Shotgun proteomics aids discovery of novel protein-coding genes, alternatice splicing, and "resurrected" pseudogenes in the mouse genome

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14 May 2014

Outline

Background

- Annotation efforts: automatic annotation systems (e.g., Ensembl) & manual annotation (e.g., VEGA, RefSeq).
- A high-throughput method providing orthogonal data for validation and confirmation of the protein-coding potential is also required.
- Efforts to combine genome annotation with protein MS: *proteomics* [Jaffe *et al.* 2004].
  - It serves as translational evidence.
- Peptide identification methods and significance measures are both required to be sensitive and accurate.

Background (contd.)

- Mascot Percolator [Brosch *et al.* 2009].
  - Mascot [Perkins *et al.* 1999]: a database search engine;
  - Percolator [Käll *et al.* 2007.]: a semi-supervised machine learning algorithm.
- Two significance measures:
  - q-value [Storey & Tibshirani 2003];
  - PEP (posterior error prob.) [Käll et al 2008]

Contribution of this paper

- A novel pipeline that integrates
  - highly sensitive & statistically robust peptide spectrum matching (PSM);
  - genome-wide protein-coding predictions

  to perform large-scale gene validation and discovery in the mouse genome for the first time.
- Validation of 32%, 17%, and 7% of all protein-coding genes, exons, and splice boundaries, resp.

Contribution of this paper (contd.)

- Strong evidence for identifying multiple AS translations from 53 genes & uncovered 10 entirely novel protein-coding genes.
  - 2 gene fusions (including a *Ins2-Igf2* fusion object).
  - 9 processed pseudogenes (unique peptide hits): not just transcribed but translated and resurrected into new coding loci.

FDR & PEP

PNAS

Statistical significance for genomewide studies

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Journal of proteome research

Posterior Error Probabilities and False Discovery Rates: Two Sides of the Same Coin

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Whereas the p value is a measure of significance in terms of the false positive rate, the q value is a measure in terms of the FDR . . .

A false positive rate of 5% means that on average 5% of the truly null features in the study will be called significant. A FDR of 5% means that among all features called significant, 5% of these are truly null on average.

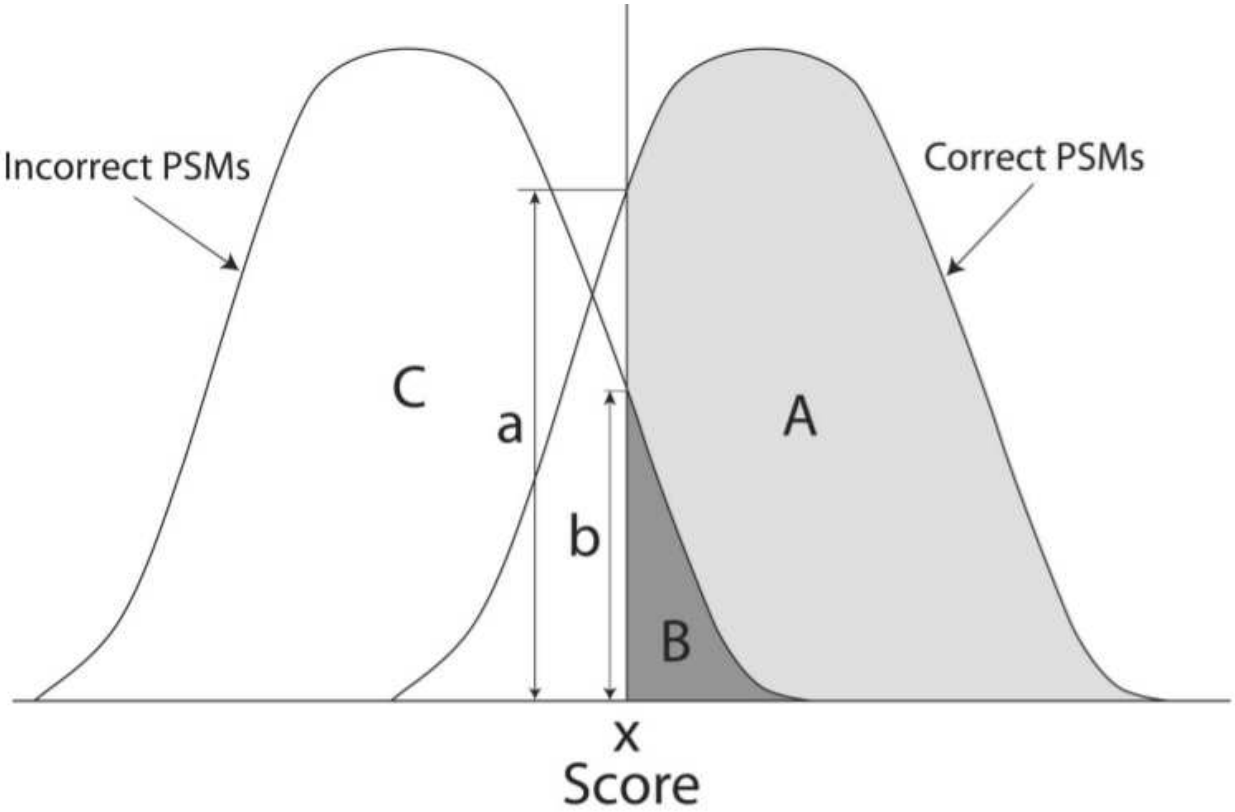
FDR & PEP

	Called significant	Called not significant	Total
Null true	F	$m_0 - F$	m_0
Alternative true	T	$m_1 - T$	m_1
Total	S	$m - S$	m

$$\frac{\text{no. false positive features}}{\text{no. significant features}} = \frac{F}{F+T} = \frac{F}{S},$$

$$\text{FDR} = \text{E}\left[\frac{F}{F+T}\right] = \text{E}\left[\frac{F}{S}\right].$$

