Candidate number	

Exercise 1

Data Analysis

You are a veterinary pathologist in an interdisciplinary research group working on the pathogenesis of prostate cancer. Your particular task in the group is to translate research results from both the molecular level as well as pathological findings in human patients with prostate cancer to findings of a new cancer pathway in breast cancer recently discovered by another group at your university with which you are collaborating.

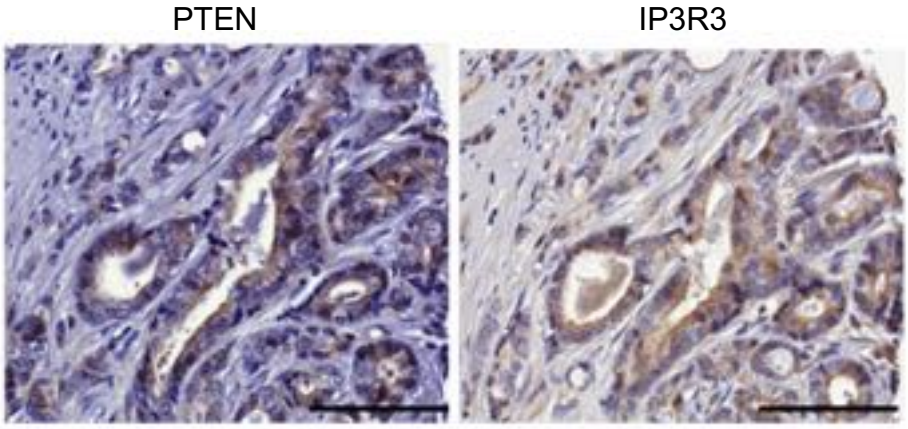


Figure 1. Representative immunohistochemistry (IHC) staining images of a human prostate tumour specimen with high levels of PTEN protein. Scale bars correspond to 50 µm.

Experiment 1

You decide to further investigate candidate proteins suggested by your collaborators to be important in the proposed cancer pathway for their relevance in PTEN+ prostate cancer. First you screen prostate tumors archived in the tissue bank of your group by IHC (Fig. 1). Eventually you decide to perform a small scale gene expression study of three tumors highly positive for PTEN.

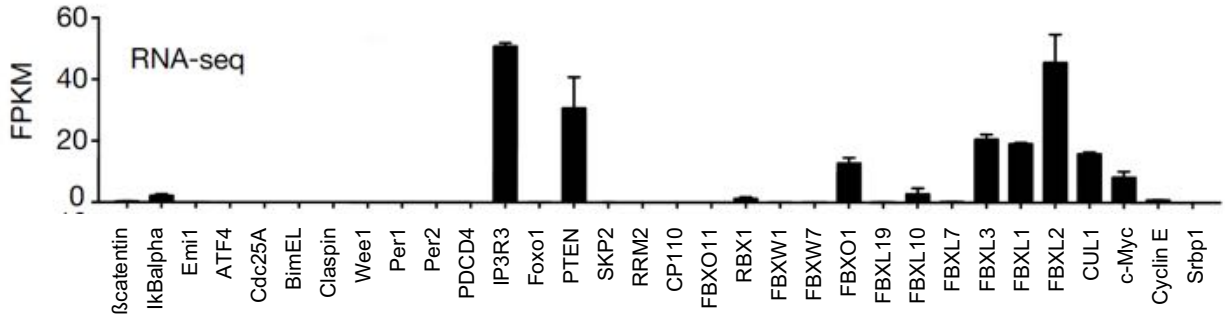


Figure 2. RNA sequencing analysis of PTEN+ prostate tumors. Only genes of interest are shown.

Candidate number	

E1 Question 1: Briefly outline the steps in the workflow of your next generation sequencing (NGS) experiment.

10p

RNA purification (1p)
Prepare cDNA from RNA by reverse transcription (2p)
Prepare DNA library by ligating adapters to both ends (1p)
Hybridize fragments to flow cell surface (1p)
Amplify to clonal cluster through bridge amplification (1p)
Use fluorescently labeled NTs and run sequencing cycles and image fluorescene (2p)
Align reads to reference sequence and quantify by bioinformatics software (2p)

(based on Illumina NGS)

To verify your expression data, you run a quantitative RT-PCR.

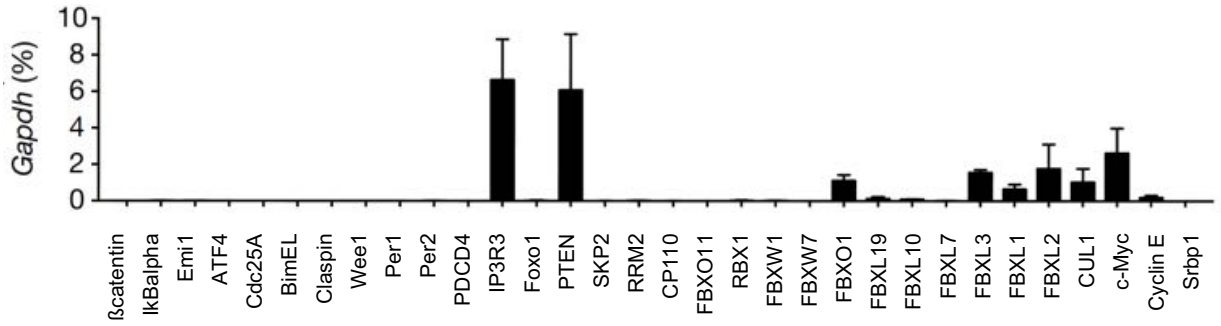


Figure 3. qRT-PCR analysis of indicated gene expression by SYBR-Green based assay.

E1 Question 2: What do you measure by quantitative RT-PCR?
5p

Abundance of mRNA in sample (5p)

E1 Question 3: Explain the principle of a TaqMan-based quantitative RT-PCR experiment. In particular, explain the differences to the technique using SYBR Green.
10p

TaqMan probes are oligonucleotides that have a fluorescent probe (2p) attached to the 5' end and a quencher (2p) to the 3' end. During PCR amplification the probe hybridize to the target sequences located in the amplicon. As polymerase replicates the template with TaqMan bound, it also cleaves the fluorescent probe due to polymerase 5'-nuclease activity. Because the close proximity between the quench molecule and the fluorescent probe normally prevents fluorescence from being detected through FRET (Förster resonance energy transfer) (2p) , the decoupling results in the increase of intensity of fluorescence proportional to the number of the probe cleavage cycles. Instead, SYBR Green is a cyanine dye (2p) that directly binds to double-stranded DNA of the PCR products and emits light upon excitation. While the TaqMan technique uses three oligos and has a higher specificity, SYBR Green only needs the two oligos for PCR amplification (2p).

Candidate number	

E1 Question 4: Why is it important to include reference genes ("housekeeping genes") in quantitative RT-PCR experiments?
5p

As in any quantitative study, it is necessary to normalize for sample to sample variations in order to obtain reliable results for which we use internal reference genes. (5p)

(The ideal reference gene is expressed at stable levels irrespective of tissue type, species, treatment, metabolism or sampling conditions. Examples are genes such as 18S (18S ribosomal RNA), ACTB (beta actin) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and multiple ones should be used)

E1 Question 5: Briefly summarize and interpret the results of Figures 1 - 3.

10p

PTEN and IP3R3 is present in epithelial cells of tumor specimen. Both genes are highly expressed in RNA-seq study together with FBXL2. However, in qPCR experiment, FBXL2 seems not to be highly expressed. In summary, the high expression of PTEN and IP3R3 can be confirmed with different method on RNA level and by IHC on protein level, whereas the results regarding the expression level of FBXL2 on the RNA level are contradictory.

