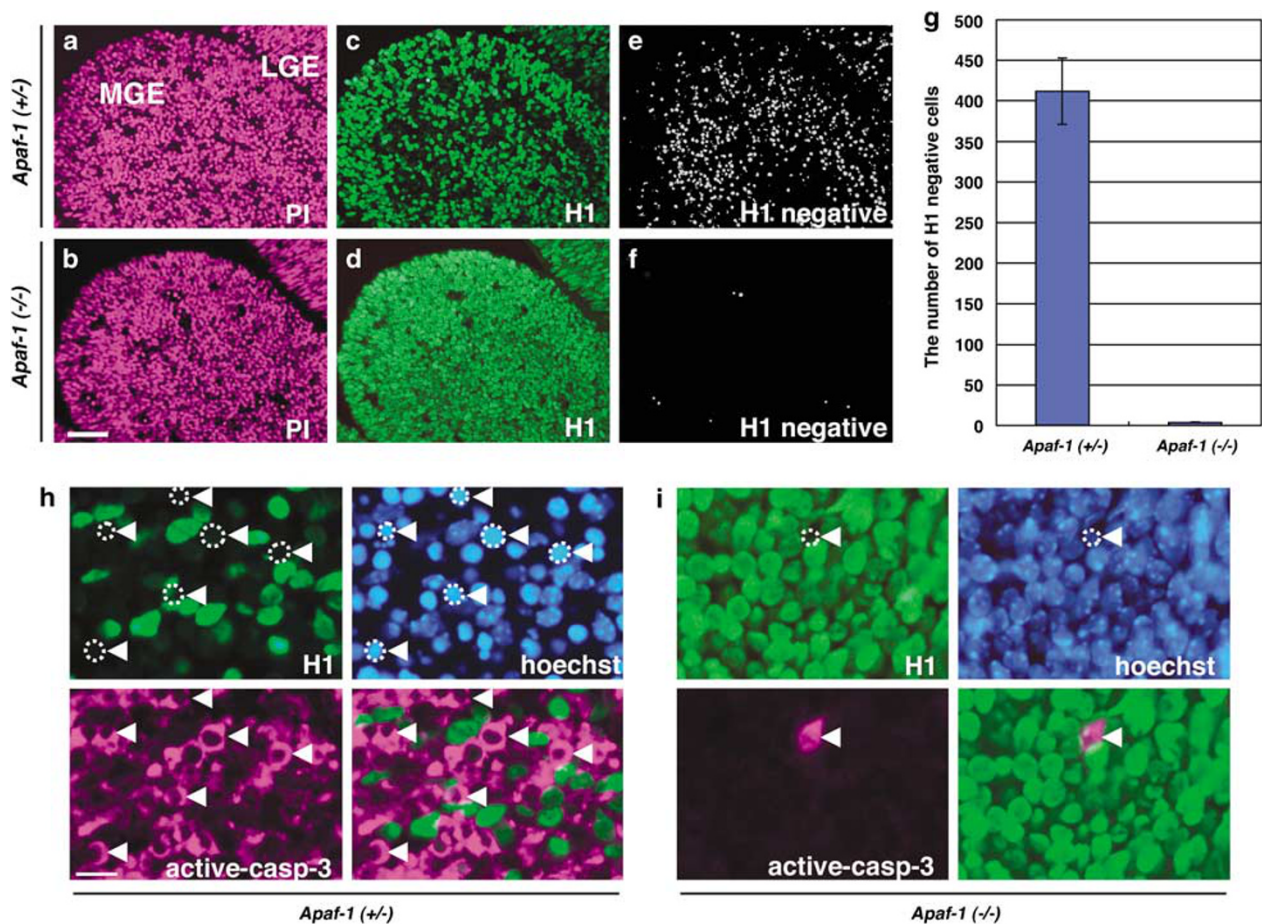
and depended on the apoptotic stimulus used. The AE-4 antibody labeled cells during this transition, marking them as H1-positive caspase-activated dying cells.

We also evaluated the caspase-activated cells in various developing neural tissues by the staining patterns of the AE-4 and anti-active caspase-3 antibodies. We found that developing olfactory sensory neurons (OSNs) uniquely exhibited a sustained H1-positive early-apoptotic status. Thus, the status of H1 can provide new insights into the post-caspase activation events in tissues.

## Results

**Apaf-1-dependent appearance of H1-negative cells in genotoxin-induced dying cells in the embryo.** According to the information provided by Abcam (Cambridge, MA, USA), the anti-H1 antibody clone AE-4 labels histone H1 variants in a broad range of species, including the H1.0, H1.1, H1.2, H1.3, H1.4, and H1.5 human H1 isoforms and H1

in *Drosophila*. To examine whether H1 undergoes caspase-dependent changes during apoptosis, we compared the staining pattern of the AE-4 antibody in wild-type mice to that in *apaf-1* mutant mice, which are deficient in caspase activation.<sup>12</sup> Apaf-1 is critical for the activation of caspase-9, and its deficiency leads to a neurodevelopmental phenotype similar to that of *caspase-9*-deficient mice.<sup>12–14</sup> Apaf-1 is also required for apoptosis induction by a DNA-damaging reagent, cytosine arabinoside (Ara-C), in neural precursor cells of the embryonic cortex.<sup>15</sup> Ara-C was injected intraperitoneally into pregnant mice bearing embryos at E12–E13, and the embryonic cortex was examined 6 h later (Figure 1). We observed numerous caspase-3-activated cells with condensed nuclei in the ventricular zone of Ara-C-treated *apaf-1* heterozygotes (Figure 1a, c, e, g and h), and, to our surprise, these cells were not immunoreactive with the AE-4 anti-H1 antibody (hereafter, we refer to cells negative for the AE-4 antibody as 'H1-negative'). In contrast, *apaf-1*-deficient homozygous embryos were resistant to the DNA damage-induced cell death by Ara-C, and, with the exception



**Figure 1** Apaf-1 required for the loss of AE-4 anti-H1 immunoreactivity in apoptotic cells in the ventricular zone of embryonic brain. Forebrain sections (5- $\mu$ m thick) were prepared from embryos that had been treated with Ara-C to induce apoptosis. Brain sections of *apaf-1* heterozygotes (a, c, and e) and homozygotes (b, d, and f) were stained with the AE-4 anti-H1 antibody (green). Nuclei were stained with PI (magenta). White dots in (e) and (f) show H1-negative cells (image processed by Leica Q Win). (g) The number of H1-negative cells in the medial ganglionic eminence (200  $\mu$ m  $\times$  200  $\mu$ m  $\times$  5  $\mu$ m) of *Apaf-1* heterozygotes and homozygotes treated with Ara-C was determined. The data are representative of three independent sections. (h and i) Double staining with the AE-4 anti-H1 (green) and anti-active caspase-3 C92-605 (magenta) antibodies in *Apaf-1* heterozygotes (h) and homozygotes (i) treated with Ara-C. Pictures of the MGE are shown. Cells that are both active caspase-3-positive and H1-negative are marked with arrowheads. Nuclei were stained with Hoechst 33342. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. Scale bars: (a) 50  $\mu$ m; (h) 10  $\mu$ m

of a few pyknotic cells, almost all the cells reacted with the AE-4 anti-H1 antibody (Figure 1b, d, f, g and i). These data indicate that cells dying of caspase-dependent apoptosis lose their reactivity for the AE-4 anti-H1 antibody *in vivo*.

