GENES & LIGHT:

many years later

Anatoly Ruvinsky

Photoperiodic studies

- Except fundamental study on domestication D.K. Belyaev also initiated several other research projects including influence of photoperiodic factors on mammalian reproduction and embryonic development.
- One such project was focused on three coat colour mutations in foxes and possible photoperiodic effects.
- Here we present a review of this particular investigation in the light of modern data.

Genetics of the *W* locus in foxes

Phenotypes of mutant foxes

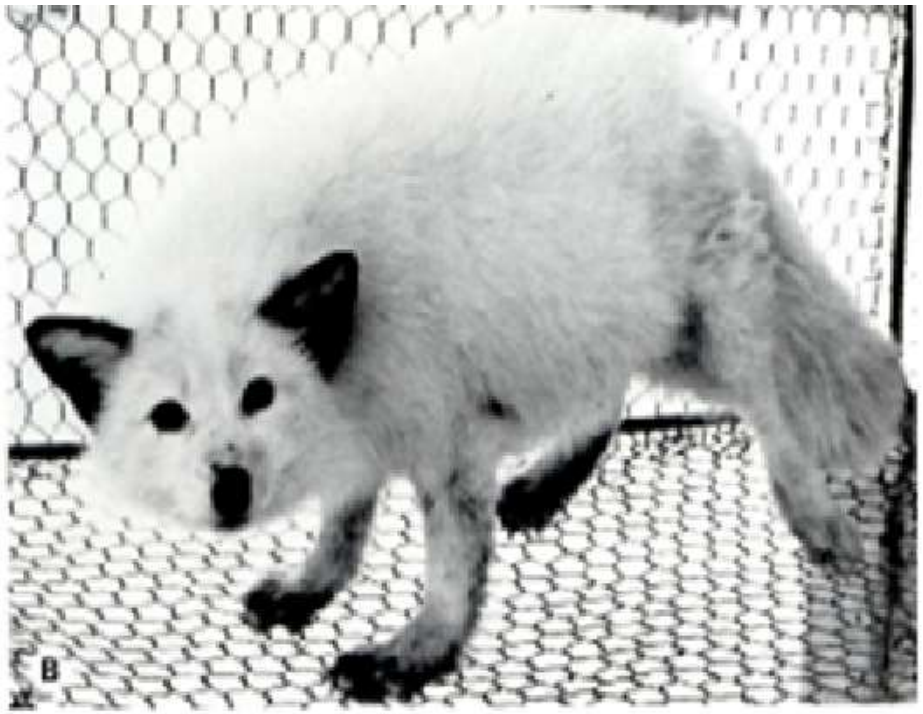
A –platinum W^P/w; B – white-faced W/w; C – Georgian white W^G/w;
D – embryos from ♂W/w x ♀W/w at day 30 of pregnancy
(Belyaev, Trut, Ruvinsky 1975).

Coat colour mutations in foxes

- Platinum and white-faced mutations are allelic and all homozygotes die after implantation around 30th day of development.
- Georgian white mutation is likely to be an allele of the same gene. Alternatively it might be a mutation of a tightly linked gene.
- Rare homozygotes for Georgian white mutation are able to implant under standard conditions. All born homozygotes died during the first 2-3 months, except one female.

Female W^G/W^G

produced 11 heterozygous offspring in test crosses



Compounds: W^G/W (left) and W^G/W^P

W locus is KIT gene

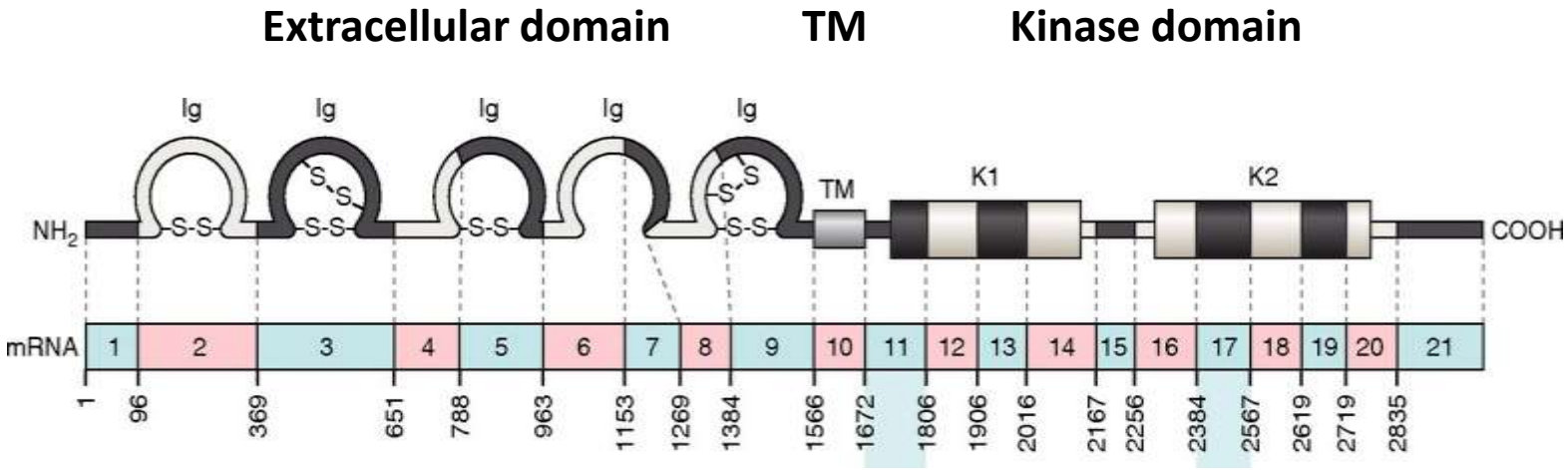
- Belyaev *et al*., 1975 tentatively defined platinum, white-faced and Georgian white mutations in foxes as alleles of *W* locus, assuming its homology to the previously studied *W* locus in mice, which was later identified as *KIT* gene (Chabot *et al*., 1988).
- Johnson *et al*., 2015 discovered that platinum mutation in foxes is indeed caused by a substitution affecting splicing of 17th exon of *KIT* gene (Chr.2) that leads to shortening of KIT protein.
- Kukekova *et al*., 2016 mapped Georgian white mutation to the region of *KIT* gene at Chr.2.
- This molecular investigation continues in order to identify the exact cause of Georgian white mutation; it looks possible that this mutation does not change the protein but rather *KIT* regulation.

