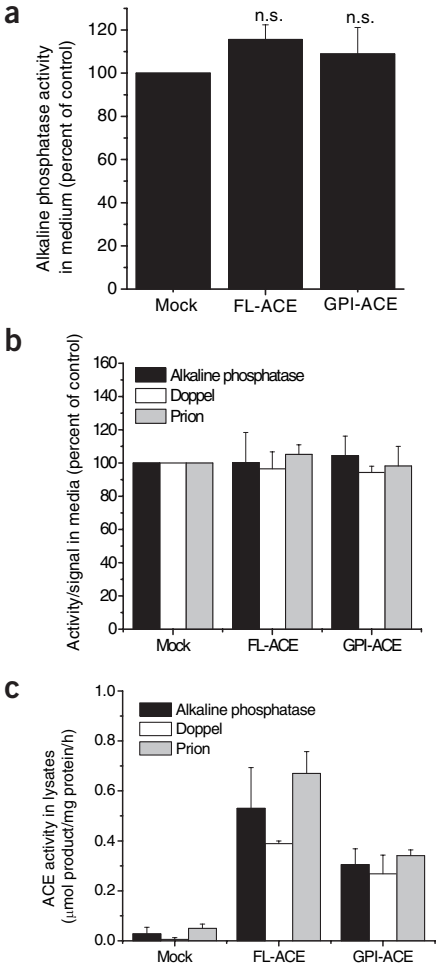

Figure 2 ACE overexpression in CHO and HEK cells does not affect the shedding of multiple GPI-anchored proteins. (**a**) CHO cells were stably transfected with vector alone (mock), full-length wild-type (FL-ACE) or GPI-ACE[10]. Endogenous alkaline phosphatase activity shed into the media was determined using p-nitrophenylphosphate as substrate. Results are means ± s.d. ($n = 3$) and are expressed as a percentage of activity shed into media of mock-transfected cells. (**b**) HEK cells stably transfected with either doppel or prion protein were transiently transfected with vector alone (mock), FL-ACE or GPI-ACE. Endogenous alkaline phosphatase activity shed into the media was determined using p-nitrophenylphosphate as substrate. Doppel and prion protein shed into media were determined by immunoblotting followed by densitometric analysis. Results are means ± s.d. ($n = 3$ or 6 (alkaline phosphatase)) and are expressed as a percentage of protein shed into the media of mock-transfected cells. (**c**) ACE activity determined using BzGly-His-Leu as substrate in lysates from the HEK cells transiently transfected with vector alone (mock), FL-ACE or GPI-ACE as in **b**. Results are means ± s.d. ($n = 3$).

The shedding of alkaline phosphatase from CHO cells transfected with either wild-type ACE or the GPI-anchored ACE was not significantly different to the shedding from mock transfected cells (**Fig. 2a**), despite the fact that the peptidase activity of ACE was increased 30–80-fold over endogenous levels in both transfected cell lines[10]. Kondoh *et al.*[6] reported that exogenously added ACE could promote the shedding of the GPI-anchored prion protein from HEK cells. We therefore examined the shedding of prion protein and its homolog doppel from HEK cells by wild-type and GPI-anchored ACE (**Fig. 2b,c**). Again, neither wild-type nor GPI-anchored ACE caused a detectable increase in the shedding of prion protein or doppel, or of the endogenous alkaline phosphatase, above that seen from mock-transfected cells.

In conclusion, using four different cellular contexts, monitoring four different GPI-anchored proteins, and using either exogenously added purified ACE from two different sources or coexpressed ACE with either a transmembrane or GPI anchor, we have found no evidence that ACE possesses considerable GPIase activity. Although it is possible that excessively large amounts of exogenously added ACE protein may result in the nonspecific release of certain membrane proteins, we were unable to find evidence for a specific release of GPI-anchored proteins in any of our systems. In addition, the overexpression of full-length ACE or a GPI-anchored form of the protein at levels in large excess of physiological situations did not enhance the shedding of GPI-anchored proteins despite the fact that the latter construct is even targeted to the membrane microdomains in which GPI-anchored proteins themselves are enriched[10]. Although there remains the possibility that ACE may be involved in the trafficking of GPI-anchored proteins and/or factors involved in their shedding, our results cast doubt upon the suggestion that ACE itself is directly responsible for the *in vivo* shedding of GPI-anchored proteins.

