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Sex- and tissue-specific expression of "similar to nothepsin" and cathepsin D in relation to egg yolk formation in *Gallus gallus*

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ABSTRACT Egg yolk constitutes the main storage compartment of the avian egg and the first nutritional source that supports embryonic growth. Most egg yolk components are synthesized by the liver of laying hens at sexual maturity and are secreted into the blood to be further transferred into the ovarian oocyte (yolky follicle) by receptor-mediated endocytosis. Egg yolk proteins are secreted as precursors and must undergo proteolytic processing to be bioactive. It is assumed that chicken cathepsin D, an aspartic protease, is a key enzyme in this process. Very recently, a novel aspartic protease, namely "similar to nothepsin," has been identified in the egg yolk. Previous experiments conducted in Antarctic fish have shown that the expression of nothepsin is tissue- and sex-specific. To gain insight into the specificities of expression of both cathepsin D and "similar to nothepsin" in *Gallus gallus*, we compared their distribution in various tissues, in male and females. Cathepsin D is ubiquitously expressed in all tissues examined, including liver of both male and female adults, and its expression is stable during sexual maturation. In contrast, "similar to nothepsin" expression is unique to the liver of adult females and is sex steroid-dependent as it increases gradually in the liver of hens during sexual maturation. The sexual dimorphic expression of the "similar to nothepsin" gene suggests that the activity of this protein is regulated by the steroid environment of laying hens and is specifically adapted for inclusion in the yolk. Further studies are needed to assess whether "similar to nothepsin" assists cathepsin D in the proteolytic processing of egg yolk proteins during follicular growth.

Key words: liver, chicken, aspartic proteinase, egg yolk

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INTRODUCTION

The extra-uterine development of a chicken embryo requires that the egg supplies all the nutrients, protective systems, and bioactive molecules that are required to ensure the development of the embryo. The yolk constitutes the main reservoir of lipids, proteins, minerals, and vitamins; the egg white is a source of water and nutrients but also antimicrobial components for the embryo, whereas the eggshell is essentially a protective barrier that prevents microbial penetration. Most yolk proteins, including vitellogenins and triglyceride-rich lipoproteins (very low-density lipoproteins), are synthesized as precursors in the liver in response to the estrogen stimulation, which occurs at sexual maturity in hens, and are then secreted into the blood (Griffin et al., 1984). These precursors are imported into the yolky follicles (oocyte) by receptor-mediated endocytosis which results in quasi-lysosomal compartments that are termed light yolk platelets (Nimpf and Schneider, 1998). Most egg yolk proteins are further processed by cathepsin D, an aspartic protease, in the endocytic compartments of the yolky follicle (Retzek et al., 1992; Elkin et al., 1995; Carnevali et al., 1999; Gerhartz et al., 1999; Carnevali et al., 2006).

Aspartic proteases participate in multiple physiological roles in animals (Benes et al., 2008). These endopeptidases are related to lysosomal digestive enzymes and are commonly active in the acidic pH range (Benes et al., 2008). In chickens, besides its involvement in egg yolk hydrolysis, cathepsin D activity has been associated with muscular dystrophy (Iodice, 1976) and coccidiosis (Rostislav et al., 2001). In mammals, cathepsin D is first synthesized as an inactive precursor before its conversion to active cathepsin D by autolysis at acidic pH, to remove the propeptide, and it is further processed by cathepsin B or L to produce a 2-chain active molecule (Zaidi et al., 2008). Like its mammalian counterpart, chicken cathepsin D is likely to be secreted as an inactive form, which is further processed into an active intermediate by autocatalytic cleavage of its propeptide (Retzek et al., 1992). The proteases that are responsible for the processing of chicken cathepsin D into the ultimate 2-chain form are not known. In Seabream, the highest activities of cathepsin D were found in early

