Current studies on molecular mechanisms of iron homeostasis in rhinoceroses

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Iron overload in captivity correlates with wild forage

Browsers (shrubs, branches) vs. Grazers (grasses)

←Black

White→

←Sumatran

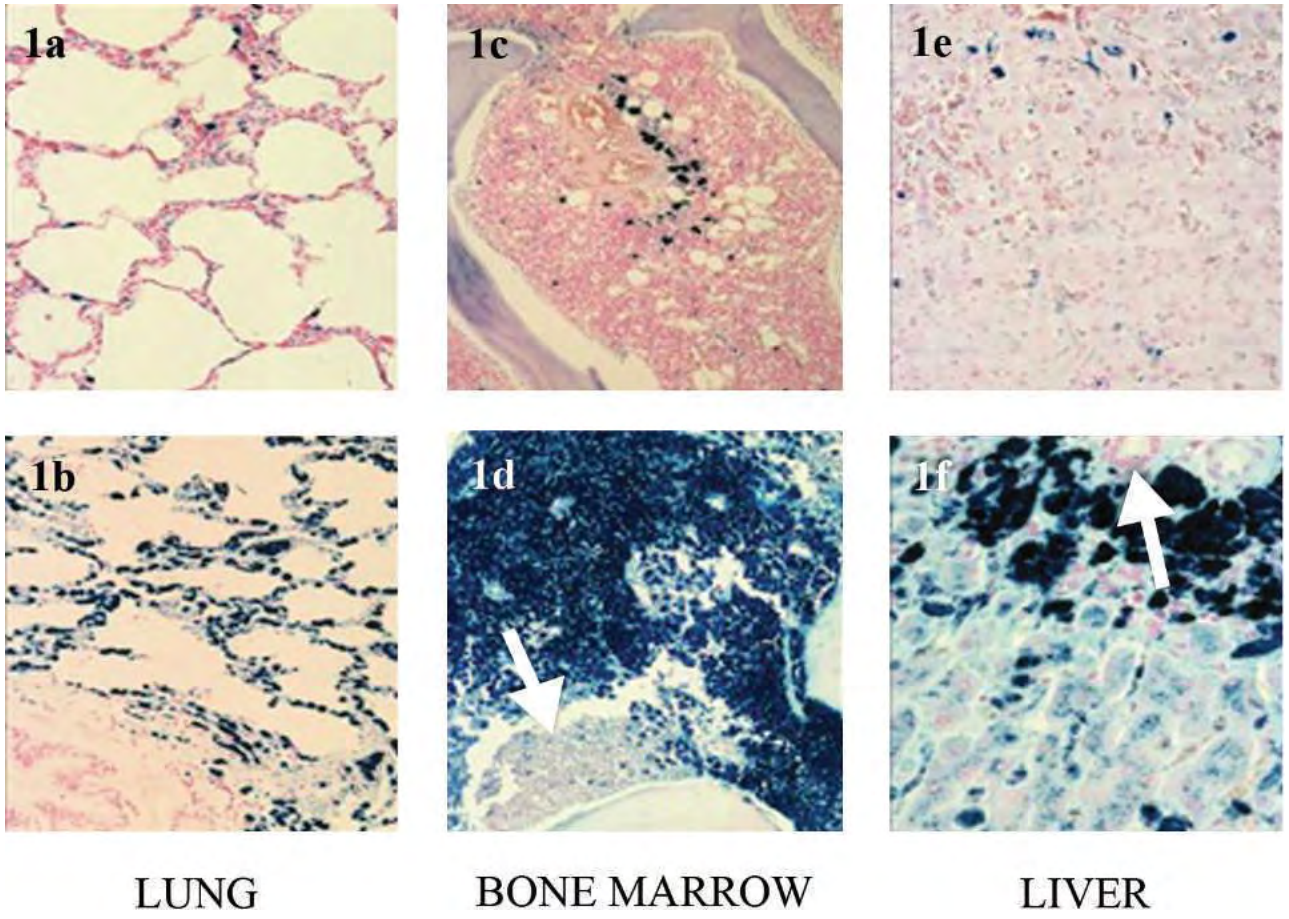
Indian→

Affected by iron overload in captivity