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Male fertility is dependent on dipeptidase activity of testis ACE

To the editor:

Testis angiotensin-converting enzyme (ACE) is an isozyme exclusively expressed by developing sperm[1,2]. This protein has only a single catalytic domain containing the HEXXH consensus-site motif typical of zinc metallopeptidases[3]. The exact role of testis ACE is unknown, but male mice lacking the protein reproduce poorly, despite normal numbers of sperm and normal motility[4–6].

Kondoh *et al.* recently analyzed glycosylphosphatidylinositol (GPI)-releasing activity in the testis and suggested that the source of this activity is ACE[7]. They further characterized the role of the ACE GPI-releasing activity in sperm-egg binding assays and concluded that this activity is crucial for mammalian fertilization. Finally, they provided evidence suggesting that the dicarboxypeptidase activity of testis ACE is dispensable for sperm-egg binding.

Using targeted homologous recombination, we prepared mice with selective genetic inactivation of the dicarboxypeptidase activity of testis ACE. Animal use was approved by the Emory University Institutional Animal Care

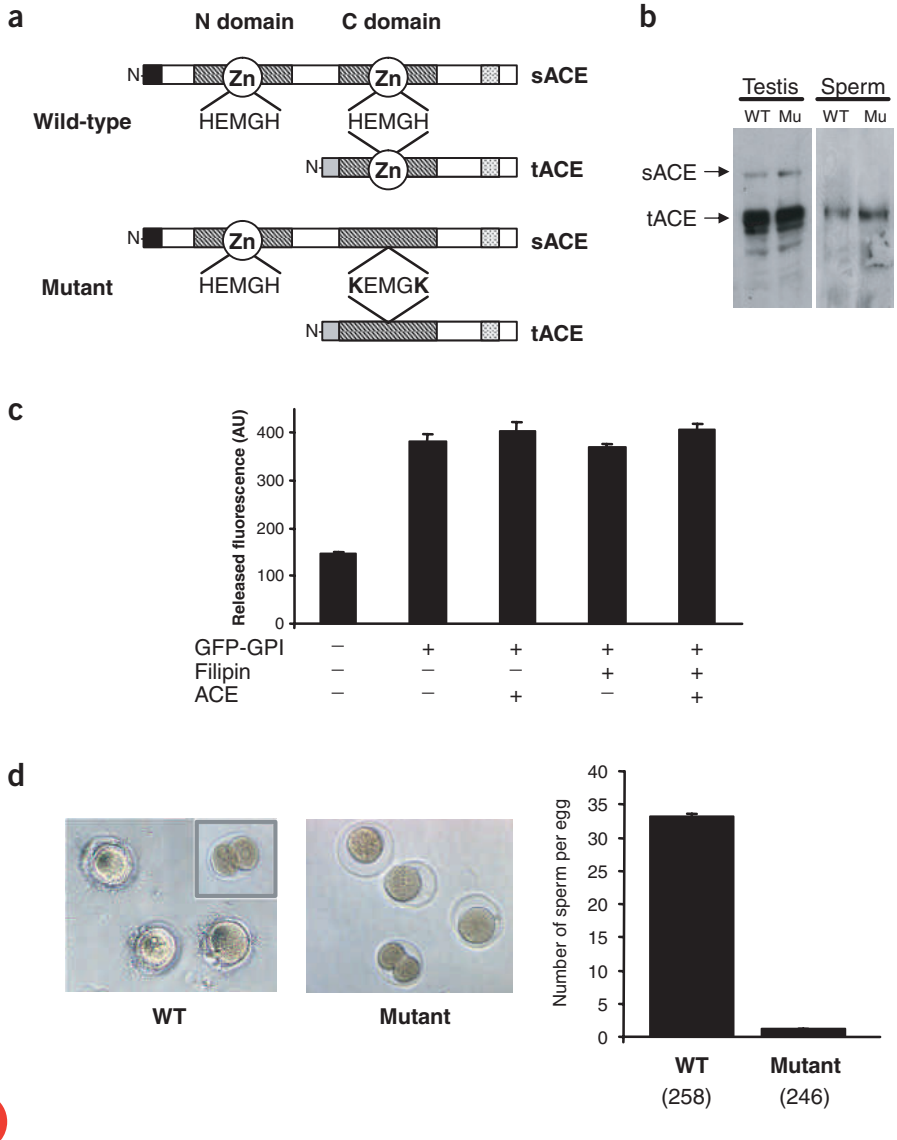

Figure 1 Creation and analysis of mutant mice. (**a**) The somatic ACE isozyme (sACE) is composed of two homologous catalytic domains (hatched boxes); each bearing the zinc-binding motif HEMGH. The testis ACE isozyme (tACE), roughly half as large, is composed of a unique amino terminus (gray box) and the C-terminal domain of sACE. To specifically inactivate tACE carboxypeptidase activity, point mutations were introduced by homologous recombination into genomic DNA to convert the HEMGH motif to KEMGK[8]. The targeting strategy also left a single *lox*P site in noncoding, intronic sequence. (**b**) We analyzed testis and sperm extracts from wild-type (WT) and homozygous mutant (Mu) mice by western blot using a rabbit antibody specific for ACE. Expression levels of the mutant protein were indistinguishable from wild-type. (**c**) HEK 293 cells were cotransfected with vectors expressing somatic ACE and a GPI-linked form of EGFP. The shedding of EGFP from the cell membrane was assessed by measuring the fluorescence released into the media. The presence of ACE did not affect the release of EGPF. Also, filipin (5 μg/ml) did not affect EGFP release (performed in triplicate, bars represent s.d.). (**d**) We incubated capacitated sperm from wild-type or homozygous mutant mice with wild-type eggs, washed and counted them. The binding of homozygous mutant sperm to eggs was nearly abolished as compared to wild-type (WT, 33.2 ± 1.2 sperm/egg versus homozygous mutant, 1.2 ± 0.1 sperm/egg). The number in parentheses represents the total number of eggs counted for each genotype over three experiments (bars represent s.d.). Two-cell embryos were included in all assays as a control for nonspecific binding (insert).

and Use Committee. We achieved this preparation by introducing genomic point mutations converting the active site of testis ACE from HEMGH (amino acids 413–417) to KEMGK (**Fig. 1a**)[8]. These genetic changes had no effect on the expression levels of testis ACE, as measured by western blot (**Fig. 1b**) and immunohistochemistry (data not shown), but reduced the ACE catalytic activity within testis to 3% of that found in wild-type testis. This small residual activity is the result of the presence in testis of some somatic ACE with retained N-terminal catalytic activity[8].

