

# **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 →

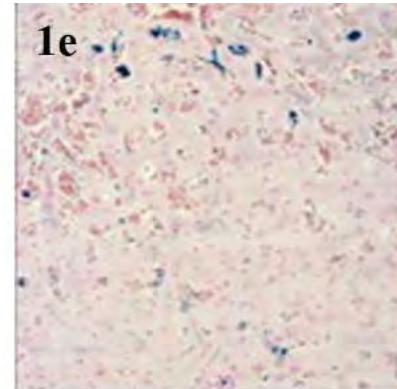
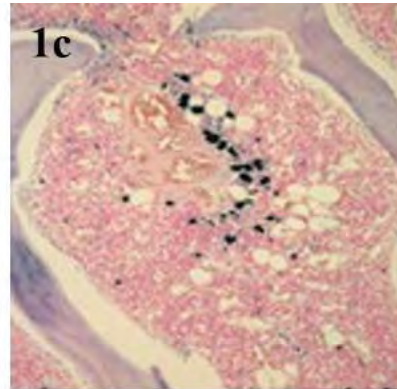
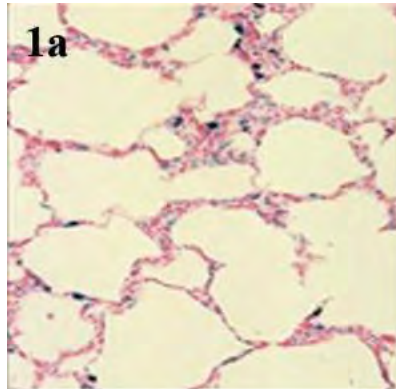


Affected by iron overload  
in captivity

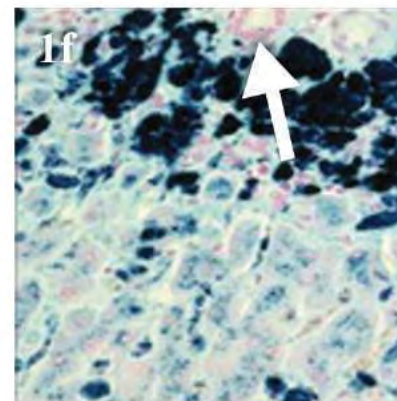
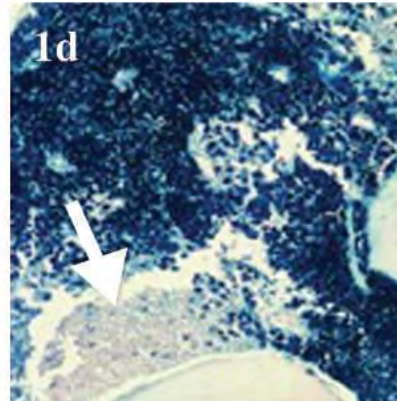
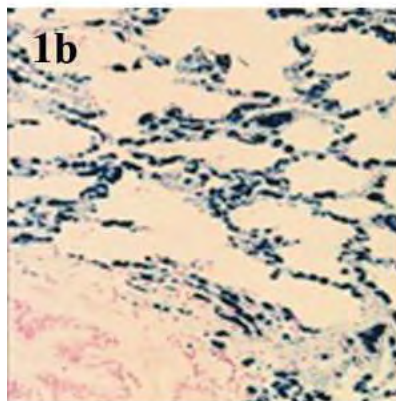
Unaffected