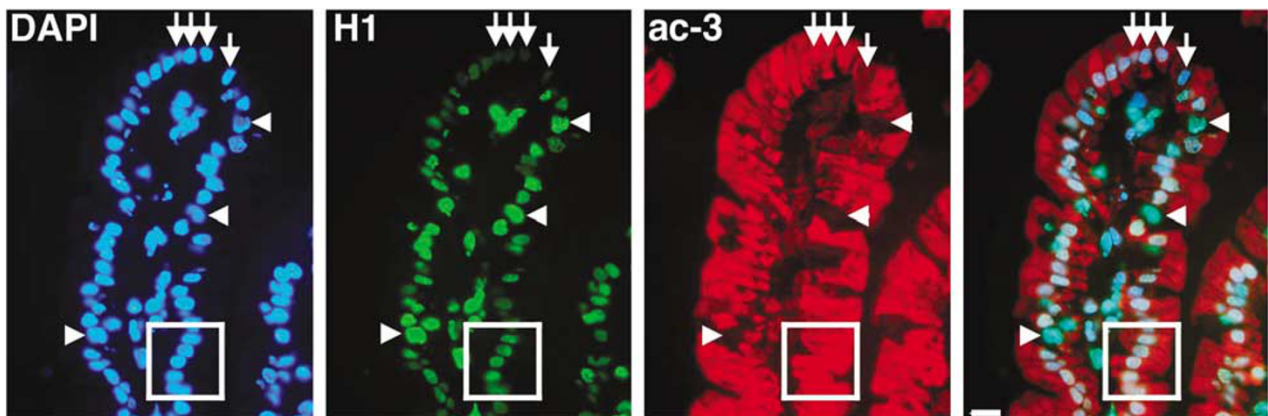
**The anti-H1 antibody AE-4 fails to immunolabel dying cells in tissues.** We next investigated whether the AE-4 anti-H1 antibody could also distinguish H1 changes during naturally occurring cell death *in vivo* (Figure 2). In the small intestine, stem cells in the crypts of the intestinal mucosa divide and differentiate while migrating along the crypt–villus axis toward the intestinal lumen. The differentiated cells are ultimately deleted by apoptosis at the villus tip.<sup>16</sup> Interestingly, double-labeling experiments in mouse showed that caspase-3 was activated in most of the villus cells, and the AE-4 anti-H1 antibody tended to weakly immunolabel migrating cells that were positive for the anti-active caspase-3 antibody (Figure 2; boxed area); conversely, the cells that were negative for active caspase-3 stained strongly with the anti-H1 antibody (Figure 2; arrowheads). At the villus tip, the known apoptotic region, many H1-negative cells were observed (Figure 2; arrows). These data suggest that the AE-4 anti-H1 antibody can distinguish between apoptotic and non-apoptotic caspase-activated cells in tissue.

**The anti-H1 antibody AE-4 fails to label specifically mammalian or *Drosophila* apoptotic cells.** We examined the properties of the AE-4 anti-H1 antibody in detail using cultured cells. In HeLa cells, apoptotic stimuli, such as a mixture of tumor necrosis factor- $\alpha$  and cycloheximide (TNF/CHX), staurosporine (STS), or etoposide led to caspase-3 activation and the loss of anti-H1 immunoreactivity, but the AE-4 anti-H1 antibody still recognized mitotic or necrotic cells (Figure 3a, b, d, and data not shown). An anti-pan-histone antibody that recognizes H1 and core histones H2a, H2b, H3, and H4 immunolabeled apoptotic cells (Figure 3c), suggesting that not all the histones changed in the apoptotic process. Treatment with the pan-caspase inhibitor zVAD-fmk suppressed the TNF/CHX-induced cell death and allowed all the cells to be immunolabeled with the AE-4 anti-H1 antibody (Figure 3a). In a *Drosophila* neuronal cell line, BG2, treatment

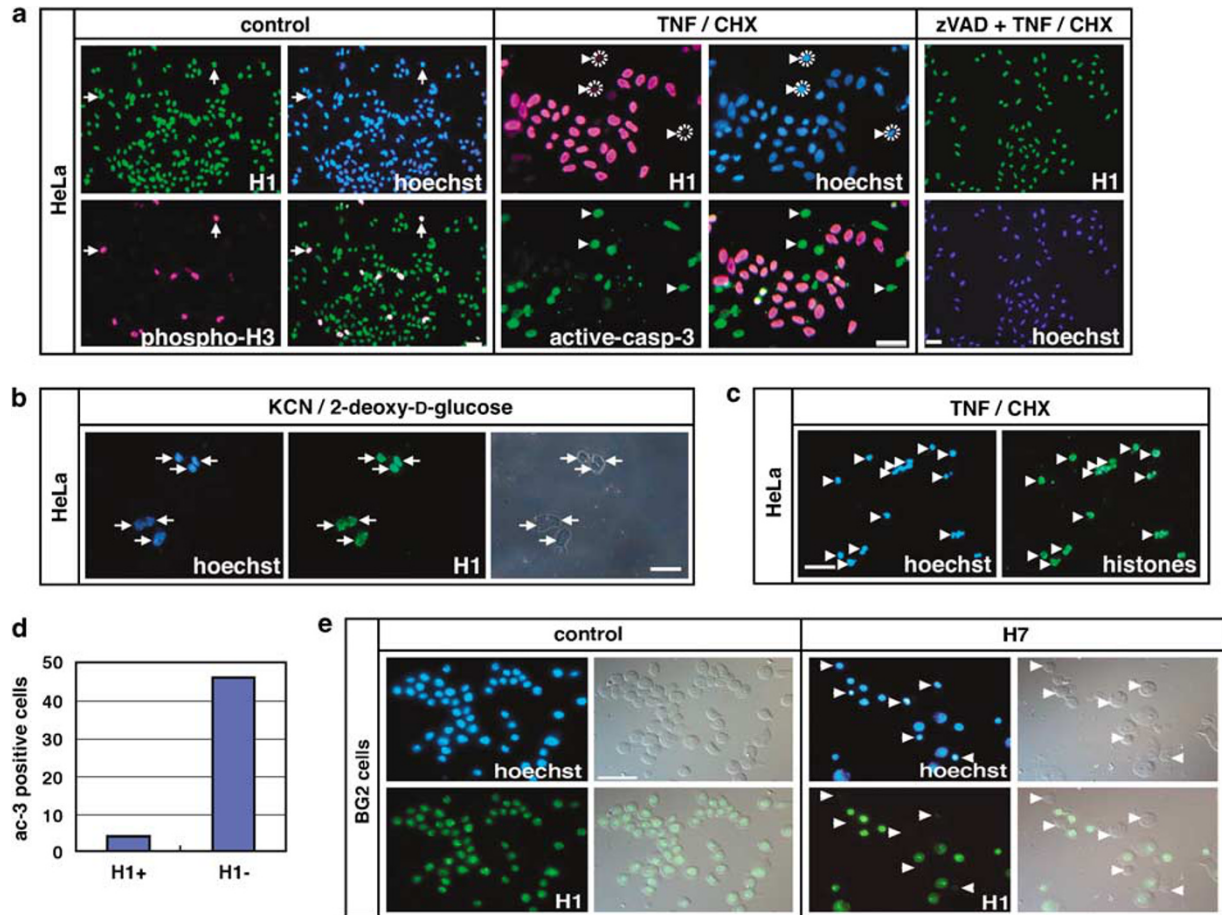
with the protein kinase inhibitor H7 induced apoptosis<sup>17</sup> characterized by pyknotic nuclei and caused the loss of anti-H1 immunoreactivity (Figure 3e). Thus, the lack of immunolabeling of dying cells with the anti-H1 antibody AE-4 is conserved in *Drosophila* and mammalian cells. Furthermore, dying cells with pyknotic nuclei failed to be immunolabeled with two additional anti-H1 antibodies prepared in mouse and rabbit (Supplementary Figure 1), suggesting that the apoptotic loss of anti-H1 immunoreactivity was not specific for the AE-4 anti-H1 antibody.