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vitellogenesis (Carnevali et al., 1999). Similarly, in lizard, a large amount of the proenzyme is accumulated in egg yolk at an early stage of oocyte development and appears to be activated at a specific stage of follicular growth to achieve the proteolytic processing of vitellogenins (De Stasio et al., 1999). Although the presence of cathepsin D in growing follicles and in liver of laying hens has been clearly stated (Retzek et al., 1992), cathepsin D is not recovered in the laid egg yolk according to 2 proteomic surveys (Mann and Mann, 2008; Farinazzo et al., 2009). These results suggest that cathepsin D is likely to be degraded following activation at the follicular site (Lah and Turk, 1982). Interestingly, both proteomic studies revealed the presence of another aspartic protease "similar to nothepsin" in egg yolk. Homologous nothepsin was found to be exclusively expressed by the liver of females in Antarctic fish (Riggio et al., 2000), whereas it is constitutively expressed by both males and female lizards (Borrelli et al., 2006). The screening of various databases to identify nothepsin-like sequences in other species revealed the presence of a pseudogene in *Gallus gallus* (named "similar to nothepsin"), whereas no similar form was apparently present in human, monkey, mouse, pig, guinea pig, *Xenopus*, bullfrog, and salmon trout (Borrelli et al., 2006). In this context, we investigated whether the expression pattern of the "similar to nothepsin" gene is tissue- and sex-specific as observed in fish. We compared the expression of "similar to nothepsin" with that of cathepsin D because both proteins are highly similar in terms of predicted activity and seem to both have a role in the egg as constituents of the yolk.

MATERIALS AND METHODS

Chicken Handling and Housing

Pullets, hens, and cocks were bred at the experimental unit Pôle d'Expérimentation Avicole de Tours according to the European Community Council Directive of November 24, 1986 (86/609/EEC) and under the supervision of S. Réhault-Godbert and J. Gautron (Authorizations # 7323 and 37–144, respectively). Animals were housed and fed according to recommendations defined by the Institut National de la Recherche Agronomique, France (**INRA**). Thirty-two growing pullets at 13, 14, 15, and 16 wk of age, eight 41-wk-old laying hens and eight 40-wk-old males (ISA Brown, Hendrix Genetics, St-Brieuc, France) were euthanized to collect tissues that were quickly frozen in liquid nitrogen and stored at −80°C.

RNA Extraction and cDNA Preparation

Tissues were harvested from the oviduct (infundibulum, white isthmus, magnum, and uterus) and other organs (liver, kidney, and duodenum) from eight 41-wk-old laying hens. Additionally, livers from 40-wk-old cocks and from 13, 14, 15, 16, and 41-wk-old prelaying/laying hens were sampled. Total RNA was extracted from frozen tissues using Nucleospin RNA II (Macherey-Nagel, Düren, Germany) for oviduct tissues, kidney, and duodenum and the RNA NOW method (Biogentex, Ozyme, Saint Quentin en Yvelines, France) for livers. Samples were then treated with DNase I (Applied Biosystems, Courtaboeuf, France). The RNA concentrations were determined by measuring absorbance at 260 nm, and the quality of RNA was assessed using the Bioanalyzer Agilent 2100 (Agilent Technologies, Massy, France). Total RNA (5 μg) was reverse-transcribed using the superscript II kit (Invitrogen, Cergy Pontoise, France) and random hexamers (GE Healthcare, Uppsala, Sweden) and stored at −80°C until further use.

Real-Time Quantitative PCR

Primers for "similar to nothepsin" (ENSGALT00000016271) 5′CTCTTATCACCGGTCCCTCA3′ (forward) and 5′AGCCACTCATGCAGAAGGTT3′ (backward) and 5′CCCCCACAGAAGTTCACTGT3′ (forward) and 5′CCCGAAGATCTGGTTCTTGA3′ (backward) for cathepsin D (ENSGALT00000010676) were diluted at 0.4 μ*M* in Upti-Therm buffer 1× (Interchim, Montluçon, France). The cDNA was amplified using Sybr Green I Master kit (Roche, Mannheim, Germany) with the LightCycler 480 apparatus (Roche Diagnostics, Meylan, France). A melting curve program was carried out from 65 to 95°C in 1 min for each individual sample. Each run included triplicates of control cDNA consisting of a pool of cDNA from all tissues. The control cDNA was diluted from 1:6.25 to 1:25,600 and relative arbitrary quantities were defined. A calibration curve was calculated using the threshold cycle (**C_T**) values of the control cDNA samples to evaluate the relative amount of samples. "Similar to nothepsin" and cathepsin D mRNA levels were corrected relative to ribosomal 18S rRNA levels for expression in the different tissues of the hens and relative to tata binding protein (**TBP**) for expression of the livers of males, females, and pullets. Levels of 18S rRNA in each sample were measured using *Taq*-Man assay reagents (Applied Biosystems, Courtaboeuf, France), and levels of TBP mRNA were determined using Sybr Green reaction and specific primers 5′GCGTTTTGCTGCTGTTATTATGAG3′ (forward) and 5′TCCTTGCTGCCAGTCTGGAC3′ (backward).

