PNAS
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Supplementary Information for

On the enigmatic disappearance of Rauber's layer

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This PDF file includes:

www.pnas.org/cgi/doi/10.1073/pnas.2002008117

Supplementary Information Materials and Methods

Whole mount cell proliferation assay in cattle embryos

Embryos were fixed for 4-6 hours in 4% PFA (paraformaldehyde)/PBS on ice. Subsequent steps were carried out in 2 ml microcentrifuge tubes with gentle rocking (40 rpm) at room temperature (R.T.) with 5 min washes unless specified. Post fixing, embryos were washed three times in 0.1% Tween-20/PBS (PBT), treated overnight (O/N) at 4˚C with 0.1% H_2O_2 in PBS and stored in 0.02% thimerosal (Sigma) in PBS at 4˚C until used.

Embryos were washed 3 x 1 h in PBT, 1 h with PBT/1% BSA (Sigma), then 1 h PBT/1% BSA/10% heat treated lamb serum (antibody dilution buffer). They were then incubated O/N at 4˚C with antibody (Ab) dilution buffer containing 1/200 dilution of anti-phospho-Histone H3 Ab (#06-570 Upstate Biotechnologies), then washed as the day before incubating with preabsorbed goat anti-rabbit horse radish peroxidase conjugated Ab (GAR-HRP; Sigma-Aldrich A6154) overnight. Preabsorption was performed by adding 10 µl Ab to 0.5 ml 0.1% Triton X-100 (AppliChem, A13880500)/PBS (PBX), 5 µl heat treated lamb serum and 2 mg of bovine embryo powder, rocking gently 1 h at 4°C, then centrifuging at 13000 x g for 10 min at 4 ˚C and adding the supernatant to 1.5 ml of Ab dilution buffer. Embryos were washed 3 x 1 h with 0.1% PBX, twice 10 min with Tris-HCl buffer pH 7.4. For staining, 3,3 diaminobenzidine tetrahydrochloride (DAB; Sigma) was prepared fresh (5 mg in 10 ml of Tris-HCL pH 7.4) and embryos incubated 1 h in the dark at 4 ˚C. The DAB solution was then replaced with fresh DAB solution containing a 1/10,000 dilution of 30% H_2O_2 (BDH). Embryos were incubated 5 to 30 min in the dark with rocking, during which time colour production was monitored. The reaction was stopped by several rinses in water, then PBS and embryos photographed whole in concavity slides. Some embryos were then embedded in agarose and histologically sectioned.

Apoptosis assay for cattle embryos

Steps were carried out at R.T. in 4 well plates (Nunc, Denmark) with gentle rocking/shaking at approximately 40 rpm unless specified Embryos were fixed for 4-6 hours in 4% PFA/PBS on ice, then progressively dehydrated (10 min 25%, 50%, 75% methanol/PBS), then washed and stored at -20˚C in 100% methanol. Embryos were treated with 3% H_2O_2 in methanol for 1 h (R.T.), before rehydrating (10 min each: 75%, 50%, 25% methanol/PBS), washing 3 x 5 min and 1 x 15 min with 1% BSA/PBX (PBX-BSA) and blocking 1 h in PBX-BSA/10% heat treated lamb serum. After 15 min in 50 mM NH_4Cl, they were rocked O/N at 4˚C with a 1/400 dilution of active caspase 3 antibody (9661, Cell Signaling Technology) in PBX-BSA/5% heat-treated (50°C, 30 min) lamb serum. Embryos were washed 3 x 5 min with PBX, 3 x 1 h with Ab dilution buffer, then O/N with 1/2000 dilution of preabsorbed GAR-HRP Ab as described for the proliferation assay.

Apoptosis assay for cell lines

Cells were plated at 3 $x10^5$ cells/well in 6 well plates, grown overnight, then media was replaced with 1 ml of PBS and cells exposed (controls not) to 90 mJ/cm^2 254 nm UV radiation in a UV

Stratalinker 1800 (Agilent Technologies) to induce apoptosis. PBS was replaced with media and cells harvested after 24 h, the pellet washed in PBS, then resuspended in 55 µl of cell lysis buffer from the EnzCheck Caspase 3 assay kit (Molecular probes). Five microliters were removed and used for protein concentration analysis using the BCA protein assay kit (Thermofisher). Fifty microlitres were used in the EnzCheck Caspase 3 assay following the manufacturer's instructions. The fluorescent product of the assay was measured using a Genesis Synergy 2 plate reader (Millenium Science) at 342 nm excitation and 441 nm emission wavelength. Fluorescent readings were normalised against protein concentration to minimise the effect of different cell numbers in each well. Experiments were performed in triplicate.