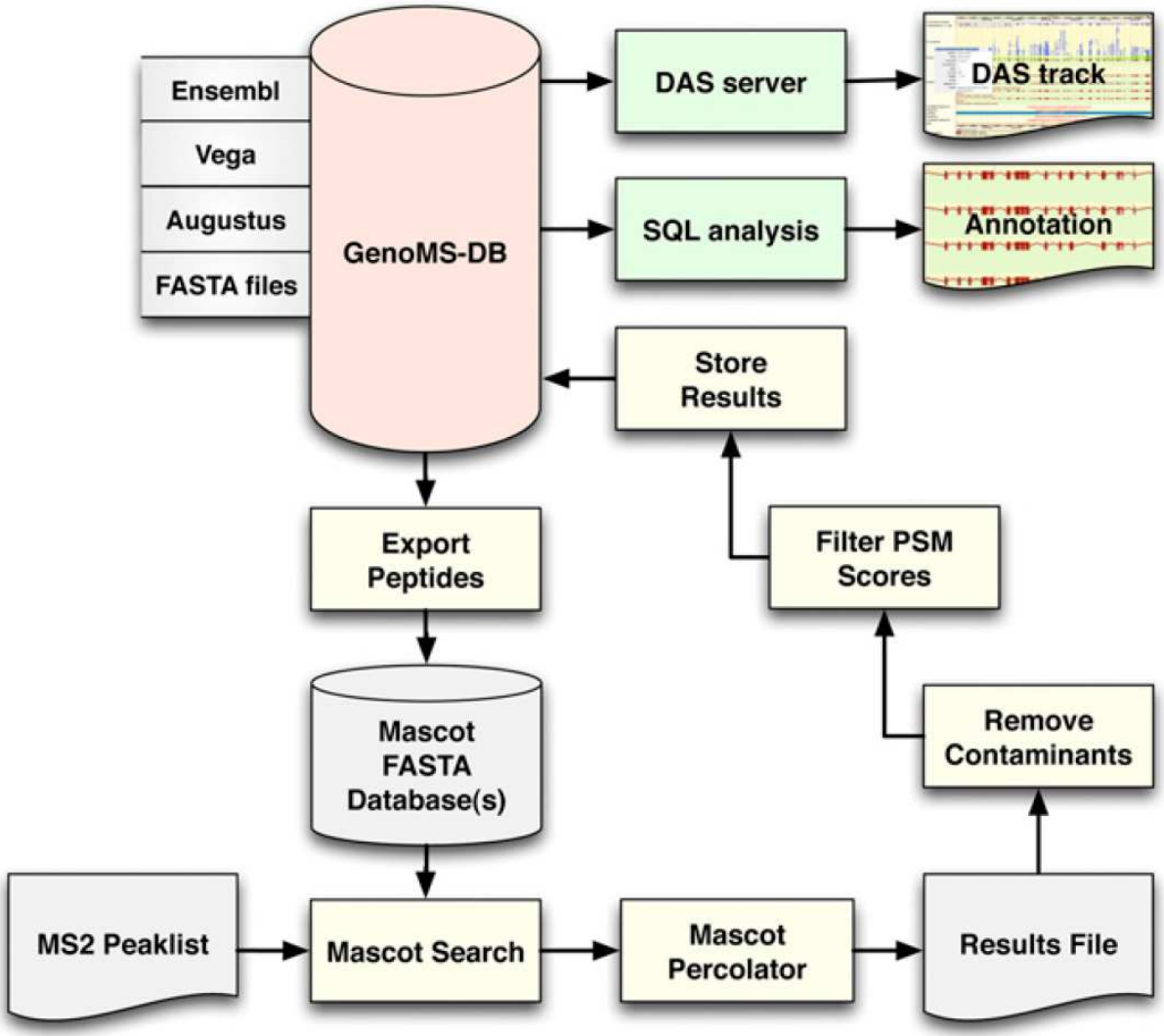
FDR & PEP



FDR = B/(A + B)

PEP = b/(a + b)

Overview of Genome Annotation Pipeline



MS/MS data

- 10,465,149 tandem MS spectra.
  - 729,583 spectra: in-house experiments
    - Nuclear protein extracts of murine ESCs & murine brain membrane fractions.
  - 9,735,566 spectra: PeptideAtlas project.
    - Sampling of mouse tissues including brain, liver, lung, heart, kidney, testes, and placenta.

GenoMS-DB database construction

- Gene products from
  - ⋆ Ensembl, VEGA, IPI digest in silico;
    - IPI: International Protein Index [Kersey *et al.* *Proteomics* 2004].
  - ⋆ predictions from Augustus.
- Ensembl Per API: to capture the peptide-genome mapping.

Automatic & manual annotation

- Perl-based Distributed Annotation System (DAS):
  - Visualize the identified peptides stored in GenoMS-DB as tracks in various genome browsers and curation tools.
- Manual annotation:
  - MS PSMs overlapping annotated loci $\rightarrow$ HAVANA.
  - Otherwise, follow the hierarchy:
    - RT-PCR $>$ species-specific transcriptional support $>$ rodent specific transcriptional support $>$ strong mammalian conservation $>$ paralogous gene transcriptional evidence.

Translated pseudogene analysis

- To select the parent of each identified translated pseudogene:
  - assign homology scoring of the putative translation of the processed pseudogene object against the SWISS-PROT data set;
  - (check) assign each of the PSMs aligning to the pseudogene loci to a parent protein by aligning to the compete UniProt database using HMMER.
- Gene orthologous to these parents: application of Ensembl website.
- Protein alignment: ClustalW2 (EBI).
- Identification of domains: InterProScan (EBI).

Generaton of high-confidence PSMs

- When considering q-value $< 1\% \rightarrow$ PEP $< 1\%$:
  - 1,124,724 peptides were identified (Ensembl, Vega).
  - 967,131 peptides were identified (Augustus).
- Only the best PEP and q-value score for each peptide sequence was considered ($\Rightarrow$ 95,606).
- Removing peptides matching common contaminants (3,260 removed).