To determine whether sperm isolated from wild-type or homozygous mutant mice contain a GPIase activity, we analyzed the partitioning of SPAM1 (PH-20), a GPI-anchored protein present on the sperm membrane, into water-soluble or detergent-soluble fractions[9]. The water-soluble fraction contains cytoplasmic proteins and soluble components of the acrosome, whereas the detergent-soluble fraction contains membrane-anchored proteins. If the sperm contained an active GPIase activity, then cleaved SPAM1 would partition into the water-soluble fraction. The results show no difference in SPAM1 partitioning between sperm from wild-type and homozygous mutant mice (data not shown). We verified proper fractionation with Erk1/2-specific and fertilin β–specific antibodies. These data suggest that the inactivation of dipeptidase activity in homozygous mutant mice had no effect on whatever GPIase activity is present in sperm.

To directly assess whether ACE can cleave GPI-anchored proteins, we transiently coexpressed a somatic ACE expression vector with an enhanced green fluorescent protein (EGFP)-GPI–anchored expression construct in HEK293 cells[10]. We monitored release of the GPI-linked EGFP by epifluorescence (**Fig. 1c**). The fluorescent signal in the ACE-cotransfected cells was equivalent to the fluorescent signal seen in cells transfected with only the EGFP-GPI construct. The addition of filipin, to disrupt the lipid rafts, did not promote the release of EGFP[11]. We obtained similar results when we transfected human somatic ACE in CHO cells and when we transfected chicken somatic ACE in either HEK-293 or AtT-20 cells (data not shown). These data do not support GPIase activity in somatic ACE.

To investigate the role of the inactivation of testis ACE dicarboxypeptidase on fertility, we mated wild-type or homozygous mutant male mice with 8–12-week-old wild-type CD1 females and checked for copulatory plugs each morning. Homozygous mutant male mice plugged CD1 females at the same rate and number as wild-type males. At 13 d after copulation, the females were killed and the number of pups *in utero* was assessed (**Table 1**). Wild-type males generated 153 embryos from 19 plugged females (8 ± 1 pups/plugged female). In contrast, homozygous mutant males produced only one embryo from 22 plugged females. These data are highly significant ($P < 0.0001$, Student *t*-test) and strongly support

Table 1 Testis ACE dipeptidase activity is required for fertility

	Total number of plugs	Total number of litters	Total number of embryos
Wild-type ($n = 6$)	19	15	153
Homozygous mutant ($n = 7$)	22	1	1

We caged wild-type or homozygous mutant males individually with a CD1 female and then assessed presence of copulatory plugs. Females were killed during the last week of gestation and the embryos counted. The average litter size for wild-type males was 10.2 ± 0.6 embryos. Homozygous mutant mice produced only a single embryo.

the hypothesis that ACE dicarboxypeptidase activity is required for male fertility.

