The HA tag is cleaved and loses immunoreactivity during apoptosis

To the editor: Since its introduction almost 20 years ago as a short immunoreactive tag[1], the hemagglutinin epitope YPYDVPDYA (HA tag) from the influenza virus A, has been extensively used in various experimental contexts of cell biology and biochemistry to track proteins of interest within cells, to isolate them and to coprecipitate their possible partners. This holds particularly true in the field of apoptosis, where the ever-increasing number of candidates participating in apoptosis signaling pathways has prompted the community to most often rely on engineered tags—such as the HA tag—rather than embarking on the risky development of specific antibodies. As an illustration, a simple full-text search "HA tag" and "apoptosis" in the Highwire press database (http://www.highwire.org) yields over 200 publications only for the past 12 months.

While studying plasma-membrane dynamics during apoptosis with a membrane-targeted fluorescent construct, we accidentally discovered that the HA tag was cleaved during apoptosis. The chimera we used was a fusion of SNAP-25, with N-terminally HA-tagged GFP (SNAP-25–HA-GFP), which becomes palmitoylated and associates with the inner leaflet of the plasma membrane[2]. The construct delineated the plasma membrane (**Supplementary Fig. 1** online), evidencing its dynamic changes, but, after 83 min of TNF-α treatment, the GFP signal diffused into the cytosol, suggesting that part if not all of the fluorescent construct 'detached' from the plasma membrane (**Supplementary Video 1** online). This phenomenon occurred in HeLa cells treated with staurosporine (**Fig. 1a**) and in Jurkat cells challenged with Fas ligand (FasL; **Supplementary Fig. 2** online) as well, and coincided with a drastic loss of HA immunoreactivity (**Fig. 1b**).

Indeed, the fluorescence release from the membrane of cells expressing SNAP-25–HA-GFP is remarkably synchronous with the development of caspase 3/7 activity (**Fig. 1a**) as it coincided with the signal of a recombinant caspase 3/7 probe consisting in a fusion of a red fluorescent protein with a mitochondrial outer membrane anchor domain tethered together by a caspase 3/7–sensitive DEVD linker. Transfection and expression of a constitutively active form of caspase 3 induced GFP release as well, further supporting the involvement of caspase 3/7 in HA cleavage (**Supplementary Fig. 3**