E1 Question 6: How do you explain potential discrepancies between Fig. 2 and 3? Suggest 1 additional experiment to verify your findings. 10p

Most likely technical problem such as primer not ideal in qPCR, calculation mistake, etc. (5p)

Additional experiment:

IHC or IFA or WB (5p)

Candidate number	

Experiment 2

FBXL2 mediates the ubiquitination and subsequent proteasomal degradation of target proteins. From previous observations you know that serum starvation causes an increase and serum re-addition induced a decrease in Ca^{2+} mobilization. First, you examine the effect of FBXL2 on Ca^{2+} mobilization in normal human fibroblasts (NHF).

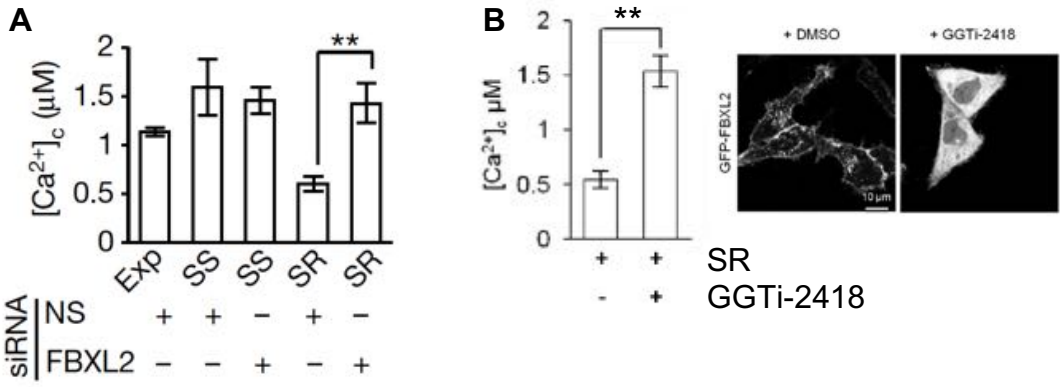


Figure 4. Concentrations of cytosolic Ca^{2+} ($[Ca^{2+}]_c$) were measured with aequorin in response to agonist stimulation (ATP) in NHFs (passage 2 and 3) exponentially growing (Exp), serum-starved (SS), or re-stimulated with serum (SR), which were transfected with an siRNA targeting FBXL2 or a non-silencing (NS) siRNA (**A**) or treated with geranylgeranyl transferase inhibitor GGTi-2418 which disrupts FBXL2 localization (insert) (**B**). Quantification of three independent experiments. ***p* < 0.001, calculated by one-way ANOVA and multiple-comparisons test. Error bars indicate s.e.m.

E1 Question 7: What molecule does siRNA interfere with?
5p

mRNA

E1 Question 8: What does p < 0.001 mean?
10p
p = probability; statistical test that when null hypothesis is true (no difference) how likely is it to obtain a result equal to (or more extreme than) what was actually observed (5p). In this case very unlikely (less than 1 of 1000 cases), therefore significant (rejection of null hypothesis) (5p).

E1 Question 9: Briefly summarize and interpret the results of Figure 4A.
10p
Re-stimulated NHF show a lower concentration of cytosolic calcium than serum-starved cells. When FBXL2 is knocked down by siRNA, there is a significant increase of cyctosolic calcium equivalent to the serum-starved condition (5p). FBXL2 is involved in calcium mobilization (5p).

Candidate number	

E1 Question 10: Why do you use two different assays (Figure 4 A and B) to study the effect of FBXL2 on calcium mobilization?
5p

To confirm results from experiment A on different level (protein level).

To verify your results, you decide to analyze the effect of FBXL2 on Ca^{2+} mobilization by gene knockout.

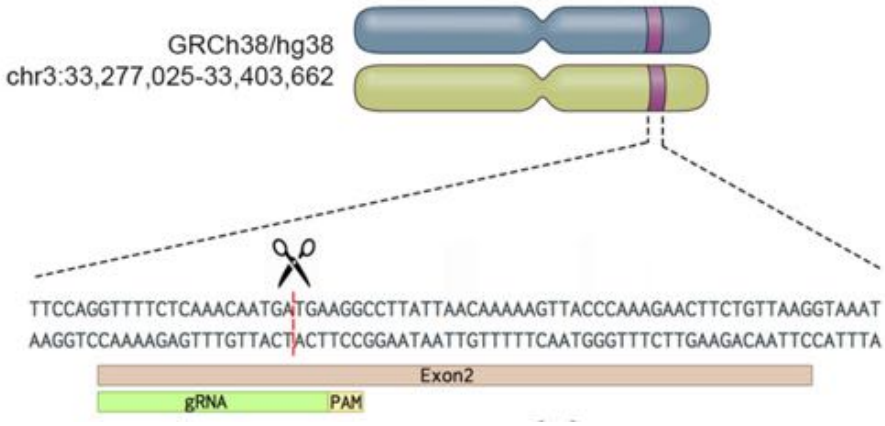


Figure 5. Schematic of the gene knockout strategy you are using.

E1 Question 11: What is the name of the technique you are using?
5p
CRISPR/CAS9 genome editing

E1 Question 12: Briefly explain the mechanism of this technique.

10p

The bacterial nuclease CAS9 facilitates a RNA guided (guide RNA) and sequence specific double strand break of the DNA in the nucleus of the target cell.

E1 Question 13: Name two alternative techniques you could use to engineer a FBXL2 null cell line.
5p

Zinc Finger
TALEN

Candidate number	

Exercise 2

Data Analysis

You are a molecular pathologist in a multidisciplinary research group whose research is focussed on the interaction of herpesviruses with the innate immune response of the host. Nucleic acids are potent triggers for innate immunity. Double-stranded DNA and RNA adopt different helical conformations, including the unusual Z-conformation. Z-DNA/RNA is recognized by Z-binding domains (ZBDs), which are present in proteins implicated in antiviral immunity. These include the Z-DNA binding protein 1 (ZBP1). ZBP1 has previously been identified as a cytoplasmic DNA sensor capable of inducing type I interferon expression and NF-κB activation. Your group is interested in the mechanism how ZBP1 activates the NF-κB pathway.

Experiment 1: First you test the importance of the Z-domains of ZBP1 for the induction of the NF-κB pathway.

