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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

RESEARCH LETTER

Subtle Protective Roles of Clusterin in Gastric Metaplasia After Acute Oxyntic Atrophy

Gastric cancer is preceded by mucosal remodeling like spasmolytic polypeptide-expressing metaplasia (SPEM) and intestinal metaplasia. The cytoprotective protein clusterin (CLU) is highly expressed in oxyntic mucosa during the emergence of metaplasia, but it is unknown whether CLU promotes or counteracts the process.[1–4] Here, we use clusterin knockout (CLU-KO) mice to examine the role of CLU in the emergence, recovery, and repair of gastric metaplasia after tamoxifen-induced acute oxyntic atrophy.[5] Detailed methodology is described in Supplementary Materials and Methods.

The stomachs of CLU-KO mice had normal gastric gland morphology and normal number of mature cell types, although with a tendency toward pit cell/foveolar and enterochromaffin-like (ECL) cell hyperplasia (Figure 1*A* and *B*). Interestingly, this could be owing to the hypergastrinemia in CLU-KO mice (Figure 1*C*).[6] We performed RNA sequencing to examine whether the absence of CLU affected the gene expression signature of the oxyntic mucosa, showing 62 differentially expressed genes (DEGs) compared with wild-type mice (Supplementary Table 1). Among them were the ECL cell marker histidine decarboxylase and the putative intestinal metaplasia marker deleted in malignant brain tumors 1. However, although several of the genes were involved in CLU-relevant processes,[1] we could not define a gene expression profile characterizing untreated CLU-KO mice compared with wild-type mice. Overall, our results indicate that CLU partly affects gastric epithelial homeostasis because the absence of CLU leads to hypergastrinemia and slightly increased numbers of pit and ECL cells.

In tamoxifen-treated wild-type mice, CLU was increased and highly expressed in proliferative and metaplastic glands with a temporal profile that correlated with emergence and repair (Figure 1*D*), suggesting that CLU participates in the metaplastic process. Unexpectedly, although they lacked CLU, tamoxifen induced metaplasia with a similar degree of parietal cell atrophy (CLU-KO, 83%; vs wild-type, 87%) (Figure 1*E* and *F*), number of apoptotic cells (Figure 1*F*), trans-differentiating chief cells, and proliferating SPEM-cells (Figure 1*G* and *H*) as in wild-type mice. Also, CLU-KO mice were not more susceptible to a lower dose of tamoxifen (Supplementary Figure 1). Hence, CLU is not a necessary promoting factor in the emergence of gastric metaplasia.

Gene expression analysis was performed to further address the role of clusterin on a molecular level (Supplementary Tables 1–11). There was an increased and prominent transcriptional difference (1026 vs 62 DEGs in untreated mice) between the emerging metaplasia (day 3) of CLU-KO and wild-type mice (Supplementary Tables 2 and 3), indicating distinct underlying molecular events. Apparently, the metaplastic mucosa of CLU-KO mice showed more mitochondrial dysfunction, which fits with the known functions of CLU as both a sensor and protector against oxidative stress.[7] Intriguingly, oxidative stress resulting from chronic inflammation might play a central role in gastric tumorigenesis,[8] suggesting that CLU deficiency will be a disadvantage. Indeed, the metaplastic mucosa of CLU-KO mice expressed a higher number of genes associated with gastric metaplasia and (cancer) stem cells,[3,8,9] and lower levels of mucosal protective factors and tumor suppressors (Figure 2*A* and Supplementary Tables 4–6).[10] Thus, although it is not clearly evident histologically, transcriptional profiling indicates that the emergence of metaplasia is more advanced in CLU-KO than wild-type mice.

During early recovery (day 7 + 10), the metaplastic mucosa of both CLU-KO and wild-type mice were still substantially changed from normal. However, when directly comparing CLU-KO with wild-type mice at this stage, there were only subtle histologic and transcriptional differences (Figure 2*A*, Supplementary Figure 2, and Supplementary Tables 6–8), possibly suggesting more similar mechanisms operating during early recovery.

