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Session 32 Page no. 315

Multiple splice variants of the ovine Mitf, c-Kit genes and evaluation of its' role in the dominant white phenotype in Merino sheep

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Abstract title: **Multiple splice variants of the ovine Mitf, c-Kit genes and evaluation of its' role in the dominant white phenotype in Merino sheep**

Author: **Saravanaperumal, S.A., Pediconi, D., Renieri, C., La Terza, A.**

Preferred presentation: **Theatre**

Preferred session: **S.32 : Animal fibre science**

Abstract text:

In this study, we investigated the role of the 2 candidate genes: *Mitf* and *c-Kit* for the genetic background of 'dominant white' phenotype in Merino sheep. Reverse transcription (RT)-PCR analysis of *Mitf* gene, revealed two splice variants hereinafter referred as SP1 and SP2, from skin biopsies. In particular, the variant SP2, commonly known as 'isoform-M', differs from SP1 with the insertion of a stretch of 18bp (CGTGTATTTTCCCCACAG, pos.560-578) in the coding region for the amino acids: ACIFPT, resulting in a Mitf isoform of 419 aa (+form) vs the 413 aa of SP1 (-form). At present, to characterize other skin-expressed *Mitf* isoforms, we are carrying out 5'and 3'RACE experiments. Preliminary results shows 3 PCR amplicons ranging in size from 1.1 to 0.45 kbp for the 5'UTR and 3 amplicons for 3'UTR ranging from 3.3 to 0.8 kbp. The cDNA encoding ovine *c-Kit* was also amplified (3.770 bp) from the same skin biopsies, of which the complete coding sequence comprises of 2828 bp, 2840 bp, respectively. In fact, here also we report two splice variants, characterized by the presence or absence of four-amino acid sequence "VTAK" (i.e., GTAACAGCAAAG pos.1534-1545). By means of RACE strategy, we obtained PCR amplicons of 0.75 kbp and 0.7 kbp for the 5'and 3'UTR regions of the *c-Kit* gene, respectively. Our future study will focus on the physiological switching of these isoforms in context with the promoter and the cell type which might have a combined influence on the gene expression programs and thus, shed light on the molecular mechanism behind dominant white phenotype in sheep.

OVERVIEW

MiTF – Microphthalmia-associated Transcription Factor

- Introduction
- cDNA Structural Coverage, Isoforms Multiplicity
- Mutations, Differential Expression (Semi-Quantitative)
- Intron-Exon Splice Junction
- Summary

c-KIT – Receptor Tyrsoine Kinase

- Introduction
- cDNA Structural Coverage, Isoforms Multiplicity
- Mutations, Differential Expression (Semi-Quantitative)
- Summary

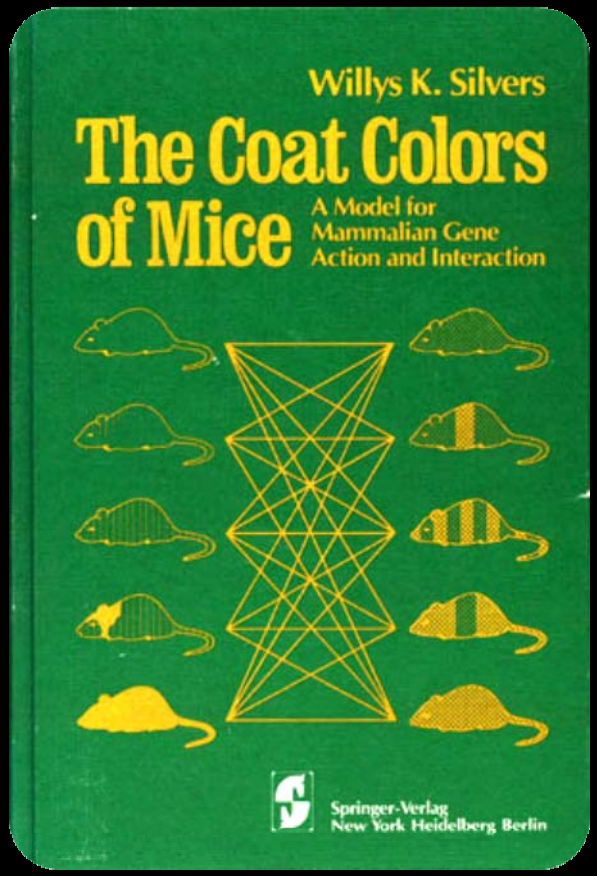
CONCLUSION

FUTURE PROSPECTIVE

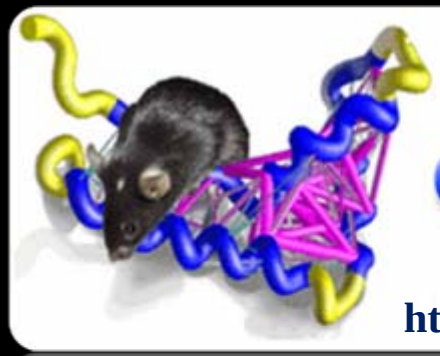
International Mouse Strain Resource

http://www.findmice.org/index.jsp

RATIONALE



Mitf
c-Kit
Scf



Color Genes

http://www.espcr.org/micemut/

International Federation of Pigment Cell Societies

Current symbol	Murine Locus (pictures)	Mouse Chrom.	KOMP TRAPS	Human Locus	Human Chromosome	Associated Disease	Protein Encoded	Function in Pigmentation / Mutant phenotype	All NCBI Databases	Zebrafish locus

