Identification of uterine ionic transport proteins involved in providing the minerals for eggshell formation in hens.

Aurélien Brionne, Joël Gautron, Yves Y. Nys

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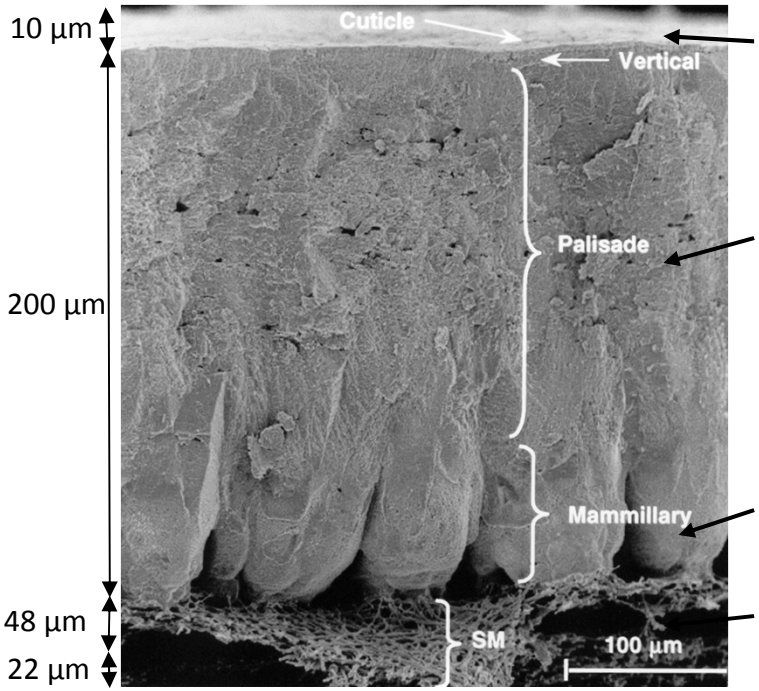
Chicken eggshell

The eggshell strength : physical protection of the egg.

Mechanical properties : 0.3 mm / 35N

The eggshell is a compact layer of calcium carbonate



Eggshell cross section, SEM

Components :

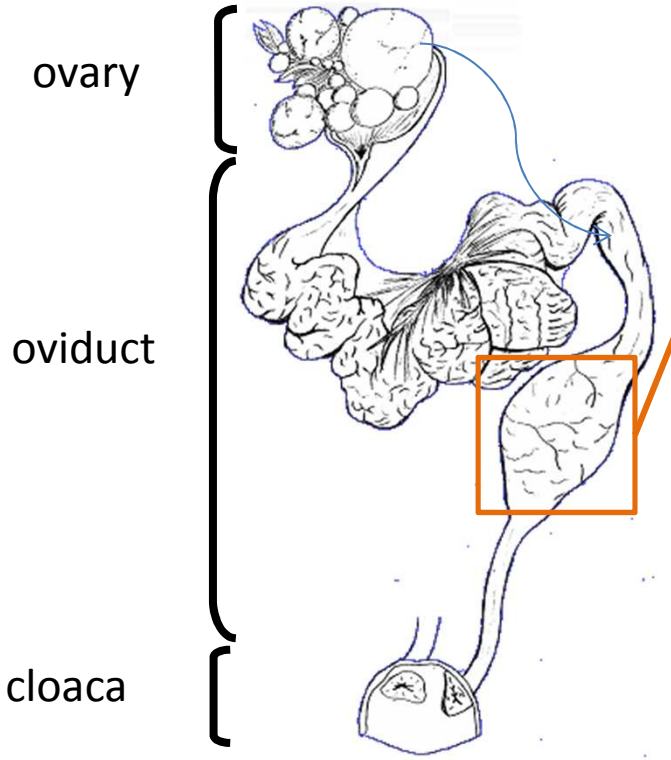
95% minerals (calcite)