In contrast, after repair (day 21), histologic analysis showed normal gland morphology and a restored number of mature cell lineages in tamoxifen-treated wild-type mice. However, the oxyntic mucosa of CLU-KO mice tended to be more hyperplastic, with slightly more parietal and neck cell-to-chief cell-transition cells per gland (Figure 2*B–D*). In addition, the gene expression profile of tamoxifen-treated CLU-KO mice was altered markedly from vehicle-treated controls (409 DEGs) (Supplementary Tables 9 and 10), which was not found in wild-type mice (44 DEGs). CLU-KO mice had continuously down-regulated mature cell lineage marker genes, including the chief cell marker *Mist1* and the pit cell gene *Muc5ac*, up-regulation of the tuft cell marker *Dclk1*, and still several dysregulated gastric metaplasia–associated and mucosal protective genes, such as *Mmp12*, *Sox9*, and *Gkn2* (Figure 2*A, E*, and *F*).

Thus, in the absence of CLU, restoration of normal mucosa, with re-expression of all cell lineage marker genes and disappearance of metaplastic gene signatures, seemed to be delayed, again suggesting that CLU-KO mice showed a more advanced form of metaplasia and a hampered repair compared with wild-type mice. Hence, instead of being a necessary promoting factor, CLU might protect against emergence and premalignant progression of gastric metaplasia.