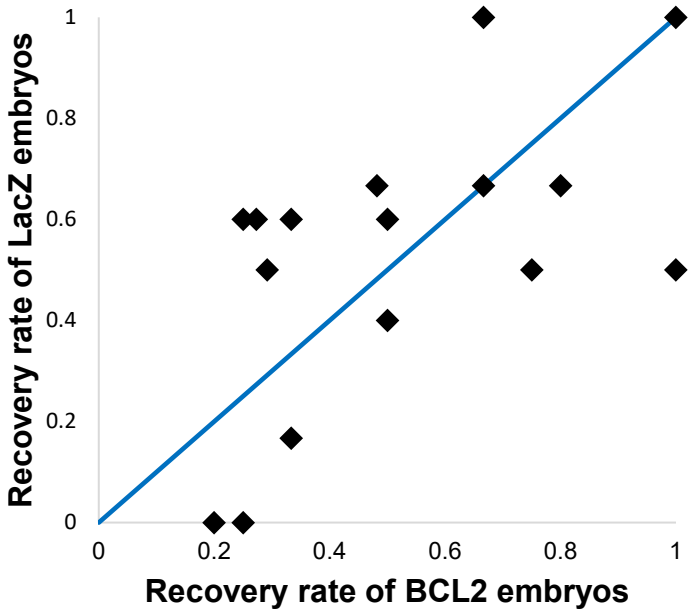

Fig. S1. Fraction of co-transferred BCL2 and LacZ transgenic embryos transferred that were recovered. No difference in the recovery rate of *LacZ* versus *BCL2* embryos recovered per cow. Each point is from an individual recipient cow, cows that showed higher recovery rates for *BCL2* embryos also had a higher recovery rate of *LacZ* embryos. A 50:50 proportion line is shown (blue).