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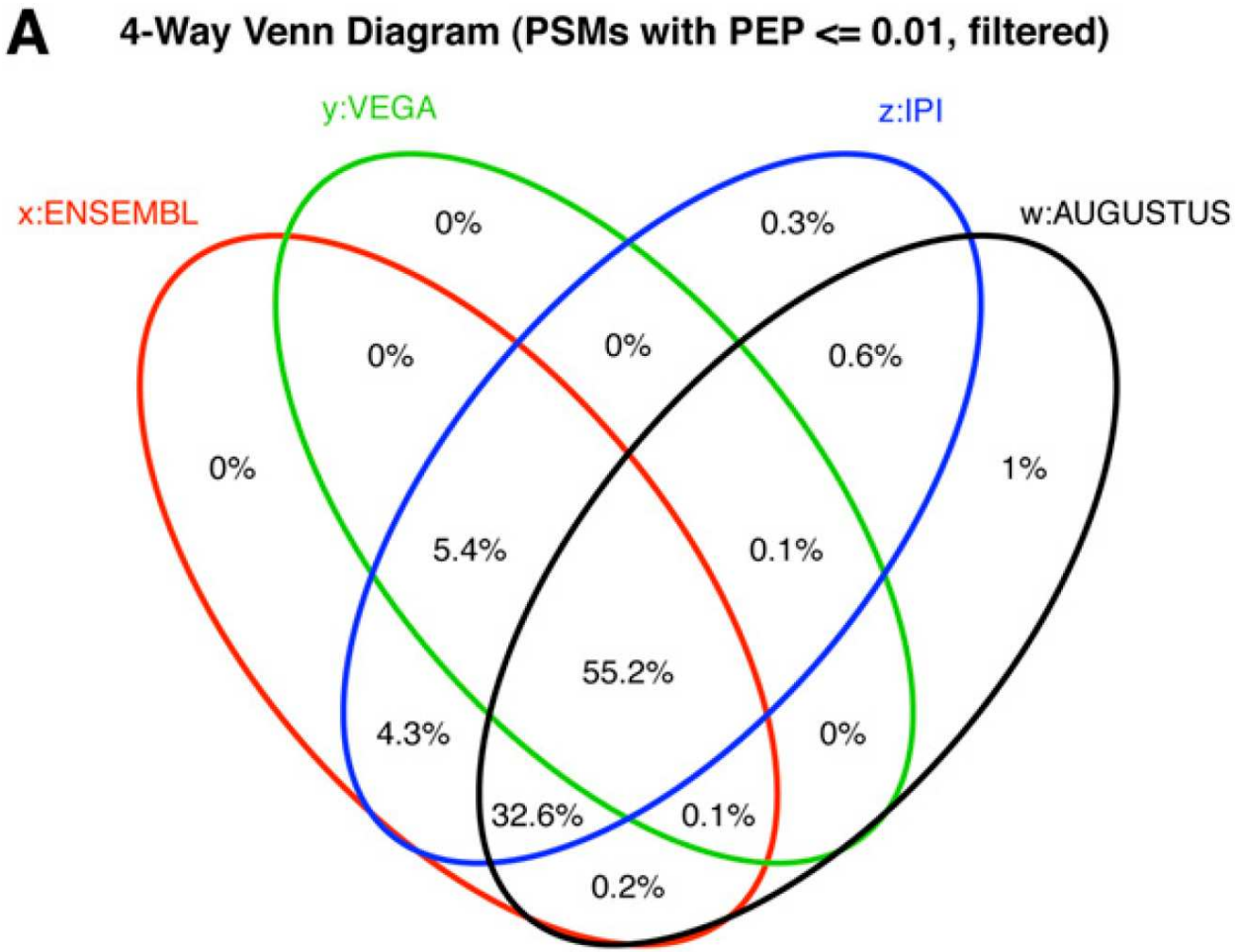
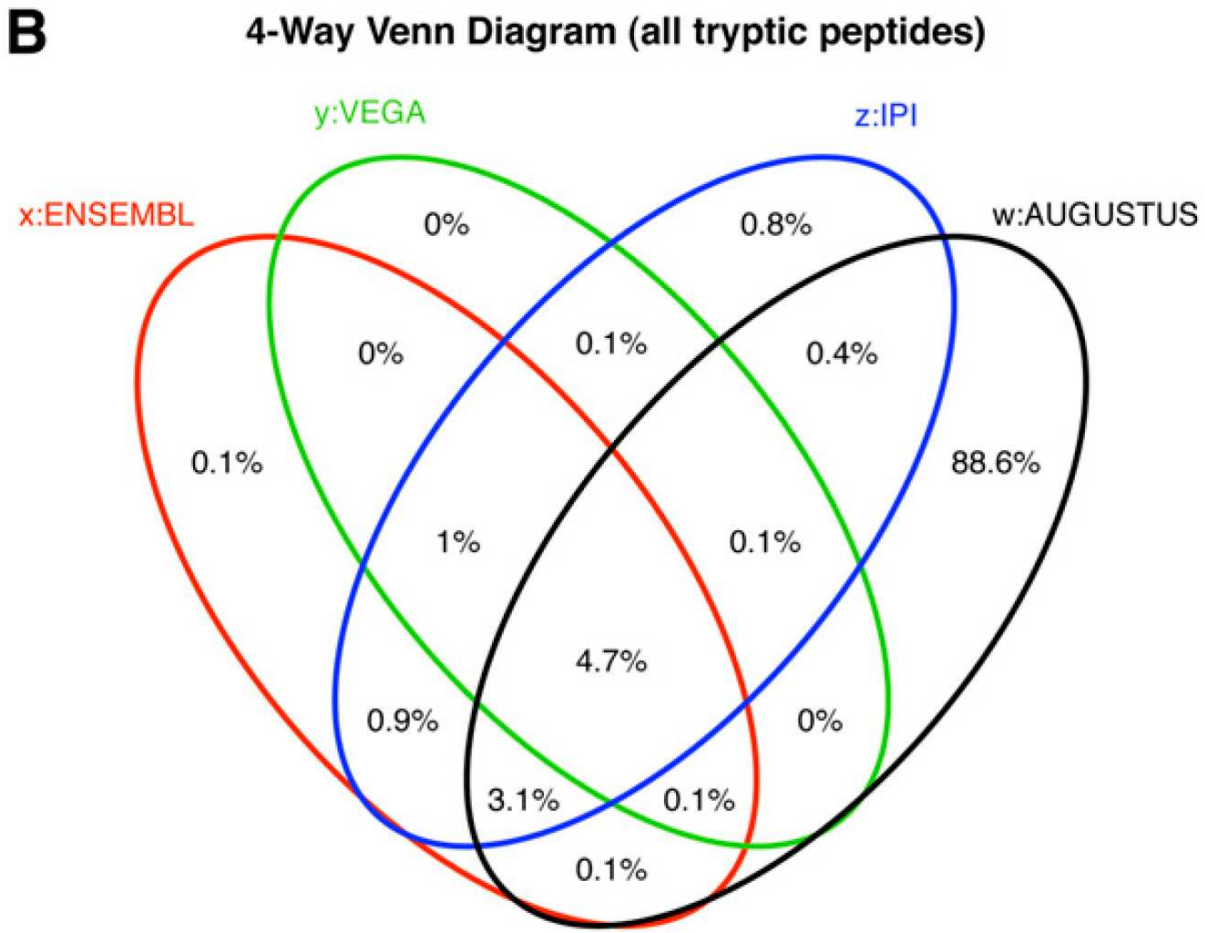
Generaton of high-confidence PSMs (contd.)