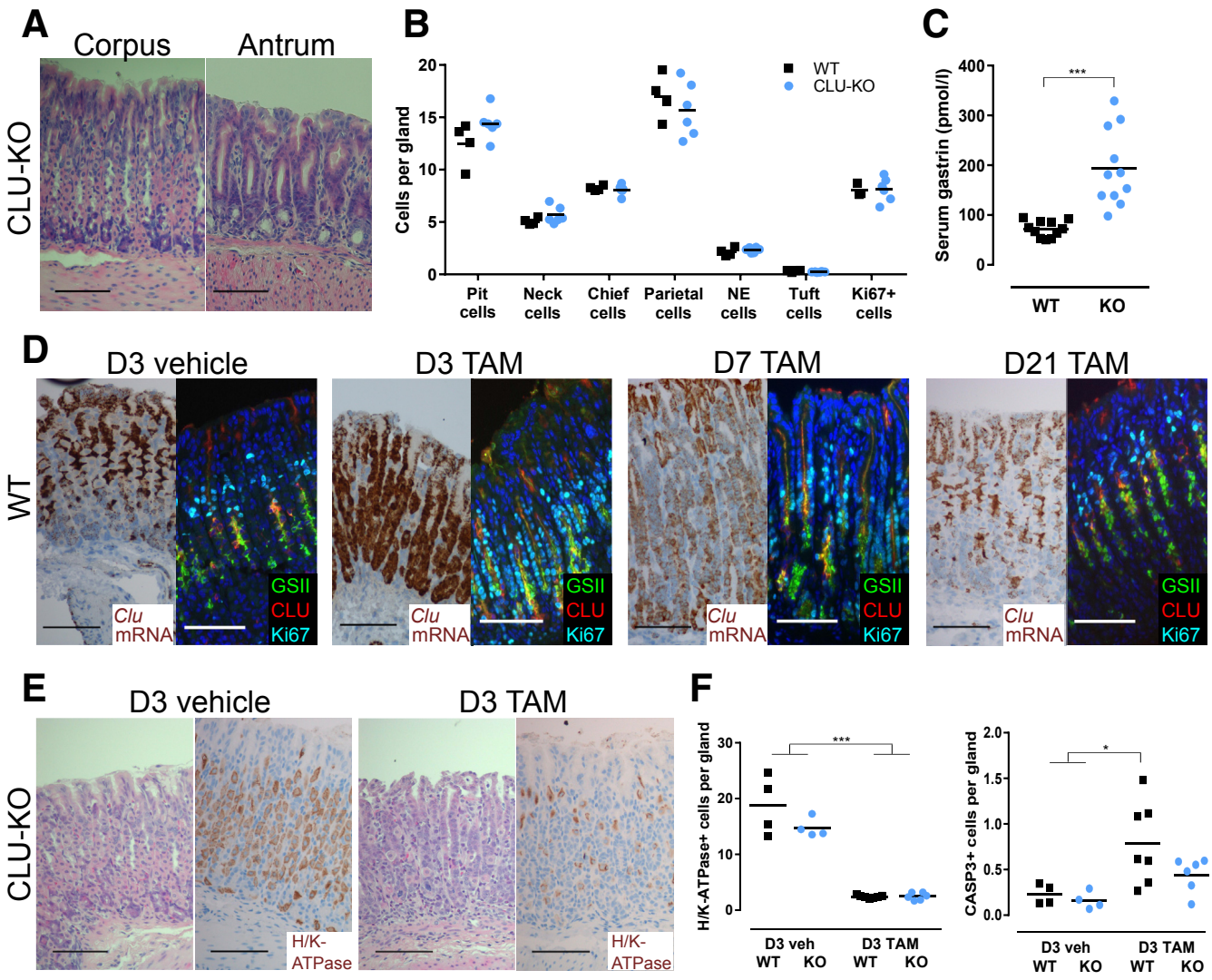

Figure 1. Clusterin is not a necessary factor for gastric epithelial homeostasis or tamoxifen-induced gastric metaplasia. (*A*) Representative H&E images showing normal gland morphology of oxyntic and antral mucosa in an untreated CLU-KO mouse. (*B*) Quantification of different mature gastric cell lineages per gland in wild-type (WT) (n = 3–4) and CLU-KO (n = 6) mice. (*C*) Serum gastrin levels (pmol/L) in untreated WT (n = 11) and CLU-KO (n = 11) mice. (*D*) *In situ* hybridization showing expression of clusterin messenger RNA (mRNA) (*Clu*; brown) and triple immunofluorescence staining showing CLU (red) expression in proliferating (Ki67; light blue) metaplastic cells (lectin *griffonia simplicifolia II* [GSII]; green) of oxyntic mucosa from vehicle- and tamoxifen-treated WT mice on days 3, 7, and 21. (*E*) Representative H&E images of oxyntic mucosa and immunohistochemistry staining showing expression of H^+/K^+-adenosine triphosphatase [ATPase] β-subunit (parietal cells; brown) in oxyntic mucosa from vehicle- and tamoxifen-treated CLU-KO mice on day 3. (*F*) Quantification of parietal cells (H+/K+-ATPase β-subunit) and apoptotic cells (cleaved caspase 3–positive [CASP3]) per gland in oxyntic mucosa from vehicle- (n = 4 in each strain) and tamoxifen-treated CLU-KO (n = 6) and WT (n = 7) mice on day 3. (*G*) Double immunofluorescence staining showing cells co-expressing (yellow) pepsinogen II (PEPII) (red) and GSII (green) and cells co-expressing Ki67 (red) and GSII (green) in oxyntic mucosa from vehicle- and tamoxifen-treated CLU-KO mice on day 3. (*H*) Quantification of cells co-expressing PEPII and GSII (yellow) or cells co-expressing Ki67 and GSII per gland in oxyntic mucosa from vehicle- (n = 4 in each strain) and tamoxifen-treated CLU-KO (n = 6) and WT (n = 7) mice on day 3. Nuclei were counterstained with hematoxylin (blue) or DAPI (blue). *Black squares*, WT; *blue circles*, CLU-KO. Each *dot* represents an individual animal and the *black lines* mark the means. *Scale bars*: 100 μm. Statistical significance: $^*P < .05$, $^{***}P < .0001$ by Student *t* test (*B* and *C*) or 1-way analysis of variance with the Sidak multiple comparison test (*F* and *H*). D, day; DAPI, 4′,6-diamidino-2-phenylindole; NE, neuroendocrine; TAM, tamoxifen; Veh, vehicle.