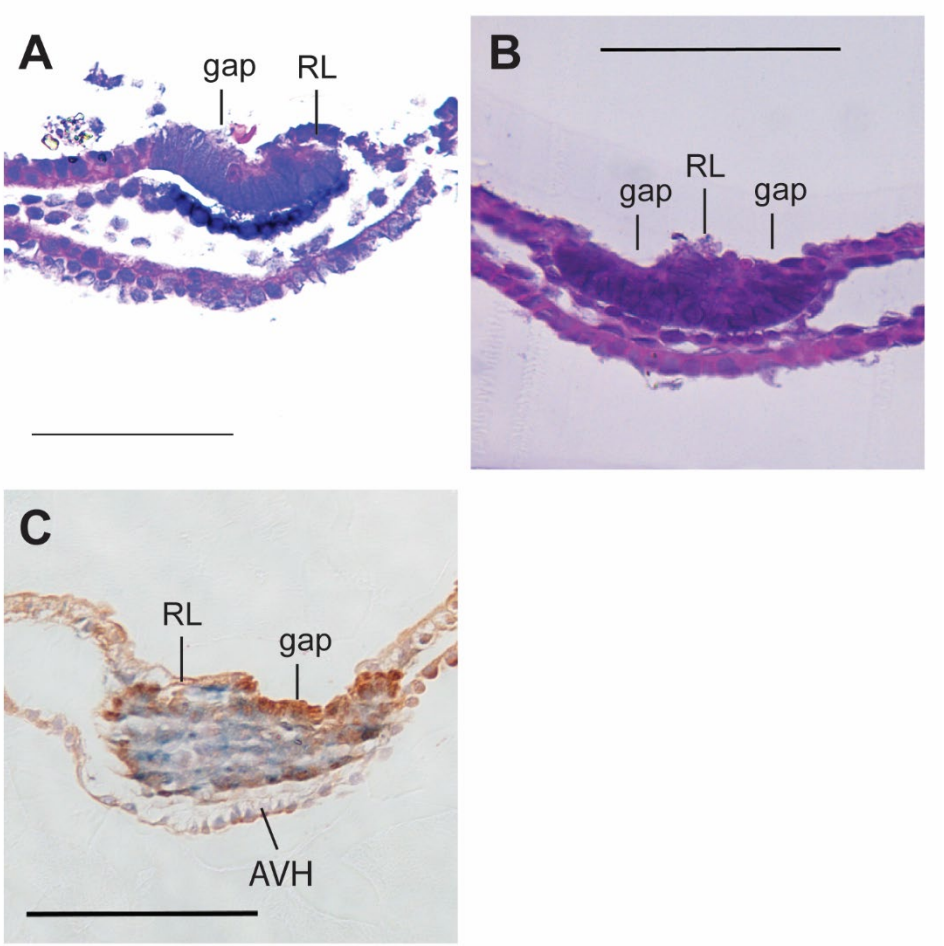

Fig. S2. Gaps in Rauber's layer and AVH formation in wild type embryos. A, B. Saggital section of embryos with Rauber's layer beginning to disintegrate and no visceral hypoblast thickening. C. Cross-section of embryo in which a gap in Rauber's layer overlies the AVH. Bar is 100 μm.