Unaffected

Iron overload in black rhinos

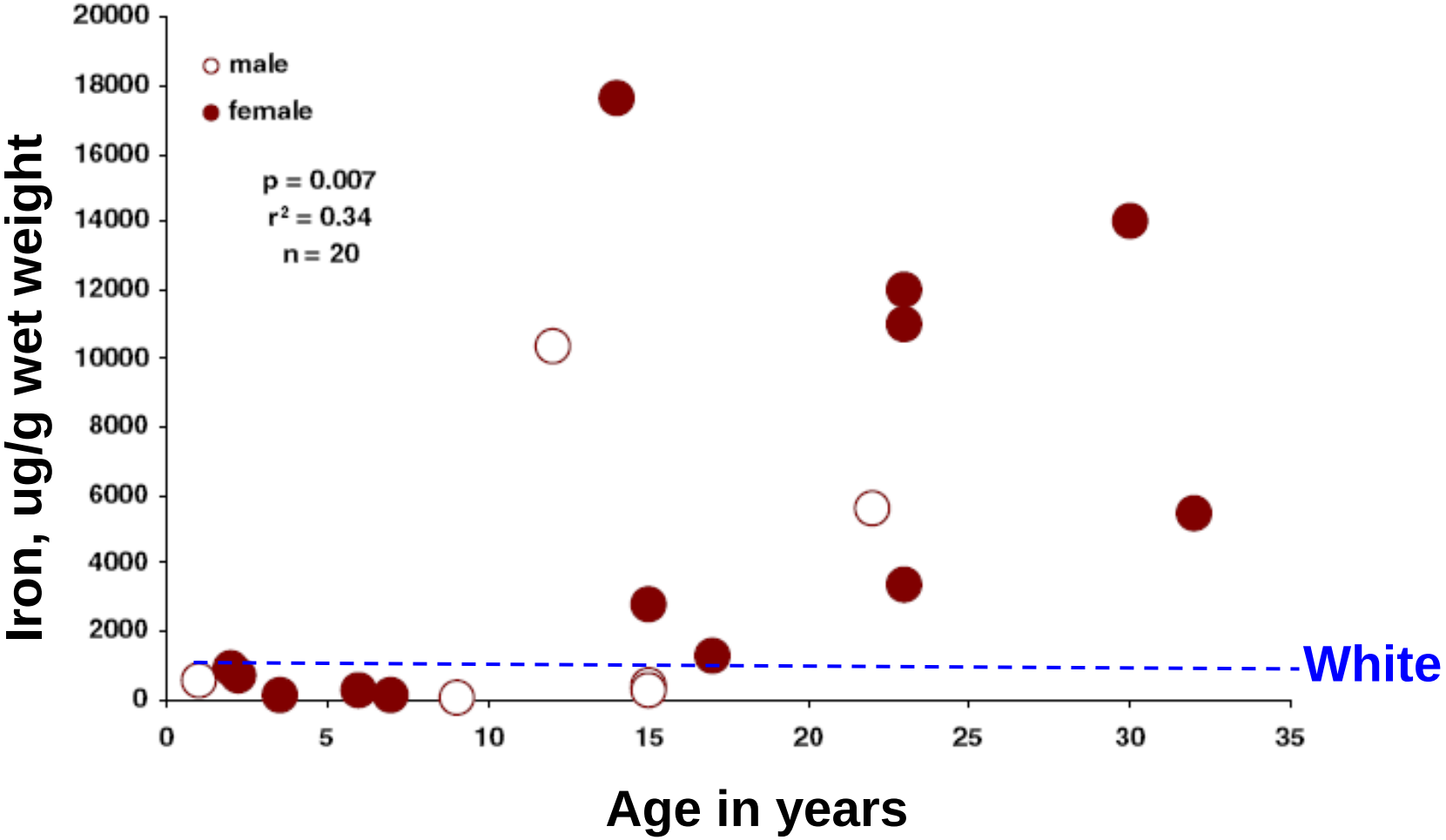
Perls stain iron deposits in tissues



Journal of Zoo and Wildlife Medicine (2012) **43(3s):**S92-S104

Iron overload in black rhinos

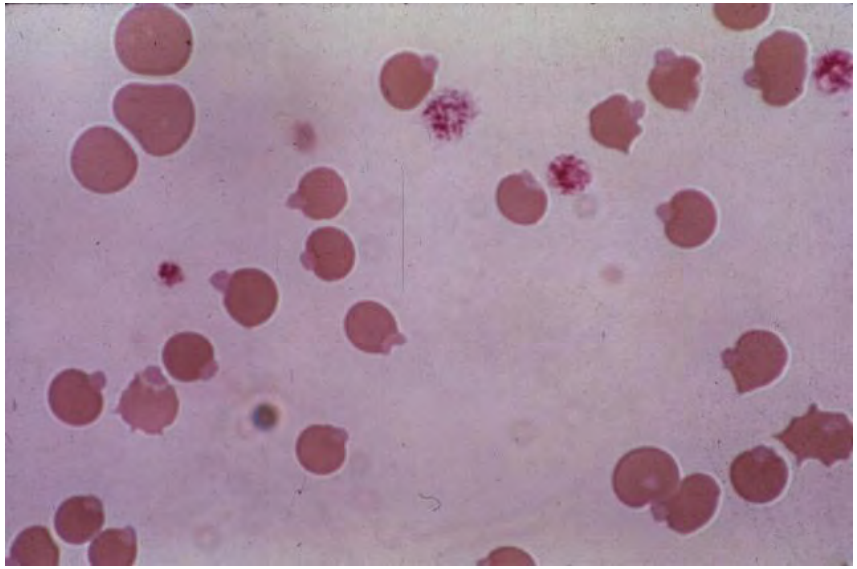
Liver iron levels increase with time in captivity



Zoo Biology (2005) **24**:51-72