KIT Proto-Oncogene Receptor Tyrosine Kinase

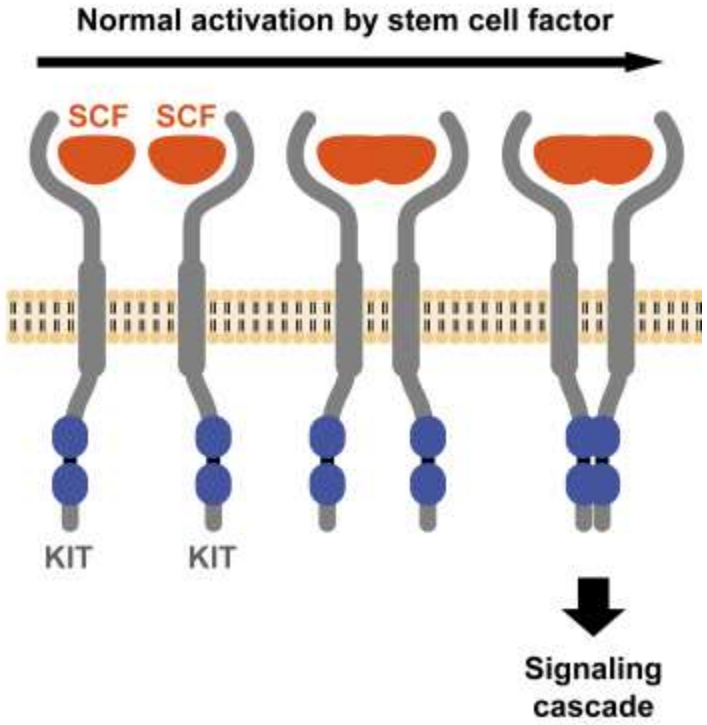
KIT gene and KIT protein

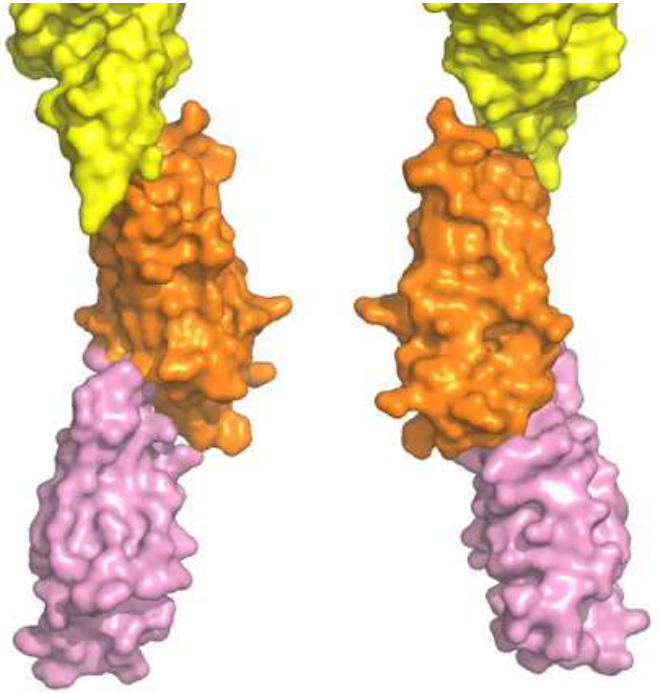
- *KIT* gene encodes a transmembrane tyrosine kinase receptor.
- This protein acts as cell-surface receptor for the cytokine KITLG, i.e. KIT ligand or SCF(stem cell factor).
- KITLG/KIT complex plays the essential role in signal transduction.