**Loss of anti-H1 immunoreactivity is a post-caspase activation event in pre-dead cells.** We next examined the loss of histone H1 immunoreactivity for the AE-4 antibody during apoptosis. Because the TNF/CHX-induced cell death in HeLa cells was quick (the entire process took only 10 min), it was difficult to study the sequence of apoptotic events after caspase activation using this system. We therefore examined various combinations of cell death stimuli and cell lines and found that the proteasome inhibitor MG132 induced a slow apoptosis process in Neuro 2a cells, a mouse neuroblastoma cell line. Neuro 2a cells were exposed to MG132<sup>18</sup> and then stained with the AE-4 anti-H1 antibody, the anti-active caspase-3 antibody, and Hoechst dye, at different time points.

On the basis of the staining pattern, the dying cells were classified into three types according to the status of their caspase activation, H1 immunoreactivity to AE-4, and chromatin condensation (Figure 4a and b). The first type (stage 1) was defined as cells showing caspase activation without nuclear changes. The second (stage 2) was defined as cells showing caspase activation, mild nuclear condensation, and H1 immunolabeling with the AE-4 antibody. The third (stage 3) was defined as cells that were caspase-activated and H1-negative. Prolonged exposure to the apoptotic stimulus led to an increased proportion of H1-negative cells (stage 3) and a decreased proportion of H1-positive cells (stages 1 and 2) among the caspase-activated cells (Figure 4b), indicating that cells dying from caspase-dependent apoptosis lost their immunoreactivity to the AE-4 antibody after nuclear condensation.



**Figure 2** Inverse staining patterns of anti-H1 and anti-active caspase-3 5A1 antibodies *in vivo*. Sections of the small intestine were stained with the AE-4 anti-H1 and anti-active caspase-3 5A1 antibodies. Arrowheads show cells that were labeled with the anti-H1, but not with the anti-active caspase-3 5A1 antibody. Arrows indicate H1-negative cells at the villus tip. Epithelial cells in white boxes were weakly labeled with the anti-H1 antibody. Scale bar, 50  $\mu$ m



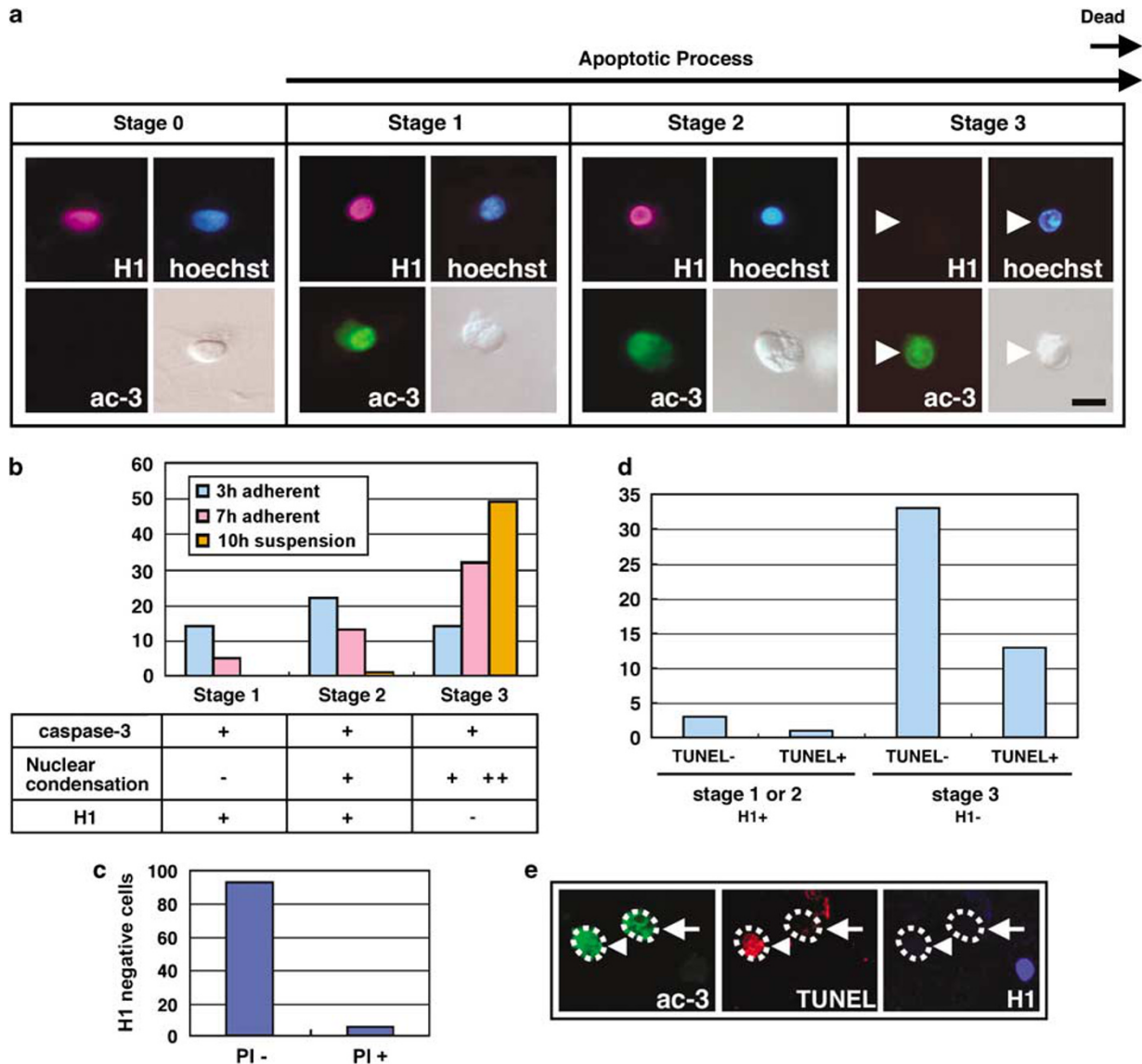
**Figure 3** Caspase-dependent loss of H1 immunoreactivity in cultured cells. (a) HeLa cells were treated with TNF/CHX for 5 h. The pan-caspase inhibitor, zVAD-fmk (100  $\mu$ M) was added to the cells 2 h before TNF/CHX treatment. The cells were then immunostained for phospho-histone H3 (a marker for mitotic cells), H1 (AE-4 monoclonal antibody), and activated caspase-3. Arrows show H1-positive mitotic cells. Arrowheads show H1-negative cells. (b) Necrotic cell death was induced by incubating HeLa cells with KCN and 2-deoxy-D-glucose for 4 h, and the cells were immunostained with AE-4. Arrows show H1-positive necrotic cells. (c) HeLa cells were treated with TNF/CHX for 5 h and then stained with an anti-pan-histone antibody. Arrowheads show apoptotic cells, characterized by pyknotic nuclei. The nuclei were stained with Hoechst 33342. (d) HeLa cells were immunostained with the AE-4 and anti-active caspase-3 antibodies. The number of H1-positive (+) and -negative (-) cells among 50 active caspase-3-positive cells exposed to TNF/CHX for 5 h was determined. (e) BG2 cells were exposed to 300 nM H7 for 16 h, followed by immunostaining with the AE-4 anti-H1 antibody. Arrowheads show apoptotic cells, characterized by pyknotic nuclei. Scale bars: (a–c) 25  $\mu$ m; (e) 8  $\mu$ m

We next compared the staining patterns of the AE-4 anti-H1 antibody and the TUNEL method, which identifies fragmented nuclear DNA in dying cells (Figure 4d and e). A considerable proportion of stage 3 (H1-negative) cells were not labeled by the TUNEL method. Conversely, all the TUNEL-positive cells were H1-negative. These data suggest that the AE-4 anti-H1 antibody could distinguish an earlier stage of apoptosis than that detected by TUNEL. Cells undergoing the dying process appeared to lose their immunoreactivity to the AE-4 anti-H1 antibody prior to DNA fragmentation.

