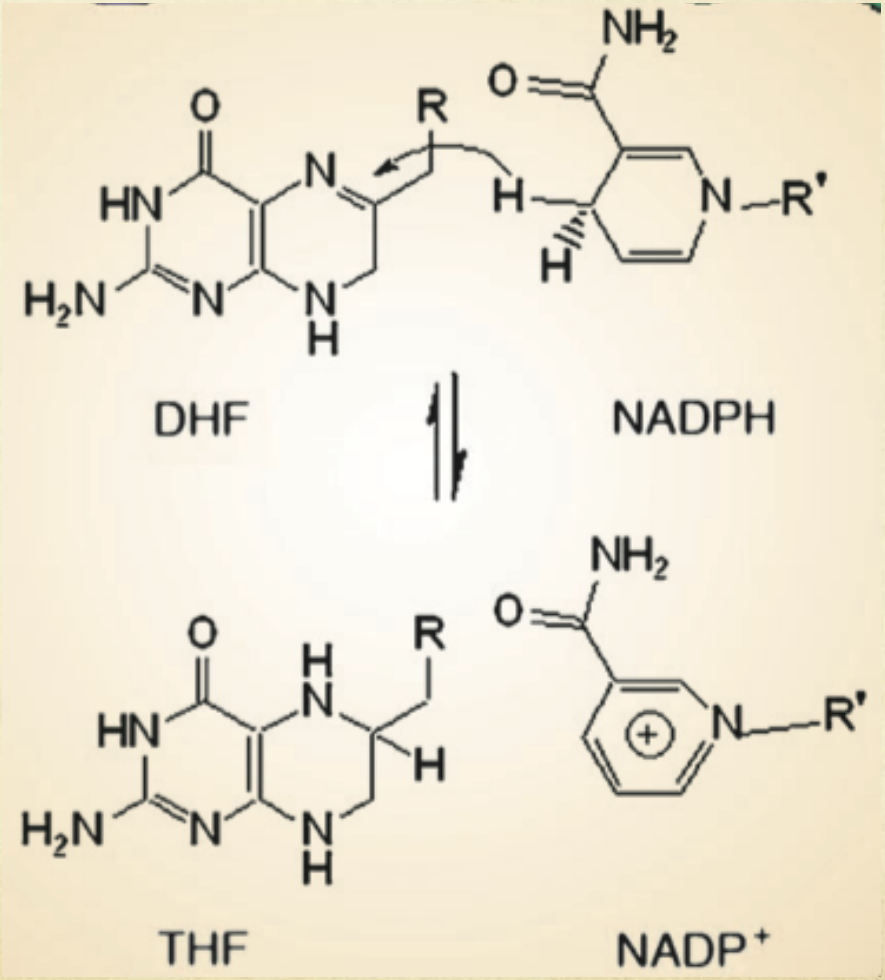
DHFR

Dihydrofolate reductase

DHFR katalüüsib redoks reaktsiooni

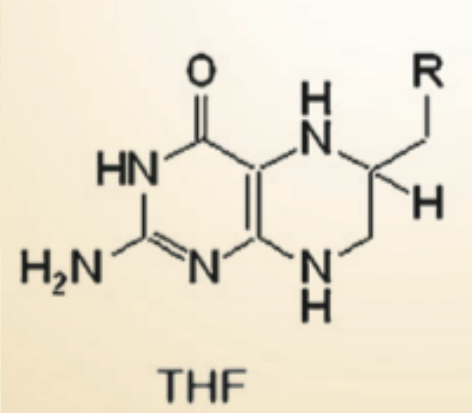
diHüdrofolaat
DHF

tetraHüdrofolaat
THF



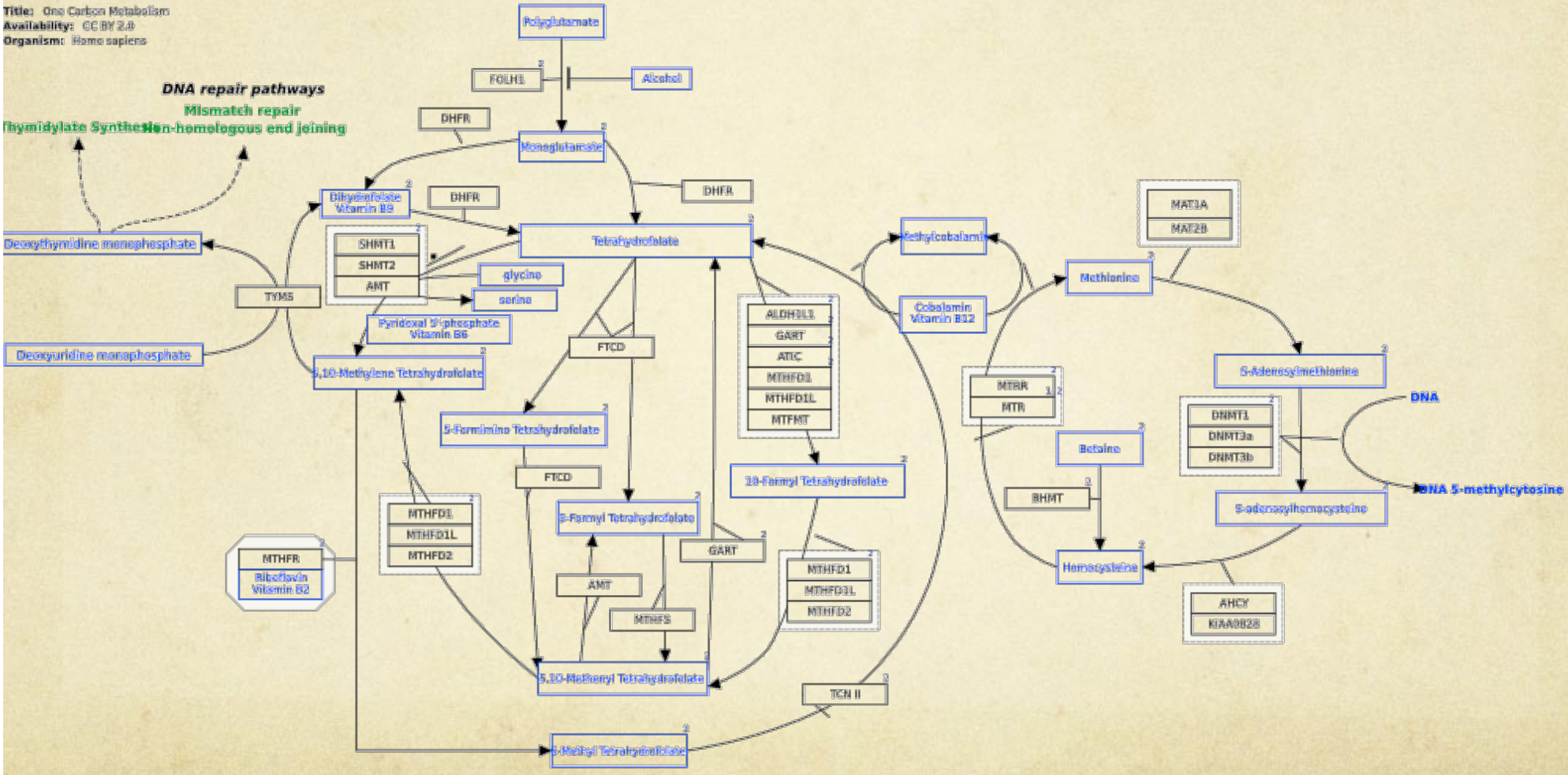
THF

- THF osaleb folaat-sõltuvate ensüümide töös ja seega on hädavajalik DNA sünteesiks ning metülatsiooniks
- Dihüdrofolaadi reduktaas on raku elutegevuseks hädavajalik ensüüm
- Eelkõige vajatakse rakujagunemisel ja teistel DNA sünteesiga seotud etappidel raku elus



THF

One Carbon Metabolism (Homo sapiens)



http://wikipathways.org/index.php/Pathway:WP241

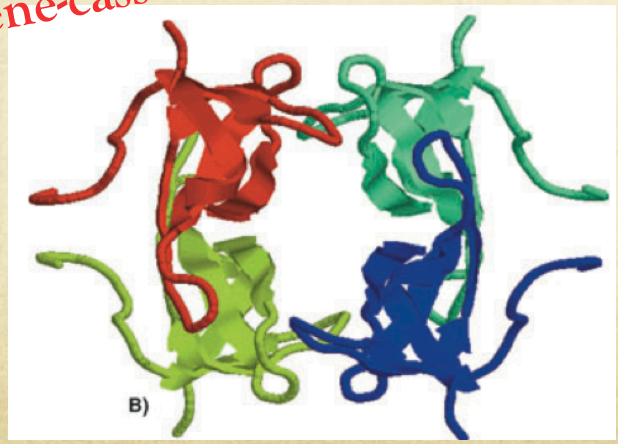
DHFR – 2 erinevat ensüümi

Chromosome coded

OPTIMIZED

- *dfrA* gene family
- monomer
- 152–189 amino acids
- identity levels of 20–90%

gene-cassetes



NON-OPTIMIZED

- *dfrB* gene family
- homo-tetramer
- 78 amino acids
- identity levels of 75%

DHFR – *dfrA* rahvamajanduslik tähtsus

- **Vähiravis** - N: acute lymphocytic leukemia, non-Hodgkin's lymphoma, osteosarcoma and choriocarcinoma
  - konkureeriv inhibiitor – MTX methotrexate
- **Rheumatoid artriidi** ravis – Low-dose MTX 'gold standard' (SNP ja individuaalne erinevus – erinevus ravis)
- **Seostatakse arenguhäirega** - Spina bifida – selgroo kanal osaliselt avatud
- **Teatud bakteriaalsete, seente ja algloomade infektsioonide mahasurumine**
  - Konkureeriv inhibiitor – TMP trimethoprim (imetaja DHFR resistentne)
- Peale TMP inhibitsiooni on inimese ja *E. coli* DHFR'i vahel veel teisigi katalüütilis/kineetilisi erinevusi

Functional significance of evolving protein sequence in dihydrofolate reductase from bacteria to humans

C. Tony Liu[a], Philip Hanoian[a], Jarrod B. French[a], Thomas H. Pringle[b,1], Sharon Hammes-Schiffer[c,1], and Stephen J. Benkovic[a,1]

- Millised on peamised erinevused inimese (hs) ja *E.coli* (ec) DHFR'i vahel?

