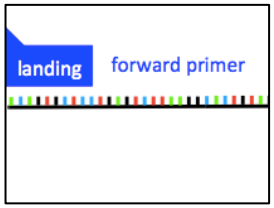
M2D4:
Cell viability;
quantitative PCR

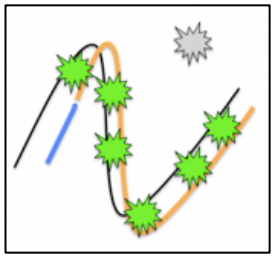
03/21/2017

In lab today

1. Finish cell viability assay



2. Design primers for qPCR



3. Set up qPCR

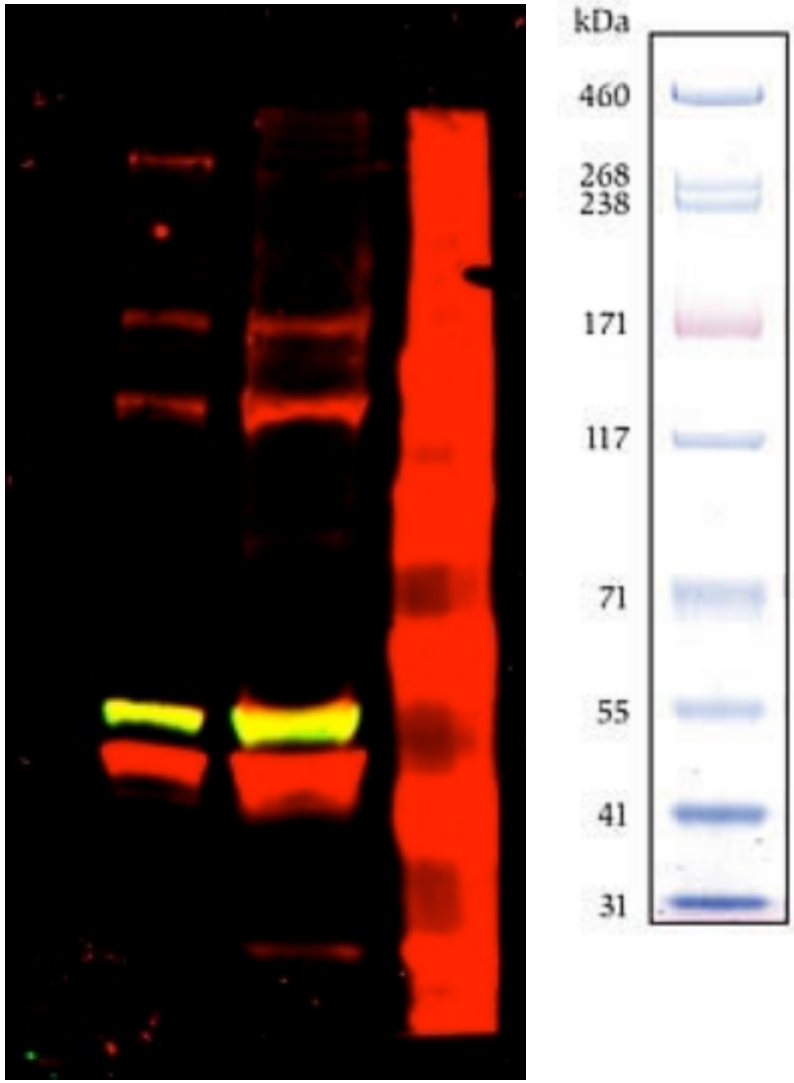
Why are there additional bands on our Western blots?

BRCA2
rabbit anti-human anti-BRCA2
donkey anti-rabbit
680 nm
red
~ 380 kDa

DLD-1 BRCA2-/-

BRCA2 →

tubulin →



Monoclonal *vs.* polyclonal antibodies

- Both types created when an antigen is injected into an animal and its immune system responds by producing antibodies specifically targeted against that antigen

- Polyclonal antibodies:
  - mixed pool of immunoglobulin molecules
  - bind to several different epitopes found on a single antigen
  - purified from serum

- Monoclonal antibodies:
  - bind to a single epitope within a target antigen
  - homogeneous cloned immunoglobulin molecules
  - made by fusing antibody-producing B-cells with immortalized B-cells

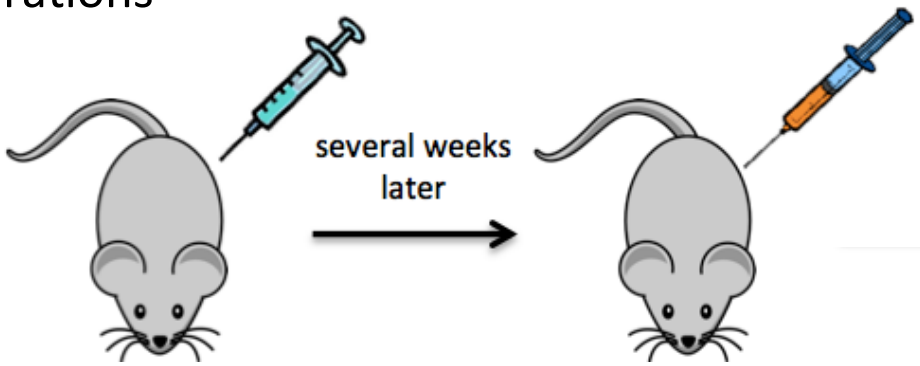
adapted from bio-rad.com

For Western blots, polyclonal antibodies

- Varying specificity to multiple epitopes
- High(er) likelihood to detect the target
- More sensitive: several antibodies per target