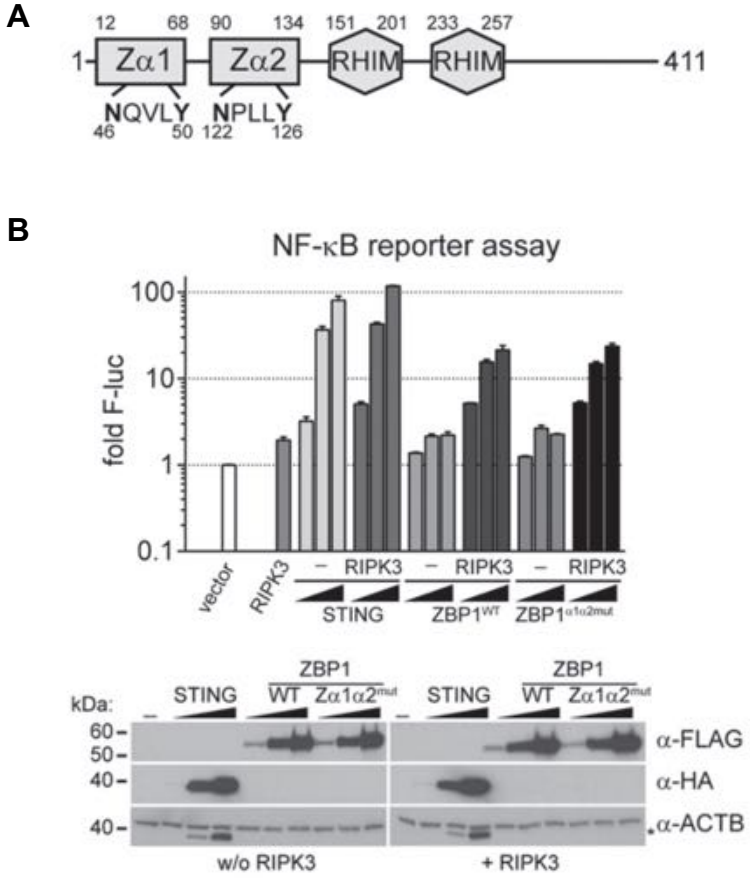


Figure 1. (A) Domain architecture of mouse ZBP1 with two N-terminal ZBDs (Zα1 and Zα2) and two RIP homotypic interaction motifs (RHIMs). (**B**) HEK293T cells were transfected with 50 ng NF-κB Firefly luciferase and 25 ng Renilla luciferase reporter plasmids, together with expression vectors for Receptor-interacting serine/threonine-protein kinase 3 (RIPK3; 0.2 ng)

and HA-tagged STING (Stimulator of Interferon Genes) or ZBP1-3xFLAG-tagged (20, 100, 500 ng) Wildtype (WT) or with mutated key conserved residues N46A and Y50A mutations into Zα1, and N122A and Y126A substitutions into Zα2 (Zα1α2mut). Luciferase activity was measured after 24 h, and the ratio of Firefly and Renilla luciferase was set to 1 for control cells transfected with empty vector. Cell lysates were analysed for expression of the indicated proteins by Western blot (bottom). Asterisk (*) indicates residual signal from the α-HA antibody.

E2 Question 1: What do you measure by Western blot and why is this experiment important in the context to your NF-κB luciferase reporter assay? 10p

The protein expression of STING and ZBP1 is measured (5p) to control for the amount of protein produced due to different plasmid quantities transfected which should correlate with the luciferase intensity measured (5p).

E2 Question 2: Why do you analyze ß-actin? 5p

Loading control of total protein

E2 Question 3: Briefly summarize and interpret the results of Fig 1B. 10p

Luciferase assay to quantifiy the influence of different proteins on the NFkB pathway (2p). The plasmid vector it set as baseline. STING in different concentrations is used as control to show that the reporter can be activated and is sensitive to concentration changes (2p). RIPK3 itself has no significant influence on reporter; also ZBP1 wildtype and mutated Z domains with no influence (2p). However, the combination of ZBP1 and RIPK3 activates the reporter in a dose dependent manner of ZBP1 (2p). This effect is not dependent on the Z domains (2p).

Next, you want to find out more about the RHIM domains of ZBP1. From previous experiments, you already know that ZBP1 interacts with RIPK3 which also contains a RHIM domain. By sequence comparison, you have identified two further candidate proteins namely RIPK1 and TIR-domain-containing adapter-inducing IFN-beta (TRIF) with RHIM domains and want to find out if they also interact with ZBP1. Notably, RIPK1 and RIPK3 are both key proteins in the necroptosis pathway.

E2 Question 4: Outline one experiment you would perform to find out whether ZBP1 interacts with RIPK1 and TRIF. 5p

FRET, Y2H, CoIP or other.
For CoIP: Extract protein and use antibody against ZBP1 to pull down ZBP1 (bait) and potential binding partners by magnetic beads. Run Western Blot with anti RIPK1 and TRIF to check for presence.

Candidate number	

E2 Question 5: Explain the basic principle of necroptosis? 5p

Caspase 8 independent cell death pathway with loss of ATP, swelling of cell and loss of cell and organelle integrity (Robbins p59)

E2 Question 6: Name one downstream key effector protein or molecule each for necroptosis and pyroptosis. 10p

Necroptosis:

MLKL or RIPK1, RIPK3 (5p)

Pyroptosis:

IL1 (5p)

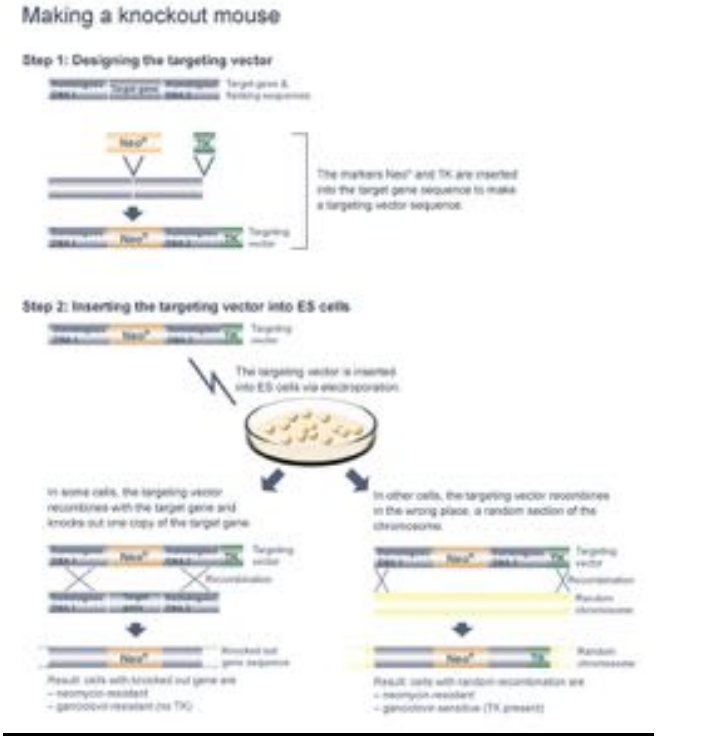
You reason that ZBP1 might also play an essential role in necroptosis and decide to perform another experiment.

Candidate number	

Experiment 2

To test the importance of ZBP1 in necroptosis in relation to RIPK1 you first decide to generate a RIPK1 knockout mouse.

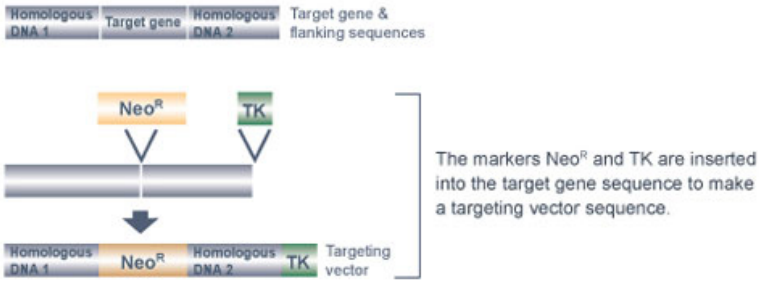
<u>E2 Question 7</u>: Give a short summary of critical steps needed to make a knockout mouse by the introduction of a targeted gene deletion into the mouse germline. Alternatively, you can also explain a CRISPR/Cas9-based approach to make a knockout mouse. 10p