We also examined the cell-membrane integrity of the H1-negative cells by propidium iodide (PI) staining. Only one-tenth of the H1-negative cells allowed PI to intercalate into the DNA (Figure 4c), supporting the idea that the AE-4 anti-H1 antibody recognized changes in H1 associated with early apoptosis.

**Loss of AE-4 immunoreactivity to H1 apparently occurs immediately after H1 translocation to the nuclear periphery.** There are two possible reasons for the loss of

anti-H1 immunoreactivity. One is that the H1 protein level decreases to an undetectable level, and the other is that H1 undergoes a specific conformational change. We examined these possibilities by purifying H1 using perchloric acid (PCA).<sup>19</sup> The H1 level was lower in dying cells than in control cells by immunoblotting and Coomassie brilliant blue staining after H1 purification; however, it did not completely disappear (Figure 5a–c). We next transfected Neuro 2a cells with a venus-tagged H1 (H1-venus) expression vector and observed the fate of the H1-venus after the cells were induced to undergo apoptotic cell death by MG132 (Figure 5d and e). Double staining with the AE-4 and anti-GFP antibodies revealed that both the AE-4-immunolabeled H1 and the anti-GFP-labeled H1-venus were initially uniformly distributed in the nucleus of normal cells (Figure 5e right; double arrows). However, during the cell-death progression, H1-venus became localized to the periphery of the nucleus, whereas the AE-4 epitope of both the endogenous H1 and exogenous H1-venus became unrecognized (Figure 5e middle, stage 2→3; double arrowheads) or only weakly

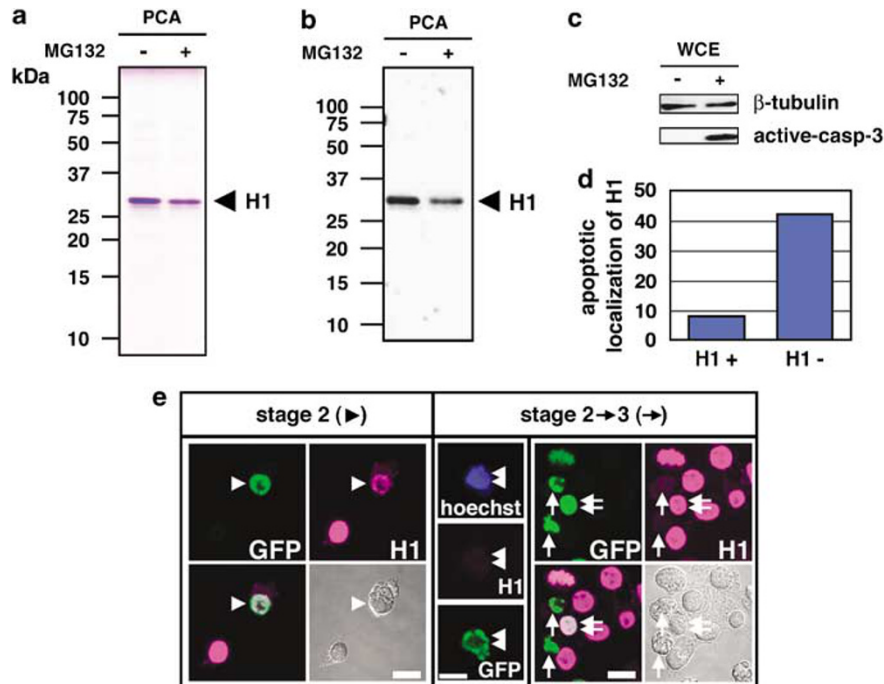


**Figure 4** Loss of AE-4 immunoreactivity to H1 in pre-dead cells. (a) Neuro 2a cells were exposed to MG132 for 7 h and stained with the AE-4 anti-H1 and anti-active caspase-3 antibodies. Nuclei were stained with Hoechst 33342. Apoptotic stimuli-induced caspase activation (stage 1), nuclear condensation distinguished by Hoechst staining (stage 2), and loss of AE-4 anti-H1 immunoreactivity (stage 3), in that order. Active caspase-3-negative cells were defined as stage 0 cells. Arrowheads show stage-3 caspase-active/H1-negative cells with a pyknotic nucleus. (b) The number of stage 1, stage 2, and stage 3 cells as defined in (a) among 50 active caspase-3-positive cells exposed to MG132 for the indicated time. (c) Neuro 2a cells were exposed to MG132 for 7 h, and then incubated with propidium iodide (PI) for 5 min. The cells were then immunostained with the AE-4 anti-H1 antibody. The number of PI-positive (+) and -negative (-) cells among 100 H1-negative cells was determined. (d) Neuro 2a cells were exposed to MG132 for 7 h and subjected to TUNEL, followed by staining with the AE-4 anti-H1 and anti-active caspase-3 antibodies. Fifty active caspase-3-positive cells were classified based on their staining patterns with TUNEL and the AE-4 anti-H1 antibody. (e) Representative staining patterns obtained with TUNEL, anti-active caspase-3, and the AE-4 anti-H1 antibodies in (d). Scale bar, 10  $\mu$ m

recognized (Figure 5e left, stage 2; arrowheads) by the anti-H1 antibody. In stage 2→3, the localization change in H1-venus did not simply reflect a change in nuclear DNA that was labeled by Hoechst dye (Figure 5e middle, stage 2→3; double arrowheads). Quantification analysis revealed that a considerable proportion of cells that showed H1-venus localization at the periphery of the nucleus was H1-negative (Figure 5d). As the AE-4 anti-H1 antibody could recognize exogenous H1-venus in Neuro 2a cells in immunoblots (Supplementary Figure 2), these data indicate that the localization change of H1 in the nucleus might have

caused the loss of AE-4 immunoreactivity. In addition, the AE-4 anti-H1 antibody did not label exogenous H1-venus in apoptotic cells with a fragmented nucleus (Figure 5e right; arrows).