  Biokeemialt –> järjestusele

- Kuidas need erinevused kajastuvad järjestuses?
- Kuna need erinevused on tekkinud? – evolutsiooniline aspekt
- Erinevuste funktsionaalne interpretatsioon kaasates struktuuri
- Katseline kinnitus/valideerimine

ecDHFR → hsDHFR
mõned eeldused ja faktid

- Inimese ja *E. coli* DHFR vahel on järjestuste identsus 26%
- Nende struktuurid on praktiliselt identsed
- => 26% identsust on piisav toetamaks sama struktuuri püsimist peale lahknemist ühisest eellasest
- Samuti on olemas järjestused, mis katavad evolutsioonilise vahemaa inimesest bakterini
- See võimaldab rekonstrueerida ensüümi muutumisega seotud olulised sündmused

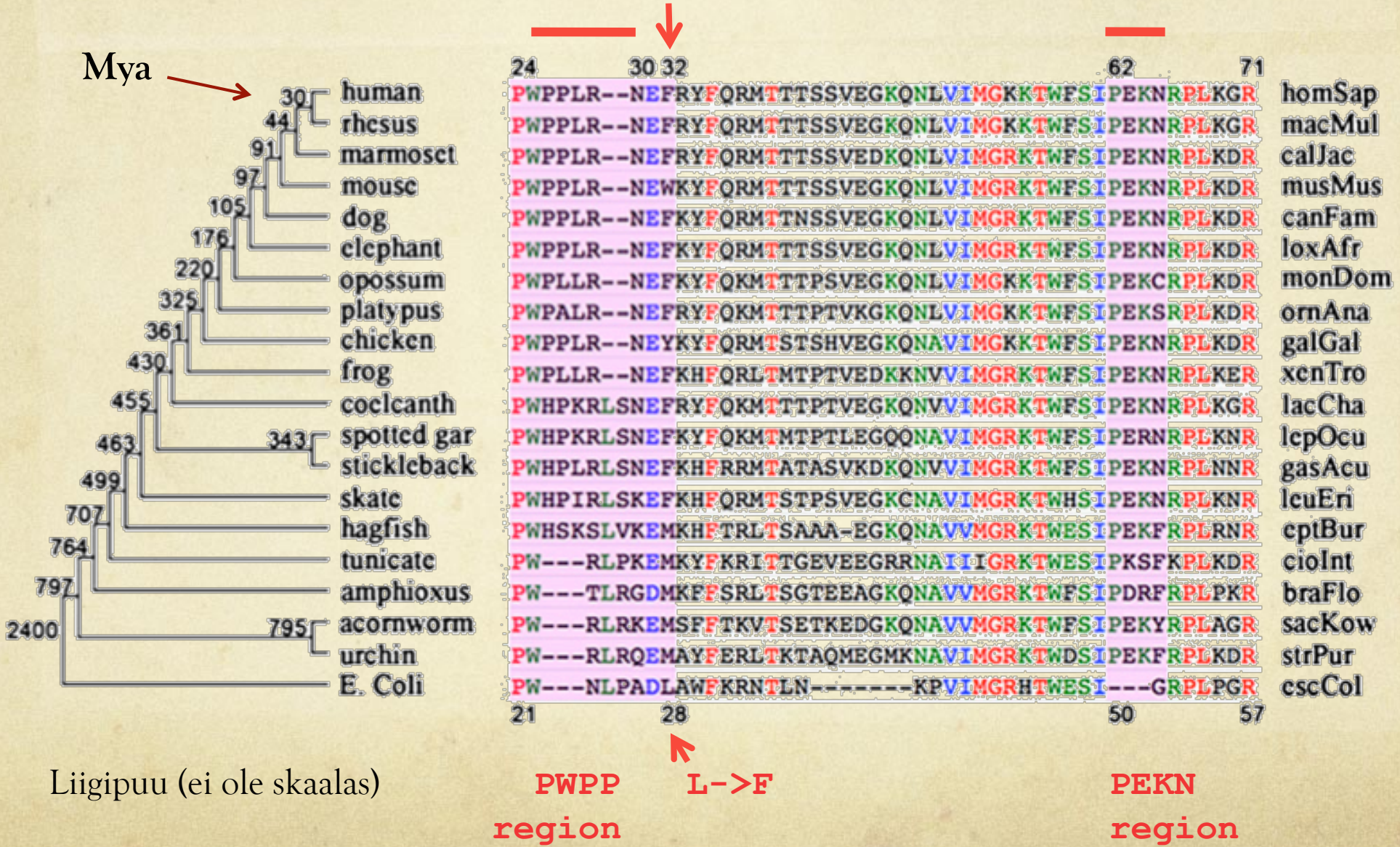
Evolutsiooniline analüüs

- Analüüsiti 233 järjestust, mille hulgas oli 99 selgroogse ja 14 bakteri järjestust
- kogu komplekt saadaval
  http://genomewiki.ucsc.edu/index.php/DHFR_dihydrofolate
- The ancestral sequence at each divergence node (Fig.1) can be reconstructed using a parsimony principle, in which **conservation** at an amino acid position **is observed** at a site **over two or more consecutive divergence nodes**, as is the case here for significant events in DHFR evolution.
- **Phylogenetically coherent events (PCEs)** are defined as changes at an amino acid **position at which both** the newly "altered site" and the unaltered long-conserved "ancestral site" **remained fixed** for **a significant amount** of subsequent geological **time**.