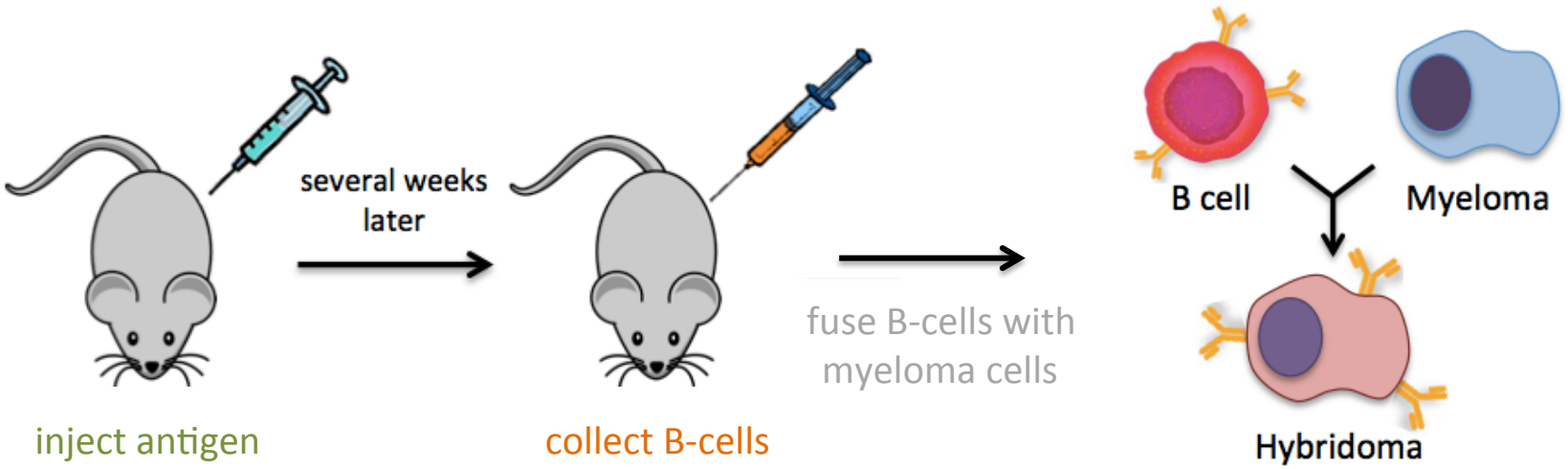
- High(er) background and cross-reactivity (multiple epitopes can be in other proteins)

- Less expensive to produce initially, but supply limited to immunized animal
- Greater variability between preparations



For Western blots, monoclonal antibodies

- Specificity for a single epitope (/region/domain)
- Less sensitive: only one antibody per target
- May cross-react with other proteins that share the recognized domain
- More expensive to produce initially, but available in an unlimited supply



Antibodies used in 20.109


primary anti-BRCA2: rabbit polyclonal


primary anti-tubulin: DM1A, mouse monoclonal


secondary anti-rabbit: donkey polyclonal, *purified**


secondary anti-mouse: goat polyclonal, *purified**

* IgG: immunoglobulin G, most common type of antibody

… for your Discussion

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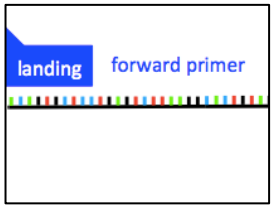

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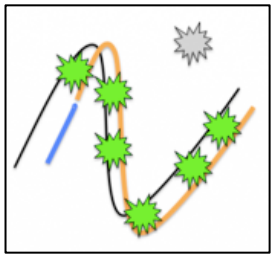


3. Set up qPCR

M2D3: you treated cells to examine viability

- etoposide:

creates double-stranded breaks

- compound 401:

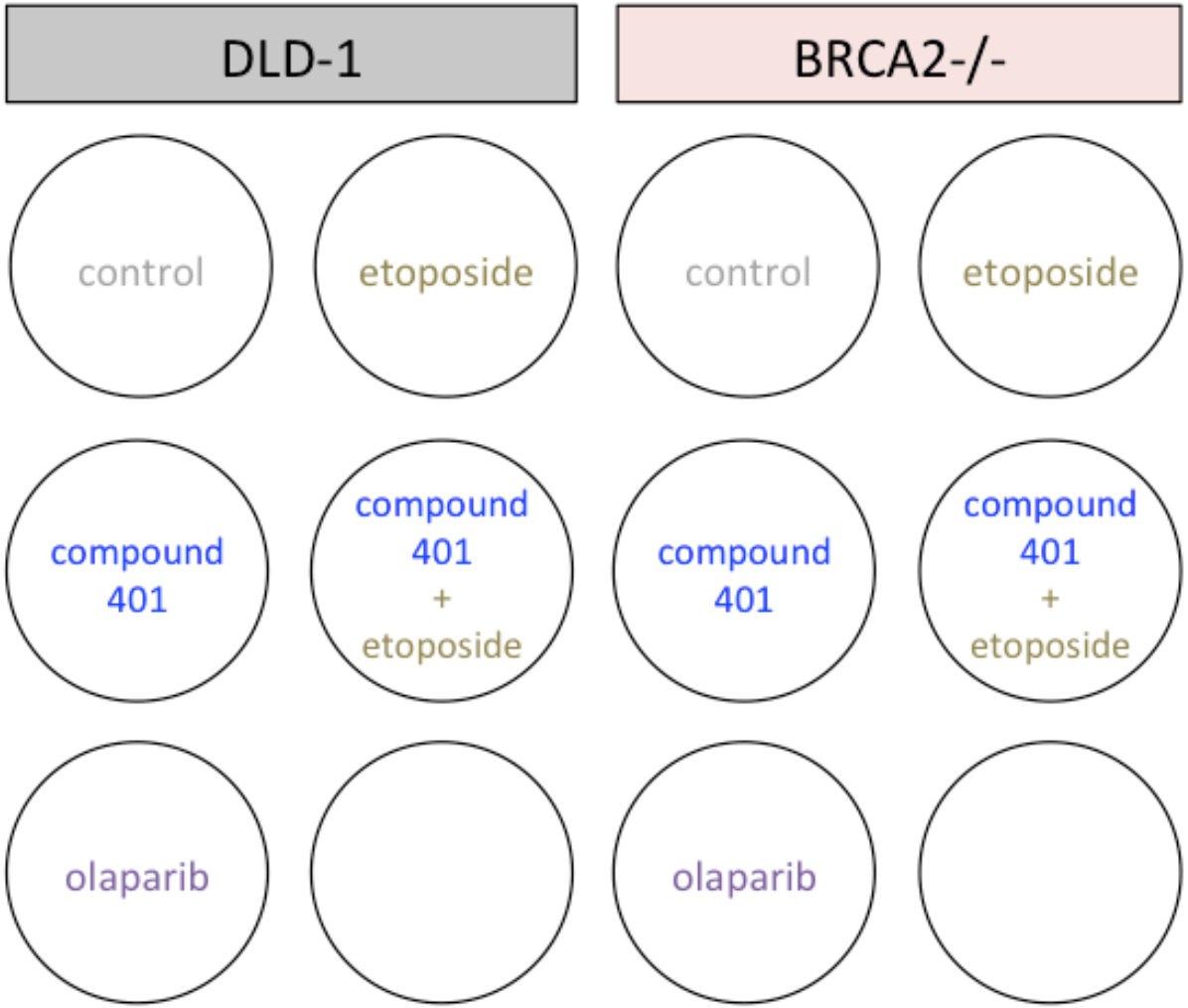
inhibitor of DNA-PK

NHEJ

- olaparib:

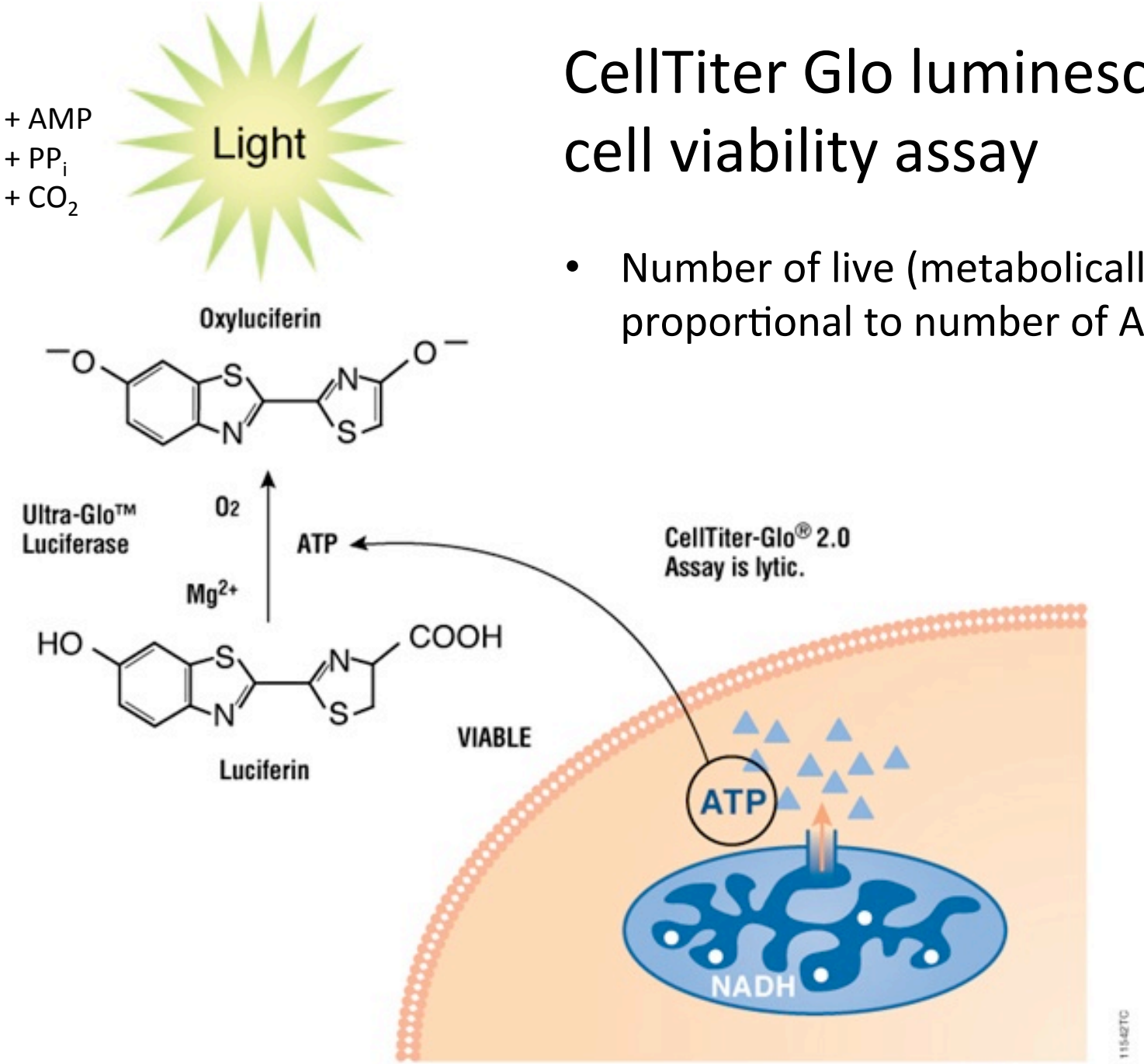
inhibitor of PARP

BER



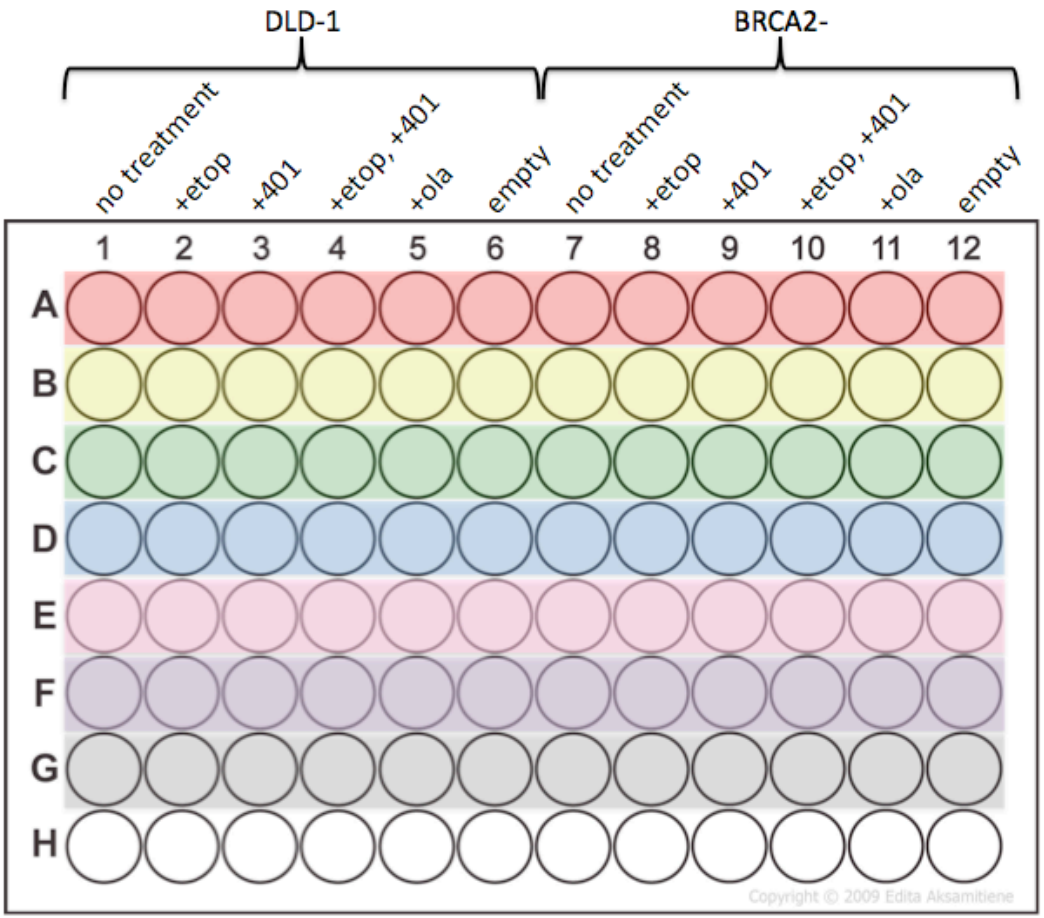
CellTiter Glo luminescent cell viability assay

- Number of live (metabolically active) cells proportional to number of ATP molecules



Practically,

- In 12-well plate,
  - fresh media
  - CellTiter Glo reagent
  - shake & incubate



- Transfer to 96-well plate
- Read luminescence with BioMicro Center plate reader

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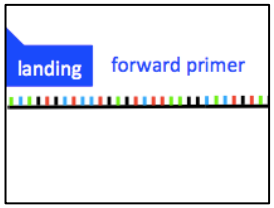

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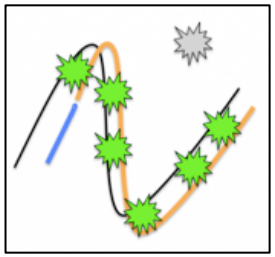