**Caspase activation and H1 changes in real time revealed by an improved indicator for activated caspase-3, 'CNL-SCAT3'.** To investigate the caspase activation and nuclear events in real time in detail, we developed CNL-SCAT3 (Caspase-dependent Nuclear Localization-SCAT3), an improved version of the fluorescence energy transfer

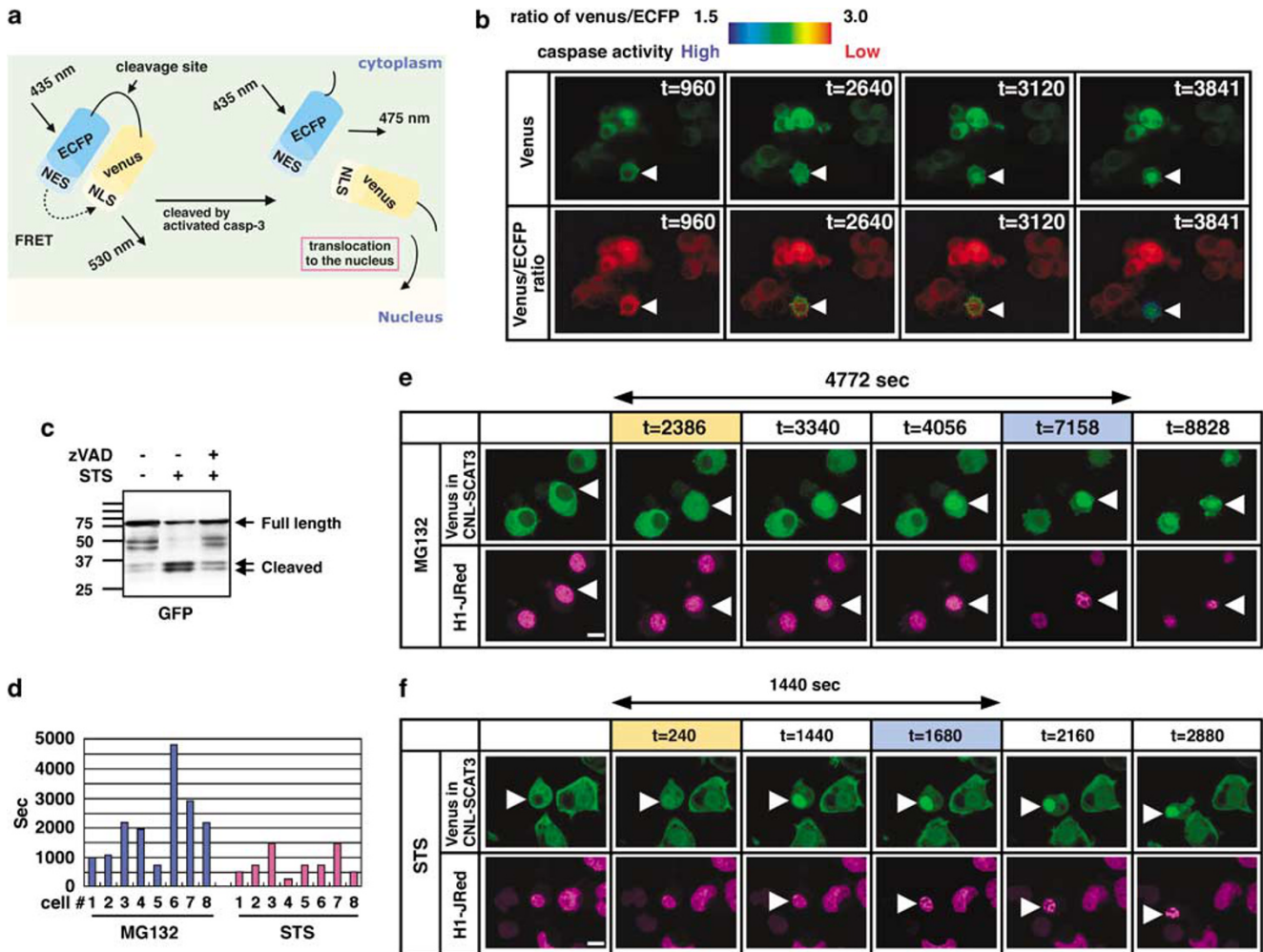


**Figure 5** Loss of AE-4 immunoreactivity to H1 was owing to both a reduced H1 level and an alteration in the H1 in apoptotic cells. (a–c) Neuro 2a cells were treated with MG132 for 7 h. Purified H1 was analyzed by Coomassie brilliant blue staining (a) and immunoblotting using the AE-4 antibody (b). Whole cell extract (WCE) aliquotted before H1 purification was analyzed by immunoblotting with an anti- $\beta$ -tubulin antibody in (c). (d) Neuro 2a cells were transfected with H1.2-venus-pTKX3 and exposed to MG132 for 7 h. The number of H1-positive (+) and -negative (–) cells among 50 that showed H1.2-venus in the nuclear periphery (defined as the apoptotic localization) was determined. (e) Neuro 2a cells were transfected with H1.2-venus-pTKX3 and exposed to MG132 for 7 h. The cells were then stained with the AE-4 anti-H1 and anti-GFP antibodies. Although most of the cells exhibiting an apoptotic localization of H1 failed to be labeled with AE-4 (middle, double arrowheads; stage 2  $\rightarrow$  3), some weakly immunolabeled cells were observed (left, arrowheads; stage 2). Chromosomal DNA in the cell (middle, double arrowheads; stage 2  $\rightarrow$  3) was visualized by Hoechst 33342. Stage 3 cells, showing nuclear condensation, lost their immunoreactivity to AE-4 (right, arrows). H1.2-venus-expressing cells with a morphologically healthy nucleus were immunolabeled with both the AE-4 anti-H1 and anti-GFP antibodies (right, double arrows). Scale bar, 10  $\mu$ m

(FRET)-based caspase indicator SCAT3.<sup>20</sup> Nuclear export signal (NES) and nuclear localization signal (NLS) sequences were fused to the N-terminal and C-terminal ends of SCAT3, respectively, enabling us to monitor caspase activity by measuring not only the FRET from enhanced cyan fluorescent protein (ECFP) to venus, but also the subcellular localization of the venus signal (Figure 6a). First, we checked whether CNL-SCAT3 was cleaved in a caspase-dependent manner (Figure 6c). Neuro 2a cells were transfected with CNL-SCAT3 and subjected to apoptosis induction. CNL-SCAT3 cleavage was observed after STS treatment and inhibited by the pan-caspase inhibitor zVAD-fmk. We then monitored the caspase activation in Neuro 2a cells exposed to MG132 (Figure 6b and Supplementary Video 1). As expected, the FRET abolishment of CNL-SCAT3 and the nuclear translocation of NLS-venus were simultaneous. Nuclear condensation could then be finely monitored because of the intense NLS-venus fluorescence in the nuclei. Thus, the change in venus localization from the cytoplasm to the nucleus in CNL-SCAT3-expressing cells could be used as a sensitive monitor of caspase activation at the single-cell level in real time.

To monitor the dynamics of caspase activation and H1 changes in a dying cell, Neuro 2a cells were cotransfected with CNL-SCAT3 and H1-JRed expression vectors, and apoptosis was induced by STS or MG132. In response to STS or MG132, H1 underwent its apoptotic localization

change after venus was translocated to the nucleus, indicating that caspase activation preceded the H1 changes during apoptosis (Figure 6e and f and Supplementary Videos 2 and 3). In addition, the time from caspase-3 activation to the H1 changes varied with the apoptotic stimulus used (Figure 6d). H1 tended to be maintained in the normal state for a relatively long time, that is,  $2079 \pm 464$  s after caspase activation by MG132 (corresponding to stage 2 described in Figure 4a); in contrast, STS induced the change in H1 localization more quickly, within  $780 \pm 155$  s after caspase activation ( $n=8$   $P<0.05$ ; Figure 6d–f). Although the H1 localization change was found to precede extensive chromatin DNA condensation, as shown in Figure 4, these live-imaging data directly showed that H1 underwent apoptotic changes after caspase-3 activation and before extensive chromatin condensation, and this step proceeded with different timing, depending on the cell-death signal used. Given that the changes in H1 are caspase-dependent, our findings indicate that the post-caspase activation process may vary with the cell-death stimulus. To examine whether the AE-4 and anti-active caspase-3 antibody staining patterns reflect different features of these cell death signal-dependent processes, we exposed Neuro 2a cells to STS and performed immunofluorescence analysis at different time points. In contrast to cells treated with MG132, almost all the caspase-activated cells were H1-negative, regardless of the exposure time to STS (Figure 4b and Supplementary Figure 3), indicating a more rapid