PCE analysis vt. suppl S13

Evolutionary Analysis: Phylogenetically Coherent Events (PCE)

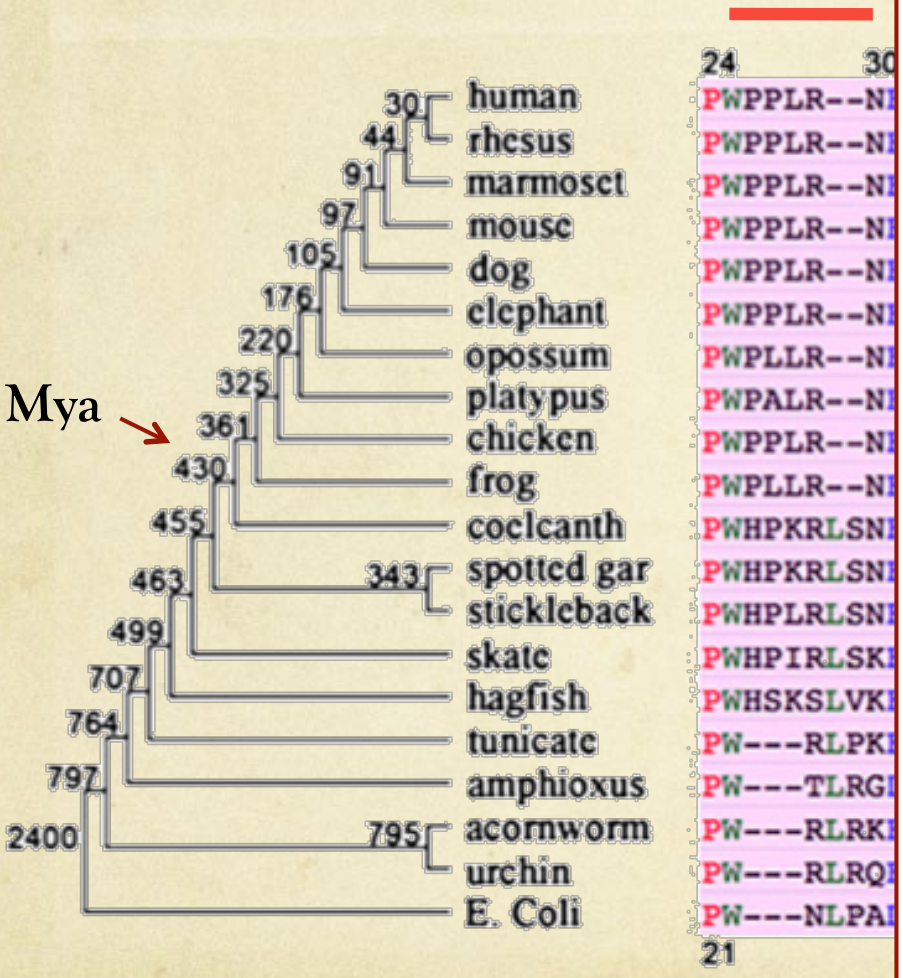
vt. suppl. S13



Liigipuu (ei ole skaalas)

www.pnas.org/cgi/doi/10.1073/pnas.1307130110

The most recent **PCE**



Liigipuu (ei ole skaalas)

PWPP region

Proline-rich region of the Met20 loop – 24-PWPPLRNEF-32 in hsDHFR

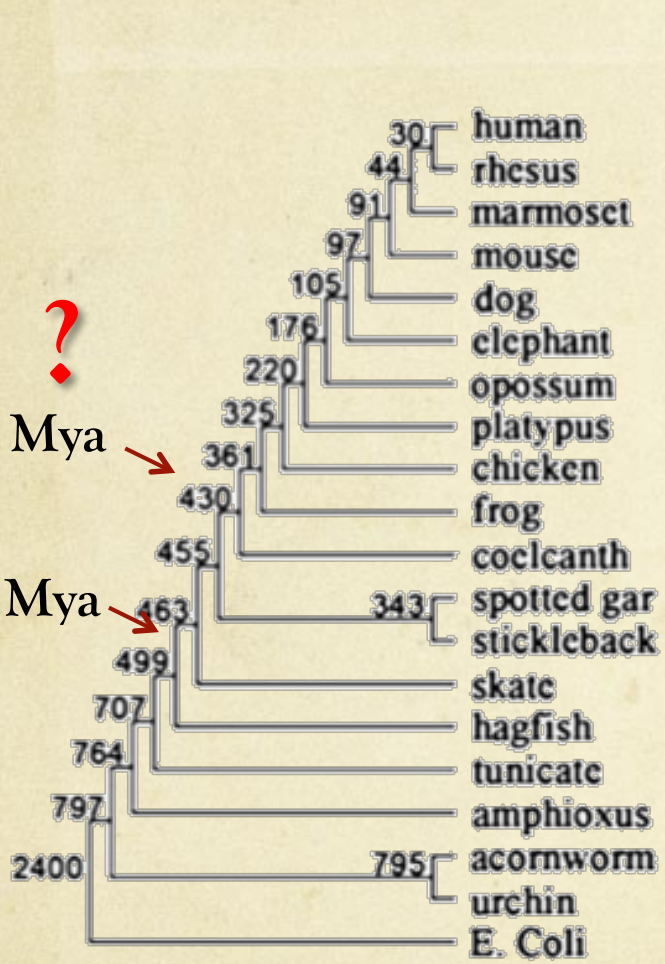
A unique evolutionary hotspot with a well-defined deletional/insertional history

The timing for the development of the PWPP region in hsDHFR is very clear: after fish and before amphibians; early to mid-Devonian, ~415–385 Mya

