Table 1 Summary of the fluorescence correlation spectroscopy measurements using 10 μM RITA

Protein	Diffusion time ± s.e.m.[a]	Change in diffusion time, percent
No protein	0.063 ± 0.011	—
GST-p53 dN(1–63)	0.356 ± 0.070	465
GST-p53 N(1–100)	0.259 ± 0.020	311
GST-p53(1–393)	0.287 ± 0.043	355
His-p53(1–393)	0.198 ± 0.007	214
His-p53(1–312)	0.111 ± 0.014	74
GST	0.076 ± 0.003	20
GST-EBNA2	0.073 ± 0.017	16

[a]All experiments were performed at least three times.

subject for future research.

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1. Issaeva, N. *et al. Nat. Med.* **10**, 1321–1328 (2004).

PrP^{Sc} in mammary glands of sheep affected by scrapie and mastitis

To the editor:

Besides colonizing the central nervous system, the infectious agent of transmissible spongiform encephalopathies, termed prion, is predominantly associated with follicular dendritic cells (FDCs) of lymphoid tissues[1,2]. Accordingly, PrP^{Sc}, a protease-resistant isoform of the host protein PrP^{C} representing the main prion constituent, is often detectable in spleen, tonsils, Peyer patches and lymph nodes of infected hosts.

Chronic inflammatory states are accompanied by local extravasation of B cells and other inflammatory cells, which may induce lymphotoxin-dependent maturation of ectopic FDCs. Consequently, scrapie infection of mice suffering from nephritis, hepatitis or pancreatitis induces unexpected prion deposits at the sites of inflammation[3]. This has raised concerns that analogous phenomena might occur in farm animals.

We have investigated this question in a flock of 818 Sarda sheep held in the Sassari region of Italy for production of wool and human foods. The European Surveillance Plan for Transmissible Spongiform Encephalopathies mandates the removal of all sheep of scrapie-susceptible genotypes in scrapie-infected flocks.Of the 818 sheep, 261 had *Prnp* alleles[4] that conferred susceptibility to prion disease. Of the latter, seven had clinically overt scrapie with PrP^{Sc} in brain, lymph nodes and tonsil. All scrapie-sick sheep and 100 randomly chosen healthy sheep were killed, and mammary glands were analyzed histologically. Of these, 10 sheep had lymphocytic mastitis, and four had coincident mastitis and scrapie. Using western blots, immunohistochemistry and histoblots, we detected PrP^{Sc} in mammary glands of all four clinically scrapie-sick sheep with mastitis (**Fig. 1a**,**b**), but not in noninflamed mammary glands from presymptomatic or scrapie-sick sheep from the same (n = 14) or a different flock (n = 1), nor in inflamed mammary glands of scrapie-uninfected sheep (n = 2). Within the inflammatory mammary lesions, PrP^{Sc} was found to be associated with lymphoid follicles by immunofluorescent labeling and by paraffin-embedded tissue (PET) blotting (**Fig. 1c**). PrP^{Sc} colocalized predominantly with $CD68^{+}$ macrophages and FDCs within inflamed mammary glands (**Fig. 2a**).

We then surveyed a second Sarda flock (272 sheep) located 30 km away from the flock described above. One sheep was found to be

