20160316 Nrf1-HA mouse liver mass spectrometry

Project: Nrf1 proteomics | Folder: Nrf1 complex completed | Owner: Dr. Brendon Smith

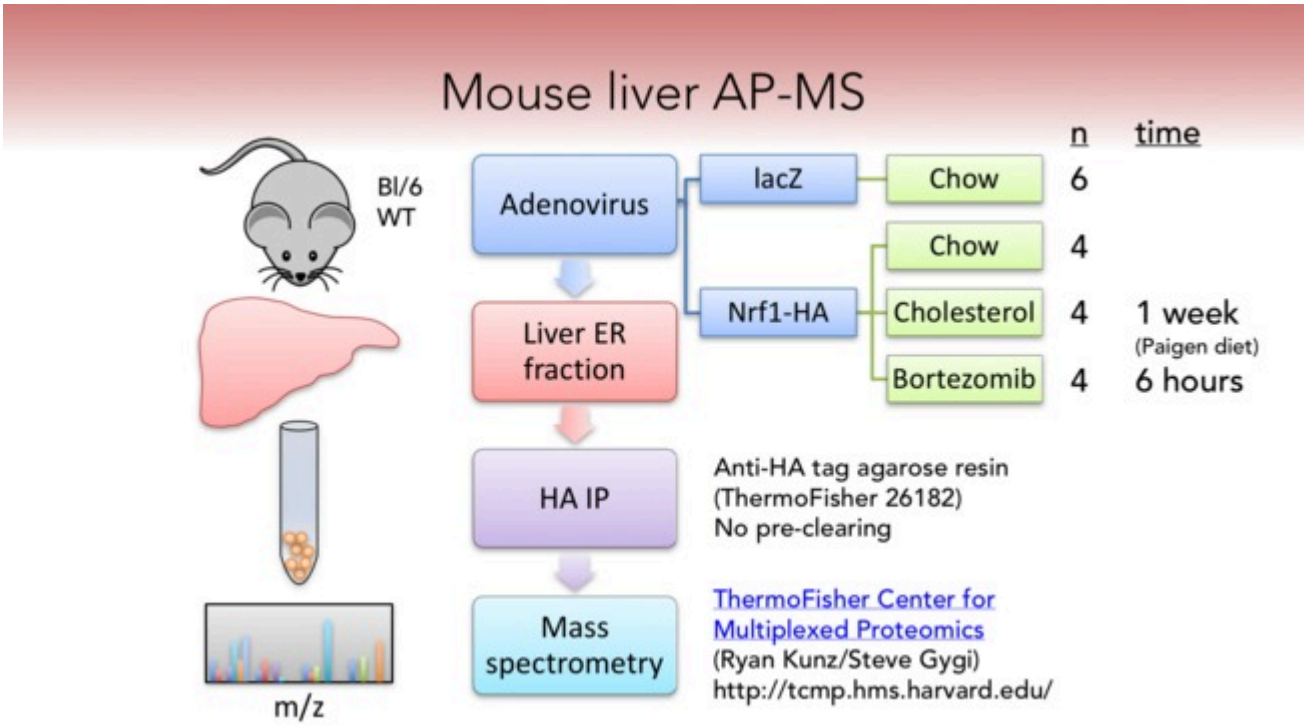
Members with editing permissions: Ediz Calay (admin), Kathryn Claiborn (admin), Martin McGrath (admin), Gokhan Hotamisligil (admin), Brendon Smith (admin), Brian Fredrick (admin)

Signed by Brendon Smith on November 16, 2016 at 12:36 | Witnessed by Kathryn Claiborn on 2016-11-18

Description

Description

In this experiment, we infected mice with Nrf1-HA or lacZ adenovirus (lacZ is a gene in the lac operon that codes for beta-galactosidase, used here as a control), and fed chow or cholesterol, with an additional bortezomib treatment group. We harvested livers, enriched the microsomal fraction, isolated Nrf1 with an HA IP (agarose resin, no pre-clearing), and performed quantitative multiplexed proteomic analysis of liver samples. See 20151216-18 Nrf1-HA mouse liver fractionation and affinity purification.



Gels

Jan 05, 2016 07:00 to Jan 05, 2016 17:00

Gels

Gel run

NuPAGE 4-12% gel 48 min 200 V

Stain: Thermo Fisher Pierce 24600 silver stain

Samples: liver lysates from 12 mice infected with Nrf1-HA adenovirus and 6 lacZ control mice. Of the 12 Nrf1-HA mice, 4 were on control diet, 4 on cholesterol diet, and 4 were treated with bortezomib. We isolated microsomal fractions from each of the liver samples, and conducted an HA affinity purification. This is 10% of our total eluate.

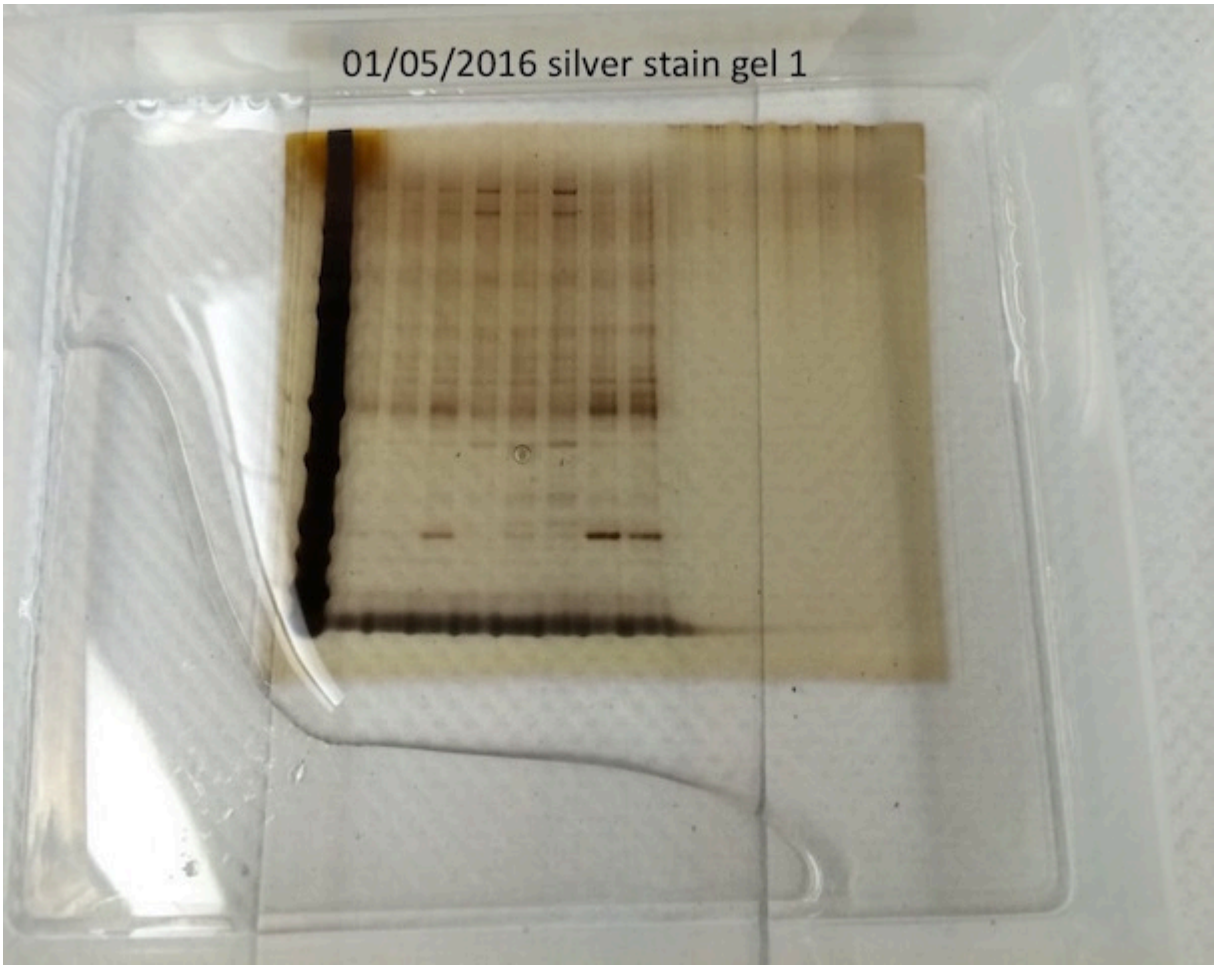
20 uL eluate aliquot for blots and gels. Samples were eluted in 2x SDS-PAGE loading buffer without beta-mercaptoethanol, so they were diluted to 1x (40 uL). I loaded 20 uL into each lane of the gel, with 5 uL molecular weight marker.

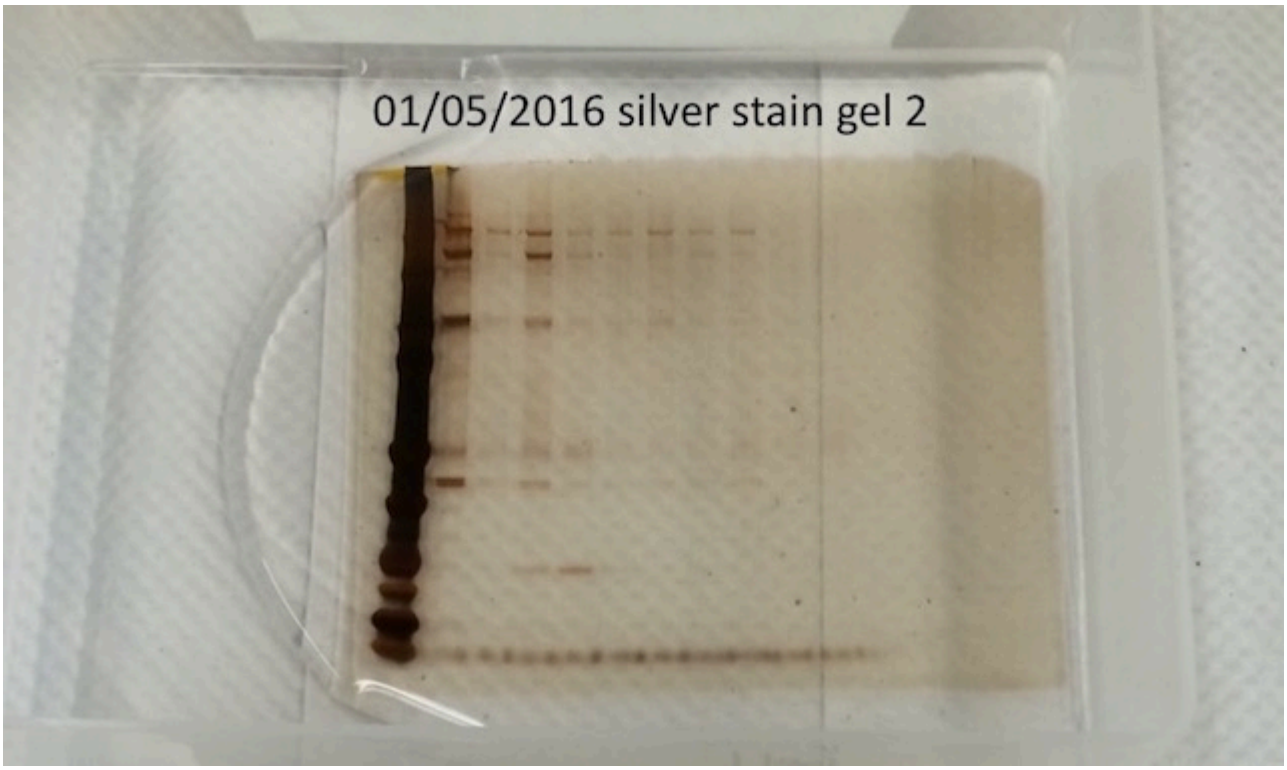
Sample prep: Made 1x RIPA with 2x (5%) 99% beta-mercaptoethanol (475 uL RIPA, 25 uL beta-mercaptoethanol), added 20 uL to each sample, mixed by repeated pipetting (didn't vortex to avoid any potential disruption of sample and protein complexes, although I used RIPA buffer which can disrupt complexes), heated, centrifuged in microcentrifuge 1 min, cooled to RT, and proceeded with electrophoresis as described in Western blot protocol. I loaded 20 uL of the diluted 1x sample, leaving an additional 20 uL for blots. I accidentally filled the lower buffer chamber before loading samples. After I realized how difficult it was to load samples, I poured off some buffer (after loading the protein ladder and lane 2 of gel 1, no samples in gel 2), which made it much easier. I didn't pipette lane 2 of gel 1 well so some may have leaked over to lane 3. I tried the 200 uL flat gel loading tips, which are annoying to use. They seem to aspirate some solution by capillary action, so it is difficult to tell if they have the correct volume, and they can forcefully dispense an air bubble that causes samples to leak out of the lanes. I completed the gels with regular Sorenson 200 uL tips. These were a little too large, and some sample would start to emerge from the tip. To minimize this, I held the sample's microtube above the gel, aspirated, and then quickly dispensed into the gel lane. In the future, I will use 20 uL barrier tips (Sorenson 14210T), which fit my 100 uL Eppendorf pipette. Some loading buffer may have bled into lanes 10-13 of gel 1 and 12-13 of gel 2.