# Iron overload in black rhinos

## Perls stain iron deposits in tissues



White



Black

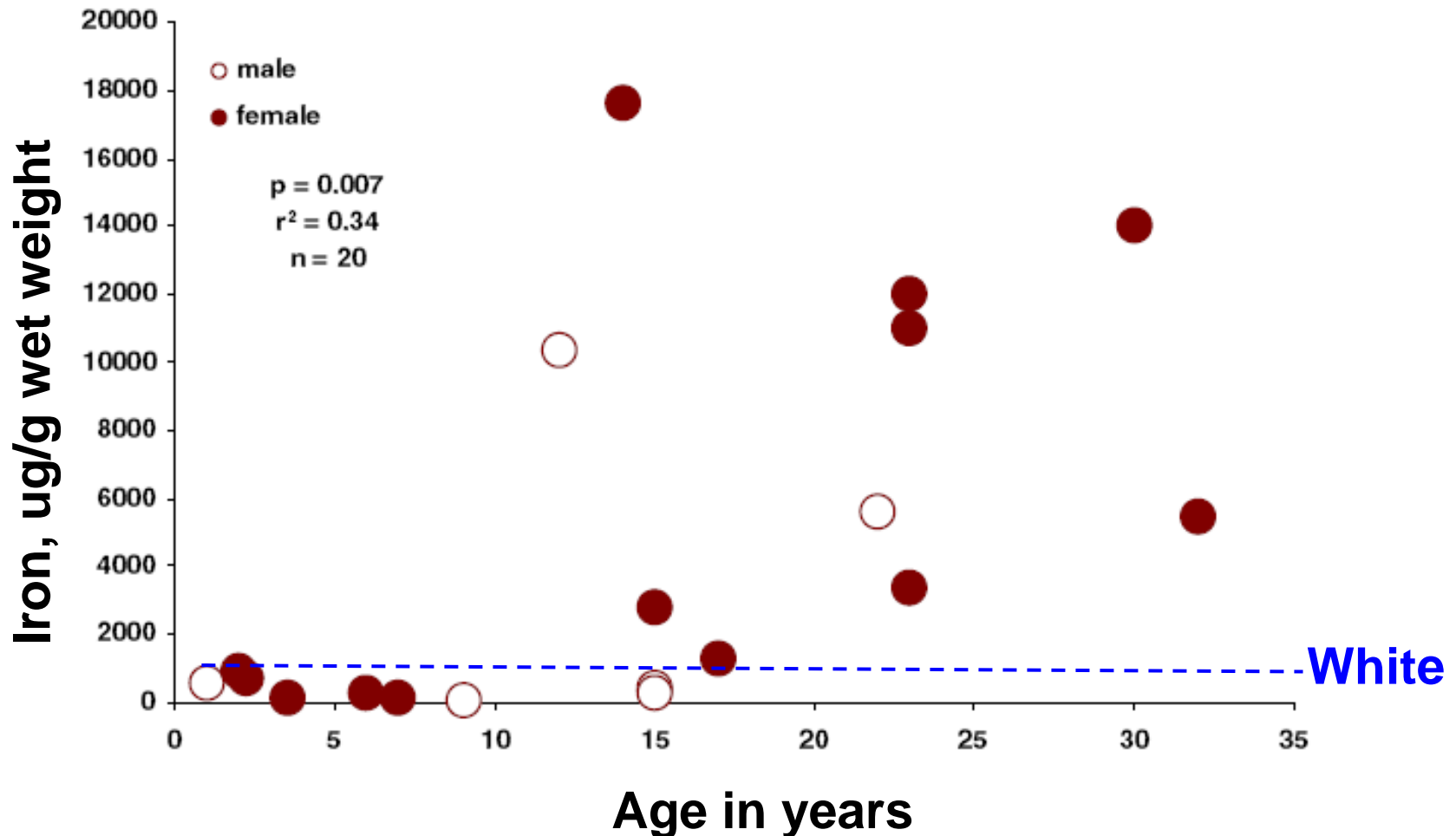
LUNG

BONE MARROW

LIVER

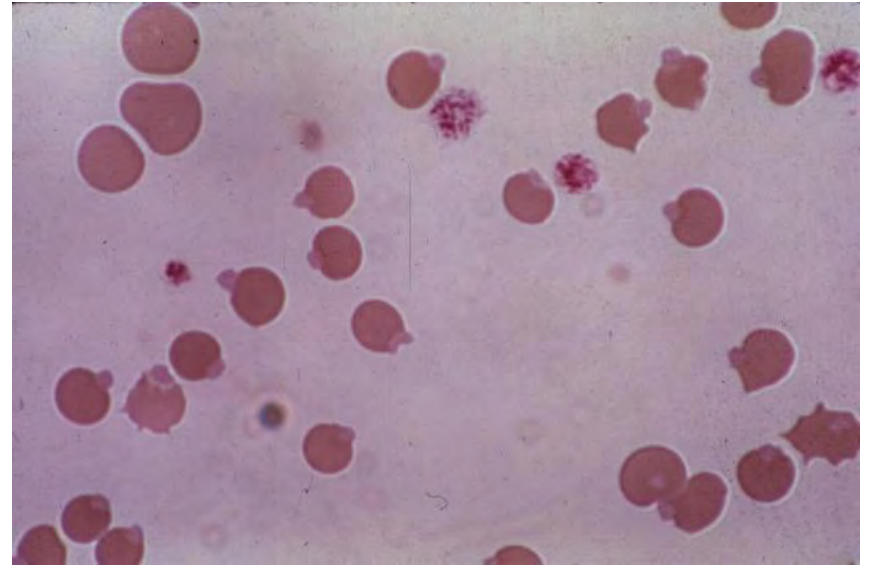
# Iron overload in black rhinos

Liver iron levels increase with time in captivity



# Erythrocyte abnormality: Hemolytic anemia in black rhinos

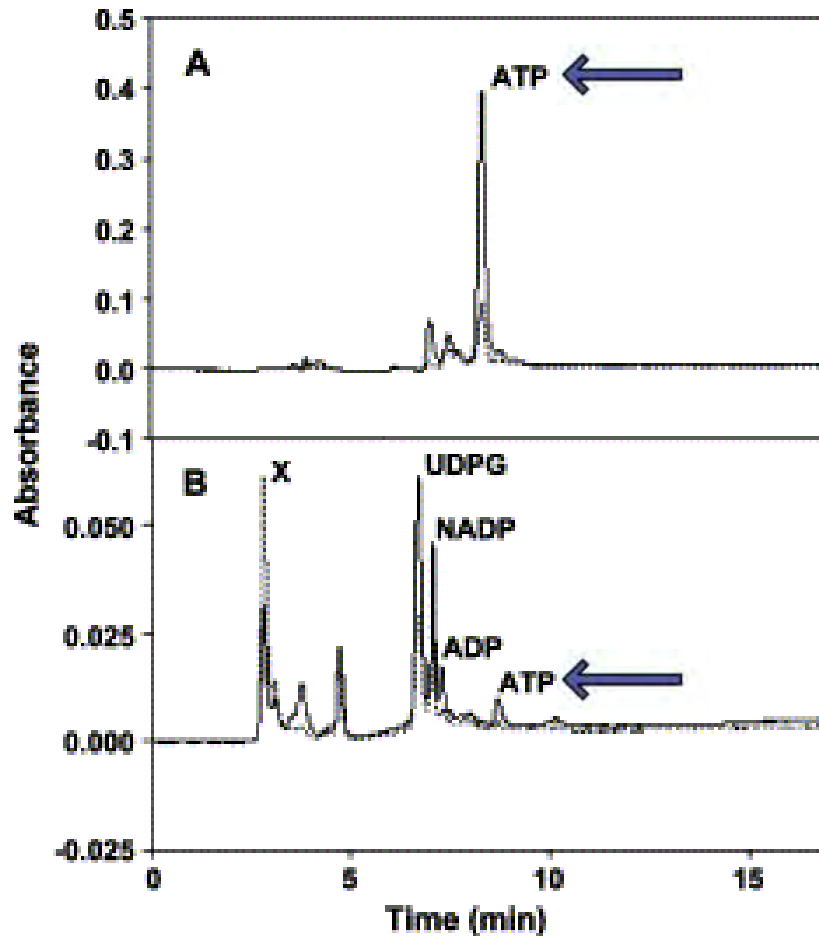