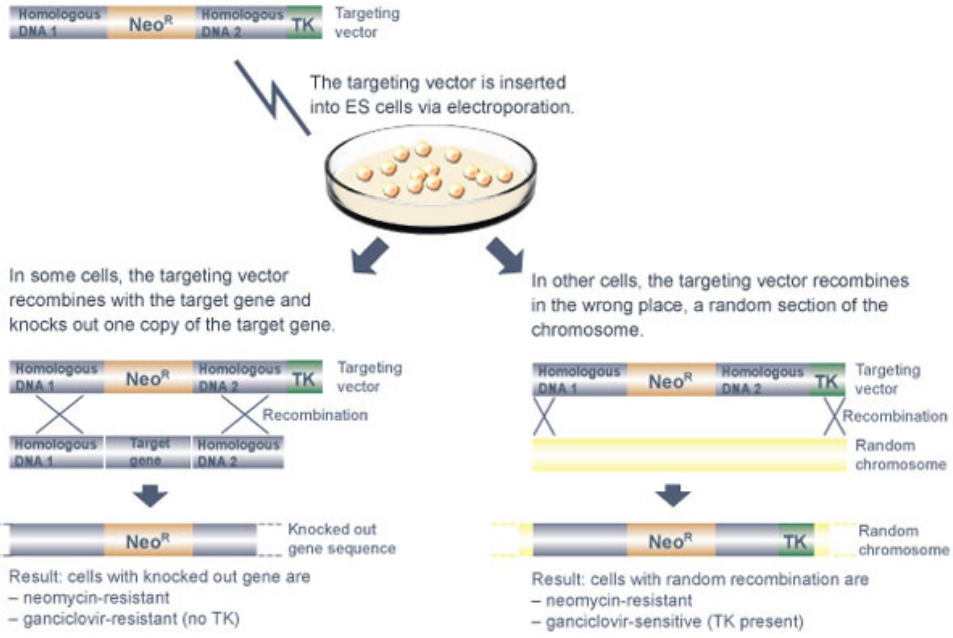
Images, tables and data are from multiple sources and are partly altered to serve the educational purpose of this mock exam. All material is intended for internal usage during Summer School 2017 only and NOT to be distributed.

Making a knockout mouse

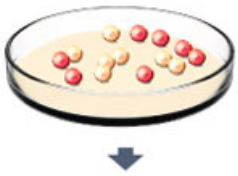
Step 1: Designing the targeting vector



Step 2: Inserting the targeting vector into ES cells

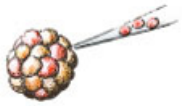


Step 3: Selecting cells



Only the cells that have successfully incorporated the targeting vector into the target gene survive in the presence of neomycin and ganciclovir (shown in red).

Step 4: Injecting cells into a new embryo



Cells containing the targeting vector are then selected and injected into a normal developing mouse embryo.

Step 5: Breeding

The resulting chimeric (spotted) mouse contains a mix of its own cells and the heterozygous knockout cells. This mouse is bred with a normal (white) mouse.

Among their offspring are mice that are capable of passing the knocked-out gene to their own offspring.

Candidate number	

Unfortunately, the homozygous RIPK1$^{-/-}$ mutation resulted in embryonic death. To overcome this problem, you make a tissue-specific conditional RIPK1 mutation. For this purpose, you use the promoter of the gene Keratin 14, which is expressed in epithelial cells.

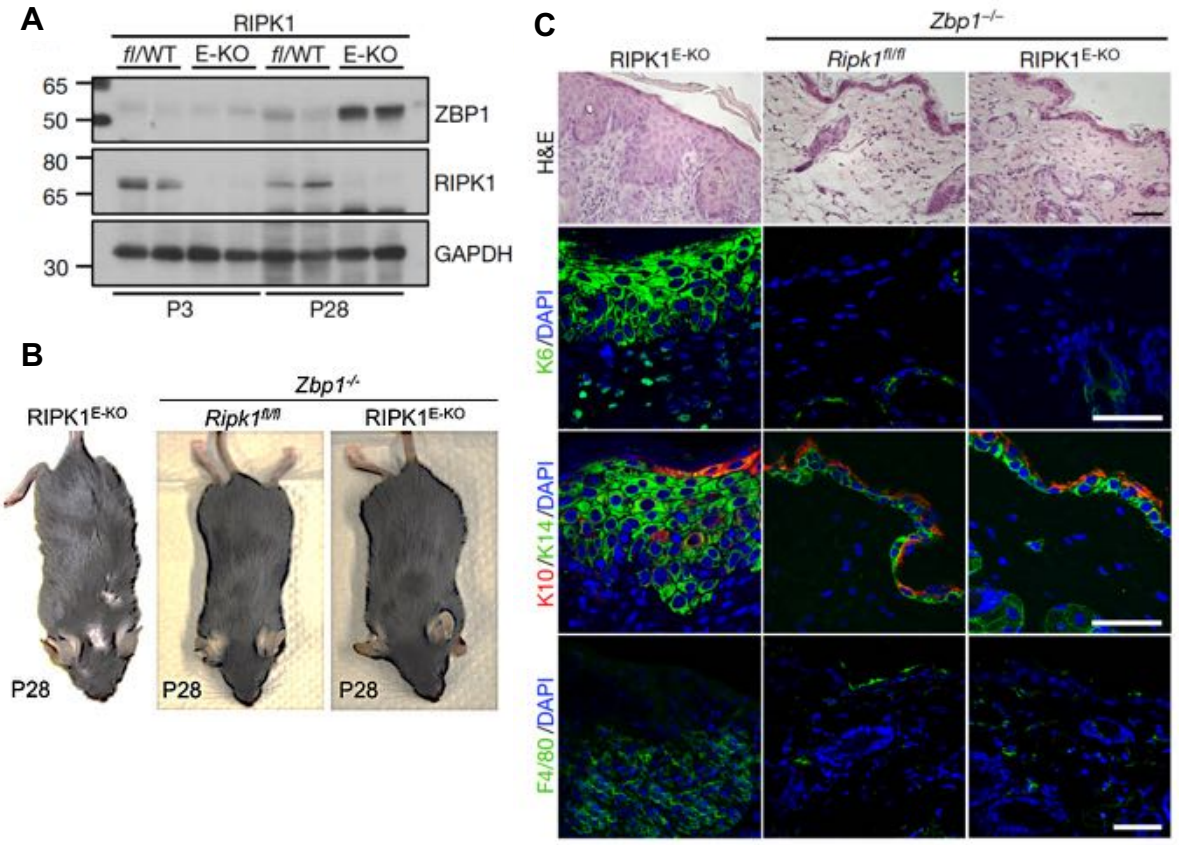
E2 Question 8: Indicate the system that can be used to generate a conditional RIPK1 mutation. 5p

Cre (loxP)-system

E2 Question 9: Explain the basic principle of the system including the use of the Keratin 14 promoter. 10p

Gene of interest is floxed on both alleles in mouse (5p). Crossing with Cre-mouse in which Cre is under K14 promotor disrupts GOI only in tissue where promotor is active (epithelium) (5p).

After you have successfully generated *Ripk1$^{fl/fl}$K14-cre$^{Tg/WT}$* mice (hereafter referred to as RIPK1$^{E\text{-}KO}$ mice) you realize that the mice develop skin inflammation (Fig 2B, C) which can be attributed to RIPK3 dependent keratinocyte necroptosis. To analyse the role of ZBP1, you cross them with *Zbp1$^{-/-}$* mice you got from a collaborator.