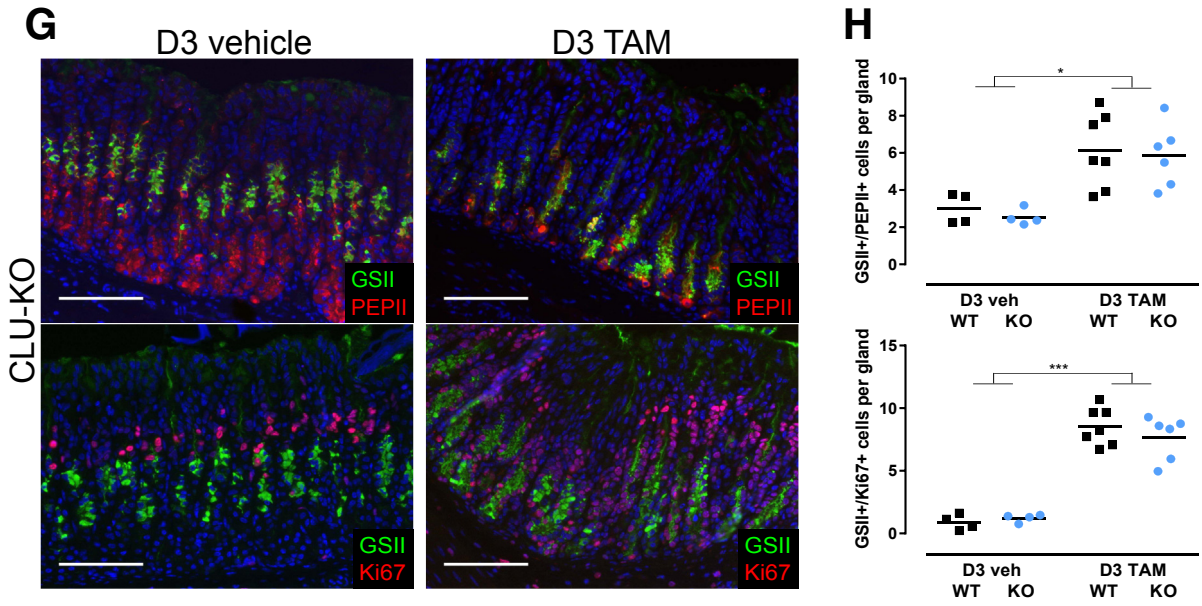

Figure 1. (*continued*).

