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Letters to the Editor

Nuclear localization of FADD protein

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Dear Editor,

FAS-associated death domain (FADD) protein is an adaptor protein that connects death receptors with caspase-8 at the cell membrane. We reported that FADD is, unexpectedly, mainly nuclear,[1] and this was subsequently confirmed by another laboratory.[2] However, a recent paper in this journal by the Strasser laboratory has challenged this conclusion.[3] They developed a series of monoclonal antibodies against FADD, one of which, clone 12E7, produced a mainly cytoplasmic signal in HeLa and L929 cells.

In the Strasser paper, cells were formaldehyde fixed and permeabilized with saponin. I compared saponin *versus* the more commonly used Triton X-100 with respect to immunofluorescence with anti-FADD 12E7 (obtained from Dr. Strasser, WEHI, Melbourne, Australia). With saponin, 12E7 localized FADD primarily in the cytoplasm, and the Transduction Labs monoclonal localized it more variably to the cytoplasm in some cells and nucleus in others (see Figure 1 below). However, with Triton X-100, 12E7 generated a primarily nuclear signal in both MCF10a and HeLa, in agreement with our published results using the Transduction Labs monoclonal antibody and monospecific anti-mouse FADD and anti-human FADD polyclonals generated/characterized in our lab.

As both the Strasser and Transduction Labs antibodies produced detergent-dependent results, we tested a series of antibodies directed against known nuclear proteins for their behavior in saponin *versus* Triton X100-permeabilized cells. C-terminal binding protein (CtBP), PML, and BRCA1 antibodies produced the expected nuclear signal using Triton X-100 permeabilization. With saponin, the nuclear signal was much weaker in all cases and was replaced by a cytoplasmic signal with CtBP. These results are consistent with the view that saponin inadequately permeabilizes intracellular membranes, which hinders access of some antibodies to the nucleus, additionally trapping certain antibodies artifactually in the cytoplasm.

The Strasser paper also asserted that FADD was cytoplasmic, based on fractionation of hypotonically lysed HeLa cells. Whereas FADD was in the nuclear fraction of MCF10a cells in our experiments, it was in their soluble cytoplasmic fraction of HeLa cells. In their protocol, nuclei were washed and re-centrifuged twice prior to lysis in SDS sample buffer, while our nuclei were not washed. We have found that FADD (a 26 kDa protein, below the exclusion limit of nuclear pores) leaks from the nucleus during repeated washes (data not shown). This is true of many other known nuclear proteins. For example, DNA polymerase-alpha appears to be cytoplasmic under standard hypotonic lysis conditions, but can be retained in the nuclear fraction using sophisticated ultracold, nonaqeuous fractionation techniques;[4,5] nuclear fractionation is thus very prone to false negatives.

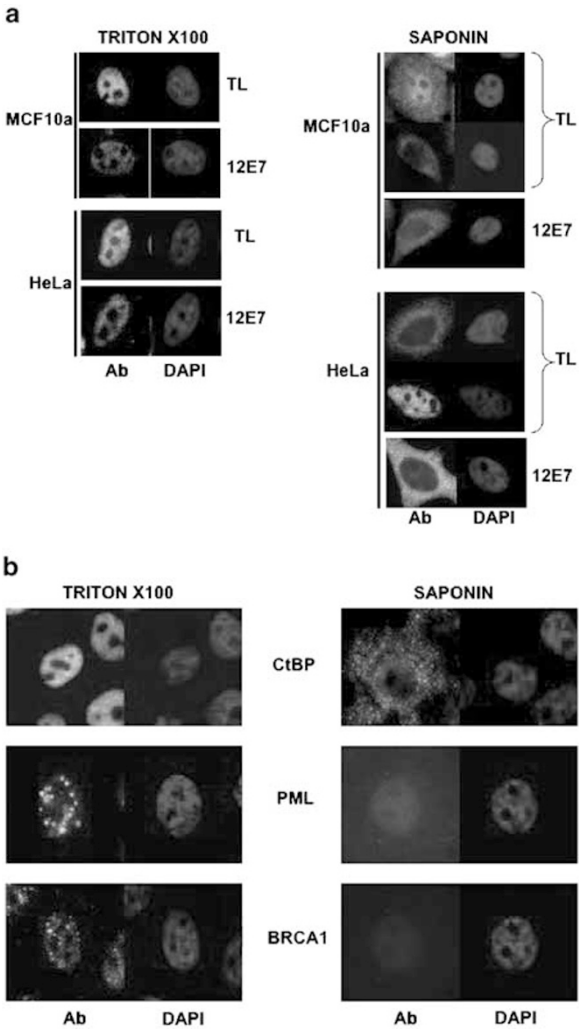

Figure 1 (**a**) Immunofluorescent localization of FADD was performed using HeLa or MCF10a cells that were fixed with formaldehyde, followed by permeabilization with 0.2% Triton X-100 or 0.3% saponin; the latter detergent was included in all subsequent incubation and washing steps. Results are shown for 12E7 and the Transduction Labs monoclonal (TL), followed by detection with anti-rat or anti-mouse labeled with Alexa 488 (Molecular Probes). (**b**) Immunofluorescent localization of other nuclear proteins on Triton X-100 *versus* saponin-permeabilized cells. Antibodies were from the following sources: CtBP: from our laboratory;[6] PML and BRCA1: from BD Pharmingen

In summary, the results with several antibodies including 12E7 indicate a nuclear localization for FADD in adherent cell lines; this localization probably differs in lymphocytes, as the Strasser[3] and Cidlowski[2] labs have reported.