Erythrocyte abnormality: Hemolytic anemia in black rhinos

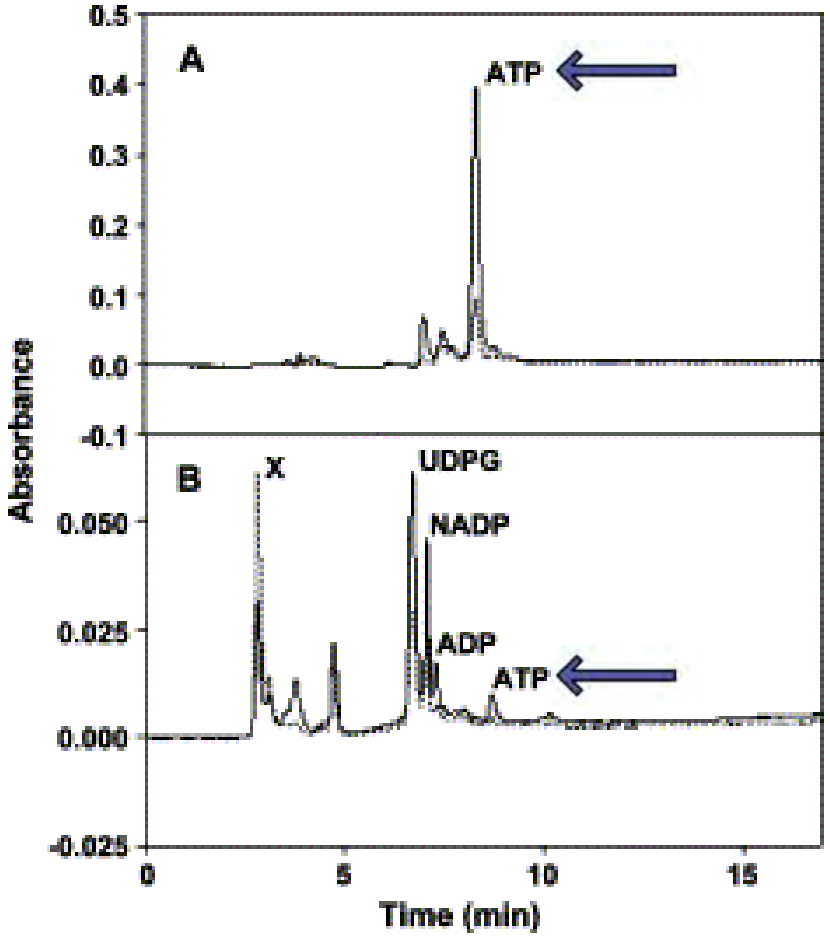
- Hemolysis of RBCs contributes to iron overload
- Can lead to death
- Potential cause: genetic mutation
  - Fragile RBC membrane
  - Prone to lysis



Hemolytic anemia horse
http://www.vetnext.com/

Erythrocyte abnormality:

ATP in black rhinos 5% of that in humans

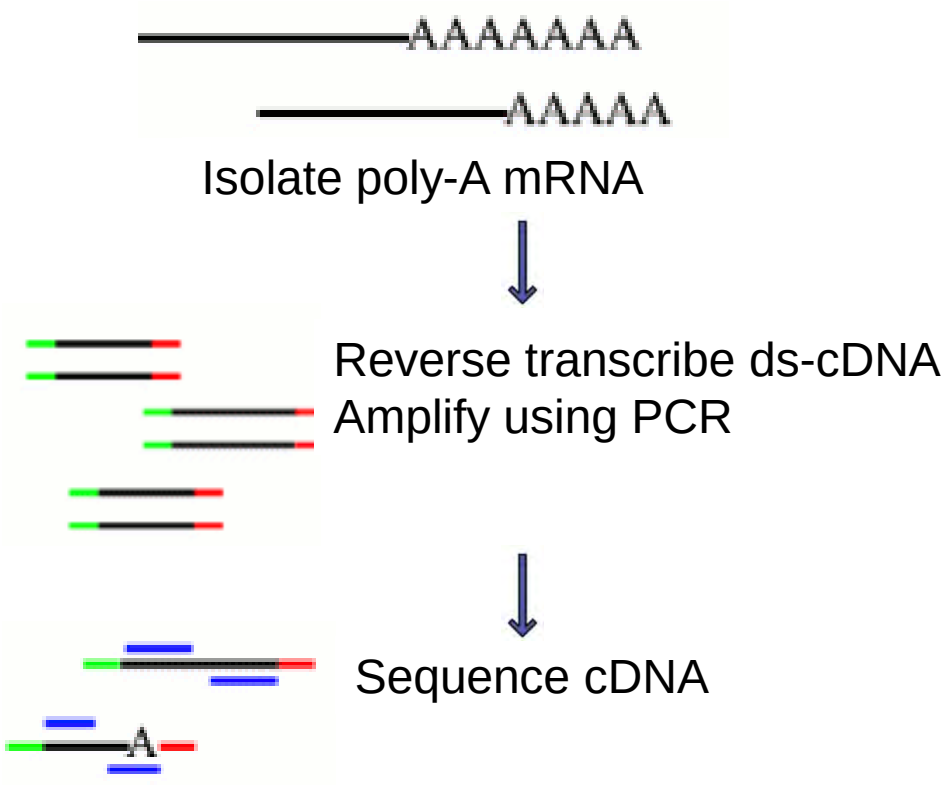


- Anion-exchange HPLC extract red blood cells
  - human (A)
  - black rhino (B)
- ATP required to maintain cell barrier
  - Low ATP levels might contribute to hemolytic anemia

Comp Biochem Physiol A Mol Integr Physiol. (2004)**138:**105-109

Search for genetic differences related to iron overload

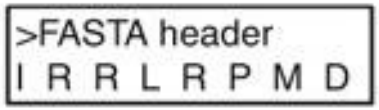
- White vs. Black rhino
- Sequencing mRNA
  - Liver mRNA
    - Iron homeostasis
  - Spleen mRNA
    - Recycling RBCs
- Acquire sequences
- Assemble
  - Trinity software