Statistical Analysis

The ratio value was calculated for each sample as "similar to nothepsin" or cathepsin D/18S RNA or TBP rRNA. The log of the ratio was used for statistical analysis using StatView software (SAS Institute Inc., version 5, Cary, NC). A one-way ANOVA was performed to detect statistically significant differences between "similar to nothepsin" and cathepsin D expression in tissues examined (means ± SEM).

RESULTS

Comparative Analysis of Cathepsin D and "Similar to Nothepsin" Protein Sequences

"Similar to nothepsin" and chicken cathepsin D share 46% of identities and 63% similarity and possess a signal peptide with a predicted cleavage site between position 16–17 and 20–21, respectively (Figure 1). Both proteases possess 2 eukaryotic and viral aspartyl proteases active signatures (Figure 1; PS00141 ASP_PROTEASE Eukaryotic and viral aspartyl proteases active site) and belong to the peptidase A1 family.

Tissue Distribution of "Similar to Nothepsin" and Cathepsin D in Laying Hens

Expressions of "similar to nothepsin" and cathepsin D genes were analyzed by real-time quantitative PCR in tissues involved in the egg formation (liver for the yolk; infundibulum for vitelline membranes; magnum for the egg white; white isthmus for shell membranes; uterus for the eggshell) in the kidney and the duodenum.

Results reveal that cathepsin D expression is ubiquitous (Figure 2A). We observed a high heterogeneity between liver samples and when considering the log ratio of the expression, no statistical differences could be found between tissues. However, significantly lower expression was observed in the magnum compared with the other oviduct tissues ($P < 0.001$) when the liver was removed from the analysis.

The relative normalized "similar to nothepsin" mean expression was considerably higher in the liver compared with all the other tissues analyzed (Figure 2B; $P < 0.001$).

Expression of "Similar to Nothepsin" and Cathepsin D in the Liver of Cocks and Hens and During Sexual Maturation of Pullets

The expression of "similar to nothepsin" and cathepsin D was analyzed in the liver of sexually mature cocks and hens. The relative normalized cathepsin D expression is significantly higher in the liver of males than in the liver of females ($P < 0.01$; Figure 3A). In contrast, the "similar to nothepsin" gene (Figure 3B) is found to be exclusively expressed by the liver of laying hens ($P < 0.001$).

To assess whether the expressions of "similar to nothepsin" and cathepsin D were regulated by sex steroids, we analyzed the kinetic of expression of both genes in the liver of prelaying pullets during sexual maturation (13–16 wk) and in the liver of mature hens (41 wk old).

As shown in Figure 4A, the relative normalized expression of cathepsin D in liver does not show any sig-