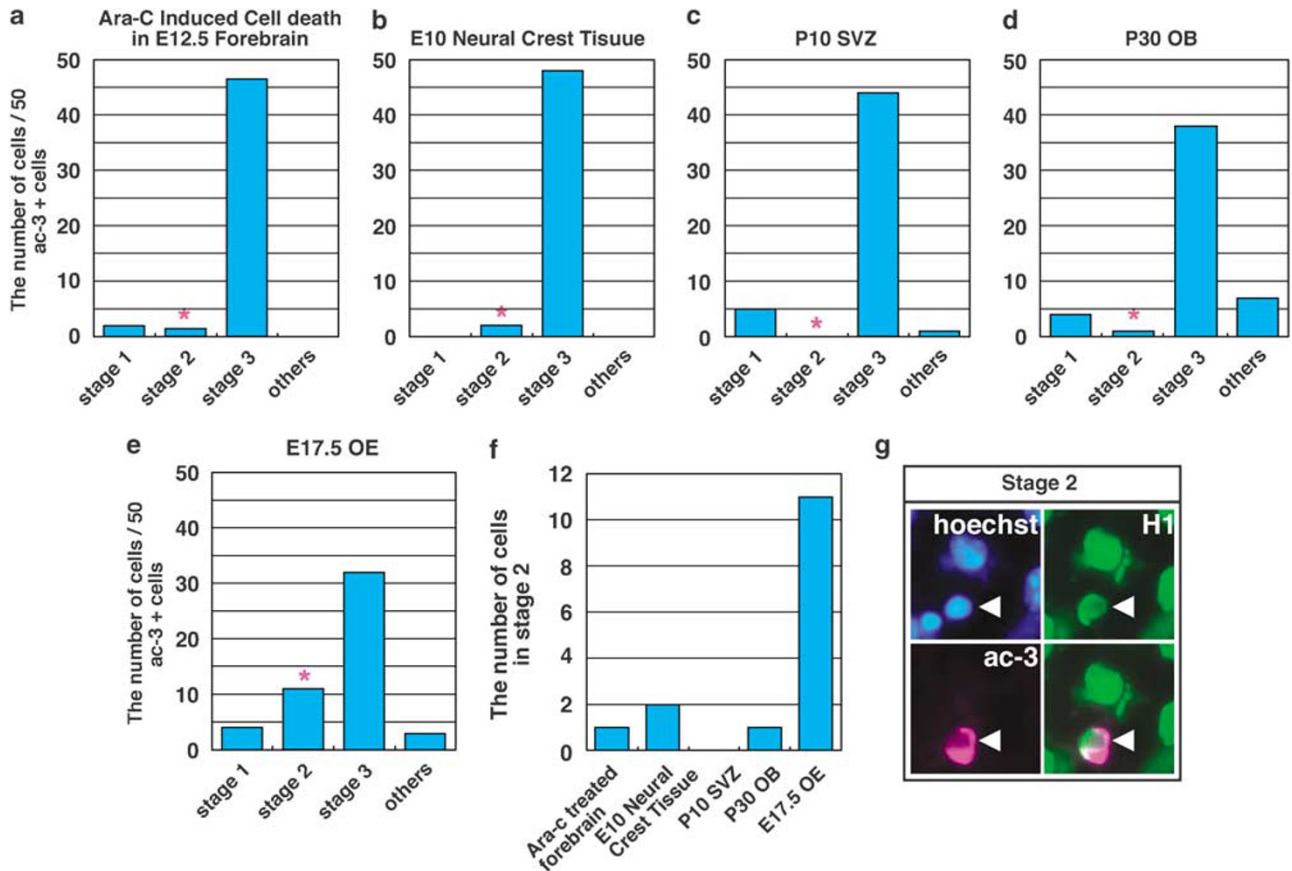
**Figure 6** Imaging analysis of caspase activation and H1 changes in Neuro 2a cells. (a) Schematic representation of CNL-SCAT3. After the caspase-3-mediated cleavage of CNL-SCAT3, venus-NLS was translocated from the cytoplasm to the nucleus. (b) Ratio images of the CNL-SCAT3 probe in Neuro 2a cells exposed to MG132. The venus signal began to be observed in the nucleus as early as the ratio change. (c) Immunoblot analysis of CNL-SCAT3-expressing Neuro 2a cells exposed to STS using an anti-GFP antibody. CNL-SCAT3 was cleaved in a caspase-dependent manner. (d–f) Neuro 2a cells were cotransfected with CNL-SCAT3 and H1.2-JRed expression vectors, and then exposed to MG132 or STS. The time interval from the initial caspase activation to the apoptotic localization of H1 in individual cells is summarized in (d). Caspase activation was determined by the change in localization of the venus signal to the nucleus. (e and f) Examples of cells monitored after treatment with MG132 (e) or STS (f). The time points marked with orange boxes in (e) and (f) correspond to the stage of initial caspase activation. Blue boxes correspond to the stage when cells began to exhibit the apoptotic change in H1 localization. Scale bar, 10  $\mu$ m

cell-death progression. These data suggest that staining with the anti-H1 antibody is useful for analyzing the characteristics of post-caspase apoptotic process both *in vitro* and *in vivo*.

**The apoptotic process in the developing olfactory sensory neuron is distinct from that in other nervous regions.** To investigate the characteristics of dying cells in developing tissues, we stained mouse neural tissues with an activated caspase-3 antibody, the AE-4 anti-H1 antibody, and Hoechst dye, and quantified the proportion of cells at each of the stages described in Figure 4a (Figure 7). Massive cell death was observed in the E10.5 neural crest tissues within the third branchial arch or in the Ara-C-treated ventricular zone, and most of these cells were categorized as stage 3 (Figure 7a and b). In adult rodents, neurons are continually generated in the subventricular zone (SVZ) of the forebrain and migrate tangentially in chains toward the

olfactory bulb (OB).<sup>21,22</sup> A few cells are lost in the P10 SVZ or the P30 OB, and a large proportion of these dying cells were also categorized as stage 3 (Figure 7c and d and Supplementary Figure 4). However, in the E17.5 olfactory epithelium (OE), H1-positive caspase-activated cells (stage 2) were frequently observed (Figure 7e–g).

We then characterized the caspase-activated cells in the developing OE using anti- $\beta$  III tubulin (a marker for neurons) and anti-OMP (a marker for mature OSNs) antibodies (Figure 8). Almost all the caspase-activated cells were OSNs (Figure 8a–c), and approximately 30% of the mature OSNs were H1-positive (Figure 8d and Supplementary Figure 5). These data suggest that the developing OSN is unique in the way caspase activation progresses to the H1 changes that are coupled with apoptotic nuclear events. The prolonged caspase-activated status before nuclear changes may reflect a unique caspase function in developing OSNs.