- Filtering peptides where isoforms attributed to amino acids that cannot be discriminated in low energy collision induced dissociation data (1,159 removed).
- Unambiguous mapping to one genomic locus ($\Rightarrow$ 76,029 remained).
- Testing whether semi-tryptic form of the peptide sequence mapped elsewhere ($\Rightarrow$ 758 cases removed).
- Testing whether one residue substitution/insertion/deletion could be identified elsewhere ($\Rightarrow$ 6,685 cases removed; 68,586 finally.)

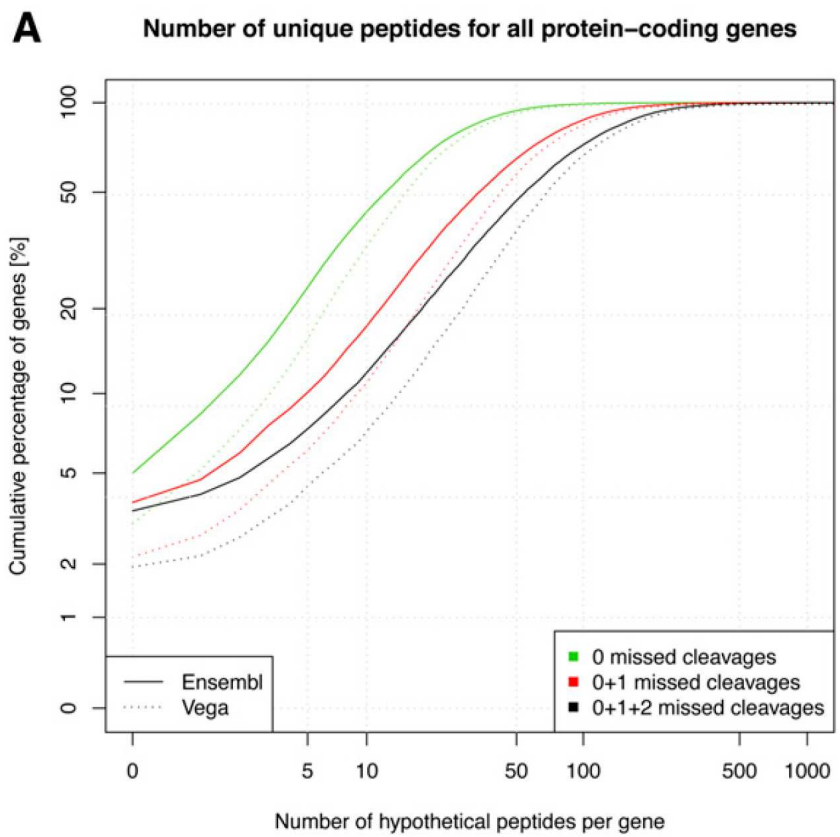
⋆ $1\% \leq \text{PEP} \leq 5\%$: exclusively used as supplement.

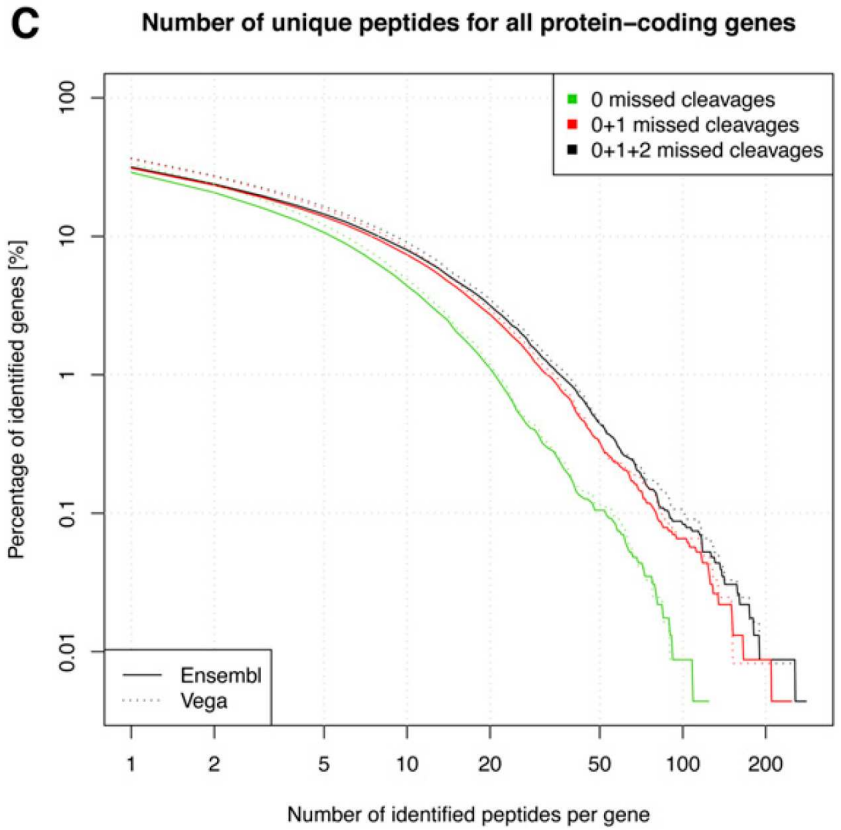
⋆ $\text{PEP} \leq 1\%$: primary annotation data source (58,574 cases).