- Hemolysis of RBCs contributes to iron overload
- Can lead to death
- Potential cause: genetic mutation
  - Fragile RBC membrane
  - Prone to lysis



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

# Erythrocyte abnormality:

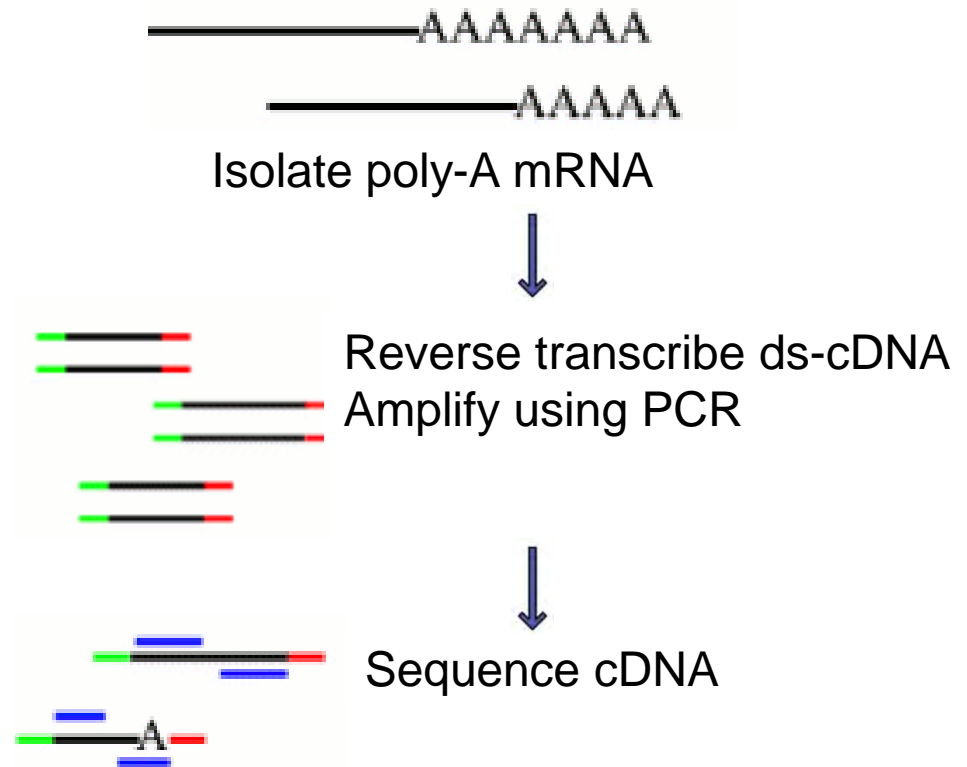
ATP in black rhinos 5% of that in humans