3. Set up qPCR

Clean up cDNA

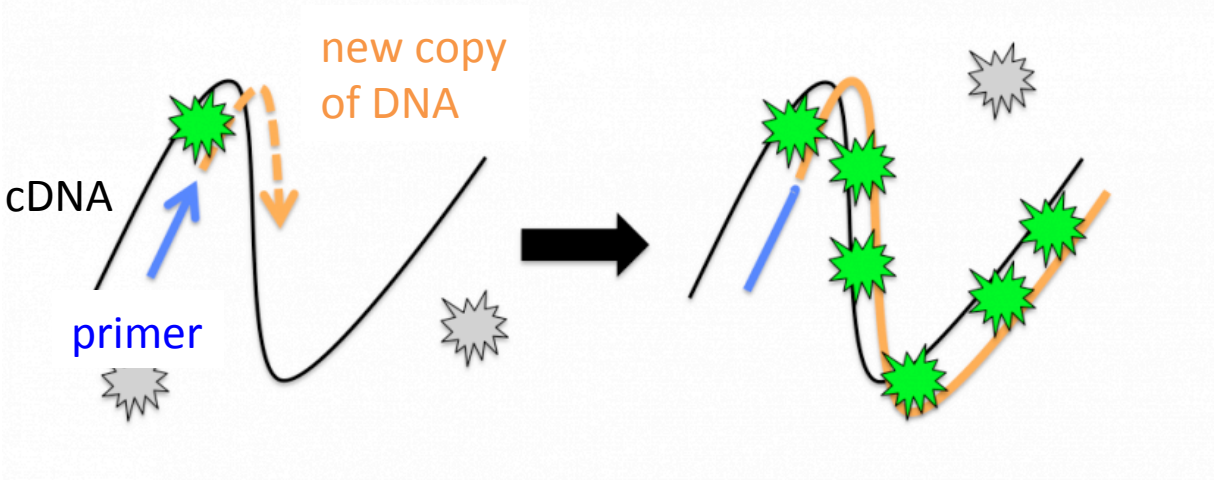
(Qiagen's PCR purification kit ~ last steps of a "mini-prep")

steps	contents	purpose
prepare P1	Tris/EDTA buffer RNase	resuspend cells weaken cell walls
lyse P2	SDS (surfactant) NaOH (alkaline lysis)	solubilize proteins, denature DNA
neutralize N3	acetic acid, chaotropic salt, potassium acetate	short (plasmid) DNA renatures
		clear lysate
concentrate	spin: bind to silica column	pellet "garbage"
wash	PB, PE	wash away dNTPs, RT-pol. ** get rid of *all* ethanol
elute	water, pH 8.0	high-purity DNA

qPCR: quantitative polymerase chain reaction

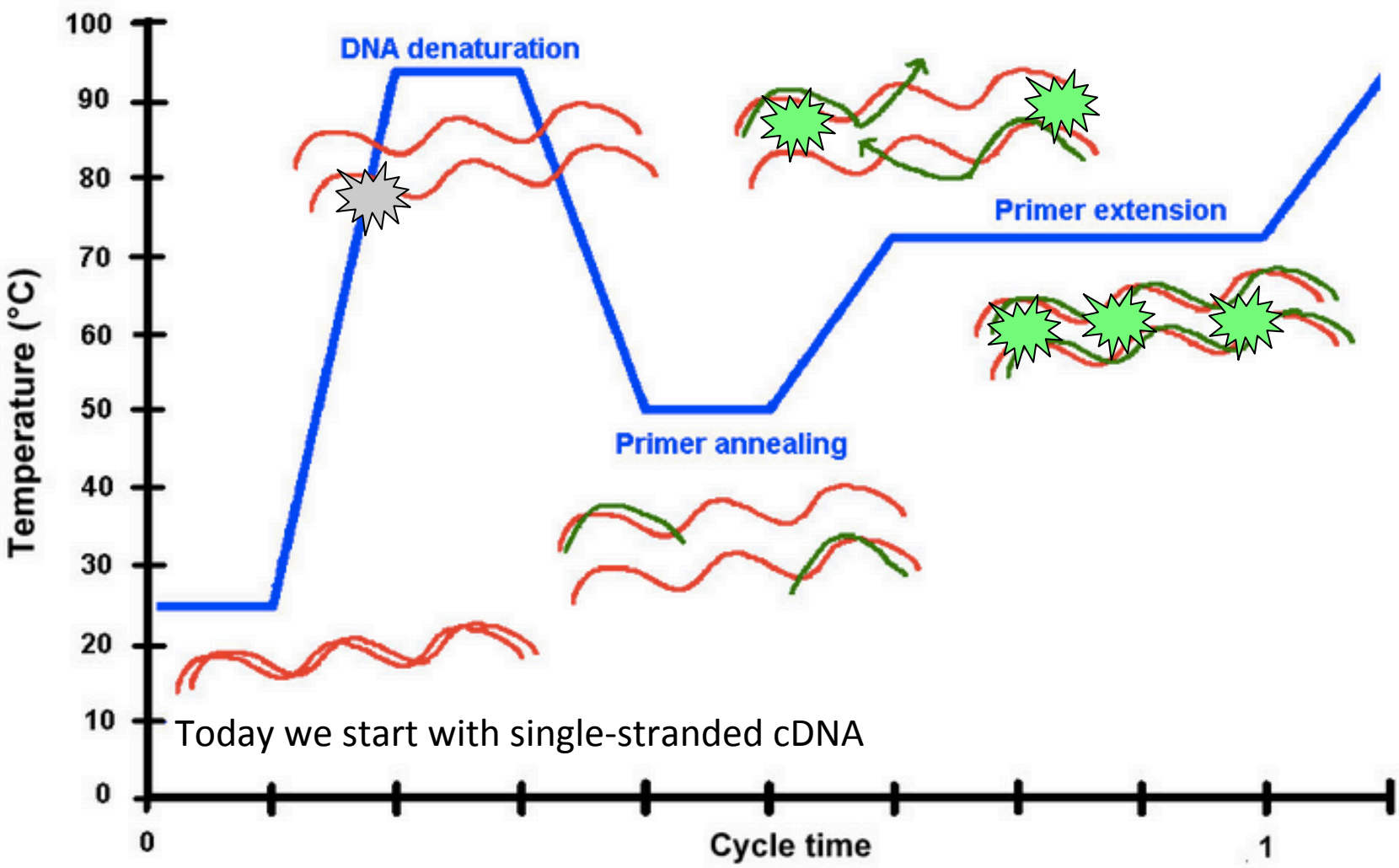
- 
  - qPCR is also referred to as RT-PCR = *real-time* PCR
  - RT-PCR on M2D3: *reverse transcriptase* PCR
    to make cDNA from RNA template

