Comment on: PACE4 (PCSK6): another proprotein convertase linked to iron homeostasis?

We thank the authors of this letter[1] for their interest in our paper[2] and for their detailed analysis of the evidence for and against the proteases responsible for the cleavage of transferrin receptor-2 (TFR2) from the cell surface in hepatocytes and erythroid cells. Their hypothesis of PACE4 (PCSK6) as the most reasonable candidate protease responsible for cleaving TFR2 on plasma membrane and releasing its soluble component is intriguing. After the identification of soluble TFR2 (sTFR2) in the media of transfected and primary cells[2] we made several attempts to identify the protease involved in TFR2 shedding. As reported in the paper,[2] and also discussed by the Guillemot *et al.*,[1] we were able to exclude some potential candidates that are already known to have a role in iron metabolism, namely furin and PCSK7, but we did not reach definitive conclusions. We, therefore, generated an artificial form of sTFR2 corresponding to the whole ectodomain of TFR2 from position 105 whose size, based on gel migration, overlaps the natural form (see Figure 1A in the original paper[2]).

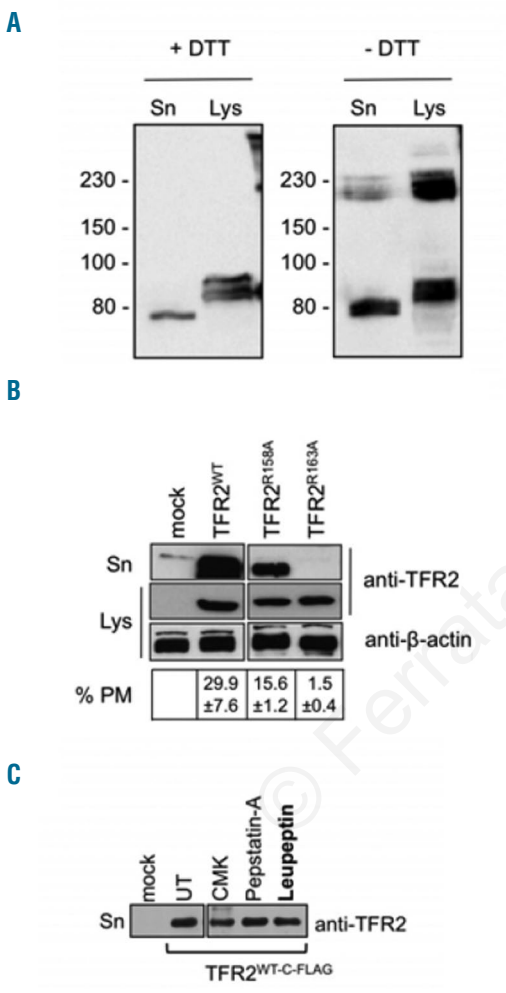

Figure 1. (A). Supernatants and cellular lysates from UT7 cells were analyzed by western blot using anti-TfR2 antibody. DTT was added (+DTT) or not to the samples (-DTT). (B). HeLa cells were transiently transfected with the empty vector (mock), wild-type TFR2, and R158A and R163A TFR2 variants. The supernatants and the cellular lysates were analyzed by western blot using anti-TFR2 antibody. Anti-actin was used for normalization. % PM is the ratio between non-permeabilized to permeabilized cells in the quantitative binding assay.[4] (C). The supernatants of HeLa cells transiently transfected with the empty vector and TFR2 construct were analyzed by western blot using anti-TFR2 antibody in the untreated condition and after 24 h of treatment with the protease inhibitors CMK (50 μM), pepstatin-A (100 μM) and leupeptin (100 μM). Sn: supernatant; Lys: total cellular lysate.

Guillemot *et al.* propose the interesting hypothesis that the proprotein convertase PACE4 might be the protease involved based on its expression and inhibition by CMK: this hypothesis deserves experimental validation. Still, the size of a product cleaved at position R163, the proposed cleavage site of PACE4, is difficult to reconcile with the size of the released form (see Figure 1A of the original paper[2]) since the product of the cleavage would be 60 amino acids shorter. In addition we observed that in non-denaturing conditions sTFR2 shows a high molecular weight band compatible with dimer formation (Figure 1A). This suggests that cysteines at positions 108 and 111, the only potentially involved in a disulfide bond in the stem region, are most likely preserved in sTFR2. This would exclude a cleavage C terminal to such a position, including the presumptive **R**QTSL**R** consensus sequence of PACE4 at positions 158-163. In addition a mutation of Arginine 158 to Alanine (R158A) that is partially defective in plasma membrane localization did not suppress TFR2 release. Unfortunately, no conclusion can be drawn on R163A, because the corresponding protein does not reach the cell surface (Figure 1B). Finally sTFR2 release was not inhibited by leupeptin (Figure 1C), which was reported to inhibit PACE4.[3] For all these reasons we did not consider PACE4 as a possible candidate. Obviously all these speculations do not exclude the possibility of indirect cleavage of TFR2 by other proteases that are targets of PACE4 (PCSK6) activation. In brief, until verified experimentally this protease remains an interesting candidate.

Alessia Pagani,[1,2] Meriem Ladli,[3,4,5] Antonella Nai,[1,2] Frederique Verdier,[3,4,5] Clara Camaschella,[1,2] and Laura Silvestri[1,2]

[1]Vita Salute University, Milan, Italy; [2]San Raffaele Scientific Institute, Milan, Italy; [3]Institut Cochin, INSERM, U1016, Université Paris Descartes, CNRS (UMR8104), Paris, France; [4]Ligue National contre le Cancer, Paris, France; and [5]LABEX (Laboratoire d'Excellence) GR-Ex, Paris, France

Correspondence: silvestri.laura@hsr.it

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References

1. Guillemot J, Seidah NG. PACE4 (PCSK6): another proprotein convertase link to iron homeostasis? Haematologica 2015;xxx
2. Pagani A, Vieillevoye M, Nai A, et al. Regulation of cell surface transferrin receptor-2 by iron-dependent cleavage and release of a soluble form. Haematologica. 2015;100(4):458-465.
3. Mains RE, Berard CA, Denault JB, Zhou A, Johnson RC, Leduc R. PACE4: a subtilisin-like endoprotease with unique properties. Biochem J. 1997;321(3):587-593.
4. Pagani A, Silvestri L, Nai A, Camaschella C. Hemojuvelin N-terminal mutants reach the plasma membrane but do not activate the hepcidin response. Haematologica. 2008;93(10):1466-1472.