cmb.molgen.mpg.de/2ndGenerationSequencing

Identify potentially deleterious mutations

SIFT sorting intolerant from tolerant substitutions



Input query:
translated
RNA sequences

Align related proteins
From NCBI database

conserved
highly conserved
unconserved

SIFT calculates conservation
value at each amino acid position

SIFT predicts effect of substitution
at a particular position

probability

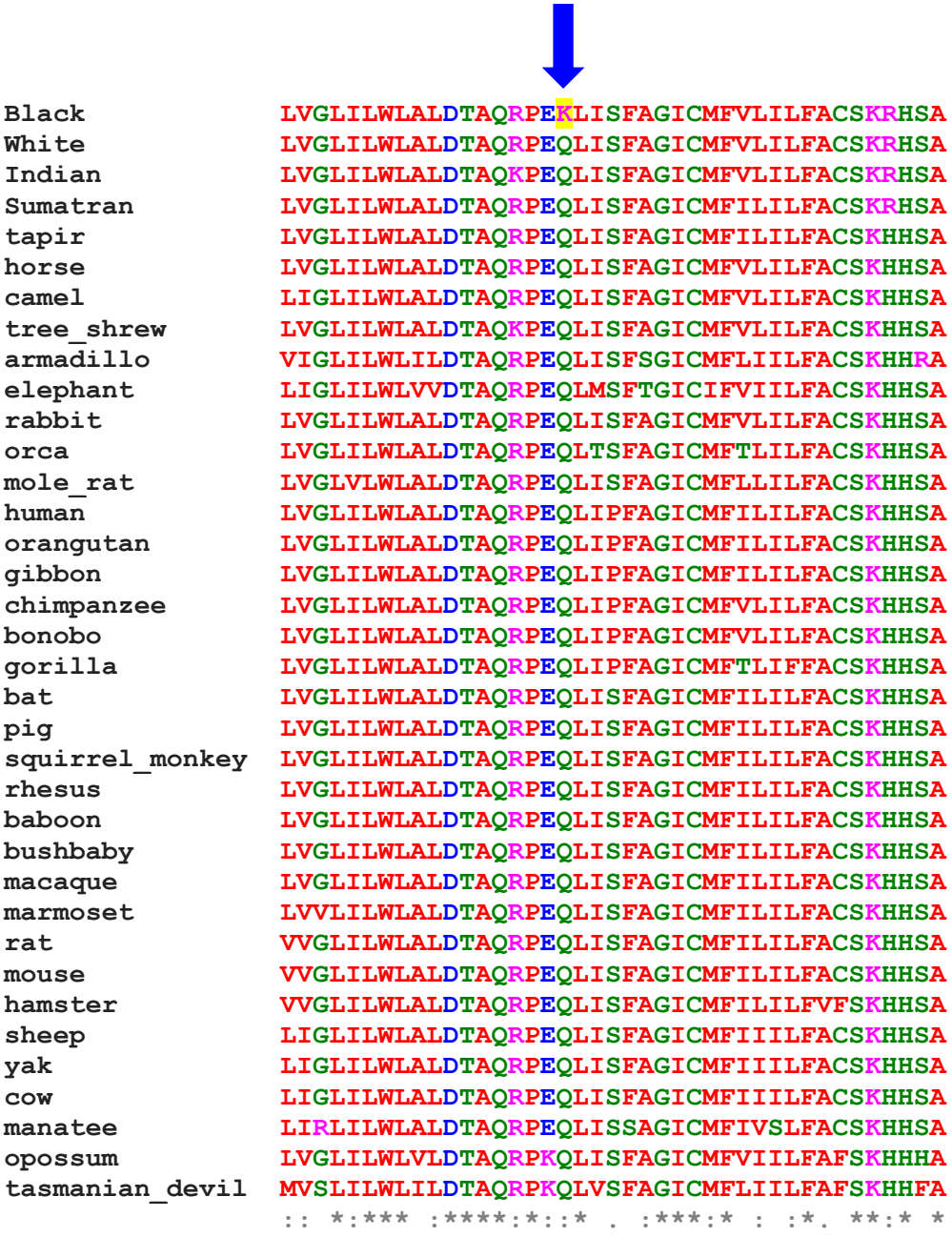
tolerated

deleterious

Nature Protocols (2009) **4:** 1073 - 1081

3 candidate mutations identified in black rhinos

Gene	Protein Function	Link to Black Rhino Phenotype	Mutation
SLC28a2	Solute carrier family 28 member 2 sodium-coupled nucleoside transporter for adenosine	Very low levels of erythrocyte ATP	Q173K Q – Glutamine K – Lysine
EPB41	Protein 4.1; structural element of erythrocyte membrane skeleton	Hemolytic anemia	G111E G – Glycine E – Glutamic acid
STEAP4	Six-transmembrane epithelial antigen of the prostate protein family obesity related insulin resistance and inflammatory processes	Suggested link elevated iron stores and insulin resistance	I433S I – Isoleucine S – Serine