- Monitor PCR as it occurs
  - using dye that is fluorescent when DNA is double-stranded
  - signal proportional to initial amount of cDNA (= original RNA)



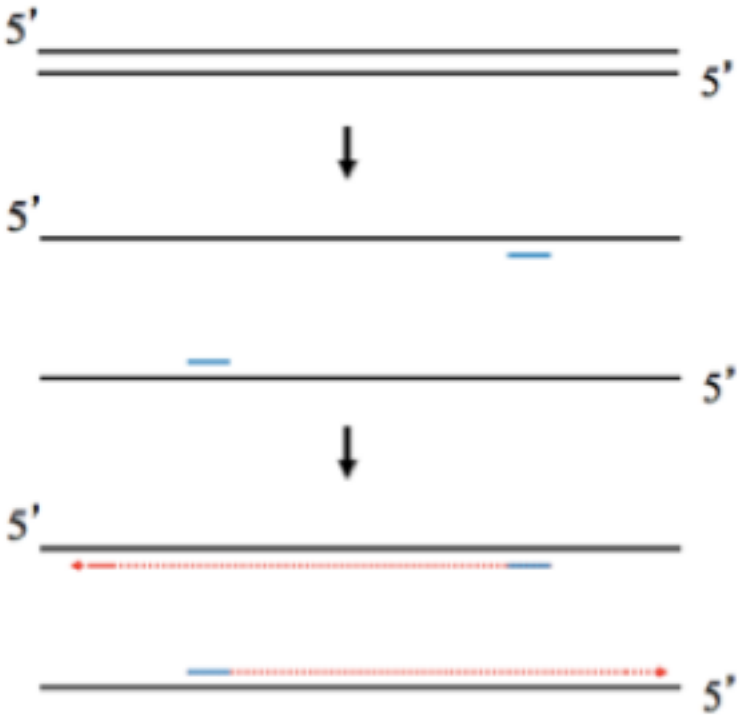
Recall from M1D1:

Polymerase chain reaction (PCR): 1 cycle



PCR ingredients and cycling conditions

PCR ingredients
fluorescent SYBR-Green dye
cDNA template
buffer and water
sequence-specific primers
DNA polymerase
dNTPs



95 °C
(15 s)

60 °C
(30 s)