A
4-Way Venn Diagram (PSMs with PEP <= 0.01, filtered)
y:VEGA
z:IPI
x:ENSEMBL
w:AUGUSTUS
0%
0.3%
0%
0%
0.6%
0%
1%
5.4%
0.1%
55.2%
4.3%
0%
32.6%
0.1%
0.2%


B
4-Way Venn Diagram (all tryptic peptides)
y:VEGA
z:IPI
x:ENSEMBL
w:AUGUSTUS
0%
0.8%
0%
0.1%
0.4%
0.1%
88.6%
1%
0.1%
4.7%
0.9%
0%
3.1%
0.1%
0.1%

Validation of Ensembl/VEGA gene annotation





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- Is there a linear model fitted?
  - gene products with more potential peptides
    $\Rightarrow$
    sampled peptides $\uparrow$

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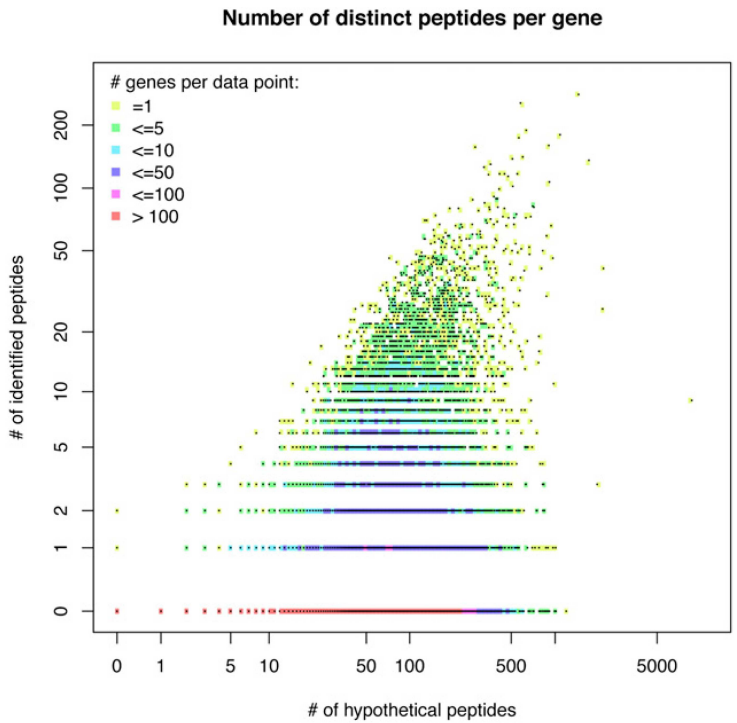
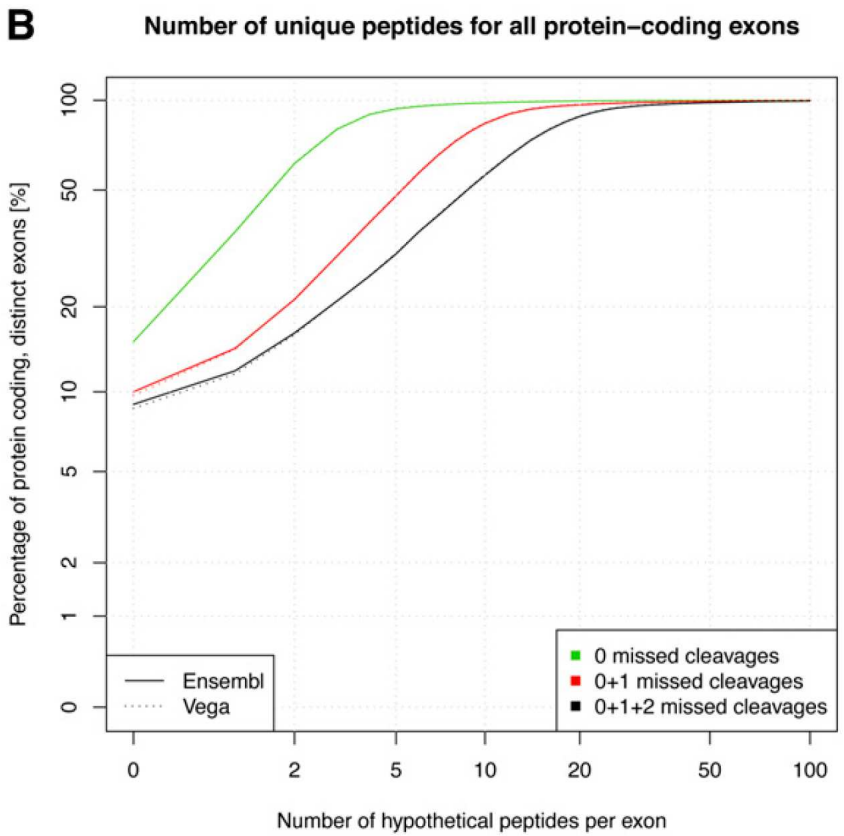
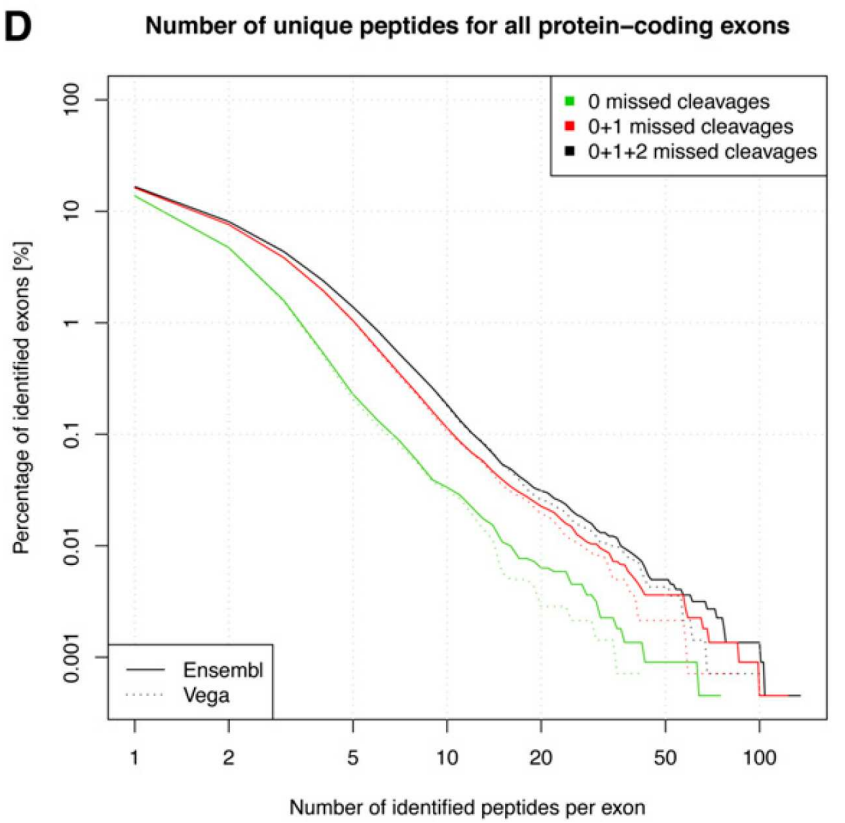


Validation of Ensembl/VEGA gene annotation (structure)





Validation of Ensembl/VEGA gene annotation (structure)

- Overall, 16.7% (7.1%) of the total Ensembl protein-coding exons (introns) could be validated by peptide identifications.