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Candidate number	

Figure 2. (**A**) Immunoblot analysis of ZBP1, RIPK1 and GAPDH in epidermal protein extracts from wild type (Ripk1$^{fl/WT}$) and RIPK1^{E-KO} mice at postnatal day 3 (P3) and P28. Lanes represent samples from individual mice. (**B**) Photographs of mice with the indicated genotypes at the age of 4 weeks. Images shown are representative of n ≥ 60 RIPK1^{E-KO} and n ≥ 40 RIPK1^{E-KO} *Zbp1*$^{-/-}$ mice. (**C**) Skin sections from 4 week old mice were stained with H&E or immunostained with the indicated antibodies. Representative images shown. Scale bars, 50 µm.

Before your double knockout mice (RIPK1^{E-KO} *Zbp1*$^{-/-}$) are ready for analysis, you establish and validate primary and secondary fluorescent antibodies that you want to use to analyse skin sections of your mice (Fig. 2C).

E2 Question 10: Briefly explain the functional principle of fluorescence vs. bioluminescence. 5p

Fluorescence is the emission of light after activation with light, whereas bioluminescence is a chemical reaction in which energy is emitted as light.

E2 Question 11: Name two fluorescent proteins often used to tag proteins of interest.

5p

GFP
RFP

E2 Question 12: Summarize and interpret the results of Figure 2.
10p

ZBP1 is increased in epithelium of RIPK1-KO mice at day 28 (2p). In the presence of ZBP1 the RIPK1-KO mice show inflammatory phenotype (necroptosis) (2p) which is absent when also ZBP1 is knocked out (2p). The necroptosis phenotype shows hyperkeratosis, thickening of epidermis and infiltration with increased numbers of F4/80+ cells (macrophages) (2p). In summary, the absence of RIPK1 induces necroptosis which can be blocked when ZBP1 is also absent (2p).

E2 Question 13: Based on Figure 2, give one hypothesis about the role of ZBP1 in necroptosis.
5p

ZBP1 plays important role in necroptosis pathway and is directly of indirectly linked to RIPK1.

E2 Question 14: Outline one experiment you would want to perform to support your hypothesis. 5p

Examples: Repeat experiment with RIPK3 KO mice. Do PPI experiment with ZBP1 to find binding partners. RNAseq study KO vs WT to find pathway changes.

Candidate number	

Exercise 3

Abstract

You are an experienced pathologist with an expertise in intestinal diseases of cattle. Today you received an email by the editor of a new journal in which you are asked to review a recently submitted manuscript. Before you decide whether you accept or decline the invitation you read the abstract of the manuscript.

Nlrp9b inflammasome restricts rotavirus infection in intestinal cells

by Dr. Zhu Whu et al.

Bovine Rotavirus, a non-enveloped DNA virus is the leading cause of severe gastroenteritis and diarrhoea in young children and calves and accounts for around 215,000 infant deaths annually worldwide. Rotavirus specifically infects the intestinal crypt cells in the host small intestine and has evolved strategies to antagonize interferon and NF-κB signalling, raising the question as to whether other host factors participate in antiviral responses in intestinal mucosa. Previous studies suggested that anti-rotavirus antibody isotype switching depends on Il-1β and Il-18, and that recombinant Il-18 enhances rotavirus infection. However, the precise role of inflammasome signalling that mediates the maturation of these chemokines in the context of enteric virus infections and the mechanism by which enteric viruses are sensed and restricted *in vivo*, especially by NOD-like receptor (NLR) inflammasomes, is largely unknown. Here we mechanistically characterize by a hypothesis-driven forward genetic CRISPR screen the intracellular NLR Nlrp9b that is specifically expressed in intestinal goblet cells and restricts rotavirus infection. Notably, rotavirus infection potently induced caspase-1 (Casp1) p10 cleavage, indicative of inflammasome activation. In addition, mice deficient in Asc or Casp1, two universal inflammasome components, exhibited higher viral loads in the intestine, increased fecal shedding of viral antigens, and more frequent incidences of diarrhoea compared to their wild-type littermates, suggesting that inflammasome signalling protects against rotavirus infection. The heightened susceptibility of Asc+/− mice to rotavirus infection implies a role of certain NLR(s) in this antiviral response. Furthermore, our Southern blot data show that, via RNA helicase Dhx9, Nlrp9b recognizes short double-stranded RNA stretches and forms inflammasome complexes with the adaptor proteins Asc and caspase-1 to promote the maturation of interleukin (Il)-18 and gasdermin D (Gsdmd)-induced pyroptosis. Conditional depletion of Nlrp9b or other inflammasome components in the intestine *in vivo* resulted in enhanced susceptibility of mice to rotavirus replication. Our study highlights an important adaptive immune signalling pathway and may present useful targets in the modulation of host defences against viral pathogens.

Candidate number	

E3 Question: Identify 10 scientific mistakes in the abstract and briefly explain why they are wrong. Each answer 10p (total 100p)

1. RNA virus, not a DNA virus

2. Bovine rotaviruses do not cause disease in children.

3. Rotaviruses infect cells on the apical half (ruminants) in the jejunum and ileum (Jubb Vol 2, p151).

4. Il18 reduces rotavirus infection.

5. Afore mentioned proteins are cytokines not chemokines.

6. One cannot mechanistically characterize a protein by a broad CRISPR screening assay. Reverse genetics is used for hypothesis-driven research.

7. Rotaviruses infect the absorptive enterocytes and ony occasionally goblet cells (Jubb Vol 2, p151). Since Nlrp9b is an intracellular protein as stated before, it is unlikely that it restricts rotavirus infection of enterocytes?

8. Asc+/- are not full Asc KO mice and are likely not sufficient to show a phenotype of increased susceptibility.

9. Northern or Western blot would be correct. Southern blot is used for DNA.

10. The inflammasome is part of the innate immunity

Candidate number	

Exercise 4

Toxicologic Pathology Study

The objective of this experimental work is to investigate the side effects induced by a new drug (Berlanin) that is expected to treat hypocorticism.

Berlanin was given orally to female Sprague Dawley rats at 25 mg/kg/day for 5, 10 and 17 days. Control female rats were treated with the vehicle only.

Day of sacrifice	**Day 5**	**Day 10**	**Day 17**
Number of control females	N=12	N=12	N=12
Number of treated females (dose)	N=12 (25 mg/kg/day)	N=12 (25 mg/kg/day)	N=12 (25 mg/kg/day)

Animals were sacrificed, organs were sampled, weighed and preserved.
Anatomic pathology was done on a selection of organs. In addition to formalin fixation, snap-frozen samples of these organs were taken and stored in -80 °C.

Organ weights are presented in Table 1 on pages 18-20. Animal body weights remained constant during the entire length of the study period.