3.3% organic matrix

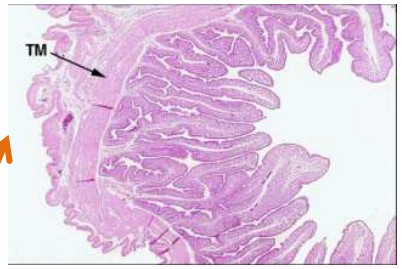
1.7% water

Eggshell formation

Hen reproductive organ

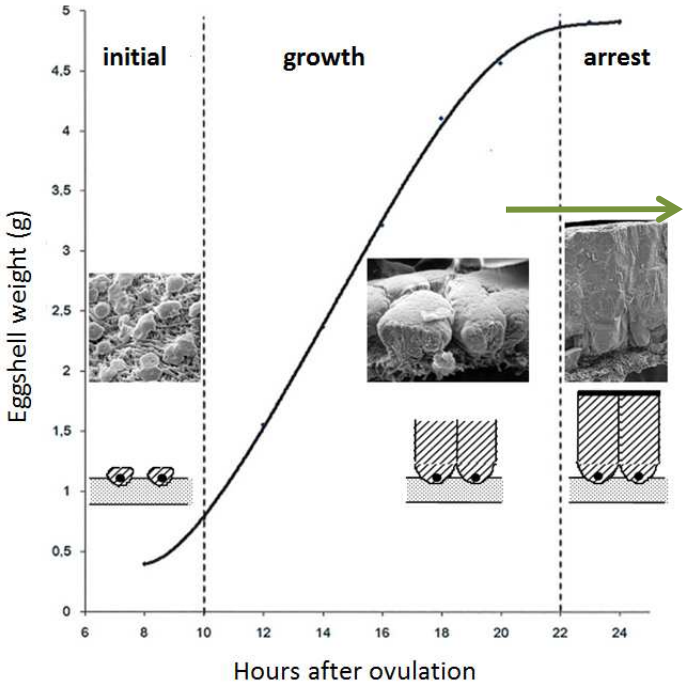


Eggshell biomineralization



- **Calcification in acellular uterine fluid**
  biologically controlled extracellular mineralization
- **Uterine mucosa gene expression**
  Mineral supplies
  Protein secretion (organic matrix)

Eggshell calcification stages



5 g of eggshell deposited in 12h.

Eggshell mineral supplies

Eggshell biomineralization

→ Need a large amount of minerals in the forming milieu (uterine fluid) which is hyper-saturated relative to calcite (x100)

Exacerbated calcium metabolism in hens (exportation of 1.5kg shell/year)

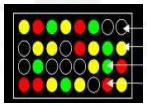
→ No calcium storage in the uterus, and hens renew the blood calcium every 12 minutes during shell formation…

Identification and characterization of proteins involved in the supply of minerals.

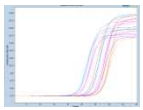
Experimental approaches

Gene expression:

Microarrays



Real time PCR



Experimental models:

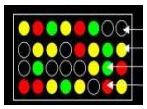
a. Uterus *vs* other parts of the oviduct
b. Uterus presence *vs* absence calcification

Data integration:

Bioinformatic analysis

Jonchère *et al. BMC Genomics* 2010, **11**:57
http://www.biomedcentral.com/1471-2164/11/57

BMC Genomics

a. 

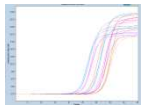
RESEARCH ARTICLE Open Access

Gene expression profiling to identify eggshell proteins involved in physical defense of the chicken egg

Vincent Jonchère[1], Sophie Réhault-Godbert[1], Christelle Hennequet-Antier[1], Cédric Cabau[1], Vonick Sibut[1,3], Larry A Cogburn[2], Yves Nys[1], Joël Gautron[1*]

Jonchère *et al. BMC Physiology* 2012, **12**:10
http://www.biomedcentral.com/1472-6793/12/10

BMC Physiology

a. 

b.

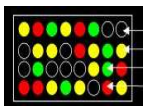
RESEARCH ARTICLE Open Access

Identification of uterine ion transporters for mineralisation precursors of the avian eggshell

Vincent Jonchère, Aurélien Brionne, Joël Gautron and Yves Nys*

Brionne *et al. BMC Genomics* 2014, **15**:220
http://www.biomedcentral.com/1471-2164/15/220

BMC Genomics

b. 

