Samples Wouter and Steven

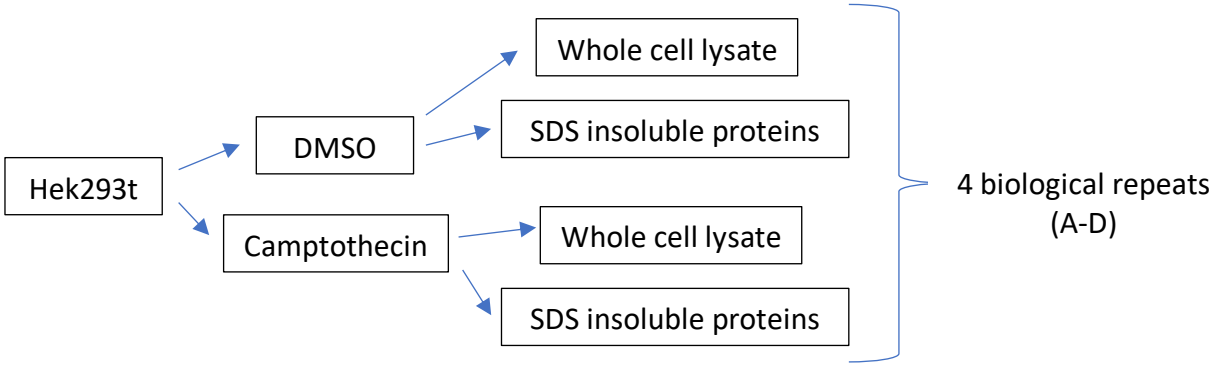
Rationale

We find a gradual accumulation of SDS insoluble proteins after different types of DNA damage. Using protein mass spectrometry we hope to:

1. Identify these SDS insoluble proteins across different types of DNA damage
2. Measure total protein level changes in a whole cell extract, hopefully get some more insight

Starting material

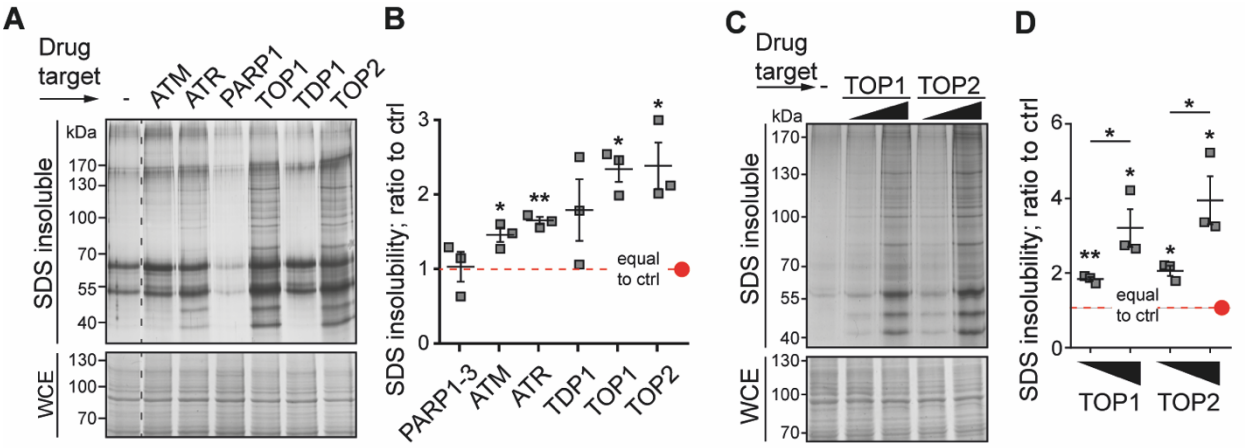
We start with vehicle (DMSO) and campthothecin treated cells (topoisomerase I inhibitor). These cells are fractionated (through centrifugation + detergent solubilization steps) to give a final 1% SDS insoluble protein fraction.