KIT protein and spliced mRNA



KITLG/KIT assembly and activation





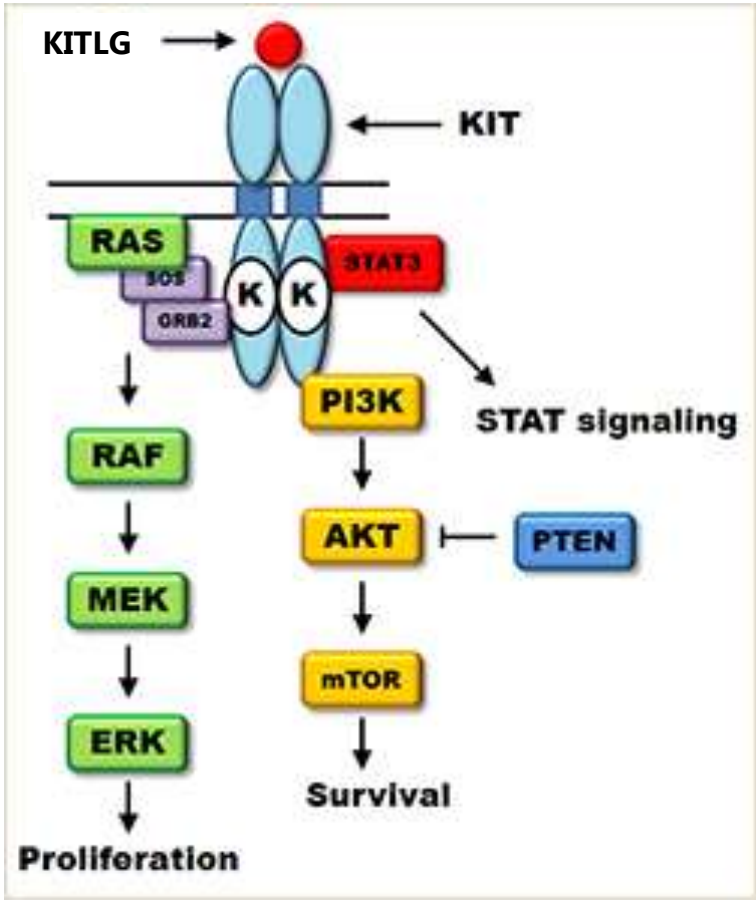
SCF=KITLG=KIT ligand

http://www.gistsupport.org/for-new-gist-patients/understanding-your-pathology-report-for-gist/diagnosing-gist.php

Movie S1. Side View of a Possible Transition of the Membrane-Proximal Region of the KIT Ectodomain upon SCF Binding.

Yuzawa S[1], Opatowsky Y, Zhang Z, Mandiyan V, Lax I, Schlessinger J. (2007) Structural basis for activation of the receptor tyrosine kinase KIT by stem cell factor. Cell 130(2):323-34.

KITLG/KIT can activate several signal pathways



KIT/KITLG promotes phosphorylation of certain protein phosphatases and several STAT transcription factors.

Mutations of *KIT* gene

Pleotropic effects of *KIT* mutations

- Mutations of *KIT* gene generate wide spectrum of pleotropic effects due to abnormal proliferation, survival and migration of several cell types like:
  - foetal and adult erythropoietic tissues; *lead to different forms of anaemia*
  - mast cells, a type of white blood derived from the myeloid stem cells, which is a part of the immune and neuroimmune systems; *lead to a variety of immune syndromes and diseases*
  - neural-crest-derived melanocytes; *lead to piebaldness*
  - interstitial cells of Cajal (ICC) from the colon, which are essential for normal peristaltic; *lead to megacolon*
- *KIT* activating mutations are often associated with a variety of tumours, mast cell disease, melanoma and leukaemia; while *KIT* inactivating mutations are rather associated with piebaldness. Embryonic or postnatal lethality is common for homozygotes.

Rabbit heterozygous for the English spotting (*En/en*) has a mutation in *KIT* (Fontanesi *et al*., 2014).

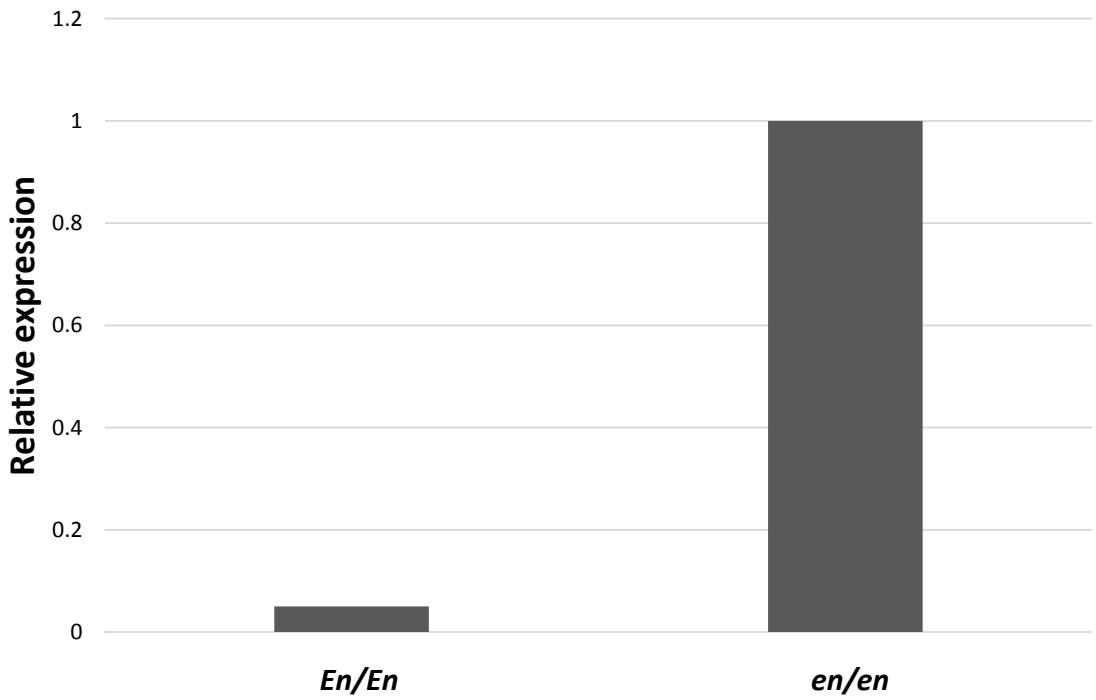
Fox heterozygous for Georgian white (W^G/w) is likely caused by a mutation in *KIT* (Kukekova *et al*., 2016).

