Massively parallel functional analysis of missense mutations in *BRCA1* for interpreting variants of uncertain significance

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Variants of uncertain significance (VUS)
How do we interpret the impact of genetic variation at scale?

<table>
<thead>
<tr>
<th>Method</th>
<th>Validity</th>
<th>Throughput</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic analysis or one-off experiments</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Computational prediction</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Massively parallel functional analysis</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>
Massively parallel functional assays for assessing function of missense variants

- Generate a library with mutations in a sequence of interest
- Multiplexed functional assay
- Sequence variants in input and selected populations
- Quantify effect sizes of individual variants

- Co-transfection
- CRISPR HDR Library
- Array-synthesized mutations
- Haploid Cells
- Population of Cells with Many Different Edits
Biochemical functions of BRCA1

BRCA1 is required for homology-directed dsDNA break repair (HDR)

BRCA1 HDR activity is required for tumor suppression

BRCA1 must dimerize with BARD1 to function in HDR

The BRCA1:BARD1 dimer has ubiquitin ligase activity
Multiplex assays for BRCA1 protein function and splicing

Experiments 1 and 2:
- BARD1-BRCA1-RING E3 ligase activity
- BARD1-BRCA1-RING interaction

Experiment 3:
Saturation genome editing to assess the effect of SNVs on splicing.
Multiplex assays for BRCA1 protein function and splicing

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BARD1-BRCA1-RING E3 ligase activity
BARD1-BRCA1-RING interaction

Experiment 3:
Saturation genome editing to assess the effect of SNVs on splicing.
Massively parallel assays for the BRCA1-RING E3 ligase and BARD1-binding activities

E3 ligase activity

5X

ATP, E1, E2
Flag-Ub
capture
elute

deep sequencing

deep sequencing

AVLIMFYWSTNQG

substituting amino acid

4-helix bundle N

loop 1

central helix

loop 2

4-helix bundle C

Zn\(^{2+}\)

Zn\(^{2+}\)

5X

E3 functional score

WT aa

no data

0

1

2.0
Massively parallel assays for the BRCA1-RING E3 ligase and BARD1-binding activities

E3 ligase activity

BARD1-binding activity
How can we leverage these measurements to estimate the likelihood that a BRCA1 variant would be pathogenic?
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to understand the homology-directed DNA repair (HDR) function of BRCA1?

Ransburgh et al. Cancer Research 2010
Experimental data build a better predictor of BRCA1 HDR function

![Graph showing Leave-One-Out Cross Validation R^2 for PolyPhen-2 and CADD]
HDR predictions for clinical BRCA1 variants

Low HDR function

High HDR function

Counts

Counts

Counts

predicted HDR score

0.0
1.0
0.33
0.77
4
2
4
2
4
4

benign

pathogenic

VUS

splice

0

0
HDR predictions for 1,287 BRCA1 variants not yet seen in patients

- Low HDR function
- High HDR function

Counts vs. Predicted HDR score graph showing:
- Green bars for benign
- Red bars for pathogenic
- Purple bars for VUS

Predicted HDR score range from 0.0 to 1.0.
HDR predictions for 1,287 BRCA1 variants not yet seen in patients

Counts

<table>
<thead>
<tr>
<th>HDR function</th>
<th>Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>High HDR function</td>
<td>100</td>
</tr>
<tr>
<td>Low HDR function</td>
<td>80</td>
</tr>
</tbody>
</table>

Predicted HDR score

- High HDR function: 0.77, 1.0
- Low HDR function: 0.33, 0.0

Counts for different predictions:
- benign: 0
- pathogenic: 0
- VUS: 0
- not yet seen: 100
Multiplex assays for BRCA1 protein function and splicing

Experiments 1 and 2:
BARD1-BRCA1-RING E3 ligase activity
BARD1-BRCA1-RING interaction

Experiment 3:
Saturation genome editing to assess the effect of SNVs on splicing.
Multiplex genome editing to measure the effects of SNVs on splicing

1. CRISPR-Cas9 construct targeting **BRCA1** exon 18

2. Repair template library to substitute **SNVs** within the exon.

Multiplex genome editing to measure the effects of SNVs on splicing

Co-transfection, Multiplex editing

Edited repair templates (N = 4,096)

5 days, collect gDNA & RNA

“Selective PCR” – only edited gDNA and cDNA

Heterogeneous population of edited cells
Multiplex genome editing to measure the effects of SNVs on splicing

Co-transfection, Multiplex editing

Cas9/gRNA

Edited repair templates (N = 4,096)

Calculate effects on splicing for each variant

Sequence, gDNA and cDNA

Variant Counts
gDNA | cDNA

Count variant gDNA and cDNA
Variants that create splice enhancers and silencers or trigger nonsense-mediated decay behave as expected.