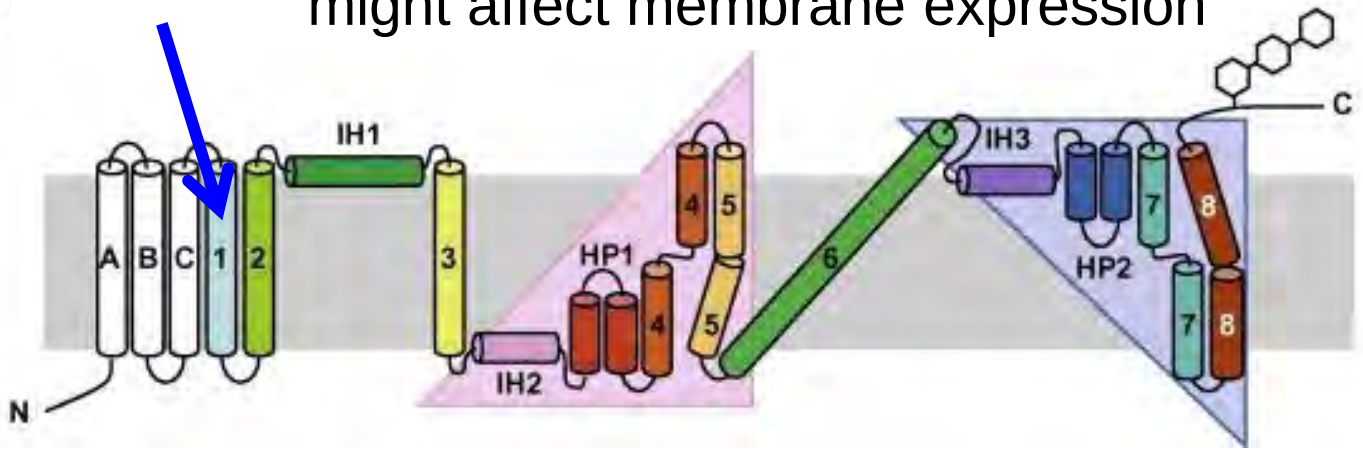


SLC28a2 Q173K

- ClustalW2 alignment
- Portion of SLC28a2 protein with amino acid position 173
- Sequence from 36 different species
- The glutamine (Q) at position 173 invariant
  - Except in black rhino
  - Replaced by lysine

Position SLC28a2 black rhino mutation

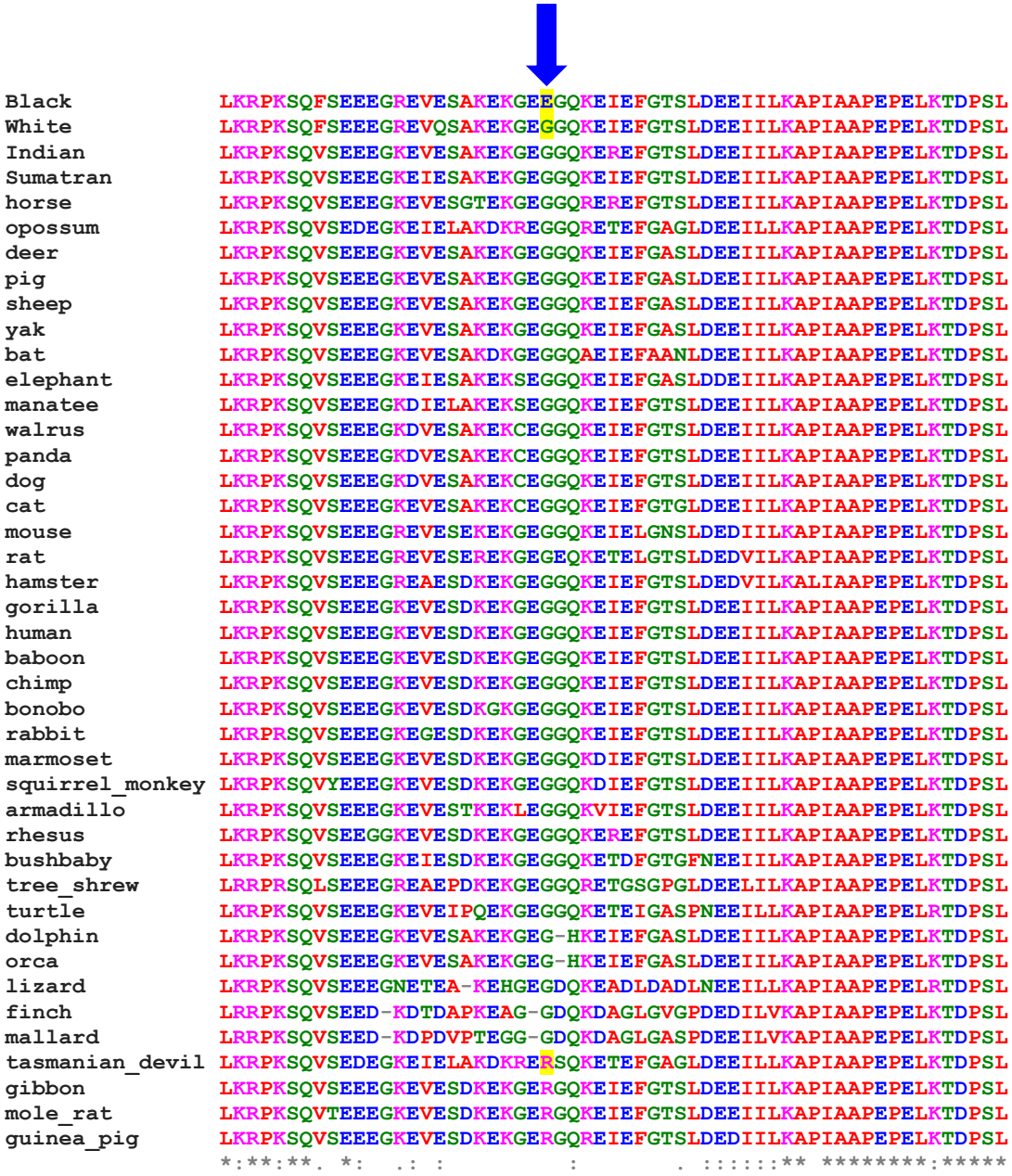
Slc28a2 Q173K in transmembrane 1 domain might affect membrane expression