**Figure 7** H1-positive dying cells (stage 2) in the developing olfactory epithelium. The number of stage 1, stage 2, and stage 3 cells, defined in Figure 4a, among 50 active caspase-3-positive cells in the Ara-C-treated E12.5 forebrain (a), E10 neural crest tissue within the third branchial arch (b), P10 subventricular zone (SVZ) (c), P30 olfactory bulb (OB) (d), and E17.5 olfactory epithelium (OE) (e). The number of stage 2 cells in the various regions is summarized in (f). (g) A representative stage 2 cell observed in the E17.5 olfactory epithelium. Arrowheads show stage 2 H1-positive caspase-activated cells with a condensed nucleus

## Discussion

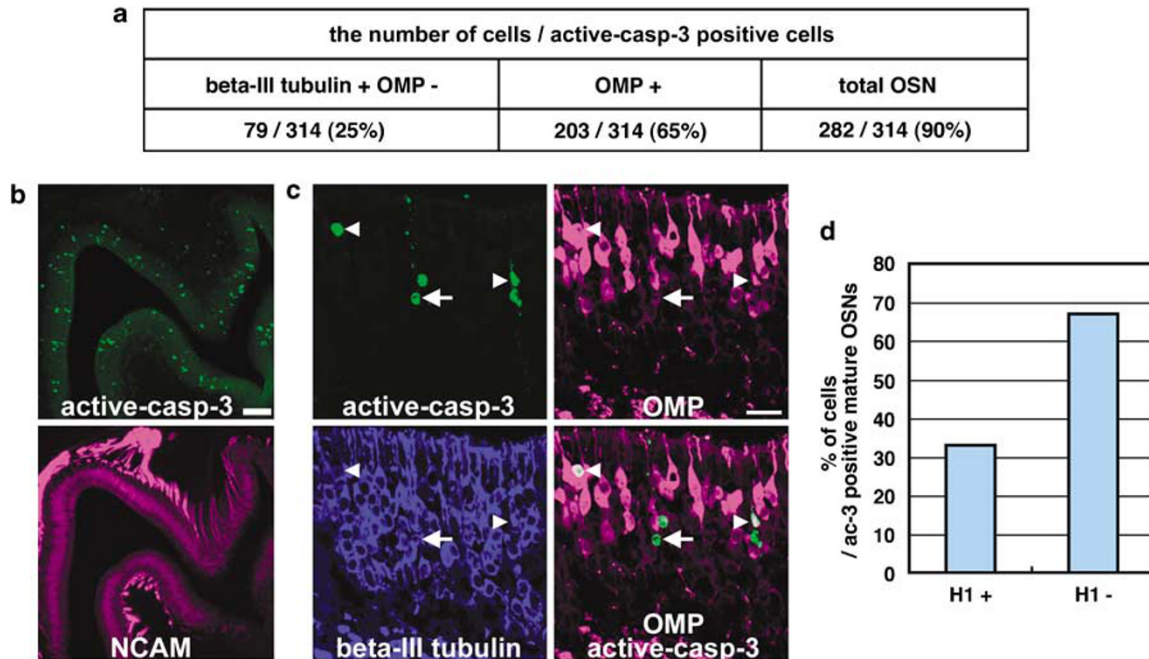
Apoptosis is an active form of cellular suicide that is essential for normal development and tissue homeostasis in multicellular organisms. Here we found that changes in H1 occur in the early phase of apoptosis both *in vitro* and *in vivo*, and these changes can serve as a useful marker of a post-caspase apoptotic event. We then investigated the post-caspase activation status of cells in the developing nervous system by staining with anti-active caspase-3 and AE-4 anti-H1 antibodies, and found that a large population of OSNs in the developing OE exhibited caspase activity without the apoptotic H1 change. Considering that these unique cells largely exhibited condensed nuclei, most of them were probably undergoing apoptotic cell death, rather than non-apoptotic events. It is possible that apoptosis progresses more slowly in OSNs than in other neural cells, and as a result, dying cells remain in the OE for a relatively long time.

Although the roles of caspase activity in the OSN remain to be clarified, recent studies in *Drosophila* indicate that caspase activity in dying cells can induce a compensatory proliferation of the neighboring cells. In the *Drosophila* wing disc, the triggering of local cell death by ectopic toxin expression induces elevated cell proliferation around the site of apoptosis, suggesting that cells can perceive apoptosis in their

vicinity and undergo extra cell divisions until the original cell number is restored.<sup>23</sup> That is, signals from the dying cells might drive this compensatory proliferation. Consistent with this idea, the initiator caspase DRONC regulates both the execution of apoptosis and the subsequent proliferation of neighboring cells, and is involved in inducing mitogen expression, including that of Wingless (Wg) and Decapentaplegic (Dpp).<sup>24–27</sup> As OSNs undergo functional turnover throughout life,<sup>28,29</sup> cell death and compensatory neurogenesis in the OE must be tightly regulated. In addition, our findings introduce the possibility that OSNs undergoing a slow apoptotic process could influence their neighboring cells for a prolonged time. An intriguing hypothesis is that caspases initiate the transduction of certain signals from the pre-dead caspase-3-activated H1-positive OSNs to their neighbors to affect the development of the OE. Although granule cells and periglomerular cells in the bulb also continue to arise from the SVZ throughout life, the developing OE exhibited higher caspase activity than the 2-month-old adult OB (data not shown). Thus, we think that the OE especially utilizes caspase functions in its development.

Recent investigations have also revealed the involvement of local caspase activity in other non-apoptotic processes. The dendritic pruning of larval neurons in *Drosophila* requires





**Figure 8** Caspase-activated cells in the developing olfactory epithelium (OE) were olfactory sensory neurons. (a) Sections of E17.5 OE were stained with anti-active caspase-3, anti- $\beta$ -III tubulin (a marker for neuronal cells), and anti-OMP antibodies (a marker for mature OSNs). A total of 314 caspase-activated cells were classified as immature neurons ( $\beta$ -III tubulin-positive (+) and OMP-negative (-)), mature neurons (OMP-positive (+)), and others. (b) Sections (80- $\mu$ m thick) of E16.5 OE were stained with anti-active caspase-3 and anti-NCAM antibodies. Many active caspase-3-positive cells were observed throughout the epithelium. (c) Sections of E17.5 OE were stained with anti-active caspase-3 C92-605, anti- $\beta$ -III tubulin, and anti-OMP antibodies. Arrows indicate a  $\beta$ -III tubulin-positive and OMP-negative immature caspase-activated neuron. Arrowheads indicate OMP-positive mature caspase-activated neurons (d). The number of H1-positive (+) and -negative (-) cells among 100 caspase-activated, OMP-positive mature OSNs. Sections of E17.5 were stained with AE-4 anti-H1, anti-active caspase-3, and anti-OMP antibodies. Scale bars, (b) 10  $\mu$ m, (c) 20  $\mu$ m

activation of the initiator caspase Dronc, and a caspase-3-like activity is present in the degenerating dendrites of neurons undergoing pruning.<sup>30,31</sup> In addition, exposure to a novel birdsong triggers an increase in activated caspase-3 at post-synaptic sites in the Zebra Finch, and the infusion of a caspase inhibitor into the auditory forebrain of these birds disrupts their long-term habituation, raising the possibility that post-synaptic caspase activation is involved in learning and memory.<sup>32</sup> Although it is unclear how cells might exhibit local caspase activity and remain non-apoptotic, one plausible mechanism for caspase-dependent signals having differential effects is that their signals might lead to variable alteration in chromatin proteins, such that some cells that are irreversibly directed die by apoptosis, and others to engage in different processes. As the H1-positive/caspase-active cells failed to intercalate PI into their nucleus (Figure 4c and data not shown), they appeared to still be alive. The double staining of tissues with anti-active caspase-3 and the AE-4 anti-H1 antibody should be useful for distinguishing cells in which caspase has non-apoptotic functions.