RESEARCH ARTICLE Open Access

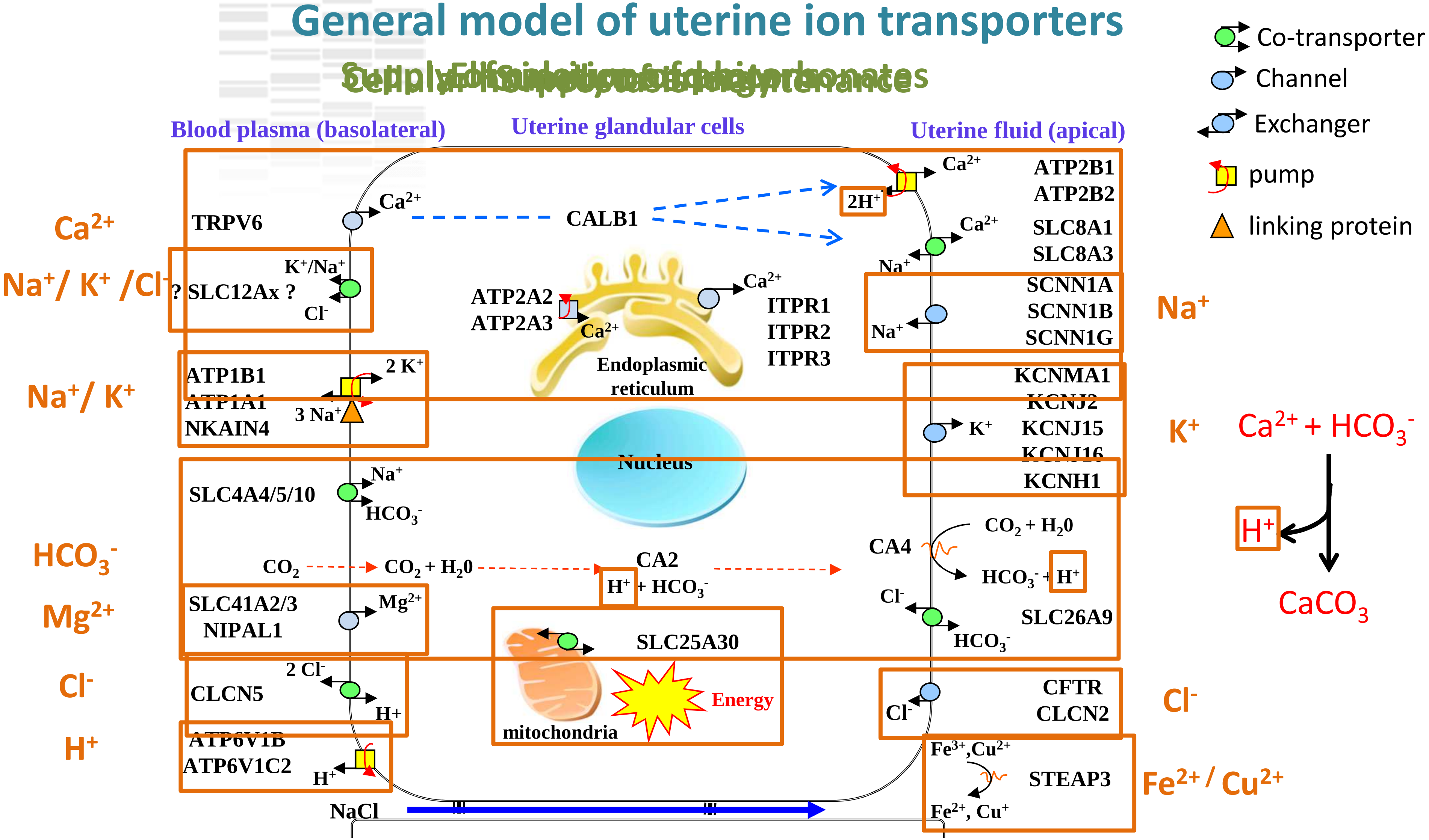
Hen uterine gene expression profiling during eggshell formation reveals putative proteins involved in the supply of minerals or in the shell mineralization process

Aurélien Brionne, Yves Nys, Christelle Hennequet-Antier and Joël Gautron*

General model describing the uterine ion transporters during eggshell calcification