Candidate number	

E4 Question 1: Based on Table 1 describe the treatment-related organ weight changes and give hypotheses regarding some likely associated histopathological changes. (total 15 points)

adrenal:

- Mean absolute adrenal weights were **statistically significantly increased** in treated animals on Day 10 and Day 17 when compared to control animals **(2 pts)**
- the magnitude of this increase seemed **proportional to the duration of Berlanin** administration **(2 pts)**
- Berlanin may stimulate the synthesis of corticoids and provoke **adrenal cortical hypertrophy;** also accept other hypertrophic changes in the adrenal gland **(1 pt)**

(max. 5 pts)

liver:

- Mean absolute liver weights were **statistically significantly increased** from Day 5 onwards when compared to control animals **(2 pts)**
- the magnitude of this increase seemed **proportional to the duration of Berlanin administration (2 pts)**.
- This might be due to **enzyme induction or any storage** (glycogen, lipids,...) **(1 pt).**

(max. 5 pts)

ovary:

- Mean absolute **ovary weights were statistically significantly increased** in treated animals on Day 10 and Day 17 when compared to control animals **(2 pts)**
- the magnitude of this increase seemed **proportional to the duration of Berlanin administration (2 pts)**
- This might be due to **cysts**, **increased production of follicles**, **blockade of maturation, or hypertrophy of any cell compartment**,...**(1 pt)**

(max. 5 pts)

Candidate number	

Table 1

Group comparison statistics for absolute organ weights (g)

Organ:	adrenal	control	test	Day 5
	Dosage mg/kg	0	25	
	number / group	12	12	
	mean	0.063	0.067	
	standard deviation	0.015	0.017	
	group difference P=0.05		0.013	
	group difference P=0.01		0.018	

Analysis of variance: F ratio = 0.46; Df=1/22; F probability= 0.51

Note: a * indicates group mean is significantly different from control at level of significance shown

Organ:	adrenal	control	test	Day 10
	Dosage mg/kg	0	25	
	number / group	12	12	
	mean	0.069	0.083	
	standard deviation	0.011	0.017	
	group difference P=0.05		0.012*	
	group difference P=0.01		0.016	

Analysis of variance: F ratio = 5.76; Df=1/22; F probability= 0.024

Note: a * indicates group mean is significantly different from control at level of significance shown

Organ:	adrenal	control	test	Day 17
	Dosage mg/kg	0	25	
	number / group	12	12	
	mean	0.067	0.101	
	standard deviation	0.007	0.014	
	group difference P=0.05		0.01*	
	group difference P=0.01		0.014*	

Analysis of variance: F ratio = 0.46; Df=1/22; F probability= 0.51

Candidate number	

Note: a * indicates group mean is significantly different from control at level of significance shown

Table 1

Organ:	liver	control	test	Day 5
	Dosage mg/kg	0	25	
	number / group	12	12	
	mean	8.64	9.4	
	standard deviation	0.99	0.65	
	group difference P=0.05		0.71*	
	group difference P=0.01		0.96	

Analysis of variance: F ratio = 5.13; Df=1/22; F probability= 0.032

Note: a * indicates group mean is significantly different from control at level of significance shown

Organ:	liver	control	test	Day 10
	Dosage mg/kg	0	25	
	number / group	12	12	
	mean	8.8	10.97	
	standard deviation	0.87	1.56	
	group difference P=0.05		1.07*	
	group difference P=0.01		1.45*	

Analysis of variance: F ratio = 17.8; Df=1/22; F probability= 0.000

Note: a * indicates group mean is significantly different from control at level of significance shown

Organ:	liver	control	test	Day 17
	Dosage mg/kg	0	25	
	number / group	12	12	
	mean	9.75	12.7	
	standard deviation	1.01	1.57	
	group difference P=0.05		1.12*	
	group difference P=0.01		1.52*	

Analysis of variance: F ratio = 30.19; Df=1/22; F probability= 0.000

Candidate number	

Note: a * indicates group mean is significantly different from control at level of significance shown

Table 1

Organ:	ovary	control	test	Day 5
	Dosage mg/kg	0	25	
	number / group	12	12	
	mean	0.078	0.087	
	standard deviation	0.011	0.028	
	group difference P=0.05		0.019	
	group difference P=0.01		0.027	

Analysis of variance: F ratio = 1.06; Df=1/22; F probability= 0.32

Note: a * indicates group mean is significantly different from control at level of significance shown

Organ:	ovary	control	test	Day 10
	Dosage mg/kg	0	25	
	number / group	12	12	
	mean	0.089	0.11	
	standard deviation	0.011	0.018	
	group difference P=0.05		0.012*	
	group difference P=0.01		0.017*	

Analysis of variance: F ratio = 11.09; Df=1/22; F probability= 0.003

Note: a * indicates group mean is significantly different from control at level of significance shown

Organ:	ovary	control	test	Day 17
	Dosage mg/kg	0	25	
	number / group	12	12	
	mean	0.093	0.11	
	standard deviation	0.011	0.04	
	group difference P=0.05		0.026*	
	group difference P=0.01		0.038	

Analysis of variance: F ratio = 30.19; Df=1/22; F probability= 0.000

Note: a * indicates group mean is significantly different from control at level of significance shown

Candidate number	

E4 Question 2: Using Table 2, on a selection of organs sampled on study day 17 and observed in control versus treated animals, outline the major treatment-related histopathological changes at the end of the study. (total 25 points)

- At the end of the study, there was a minimal to mild adrenal cortical hypertrophy in all treated animals **(5 pts).**
- There was also a minimal to mild hepatocellular hypertrophy in 8/12 treated animals. **(5 pts)**
- An increased secretory activity of the mammary gland was related to treatment (10/11 treated animals versus 3/12 controls) **(5 pts)** and
- ovarian cysts were noted in a majority (9/12) of treated rats whereas these were only occasional in control females (1/12).**(5 pts)**
- Administration of Berlanin also induced estrus in 11/12 females.**(5 pts)**

Also accept instead of exact numbers of animals statements such as “most”, “majority” etc.

Candidate number	

lesion incidence summary with expanded severity levels

		control	test	day 17
no in group		12	12	
Adrenal				
Hypertrophy, cortex, diffuse				
	none	12	0	
	minimal	0	3	
	mild	0	9	
	total	12	12	
Liver				
Infiltration, mononuclear cells				
	none	10	12	
	minimal	2	0	
Hypertrophy, hepatocellular				
	none	12	4	
	minimal	0	7	
	mild	0	1	
	total	12	12	
Mammary gland				
Secretory activity				
	none	9	1	
	present	3	10	
	total	12	11	
Ovary				
cyst				
	none	11	3	
	present	1	9	
	total	12	11	

Candidate number	

Table 2

lesion incidence summary with expanded severity levels

		group		
		no.	12	12	all females
		dosage mg/kg	0	25	
Uterus					
	proestrus				
		none	11	12	
		present	1	0	
	estrus				
		none	9	1	
		present	3	11	
	metestrus				
		none	8	12	
		present	4	0	
	dietestrus				
		none	8	11	
		present	4	1	
		total	12	12	

Candidate number	

E4 Question 3: How do the histopathological findings correlate to organ weight changes?
(total 15 points)

- Increased liver weights are correlated to liver hypertrophy. (enzyme induction or peroxisome proliferation are most probable causes) **(5 pts)**
- Increased ovary weights are correlated to ovarian cysts. **(5 pts)**
- Increased adrenal weights correspond to adrenal cortical hypertrophy. **(5 pts)**

You decide to use the snap-frozen pituitary glands to investigate selected hormones. For this purpose you want to run a ”sandwich ELISA” that is commercially available.

E4 Question 4: What do you measure with a “sandwich ELISA”?

(total 5 points)

enzyme-linked immunosorbent assay: an antibody technique for accurately measuring the amount of a protein, e.g. hormone

E4 Question 5: Briefly outline the principle of a “sandwich ELISA”.