Validation of Ensembl/VEGA gene annotation (AS)

- Until recently, only limited evidence of expression of AS transcripts was available at the protein level.
- The majority of protein sequence is shared between the variant transcripts, differing only in small parts ($\Rightarrow$ **signatures**) of the translation products.
- Here, a total of 370 peptides enabled discrimination of 112 Ensembl transcripts in 53 genes.
  - 3.4% of all protein-coding genes with annotated multiple coding AS forms that can be discriminated by a peptide.

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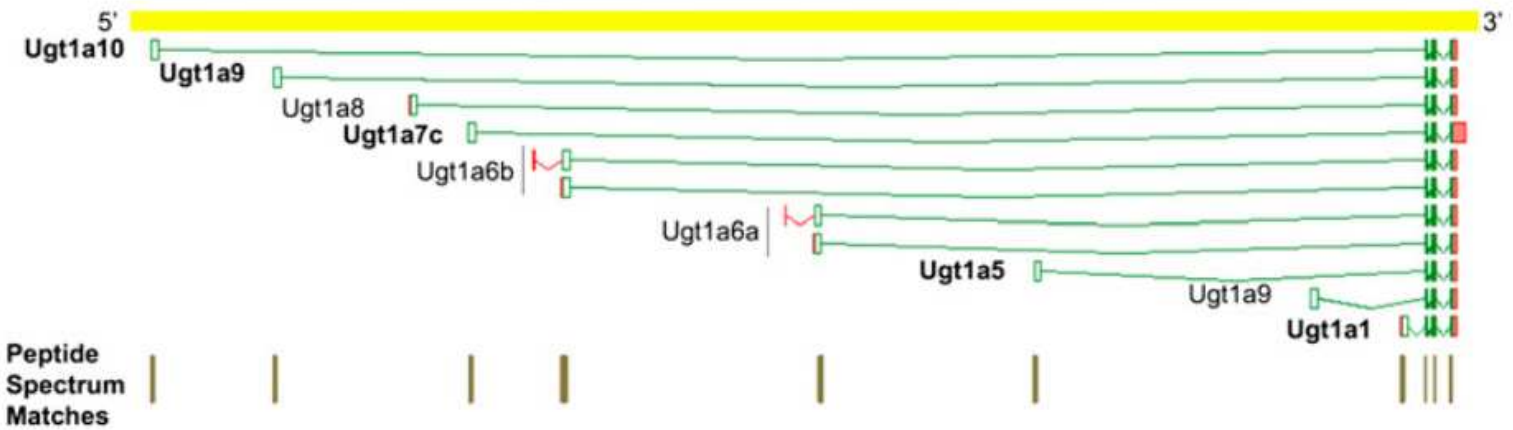


Figure 5. MS PSMs confirm the protein-coding potential of five alternatively translated products of the UDP-glucuronosyltransferase 1 family, polypeptide A6 (highlighted in bold). Ambiguous PSMs are shown for the two alternatively spliced transcripts of the Ugt1a6a and Ugt1a6b genes, respectively; and as clusters for each of the 3′ exons.

Manual identification of protein-coding novel loci and AS variants

- The more stringent criteria for the peptide identification.
  - PEP $\leq 1\%$ ($\Rightarrow$ q-value $< 0.14\%$).
  - For peptides not support by Ensembl & VEGA:
    - ≥ 2 peptides had to be identified (one having PEP < 0.01 and the second < 0.05).
- 36 MS PSMs were identified; 10 novel protein-coding loci were supported.

Table 1. Summary of novel protein-coding objects identified by PSMs

Transcript stable ID	Chromosome	Genomic clone	Mass spec tags aligning	Description	Additional Evidence
OTTMUST00000090068	6	AC165974.4	IVAAQQELLAQR RPDPGPSPLGAIPELGCRR RPDPGPSPLGAIPELGCR ENAGLLER IVAAQQELLAQRR LSRENAGLLER	Uni-exon novel orphan CDS	Strong mammalian conservation
OTTMUST00000090127	14	AC165148.2	AAEDEEVPAFFK DVAHLGPDPHR	Uni-exon novel orphan CDS	Mouse-specific transcriptional evidence
OTTMUST00000090128	7	AC113298.14	ASSAAAAAALSR AGAPGPASSPALLVLR	Uni-exon novel orphan CDS	Rodent-specific transcriptional evidence
OTTMUST00000090124	15	AC164597.11	FAKPPPPLLTSSESSTVEPPHMAR FGLHTEDLYER	CDS highly similar to de novo prediction EDL29334	Rodent-specific transcriptional evidence
OTTMUST00000090118	7	AC108827.10	SFVSHSHLQSHGR AFTHPSTVVLHK	CDS highly similar to de novo prediction EDL12440	Paralogous gene transcriptional evidence
OTTMUST00000090119	7	AC108827.10	AFAQSSSLQYHK NPPASAFQVVGLKACTTTAWPG	CDS highly similar to de novo prediction EDL12440	Paralogous gene transcriptional evidence
OTTMUST00000090503	13	AC154437.2	IITITGTQDQIQNAQYLLQNRR SLHELNPR	*Hnrnpk-2210016F16Rik* fusion object	Mouse-specific transcriptional evidence
OTTMUST00000090122	7	AC013548.13	ILGTSDSPVLFIHRPGTSGTTK APPALEGAANIDPASGSSSGQFRK LLVQPELQKPK	*Ins2-Igf2* fusion object	Mouse-specific transcriptional evidence
OTTMUST00000089966	5	AC162528.5	MDATPQDPDADFQELAK VATEQSTAEHQGPER AHSVENPAGQAPEAKPQPK FDQEAYAQTER EAPQSDSVGQQAGR ATQVSLLSARPEVATKPAVPAR GVASGHGSAVVSK HDLDAAPATK YDIVHASGER SGTEDMLEPSR	5′ Extension of novel protein (2900026A02Rik) CDS	Strong mammalian conservation
OTTMUST00000090346	X	AL450395.7	VKQEEQLQSVPAKEK YSLQPWQSTPFEQVSVTPDHDPA AAAASWSPPIDPPTSR SGLPVPSTSISSATAEDDVSPK SSEGQLPSTQPSQAFDVAK DIGQPTTTEAEVTTVQK	*Gm14569* locus	Strong mammalian conservation