- 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

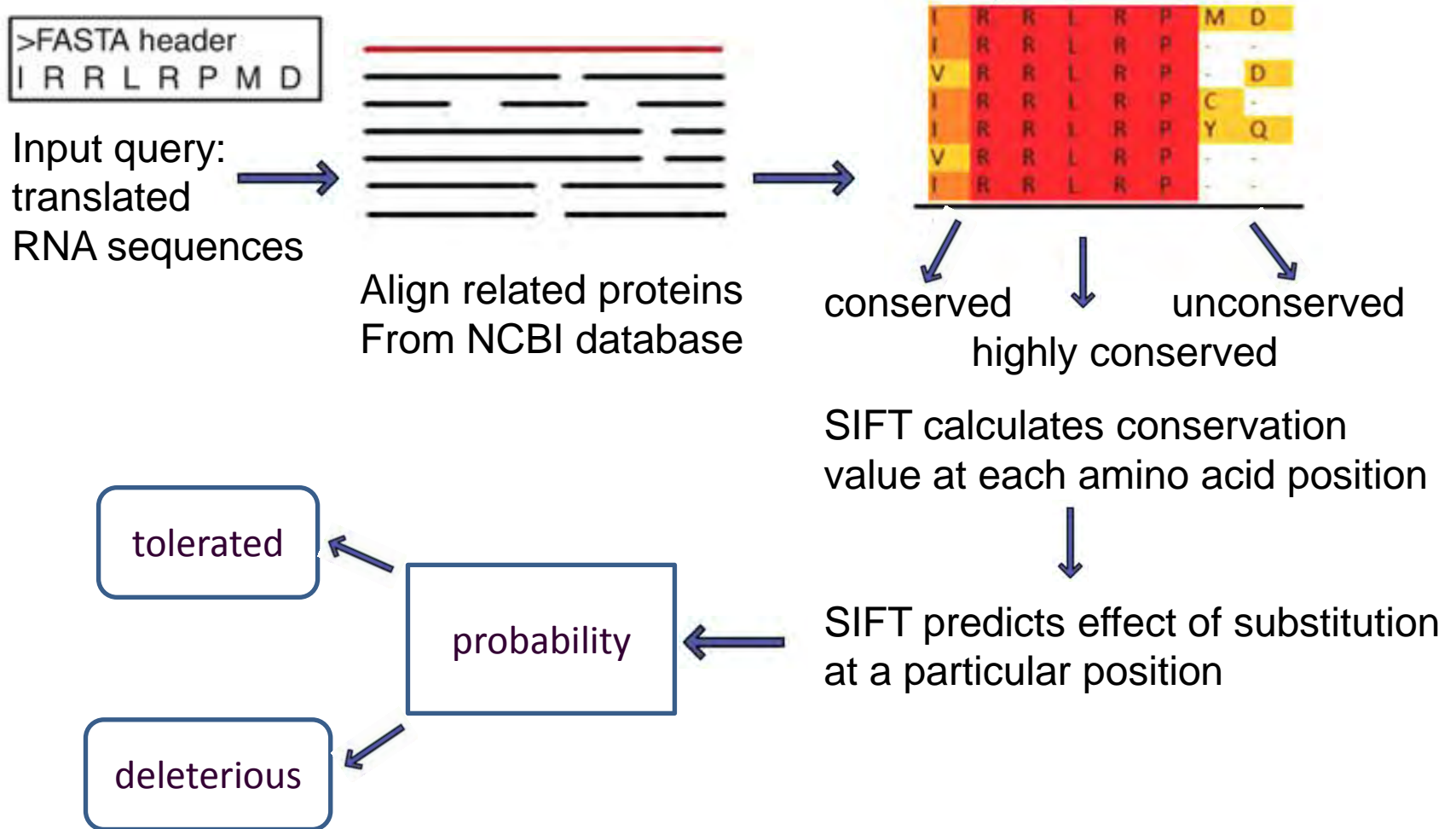
# Search for genetic differences related to iron overload