- Co-transporter
- Channel
- Exchanger
- pump
- linking protein

General model of uterine ion transporters



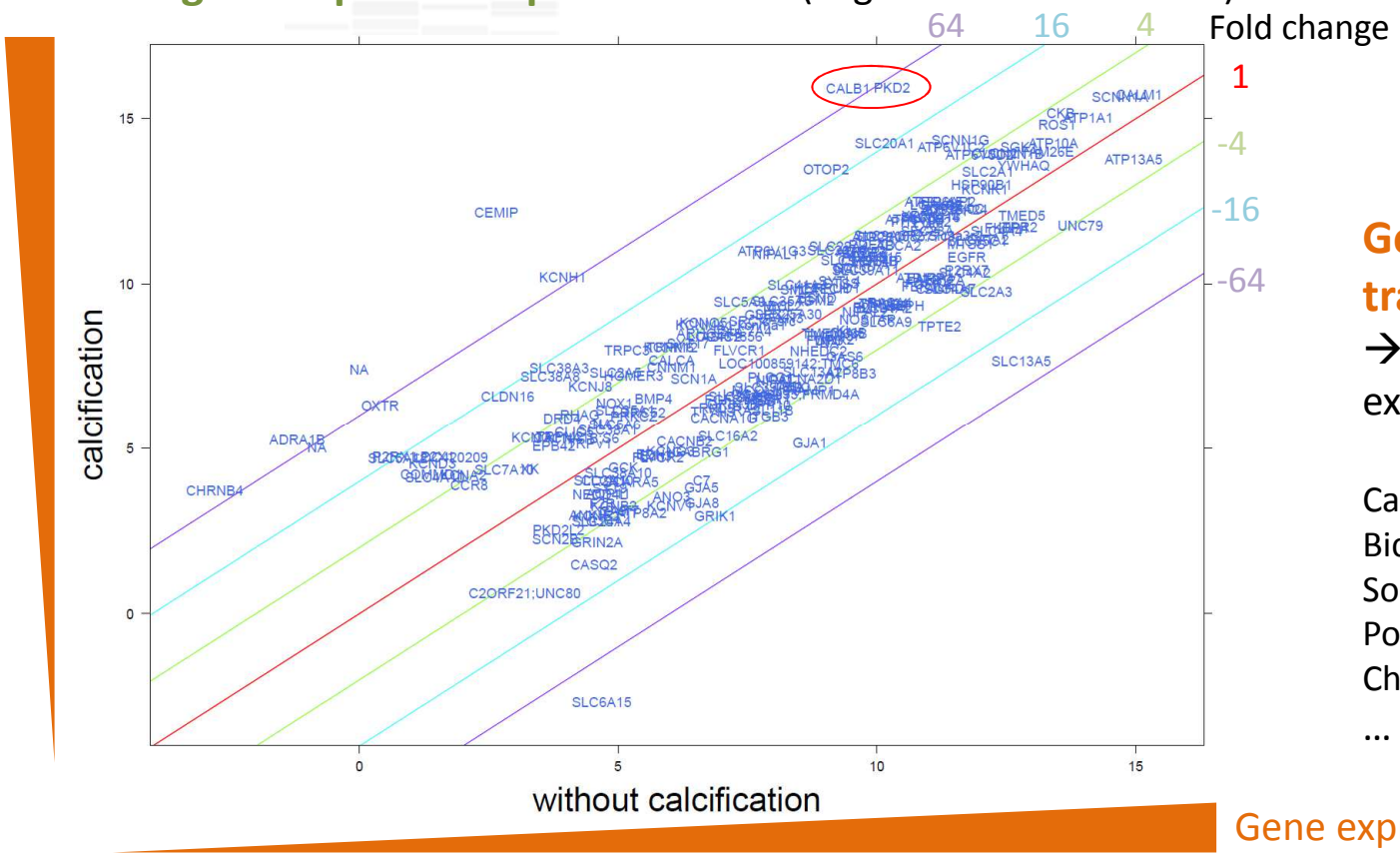
Co-transporter
Channel
Exchanger
pump
linking protein
Blood plasma (basolateral)
Uterine glandular cells
Uterine fluid (apical)
Ca2+
TRPV6
Ca2+
CALB1
2H+
Ca2+
ATP2B1
ATP2B2
Ca2+
SLC8A1
SLC8A3
Na+
Na+/ K+ /Cl-
K+/Na+
? SLC12Ax ?
Cl-
ATP2A2
ATP2A3
Ca2+
Ca2+
ITPR1
ITPR2
ITPR3
Endoplasmic reticulum
SCNN1A
SCNN1B
SCNN1G
Na+
Na+
Na+/ K+
ATP1B1
ATP1A1
NKAIN4
2 K+
3 Na+
KCNMA1
KCNJ2
KCNJ15
KCNJ16
KCNH1
K+
K+
Ca2+ + HCO3-
H+
CaCO3
Nucleus
HCO3-
SLC4A4/5/10
Na+
HCO3-
CO2
CO2 + H20
CA2
H+ + HCO3-
CA4
CO2 + H20
HCO3- + H+
Cl-
SLC26A9
HCO3-
Mg2+
SLC41A2/3
NIPAL1
Mg2+
SLC25A30
Energy
mitochondria
Cl-
2 Cl-
CLCN5
H+
CFTR
CLCN2
Cl-
Cl-
H+
ATP6V1B
ATP6V1C2
H+
NaCl
Fe3+,Cu2+
STEAP3
Fe2+, Cu+
Fe2+ / Cu2+