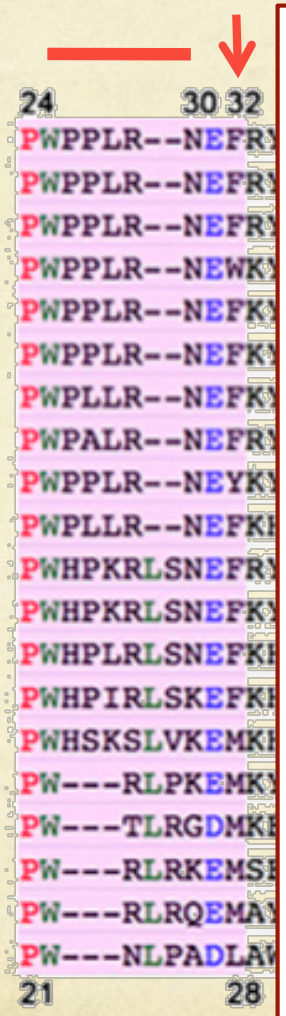
www.pnas.org/cgi/doi/10.1073/pnas.1307130110

vt. suppl. S13

The second strong **PCE**



Liigipuu (ei ole skaalas)



PWPP region

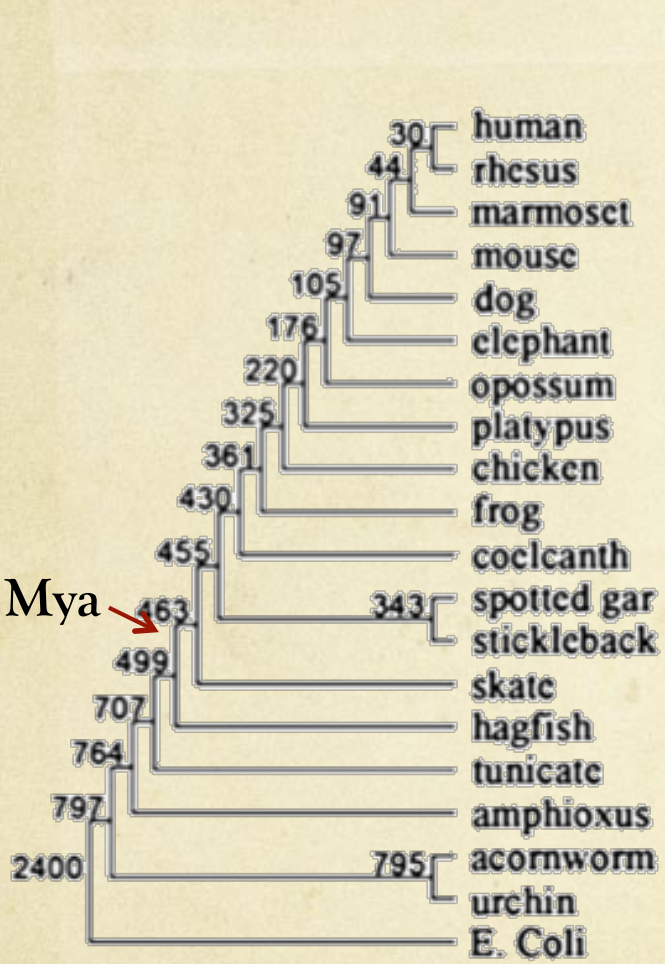
L->F

the transition from L28 in ecDHFR to F32 in hsDHFR, which occurred around the same time (~415–385 Mya) and has persisted as phenylalanine ever since

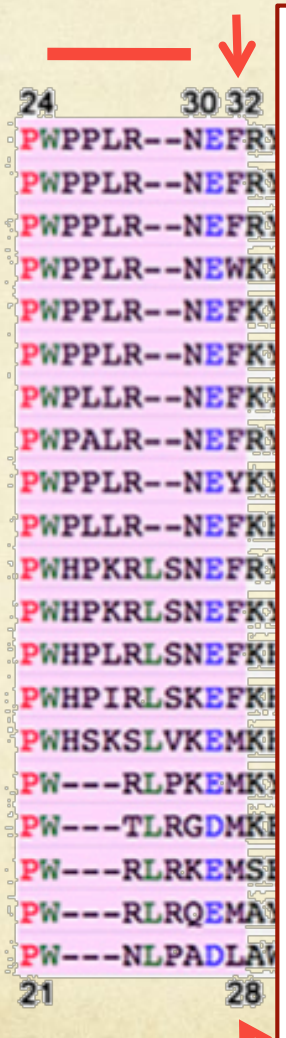
www.pnas.org/cgi/doi/10.1073/pnas.1307130110

vt. suppl. S13

The second strong **PCE**



Liigipuu (ei ole skaalas)