I prepared the fixing and stop solutions during the electrophoresis run.

Gel results

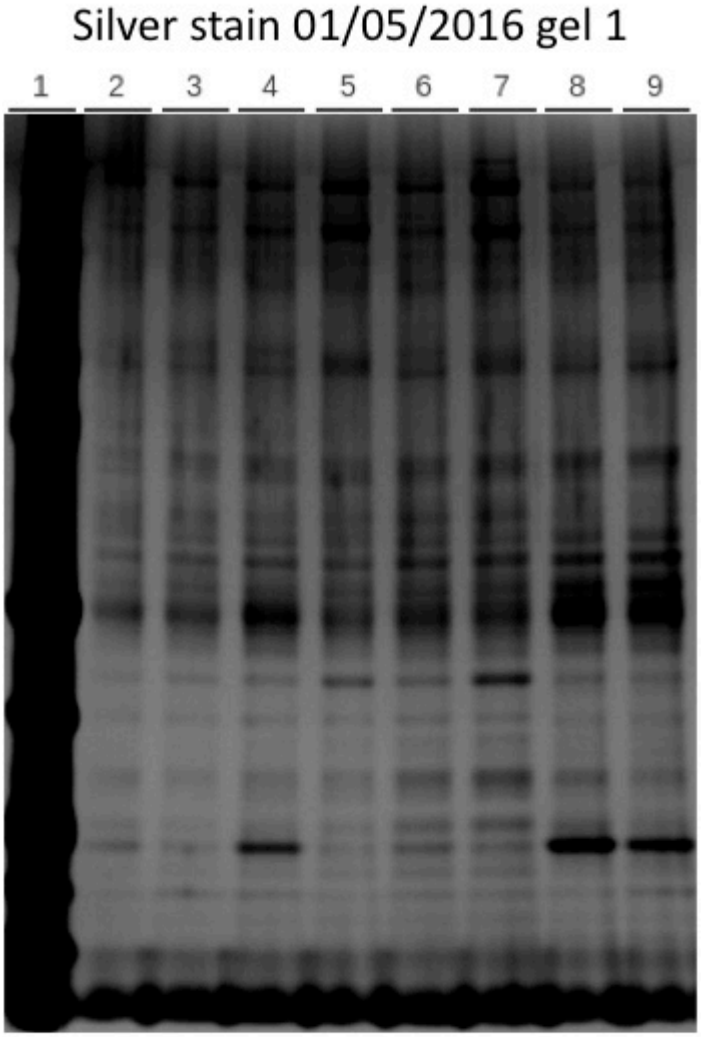
Gel 2 (samples for which Scott completed the fractionation and HA IP) developed more slowly than gel 1 (samples for which I completed the fractionation and started the HA IP, and Scott completed the IP). Could be due to differences in how we performed the procedures. I took a picture at 10 min. Images below adjusted/scaled to 640x480 pixels.

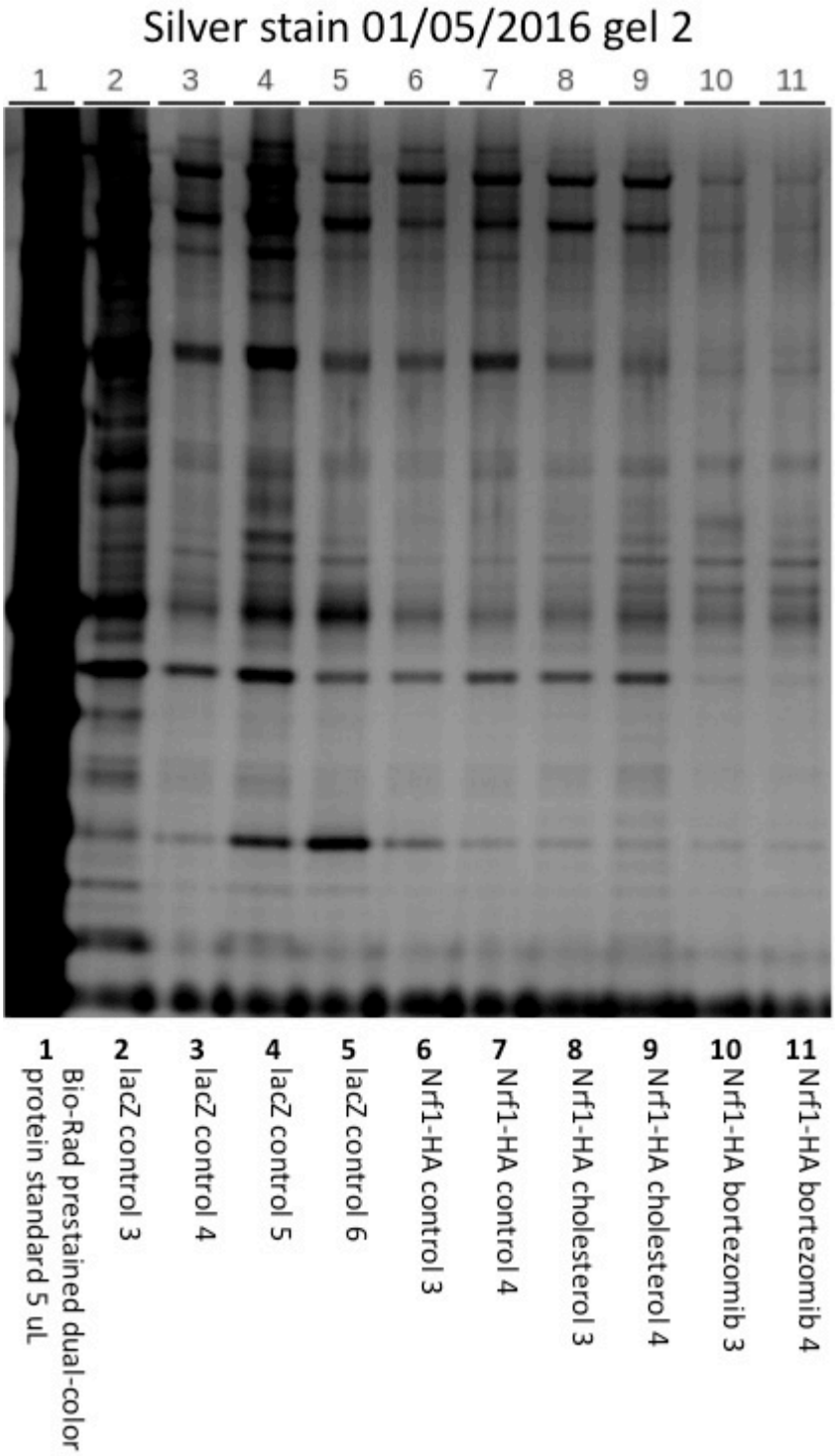




Protein ladder was totally overexposed and should be diluted. We got a much better yield from the microsomal fraction than we previously did with the HEK cells. As before, we don't have prominent Nrf1 bands, but many other bands, some of which are probably nonspecific.

Images from ProteinSimple, labeled in PowerPoint:


Silver stain 01/05/2016 gel 1
1 2 3 4 5 6 7 8 9
1 Bio-Rad Prestained dual-color protein standard 5 uL
2 lacZ control 1
3 lacZ control 2
4 Nrf1-HA control 1
5 Nrf1-HA control 2
6 Nrf1-HA cholesterol 1
7 Nrf1-HA cholesterol 2
8 Nrf1-HA bortezomib 1
9 Nrf1-HA bortezomib 2



I sent an image of gel 2 to Ryan Kunz, who said the amount of protein looks good. It was not adequate though (see below).

Western blots

Western blots 1/7 and 3/3

Western blots demonstrated purity of mouse liver microsomal fractions, as determined by the presence of calreticulin and absence of

COX IV (mitochondria) and Lamin A/C (nucleus), and relative to the cross-contaminated HEK-293 A Nrf1-HA 1-741 cell fractions generated by the same method.

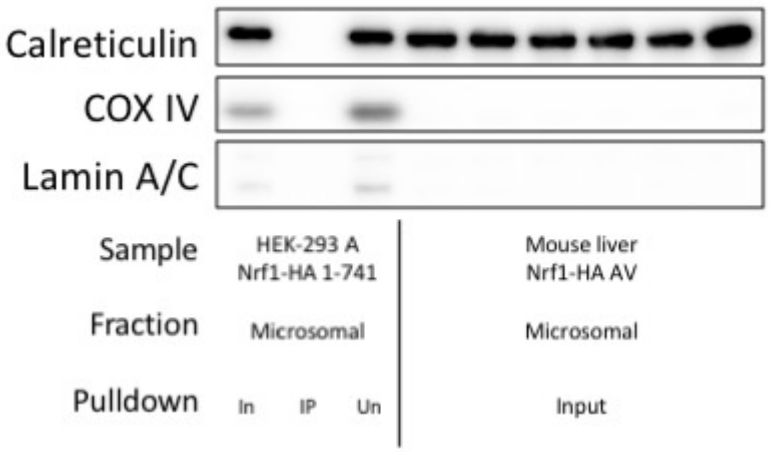


Image shows lanes 7-15 from 3/3/16 blot 2 (Calreticulin and COX IV) and blot 4 (Lamin A/C).