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



# Identify potentially deleterious mutations

## SIFT sorting intolerant from tolerant substitutions





## 3 candidate mutations identified in black rhinos

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



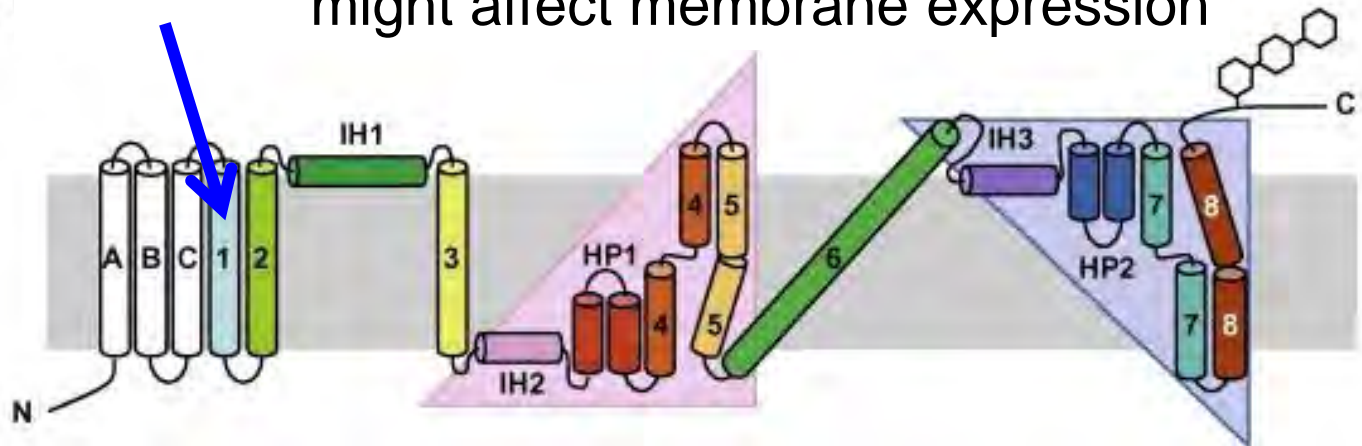
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Black      LVGLILWLALDTAQRPEQLISFAGICMFVLILFACSKRHSA
White     LVGLILWLALDTAQRPEQLISFAGICMFVLILFACSKRHSA
Indian    LVGLILWLALDTAQRPEQLISFAGICMFVLILFACSKRHSA
Sumatran  LVGLILWLALDTAQRPEQLISFAGICMFILILFACSKRHSA
tapir     LVGLILWLALDTAQRPEQLISFAGICMFILILFACSKHHS
horse     LVGLILWLALDTAQRPEQLISFAGICMFVLILFACSKHHS
camel     LIGLILWLALDTAQRPEQLISFAGICMFVLILFACSKHHS
tree_shrew LVGLILWLALDTAQRPEQLISFAGICMFVLILFACSKHHS
armadillo VIGLILWLALDTAQRPEQLISFSGICMFLIILFACSKHHS
elephant  LIGLILWLALDTAQRPEQLMSFTGICIFVILFACSKHHS
rabbit    LVGLILWLALDTAQRPEQLISFAGICMFVLILFACSKHHS
orca      LVGLILWLALDTAQRPEQLTSFAGICMFTLILFACSKHHS
mole_rat  LVGLVWLALDTAQRPEQLISFAGICMFLILFACSKHHS
human     LVGLILWLALDTAQRPEQLIPFAGICMFILILFACSKHHS
orangutan LVGLILWLALDTAQRPEQLIPFAGICMFILILFACSKHHS
gibbon    LVGLILWLALDTAQRPEQLIPFAGICMFILILFACSKHHS
chimpanzee LVGLILWLALDTAQRPEQLIPFAGICMFVLILFACSKHHS
bonobo    LVGLILWLALDTAQRPEQLIPFAGICMFVLILFACSKHHS
gorilla   LVGLILWLALDTAQRPEQLIPFAGICMFTLIFFACSKHHS
bat       LVGLILWLALDTAQRPEQLISFAGICMFILILFACSKHHS
pig       LVGLILWLALDTAQRPEQLISFAGICMFILILFACSKHHS
squirrel_monkey LVGLILWLALDTAQRPEQLISFAGICMFILILFACSKHHS
rhesus    LVGLILWLALDTAQRPEQLISFAGICMFILILFACSKHHS
baboon    LVGLILWLALDTAQRPEQLISFAGICMFILILFACSKHHS
bushbaby  LVGLILWLALDTAQRPEQLISFAGICMFILILFACSKHHS
macaque   LVGLILWLALDTAQRPEQLISFAGICMFILILFACSKHHS
marmoset  LVVILWLALDTAQRPEQLISFAGICMFILILFACSKHHS
rat       VVGLILWLALDTAQRPEQLISFAGICMFILILFACSKHHS
mouse     VVGLILWLALDTAQRPEQLISFAGICMFILILFACSKHHS
hamster   VVGLILWLALDTAQRPEQLISFAGICMFILILFVFSKHHS
sheep     LIGLILWLALDTAQRPEQLISFAGICMFIILFACSKHHS
yak       LIGLILWLALDTAQRPEQLISFAGICMFIILFACSKHHS
cow       LIGLILWLALDTAQRPEQLISFAGICMFIILFACSKHHS
manatee   LIRLILWLALDTAQRPEQLISSAGICMFIVSLFACSKHHS
opossum   LVGLILWLALDTAQRPKQLISFAGICMFVILFAFSKHHA
tasmanian_devil MVSILWLALDLTAQRPKQLVLSFAGICMFLIILFAFSKHHA
:: **  * : **** : ***** : : * . : **** : * : * . ** : *
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# 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