PWPP region

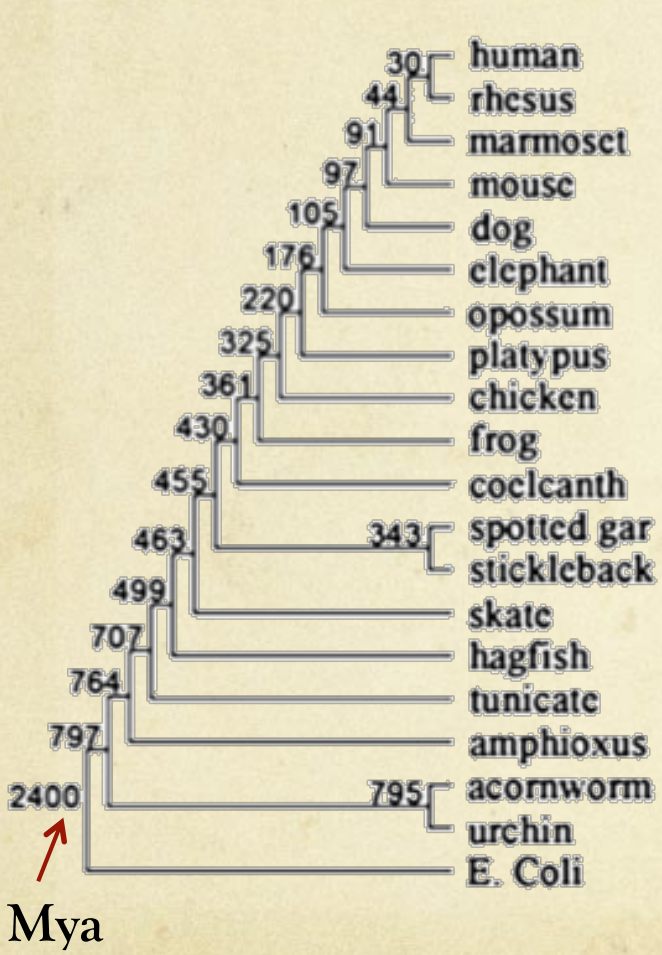
L->F

the transition from L28 in ecDHFR to F32 in hsDHFR occurred ~**490–460 Mya** and has persisted as phenylalanine ever since

www.pnas.org/cgi/doi/10.1073/pnas.1307130110

vt. suppl. S13

The most ancient **PCE**

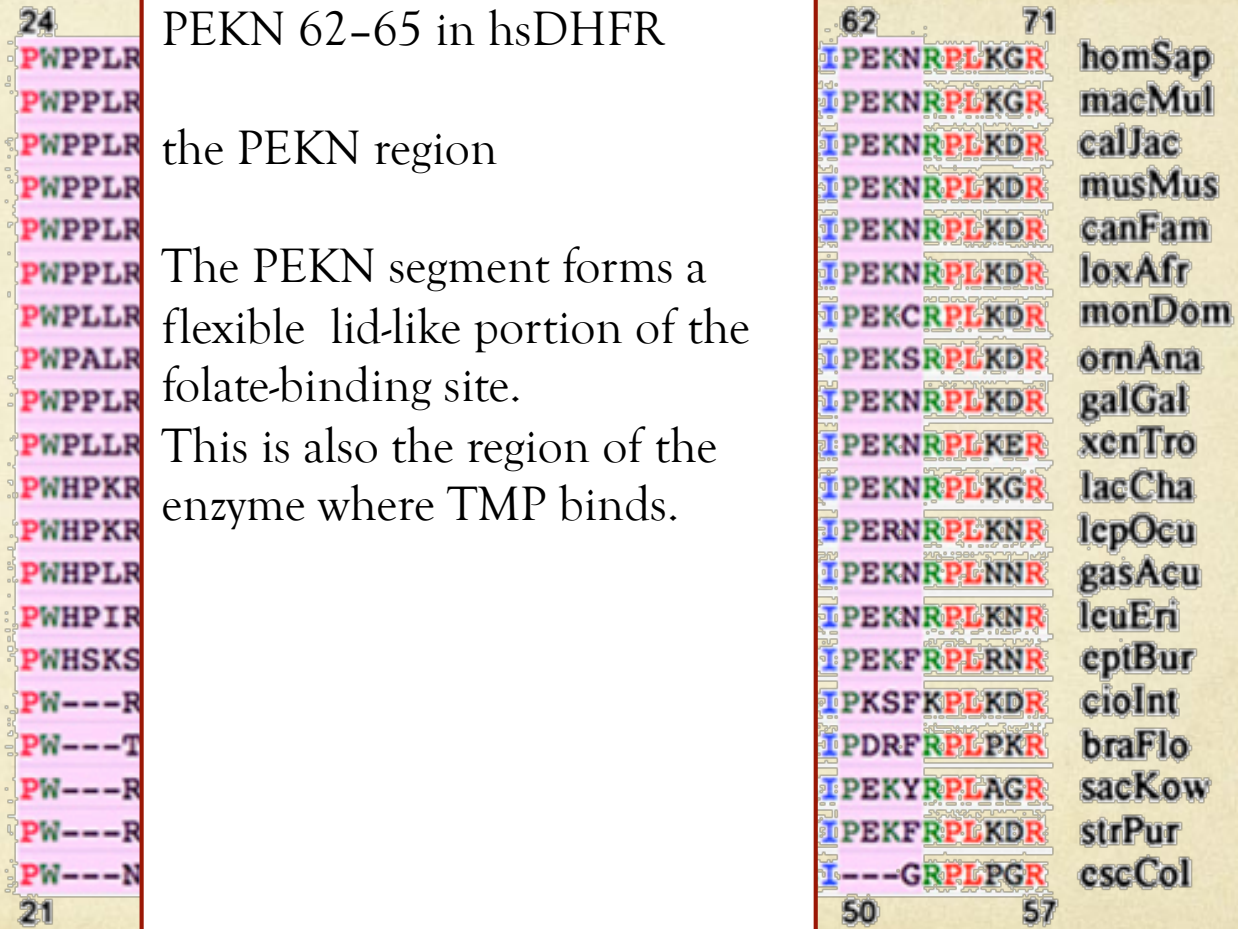


Liigipuu (ei ole skaalas)

around G51 in ecDHFR and PEKN 62-65 in hsDHFR

the PEKN region

The PEKN segment forms a flexible lid-like portion of the folate-binding site.
This is also the region of the enzyme where TMP binds.