Described loci: 367 loci

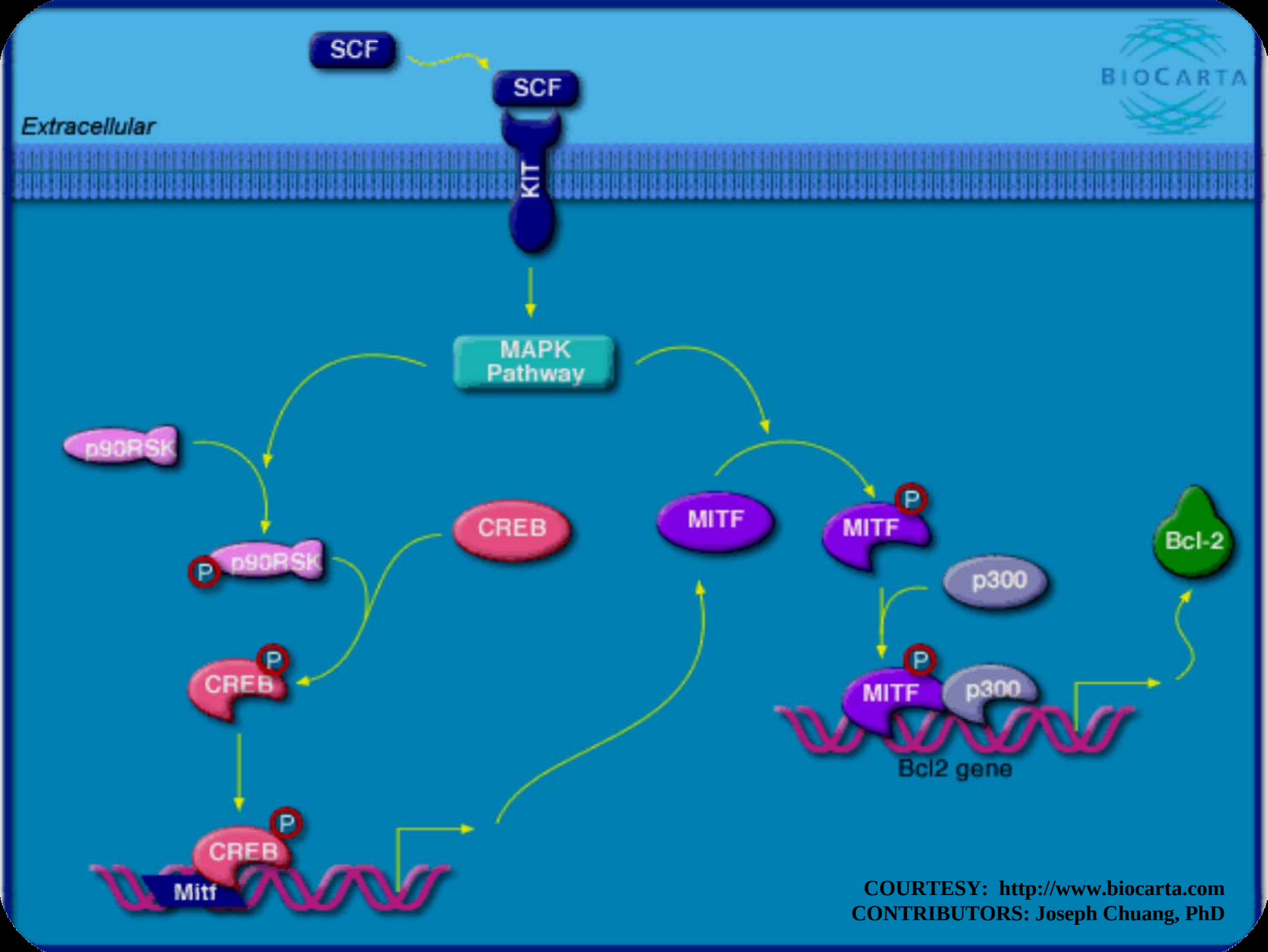
Summary of cloned color genes: 158

Summary of non-cloned color genes: 209

Last modified on July 21, 2009

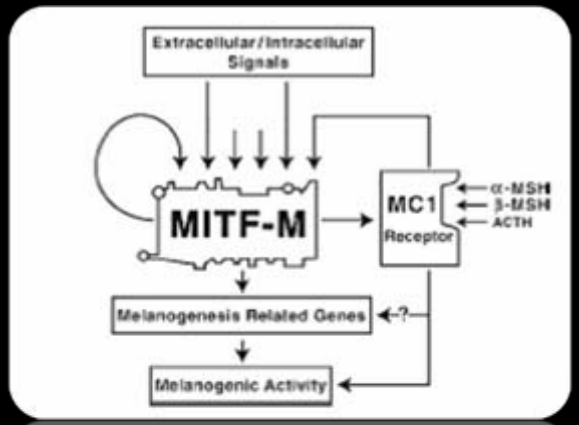
“The inheritance of white coat colour in merino sheep is dependent by a single gene segregation, without any modifying effects. It is completely dominant over pigmented animals….” C. Renieri et al., (Small Rum. Res. 2007)

Cloning and Identification of *differentially expressed, multiple splice variants* of the ovine Mitf, c-Kit & Scf genes and evaluation of its' role in the *dominant white phenotype* in Merino Sheep

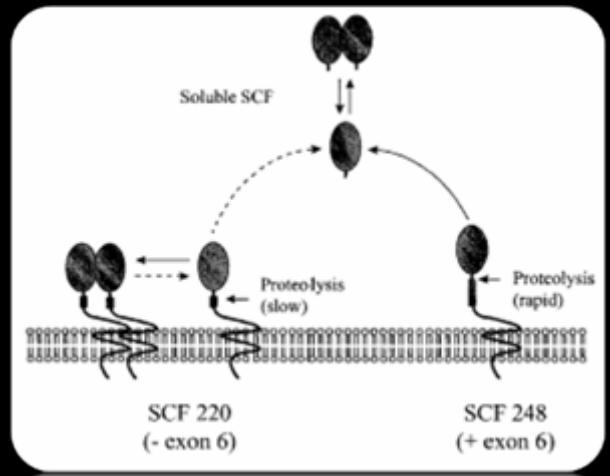


COURTESY: http://www.biocarta.com
CONTRIBUTORS: Joseph Chuang, PhD

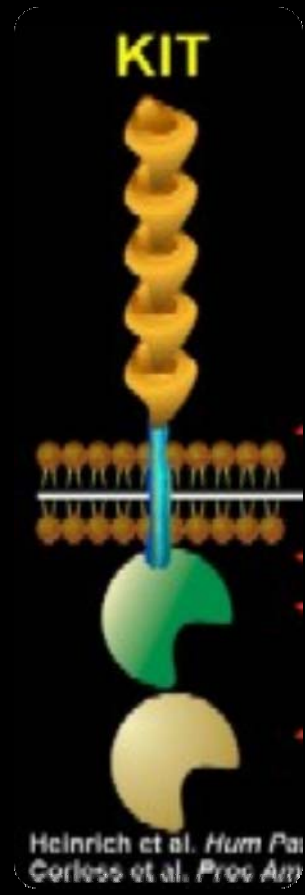
GENES UNDER INVESTIGATION



MITF



SCF



c-KIT

MITF – Microphthalmia Transcription Factor, signal protein

c-KIT – Transmembrane Tyrosine kinase Receptor, Dominant white spotting

Steel/SCF – Stem Cell Factor, ligand for/of c-kit

OBJECTIVE

1. **To Understand the molecular basis behind dominant white phenotype**

2. **Marker development to assist breed identification i.e., Traceability**

SAMPLING

WHITE

BLACK

BROWN

SCHEMATIC REPRESENTATION OF WORKPLAN

Skin biopsies from Merino SHEEP's
(Aziende la Campana Montefiore dell'Aso (AP)
La Maridiana Umbertide (PG)

↓

Designing degenerate primers for conserved domains of MITF / c-Kit / Scf

↓

RNA isolation

↓

cDNA synthesis

↓

RT-PCR amplicfication

↓

Cloning and sequencing of amplicon(s) of interest

↓

Genotyping – detecting allelic variant

Microphthalmia-associated transcription factor (MITF)

Belongs to the supergene family of the basic helix-loop-helix with leucine zipper transcription factors

GENE SUMMARY

This gene encodes a transcription factor that contains both basic helix-loop-helix and leucine zipper structural features. It regulates the differentiation and development of melanocytes, retinal pigment epithelium and is also responsible for pigment cell-specific transcription of the melanogenesis enzyme genes. Alternatively spliced transcript variants encoding different isoforms have been identified