Georgian white foxes versus rabbits with the English spotting

Georgian white mutation in foxes has several similarities with the English spotting in rabbits:

- distribution of white and black spots in hetero- and homozygotes
- incomplete dominance
- low viability of homozygotes during early postnatal period
- abnormal peristaltic potentially leading to megacolon often during transition to solid food
- lack of substitutions in the coding part of *KIT* gene, which might generate structural changes of KIT protein

Relative level of *KIT* expression in the ascending colon of *En/En* and *en/en* rabbits



En/En homozygotes are subvital and suffer from megacolon (Fontanesi *et al.,* 2014) that is similar to colon malfunction of Georgian white homozygotes.

The English spotting in rabbits

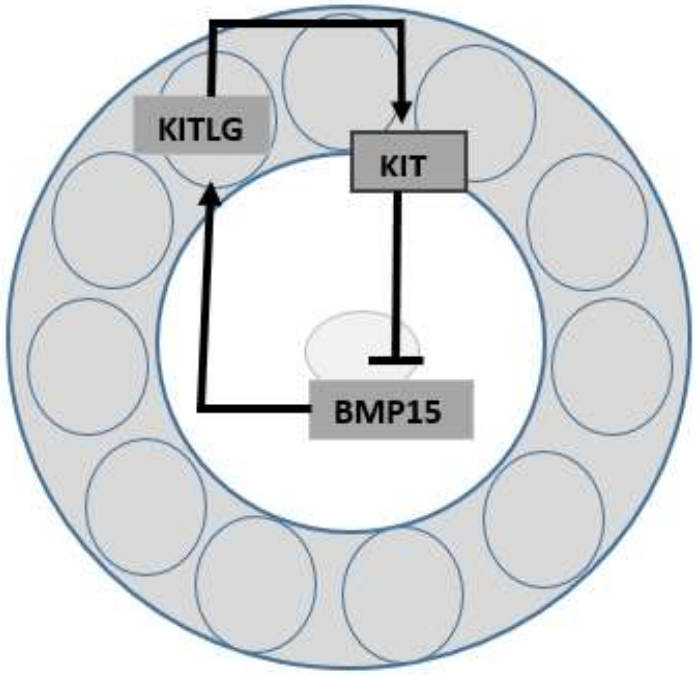
(Fontanesi *et al*., 2014)

- It was established that the English spotting is caused by a mutation of *KIT* gene. It seems likely that a regulatory mutation is responsible for the observed phenotypic manifestations.
- Low relative *KIT* expression in cecum and ascending colon of *En/En* rabbits is a proof of its role in developing gut complications and severely reduced viability, which is so typical for the homozygous rabbits.
- *KIT* expression in the colon is mainly prominent in interstitial cells of Cajal (ICC).
- ICC and certain neuronal cells are known to play an important role in the gut motility. Electron microscopy showed significant neuronal and ICC abnormalities in *En/En* rabbits tissues.
- KIT protein is involved in driving migration of melanoblasts and neuroblasts from the neural crest to their final destinations.
- **A similar set of phenotypical features is common for Georgian white mutation in foxes. It is not unreasonable to expect that this mutation is also caused by a change in the regulatory region of *KIT* gene.**

KIT and other proteins in development

KITLG-KIT-BMP15 feedback regulatory system

(Hutt *et al.*, 2006)



- Oocyte-originated BMP15 activates *KITLG* expression in granulose cells.
- KIT/KITLG assembly and activation of KIT signalling through the oocyte's membrane.
- KIT signalling inhibits *BMP15* expression, thus affecting BMP15 concentration.
- **Low KIT level in some mutant homozygotes may lead to overproduction of BMP15 and KITLG.**
- Other genes and proteins are likely involved in KIT/KITLG signalling, that may operate differently in distinct tissues.