As apoptotic stimuli only partially reduced the amount of H1 protein in the nucleus, the loss of immunoreactivity to AE-4 could not be completely attributed to H1 degradation; we therefore hypothesized that the remaining H1 undergoes some conformational change. Even in cells expressing transfected H1.2-venus, the AE-4, but not the GFP, immunoreactivity was lost after H1.2-venus changed its localization in the nucleus during apoptosis, supporting the idea that H1-

specific conformational changes occur during apoptosis. As not only AE-4, but also two additional anti-H1 antibodies, failed to immunolabel H1 in apoptotic cells, H1 might undergo global structural changes, possibly forming an unidentified molecular complex, during apoptosis.

To investigate the correlation of caspase activation with H1 alteration in detail, we developed an improved activated caspase-monitoring protein named CNL-SCAT3, and used it to examine the real-time dynamics of caspase activation and H1 change. A previous study suggested that histone H1.2 promotes cytochrome *c* release from mitochondria in the cytoplasm after exposure to X-ray irradiation.<sup>33</sup> The authors indicated that H1.2 was translocated into the cytoplasm only in cells in which apoptosis was induced by DNA double-strand breakage. However, we did not observe cytoplasmic H1 in cells undergoing etoposide-induced apoptosis. In addition, exogenous H1.2-venus protein remained associated with the chromosomal DNA of dying cells after exposure to TNF/CHX, STS, or MG132. It is nonetheless possible that a small quantity of cytoplasmic H1 is sufficient to stimulate cytochrome *c* release.

We propose that the AE-4 anti-H1 antibody can be used as a new marker for distinguishing the status of caspase-activated cells in tissue. AE-4 specifically indicated the early stage of apoptosis in dying cells, in contrast to TUNEL, which labels fragmented DNA in late apoptotic or even necrotic cells. As H1 is a ubiquitous and abundant protein, the detection of its alteration by a specific antibody should be a very useful method for examining post-caspase activation events in

various tissues. Monitoring of the H1 state should provide new insight into the progression of the apoptotic processes that occur after caspase activation and their functions during development.

### Materials and Methods

**Plasmids.** To construct H1.2-JRed, the coding sequence for human histone H1.2 was inserted into the pJRed-N vector (Evrogen). H1.2-venus was constructed by replacing the ECFP of SCAT3 with H1.2. H1.2-venus was subcloned into the pTKX3 vector. CNL-SCAT3 was generated by adding the NES NSNELALKLAGLDINKTE of protein kinase inhibitor  $\alpha$  to the N terminus of NLS-SCAT3 in the pcDNA3.1(-) vector.<sup>20</sup>

**Mice.** C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan) and used for the immunohistochemistry experiments as previously described.<sup>34,35</sup> *Apaf-1*(+/−) and *apaf-1*(−/−) mice were generated by interbreeding heterozygotes.<sup>12</sup> To induce proliferative cell death in embryonic mice, Ara-C at 250 mg/kg body weight was injected into pregnant mice between gestational days 12 and 13.<sup>15</sup> Embryos were isolated 6 h later and fixed as described previously.<sup>35</sup>

**Imaging analysis.** Cells were plated on poly-L-lysine-coated glass coverslips and transfected with plasmid vectors. Confocal images of FRET-based caspase activation were acquired with the MetaMorph system (Molecular Devices) using an inverted microscope DMI6000B (Leica) and spinning disk-type confocal unit (CSU10, Yokogawa). Double imaging of H1-JRed and Venus in CNL-SCAT3 was carried out using the TCS SP5 confocal system (Leica).

**Immunofluorescence staining in tissues.** The staining method has been described.<sup>34,35</sup> The following antibodies were used at the indicated dilutions: mouse monoclonal anti-H1 clone AE-4 (Santa Cruz, 1 : 1000; or Upstate, 1 : 200), rabbit monoclonal anti-active caspase-3 5A1 (Cell Signaling; 1 : 800), rabbit monoclonal anti-active caspase-3 C92-605 (BD; 1 : 1000), rabbit polyclonal anti-phospho-histone H3 for mitotic cells (Upstate; 1 : 200), mouse monoclonal anti-histones (Chemicon; 1 : 500), rabbit polyclonal anti-GFP (MBL; 1 : 400), goat polyclonal anti-OMP (Wako Chemicals; 1 : 1000), rat monoclonal anti-NCAM (Chemicon; 1 : 1000), and mouse monoclonal anti-mouse class III  $\beta$  III tubulin (Covance; 1 : 500).

**Cells, immunostaining, preparation of cell lysates, and immunoblotting.** The mouse neuroblastoma cell line Neuro 2a was cultured in DMEM containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine. HeLa cells were cultured in DMEM containing 10% FBS. *Drosophila* BG2 cells were cultured in Schneider's medium containing 10% FBS. After treatment with the apoptotic reagent, the cells were fixed with 4% paraformaldehyde in PB (PFA/PB) for immunostaining. For immunoblotting, cells were lysed in 2 × SDS sample buffer (120 mM Tris-HCl pH 6.8, 4% SDS, and 20% glycerol). The lysates were then separated by 15 or 10% SDS-PAGE. The following antibodies were used for immunoblotting at the indicated dilutions: monoclonal mouse anti-H1 clone AE-4 (Upstate; 1 : 200), monoclonal mouse anti-H1 clone C14093 (MBL; 1 : 100), rabbit polyclonal anti-H1 (kindly provided by K Ohsumi; 1 : 500),<sup>36</sup> rabbit monoclonal anti-active caspase-3 C92-605 (BD; 1 : 1000), rabbit polyclonal anti-active caspase-3 (AC-3), generated as described<sup>37</sup> (1 : 1000), mouse monoclonal anti- $\beta$ -tubulin (Chemicon; 1 : 1000), and rabbit polyclonal anti-GFP (MBL; 1 : 500). A horseradish peroxidase-conjugated antibody (anti-rabbit or anti-mouse IgG; Transduction Laboratories; 1 : 2000) was used as the secondary antibody.

**Propidium iodide staining.** Cells were plated on poly-L-lysine-coated glass coverslips and treated with MG132 for 7 h. PI (2  $\mu$ g/ml; Molecular Probes Inc.) was added to each well, and the plates were incubated for 5 min and washed with PBS, followed by immunostaining with the AE-4 antibody as described above.

**Treatment of HeLa cells with various reagents.** To induce apoptosis, HeLa cells were exposed to 50 ng/ml TNF and 10  $\mu$ g/ml CHX, 1  $\mu$ M STS, or 200  $\mu$ M etoposide. To inhibit caspase activation, 100  $\mu$ M zVAD-fmk was added 2 h before the TNF/CHX. Necrotic cell death was induced by incubation with 4 mM KCN and 50 mM 2-deoxy-D-glucose for 4 h, as described.<sup>38</sup>

**Purification of H1 protein.** H1 was directly extracted from Neuro 2a cells with 5% PCA and then precipitated with 20% trichloroacetic acid as previously described.<sup>19</sup> The protein yield was normalized by western blotting against  $\beta$ -tubulin in the whole cell extract before purification.

**Acknowledgements.** We thank T Yagi (Osaka University) for many helpful discussions, H Sakaguchi for technical support, and members of the Yagi laboratory for encouragement. We also thank T Iwawaki (Riken) for the pTKX3 vector and K Ohsumi (Tokyo Institute of Technology) for the rabbit polyclonal anti-H1 antibody. This work was supported in part by grants from the Japanese Ministry of Education, Science, Sports, Culture and Technology (to MM) and by a RIKEN Bioarchitect Research Grant (to MM). This work was also supported in part by research fellowships of the Japan Society for the Promotion of Science for Young Scientists (to SO).

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)