ISOFORMS OF MITF

Mitf - A,C, Mc, E, H, D, B, M and J

FUNCTION

DNA binding
Transcription activator activity

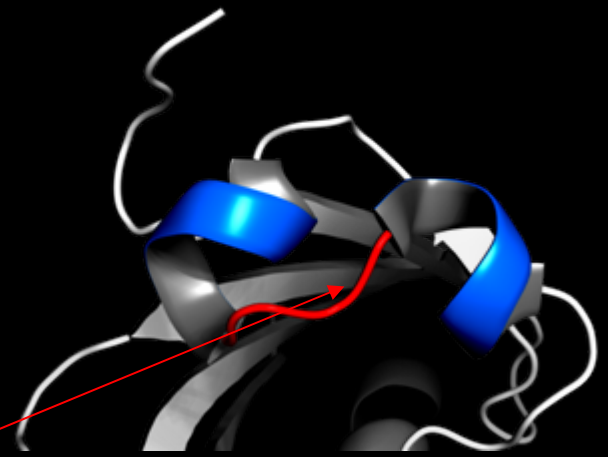
BIOLOGICAL PROCESS

Melanocyte differentiation
Multicellular organismal development
Regulation of trascription,
DNA-dependent Sensory perception of sound

Schematic representation of the MITF mRNA structure detected in the Skin biopsies of White Merino Sheep (Ref. Seq. MAMMALS - DOG, HUMAN, MOUSE)

5’	5’ Untranslated Region
B1b	Common Domain, Location of 1st Methionine
A	Transcriptional activation domain
bHLH LZ	Basic Helix Loop Helix Leucine Zipper Domain Structure
S	Serine-rich region
3’	3’ Untranslated Region

In Human,
The 18bp insert
CGTGTATTTTTCCCACAG
corresponding to the aminoacids ACIFPT
i.e., pos. 1 of Exon-6 in Isoform 4_M &
pos. 2644-2661of Intron 5-6 in Isoform 5
on chromosome 3



The MITF with the 18-base insert binds target E-boxes in promoter DNA with a slightly higher affinity and transcribes tyrosinase more efficiently than the MITF without the insert
(Hemesath et al., 1994; Murakami et al., 2007)

SUMMARY

1. **Entire Coding Region of cDNA - 1260bp & 1242bp.**

2. **Sequence Identity of the coding region showed 97%, 93% and 91% for Cattle, Pig, and Dog & Human, respectively.**

3. **In 5'UTRs – 4 Unique UTRs' for 4 different protein N-termini ie., MITF-E, M, Truncated Form-1 & 2.**

4. **In 3'UTRs – 3 Isoforms out of 4, yet to be characterized, a longer 3.4kb.**

5. **Two Splice variants (NCBI GeneBank, 01.12.2009) – SP1, SP2, commonly known as 'isoform-M', CGTGTATTTTCCCCACAG, pos.560-578 in the coding region for the amino acids: ACIFPT, resulting in a Mitf isoform of 419 aa (+form) vs the 413 aa of SP1 (-form).**

Conti ……

6. **Semi-quantitative, Relative Expression of the Two Isoforms, significant difference was observed. Further, result needs to be quantified with qRTPCR.**

7. **A transition (synonymous) of C to T was observed in the (+) form at nuc. pos. 537. This change is present just 17bp before the bHLH domain a.a pos. 196 to 256 (starting at nuc. pos. 588).**

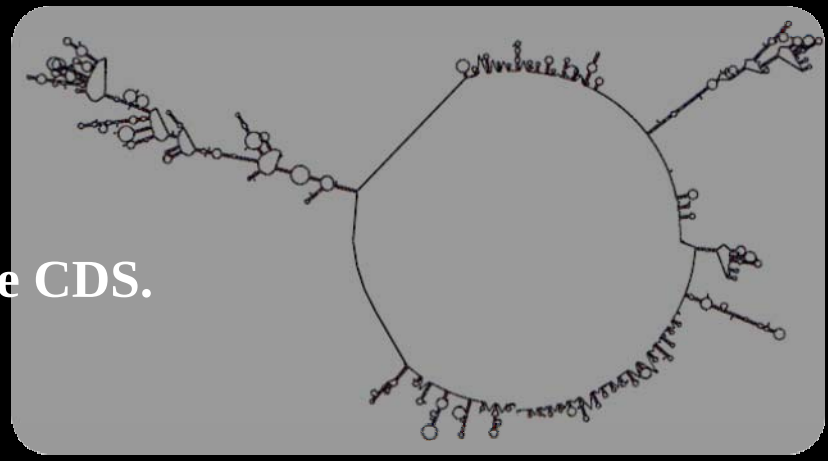
Identification of microphthalmia-associated transcription factor isoforms in dogs (Tsuchida, S., et al., The Veterinary Journal, 2008, doi:10.1016/j.tvjl.2008.06.004).z

A mutation in or near the insertion in the basic region of MITF gene might affect dimer formation or DNA binding (Yasumoto et al., 1998, Pig Cell. Res. 11, 329-336).