PEKN region

vt. suppl. S13

www.pnas.org/cgi/doi/10.1073/pnas.1307130110

Muudetud ensüümid

- Muudetud ensüümide aluseks oli *E. coli* DHFR
- Konstruktid:
  - N23PP — PWPP region
  - N23PP/L28F — + L-> F
  - N23PP/G51PKEN — + PKEN region
  - N23PP/L28F/G51PEKN
- 3D struktuur määrati konstruktile N23PP/G51PEKN (PDB ID 4GH8)

1. Kinetic Implications of PCEs

- Kuidas mõjutavad PCE regioonide ülekandmine inimese DHFR'ilt *E. coli* DHFR'le uue mutantse ensüümi omadusi?

- Singel mutant **N23PP**
  - võrreldes ecDHFR'a **väheneb**
    reaktsiooni efektiivsus **~30X**
  - Pro rikas ala vähendab Met20 luubi liikuvust ja hoiab seda suletud konfirmatsioonis (oman hsDHFR'e)

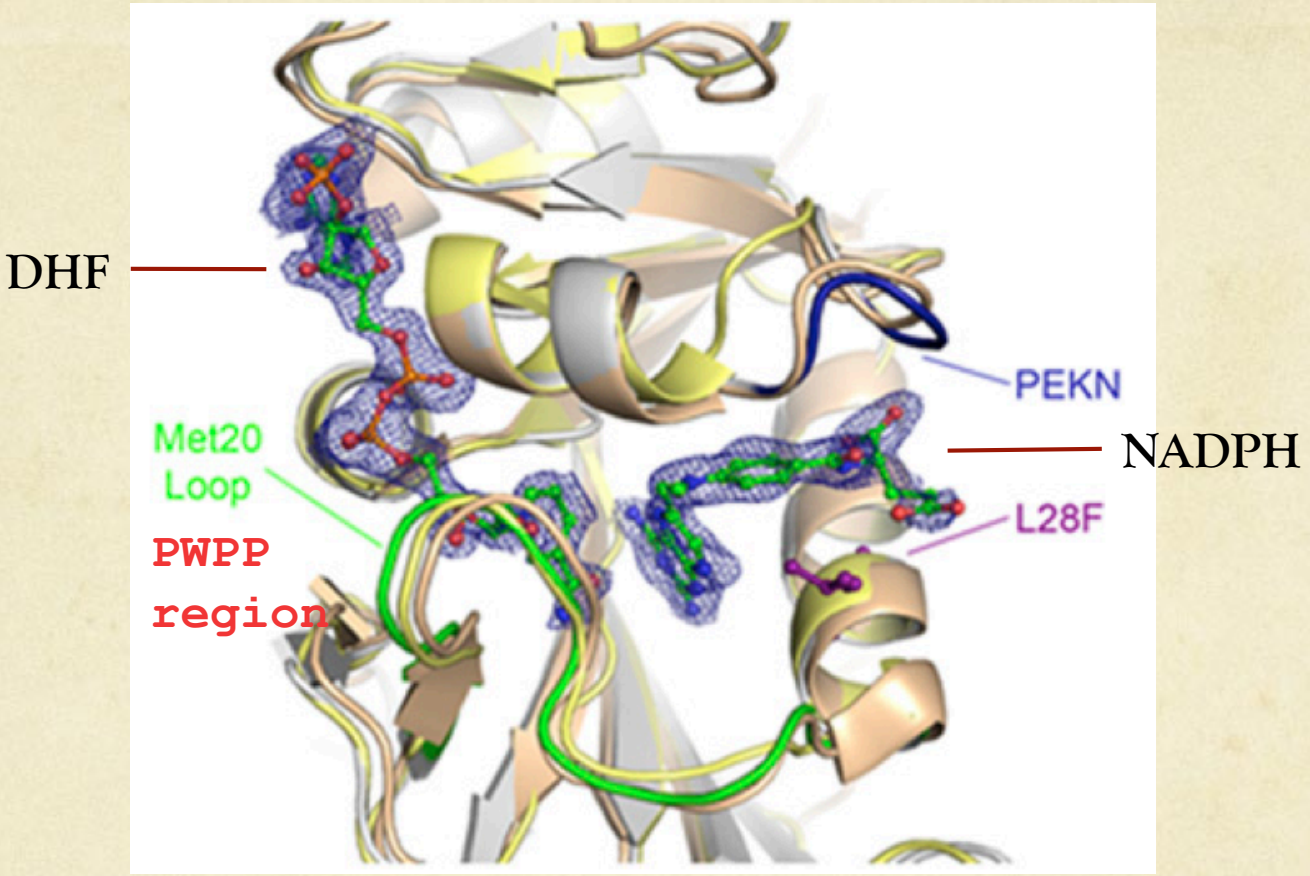
2. Kinetic Implications of PCEs

- double mutant: **N23PP/L28F**
  - was **unable to recover** the WT catalytic rate
- double mutant: N23PP/**G51PKEN**
  - the addition of the PEKN lid-domain into WT ecDHFR **completely negates** the negative catalytic influence of the PWPP element
  - By itself, the G51PEKN ecDHFR mutant exhibited a hydride transfer rate that are essentially identical to WT ecDHFR
- hsDHFR is more catalytically efficient than both WT ecDHFR and the N23PP ecDHFR mutant.