Jonchère, *et al., BMC physiology*, 2012; Brionne, *et al., BMC Genomics*, 2014

Exhaustive uterine gene expression quantification by RNAseq during shell calcification (presence / absence)

IMPACT
ANR-13-BSV6-0007-01

Uterine gene expression quantification (Log2 normalized counts)



Genes related to ions transport

→ 226 genes differentially expressed

Calcium: 65
Bicarbonates: 7
Sodium: 37
Potassium: 32
Chloride: 13
…

- ☞ **Our complementary study identified numerous additionnal ionic tranporters**
- ☞ **Need to quantify their respective role**
  - → **Experimental models: reinduction of shell formation**

Calcification cycle disruption and reinduction

Experimental model (Nys et al., 1991)

- Suppression of shell formation by egg expulsion (several days)

↘ CALB1 mRNA and protein levels

- Resumption of eggshell calcification in hens previously laying shell-less eggs.

↗ CALB1 mRNA and protein levels (fold change: 9; 2.4, respectively)

→ Use of this model to reveal which transporter has a predominant role amongst the numerous candidate.

Gene expression (qRT-PCR)

→3 genes involved in the calcium secretion pathway (PKD2, CALB1, ATP2B1)

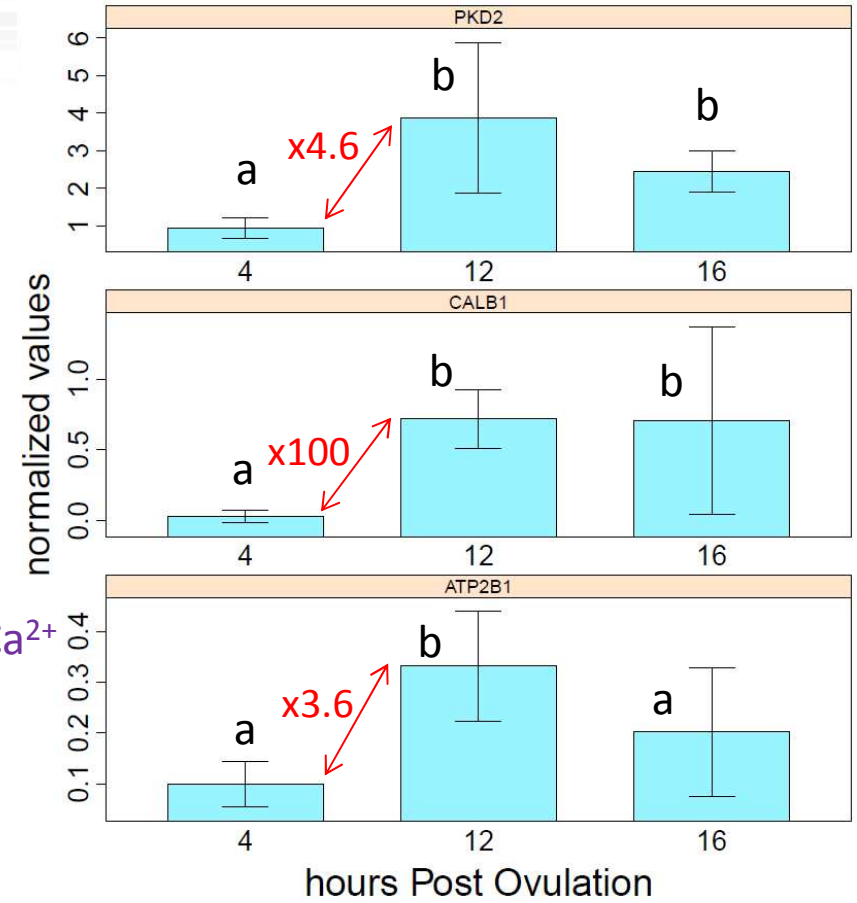
Calcification cycle disruption and reinduction