Conti ……

8. **Post-transcriptional regulation of mRNA levels (control point in gene expression) – Two cis-acting adenylate-uridylate-rich elements (ARE, AUUUA) are present at pos. 1442–1446bp & 1450–1454bp in the 3'UTR.**

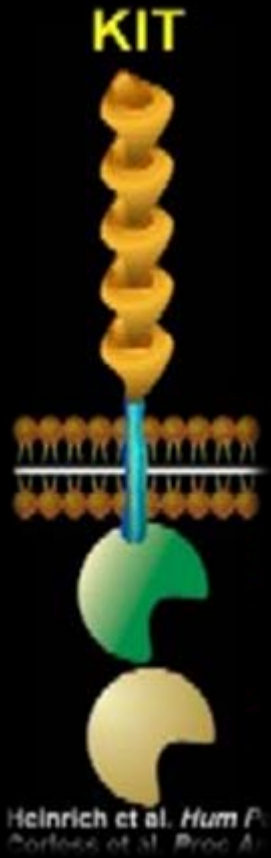
9. **2.6kb Intron-Exon Splice Junction point (+18/-18bp, hotspot for repeat elements).**

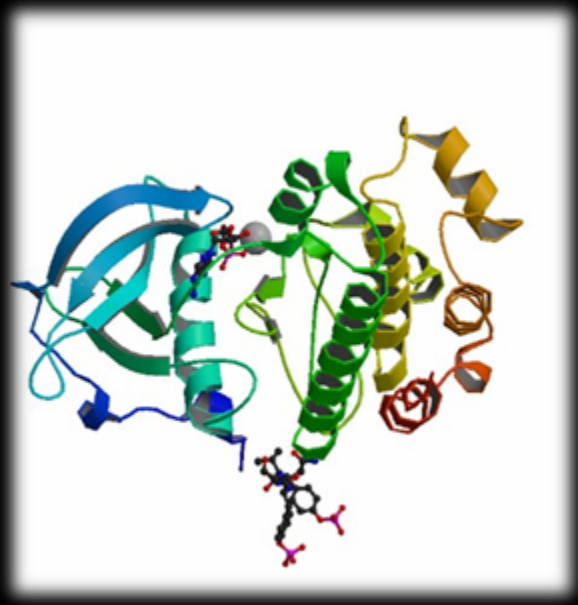


10. **Few mutations were observed in the CDS.**

11. **mRNA Expression (Phenotypic) – GeneDuplication (GD), Alternative Splicing (AS).**

Molecular Cloning and Characterization of c-Kit





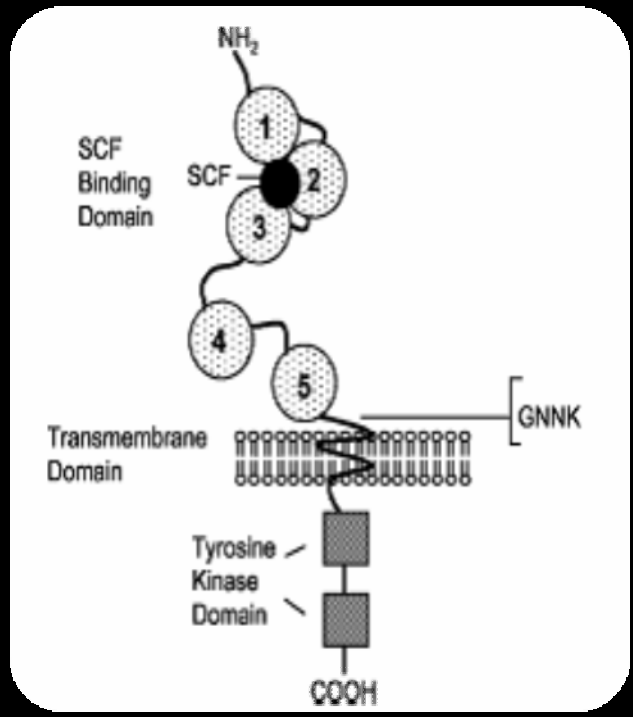
c-Kit (Receptor Tyrosine Kinase)

‘c’ for cellular, and ‘kit’ for kitten, a protein

Belly-spot, Steel Factor Receptor, Dominant White Spotting, c-kit proto-oncogene protein

Type 3 transmembrane receptor for MGF (mast cell growth factor, also known as stem cell factor)

Architecture c-Kit



ISOFORMS OF c-Kit:

Longer, isoform 1 (GNNK+)

Shorter, isoform 2 (GNNK –)