# 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

Black LKRPKSQFSEEEGREVESAKEKGGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 White LKRPKSQFSEEEGREVQSACEKGGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 Indian LKRPKSQVSEEEGKEVESAKEKGGGGQKEREFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 Sumatran LKRPKSQVSEEEGKEIESAKEKGGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 horse LKRPKSQVSEEEGKEVESGTEREGGGQREIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 opossum LKRPKSQVSEDEGKEIELAKDKREGGQRETEFGAGLDEEIIILKAPIAAPEPELKTDPSSL  
 deer LKRPKSQVSEEEGKEVESAKEKGGGGQKEIEFGASLDEEIIILKAPIAAPEPELKTDPSSL  
 pig LKRPKSQVSEEEGKEVESAKEKGGGGQKEIEFGASLDEEIIILKAPIAAPEPELKTDPSSL  
 sheep LKRPKSQVSEEEGKEVESAKEKGGGGQKEIEFGASLDEEIIILKAPIAAPEPELKTDPSSL  
 yak LKRPKSQVSEEEGKEVESAKEKGGGGQKEIEFGASLDEEIIILKAPIAAPEPELKTDPSSL  
 bat LKRPKSQVSEEEGKEVESAKDKGGGGQAEIEFAANLDEEIIILKAPIAAPEPELKTDPSSL  
 elephant LKRPKSQVSEEEGKEIESAKEKSEGGQKEIEFGASLDEEIIILKAPIAAPEPELKTDPSSL  
 manatee LKRPKSQVSEEEGKDIELAKEKSEGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 walrus LKRPKSQVSEEEGKDVESAKEKCEGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 panda LKRPKSQVSEEEGKDVESAKEKCEGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 dog LKRPKSQVSEEEGKDVESAKEKCEGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 cat LKRPKSQVSEEEGKEVESAKEKCEGGGQKEIEFGTGLDEEIIILKAPIAAPEPELKTDPSSL  
 mouse LKRPKSQVSEEEGREVESEKEKGGGGQKEIELGNSLDEEIIILKAPIAAPEPELKTDPSSL  
 rat LKRPKSQVSEEEGREVESEREKGGGGQKETELGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 hamster LKRPKSQVSEEEGREAESDKEKGGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 gorilla LKRPKSQVSEEEGKEVESDKEKGGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 human LKRPKSQVSEEEGKEVESDKEKGGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 baboon LKRPKSQVSEEEGKEVESDKEKGGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 chimp LKRPKSQVSEEEGKEVESDKEKGGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 bonobo LKRPKSQVSEEEGKEVESDKEKGGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 rabbit LKRPKSQVSEEEGKEGESDKEKGGGGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 marmoset LKRPKSQVSEEEGKEVESDKEKGGGGQKIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 squirrel\_monkey LKRPKSQVSEEEGKEVESDKEKGGGGQKIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 armadillo LKRPKSQVSEEEGKEVESTKEKLEGGQKIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 rhesus LKRPKSQVSEEEGKEVESDKEKGGGGQKEREFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 bushbaby LKRPKSQVSEEEGKEIESDKEKGGGGQKETDFGTGFNEEIIILKAPIAAPEPELKTDPSSL  
 tree\_shrew LRRPKSQLSEEEGREAEPPDKEKGGGGQRETTGSGPGLDEEIIILKAPIAAPEPELKTDPSSL  
 turtle LKRPKSQVSEEEGKEVEIPQEKGGGGQKETEIGASPNEEIIILKAPIAAPEPELKTDPSSL  
 dolphin LKRPKSQVSEEEGKEVESAKEKGGG-HKEIEFGASLDEEIIILKAPIAAPEPELKTDPSSL  
 orca LKRPKSQVSEEEGKEVESAKEKGGG-HKEIEFGASLDEEIIILKAPIAAPEPELKTDPSSL  
 lizard LKRPKSQVSEEEGNTEA-KEHGEGDQKEADLDADLNEEIIILKAPIAAPEPELKTDPSSL  
 finch LRRPKSQVSEED-KDTDAPKEAG-GDQKDAGLGVGPDDEDILVKAPIAAPEPELKTDPSSL  
 mallard LRRPKSQVSEED-KDPDVPTTEGG-GDQKDAGLGPDEEIIILKAPIAAPEPELKTDPSSL  
 tasmanian\_devil LKRPKSQVSEDEGKEIELAKDKREISQKETEFAGLDEEIIILKAPIAAPEPELKTDPSSL  
 gibbon LKRPKSQVSEEEGKEVESDKEKGERGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 mole\_rat LKRPKSQVSEEEGKEVESDKEKGERGQKEIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
 guinea\_pig LKRPKSQVSEEEGKEVESDKEKGERGQREIEFGTSLDEEIIILKAPIAAPEPELKTDPSSL  
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	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