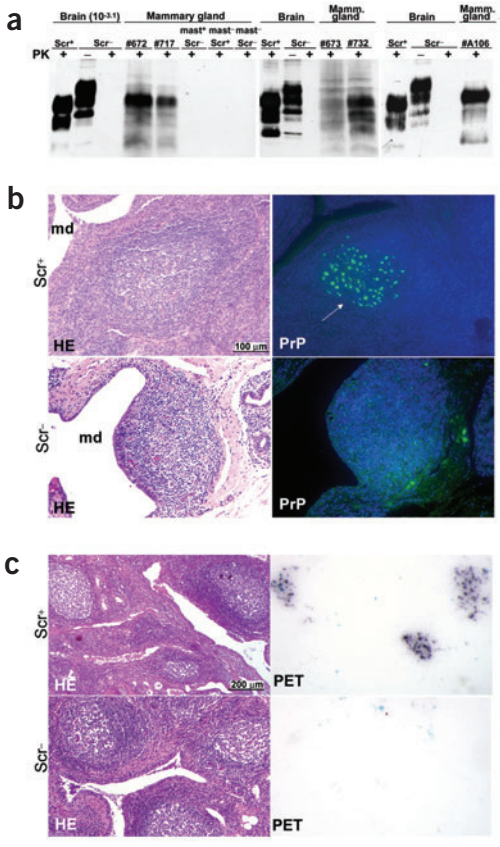

Figure 1 Prion protein in inflamed mammary glands. (**a**) Western blots with a PrP-specific antibody. Lanes 1–3, 9–11, 14–16 from left: native and proteinase K (PK)-digested brain homogenates (diluted 1/1,400) from a scrapie-infected (Scr^{+}) and a scrapie-free sheep (Scr^{-}). Lanes 6–8: mammary glands from a scrapie-free sheep with follicular mastitis (Scr^{-}, $mast^{+}$), a scrapie-positive sheep from a flock with neither MVV seropositivity nor mastitis (Scr^{+}, $mast^{-}$), and a sheep with neither mastitis nor scrapie (Scr^{-}, $mast^{-}$). Each one of five scrapie-infected sheep with mastitis had mammary PrP^{Sc} (lanes 4, 5, 12, 13, and 17). Non-scrapie-infected brain and mammary gland extracts showed no PrP^{Sc} upon PK digestion (lanes 3, 6–8, 11 and 16). (**b**) Mammary gland micrographs from MVV-seropositive sheep with mastitis and coincident scrapie (Scr^{+}), or with mastitis but no scrapie (Scr^{-}). Lymphoid follicles are adjacent to milk ducts (md). Immunofluorescence stains show abundant PrP deposits within mammary lymphoid follicles (arrow) from scrapie-positive but not from scrapie-free sheep. Scale bars, 100 μm. (**c**) PK-treated paraffin-embedded tissue blots of mammary gland sections show punctate PrP^{Sc} deposits colocalizing with lymphoid follicles in scrapie-infected (Scr^{+}), but not in scrapie-free (Scr^{-}) sheep with mastitis. Scale bars, 200 μm.