Mol Aspects Med (2013) **34:**529-47

Protein 4.1, a component of the erythrocyte membrane skeleton

- Stabilizes erythrocyte shape and membrane mechanical properties, such as deformability and stability
- In humans, rare deletions cause complete loss of protein 4.1R, severe hemolytic anemia
  - A disease common in captive Black rhinos
- Knock-out mouse model
  - Decreased deformability of erythrocyte plasma membrane, increased hemolysis leading to hemolytic anemia



EPB41 G111E

Amino acid		Charge	# Spp
E	glutamic acid	acid	1
G	glycine	neutral	37
R	arginine	basic	4

Not a conservative substitution
Black rhino only acidic side chain

Position EPB41 black rhino mutation

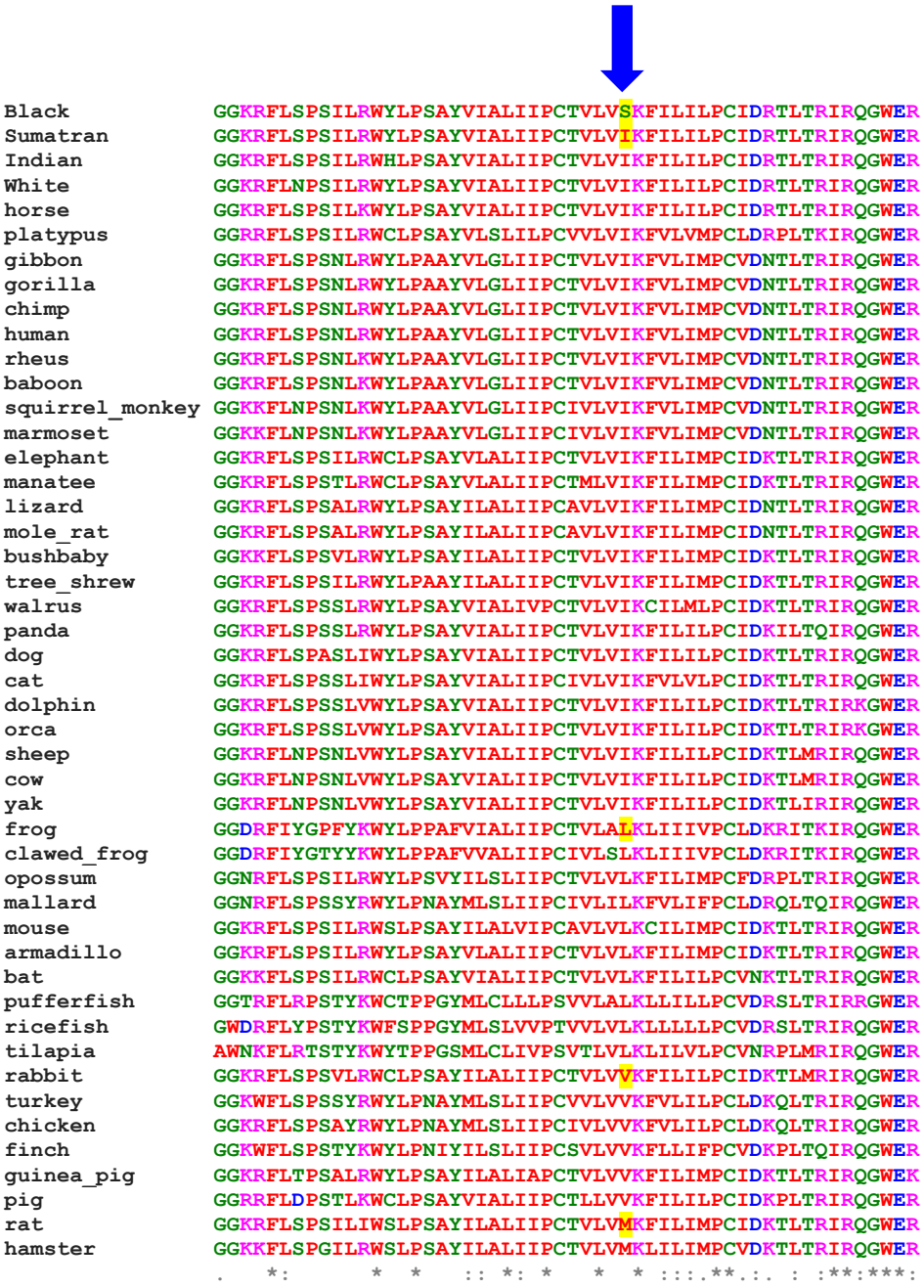
G111E near start site for erythroblasts translation
Might affect translation initiation