# 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



# STEAP4 I433S

Black GGKRFLSPSILRWYLPAYVIALIIPCTVLVSKFILILPCIDRTLTRIRQGWER  
 Sumatran GGKRFLSPSILRWYLPAYVIALIIPCTVLVSKFILILPCIDRTLTRIRQGWER  
 Indian GGKRFLSPSILRWHLPAYVIALIIPCTVLVSKFILILPCIDRTLTRIRQGWER  
 White GGKRFLNPSILRWYLPAYVIALIIPCTVLVSKFILILPCIDRTLTRIRQGWER  
 horse GGKRFLSPSILRWYLPAYVIALIIPCTVLVSKFILILPCIDRTLTRIRQGWER  
 platypus GGKRFLSPSILRWYLPAYVIALIIPCTVLVSKFILILPCIDRTLTRIRQGWER  
 gibbon GGKRFLSPSNLRWYLPAYVGLIIPCTVLVSKFVLMPCVDNTLTRIRQGWER  
 gorilla GGKRFLSPSNLRWYLPAYVGLIIPCTVLVSKFVLMPCVDNTLTRIRQGWER  
 chimp GGKRFLSPSNLRWYLPAYVGLIIPCTVLVSKFVLMPCVDNTLTRIRQGWER  
 human GGKRFLSPSNLRWYLPAYVGLIIPCTVLVSKFVLMPCVDNTLTRIRQGWER  
 rhesus GGKRFLSPSNLRWYLPAYVGLIIPCTVLVSKFVLMPCVDNTLTRIRQGWER  
 baboon GGKRFLSPSNLRWYLPAYVGLIIPCTVLVSKFVLMPCVDNTLTRIRQGWER  
 squirrel\_monkey GGKRFLNPSNLKRWYLPAYVGLIIPCTVLVSKFVLMPCVDNTLTRIRQGWER  
 marmoset GGKRFLNPSNLKRWYLPAYVGLIIPCTVLVSKFVLMPCVDNTLTRIRQGWER  
 elephant GGKRFLSPSILRWYLPAYVIALIIPCTVLVSKFILILPCIDRTLTRIRQGWER  
 manatee GGKRFLSPSTLRWYLPAYVIALIIPCTMLVSKFILILPCIDRTLTRIRQGWER  
 lizard GGKRFLSPSALRWYLPAYVIALIIPCAVLVSKFILILPCIDNTLTRIRQGWER  
 mole\_rat GGKRFLSPSALRWYLPAYVIALIIPCAVLVSKFILILPCIDNTLTRIRQGWER  
 bushbaby GGKRFLSPSVLRWYLPAYVIALIIPCTVLVSKFILILPCIDRTLTRIRQGWER  
 tree\_shrew GGKRFLSPSILRWYLPAYVIALIIPCTVLVSKFILILPCIDRTLTRIRQGWER  
 walrus GGKRFLSPSSLRWYLPAYVIALIIPCTVLVSKCILMLPCIDRTLTRIRQGWER  
 panda GGKRFLSPSSLRWYLPAYVIALIIPCTVLVSKFILILPCIDKILTQIRQGWER  
 dog GGKRFLSPASLIWYLPAYVIALIIPCTVLVSKFILILPCIDRTLTRIRQGWER  
 cat GGKRFLSPSSLRWYLPAYVIALIIPCTVLVSKFILILPCIDRTLTRIRQGWER  
 dolphin GGKRFLSPSSLRWYLPAYVIALIIPCTVLVSKFILILPCIDRTLTRIRQGWER  
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 sheep GGKRFLNPSNLVWYLPAYVIALIIPCTVLVSKFILILPCIDKTLMRIRQGWER  