In=input (sample loaded into IP)

IP=HA IP with anti-HA magnetic beads (mouse liver sample IPs, not shown here, were performed with anti-HA agarose)

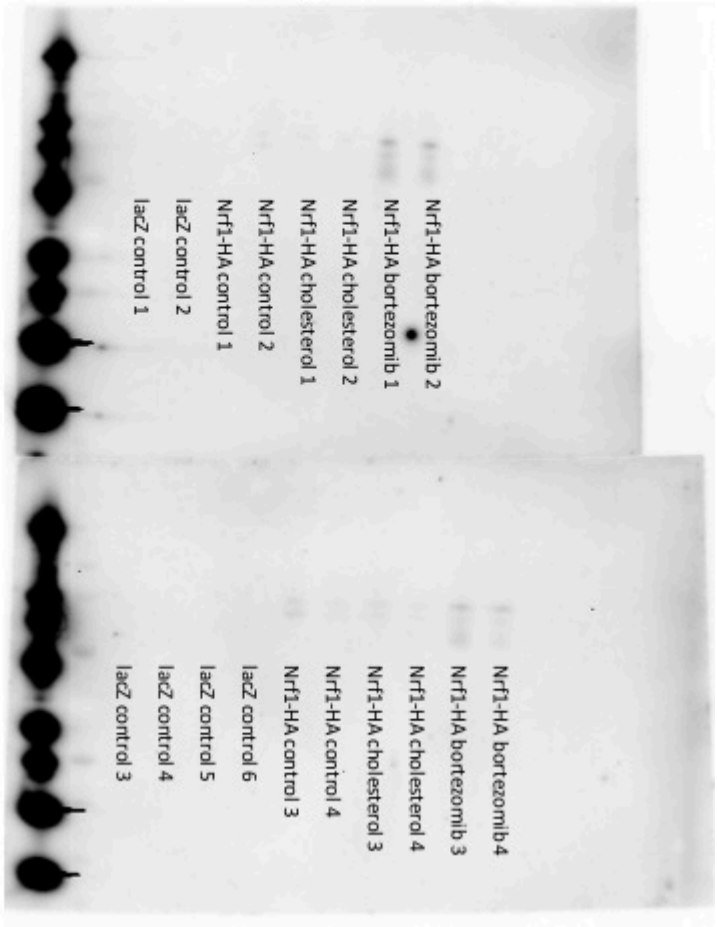
Un=unbound (sample not bound to agarose after IP)

There was little detectable Nrf1 and HA in the mouse liver samples on these blots, but it was visible with long exposures. I may need to add more sample to see Nrf1. See 20160227 HEK-293 A Nrf1-HA 1-741 subcellular fractionation for full details on Western blots from that date.

Calreticulin (ER), COX IV (mitochondria) and Lamin A/C (nucleus) are essentially loading controls for specific cellular compartments, so I don't need an additional loading control like Tubulin. Note that I later found that the COX IV antibody I used did not cross-react with mouse. See Western blots 11/10 for updated images.

Scott also ran Western blots for Nrf1 on the IP eluates, and identified Nrf1 in the mice infected with Nrf1-HA but not lacZ (imaged on Thursday 1/7/16 1:35 pm). Note that I discussed these blots with him when he was imaging them on 1/7, and he told me he had used 2.5% of the eluate (2.5 uL) and an Nrf1 antibody, but he later said that these were blots with 5% of the eluate using an HA antibody. I think the former is probably correct (2.5% eluate, Nrf1 antibody).

Western blot
SBW011-_2015-12-16_Nrf1-HA_AdVir_WTMice_ERFrac_HA-IP_HA-Ab
Original file name: Scott-2016-01-07_13-35-59



Western blots 10/1-10/6

Western blots 10/1-10/6

Steps 10/1

Sample Prep	10/1/16 14:00		Made 1 mL 4x loading buffer with 10% 2-ME under hood Diluted by half to 2x in npH2O for IP samples Sample prep inputs: I had previously prepped 8 of the input samples for 2/27 blots. I noted a clear gelatinous precipitate in be glycerol in loading buffer. For remaining samples that had not been previously run: add 40 uL 2x sample buffer to 10 fresh tube Sample prep IP: Transferred 10 uL IP samples to PCR tube strips because of small volumes, and added 10 uL 2x loadin lacZ 3,5, con 2, chol 4 <10 uL Chol 3 none C1q deficient serum: Calbiochem 23440, lot 2725466, 62 mg/mL (is this the C1q concentration?). Dilute 100 uL 4x loading buffer for final dilution of 1:20. Purified C1Q: 0.6 mL, 1.1 mg/mL, lot SLBQ8480V. I'm not sure how much to use, try diluting 1:100 in 1 npH2O, add 3 uL C1q to 297 uL 10 mM HEPES, aliquot and freeze remaining protein), freeze this and t C1q for blots.
Electrophoresis Prep			
Electrophoresis			150 V 70-75 minutes room temp **The XCell midi cell would not start.** I started off at 50 V. I could see some bubbles on the wires along holding the IP samples was. I could not see any bubbles along the side of module 1 (not labeled with a I think the wiring in the first module must be faulty. I carefully unlocked the gel tension wedge, remov them over to a new module (labeled 1), and carefully refilled the module with running buffer. I started first must be faulty. I set it aside and will order a replacement.
Transfer Prep			Make 4 L NuPAGE 20% MeOH transfer buffer Label blots with alcohol proof permanent marker, especially for strips, before transfer. Use scissors to cut blots, not razor blade.

Transfer			18 hours 30 V. I tried to use eight sheets of filter paper on each side for gel 1. It was a little too thick, b this. I used four per side for gels 2 and 3. Ponceau S staining revealed air bubbles on blot 2, and I could not see the IP samples on blot 3, which **The key to avoiding air bubbles is to clasp the transfer cassette when fully submerged under bu** I accidentally let the membranes dry out while I figured out what to do. Don't put blots under transfer
Western blot			**See Labguru experiment "20160928 HEK-293 A Nrf1-HA 1-741 detergent fraction performed with fractionation samples on 10/5.**

Gel setup 10/1

I used the sample setup in order to fit on 20 well gel, and also because I didn't have any more cholesterol 3 IP sample and I wanted to match the setup on both blots.

Gel	Lane	Sample	Amount (uL)	
1 2 input	20	HA bor 4	10	
1 2 input	19	HA bor 3	10	
1 2 input	18	HA bor 1/2	10	
1 2 input	17	HA bor 2/1	10	Bortezomib 2 in gel 1. Corrected on gel 2.
1 2 input	16	HA chol 4	10	
1 2 input	15	HA chol 2	10	
1 2 input	14	HA chol 1	10	
1 2 input	13	HA cont 4	10	
1 2 input	12	HA cont 3	10	
1 2 input	11	HA cont 2	10	
1 2 input	10	HA cont 1	10	
1 2 input	9	lacZ 5	10	
1 2 input	8	lacZ 4	10	
1 2 input	7	lacZ 3	10	
1 2 input	6	lacZ 2	10	

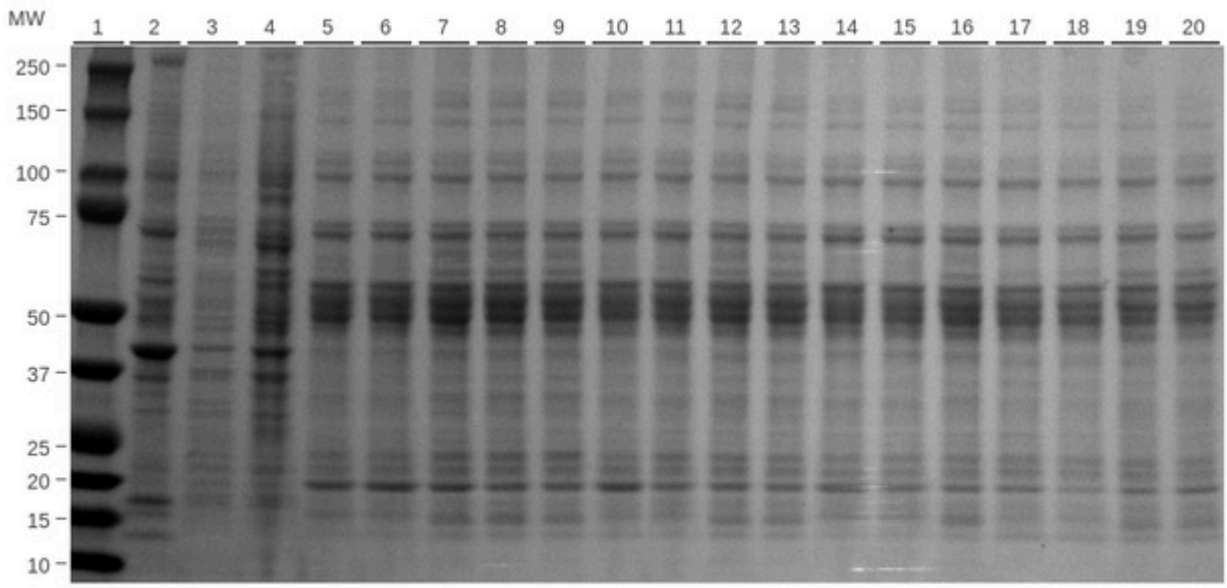
1 2 input	5	lacZ 1	10	
1 2 input	4	HEK-293 A Nrf1-HA 1-741 2/28/16 epoxomicin nuclear input	10	
1 2 input	3	HEK-293 A Nrf1-HA 1-741 2/28/16 epoxomicin microsomal input	10	
1 2 input	2	Nrf1 KO iMEF	10	
1 2 input	1	Ladder	10	
3 IP	20	HA bor 4	10	
3 IP	19	HA bor 3	10	
3 IP	18	HA bor 2	10	
3 IP	17	HA bor 1	10	
3 IP	16	HA chol 4	10	
3 IP	15	HA chol 2	10	
3 IP	14	HA chol 1	10	
3 IP	13	HA cont 4	10	
3 IP	12	HA cont 3	10	
3 IP	11	HA cont 2	10	
3 IP	10	HA cont 1	10	
3 IP	9	lacZ 5	10	
3 IP	8	lacZ 4	10	
3 IP	7	lacZ 3	10	
3 IP	6	lacZ 2	10	
3 IP	5	lacZ 1	10	
3 IP	4	C1q depleted serum	10	
3 IP	3	C1q from human serum	10	
3 IP	2	Nrf1 KO iMEF	10	
3 IP	1	Ladder	10	

Images

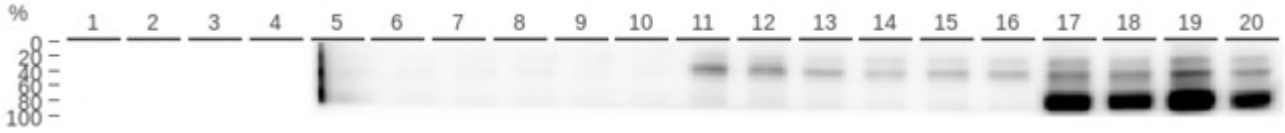
Blots imaged with samples from "20160928 HEK-293 A Nrf1-HA 1-741 detergent fractionation and Nrf1 IP." Images separated and cropped here. Full images available in attached files.