FUNCTION

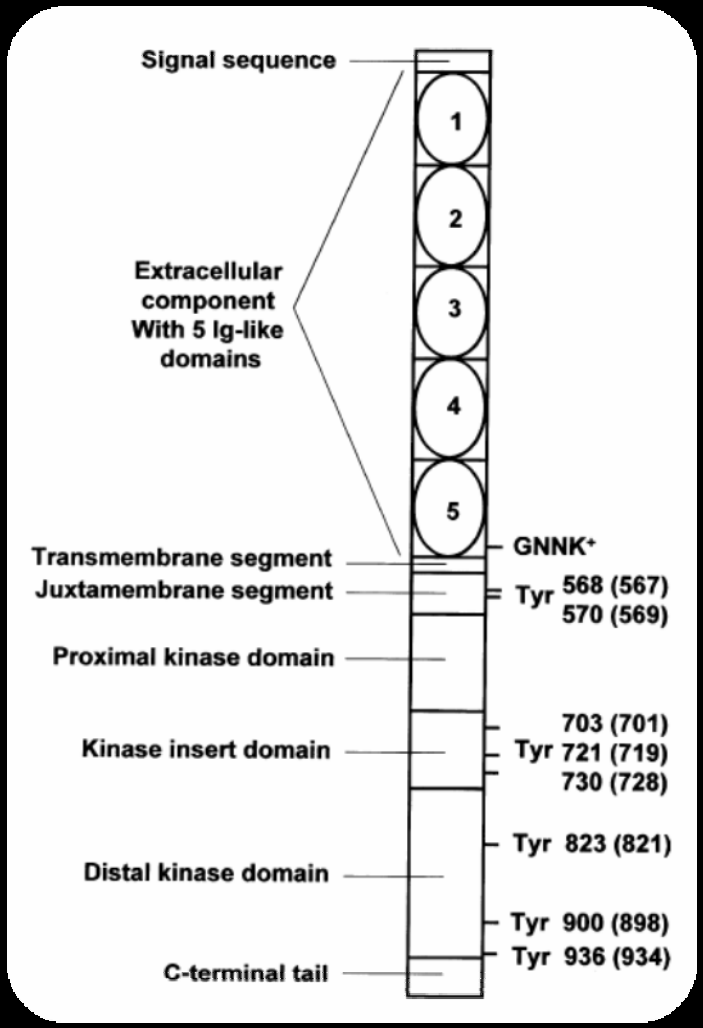
Receptor activity, Receptor Signaling protein tyrosinase kinase activity, Stem cell factor receptor activity

BIOLOGICAL PROCESS

Cytokine and chemokine mediated signaling pathway, Peptidyl-tyrosine phosphorylation, Pigmentation during development, Protein amino acid dephosphorylation & phosphorylation, Signal transduction, Transmembrane receptor protein tyrosine kinase signaling pathway

SCF/c-kit signaling is required for cyclic regeneration of the hair pigmentation unit.

Membrane-bound steel factor induces more persistent tyrosine kinase activation and longer life span of c-kit gene-encoded protein than its soluble form. More sustained signalling was mediated by membrane associated SCF and the soluble SCF can act to down regulate c-Kit - Miyazawa et al., 1995, Blood 85, 641-649.



Tyrosine phosphorylation sites and organization of Kit

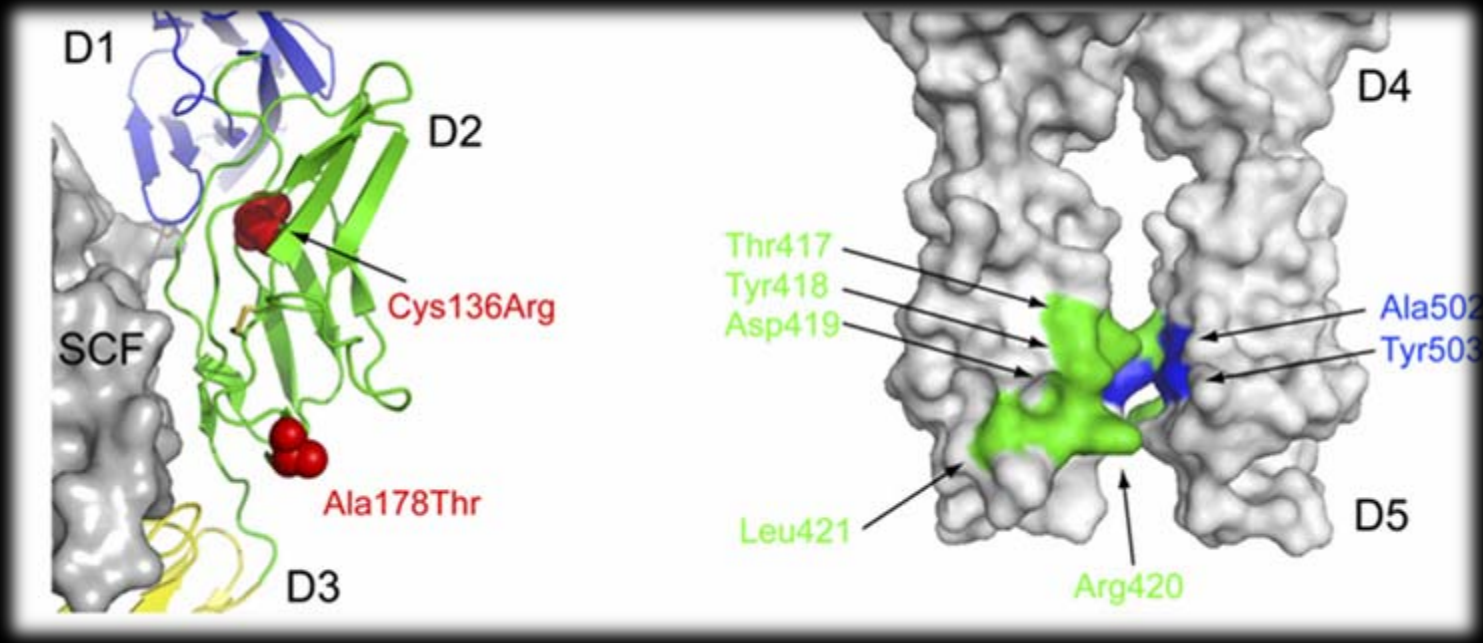
The relative length of the domains is to scale. The human (mouse) numbering system is displayed.