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Nuclear localisation of FADD – rebuttal

Cell Death and Differentiation (2004) **11,** 1362–1363. doi:10.1038/sj.cdd.4401513

Dear Editor,

We recently published in your journal a paper describing the subcellular localisation and post-translational modifications of caspase-8 and its adaptor FADD (also called Mort1) in unstimulated cells, and in lymphocytes that had been activated with mitogens or treated with FasL.[1] In their letter, Frisch *et al.* question some of the conclusions from our work, specifically our conclusion that FADD is located exclusively in the cytoplasm, since they found significant amounts of FADD in the nucleus, at least in adherent cells.

Firstly, it is noteworthy that most results and conclusions from our publication,[1] namely mitogenic activation- and FasL-induced post-translational modification and recruitment of caspase-8 and FADD to distinct clusters at the plasma membrane, have not been challenged. Furthermore, the quality of our novel monoclonal antibodies to mouse caspase-8 and FADD has not been challenged by Frisch *et al.*, but was in fact reinforced, since they have used these antibodies in their experiments.

We would now like to address in depth the specific issues raised by Frisch *et al.*: (1) our method for immunofluorescent staining does not allow efficient detection of nuclear proteins, (2) our method for subcellular fractionation entails the risk of losing proteins from the nucleus and (3) a substantial amount of FADD is localised in the nucleus.

(1) Frisch *et al.* state that immunofluorescent staining of paraformaldehyde-fixed cells permeabilised with saponin does not allow detection of proteins in the nucleus. We now show that our method readily detects p53 in the nucleus of HeLa cells (Figure 1i). In fact, the staining for p53 was at least as strong as that seen in cells permeabilised with Triton-X-100, the procedure recommended by Frisch *et al* (compare Figure 1i with 1k). It is noteworthy that permeabilisation with saponin has also been used for detection of Ikaros protein in the nucleus.[2] We used both methods of cell permeabilisation to detect FADD localisation using our mAb 12E7 (Figures 1a–h). In cells permeabilised with saponin, FADD was found both in the cytoplasm (Figure 1a) and in the nucleus (Figure 1b), with cytoplasmic localisation being considerably more evident. In contrast, and in agreement with the results presented by Frisch *et al.*, when cells were permeabilised with Triton-X-100, FADD was found predominantly in the nucleus and only to a lesser extent in the cytoplasm (Figures 1e and f). The reasons for this discrepancy are not clear, but it is possible that epitopes recognised by this antibody are only exposed on nuclear FADD after treatment with certain detergents, perhaps due to association of nuclear FADD with other proteins.

(2) Frisch *et al.* state that our method for subcellular fractionation may be inadequate because some low molecular weight proteins, such as FADD, may be lost from the nucleus because of diffusion through nuclear pores during washing. We recognise that this is a potential risk, but we note that standard nuclear purification protocols

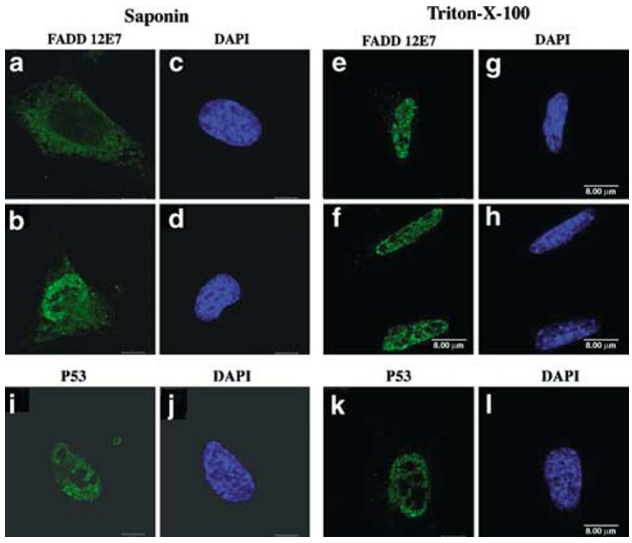

Figure 1 Analysis of FADD subcellular localisation by confocal microscopy. (**a**, **b**) FADD (staining with mAb 12E7) is localised in both the cytoplasm and nucleus of HeLa cells permeabilised with 0.3% saponin. (**g**, **h**) FADD is found in the nucleus and to a lesser extent in the cytoplasm of HeLa cells permeabilised with 0.2% Triton-X-100. (**i**, **k**) HeLa cells stained with anti-P53 mAb (clone 248), showing nuclear localisation after permeabilisation with 0.3% saponin (**i**) or 0.2% Triton-X-100 (**k**). Cells were stained with anti-FADD mAb 12E7 (green), detected by biotinylated mouse anti-rat IgG antibodies and FITC-streptavidin (**a**, **b**, **e**, **f**), or with anti-P53 mAb (248, a gift of Professor D Lane, University of Dundee, Dundee, Scotland) detected with FITC-labelled goat anti-mouse IgG antibodies (**i**, **k**) plus DAPI (blue) (**c**, **d**, **g**, **h**, **j**, **l**). Bars represent 8 μm