Blot 1: input

Ponceau S



Nrf1 (mouse samples only to avoid signal saturation by 293 cell lysate)

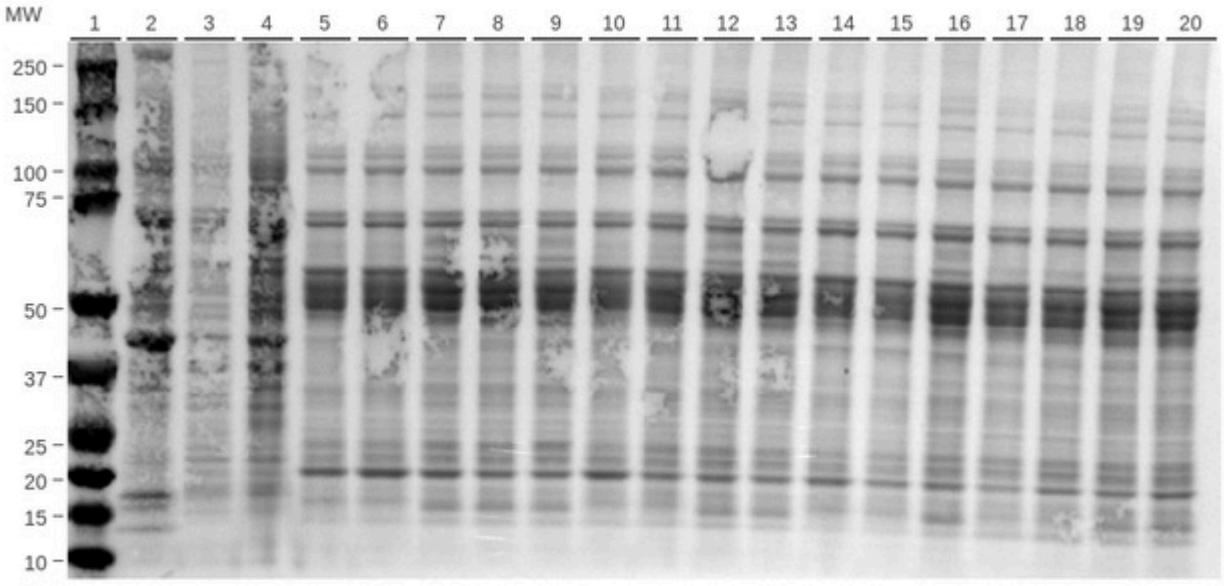


Calreticulin

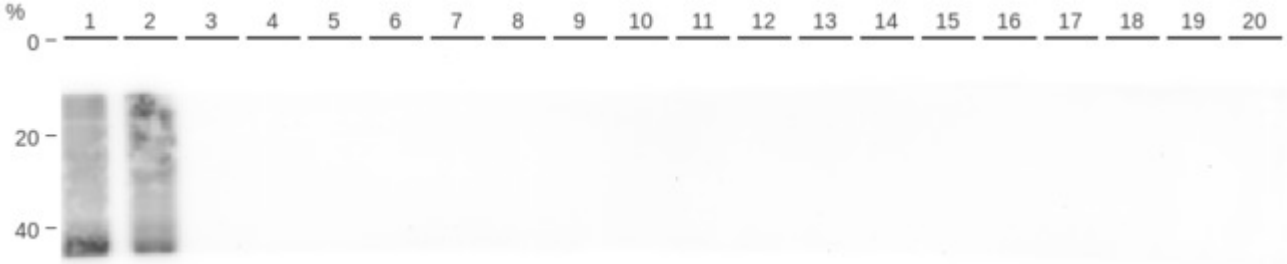
COX IV

Blot 2: input

Ponceau S



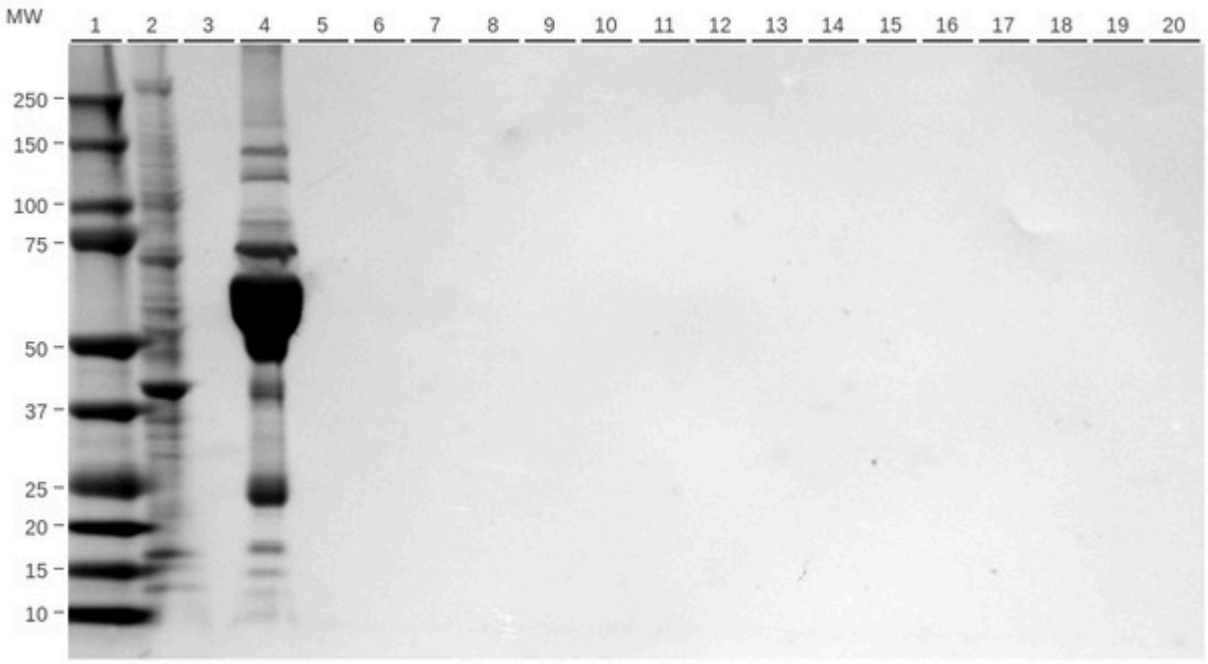
Na K ATPase



Lamin A/C

Histone H3

Blot 3: IP


MW
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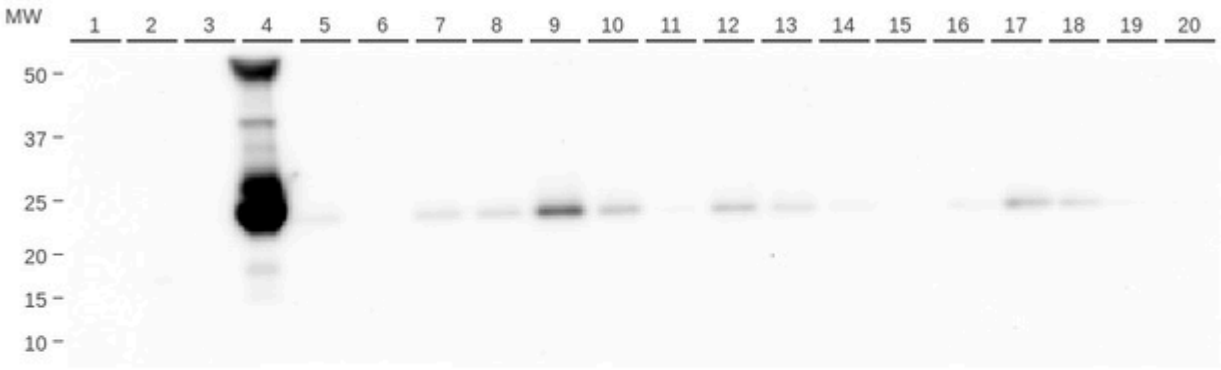
C1q



Discussion

Note that I later found that the COX IV and Na,K ATPase antibodies I used did not cross-react with mouse.

C1q results were difficult to interpret and compare with mass spec. The C1q depleted serum had strong nonspecific signal, so probably not a good control to use.

The strips were too narrow at times (see results). Two strips per blot would be better.

I should also image each strip separately instead of combining them so I don't have to do so much image editing.

Samples & Reagents

Antibodies (5)

Name	Alternative name	Clonality	Raised In	Reacts with	Manufacturer	Remarks	Stocks
COX IV (3E11) Rabbit mAb #4850				Human, Rat, Monkey, Bovine, Pig, Zebrafish	Cell Signaling Technology		
Calreticulin (D3E6) XP® Rabbit mAb		Monoclonal	Rabbit	Human, Mouse, Rat	Cell Signaling Technology		
Lamin A/C (4C11) Mouse mAb #4777					Cell Signaling Technology		

Name	Alternative name	Clonality	Raised In	Reacts with	Manufacturer	Remarks	Stocks
Histone H3 (D1H2) XP® Rabbit mAb #4499	Histone H3	Monoclonal	Rabbit		Cell Signaling Technology		
Na,K-ATPase α1 (D4Y7E) Rabbit mAb		Monoclonal	Rabbit	Human	Cell Signaling Technology		

Consumables (1)

Name	Alternative name	Manufacturer	MW	Type	Remarks	Stocks
Pierce™ Fast Western Blot Kits, SuperSignal™ West Dura, Rabbit		ThermoFisher Scientific				

Western blots 11/10

Western blots 11/10

Western blots to re-do the mouse liver input samples with COX IV and Na,K-ATPase antibodies that cross-react with mouse.

Steps

Step	Start	End	Notes
Sample Prep	11/10/16 2:00 PM	11/10/16 2:30 PM	Made undiluted C1q and 1 from the 100 uL C1q and a uL 4x sample buffer with 1
Electrophoresis Prep			
Electrophoresis			4-12% NuPAGE 20 well mid Stopped after 60 minutes b went to the Barabasi lectur Gels sat in tank for 45 minu affect performance.
Transfer Prep	11/10/16 6:00 PM	11/10/16 7:00 PM	
Transfer	11/10/16 7:00 PM	11/11/16 11:00 AM	16 hours 34 V 4C (added or Tris-glycine transfer buffer

Western blot	11/11/16 2:20 PM		Blot 1 input 250-50 kDa strip: Na K ATP reprobe Lamin A (74 kDa) a in 5 mL for blot strip) 50-10 kDa strip: COX IV CST C1q (~30 kDa) ThermoFisho strip) Blot 2 IP 250-50 kDa strip: Nrf1 (120 50-10 kDa strip: C1q (~30 k in 5 mL for blot strip) Sequence: Rabbit strips: Blot 1 strip 1- Briefly strip Blot 1 strip 1-2 Mouse strips: Blot 1 strip 1 **I used the VWR 5 mL tube** **and they fit in our 15 mL** **Weak signal from blots 1-** **after the 4x5 wash buffe** **secondary HRP antibody** **have turned out.** **Purified C1q may be aggr**
Imaging	11/11/16 4:00 PM	11/11/16 5:00 PM	
Stripping			
Reprobing	11/11/16 5:00 PM	11/11/16 7:00 PM	Nrf1 IP blot 2-1 didn't turn

Gel setup

Gel	Lane	Sample	Amount (uL)	Notes
1 input	20	HA bor 4 input	10	
1 input	19	HA bor 3 input	10	