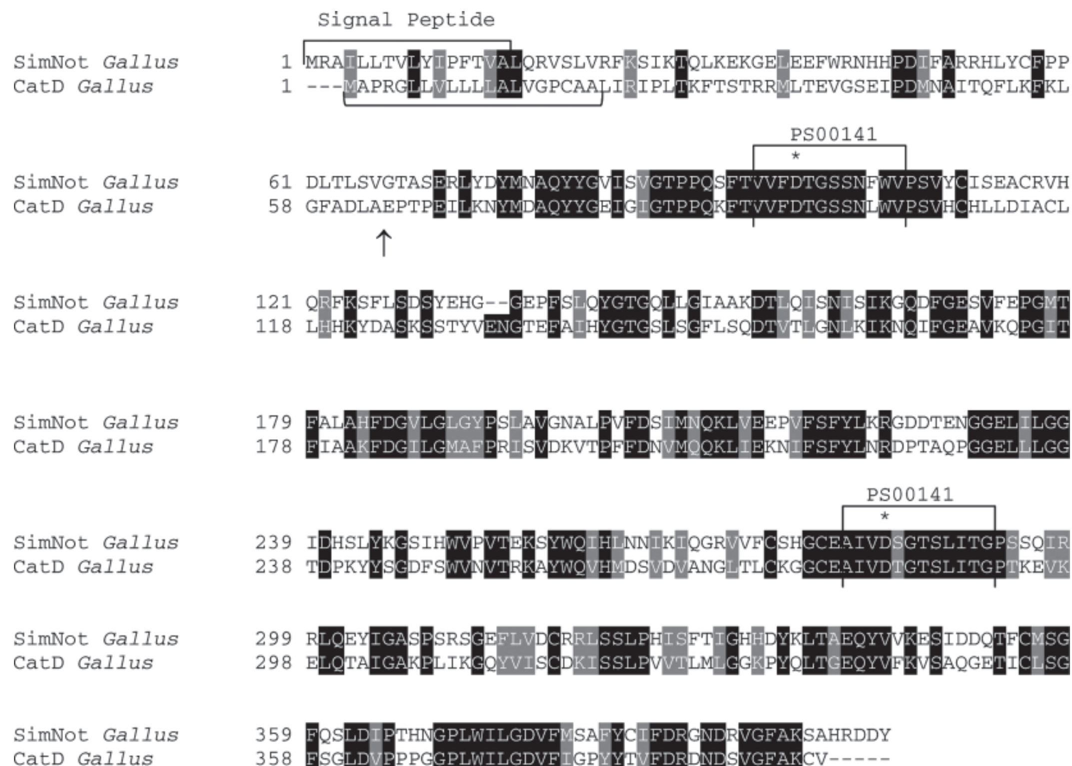

Figure 1. Sequence alignment of "predicted: similar to nothepsin" (SimNot Gallus, XP_416090) and cathepsin D precursor (CatD Gallus, NP_990508). Identical residues and homologous residues are shaded black and gray, respectively. This alignment was performed using CLUSTAL W (1.8) and BOXSHADE (3.21). Signal peptides were predicted using SignalP 3.0. The arrow illustrates the cleavage site of the cathepsin D propeptide. Asterisks indicate aspartate active sites (D) included in 2 specific signatures of aspartate proteases as defined in PROSITE entry PS00141 (http://prosite.expasy.org/cgi-bin/prosite).