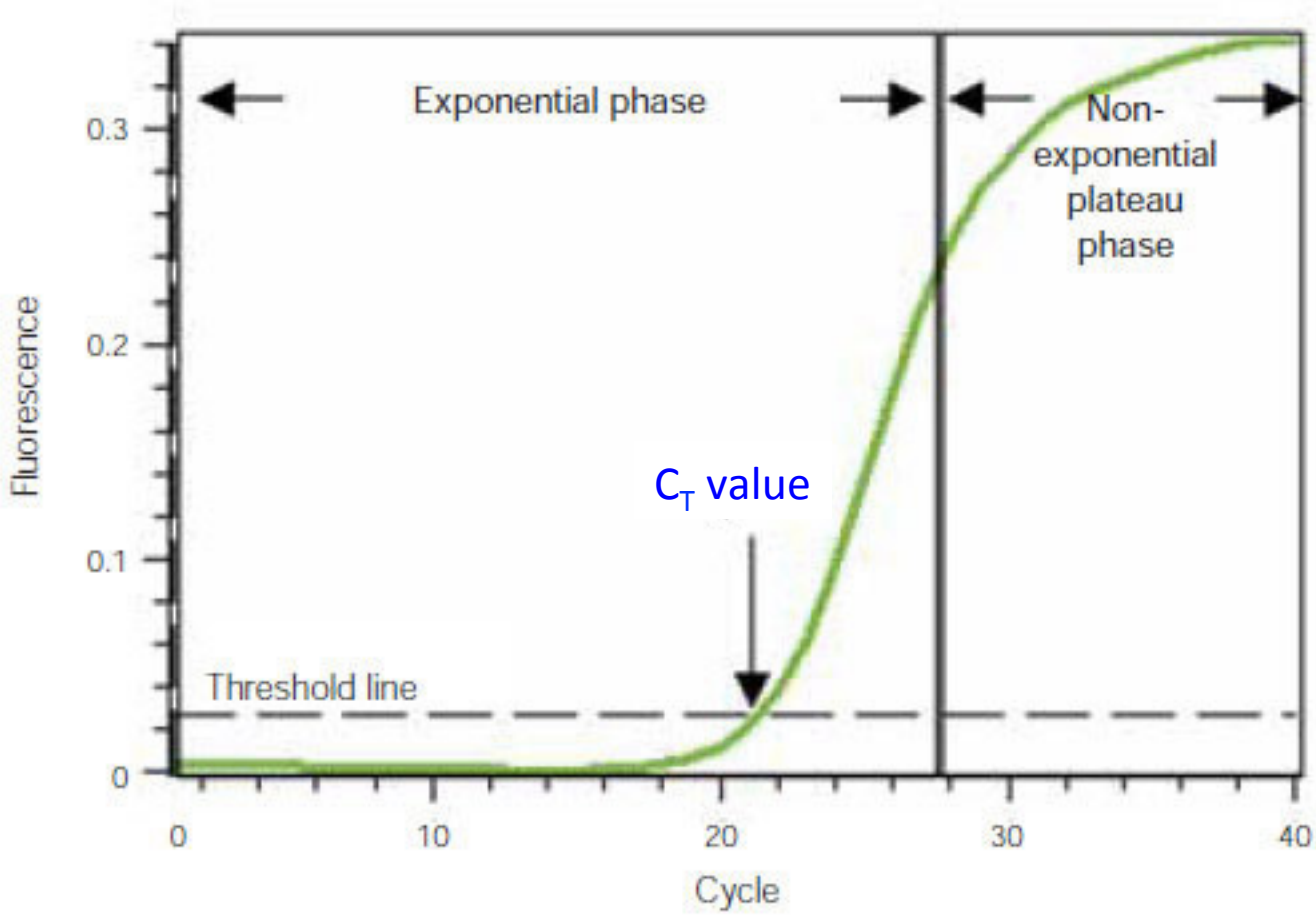
72 °C
(30 s)