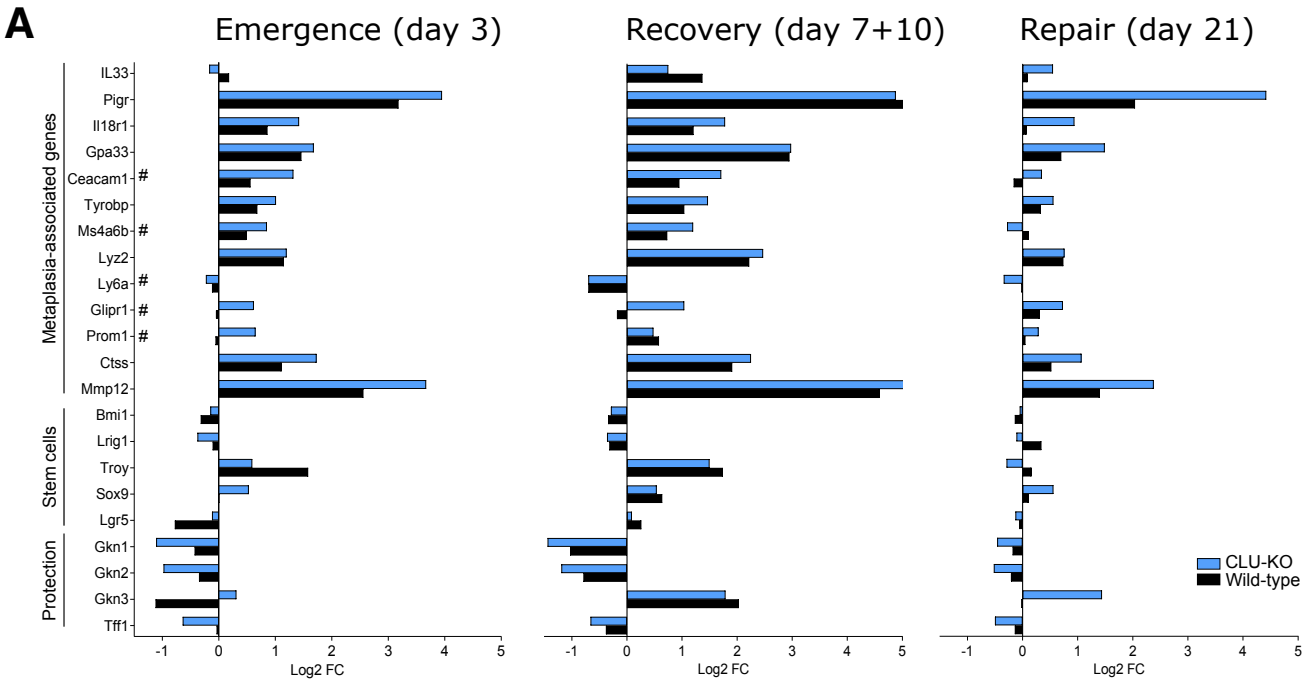

Figure 2. Tamoxifen induces transcriptionally more advanced gastric metaplasia with delayed repair in CLU-KO mice compared with wild-type (WT) mice. (*A*) Gene expression levels (log_2 fold-change [FC]) of metaplasia-associated genes, (cancer) stem cell-associated, and mucosal protective factors expressed at different levels (statistically significant for at least 1 time point) in tamoxifen-treated CLU-KO (n = 4–6) (blue) and WT (n = 3–7) (black) mice compared with vehicle-treated controls (n = 9 in each strain) at emergence (day 3), recovery (day 7 + 10), and repair (day 21) (Supplementary Table 6). #Metaplasia-associated genes that were dysregulated only in CLU-KO mice on day 3. (*B*) Representative H&E images of oxyntic mucosa from tamoxifen-treated WT and CLU-KO mice on day 21. (*C*) Quantification of parietal cells H^+/K^+ -ATPase β subunit and cells co-expressing Ki67 and lectin *griffonia simplicifolia II* [GSII] per gland in oxyntic mucosa from tamoxifen-treated WT (n = 5) and CLU-KO (n = 4) mice on day 21. (*D*) Quantification of cells expressing pepsinogen II (PEPII; red), GSII (green), or co-expressing PEPII and GSII (yellow) per gland in oxyntic mucosa from tamoxifen-treated WT (n = 5) and CLU-KO (n = 4) mice on day 21. Data are presented as means - SD. (*E*) Gene expression levels (log_2 FC) of mature gastric cell lineage marker genes in tamoxifen-treated CLU-KO (n = 4) (blue) and WT (n = 5) (black) mice compared with vehicle-treated controls (n = 9 in each strain) at repair (day 21) (Supplementary Tables 9 and 10). (*F*) Immunohistochemistry staining showing expression of DCLK1 (brown) and quantification of cells expressing DCLK1 per gland in oxyntic mucosa from vehicle- and tamoxifen-treated WT and CLU-KO mice (n = 4 in each group) on day 21. Nuclei were counterstained with hematoxylin (blue). *Black squares*, WT; *blue circles*, CLU-KO. Each *dot* represents an individual animal and the *black lines* mark the means. Statistical significance: $^*P < .05$, $^{***}P < .0001$ by Student *t* test (*C* and *D*) or 1-way analysis of variance with the Sidak multiple comparison test (*F*). *Scale bars*: 100 μm (*B*) and 50 μm (*F*). D, day; TAM, tamoxifen; Veh, vehicle.