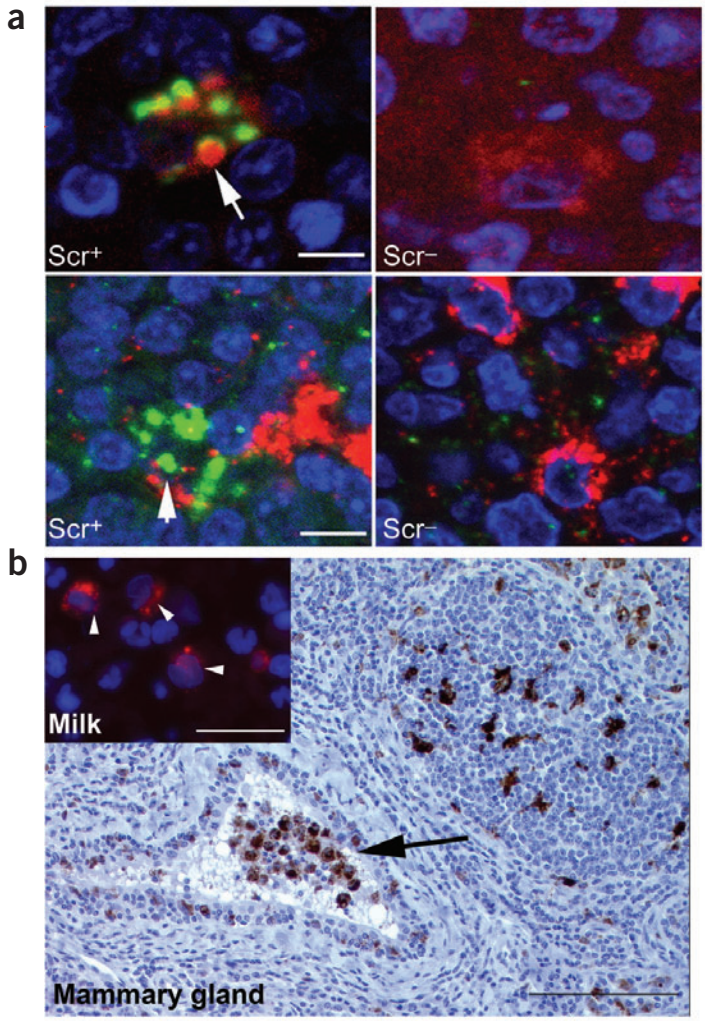

Figure 2 Mammary PrP^{Sc} localizes to macrophages and FDCs. (**a**) Mammary gland from a sheep with coincident mastitis, MVV seropositivity and scrapie (sheep #732). Confocal laser scanning micrographs of lymphoid follicles immunostained for PrP (green), nuclear DNA (blue) and macrophages (red, top panels) or FDC (red, bottom panels). PrP^{Sc} associates with $CD68^+$ macrophages and FDCs in scrapie-positive (Scr^+, arrows) but not in scrapie-free sheep (Scr^-). Scale bars, 6.3 µm (top) and 7.5 µm (bottom). (**b**) $CD68^+$ macrophages (arrow) and degenerating leukocytes within milk ducts and in adjacent lymphoid follicles of an inflamed mammary gland, as well as in milk sediment (inset, arrowheads). Scale bar, 100 µm (mammary gland) or 20 µm (milk cells).

scrapie-sick and was killed: necropsy showed lymphofollicular mastitis and PrP^{Sc} in the brain and tonsil. Again, PrP^{Sc} was present in the mammary gland (**Fig. 1a**). These results indicate that coincidence of natural chronic inflammatory conditions and natural scrapie can expand the deposition of PrP^{Sc} to unexpected tissues of sheep.

By plotting western blot signals against serially diluted scrapie-infected brain and spleen, we determined that the median mammary PrP^{Sc} concentration was 0.1% of that of spleen and 0.05% of brain. But because mammary lymphoid follicles were stochastically distributed, local PrP^{Sc} loads varied markedly. Hence these figures may underestimate PrP^{Sc} in sites of abundant follicles, and overestimate it in sites with few or no follicles.

Common causes of lymphofollicular mastitis in sheep include Maedi-Visna virus (MVV) and mycoplasma[5]. We could not culture mycoplasma from mastitic glands, whereas we found that four of the five sheep with scrapie and mastitis were seropositive for MVV and that the three scrapie-sick sheep without mastitis were seronegative for MVV. In the clinically healthy group, 7 of 10 sheep with mastitis, but only 32 of 90 sheep without mastitis, were seropositive for MVV. Hence, MVV seropositivity correlated with lymphoid follicular mastitis (Fisher exact test, $P = 0.01$) as reported previously[6,7].

MVV and related small-ruminant lentiviruses are endemic in most, if not all, European populations of small ruminants[6]. The above data suggest that common viral infections of small ruminants may enhance the spread of prions. MVV is found within mammary epithelial cells and macrophages[8], and has been experimentally passed to lambs through milk[9]. Milk is believed to represent a major route of transmission for the natural spread of MVV[5]. The PrP deposits in $CD68^+$ cells of mammary lymphoid follicles, in concert with the copious shedding of macrophages into milk of mastitic sheep (**Fig. 2b**)[9,10], raises the question whether coexistence of prion infection and inflammation in secretory organs may lead to prion contamination of secretes, and may represent a cofactor for horizontal prion spread within flocks.

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1. Mabbott, N.A., Mackay, F., Minns, F. & Bruce, M.E. *Nat. Med.* **6**, 719–720 (2000).
2. Montrasio, F. *et al. Science* **288**, 1257–1259 (2000).
3. Heikenwalder, M. *et al. Science* **307**, 1107–1110 (2005).
4. Goldmann, W. *et al. Proc. Natl Acad. Sci. USA* **87**, 2476–2480 (1990).
5. Pepin, M., Vitu, C., Russo, P., Mornex, J.F. & Peterhans, E. *Vet. Res.* **29**, 341–367 (1998).
6. Peterhans, E. *et al. Vet. Res.* **35**, 257–274 (2004).
7. de Andres, D. *et al. Vet. Microbiol.* **107**, 49–62 (2005).
8. Carrozza, M.L., Mazzei, M., Bandecchi, P., Arispici, M. & Tolari, F. *J. Virol. Methods* **107**, 121–127 (2003).
9. Preziuso, S. *et al. Vet. Microbiol.* **104**, 157–164 (2004).
10. Lerondelle, C. & Ouzrout, R. *Dev. Biol. Stand.* **72**, 223–227 (1990).