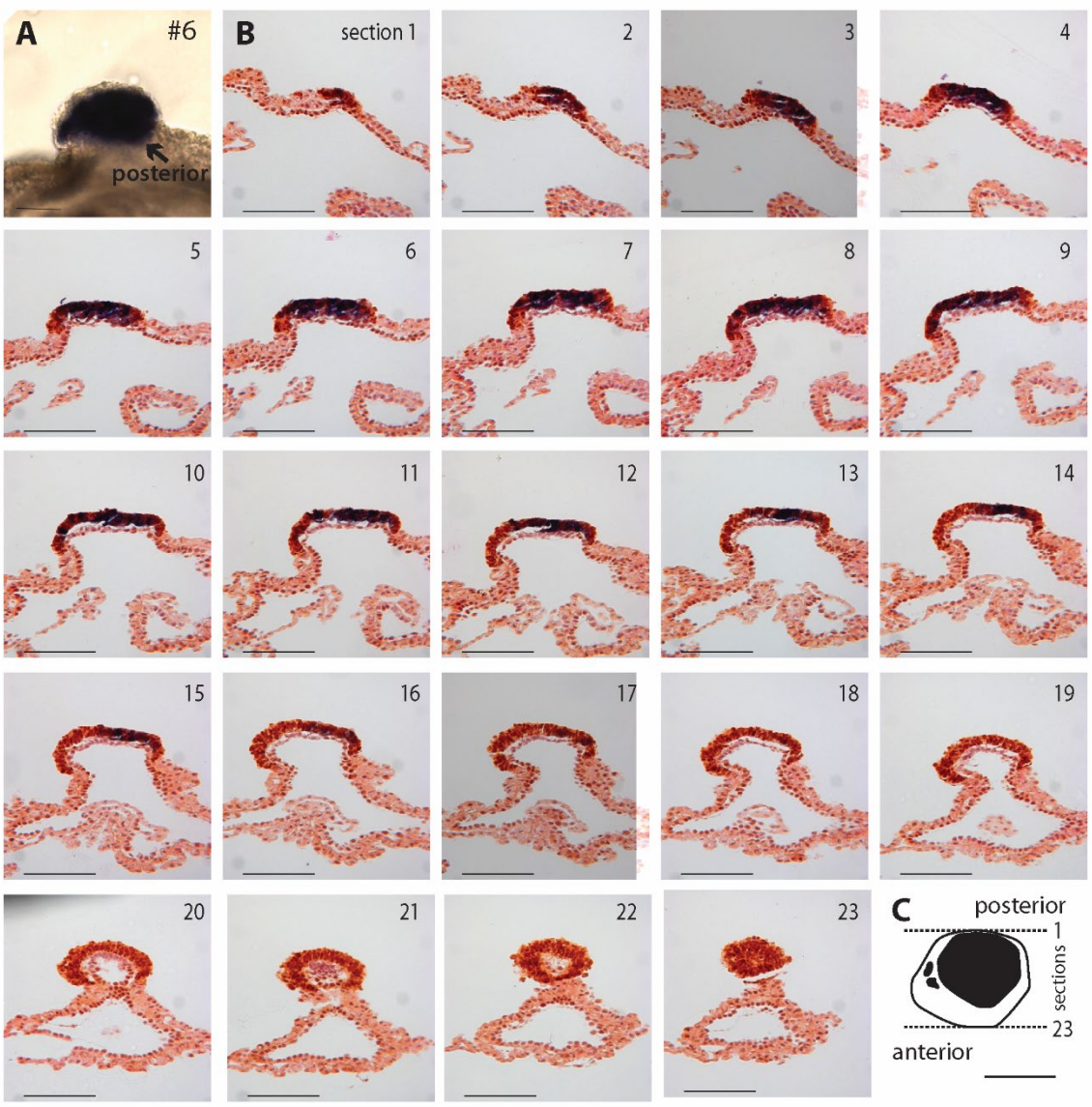

Fig. S3. BCL2-tg embryo #6 stained for BRACHYURY mRNA (dark blue) and OCT4 protein (brown). A. Rear/side whole mount view of E15 embryo #6 exhibiting an abnormal anterior extension of BRA expression. B. Series of 23 oblique transverse 8 µm sections as shown in panel C. C. Dorsal view-reconstruction of BRA stain (in black). Scale bar, 100 µm.

Table S1. Correlation between polar trophoblast fate and proamniotic cavity formation.

ORDER[1] Family	Species (*Genus*)	Polar TB kept?	Proamni-otic cavity	Amnion formation	Ref.
	EUARCHONTOGLIRES				
Primates					
Hominidae	Humans	Yes	Yes	Cavitation	(1, 2)
Cercopithecidae	Rhesus Macaque, Proboscis monkey, "Old world monkeys"	Yes	Yes	Cavitation	(2-4)
Callitrich/Ceb*idae*	Marmoset, Capuchin, "New world monkeys"	Yes	Yes	Cavitation	(4)
Tarsiidae	Tarsier	No	No[2,3]	Folding	(4, 5)
Lorisidae	Loris	No	No	Folding	(4, 6)
Galagidae	Bushbaby (*Galago*)	No	No	Folding	(7)
Dermoptera					
Cynocephalidae	Sunda colugo (*Galeopithecus*)	Yes	Yes	Cavitation	(8)
Lagomorpha					
Leporidae	Rabbit	No	No	Folding	(9)
Rodentia					
Muridae	Mouse, Rat	Yes	Yes	Folding	(9)
Cricetidae	Vole	Yes	Yes	Folding	(10)
Heteromyidae	Kangaroo Rat	Yes	Yes	Folding	(9)
Caviidae	Guinea Pig	Yes	Yes	Cavitation	(11, 12)
Chinchillidae	Chinchilla	Yes	Yes	Cavitation	(12)
Sciuridae	(13-striped-)/ Ground Squirrel	No	No	Folding	(13, 14)
Scandentia					
Tupaiidae	Tree shrew	No	No	Folding	(9),(15)
	LAURASIATHERIA				
Cetartiodactyla					
Bovidae	Cattle, Sheep	No	No[2]	Folding	(16, 17)
Cervidae	Deer	No	No	Folding	(18)
Suidae	Pig	No	No[2]	Folding	(19-21)
Carnivora					
Felidae	Cat	No	No	Folding	(22)
Canidae	Dog	No	No	Folding	(22, 23)

ORDER[1] Family	**Species (*Genus*)**	**Polar TB kept?**	**Proamni-otic cavity**	**Amnion formation**	**Ref.**
Mustelidae	Ferret	No	No	Folding	(24)
Pholidota					
Manidae	Pangolin	No	No	Folding	(9)
Perrisodactyla					
Equidae	Horse	No	No	Folding	(25)
Chiroptera					
Vespertilionidae	Vesper bats, African yellow bat	Yes	Yes	Cavitation	(9, 26, 27)
Miniopteridae	Natal clinging bat (*Miniopterus*)	Yes	Yes	Folding	(27-29)
Noctilionidae	Lesser bulldog bat	Yes	Yes	Cavitation	(30)
Phyllostomidae	Leaf-nosed bats (*Macrotus, Glossophaga, Carollia*)	Yes	Yes	Cavitation	(31-33)
Pteropodidae	Flying fox (*Pteropus*)	Yes	Yes	Cavitation	(9)
Eulipotyphla					
Erinaceae	Hedgehog (Erinaceus)	Yes	Yes	Cavitation	(34)
Soricidae	Shrew, Musk shrew *(Suncus)*	No	No	Folding	(35, 36)
Talpidae	Mole (*Talpa*)	No	No	Folding	(9)
	AFROTHERIA				
Afrosoricida					
Tenrecidae	Tenrec (*Hemicentetes*)	Yes	Yes	Cavitation	(37, 38)
Chrysochloridae	Golden mole (*Eremitalpa*)	No	No	Folding	(39)
Macroscelidea					
Macroscelicidae	Elephant shrew	Yes	Yes	Cavitation	(40)
	XENARTHRA				
Cingulata					
Dasypodidae	9-banded Armadillo	Yes	Yes	Cavitation	(41)