(total 13 points)

one antibody captures antigen to plate (4 pts), another antibody detects antigen (4pts) and it is then bound by an enzyme-linked secondary antibody (2 pts); the substrate is added that is converted by enzyme to detectable form enzyme-linked immunosorbent assay (3 pts)

Candidate number	

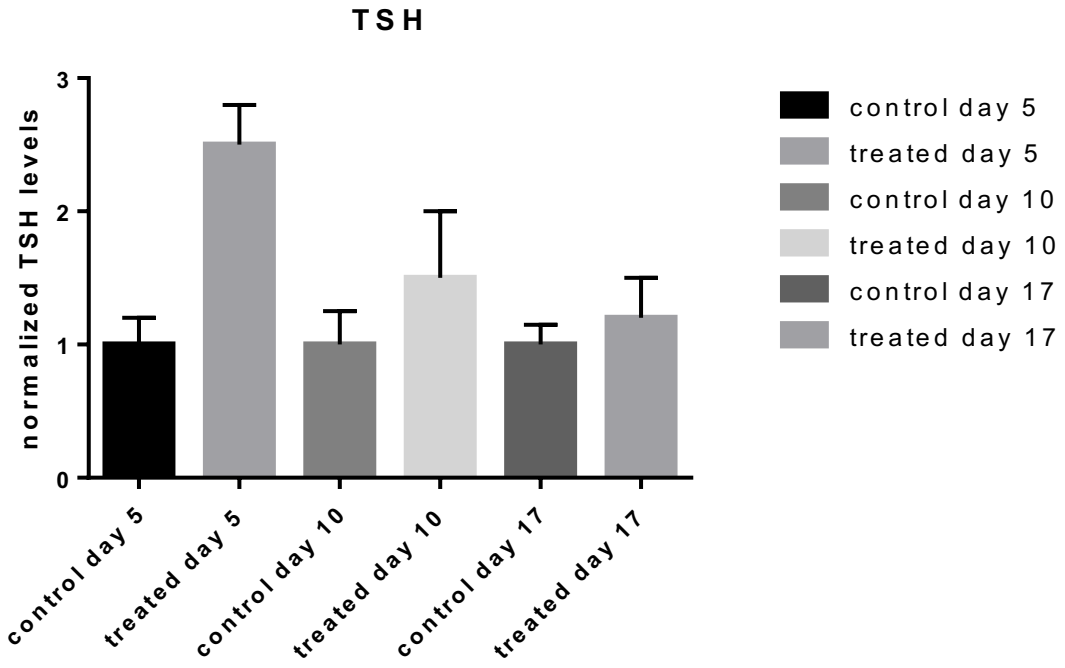


Figure 1: Relative expression of normalized Thyroid Stimulating Hormone (TSH) of pituitary glands on different sacrifice times for control versus treated animals. Presented is the ratio of TSH to actin as reference.

Figure 1 shows the results for Thyroid Stimulating Hormone (TSH).

E4 Question 6:

12p

1. **Summarize and interpret the ELISA results (Figure 1).**

TSH protein levels are clearly increased in the pituitary cells on Day 5 (3p) and the levels drop on days 10 and day 17 (3p);

you can also mention that it is questionable whether day 10 is significant, and that a statistical test should be included to show significance levels.

2. **Give a hypothesis how they could be linked to the previous findings in the liver.**

Increased liver catabolism leading to low blood levels of thyroid hormones (3p), therefore stimulation of synthesis of TSH synthesis (3p) in the pituitary following a feed-back mechanism

Candidate number	

E4 Question 7: What morphological change would you expect in the thyroid gland?

5p

follicular epithelium hypertrophy (diffuse) or hyperplasia

In additional experiments Berlanin was eventually shown to alter the synthesis of thyroid T3 hormone, to modify the synthesis of glucocorticoids, and to interact with the synthesis of sexual steroids. Thus, Berlanin lacks specificity to treat hypocorticism, unfortunately.

E4 Question 8: State a hypothesis to explain this lack of selectivity.

10p

steroid (sexual/gluco) and T3 receptors belong to the same superfamily of nuclear (hormone) receptors. Berlanin is probably a non-selective modulator of these receptors

Candidate number	

Exercise 5

A clinical/forensic case

Introduction:

You are very excited to be in Berlin, taking part at the ECVP Summer School 2017. You decided to travel with your dog Molly and board her during the day. Luckily you were able to find a boarding kennel nearby. *Happy Dog* is located just a couple of blocks from the seminar venue in Berlin-Zehlendorf. What you liked most about *Happy Dog* is that they take dogs for long walks to the close forest named *Grunewald* which has several lakes so the dogs can swim and run free.

Figure 1. Map of Berlin-Zehlendorf with walking route on July 25th around *Lake Schlachtensee* in *Grunewald Forest*. Insert on the right shows location of *Lake Schlachtensee* in *Grunewald Forest* and location of *Lake Tegel* (Tegeler See).

On the 25th of July, while you are in the middle of a mock exam in Comprehensive Pathology you receive a disturbing phone call from the secretary of *Happy Dog* and are asked to pick up your dog asap. You arrive at their office and spot a family with two daughters standing next to their minivan lifting something big into the trunk of the car which appears to be the body of a Golden Retriever wrapped in a blanket. You hear them talking in German and don't immediately understand what has happened. After you picked up Molly – who luckily is safe and sound - you learn that the family's dog had just died. After a long stroll around *Lake*

Candidate number	

Schlachtensee (Fig. 1) together with Molly and the other boarded dogs, the Golden Retriever collapsed and died with massive hemoptysis and respiratory failure in the ICU of Dr. Wonderful's Mobile Vet Service that was called to the scene at *Lake Schlachtensee*. After you have talked for several minutes with the family, you can convince them to further investigate the case and recommend a necropsy at the Insitute of Veterinary Pathology in Berlin. The family's oldest daughter, which is a first-year medical student, insists that you keep her informed about the necropsy results which you promise to do. You learn that the dog had a clean medical history and no observed clinical signs prior to death. However, the daughter tells you that she had the impression that her dog was more quiet over the past few days, but she interpreted it with the adjustment to the new dog walking service *Happy Dog* they only switched to recently.

Back at the hotel in the evening you sit together with your fellow pathologists at the bar and talk about what happened today. You learn from the waiter at the bar that multiple dogs mysteriously had died at *Lake Tegel* (Tegeler See) in the past few weeks: "It was all over the news. Very sad". You search the internet to find out what has been reported. Most is written in German but you find a podcast from a radio station in Berlin and listen to it:

http://bit.ly/radiospaetkauf

Note: If the embedded file does not play, try the link. Only listen to the first radio feature. To answer the questions below you do not need to listen to the podcast.

E5 Question 1: If the dog was poisened by cyanobacterial toxins which are the histopathological lesions you expect to find? Name two.
10 p

Hepatocellular necrosis, apoptosis, perisinusoidal hemorrhage (5p)

Necrosis of renal tubules (5p)

(Jubb Vol 2 p330)

E5 Question 2: Name two organs that frequently show lesions after intoxication with phosphorus such as it is present in rodenticides.
10p
Liver (5p)

Kidney (5p)

Also: heart, GIT (Jubb Vol 2 p332)

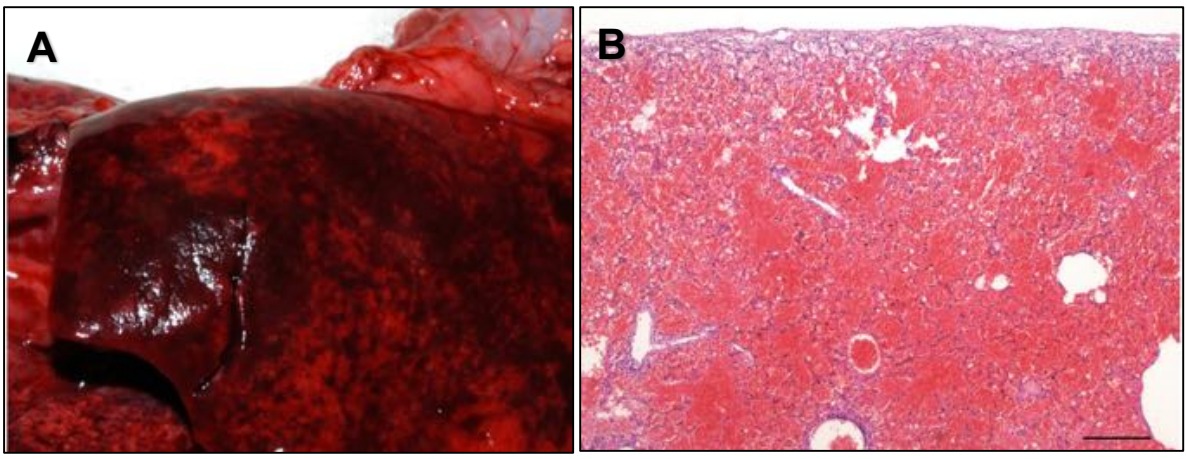
Candidate number	

Table 1: Haematology, blood chemistry and urine parameters taken before the death of the dog.