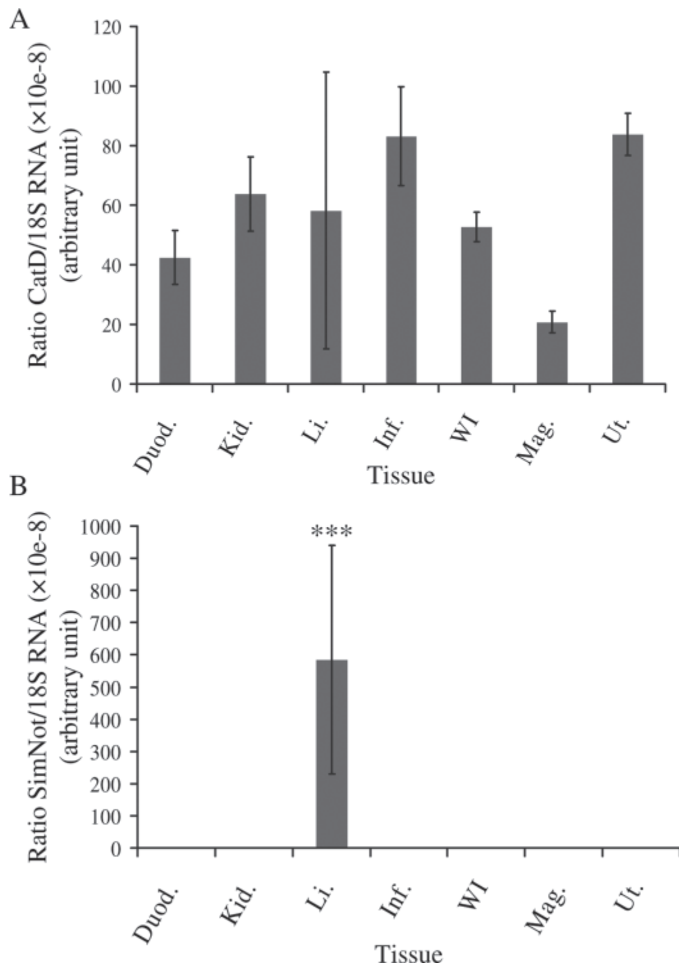

Figure 2. Tissue expression of cathepsin D and "similar to nothepsin" in 41-wk-old laying hens (mean ± SEM). A) Cathepsin D (catD). B) "Similar to nothepsin" (SimNot). Duod., duodenum; Kid., kidney; Li., liver; Inf., infundibulum; WI, white isthmus; Mag., magnum; Ut., uterus. Real-time quantitative PCR was conducted as described in Materials and Methods. Asterisks indicate significant differences (***$P < 0.001$; n = 8).

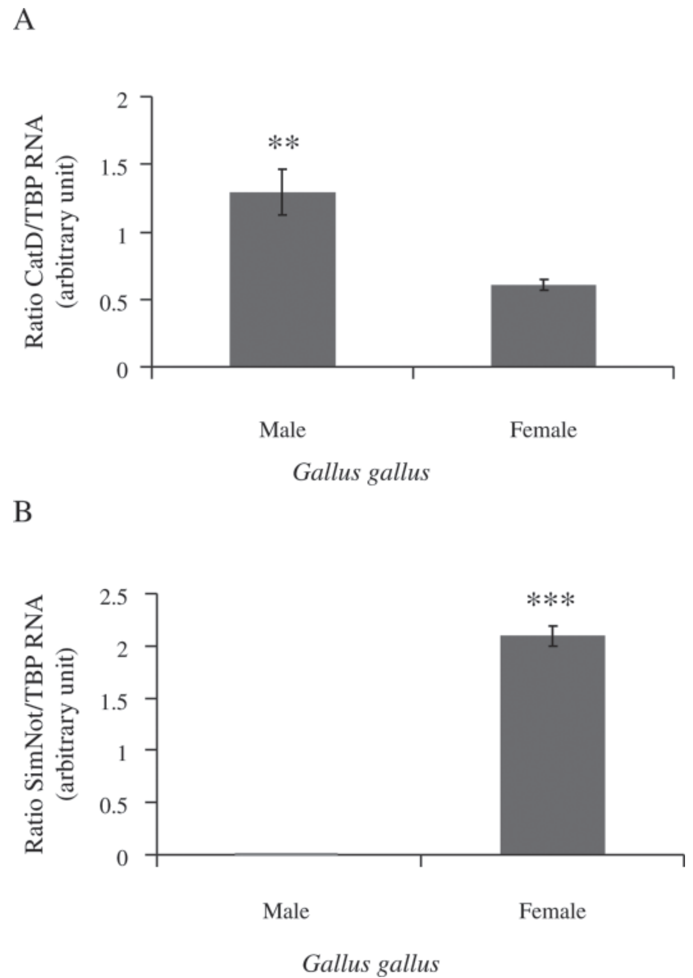

Figure 3. Expression of cathepsin D and "similar to nothepsin" in the liver of 41-wk-old males and females. A) Cathepsin D (catD). B) "Similar to nothepsin" (SimNot). Real-time quantitative PCR was conducted as described in Materials and Methods. Asterisks indicate significant differences (**$P < 0.01$, n = 8; or ***$P < 0.001$, n = 8).

nificant statistical differences from 13 wk to 41 wk of age. In contrast, the relative normalized "similar to nothepsin" expression increased strongly from 13 wk of age to reach its maximum expression from 16 wk of age onwards ($P < 0.001$; Figure 4B).