Gene expression (qRT-PCR)

Channel for Ca^{2+} entry in cell (PKD2)

Intracellular Ca^{2+} binding protein (CALB1)

Active secretion of Ca^{2+} in uterine fluid (ATP2B1)



calcification

→ **PKD2, CALB1 and ATP2B1 are highly overexpressed after reinduction of the calcification.**

Summary

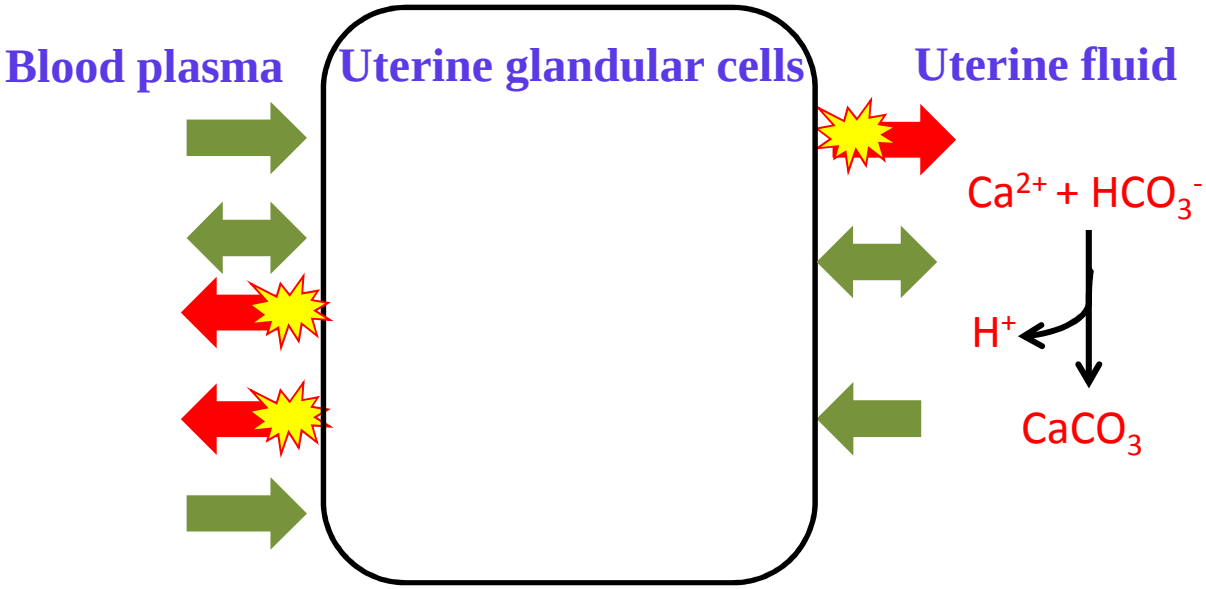
→ Coherent model describing ion transporters in the uterine glandular cells during eggshell calcification

Ca^{2+} and HCO_3^- supplies

Maintain cellular homeostasis

Elimination of H^+

Supply of energy



→ The relative contribution of the numerous identified candidate genes is under study

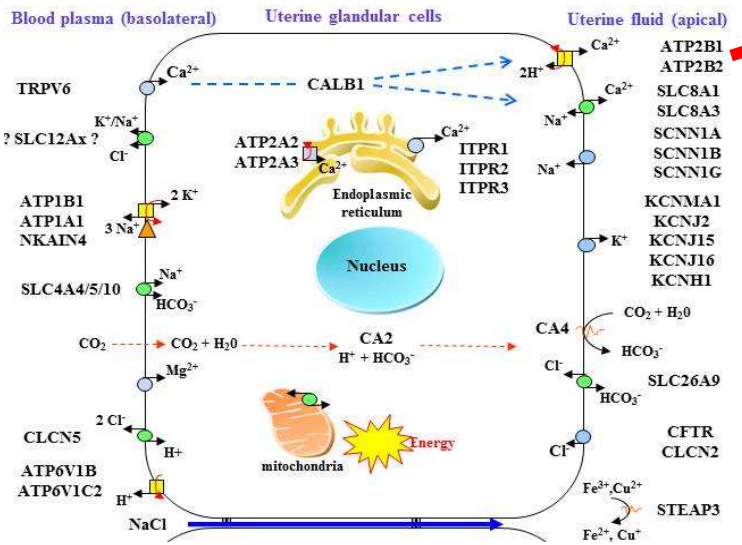
Tools for exploring the regulation of ionic secretion and understand the origin of lower eggshell strength induced by lower material deposition