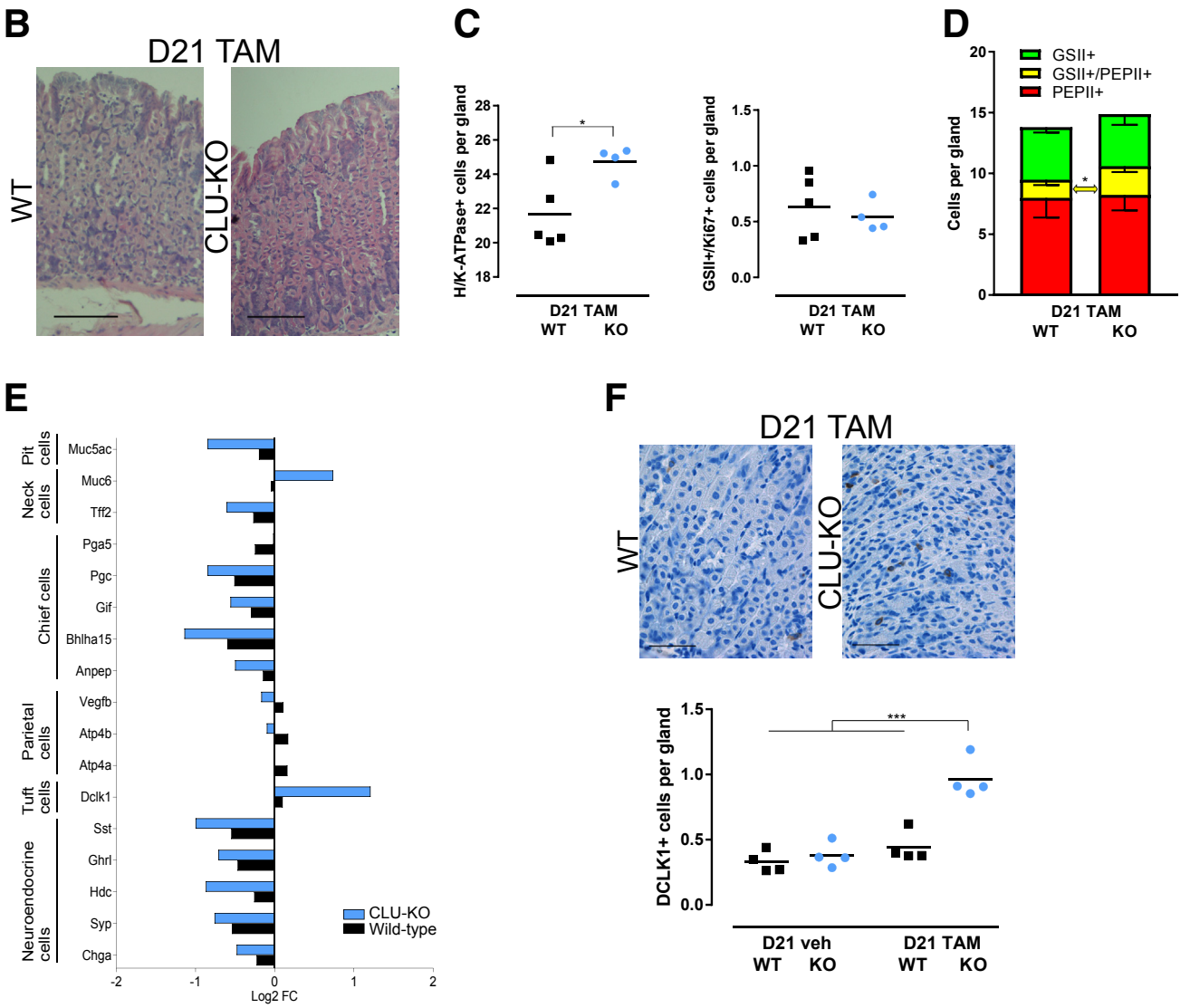

Figure 2. (*continued*).

PÅL VANGE,[1] *TORUNN BRULAND,*[1,2] *BJØRN MUNKVOLD,*[1] *ELIN SYNNØVE RØYSET,*[1,3] *MARTIN GLEAVE,*[4] *INGUNN BAKKE,*[1,2]

[1]Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, NTNU–Norwegian University of Science and Technology, Trondheim, Norway

[2]Clinic of Medicine, St. Olav's University Hospital, Trondheim, Norway

[3]Department of Pathology, St. Olav's University Hospital, Trondheim, Norway

[4]Vancouver Prostate Centre, Vancouver, Canada

Correspondence author e-mail: ingunn.bakke@ntnu.no.

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Abbreviations used in this letter: **CLU, clusterin; CLU-KO, clusterin knockout; DEG, differentially expressed gene; ECL, enterochromaffin-like cell; SPEM, spasmolytic polypeptide-expressing metaplasia.**

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Author contributions
Pål Vange, Torunn Bruland, and Ingunn Bakke contributed to the conception and design of the study; Martin Gleave provided transgenic mice; Pål Vange, Ingunn Bakke, and Bjørn Munkvold performed the experiments; Pål Vange, Torunn Bruland, Bjørn Munkvold, Elin Synnøve Røyset, and Ingunn Bakke contributed to the assembly, analysis, and interpretation of data; Pål Vange, Torunn Bruland, and Ingunn Bakke wrote the manuscript; and Pål Vange, Torunn Bruland, Bjørn Munkvold, Elin Synnøve Røyset, Martin Gleave, and Ingunn Bakke were involved in the revision and final approval of the submitted and published version of the manuscript.

Conflicts of interest
The authors disclose no conflicts.

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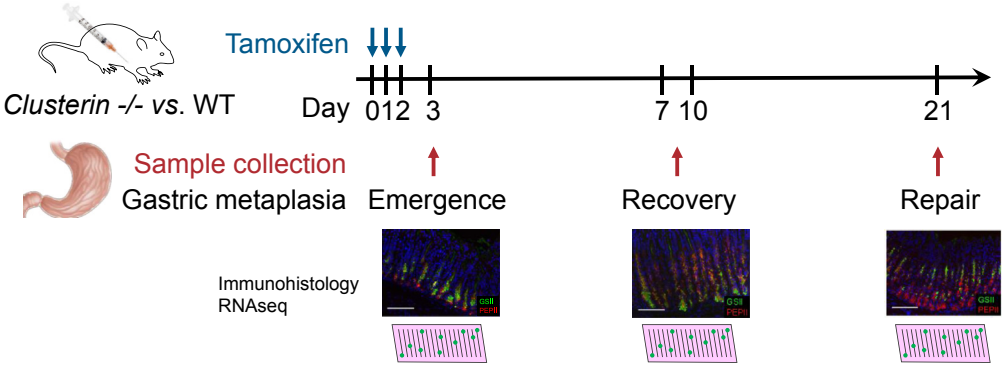

Lack of **clusterin** in homeostasis caused subtle changes with hypergastrinemia, development of slightly more advanced acute metaplasia and hampered repair

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Supplemental Graphical Summary.