DISCUSSION

Until recently, chicken cathepsin D was thought to be the major aspartic protease of egg yolk (Retzek et al., 1992; Elkin et al., 1995; Gerhartz et al., 1999). However, "similar to nothepsin," a novel predicted aspartic protease, has been recently identified in egg yolk by proteomic analyses (Mann and Mann, 2008; Farinazzo et al., 2009). Protein sequences of these 2 aspartic proteases are very similar (Figure 1). Although the involvement of cathepsin D in the hydrolysis of yolk precursors (very low-density lipoproteins and vitellogenins) during oocyte's growth is well established (Retzek et al., 1992; Carnevali et al., 2006), there is to date no information related to the functional specificities of "similar to nothepsin."

We have shown that chicken cathepsin D is expressed in all the tissues tested (Figure 2A). In mammals, cathepsin D was initially described as a ubiquitous lysosomal enzyme. It is involved in numerous biological processes (Tsukuba et al., 2000; Benes et al., 2008), and more importantly in chickens, cathepsin D participates in the intra-oocytic processing of egg yolk precursors (De Stasio et al., 1999). Cathepsin D has been described elsewhere to be expressed by both the liver of laying hens and the ovarian follicle (Retzek et al., 1992). Additionally, we show that cathepsin D is expressed by the liver of sexually mature cocks and that its expression is higher when compared with females (Figure 3A). Furthermore, the fact that the expression of chicken cathepsin D is stable during the sexual maturation of pullets (Figure 4A) indicates that the hepatic expression of cathepsin D in females is not regulated by estrogens. We thus hypothesize that the expression of cathepsin D by the ovarian follicle is probably more

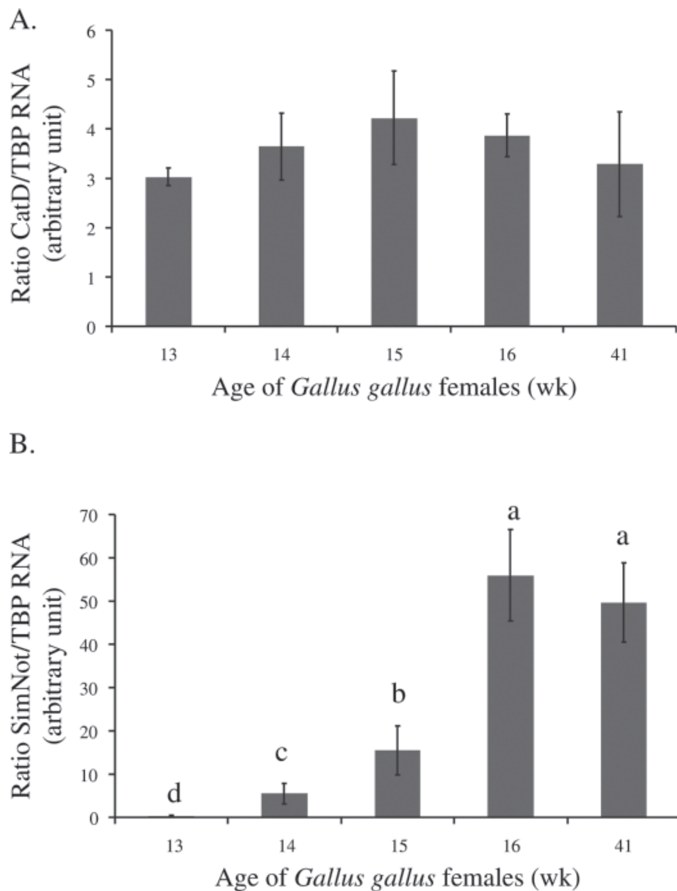

Figure 4. Expression of cathepsin D and “similar to nothepsin” in the liver of pullets during sexual maturation (13 to 16 wk old; mean ± SEM). A) Cathepsin D (catD). B) “Similar to nothepsin” (SimNot). Real-time quantitative PCR was conducted as described in Materials and Methods. [a–d]Means that do not share a common letter are significantly different ($P < 0.01$; n = 8).

relevant in the process of egg yolk formation than that of the liver. In fact, although cathepsins D from both sources have similar activity on egg yolk precursors (Retzek et al., 1992), it is possible that the cathepsin D from the hepatic source is not transferred to the yolky follicle but rather has other physiological functions. In fact, the ubiquitous expression of cathepsin D corroborates the likelihood that cathepsin D has various functions in the physiology of both female and male chickens.