Manual identification of protein-coding novel loci and AS variants

B

```
Mouse     MASAAEPPGTAAEYLQELTRIVAAQQELLAQRRRRIEELERQVAR
Rat       MASAAEPPGTAAEYLQELTRIVAAQQELLAQRRRRIEELERQVAR
Human     MASAAEPPGQAAEYLQELTRIVAAQQELLARRRRRIEELERQVAR
                 2                                3
Mouse     LSRENAGLLERHRRHLAACARRPDPGP----SPLGAIPELGCRRD
Rat       LSRENAGLLERHRRHLAACARRPDPGP----SPLGAIPELGSRRD
Human     LSRENAGLLERHRRHLAACARRPDPGPGPGPQPLGAIPELGGRRD

Mouse     K*
Rat       K*
Human     K*
```

Manual identification of protein-coding novel loci and AS variants

B

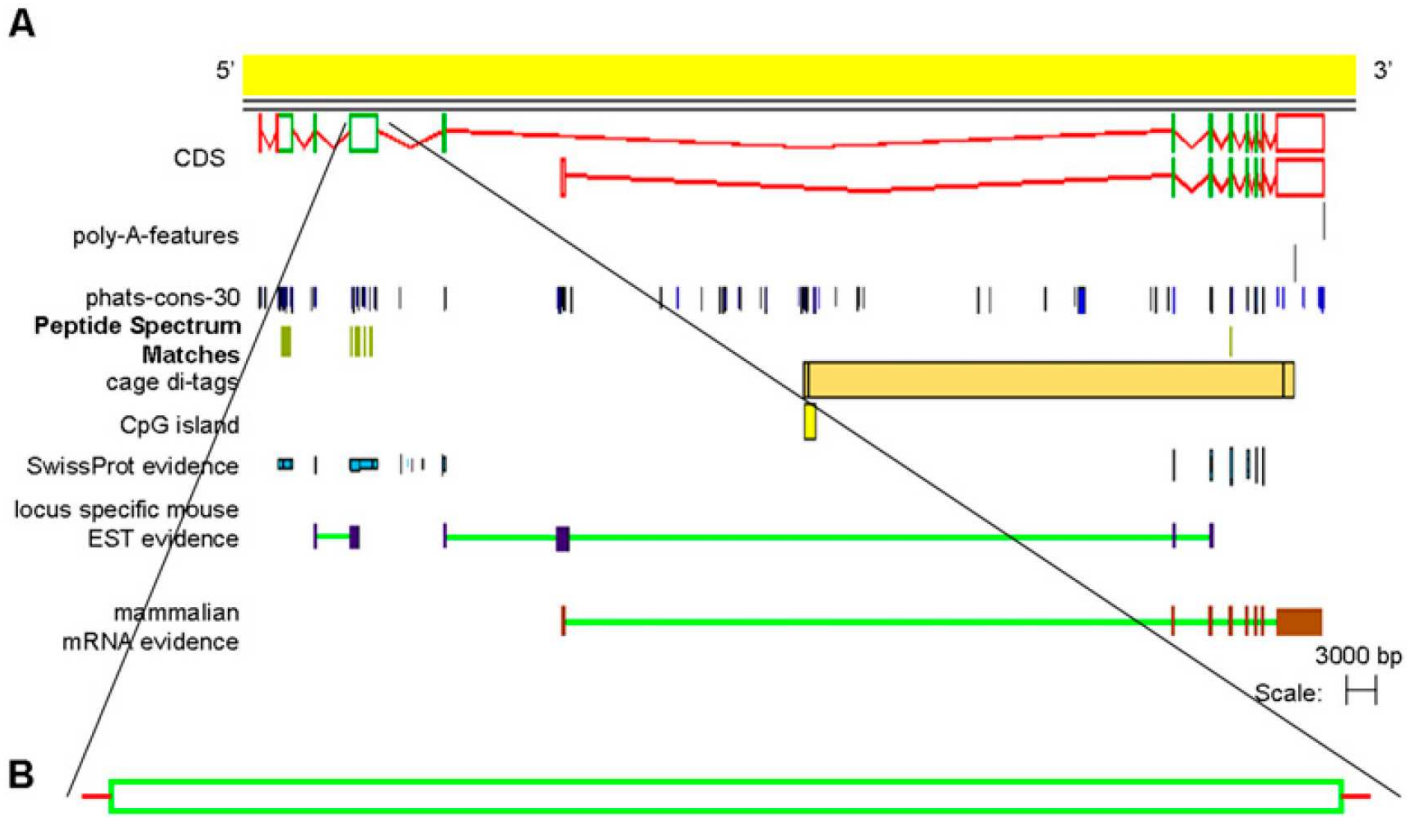
Insulin 2B peptide

```
Mouse   MALWMRFLPLLALLFLWESHPTQAFVKQHLCGSHLVEALYLVCGERGFFYTPMSRREVED
Human   MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAED

Mouse   PQDSAPSSPAATSSPRLEGPGTTTRAPPALEGAANIDPASGSSSGQFRKRILGTSDSPVL
Human   LQASALSLSSSTST-WPEGLDATARAPPALVVTANIGQAGGSSSRQFRQRALGTSDSPVL

Mouse   FIHRPGTSGTTKRLEYRGRVIKTELTVQEEEEEEENDYDDCRPLTQGSESPKAKLLVQPE
Human   FIHCPGAAGTAQGLEYRGRRVTTELVWEEVDSSPQPQGSESLPAQPPAQPAPQPEPQQAR

Mouse   LQKPKSRPVPMGIPVGKSMLV-LLISLAFALCCIAAYGPGETLCGGELVDTLQFVCSDRG
Human   EPSPEVSCCGLWPRRPQRSQN*GWQPAPASAPTARQRDTNGNPNGEVDAGASHLLGLRLV

Mouse   FYFSRPSS-RANRRSRGIVEECCFRSCDLALLETYCATPAKSERDV-STSQAVL-PDDFP
Human   LHCCLPPQ*DPVRRGAGGHPPVRLWGPRLLLQQARKPCEPSQPWHR*GVLFPQL*PGPPG
```

Preptin peptide

```
Mouse   RYPVGKFFQYDTWRQSAGRLRRGLPALLRARRGRM-LAKELKEFREAKRHRPLIVLPPKD
Human   DVLCYPRQVREGRVDPSDRASGQLPQIPRGQVLPI*HLEAVHPAPAQGPACPPACPPGSR

Mouse   PAHGG---------ASSEMSSNHQ*---------------
Human   ARQGARGVQGGQTSPSPDCSTHPRPRPRGRPPRDGQQSEV
```

Manual identification of protein-coding novel loci and AS variants



Resurrected pseudogenes

- Retrotransposed/processed pseudogenes have generally been considered as "dead on arrival".
- While the increasing number of transcribed retrotransposed genes creates additional candidate protein-coding loci [Bärtsch *et al.*, BMC Genomics 2008], there is no evidence that proteins originates from such loci.
- The MS data in this paper provides support for the translation of nine processed pseudogenes in the reference mouse genome.