Value	Reference	
HCT	**29%**	**(40-55)**
WBC	**28.9 x 10^9/l**	**(6-10)**
PLT	**111 x 10^9/l**	**(150-400)**
PT	**8.9 s**	**(6.3-8.5)**
PTT	**25.0 s**	**(9.6-16.1)**
Na^+	**136 mmol/l**	**(144-155)**
K^+	**4.9 mmol/l**	**(4.1-5.3)**
P_i	**4.7 mmol/l**	**(0.93-1.93)**
Urea	**68.9 mmol/l**	**(3.5-11.1)**
Creatinine	**986 µmol/l**	**(53-120)**
Bilirubin	**4.5 µmol/l**	**(0.6-4.3)**
ALT	**130 IU**	**(24-124)**
AST	**59 IU**	**(20-73)**
Glucosuria ++		
Proteinuria +		
Bacteria in urine: Negative		

Necropsy

A necropsy was performed the next day. Macroscopical lesions were only observed in the lung of the dog. Lungs were wet and heavy and had a dark-red colour (Fig. 2A). Samples of the lung and all other internal organs were fixed in formalin for histopathological evaluation. Histopathological lesions were only present in lungs and kidneys.



Candidate number	

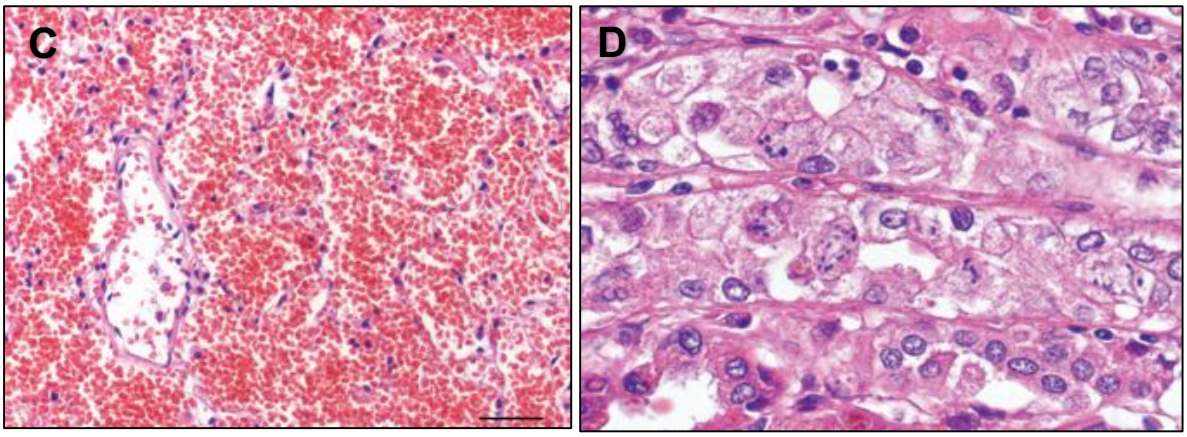


Figure 2. Lung at necropsy (**A**) and HE stainings of lung (**B** and close-up, **C**) and kidney (**D**) tissue

E5 Question 3: Give a list of five etiological differential diagnoses for the macroscopic findings of the lung as shown in Fig 2A.

15p

1. Trauma (3p)

2. Anticoagulant rodenticide toxicity (3p)

3. Sepsis (3p)

4. Disseminated intravascular coagulation (3p)

5. Leptospirosis (3p)

Also: Thrombocytopenia, bronchopneumonia, drug reactions, ruptured pulmonary arterial aneurysms (Jubb Vol 2 p490)

E5 Question 4: Evaluate the lung and kidney histology (Figure 2) and provide morphologic diagnoses (no description):
10p
Morphologic diagnosis Fig. 1C:

Lung: Massive diffuse intraalveaolar hemorrhage (5p)

Morphologic diagnosis Fig. 1D:

Kidney: Severe multifocal acute tubular epithelial necrosis (5p)

Candidate number	

E5 Question 5: Briefly summarize and interpret the results of the blood and urine analysis shown in Table 1, taking into account the necropsy findings and the case history. 15p

Reduced hematokrit and thrombocytopenia. White blood cells increased → suggests acute infection (3p). Kidney parameters (urea, creatinine) dramatically increased → suggests kidney damage (3p). Also glucosuria and proteinuria present. Slight increase of hepatic parameter (bilirubin, ALT, AST), but no lesions found in histopathology. Lesions in kidney prominent in histopathology. Hemorrhage in alveolae without visible lesions of endothelial cells or inflammation etc. (3p) Macroscopically laceration of lung parenchyma visible. Blunt forced trauma during CPR due to rip fracture likely, which can also be cause of extensive pulmonary hemorrhage (3p). However, taken the case history (dog likely was 100% well for few days, contact with lake water in wooden area) together with clinical record, high WBC, acute necrosis in kidney, infection/sepsis more likely as cause of pulmonary hemorrhage and cause of death (3p).

E5 Question 6: Regarding your top differential diagnosis in this case, name the etiological agent and briefly outline the pathogenesis. 15p

Etiology:

Leptospira interrogans (serovars canicola, bratislava, australis, autumnalis, pomona, grippotyphosa or icterohaemorrhagiae) (3p)

Pathogenesis:

Infection through direct contact of mucosae or skin with leptospira-infected urine or water (3p). Leptospira are motile (3p) and might enter through abrasions and probably also able to penetrate mucus layers and move across mucosae by going directly through mucosal epithelial cells or between the cells through intracellular junctional complexes. Able to penetrate the vascular wall and spread systemically. Penetration of blood vessels result in systemic petechial hemorrhages (3p). The natural reservoir of pathogenic leptospires is the proximal convoluted tubules of the kidney (3p). When proximal tubular cells die, they may release bacteria into the urinary space where they are carried in urine and spread into the environment via urination. (McGavin; see also Jubb Vol 2, p433)
Pathogenesis of leptospiral pulmonary haemorrhage syndrome unknown (compare: Schuller et al. 2015 European consensus statement on leptospirosis in dogs and cats. J Small Anim Pract. 56:159-79. doi: 10.1111/jsap.12328.)

E5 Question 7: Name two special stains that you would recommend in order to support your diagnosis. Fill in the table below, indicating the rationale for each special stain: 10p

Organ	Special stain	Demonstration of
Kidney	Levaditi of Warthin-Starry	spirochetes

Candidate number	

Kidney	Giemsa	spirochetes

E5 Question 8: **Name three molecular techniques that you could use to confirm your etiological diagnosis:**

15p

IF or IHC (5p)

MAT (microscopic agglutination test) (5p)

PCR (5p)

Also: culture