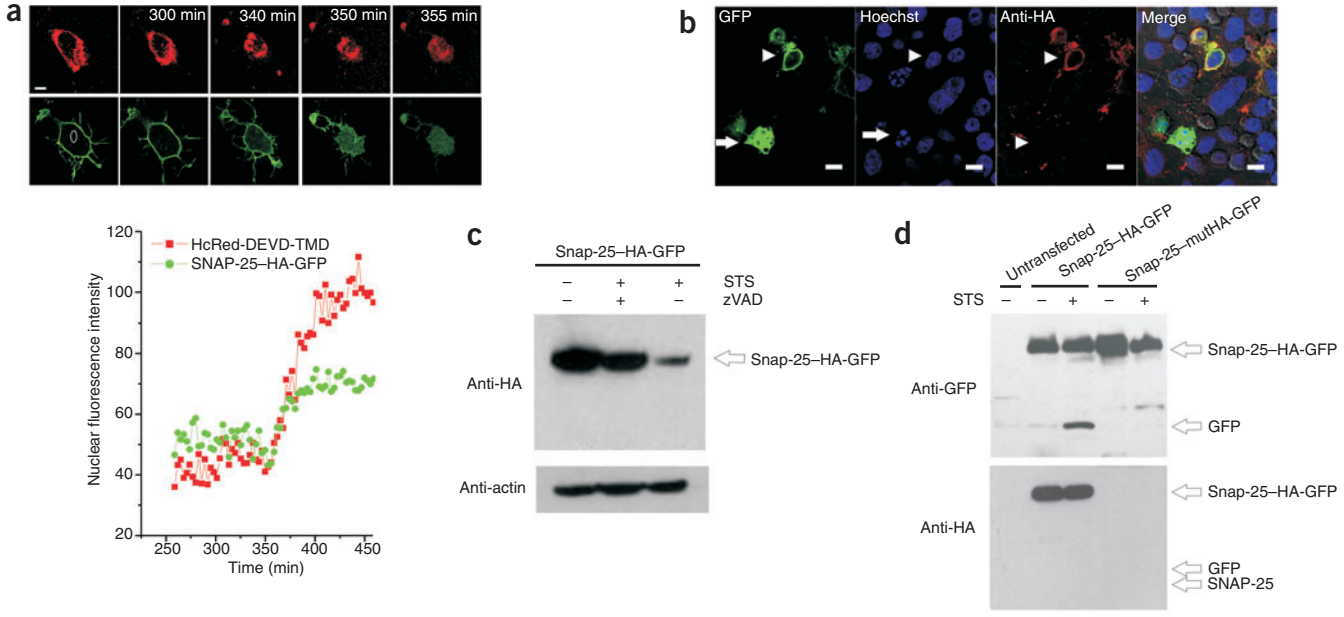

Figure 1 | The HA tag is cleaved by caspase 3/7. (**a**) Dual-channel confocal images of a single live transfected HeLa cell expressing SNAP-25–HA-GFP (green) and the caspase 3/7 probe HcRed-DEVD-TMD (red) anchored to the outer mitochondrial membrane at the indicated times after challenge with 500 nM staurosporine (top). Quantification of the average signal intensity in the nuclear region (ellipse) of both channels in each frame (bottom). There is simultaneous release of both probes starting at 350 min, at the onset of caspase 3/7 activity. (**b**) Fluorescence confocal image of staurosporine-treated (500 nM, 6 h) HeLa cells expressing SNAP-25–HA-GFP (GFP), and stained with Hoechst and anti-HA as indicated. Merged image shows the coincidence of cytosolic GFP release, HA immunoreactivity loss and nuclear degradation in an apoptotic cell (arrow) compared to a still unaffected cell (arrowhead). (**c**) Western blot of transfected HeLa cells expressing SNAP-25–HA-GFP and treated 6 h with 500 nM staurosporine (STS). Staurosporine treatment induces a loss of HA immunoreactivity that is antagonized by zVAD-fmk. (**d**) Western blot of HeLa cells transfected with indicated constructs and treated with 500 nM staurosporine (STS) for 3 h where indicated. The diffusion of the GFP signal evidenced in **a** corresponds to a proteolytic cleavage that releases the GFP moiety. The cleavage is accompanied by a loss of HA immunoreactivity. SNAP-25–mutHA-GFP is not cleavable and does not react with anti-HA. SNAP-25–mutHA-GFP is also silent in anti-HA immunofluorescence, that is, under nondenaturing conditions (**Supplementary Fig. 4**).

online). Altogether, these data suggested that release of GFP from the plasma membrane could result from a direct proteolytic cleavage of SNAP-25–HA-GFP by caspase 3/7. Notably, we realized that the HA tag itself contained a DVPD sequence, which had been documented as a caspase 3/7 cleavage site in two other proteins[3,4].

A western blot of HeLa cells transfected with the SNAP-25–HA-GFP and treated for 6 h with staurosporine in combination with or without the caspase inhibitor zVAD-fmk indicated that staurosporine induced a loss of the HA signal that is prevented by zVAD -fmk, consistent with the idea that HA is attacked during apoptosis in a caspase-dependent manner (**Fig. 1c**). Even with a shorter 3-h treatment, staurosporine caused in a fraction of the cell population a proteolytic cleavage of SNAP-25–HA-GFP that released GFP and abolished the HA immunoreactivity of the cleavage products (GFP and SNAP-25; **Fig. 1d**). We found that the cleavage takes place within the DVPD sequence of HA tag as the same construct containing the caspase 3/7–insensitive DVPE mutant (SNAP-25–mutHA-GFP) was not cleaved (**Fig. 1d**). The HA epitope mutant YPYDVPEYA did not react with the reference antibody against HA, mAb12CA5 (ref. 1; **Fig. 1d** and **Supplementary Fig. 4** online).

HA cleavage by caspase 3/7 appears to be neither distinctive of SNAP-25–HA-GFP nor influenced by the subcellular position of the HA-tagged construct: soluble cytosolic HA-GFP or HA-GFP targeted to the mitochondrial matrix (mtHA-GFP) were both cleaved in HeLa cells treated 10 h with staurosporine (**Supplementary Fig. 5** online). Collectively, these results indicate that the HA tag is cleaved by caspase 3/7, and notably, that HA tag cleavage results in a total loss of immunoreactivity (**Fig. 1d** and **Supplementary Figs. 4** and **5**). These observations thus indicate that the use of HA to tag proteins and constructs to study cell death–related and apoptotic mechanisms can result in serious artifacts. For instance, it seems likely that in cell populations transiently transfected with HA constructs, the selective loss of HA immunoreactivity in apoptotic cells causing a restriction of the signal to the fraction of the nonapoptotic cells can easily lead to the erroneous identification of antiapoptotic proteins. In stable cell clones, the development of such an immunoreactivity 'partition' during apoptosis can also be interpreted as an indication that the tagged protein is downregulated. Finally, HA immunoprecipitation performed on cell populations treated to undergo apoptosis will selectively coprecipitate partners that are distinctive of the fraction of cells that precisely have not responded to the apoptotic challenge.

From a more positive point of view, our observations open the way to the use of the HA tag as a *bona fide* caspase 3/7 sensor responding by immunoreactivity loss with a good signal-to-noise ratio. They also indicate that immunoprecipitation of HA-tagged constructs can be helpful to identify early interaction events selectively taking place upstream of caspase 3/7 activation during the apoptotic process.

Note: Supplementary information is available on the Nature Methods website.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

Laura Schembri[1], Renée Dalibart[1], Flora Tomasello[1], Patrick Legembre[2], François Ichas[1] & Francesca De Giorgi[1]

[1]INSERM E347, Institut Bergonié, 229 Cours de l'Argonne, 33076 Bordeaux, France. [2]CNRS UMR5164, Université Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux, France.
e-mail: degiorgi@bordeaux.inserm.fr

1. Field, J. *et al. Mol. Cell. Biol.* 8, 2159–2165 (1988).
2. De Giorgi, F. *et al. Methods Cell. Biol.* 58, 75–85 (1999).
3. Pochampally, R. *et al. Oncogene* 17, 2629–2636 (1998).
4. Katsuda, K. *et al. Oncogene* 21, 2108–2113 (2002).