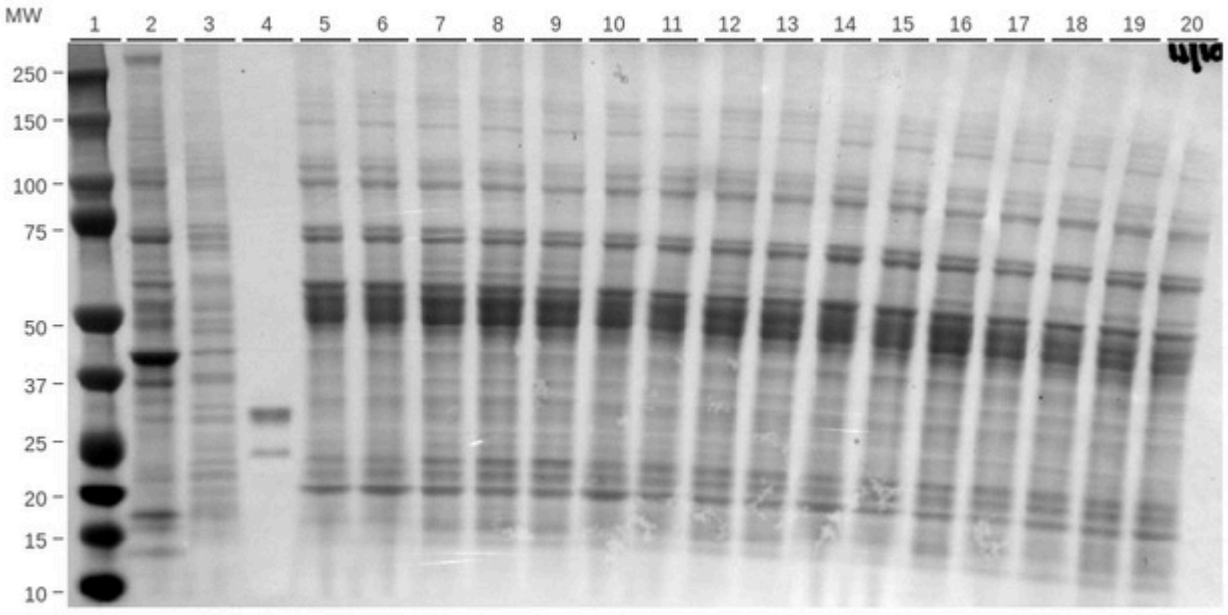
1 input	18	HA bor 2 input	10	
1 input	17	HA bor 1 input	10	
1 input	16	HA chol 4 input	10	
1 input	15	HA chol 2 input	10	
1 input	14	HA chol 1 input	10	
1 input	13	HA cont 4 input	10	
1 input	12	HA cont 3 input	10	
1 input	11	HA cont 2 input	10	
1 input	10	HA cont 1 input	10	Gel 1: Air bubble came out of pipette tip, may have slightly less here.
1 input	9	lacZ 5 input	10	
1 input	8	lacZ 4 input	10	
1 input	7	lacZ 3 input	10	Gel 1: Air bubble came out of pipette tip, may have slightly less here. Gel 2: 5 uL
1 input	6	lacZ 2 input	10	
1 input	5	lacZ 1 input	10	Gel 1: Placed pipette tip filled with C1q 1:5 here accidentally before moving to lane 4 to dispense. Shouldn't affect results. Gel 2: added all that was left of sample ~8 uL, added 8 uL exactly for rest of IP samples.
1 input	4	Purified C1q 1:5	10	
1 input	3	HEK-293 A Nrf1-HA 1-741 2/28/16 epoxomicin microsomal input	10	

1 input	2	Nrf1 KO iMEF	10	Gel 2: some spilled out when pipetting
1 input	1	Ladder Bio-Rad precision plus dual color	10	
Gel	Lane	Sample	Amount (uL)	Notes
2 IP	20	HA bor 4 IP	8	
2 IP	19	HA bor 3 IP	8	
2 IP	18	HA bor 2 IP	8	
2 IP	17	HA bor 1 IP	8	
2 IP	16	HA chol 4 IP	8	
2 IP	15	HA chol 2 IP	8	
2 IP	14	HA chol 1 IP	8	
2 IP	13	HA cont 4 IP	8	
2 IP	12	HA cont 3 IP	8	
2 IP	11	HA cont 2 IP	8	
2 IP	10	HA cont 1 IP	8	
2 IP	9	lacZ 5 IP	8	
2 IP	8	lacZ 4 IP	8	
2 IP	7	lacZ 3 IP	5	Gel 2: only 5 uL left
2 IP	6	lacZ 2 IP	8	
2 IP	5	lacZ 1 IP	8	Gel 2: added all that was left of sample ~8 uL, added 8 uL exactly for rest of IP samples.
2 IP	4	Purified C1q 1:5	10	
2 IP	3	Purified C1q undiluted	10	
2 IP	2	Nrf1 KO iMEF	10	Gel 2: some spilled out when pipetting
2 IP	1	Ladder Bio-Rad precision plus dual color	10	

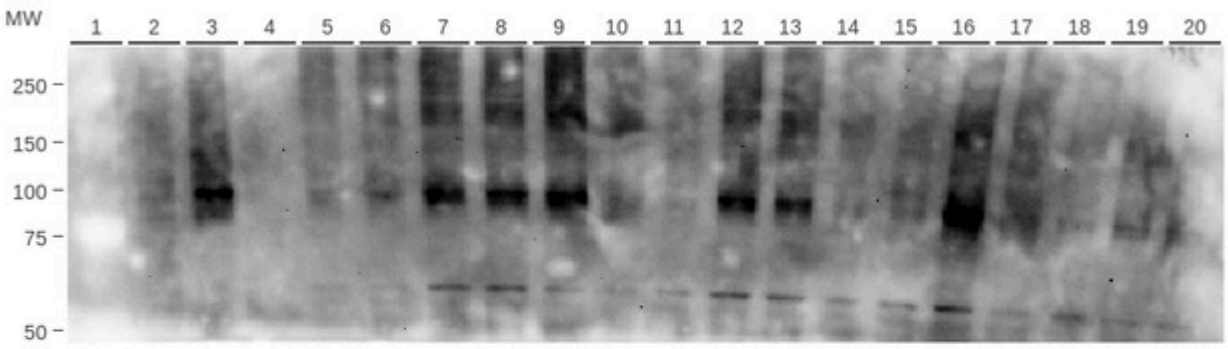
Images

Blot 1 (input)

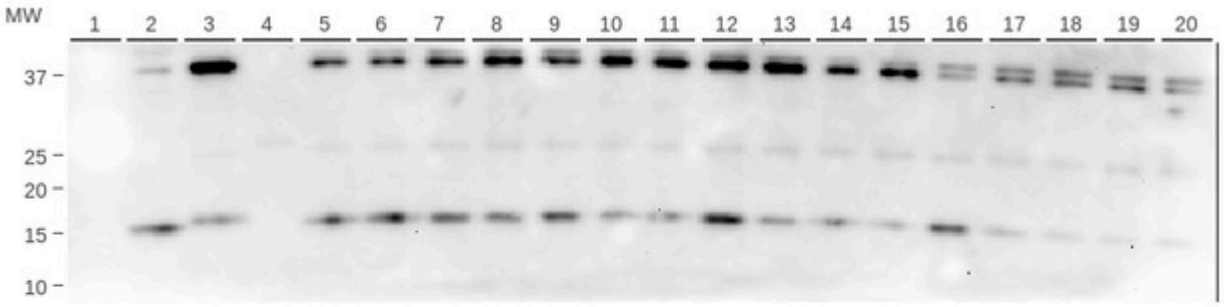
Ponceau S



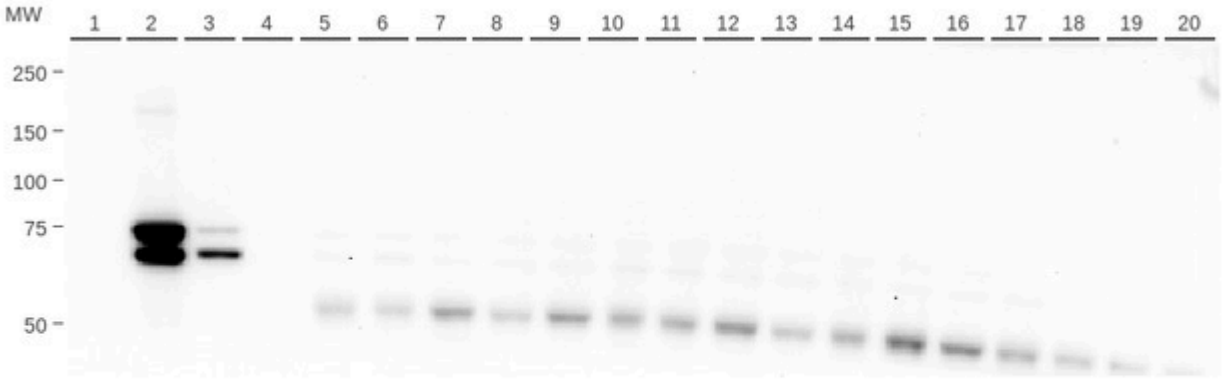
Na,K-ATPase (specific band at 100 kDa)



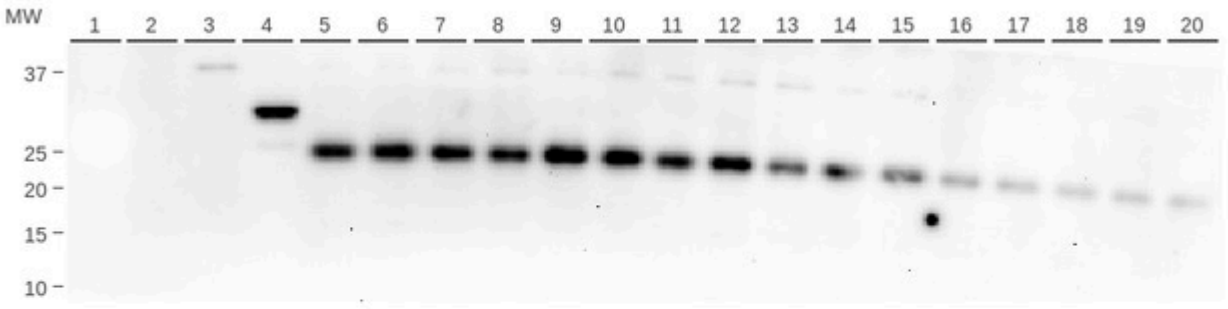
COX IV (specific band at 17 kDa)



Lamin A/C (specific bands at 75 and 63 kDa, nonspecific band at 50 kDa)