We performed an *in vitro* sperm-egg binding assay to assess the functionality of the sperm. We collected cauda epididymal sperm from homozygous mutant or wild-type male mice and capacitated the sperm for 45 min. We observed no difference in the sperm count, morphology or motility after capacitation. We incubated capacitated sperm with wild-type unfertilized eggs and, as a control, two-cell embryos for 45 min, after which we washed eggs and embryos until one to two sperm remained bound to the two-cell embryos. We counted the remaining sperm bound to the zona pellucida of unfertilized eggs. As expected, sperm from wild-type males readily bound to the zona pellucida (33.2 ± 1.2 sperm/egg). However, sperm from homozygous mutant males showed background levels of binding (1.2 ± 0.1 sperm/egg; **Fig. 1d**).

Our data are consistent with the results of ACE-null mice, and extend the analysis by showing that it is dicarboxypeptidase activity, and not the mere presence of testis ACE protein, that is crucial for male fertility. These data are contrary to the results recently published by Kondoh *et al.* There are now several lines of evidence suggesting that ACE does not directly cleave GPI-anchored proteins. We present *in vitro* evidence showing that c-expressing GPI-anchored EGFP and somatic ACE in cells do not release EGFP from its GPI anchor. Additional evidence from Leisle *et al.* (this issue) shows that, in four different cellular contexts, ACE is unable to cleave GPI-anchored proteins. The mutations made in homozygous mutant mice do not seem to affect sperm GPIase activity, as measured by SPAM1-release data. Yet, homozygous mutant male mice are nearly infertile. These data indicate a crucial role for ACE dicarboxypeptidase activity, not GPIase activity, in reproduction.

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Kondoh replies:

Leisle *et al.* and Fuchs *et al.* argue that, contrary to our published findings[1], angiotensin-converting enzyme (ACE) does not directly release GPI-anchored proteins *in vitro* and *in vivo*. Several reasons could account for the differences between the studies.

The two groups coexpressed ACE and several GPI-anchored proteins in cell lines, measured released product in the media and compared the results with those of mock controls. But as can be seen from the data of Fuchs *et al.*, spontaneous release of EGFP-GPI was extremely high, a phenomenon that we have also seen before[2]. As HEK293 cells do not seem to express endogenous ACE, this spontaneous release might be caused by other factors, such as endogenous GPI-PLD[3], GPI-PLC–like activity[4] or secretion of unanchored products. So, the background release of GPI-anchored proteins should not be compared with the results after adding ACE; instead, it should be subtracted from them.

As ACE is not the only factor with GPIase activity, the intracellular localization of these other factors might be relevant to the differences between our studies. For example, GPI-PLD acts at the level of intracellular organelles[5]. As ACE acts at the cell surface, substrates may not reach it in sufficient amounts to yield a substantial signal.

In our experiments, we managed to detect the initial amount of substrate and the magnitude of the background. By using PI-PLC as a control, the rate of enzymatic activity can be simply indicated as percent shedding. As the reaction takes place at the cell surface, we could ignore intracellular events. Last, we could evaluate the effect soon after performing the enzymatic reaction, excluding the possibility of subsequent modifications that could affect the size of the signal.

Leisle *et al.* showed that GPI-anchored alkaline phosphatase was not released from the cell surface by purified porcine kidney and insect ACEs even after filipin treatment. It is possible that cofactors required for efficient GPIase activity were lost during their purification of the ACEs, but remained in the rabbit lung ACE sample that we used. We previously assessed the sensitivity of the shedding assay by applying various doses of rabbit lung ACE and found that it was 20-fold weaker than that of the *in vitro* PLAP conversion assay.

By using rabbit lung ACE, Leisle *et al.* found limited but significant release of alkaline phosphatase from filipin-treated porcine brush-border membrane, but not from MDCK cells. In contrast, we showed shedding of six GPI-anchored proteins in three different cell types using the same enzyme. So, this difference might be the result of differences in the cells used. We have also found that serum inhibits the shedding activity of ACE. Complete inhibition of EGFP-GPI shedding from F9 cells occurs in the presence of very small amounts of serum (unpublished data).

Third, we reported that GPI-anchored proteins were not released from ACE-null sperm and that the egg-binding defect of mutant sperm was rescued by peptidase-inactivated ACE or PI-PLC treatments, and by wild-type ACE. We concluded that the GPIase activity of ACE has a crucial role in fertilization. Fuchs *et al.* created a mutant mouse that car-