* defined from Ke et al. 2011

Effects of SNVs across *BRCA1* exon 18

![Graph showing effects of SNVs across *BRCA1* exon 18]

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*Findlay, Boyle et al., Nature (2014).*
Effects of SNVs across \textit{BRCA1} exon 18

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{MutPred Splice annotations:}
\begin{itemize}
\item \textbf{C49G} \ “Splice Affecting Variant”
\item \textbf{A53G} \ “ESE Loss / ESS Gain”
\item \textbf{A56G} \ “ESE Loss”
\item \textbf{G63T} \ “Cryptic 5’ SS”
\item \textbf{T67G} \ “Cryptic 5’ SS” \textit{aka} VUS V1714G
\end{itemize}
\end{figure}

Findlay, Boyle et al., \textit{Nature} (2014).
In summary

Parallelized assays for the protein function of the RING domain of BRCA1

Saturation genome editing to understand the effect of missense variants on splicing
Next steps…

Suggestions?
Challenges for scaling up

Library construction and variant delivery

Parallelizable assays for protein function

Sequencing of variants

Computational variant scoring pipeline

Calculate likelihood estimates for pathogenicity
How the results from massively parallel assays could get to the bedside...

- More scans
- Better databases
- Better variant effect prediction
Thanks to:

Genome Sciences
University of Washington

Shendure lab

Fields lab
Dave Young
Justin Gullingsrud

Fowler lab

Parvin lab
Muhtadi Islam
The Ohio State University

Kitzman lab
University of Michigan

Funding from the Yeast Resource Center
NIH P41
The effects of missense SNVs on splicing and protein function are difficult to predict.

Stop Gain ✓ Frameshift ✓ Missense ?

Splicing effects

Multiplex genome editing to determine effects of SNVs on splicing of exon 18 of BRCA1
Findlay et al. Nature 2014

Learning the Sequence Determinants of Alternative Splicing from Millions of Random Sequences
Rosenberg et al. Cell 2015
Scoring full-length BRCA1 variants for HDR function in human cells

HDR rescue assay

- I-SceI-GFP
- donor GFP
- + I-SceI to induce break
- HDR
- GFP
- broken GFP
- + functional BRCA1 variant
- + siRNA to target BRCA1 3'UTR
- + nonfunctional BRCA1 variant
- error prone break repair

% HDR rescue

0.00 0.25 0.50 0.75 1.00 1.25

WT Vector only R7C M18T L22S C39Y H41R C44F C44S K45Q C61G C64G D67Y

benign pathogenic control

Muhtadi Islam and Jeff Parvin
Scoring full-length BRCA1 variants for HDR function in human cells

HDR rescue assay

I-SceI-GFP  donor GFP
Construction of the barcoded single amino acid substitution BRCA1-RING library
Multiplex genome editing to measure the effects of SNVs on splicing

Each edited exon receives

1. A random SNV
2. A fixed mutation

3% of $10^6$ cells = 30,000 events
Prospective functional map for 1,287 BRCA1 RING variants

- Predicted HDR rescue score
  - 0.0 1.0
  - 0.33 0.53 0.77

- 59 BRCA1 RING variants likely HDR
- 1,238 BRCA1 RING variants likely HDR
- 20 BRCA1 RING variants non-functional

- Midpoint between mean pathogenic and benign scores

- Max pathogenic HDR rescue score (experimental)
- Min benign HDR rescue score (experimental)

- Zn²⁺: N-4 helix bundle, loop 1, central helix, loop 2, C-4 helix bundle

- Substituting amino acid:

- COSMIC, EVS, pathogenic, VUS, benign

- No data

- WT aa

- PREDICTED HDR rescue sore

- Colors:
  - Yellow: WT aa
  - Red: Higher predicted HDR rescue score
  - Gray: No data
Massively parallel assays for BRCA1-RING E3 ligase activity

E3 ligase activity

[Diagram showing amino acid substitutions and functional scores]

- Damaging
- Neutral
- Enhancing

E3 functional score

Substituting amino acid

M18

Deep sequencing

Calculate variant frequency

Calculate selected/input ratio for each variant for each round

Calculate slope of log2 ratios over 5 rounds of selection

**E3 functional score**

Substituting amino acid

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E3 ligase activity

ATP, E1, E2, Flag-Ub → capture → elute

5X

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E3 ligase activity

- ATP, E1, E2, Flag-Ub
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E3 ligase activity

Flag-Ub capture
elute

5X
ATP, E1, E2

deep sequencing

BARD1-binding activity

Yeast two-hybrid

transformation, Time 0
depth sequencing

Time 1
depth sequencing

Time 2
depth sequencing

Time 3
depth sequencing
Genetic testing is big business

More companies, lower costs, more genes*

*41.7% of tests revealed a VUS in at least one gene

Tung et al. Frequency of mutations in individuals with breast cancer referred for BRCA1 and BRCA2 testing using next-generation sequencing with a 25-gene panel. Cancer 2015
We need new technologies to deliver on the promises of genetic medicine

Massively parallel functional analyses are a possible solution

https://www.whitehouse.gov/precision-medicine

Starita et al. Genetics, 2015
Rosenberg et al. Cell, 2015
Fowler et al. Nature Methods, 2010