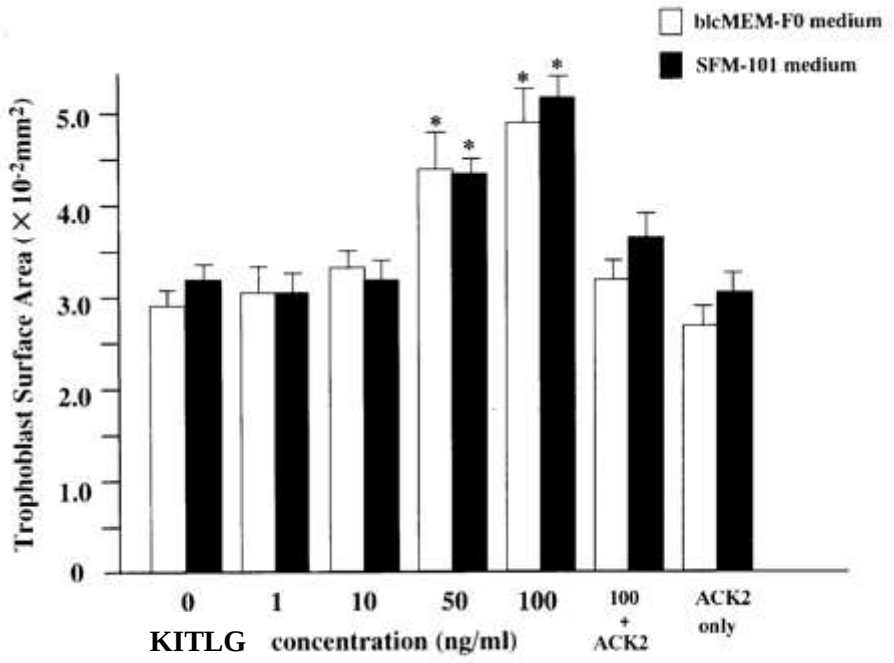
Oocyte maturation and preimplantation development in canids

- In canine species including foxes, unlike many other mammals, oocytes are ovulated prior to resumption of the first meiotic division.
- In foxes oocyte undergo MI and reach maturation within 24-48 hours after ovulation.
- Asynchronous ovulation typical for canids creates a diverse population of oocytes, which might be fertilised within 2-3 days and continue asynchronous development.
- Hence some blastocysts might be ready for implantation earlier than others.

Developmental effects of KIT and KITLG

- Turning off *KIT* gene, using siRNA, significantly decelerates development from 2-cell stage onwards.
- KITLG produced by both blastocyst and endometrial cells exerts paracrine and autocrine effects.
- Trophoblast surface enlarges significantly in response to higher concentration of KITLG in the medium.

Trophoblast surface and concentration of KITLG



ACK2 (anti-c-KIT antibodies 10 ng/ml), Mitsunari *et al.*, 1999.

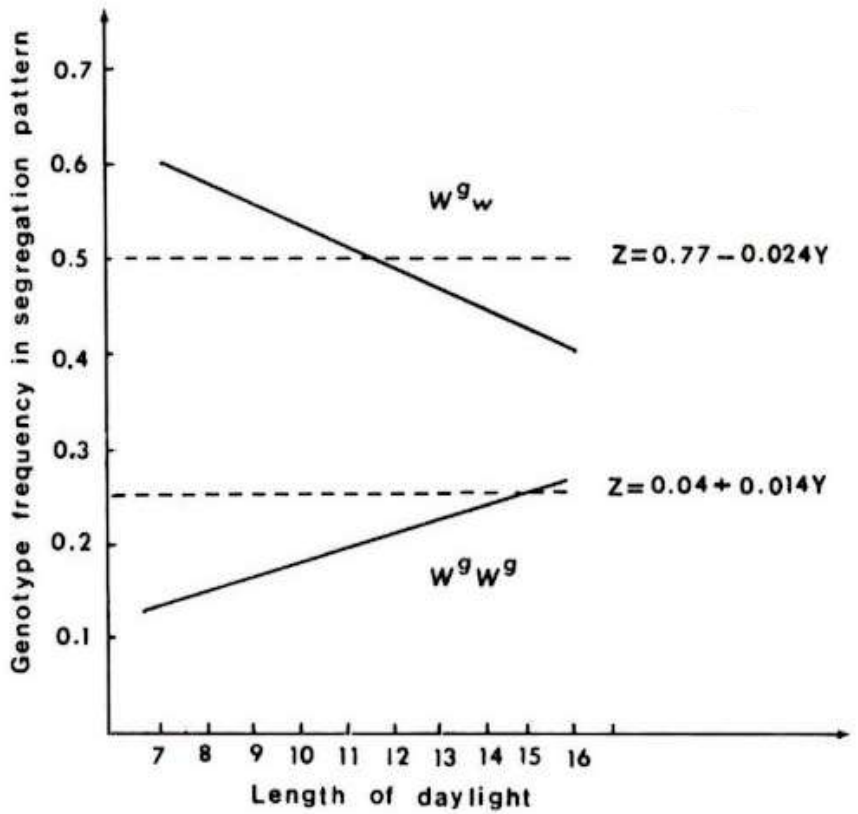
Question and possible Answer

- **Q.** How could homozygosity for Georgian white mutation reduce blastocyst ability to implant?
- **A.** Low KIT production diminishes signalling capacity of KIT/KITLG and slows down development of W^G/W^G blastocysts. As the result **mutant homozygotes could often be late for implantation**.

