85/100, Good job. I'm not a BARD1 expert, but if the BARD1-dependent ubiquitinome is not well characterized, this is definitely the way to do it. I especially liked Aim 5 where you compare targets upregulated by BARD1 overexpression and DNA damage to see whether some BARD1 targets are potentially unrelated to DNA damage.

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Dr. Weiner
BIOC 451
Final Proposal (P. 1)

Title: Elucidating the Targets of BARD1 E3 Ligase Activity Through Changes in the Ubiquitinome

Abstract: Breast Cancer 1 (BRCA1)-associated RING domain protein-1 (BARD1) is a tumor suppressor protein most commonly understood as a participant in a dimer with the BRCA1 protein. Together, their E3 ligase function leads ubiquitination of other molecules and prevents tumor formation through the destruction of tumorigenic elements. However, little is known about the other ubiquitination targets of BARD1, and so I propose to elucidate the effect of BARD1 on the ubiquitinome.

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but you haven't yet mentioned any BARD1 targets specifically, so do you mean "any" or "other" targets?

Introduction: The BRCA1-associated RING domain protein-1 (BARD1) tumor suppressor is known *in vivo* to form a heterodimeric complex with the BRCA1 protein, another known tumor suppressor implicated in hereditary breast and ovarian carcinomas[1]. BARD1 shares sequence homology with the N-terminal RING motif and C-terminal BRCT domains of BRCA1, which are highly conserved[2]. This heterodimeric complex has E3 ubiquitin ligase activity and ubiquitinates RNA polymerase II to prevent the transcription of damaged DNA. BARD1 is a necessary part of heterodimer BRCA1/BARD1 for this activity, and damaged BARD1 leads to decreased ubiquitination of RNA polymerase II due to reduced E3 ligase activity[3].

Little is known about BARD1 activity independent of BRCA1, although genotoxic stress leads to BARD1 upregulation. It is involved in apoptosis through binding and stabilizing p53, which indicates tumor suppressing activity[4]. Downregulation of BARD1 leads to oncogenic

activity, and it normally decreases mitotic activity by downregulating cyclin B/Cdk1 and causing cell cycle arrest at the G2-M boundary, although the exact mechanism for this process is unknown[4].

Although BARD1 plays an important role in cells as a heterodimeric complex with BRCA1, it also has important independent tumor suppressor activities. Depletion of BARD1 leads to early embryonic lethality in mice and genomic instability[5]. Although BARD1 has little E3 ligase activity with BRCA1/BARD1 targets on its own, its involvement in increasing the E3 ligase activity of BRCA1 indicates that it may have independent E3 ligase activity with other protein targets[6].

these two statements almost seem at odds with each other

The ubiquitin-proteasome system (UPS) is a regulation mechanism that regulates protein degradation within the cell. The UPS includes a cascade of enzymatic reactions involving ubiquitin, ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), ubiquitin-protein enzymes (E3s), deubiquitinating enzymes (DUBs), and the 26S proteasome[7]. The E3 ubiquitin protein ligases contain a RING finger zinc-binding domain that interacts with E2 enzymes and allows the E3 ligase to promote the attachment of ubiquitin to targets (ubiquitination)[8]. Repeat ubiquitination leads to a polyubiquitin chain on the target and polyubiquitination using the K48 or K11 site of ubiquitin leads to degradation of the target by the 26S proteasome complex[7]. Failure of E3 ligase activity can lead to cancer development and, since some E3 ligases can ubiquitinate multiple oncogenic targets, it is important to understand the targets of BARD1[7].

The targets of an E3 ligase can be discovered by analyzing alterations in ubiquitinated proteins (ubiquitinome) using stable isotope labeling with amino acids in cell culture (SILAC) in different cell conditions[9]. Through SILAC, two cell populations are grown: one in a medium that contains a 'light' (normal) amino acid and the other in a medium that contains a 'heavy' amino acid[10]. Cells are passaged multiple times to ensure that heavy amino acids are fully incorporated into the proteome[10]. Cells from both populations are mixed and their proteomes are extracted and measured by liquid chromatography (LC) and mass spectrometry (MS)[10]. Peptides then appear as pairs, with the higher mass containing the heavy amino acid. The ratio of the SILAC peptide pair indicates the relative abundance of the protein between the proteomes[9] [10]. The goal of this proposal is to identify further BARD1 ubiquitination targets, or ubiquitinome, during genotoxic stress to better understand the mechanisms through which the protein acts to preserve the cell. OK

Experimental:

Aim 1: Induce genotoxic stress in BARD1 and verify BARD1 elevation in cells

BARD1 has previously been shown to be upregulated due to genotoxic stress and this should be confirmed before proceeding with the rest of the project. Mouse mammary epithelial TAC-2 cells should be grown to subconfluence, and then treated with DNA-damaging chemotherapeutic agent doxorubicin (10, 50, 100 μg/ml) for 6 to 12 hours to induce genotoxic stress[11]. Protein extracts should be prepared from the TAC-2 cells and analyzed by Western blotting using anti-p53, anti-BARD1, and anti-β-tubulin antibodies. Increasing dosage of

doxorubicin should increase the amount of p53 and BARD1 in the cells while the amount of β-tubulin, the control protein, remains constant.