C1q input


MW
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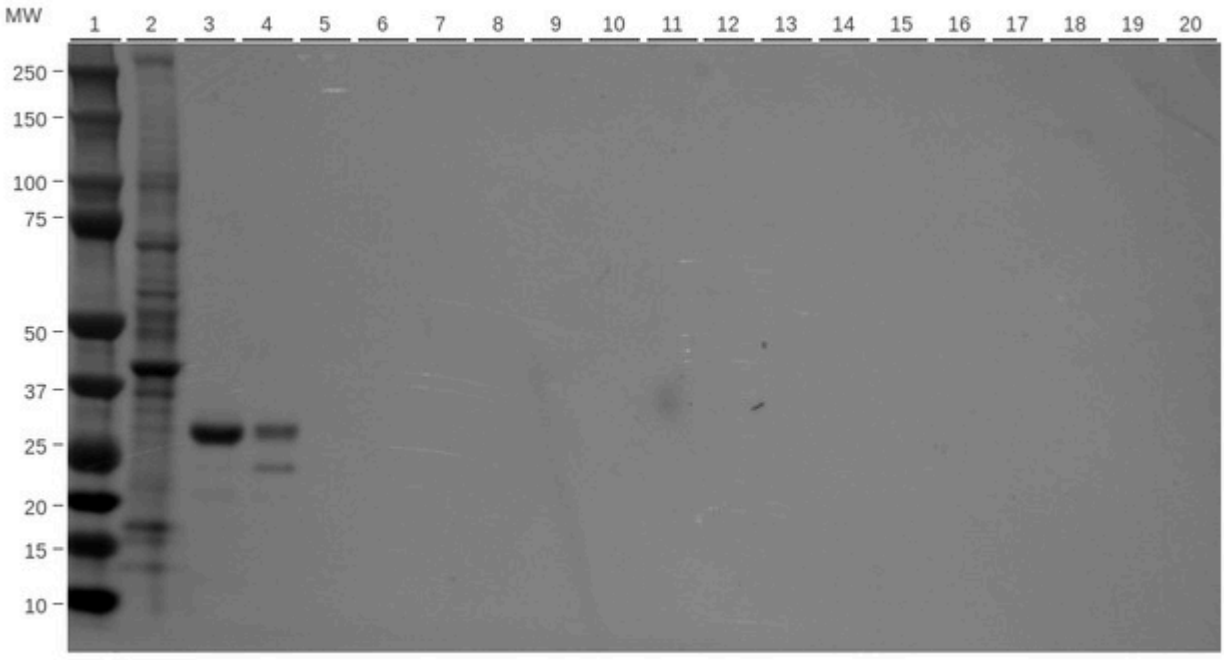
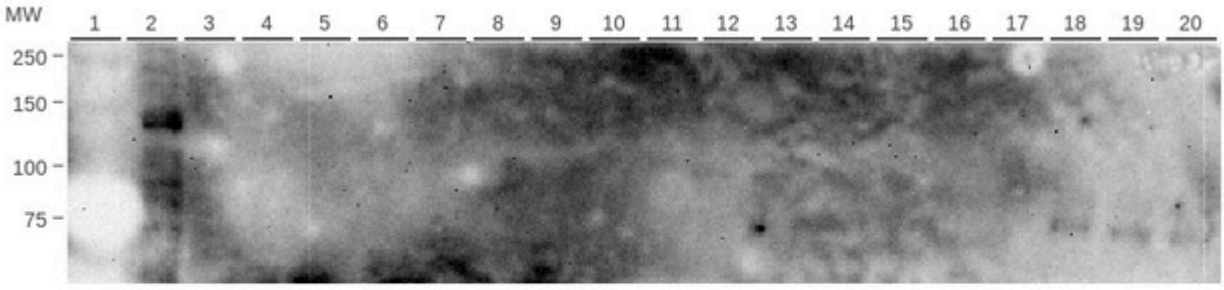
Blot 2 (IP)

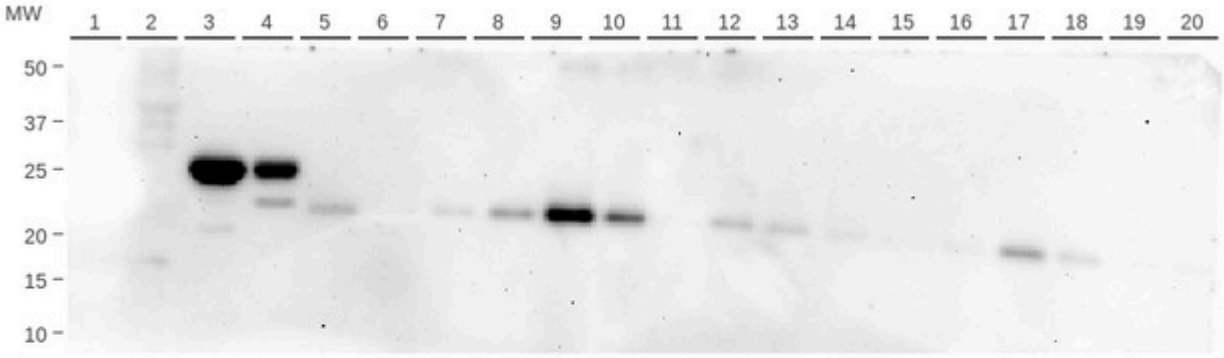
Ponceau S



Nrf1


MW
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
250
150
100
75

C1q IP


MW
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
50
37
25
20
15
10

Discussion

The fractions are not as pure as I thought. I could include total liver lysate as a control, but it's not totally equivalent because the microsomal fraction is enriched. I could try matching the calreticulin signal to get an approximation.

Couldn't see Nrf1 in the IP samples. Probably not enough protein, or degraded.

1:5 C1q was an appropriate dilution.

Samples & Reagents

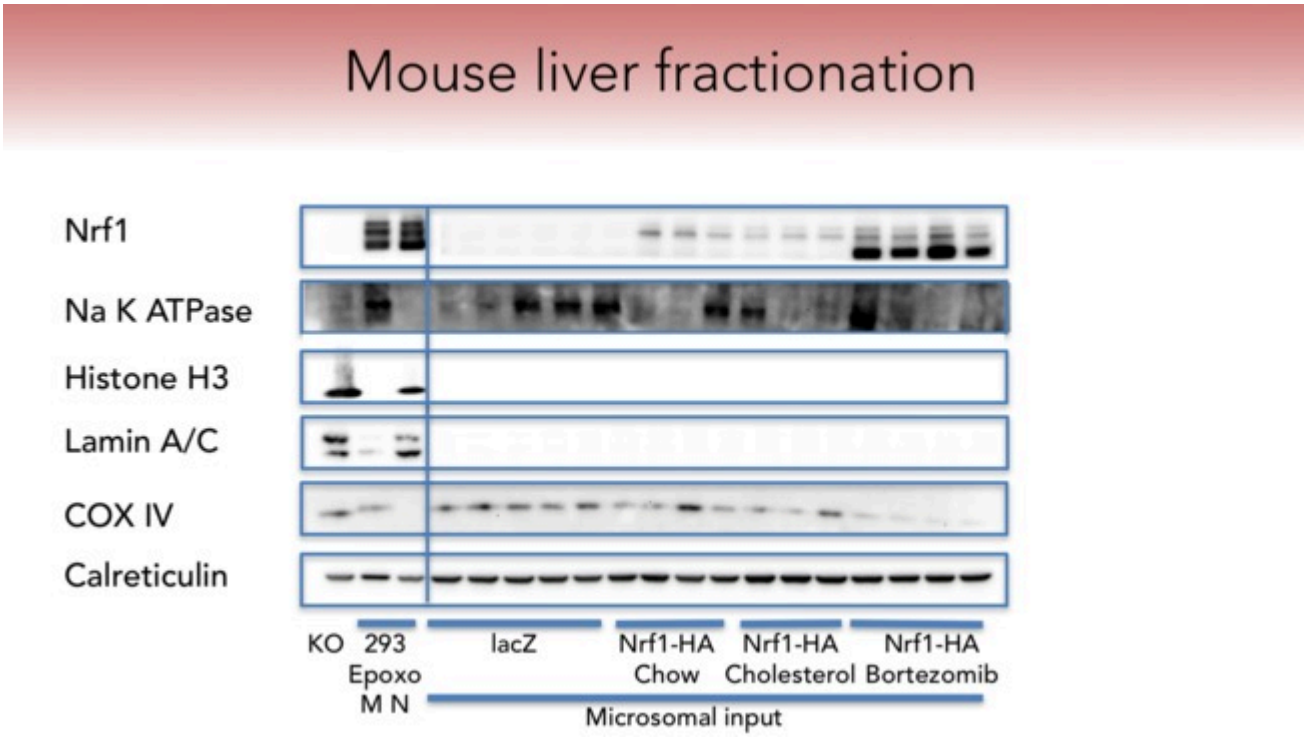
Antibodies (5)

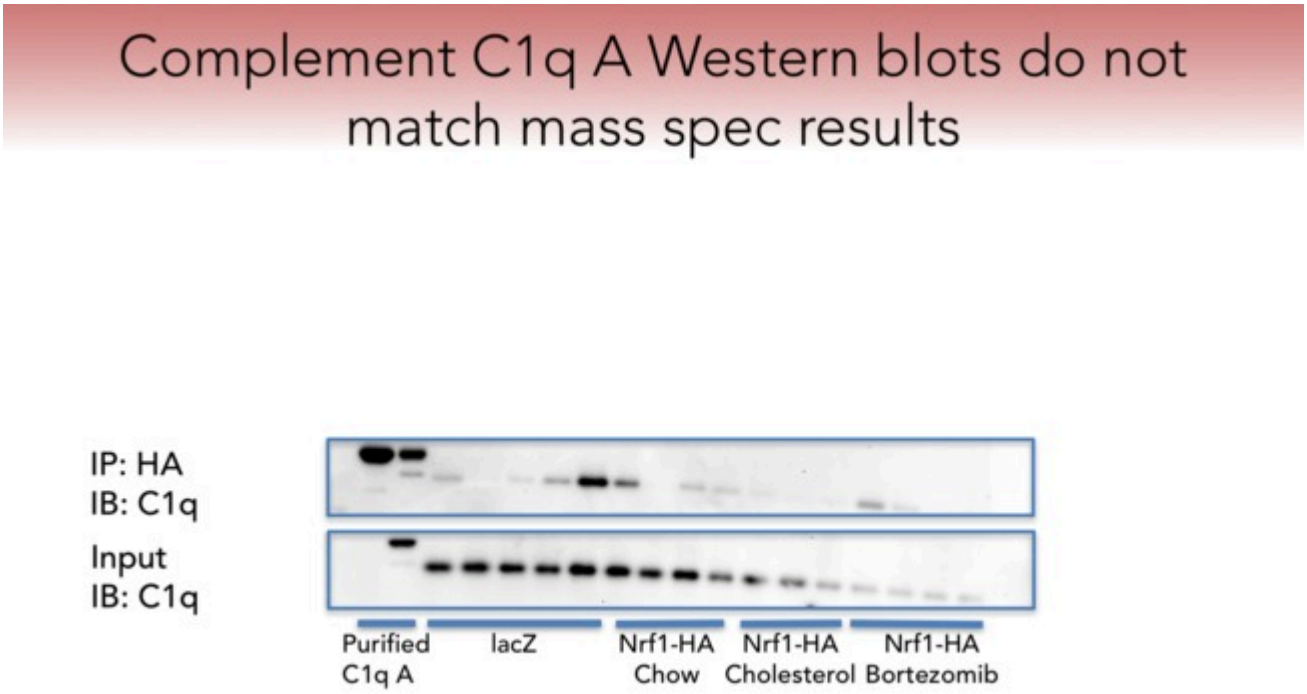
Name	Alternative name	Clonality	Raised In	Reacts with	Manufacturer	Remarks	Stocks
COX IV 4844	Cytochrome C oxidase subunit 4	Polyclonal	Rabbit	Human, Mouse, Rat, Monkey, Bovine	Cell Signaling Technology		
Na,K-ATPase 3010		Polyclonal	Rabbit	Human, Mouse, Rat, Monkey, Hamster, Zebrafish	Cell Signaling Technology		
TCF11/Nrf1 (D5B10) Rabbit mAb 8052	TCF11	Monoclonal	Rabbit	Human, Mouse, Monkey	Cell Signaling Technology		
C1q Antibody (JL-1)	C1q A	Monoclonal	Mouse	Mouse, Human, Rat	ThermoFisher		
Lamin A/C (4C11) Mouse mAb #4777					Cell Signaling Technology		

Consumables (1)

Name	Alternative name	Manufacturer	MW	Type	Remarks	Stocks
Pierce™ Fast Western Blot Kits, SuperSignal™ West Dura, Rabbit		ThermoFisher Scientific				

Western blot summary





Thermo Fisher Center for Multiplexed Proteomics

Jan 11, 2016 10:00 to Jan 11, 2016 11:00

Thermo Fisher Center for Multiplexed Proteomics

TCMP sample submission

I delivered the 18 samples to Ryan Kunz on dry ice on January 11, 2016 11 am.