 cow GGKRFLNPSNLVWYLPAYVIALIIPCTVLVSKFILILPCIDKTLMRIRQGWER  
 yak GGKRFLNPSNLVWYLPAYVIALIIPCTVLVSKFILILPCIDKTLIRIRQGWER  
 frog GGDRFIYGFYKRWYLPAYVIALIIPCTVLVSKLIIIVPCLDKRITKIRQGWER  
 clawed\_frog GGDRFIYGTYYKRWYLPAYVIALIIPCTVLVSKLIIIVPCLDKRITKIRQGWER  
 opossum GGNRFLSPSILRWYLPAYVIALIIPCTVLVSKFILILPCIDRPLTRIRQGWER  
 mallard GGNRFLSPSSYRWYLPAYVIALIIPCTVLVSKFILILPCIDRPLTRIRQGWER  
 mouse GGKRFLSPSILRWYLPAYVIALIIPCTVLVSKFILILPCIDKTLTRIRQGWER  
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 bat GGKRFLSPSILRWYLPAYVIALIIPCTVLVSKFILILPCIDKTLTRIRQGWER  
 pufferfish GGTRFLRPSSTYKWTTPPGYMLCLLPSVVLAKLLLLPCVDRSLTRIRQGWER  
 ricefish GWDRFLYPSSTYKWFSPPGYMLSLVPTVVLVSKLIIIVPCVDRSLTRIRQGWER  
 tilapia AWNKFLRSTYKWTTPPGYMLCLVPSVTLVSKLIIIVPCVDRSLTRIRQGWER  
 rabbit GGKRFLSPSVLRWYLPAYVIALIIPCTVLVSKFILILPCIDKTLMRIRQGWER  
 turkey GGKRFLSPSSYRWYLPAYVIALIIPCTVLVSKFILILPCIDKTLTRIRQGWER  
 chicken GGKRFLSPSAYRWYLPAYVIALIIPCTVLVSKFILILPCIDKTLTRIRQGWER  
 finch GGKRFLSPSTYKRWYLPAYVIALIIPCTVLVSKFILILPCIDKTLTRIRQGWER  
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 pig GGKRFLDPSSTLKWYLPAYVIALIIPCTLLVSKFILILPCIDKPLTRIRQGWER  
 rat GGKRFLSPSILRWYLPAYVIALIIPCTVLVSKFILILPCIDKTLTRIRQGWER  
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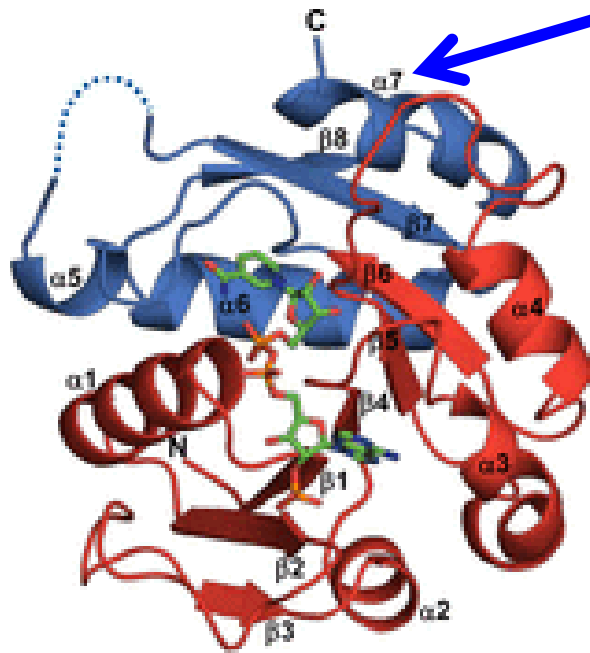
Amino acids			# Spp
S	Serine	Nucleophilic	1
I	Isoleucine	Hydrophobic	28
L	Leucine	Hydrophobic	10
V	Valine	Hydrophobic	6
M	Methionine	Hydrophobic	2

Not a conservative substitution

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# Position STEAP4 black rhino mutation



- STEAP4 I433S located in the  $\alpha 7$  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
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  - RNA sequencing and SIFT analysis
- Beto Palacios and Damond Ng
  - PCR and DNA sequencing