G refers to glycine; N, asparagine; K, lysine; and Ig, immunoglobulin

Signaling by Kit protein-tyrosine kinase - The stem cell factor receptor , Robert Roskoski Jr., BBRC, 337 (2005) 1–13

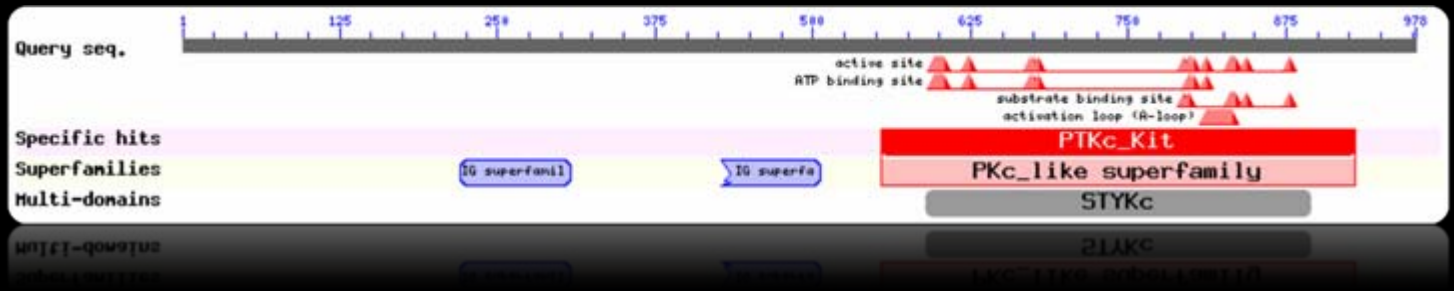
5 mutation found in KIT Juxtamembrane region controlling receptor kinase activity in Canine mast Cell Neoplasms, Yongsheng et al., 1999, THE J. INVES. DERM, Vol. 112 (2), 165-170

KIT activation is compromised by in-frame deletions, point mutations, in-frame duplications, and insertions that collectively lead to formation of activated forms of KIT (Satoru Yuzawa et al., Cell 130, 323–334, July 27, 2007).



A) LOSS-of-Function **B) GAIN-of-Function**

Schematic representation of the c-Kit mRNA structure detected in the Skin biopsies of White Merino Sheep (Ref. Seq. MAMMALS - GOAT, PIG, DOG, HUMAN, MOUSE)



5’ 5’ Untranslated Region

CDS Coding Region, Location of 1st Methionine

IG Immunoglobulin Like Domains, Cell Surface recognition

ATP BS ATP Binding Site

SBS Substrate Binding Site

3’ 3’ Untranslated Region

GENE DUPLICATION	REPORTED MUTATION(S)	ANIMALS
c-KIT	Tandem duplication > partially dominant phenotype – Regulatory, affect KIT Expression A splice mutation G to A at the first nucleotide in intron 17, skipping of exon 17 – Impaired or Absence of Tyrosine Kinase Activity.	PIG Stefan Marklund et al., Genome Res. 1998, 8: 826-833.
c-KIT	Genome duplication *kita* and *kitla* – Maintained Congruent Expression Patterns *kitb* and *kitlb* – Evolved Divergent Expression Patterns	ZEBRA FISH Hultman et al., PLoS Genetics, 2007, 3 (1): 0089-0102.
ASIP-AHCY-ICTH	A 190kb tandem duplication of ASIP with AHCY, ICTH in white merinos over recessive black could have an important role in the evolution of sheep pigmentation	SHEEP Norris and Whan, Gen. Res., 2008, 18:1282–1293.