NOTE: they actually did two 9 plexes (lacZ1-3 in run 1, lacZ 4-5 in run 2), instead of an 8 plex and a 10 plex as I specified in the order form. This change is reflected in the sample list below.

Harvard 33 digit billing #: 275.23493.8100.207027.264705.0001.29362

==Sample Information==

Species: Mouse

Type of analysis: Quant-IP

Number of Sample Sets (How many "plexes" are you submitting?): 2

==Plex A==

A1: lacZ 1

A2: lacZ 2

A3: lacZ 3

A4: HA control 1

A5: HA control 2

A6: HA cholesterol 1

A7: HA cholesterol 2

A8: HA bortezomib 1

A9: HA bortezomib 2

==Plex B==

B1: lacZ 4

B2: lacZ 5

B3: lacZ 6

B4: HA control 3

B5: HA control 4

B6: HA cholesterol 3

B7: HA cholesterol 4

B8: HA bortezomib 3

B9: HA bortezomib 4

TCMP results

Email from TCMP with results

Subject: TCMP gel band final report and data

Rodrigues, Rachel Beth <Rachel_Rodrigues@hms.harvard.edu> Fri, Mar 18, 2016 at 10:58 AM
To: "Smith, Brendon" <brsmith@hsph.harvard.edu>
Cc: "Kunz, Ryan Carl" <Ryan_Kunz@hms.harvard.edu>, "Gygi, Steven" <steven_gygi@hms.harvard.edu>
Hi Brendon,

Attached please find an experimental summary (powerpoint) and protein quant data (excel) for the two 10-plex experiments you submitted to the TCMP facility. The quant data is provided in both raw and normalized formats. We performed global normalization based on total summed TMT signal for each channel within each experiment but not between them. Please be in touch if you have any questions about the report or data.

Thank you,

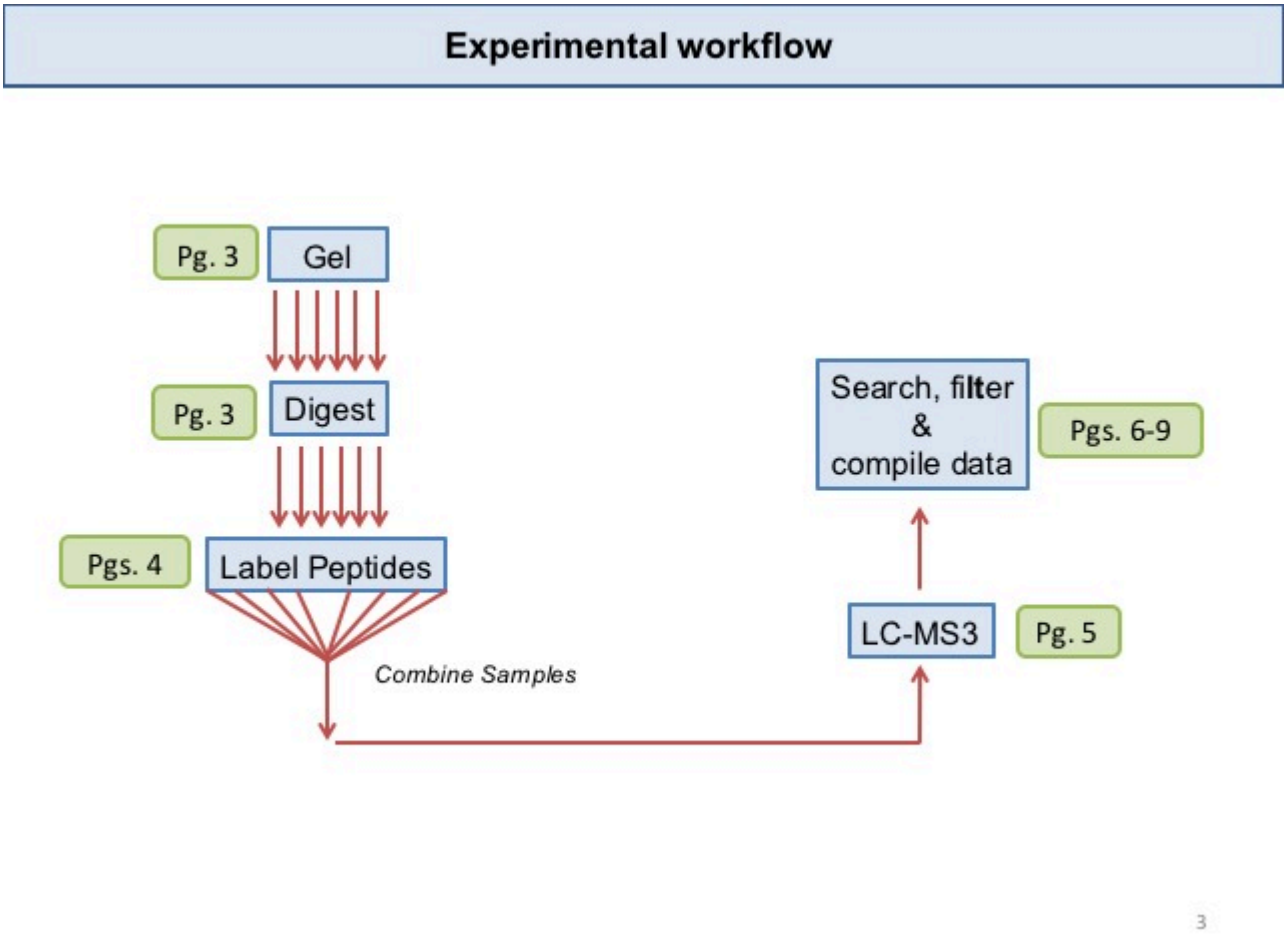
Rachel

_____

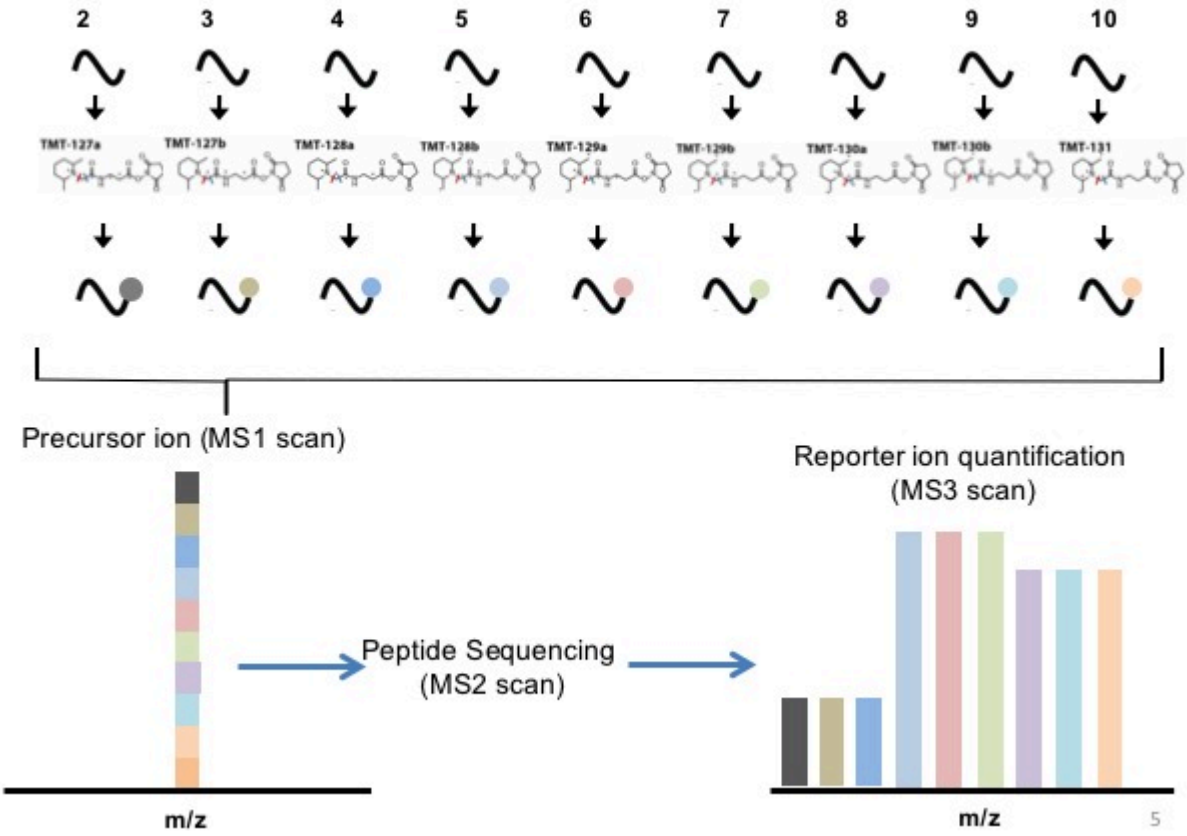
Rachel Rodrigues, PhD
Harvard Medical School
Boston, MA 02130

The TCMP provided an Excel workbook and a PowerPoint file describing the methods. See proteomics protocol for more details on methods.

Methods



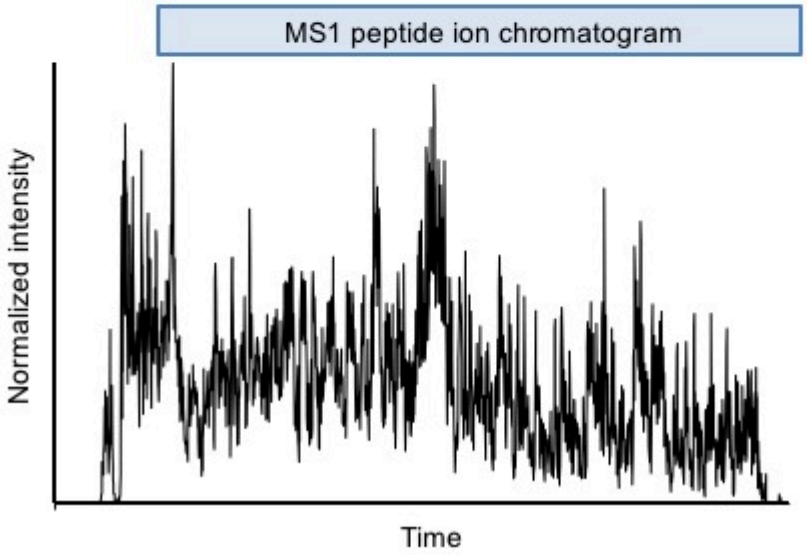
Tandem Mass Tags for Multiplexed Proteomics



5

MS analysis

- Peptides were analyzed on an Orbitrap Fusion mass spectrometer
- Peptides were separated using a gradient of 6 to 28% acetonitrile in 0.125% formic acid over 180 minutes.
- Peptides were detected (MS1) and quantified (MS3) in the Orbitrap
- Peptides were sequenced (MS2) in the ion trap.