Aim 2: Generation of TAC-2 cells with elevated BARD1 expression

Generating stable cell lines with elevated BARD1 is difficult, possibly due to apoptotic tendencies of cells with highly elevated BARD1[11]. Instead, TAC-2 cells should be transiently transfected with BARD1. A BARD1 expression clone will be produced by cloning the coding sequence of the mouse BARD1 cDNA into the pcDNA3 expression vector[11]. This vector will be used to transfect TAC-2 cells. or you could introduce BARD1 on an inducible promoter.

Flow cytometry will be used to determine transfection efficiency. Cells will be permeabilized and fixed prior to intracellular staining. The fixed cells will be conjugated with anti-BARD1 antibodies and FITC-coupled secondary antibodies for flow cytometry.

Aim 3: Determining ubiquitinome change in cells with elevated BARD1 expression

SILAC and MS will be used to determine change in the ubiquitinome needs to be determined through SILAC. TAC-2 cells will be passaged in culture medium with heavy $^{13}C_6$-lysine and $^{13}C_6$-arginine multiple times to ensure that all proteins are isotope labeled. Simultaneously, TAC-2 cells will also be passaged in culture medium with light $^{12}C_6$-lysine and $^{12}C_6$-arginine multiple times to ensure that all proteins are isotope labeled. After passaging is complete, cells in heavy media will be transiently transfected with a BARD1 expression vector.

After 6 days, the cells will be treated with proteasome inhibitor MG13 for 5 hours to prevent degradation of ubiquitinated proteins and harvested.

Cell lysates are then mixed in a 1:1 ratio and subjected to a trypsin digest. The C-terminal sequence of ubiquitin is KESTLHLVLRLRGG, with the last glycine conjugated to a lysine on residue in the target protein[9]. After a trypsin digest, this sequence is cleaved after the third to last amino acid, arginine, leaving a glycine-glycine dipeptide remnant conjugated to the lysine[12]. Peptides that had been ubiquitinated will be enriched using an antibody for lysine conjugated to the G-G ubiquitin remnant. A 1:1 ratio of cell lysates from untreated TAC-2 heavy and light media cell cultures will also be mixed and subjected to the trypsin digest.

The enriched peptides from the experimental and control cultures will then be separated for identification using ultra-performance liquid chromatography (UPLC) and reversed phase high-performance liquid chromatography (RP-HPLC). Eluted peptides are scanned using MS followed by tandem mass spectrometry (MS/MS) scans of the most abundant ions for each peptide. These ions in each peptide are then cross-referenced against a MS protein database to identify each protein. Differences in relative abundance of proteins between the heavy and light media will be used to indicate change in the ubiquitinome due to upregulated BARD1.

Aim 4: Determining ubiquitinome change in cells treated with doxorubicin

This experiment will be very similar to the one conducted in Aim 3, except cells will not be transiently transfected with BARD1. For this experiment, two TAC-2 cell cultures will be passaged simultaneously in a culture mediums with heavy $^{13}C_6$-lysine and $^{13}C_6$-arginine and a

culture medium with light $^{12}C_6$-lysine and $^{12}C_6$-arginine multiple times to ensure that all proteins are isotope labeled. After passaging is complete, cells will be treated with 100 µg/ml of doxorubicin for 6 to 12 hours and then treated with MG13 for 5 hours before cell harvest.

Cell lysates will be mixed in a 1:1 ratio and digested using trypsin to cleave ubiquitin, before enrichment using an antibody specific for the G-G dipeptide remnant of ubiquitin conjugated to lysine on the target. A 1:1 ratio of cell lysates from untreated TAC-2 heavy and light media cell cultures will also be mixed and subjected to the trypsin digest. These enriched peptides of the experimental and control cultures will then be separated and identified using a combination of UPLC, HPLC, and cross-reference against an MS protein database to identify proteins ubiquitinated. Differences in relative abundance of proteins between the heavy and light media will be used to indicate change in the ubiquitinome due to upregulated BARD1.

Aim 5: Determine ubuitinome change common to genotoxic stress and elevated BARD1, along with pathways implicated

The last part of this proposal is to cross-examine the date from Aim 4 and Aim 5. This will do three things. The first is to determine BARD1 targets relevant to both upregulated BARD1 and genotoxic stress to determine BARD1 activity during genotoxic stress. The second is to determine BARD1 targets during upregulated BARD1 but not genotoxic stress, indicating other pathways to investigate for BARD1 activity. The third is to determine targets which are upregulated during genotoxic stress due to ligases other than BARD1. Furthermore, to better

nice idea

understand the implications of the BARD1 targets, QIAGEN's Ingenuity® Pathway Analysis will be used to analyze data and determine affected disease networks and pathways.

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