In contrast, the expression of “similar to nothepsin” is very specific to the liver of laying hens (Figure 2B, Figure 3B). The expression of “similar to nothepsin” increases gradually during sexual maturation of pullets (Figure 4B), coincident with the increase of oestradiol plasma concentration during the 2 wk preceding the onset of egg laying. The expression of “similar to nothepsin” in *Gallus gallus* is therefore dependent on sex-steroids. In fact, expression of its homologous nothepsin in liver of zebrafish males was shown to be stimulated after estrogen treatment (Riggio et al., 2002).

Our data clearly indicate that the nothepsin gene has not been lost or inactivated in chicken during evolution, as it has been suggested from phylogenetic studies (Borrelli et al., 2006). They reveal that when considering this specific gene, *Gallus gallus* is closer to fish than to lizard in terms of egg physiology, although *Gallus gallus* and lizard are more phylogenetically related than *Gallus gallus* and fish (Borrelli et al., 2006). Phylogenetic investigation of estrogen responsive-elements linked to “similar to nothepsin” gene might help to explain the different sensibility of liver to estrogen between species.

Further studies will also be needed to explore the role of “similar to nothepsin” in egg yolk. The protein sequence analysis of “similar to nothepsin” suggests that this protein is expressed as a precursor (Figure 1), similar to cathepsin D. In mammals, lysosomal cathepsin D is processed by 2 cysteine proteinases, cathepsin B and L (Zaidi et al., 2008). There is no evidence of the presence of these cathepsins in egg yolk (Mann and Mann, 2008; Farinazzo et al., 2009), which suggests another mechanism of activation for yolk cathepsin D and presumably “similar to nothepsin” compared with lysosomal cathepsin D (Tsukuba et al., 2000; Benes et al., 2008). Further studies conducted with purified proteins will be needed to answer this question. The property of aspartic proteases to be inhibited by pepstatin has been used to purify cathepsin D from the liver and follicles (Barrett, 1970; Retzek et al., 1992). Using pepstatin-chromatography, cathepsin D, but not “similar to nothepsin,” could be recovered in the fraction retained by the affinity chromatography (Retzek et al., 1992). Comparable results were obtained when we used this pepstatin-affinity chromatography on egg yolks collected from freshly laid eggs (data not shown). In fact, in our preliminary study, we found only trace amounts of cathepsin D bound to pepstatin-sepharose and we could not identify “similar to nothepsin” in this fraction. We hypothesize that chicken cathepsin D plays a role in the early stages of the yolk formation, that is, in the small growing follicles, which accumulate egg yolk precursors, and that thereafter, cathepsin D is further degraded into inactive peptides (Lah and Turk, 1982) in more mature follicles or laid eggs. This hypothesis could explain why Mann et al. and Farrinazzo et al. could not identify cathepsin D in egg yolk proteomes (Mann and Mann, 2008; Farinazzo et al., 2009). The fact that “similar to nothepsin” from liver, follicles (Retzek et al., 1992), and egg yolk (data not shown) is not recovered after pepstatin-sepharose suggests that pepstatin is not an inhibitor of “similar to nothepsin,” or more interestingly, that “similar to nothepsin” is still present as a inactive precursor form in these various samples. Indeed, most protease precursors cannot interact with their cognate inhibitor or substrate because their active site is inaccessible, due to the presence of a propeptide (Mása et al., 2006).

Both active cathepsin D and “similar to nothepsin” could act in a synergistic manner to process egg yolk proteins in endocytic vesicles because they are both presumably active at acidic pH. On the other hand, the presence of the precursor of “similar to nothepsin” in laid eggs could imply that this protein is activated later

to play a role at a specific stage of embryonic development, whereas cathepsin D would rather be associated with follicular growth and processing of egg yolk precursors within the oocyte (yolky follicle).

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