Photoperiodic influence

Changes of W^G/w and W^G/W^G frequencies in offspring from W^G/w x W^G/w crosses depending on daylight length

(Belyaev *et al*.,1975)



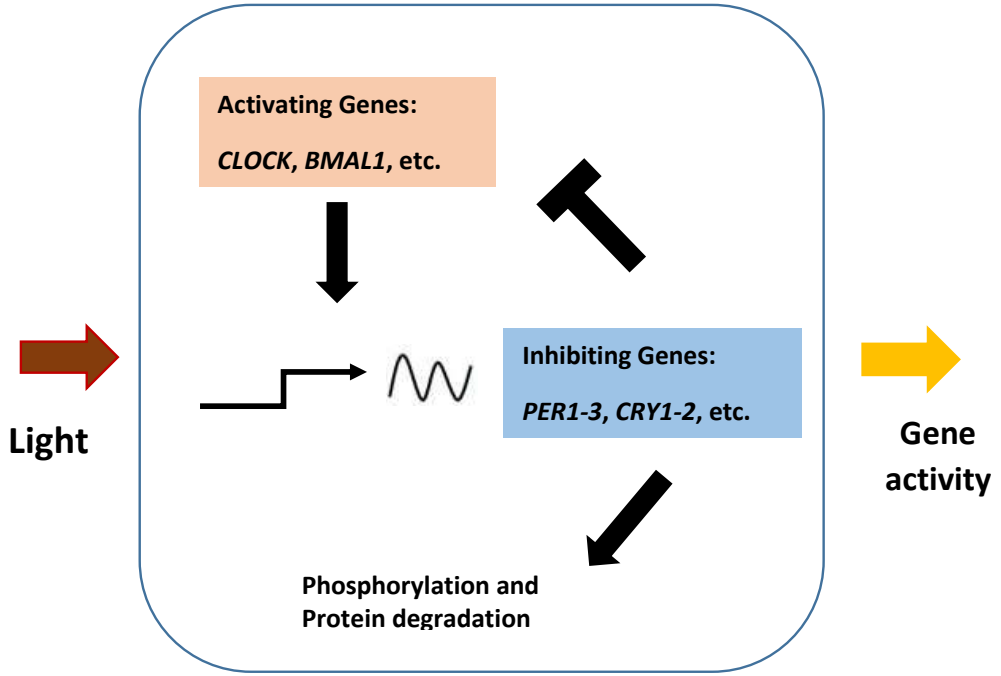
Question and possible Answer

- **Q.** How could artificially elongated light day selectively compensate slow development of homozygous (W^G/W^G) blastocysts?
- **A**. If this photoperiodic change could stimulate development of W^G/W^G blastocysts; promote endometrial decidualization and/or slow down the final maturation stage of blastocysts, then the desirable outcome can be achieved.

Photoperiodic changes

- Photoperiodic changes have critical influence on mammalian reproduction and other essential functions particularly in higher latitudes.
- The hypothalamo-pituitary-gonadal (HPG) axis together with the pineal gland are the key response system to photoperiodic changes, which controls expression of many genes, dynamics of relevant hormones like estrogens, progesterone, prolactin, melatonin and numerous other biologically active molecules.
- Ongoing physiological adjustments are based on response of cellular circadian clocks to changing photoperiodic conditions.

Regulation of circadian rhythms in vertebrates: a general scheme



Circadian oscillators generate a free-running period ~24 h in constant environmental conditions. Environmental signals can reset the oscillation pattern, through input pathways. The oscillators send phase information through the output pathways to control the expression of clock-controlled genes and rhythm of microprocesses. Circadian oscillators are composed of positive and negative elements that form auto-regulatory feedback loops (de Paula *et al*., 2008). Progesterone and estradiol might be involved in modification of uterine circadian rhythm via direct regulation of the expression of clock genes (Hirata *et al*., 2009).

The circadian clock oscillations

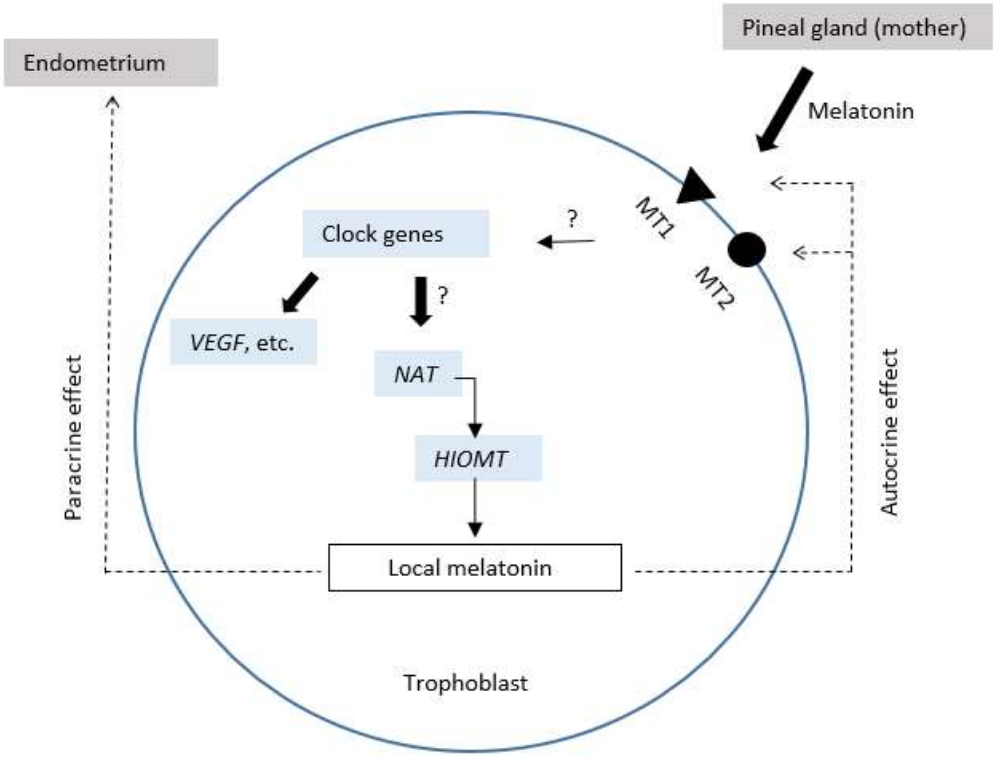
(Tasaki *et al*., 2013)