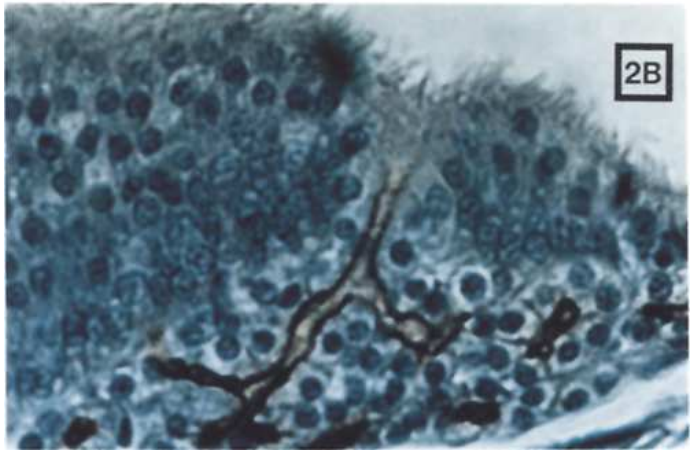
Thank you for your attention.

Eggshell mineral supplies

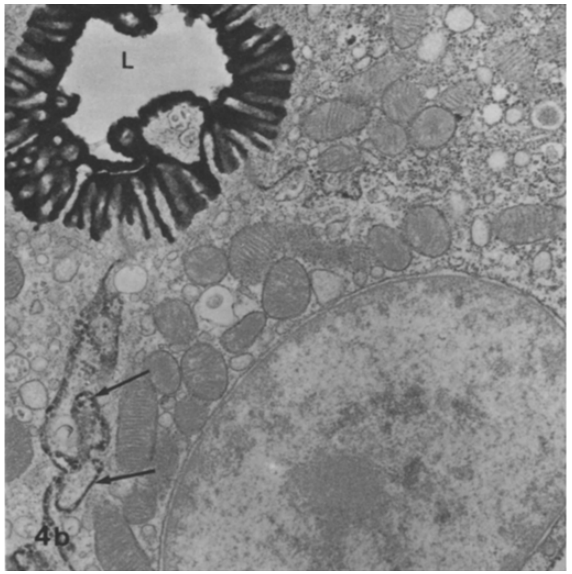
Uterine fluid ion concentration (adapted from Nys, 1999)

	PLASMA	flux	UTERINE fluid Initial	UTERINE fluid Growth
Na	140	←	144	80
Cl	130	←	71	45
K^+	4	→	12	60
Ca^{++}	1.2	→	6 to 10	
HCO_3^-	23	→	6 to 110	
PCO_2	50-60 + H_2O	Carbonic Anhydrase	90 to 110	
pH	7.4	←	7.6	7.1