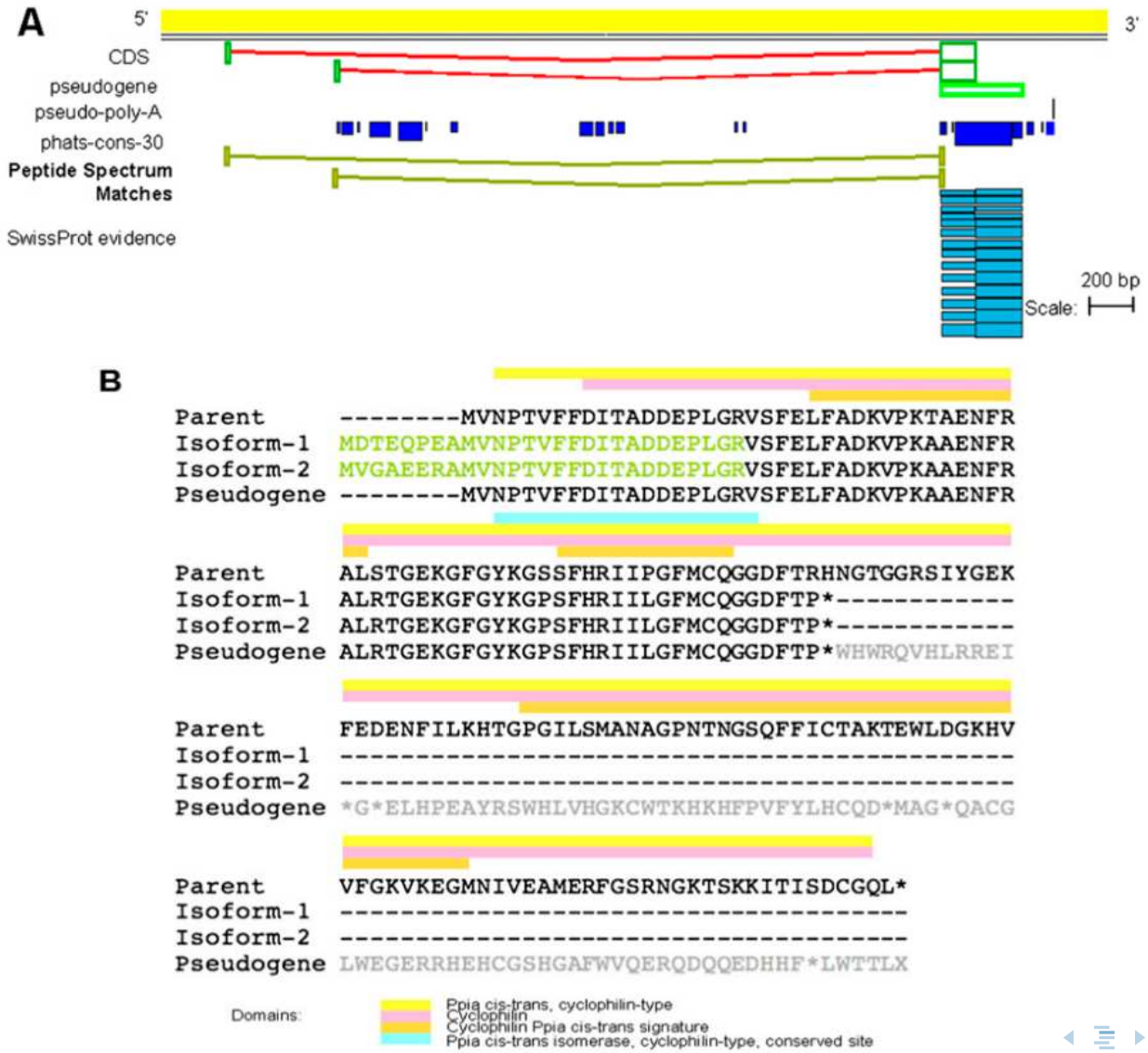
Resurrected pseudogenes (contd.)

- Each pseudogene is supported by ≥ 2 peptides.
- Unique mapping in the genome.
- Each PSM shows ≥ 2 amino acid substitutions compared with the translated parent protein sequence.
  - Each supporting PSM needed to be detected in ≥ 2 different tissues.

Resurrected pseudogenes (contd.)

- To ensure high confidence that these MS PSMs do indeed represent translations of these pseudogenic loci and NOT polymorphisms of the parent locus:
  - The residues substituted in our PSMs in comparison with the parent polypeptide are conserved in the amino acid sequences of the 1:1 rat and human orthologs;
  - No evidence of SNP/INDEL at these codon positions of the parent mouse locus.

Resurrected pseudogenes



Resurrected pseudogenes (contd.)

- Among the nine identified pseudogenes:
  - Only 2 shows syntentic ortholog in rat.
  - **None** possess human orthologs.
- However, the genes surrounding each translated mouse pseudogene show strong syntetic conservation with the equivalent rat and human loci (data not shown).
- Hypotheses to explain the detection:
  - Only relics of translation; generated until sufficient mutations are accrued $\Rightarrow$ NMD targets.
  - positive selection.
- Further investigation is required.

Discussion

- The mouse proteome is far from being saturated by MS-based peptide identifications.
  - However, MS data have become a richer and more valuable resource for genome annotation than 10 year ago.
- For the nine putative translated pseudogenic loci, whether they are able to produce functional protein is unclear.
- Among the 10 novel protein-coding loci, 8 of them can be found in the reference human genome.
  - **Note:** None of them was identified by either RefSeq or Ensembl annotation.

Thank you.