- The peripheral circadian oscillator plays an essential role in synchronizing local physiology via regulation of the expression of clock-controlled genes.
- Among 12,252 genes from endometrial cells showing observable expression 7,235 genes displayed significant phase alterations, of which 11 genes were directly connected with implantation and 24 genes affected formation of placenta.
- As a large fraction of mammalian genes are involved in circadian oscillations, hence sharp photoperiodic changes can influence dynamics of numerous biological processes including reproduction.
- Among them is essential PI3K (phosphatidylinositol-3-kinase) signal pathway participating in circadian rhythm modulation by interactions with BMAL1 and CLOCK; PI3K/Akt is directly affected by signals coming through KITLG/KIT inside cells, thus influencing apoptosis/survival and proliferation.

Melatonin, blastocysts and implantation

- Pineal melatonin is produced during darkness and is essential factor synchronizing ~24 h circadian oscillations with photoperiodic changes.
- Local melatonin is produced by different tissues including endometrium and blastocysts.
- Local melatonin activates several genes critically important for implantation: *HBEGF* (heparin binding EGF like growth factor) and its receptor *ErbB1*; *PRA* (progesterone receptor A), *p53* (protein 53) and *LIF* (leukaemia inhibitory factor).
- Low concentrations of local melatonin (10^{-9}M) accelerate blastocyst development and reduce embryonic mortality (Tian *et al*., 2010).

Autocrine and paracrine effects of melatonin over circadian system in trophoblast and endothelial cells

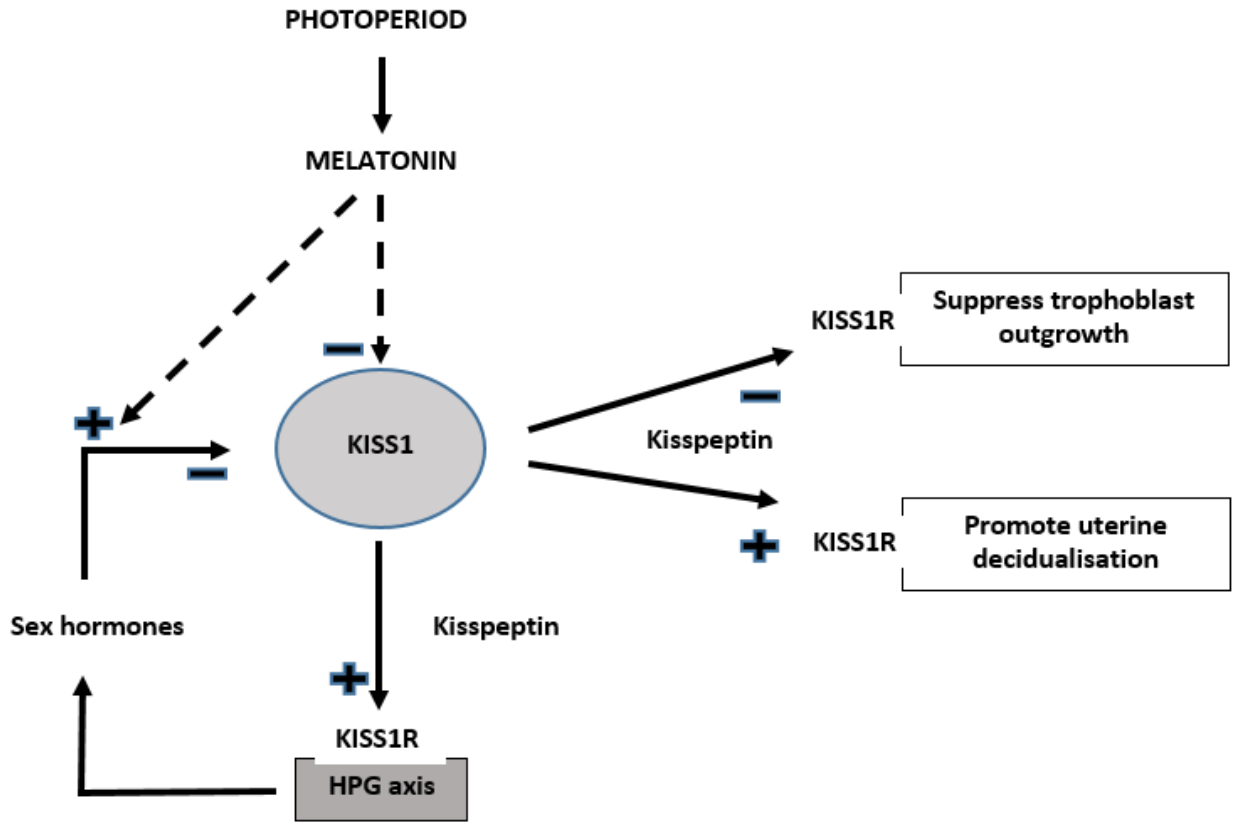


There are two melatonin receptors: MT1 and MT2 and both are G protein-coupled receptors (Valenzuela *et al.*, 2015). The critical enzymes for the synthesis of melatonin are arylalkylamine N-acetyltransferase (AA-NAT) and hydroxyindole O-methyltransferase (HIOMT).