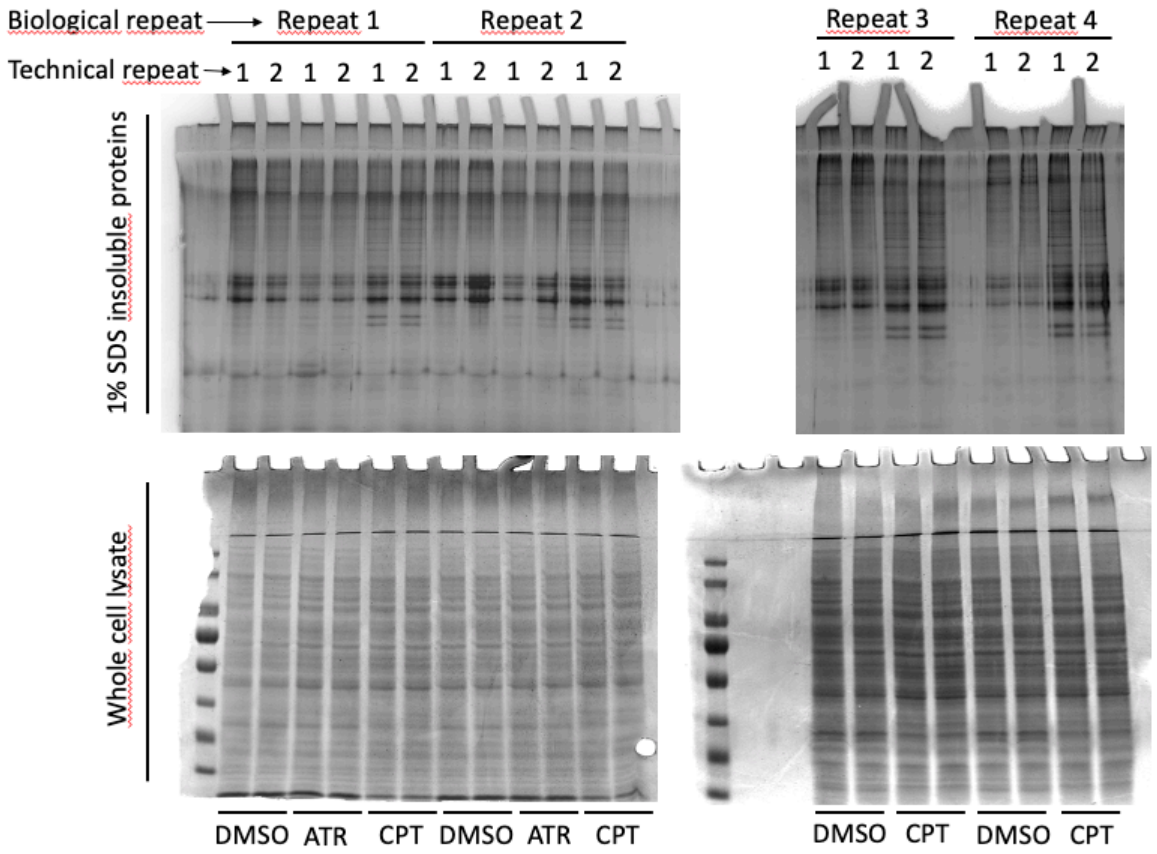
SDS insoluble proteins are finally dissolved in an 8M urea buffer, see recipe below:

Buffer U	Final Concentration	Volume (per ml)
Urea	8 M	n/a
20% (w/v) SDS	2% (w/v)	100 µl
1 M DTT	50 mM	50 µl
2 M Tris-HCl, pH = 7.4	100 mM	50 µl
Ammonium Chloride	25 mM	n/a
Ultrapure H_2O	n/a	Add to 1000 µl
Total	n/a	1000 µl

This is what we normally see: TOP1 = CPT treatment. A roughly 4-fold increase with a high dose of CPT. Silver stain is performed with a kit from Pierce (https://www.thermofisher.com/order/catalog/product/24612#/24612), staining takes ~1 minute to reach this intensity.