3. Kinetic Implications of PCEs

- The **N23PP/L28F/G51PEKN** ecDHFR **triple** mutant exhibits an **enhanced catalytic efficiency** that is commonly found in human and other vertebrate DHFRs
- hsDHFR is catalytically more efficient than WT ecDHFR

Struktuur



Superposition of N23PP/G51PEKN E. coli DHFR double mutant with human and *E. coli* DHFRs. The ecDHFR double mutant (silver, PDB ID 4GH8) is shown superimposed onto native human DHFR (bronze, PDB ID 1U72) (27) and native E. coli DHFR (gold, PDB ID 1RH3)

Computational Investigation of Enzyme Reaction

- Further mechanistic insights they provide EVB MD simulations
- The calculated relative free energy barriers agree with the experimental values

Table 1. Summary of experimental pre–steady-state k_{hyd}, $\Delta G^{\neq}$, pK_a values, and k_{cat} for various DHFR species at 298 K

	k_{hyd} max, s^{-1}	Expt k_{hyd} $\Delta G^{\neq}$, kcal/mol	Theoretical $\Delta G^{\neq}$ for k_{hyd}, kcal/mol	Kinetic pK_a	k_{cat}, s^{-1}
WT ecDHFR (5)	950 ± 50	13.4	13.4	6.5 ± 0.1	12
N ecDHFR (19)	~35	15.3	14.7	6.6 ± 0.1	2.5 ± 1
G ecDHFR	1,100 ± 80	13.3	NA	6.77 ± 0.07	8.9 ± 0.4
NG ecDHFR	1,100 ± 100	13.3	13.2	6.20 ± 0.06	26.9 ± 1.6
NLG ecDHFR	5,100 ± 1200	12.4	NA	5.9 ± 0.1	17.6 ± 5.1
WT mouse (22)	~2,400–9,000	12.1–12.8	NA	6.40 ± 0.05	17 ± 2
WT human (20)	3,000	12.7	13.1	5.9–6.2	12.5

N – N23PP;
L – L28F;
G – G51PEKN;

G51PEKN Alters Cofactor Bindings

- Isothermal titration calorimetry (ITC)
- Hinnatakse dissotsatsiooni konstandid ($\mathbf{K_d}$)
- **E:NADPH <–> E <–> E:NADP$^+$**

  ning $\mathbf{K_p} = K_d$ (E:NADPH)/K_d(E:NADP$^+$)
- The ratio has dramatically shifted from
  K_p ~ 0.0075 (favors NADPH) in WT ecDHFR
  to a **more "human-like"** value (K_p ~11.6; favors NADP$^+$)
- K_p value shifts ~**100-fold** toward hsDHFR, other amino acid sequence divergence(s) is (are) responsible for **the other 20-fold** differences
- **NB!** Eukarüootses rakus [NADP$^+$] << [NADPH] (100x madalam kons)

  Prokarüoodis [NADP$^+$] ≈ [NADPH]

G51PEKN Alters Inhibitor Bindings

- *TMP is an antibiotic that is a million times more selective for bacterial DHFRs; E:TMP has K_d = 6 pM over mammalian DHFRs K_d ~1–10 mM*
- The binding constant between ecDHFR and TMP is greatly weakened because of the G51PEKN mutation

Table 2. Dissociation constants (K_d; reciprocal of the binding constants) of binary DHFR complexes of E:TMP, E:NADPH, and E:NADP$^+$ in aqueous medium at pH 7.0 and 298 K

	TMP K_d, M	NADPH K_d, M	NADP$^+$ K_d, M	$K_p = K_d$(NADPH)/ K_d(NADP$^+$)
WT ecDHFR (5, 7)	6×10^{-9}	1.75×10^{-7}	2.3×10^{-5}	0.0076
G ecDHFR	$(1.65 \pm 0.2) \times 10^{-6}$	$(9.2 \pm 0.8) \times 10^{-7}$	$(1.1 \pm 0.2) \times 10^{-6}$	0.88
NG ecDHFR	$(1.2 \pm 0.5) \times 10^{-6}$	$(2.6 \pm 0.4) \times 10^{-6}$	$(4.0 \pm 0.3) \times 10^{-6}$	0.67
NLG ecDHFR	$(5.0 \pm 0.7) \times 10^{-6}$	$(3.0 \pm 0.1) \times 10^{-6}$	$(7 \pm 1) \times 10^{-6}$	0.45
WT human (9, 23)	10^{-6}	2.2×10^{-5}	1.9×10^{-6}	11.6

N – N23PP;
L – L28F;
G – G51PEKN;

Kokkuvõte

- Kasutades evolutsioonilist lähenemist, leidsid Liu et al. **kolm (3)** fülogeneetiliselt koherentset sündmust (**PCE**s), mis eristasid *E. coli* ja inimese DHFR'i
- Nendest 2 jäi Met20 luupi (PWPP regioon ja L>F) ning kõige vanem PEKN, jäi koofaktori seondumis domääni
- Viies need 3 regiooni ecDHFR'i oli võimalik konstrueerida inimese DHFR'le sarnaste omadustega ensüüm
- Muudetud ensüümi 3D struktuur oli väga sarnane teadaolevate DHFR'i struktuuridega