SUMMARY

1. Entire Coding Region of cDNA - 2938bp & 2926bp.

2. Two Splice variants (NCBI GeneBank, 01.12.2009) – KIT1, KIT2, known as '+' & – form respectively, differs with GTAACAGCAAAG, pos. 1532-1543 in the coding region for the amino acids: GNSK, pos. 511-514, resulting in a isoform of 978 aa (+form) vs the 966 aa (-form).

Src Family Kinases Are Involved in the Differential Signaling from Two Splice Forms of c-Kit, Voytyuk, et al., JBC, 2003, Vol. 278 (11), 9159–9166.

3. Sequence Identity of the cDNA showed 98%, 97%, and 84% for goat, cattle & pig, and human, respectively.

Conti ……

4. In 5’UTRs – 2-3 (?) Unique UTRs’ .

5 . In 3’UTRs – so far , single 775bp fragment has been characterised.

6. Semi-quantitative, Relative Expression of the Two Isoforms

significant difference was observed, yet to be confirmed with qRTPCR.

7. Observed 5 mutations in the N-glycosylation region –1511bp (480a.a).

CONCLUSION

1. **Significant difference has been observed between mRNA Transcripts of Mitf, c-Kit with (+18bp/+12bp) and without (-18bp/-12bp) the insertion; Low Level of Expression of (+) form in Skin, Further, result needs to be quantified with qRTPCR.**

2. **First time, Two Novel Isoforms of Mitf has been Identified in Skin (Manuscript under preparation).**

3. **Mutations (SNPs) Observed in the CDS of Mitf, seems no Significance.**

4. **In Contrast, Significant Mutations were detected in c-Kit Extracellular IG Domain – 'Attraction of Substrate Binding', Yuzawa et al., 2007, Cell 130, 323–334.**

5. **Structural Prediction of Mitf, c-Kit Implies on the Stability and Self-regulation of mRNA Transcripts.**

FUTURE PROSPECTIVE

1. **Gene Expression & Regulation – Promoter and 3'UTR regulatory elements (miRNA Silencing), "regulatory mechanisms underlying hair follicle pigmentation".**

2. **Gene Duplication (GD), Alternative Splicing (AS) – c-Kit, Mitf & *ASIP*.**

3. **To Find out Potential Mutation(s) in the CDS – SNPs.**

4. **Bioinformatics & Wet Lab – Docking and Cell Line Assay.**

5. **To Investigate 'Wrestling' Genes or the other Biochemical Pathways of Pigmentation Biology.**

Results will be applied in breeding programmes to reduce the incidence of disease and to improve product quality and production efficiency

RESEARCH GROUP

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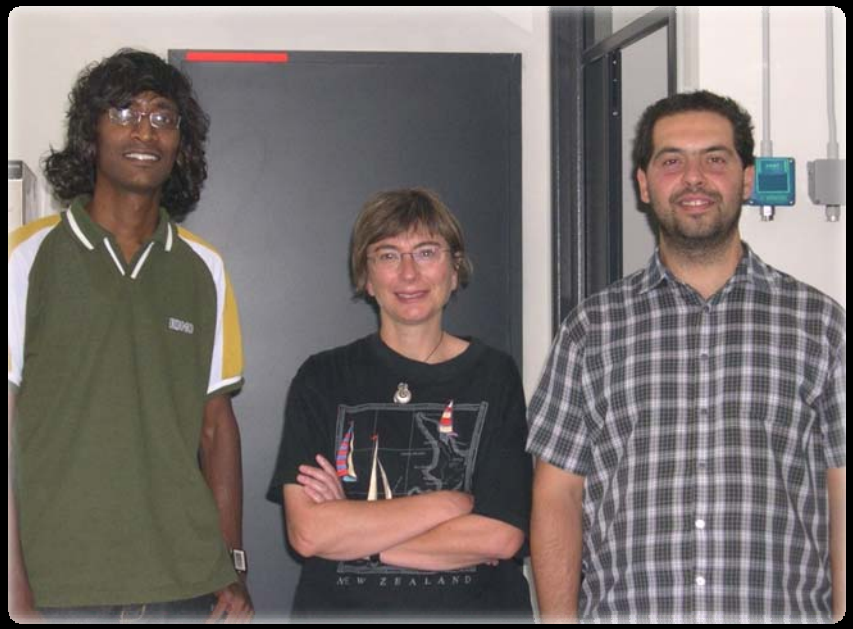
Dr. Dario Pediconi, Post doc

Chandramohan Bathrachalam, PhD candidate



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THANK YOU