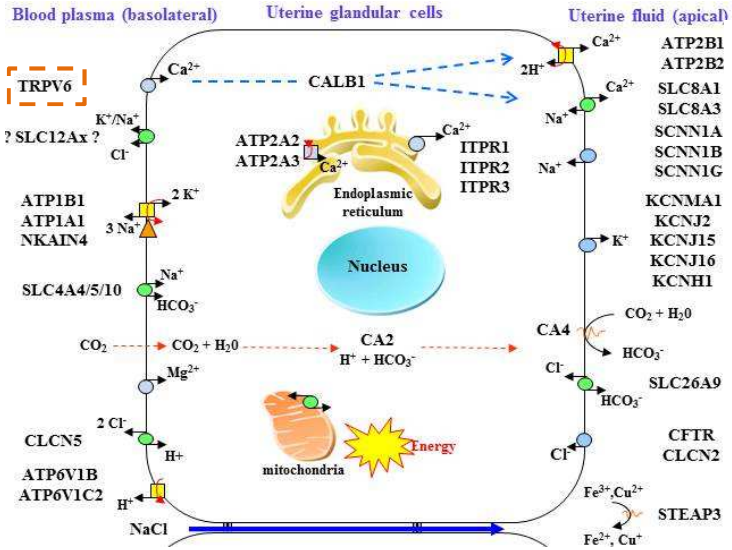
Wasserman, R.H., *et al.*, 1991

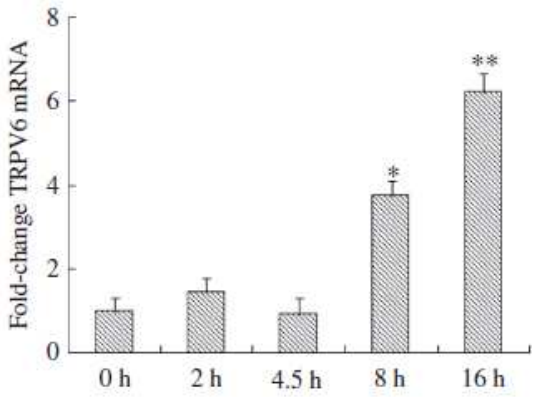


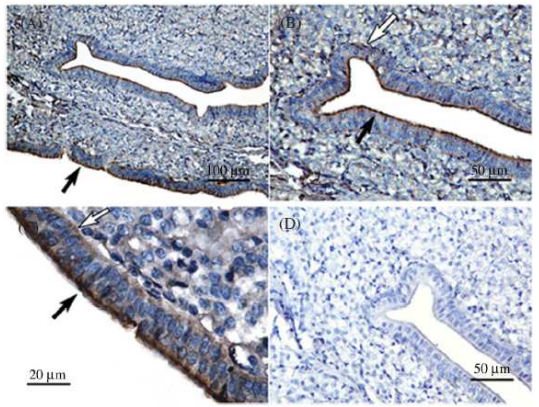
Yamamoto, T., *et al.*, 1985

TRPV6

Calcium ion entry into the glandular cell through TRPV6







Gene expression and tissue location of TRPV6 during calcification (Yang *et al.*, 2013)

Nys Y., et al., 1991

Estrogen and a Calcium Flux Dependent Factor Modulate the Calbindin Gene Expression in the Uterus of Laying Hens

Y. Nys,* K. Baker,† and D. E. M. Lawson‡

*INRA, Station de Recherches Avicoles, 37380 Nouzilly, France; †AFRC, Shinfield, Reading, Berks, RG2 9AT, United Kingdom; and ‡AFRC, Babraham Cambridge CB2 4AT, United Kingdom

Accepted November 12, 1991

TABLE 2

Uterine Concentration of Calbindin and its mRNA at Various Stages of Egg Formation in Hens Laying Hard Shelled or Shell-less Eggs

Physical stage (hr after ovulation):	Hens calcifying an egg 4	8	12	Shell-less egg* (12-hr stage)
Uterine calbindin (μg/mg protein)	54 ± 6[b]	57 ± 3[b]	60 ± 11[b]	27 ± 2[a]
Uterine calbindin mRNA (% 12-hr stage value)	4 ± 1[a]	46 ± 6[b]	100 ± 15[c]	13 ± 2[a]

x25

Note. See note to Table 1.

* Shell formation had been suppressed by premature expulsion of the eggs for 8 days.

TABLE 4

The Effects of Resumption of Shell Formation and Parathyroidectomy (PTX) on Uterine Calbindin in Hens Previously Laying Shell-less Eggs

Physiological stage:	Resumption of the first shell after experimental suppression of shell formation* 4	12	24 (oviposition) Sham PTX	PTX[a]
Uterine calbindin (μg/mg protein)	19 ± 6[a*]	48 ± 4[b]	44 ± 3[b]	31 ± 5[ab]
Uterine calbindin mRNA (% control, 4-hr stage)	100 ± 50[a]	892 ± 85[b]	412 ± 76[b]	323 ± 48[b]

x2.5

x9

[a] The hens were parathyroidectomized 4–6 hr after ovulation.

* Means without a common superscript are significantly different ($P < 0.05$, Tukey's test).