[1] Within orders, successive families are, where possible, arranged in increasing order of diversity.
[2] Cavitation within epiblast and/or between epiblast and Rauber's layer is very transient and disappears as Rauber's layer is lost.
[3] See section 37b, 39a and 40b of ref. (5) for examples of transient cavitation with cavities opening out to the uterine fluid.

Table S2. In vitro development to Day 7 of zona free nuclear transfer transgenic embryos (runs 1-4).

SCNT Run	*Cell Line*	*Eggs fused*	*2-Cell*	*Early Development[a] (%)*	*P[c]*	*Late Development[b] (%)*	*P[c]*
1	BCL2 5	69	67	28 (42)	0.98	22 (79)	0.32
	BCL2 51	70	65	29 (45)	1.00	19 (66)	1.00
	LacZ 7	73	69	30 (43)		19 (63)	
2	BCL2 5	95	94	71 (76)	0.07	34 (48)	1.00
	LacZ 1	99	98	61 (62)		30 (49)	
3	BCL2 53	57	50	31 (62)	0.84	14 (45)	0.32
	Lac Z 1	19	18	10 (56)		7 (70)	
4	BCL2 2	70	70	49 (70)	1.00	26 (53)	0.08
	BCL2 53	62	60	36 (60)	0.37	25 (69)	0.96
	LacZ 1	69	68	47 (69)		34 (72)	

a Number and percentage of cleaved embryos developing to at least morula stage;
b Number of grade 1 or 2 blastocysts and as a percentage of those embryos having developed to at least morula stages;
c Significance of difference between BCL2 lines and LacZ control lines as determined by Fisher's exact test.

Table S3. Extrinsic apoptosis pathway gene expression in Stage 4 and 5 manually dissected cattle embryos as determined by RNA sequencing (Ref. (42) with Stage 4 TB data not previously published); EPI, epiblast; TB, mural Trophoblast. Values are FPKM (Fragments Per Kilobase of transcript per Million mapped reads) and a value of one corresponds approximately to one transcript per cell.**Movie S1 (separate file).** Type or paste legend here.

Gene [a]	St.4 EPI [b]	St.5 EPI	St.4 TB	St.5 TB	Access. No.
FAS/FASL					
FAS ligand	**0.1**	**0.6**	1.5	0.1	NM_001098859
FAS	2.7	2.7	**14.0**	**34.9**	NM_174662
TRAIL/DR4 and 5					
TRAIL (TNFSF10)	**0.0**	**0.0**	0.0	0.0	NM_001319901
TNFRSF10a (Trail-R1/DR4)	0.0	0.0	**0.0**	**0.0**	XM_005210265
TNFRSF10b (Trail-R2/DR5)	19.2	18.0	**50.8**	**11.6**	NM_001102327
TNF/TNFR					
TNF	**0.1**	**0.1**	1.9	0.8	NM_173966
TNFR1 (TNFRSF1A)	18.4	15.6	**2.2**	**1.8**	NM_174674
TL1A/DR3					
TL1A (TNFSF15)	**4.0**	**0.4**	13.2	3.7	NM_001205782
DR3 (TNFRSF25)	0.2	0.1	**0.3**	**0.1**	NM_001144077
DISC					
FADD	19.8	2.5	**0.6**	**0.5**	NM_001007816
CFLIP	1.7	4.7	**4.9**	**10.9**	NM_001012281
CASP8	0.8	1.3	**3.1**	**3.3**	NM_001045970

a Ligand/receptor pairs are listed with ligand above receptor(s); DISC refers to the death-inducing signaling complex mediating the extrinsic apoptotic pathway that cannot be inhibited by BCL2 (42).
b The dissected stage 4 epiblast disc includes epiblast as well as the underlying visceral hypoblast.

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