Kisspeptins, photoperiod and implantation

- Adjustment of reproductive system to photoperiodic conditions depends on kisspeptin neurons which are active participants of many processes controlled by HPG axis.
- Kisspeptins, encoded by *KISS1*, together with its receptor KISS1R are very active in pregnancy and critical during implantation (including interactions with *BMP15*, *LIF*).
- Kisspeptins, by controlling apoptosis, decelerate trophoblast outgrowth during the final stage of blastocyst development.
- At the same time kisspeptins promote decidualization of endometrium, thus affecting fine tuning of blastocysts and endometrium.

A model of *KISS1* regulation and its possible effects on implantation



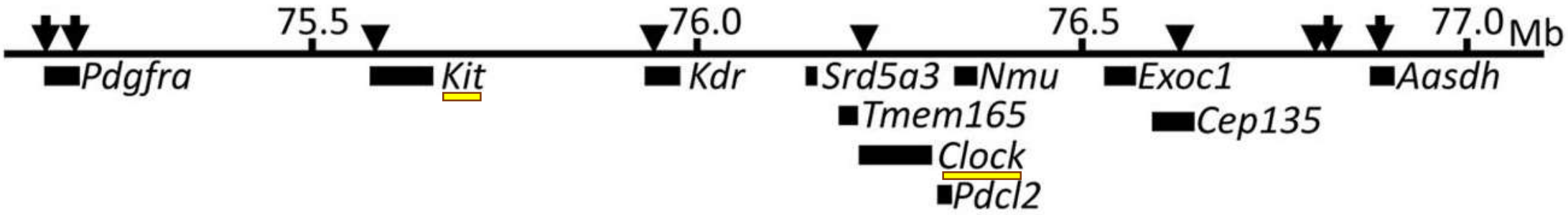
The *KISS1* gene product, kisspeptin, stimulates the hypothalamo-pituitary-gonadal (HPG) axis during the light period via the KISS1R receptor. In the arcuate nucleus, *KISS1* expression is negatively regulated by sex hormones and melatonin (directly at KISS1 neurons or indirectly through sex hormones?). Body energy stores as well as age and gender are assumed but not shown here. A specific feature of this regulatory system is based on ability to slow down trophoblast outgrowth and promote decidualization of uterine endometrium prior to the beginning of implantation in pregnant females (Revel *et al*., 2006; Saadeldin *et al*., 2012; Bilban *et al*., 2004; Zhang *et al*., 2014). Activation of *LIF* gene, which is absolutely essential for implantation, cannot be achieved without active *KISS1* (Calder *et al*., 2014).

Photoperiodic changes and W^G/W^G survival: some possible explanations

- KITLG level is significantly higher during long-day than short-day; this might accelerate trophoblast development of W^G/W^G blastocysts in the long-day experiment; gain for W^G/W^G (Photoperiodism: 1415854_at: Kitl)
- By reducing melatonin suppression of *KISS1* long-day experiment might lead to higher kisspeptin level and consequently deceleration of trophoblast outgrowth at the final stages of blastocyst development and promotion of endometrial decidualization; gain for W^G/W^G (Simonneaux and Bahougne 2015)
- Long-day conditions also upregulate estrogene-dependent expression of *NDRG4* gene which is associated with decidualization and the activation of delayed implantation; that is locally critical during implantation particularly for PI3K/Akt deficient blastocysts, like homozygotes with low KIT; gain for W^G/W^G (Yang *et al.*, 2016)

Syntenic group of genes

Syntenic group of genes existing at least since the common ancestor of mammals and bonny fish



It includes: 3 nearby located genes with common origin that determine growth factors (tyrosine kinase receptors) - *PDGFRA*, *KIT*, *KDR*; *SRD5A3* – gene of steroid 5 alpha-reductase 3; *TMEM165* – gene of transmembrane protein 165; *CLOCK* - circadian locomotor output cycles kaput; *PDCL2* – gene of phosducin like 2; *NMU* – gene of neuromedin U; *EXOC1* – gene of exocyst complex component 1. The distance between *KIT* and *CLOCK* in the mouse and some other vertebrates is less than 1 Mb (Mizuno *et al*., 2015).

Conclusions

- Comparison of pleiotropic effects of Georgian white mutation in foxes with the English spotting in rabbits tentatively indicates a regulatory nature of these mutations.
- Significantly reduced implantation capability of W^G/W^G blastocysts might be caused by slow development and hence missed opportunity to fit into optimal implantation time.
- Nearly all W^G/W^G homozygotes died within 2-3 months of postnatal life and this lethality is possibly caused by low *KIT* expression in interstitial Cajal cells (ICC) of the colon.
- Additional illumination of females from W^G/w x W^G/w crosses allows practically all W^G/W^G blastocysts to overcome implantation difficulties possibly by accelerating their development, slowing down trophoblast outgrowth of almost mature blastocysts of all genotypes, promoting decidualization and delaying implantation.