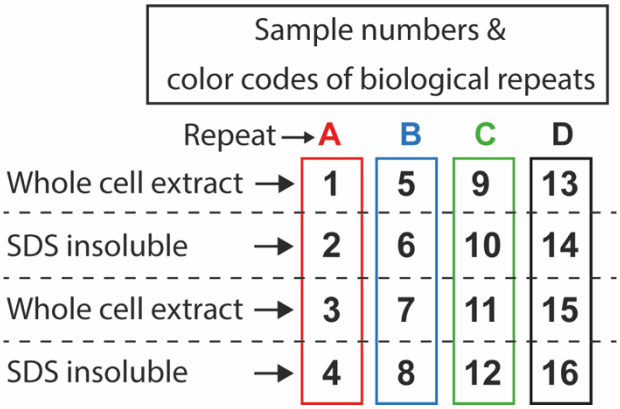


The samples for this mass spectrometry experiment are made separately, with 500 ug starting protein for each technical repeat. I made some quick and dirty figures (underneath). In the first two repeat we included ATR inhibition but it did not work so well for those so we decided to first go with just CPT inhibition. For that reason, biological repeats 3 and 4 only have DMSO and CPT. This is only relative quantification, not absolute. This is because with the silver stains I never include a ladder, for it normally gives substantial artefacts in the staining.



To correct for any possible effects due to urea itself, for this set of experiments whole cell extracts were lysed in the same 8 M urea buffer (dry pelleted cells, shaking in this buffer overnight at RT). No extra shearing/digestion of DNA is performed (perhaps that is needed?). I pooled technical repeats together for both the whole cell lysates and the insoluble protein fractions.

I have color- and number coded these samples as follows:



S-trap Protocol as how I executed it:

I started with 60 ul of starting volume for each sample so I adjusted all volumes as per instructions of Protifi.

I did not add extra SDS, as Protifi states: 'Add SDS to your buffer to at least a 2% w/v final concentration. Then proceed as normal: add phosphoric acid, then binding buffer and everything will be great!'

I replaced TEAB with Tris in the same concentrations, as Protifi states that that is OK.

I found that when I set the pH of my Tris with phosphoric acid and add it to the methanol to make the binding buffer, that this results in a white precipitate. I therefore set the pH with HCl.

- 1.7 mL sample tubes
- Protein fractions (see page 1)
- Iodoacetamide
- Trypsin, proteomics grade (Sigma)
- Digestion buffer (50 mM Tris)
- S-Trap protein binding buffer (90% aqueous methanol containing a final concentration of 100 mM (Tris, pH 7.1).
- 12% phosphoric acid

I started with alkylation of my samples and continued the protocol from there

4) Reduced and alkylated disulfides:

- Alkylated cysteines by addition of iodoacetamide to a final concentration of 40 mM
    - From a 0.5 M stock I added 5.2 ul to 60 ul sample
- Incubated in the dark for 30 min.

5) To the SDS lysate, I added ~12% aqueous phosphoric acid at 1:10 for a final concentration of ~1.2% phosphoric acid (I did 6.5 µL into 65.2 µL). Mix.

6) Added 430,2 µL of S-Trap buffer to the 71.7 µL of acidified lysis buffer from step 5 giving a total volume of 501.4 µL. Mix.

7) With the S-Trap micro in a 1.7 mL tube for flow through, I added the acidified SDS lysate/MeOH S-Trap buffer mixture into the micro column. As the initial SDS lysate volume was higher, I loaded the column multiple times until the full volume has been bound. (E.g. for and initial SDS lysate volume of 50 µL, pass 192.5 µL through twice.)

8) Centrifuged the micro column at 4,000 g until all SDS lysate/S-Trap buffer has passed through the S-Trap [7] column. Protein will be trapped within the protein-trapping matrix of the spin column. [7]

9) I washed captured trotein by adding 150 µL S-Trap buffer; repeat centrifugation. Repeat wash three times.
I rotated the S-Trap micro units like a screw 180 degrees between the centrifugations of steps 8 and 9.

10) Moved S-Trap micro column to a clean 1.7 mL sample tube for the digestion. [8]

11) Added 20 µL of digestion buffer containing protease at 1:19 ratio into the top of the micro column. I used 1 µg of trypsin per column. I ensured there is no air bubble between the protease digestion solution and the protein trap.

12) Capped the S-Trap tip column loosely to limit evaporative loss. [8]
13) Incubated for 1 hr at 47°C for trypsin in an unmoving thermomixer.
14) Eluted peptides with 40 µL each of 50 mM Tris and then 0.2% aqueous formic acid. Added the first Tris elution to the trypsin solution prior to any centrifugation. Centrifuged elutions through at 4,000 g. Recovered hydrophobic peptides with an elution of 35 µL 50% acetonitrile containing 0.2% formic acid. Pooled elutions.