Int J Cell Biol (2011) **2011:**943272

STEAP4 – member of six-transmembrane epithelial antigen of the prostate protein family

- Associated with obesity, insulin resistance, inflammation
  - K/O mouse has metabolic syndrome
  - Related to described black rhino issues
- High expression in adipose tissue
  - In captivity rhinos have greater fat stores
- N-terminal domain has oxidase activity
  - Allow cellular uptake of iron and copper
    - Both essential for glucose and lipid metabolism

Mol Cancer Res (2012) **10:**573-87

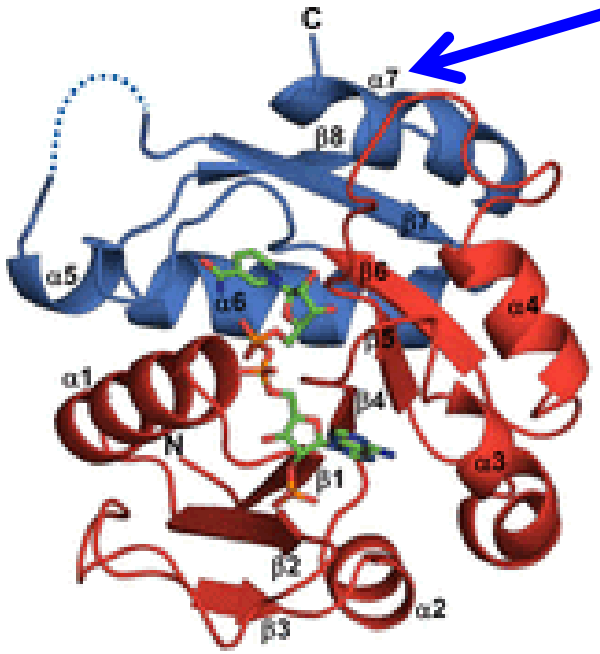


STEAP4 I433S

Amino acids			# Spp
S	Serine	Nucleophilic	1
I	Isoleucine	Hydrophobic	28
L	Leucine	Hydrophobic	10
V	Valine	Hydrophobic	6
M	Methionine	Hydrophobic	2

Not a conservative substitution

Position STEAP4 black rhino mutation



- STEAP4 I433S located in the α7 helix
  - Near site oxidation activity
  - A functionally significant location
- A defect in STEAP4 might explain insulin resistance in black rhinos
- In humans, metabolic syndrome causes mild iron overload

STEAP4 structure:
J Biol Chem (2013)**288**:20668-82

Black rhino insulin resistance:
Journal of Zoo and Wildlife Medicine (2012) **43(3s):**S61-S65

Conclusions and future plans

- Novel genetic techniques identify causes of hereditary disease
  - SLC28a2, EPB41 and STEAP4
  - Mutations are probably deleterious and located in functionally significant portions of the proteins
- Characterize candidate mutations
  - Express altered proteins and assay their function
- Expand to other affected rhino populations
  - Sumatran rhino tissue for mRNA isolation
    - RNA sequencing and SIFT
    - Identify and analyze candidate mutations
- Understanding the affect of these mutations could lead to improved care and treatment of iron overload in captive black rhinos

Acknowledgements

- Tom Ganz and Ella Nemeth
  - Helpful discussion and direction
- Don Paglia
  - Directing sample collection and insight into iron overload in rhinoceroses
- Pauline Lee
  - Directing RNA sequencing and SIFT analysis
- Ryan Thompson and Sarah LaMere
  - RNA sequencing and SIFT analysis
- Beto Palacios and Damond Ng
  - PCR and DNA sequencing