40 cycles

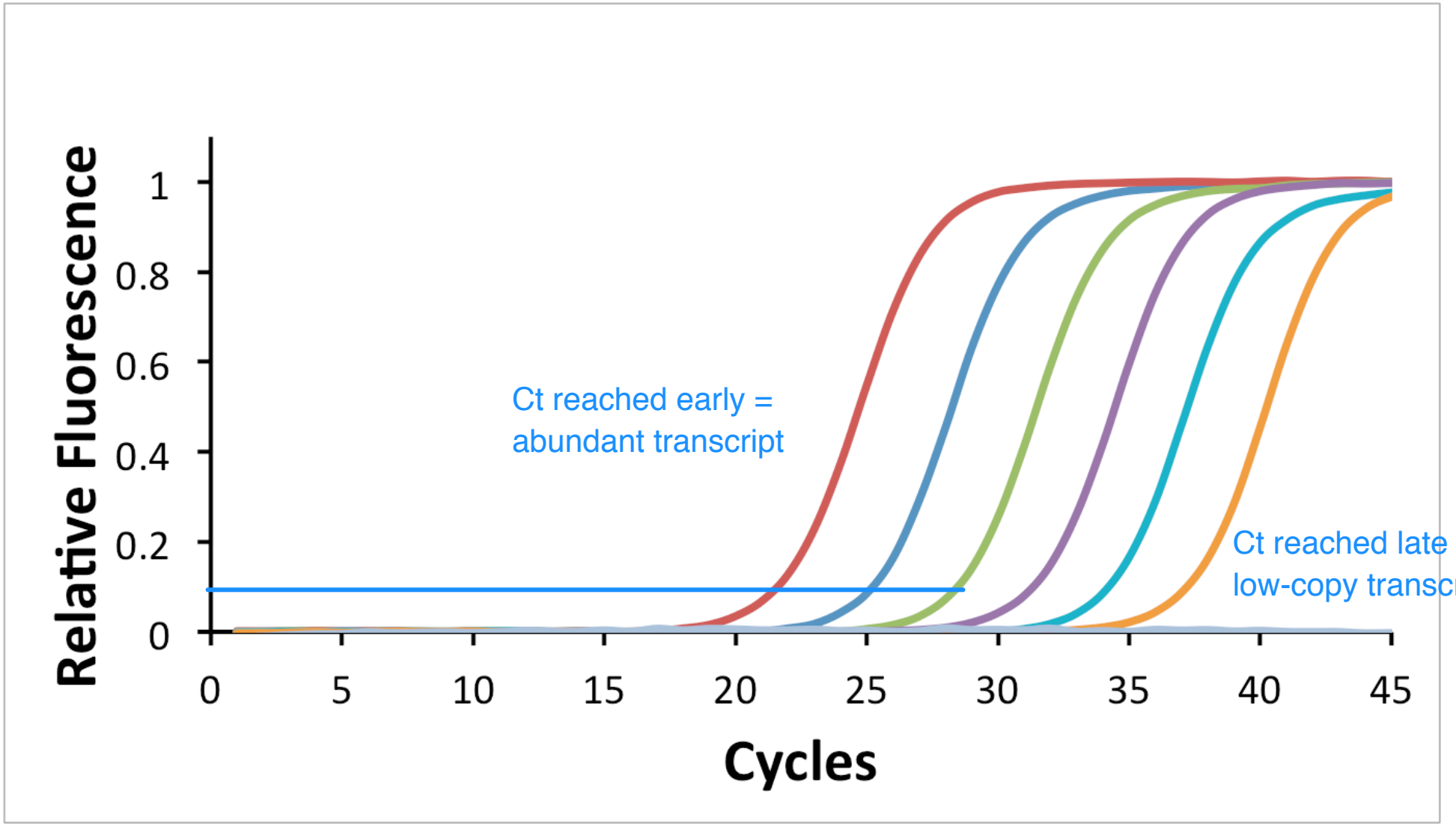
qPCR data

- Fluorescence *vs*. cycle number
- C_T threshold cycle

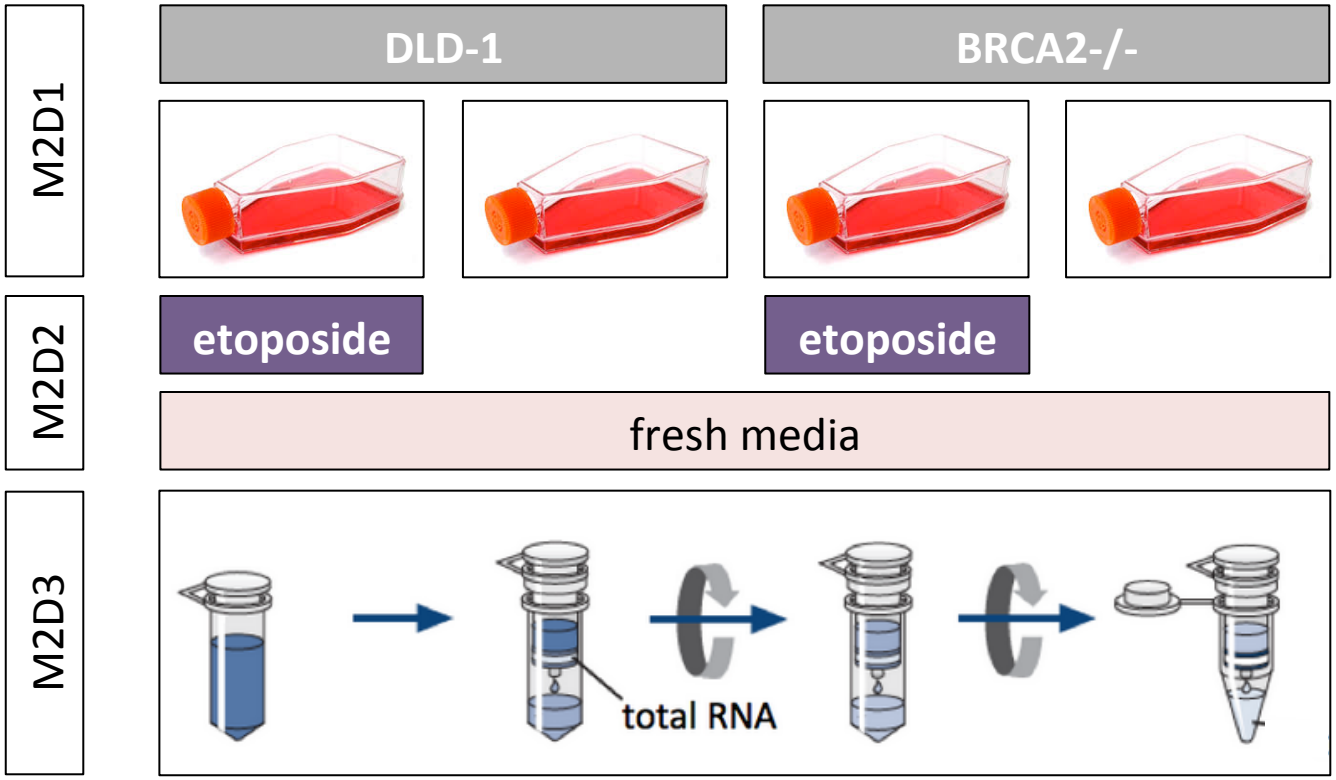
  relative measure of the initial number of copies of template cDNA



C_T value ~ amount of template present at the start of the amplification reaction



How does cancer chemotherapy affect gene expression?



and cDNA

quantify levels of GAPDH
p21

Why look at this subset of transcripts?

- p21 (also CDKN1A) cyclin dependent kinase
  - regulator of cell cycle progression at G1; arrest in G1 in response to stress
  - tightly controlled by tumor suppressor p53

- GAPDH (glyceraldehyde 3-phosphate dehydrogenase)
  - catalyzes glycolysis
  - housekeeping gene: stably, constitutively, highly expressed in cells

- BRCA2 exon 11

Journal club presentations in 16-336

- Upload your slides to Stellar by 1pm Thursday
  - The first student to upload will choose his/her presentation slot
- 10 minutes (+/- 30 s)
  - Practice
- Video-recorded
  - Debrief with Noreen Lyell
- Q&A

- Snacks!

Enjoy spring break!

- M1 Data Summary revision due Monday, March 27
- M1 blog post due Monday, April 3
- M2D6 homework: M2D1-M2D4 Methods