6

Final report for Brendon Smith
TMT10 quantitative gel protein profiling experiments

THERMO FISHER CENTER FOR MULTIPLEXED PROTEOMICS

Date	2/22/2016
Submitted By	Brendon Smith
PI/Institute	G. Hotamisligil
Number of Samples	18
Experiment Type	decaplex (X2)
Species	mouse
TCMP ID	tc-d278g, tc-d279g

Sample Description	Sample Name - d278g	Tag	Channel (m/z)
A10 - mix	A1	T1	126.1275099
A9 - LacZ 3	A2	T2	127.1245742
A3 - HA control 1	A3	T3	127.1308484
A4 - HA control 2	A4	T4	128.1279205
A5 - HA cholesterol 1	A5	T5	128.1342117
A1 - LacZ 1	A6	T6	129.1312653
A2 - LacZ2	A7	T7	129.1375705
A6 - HA cholesterol 2	A8	T8	130.1346157
A7 - HA bortezomib 1	A9	T9	130.1409215
A8 - HA bortezomib 2	A10	T10	131.1379687

1

Final report for Brendon Smith
TMT10 quantitative gel protein profiling experiments

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Date	2/22/2016
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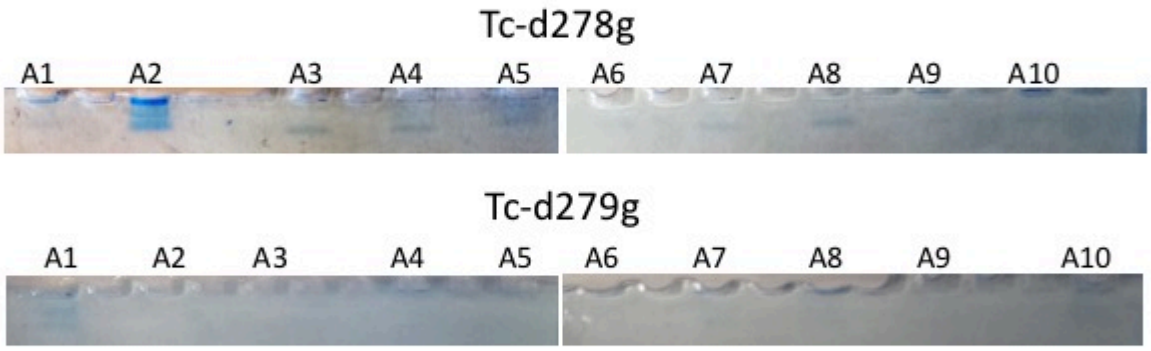
Number of Samples	18
Experiment Type	decaplex (X2)
Species	mouse
TCMP ID	tc-d278g, tc-d279g

Sample Description	Sample Name - d279g	Tag	Channel (m/z)
B3 - LacZ5	A1	T1	126.1275099
B8 - HA Cholesterol 4	A2	T2	127.1245742
B1 - Mix	A3	T3	127.1308484
B5 - HA control 3	A4	T4	128.1279205
B9 - HA bortezomib 3	A5	T5	128.1342117
B10 - HA bortezomib 4	A6	T6	129.1312653
B2 - LacZ4	A7	T7	129.1375705
B4 - LacZ6	A8	T8	130.1346157
B7 - HA cholesterol 3	A9	T9	130.1409215
B6 - HA control 4	A10	T10	131.1379687

2

Sample Preparation

Samples were submitted in gel loading buffer. 5µl of each sample was combined for the "mix" sample to be included in both experiments. 30µl of each sample was loaded into 10% Bis/Tris gels and run at 120V for 12 minutes. Gels were stained for 2 hour with Coomassie and destained overnight in water. No bands were visible in tc-d279g gels. Remaining sample was loaded into new gels, stained, and destained overnight.



Gel bands or approximate areas were cut out, destained, reduced, and alkylated. In-gel Trypsin digests were performed and peptides were extracted and labeled with TMT10 reagents. Labeling reactions were combined, cleaned, and dried down. Peptides were resuspended in 5% Acetonitrile, 5% formic acid and ½ of the sample was shot on an Orbitrap Fusion Mass spectrometer.

Data analysis

MS2 spectra were **searched using the SEQUEST algorithm** against a Uniprot composite database derived from the mouse proteome containing its reversed complement and known contaminants. Peptide spectral matches were **filtered to a 1% false discovery rate (FDR)** using the target-decoy strategy combined with linear discriminant analysis.

Proteins were quantified only from peptides with a summed SN threshold of >=200 and MS2 isolation specificity of 0.5.

Quantified proteins do not include contaminants or reverse hits.

Protein normalization

Protein Quant data is provided in two forms, raw signal to noise and globally normalized.

For normalized data, normalization factors are calculated based on the total summed signal to noise in each TMT channel.

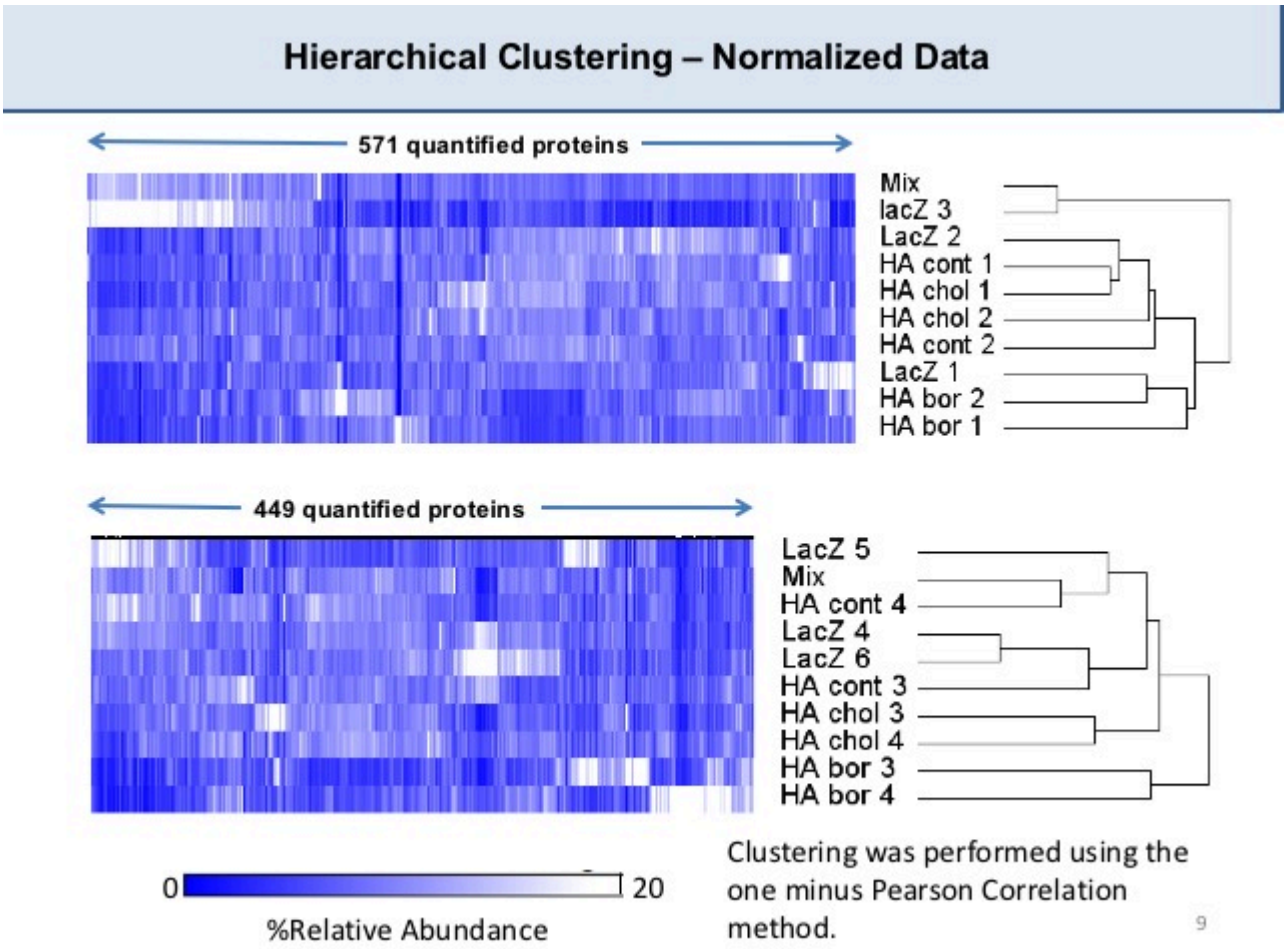
Normalization was performed within each gel, but not between them.

The large normalization factors for tc-d278g are due to the large difference in intensity between the second gel band compared to the other samples.

Normalization Factors										
	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
d178g	4.3	1	7.7	7	6.8	8.2	12.3	5.3	6.7	5.3
d179g	1	3.8	3.5	3.6	1.7	2.8	1.8	2.2	2.4	2.5

Hierarchical clustering

From 031616_Report_tc-d278g_tc-d279_Smith_B.pptx: "Clustering was performed using the one minus Pearson Correlation method." The report does not indicate if the relative abundance displayed in the heat map is relative (within each protein among samples) or global (color scale based on all abundance values in the dataset). I think it is probably relative.



Meeting 6/21/16

Meeting 6/21/16

Meeting with Ryan Kunz to discuss results, Tuesday, June 21, 2016

I called Ryan to set up the meeting after I had made progress with data analysis and come up with questions (see below for my data analysis).

Coomassie gel

Unfortunately, we are still limited by Coomassie stained gels when working with the TCMP. There simply isn't enough protein in our samples to yield clear Coomassie bands, and the staining was uneven. Although we load the same amount of total protein into our IP, we get dramatically different amounts of protein out. Ryan acknowledged this as a challenge of IPs. There isn't a great way of quantifying protein after the IP, and we would be wasting precious sample in the protein quant assay. He showed me an example of a gel he ran recently with IP samples. There were consistently bright blue bands in all the lanes, so I know it's possible. I need better reagents to get a more concentrated protein extract.

Dataset

Proteins were either quantified in all of the samples, or none. This doesn't make sense to me, because we had lacZ control mice that didn't even express Nrf1-HA. We demonstrated that Nrf1 was not present in the IP eluates (see Scott's Western blots). If we didn't pull down Nrf1 in the lacZ samples, why are all the same proteins present? Proteins in the dataset are presumably either pulled down with Nrf1, or bound nonspecifically to the HA agarose resin. Does the presence of these proteins in the lacZ samples indicate that the entire dataset is background?

?A signal of 0 across all the channels indicates that there were no peptides corresponding to the protein detected in the run. Sometimes, a few peptides will be detected in one run, and none in another run. When peptides corresponding to a protein are detected, there will be signal across all the TMT channels. There is always a small amount of background signal, but samples in which the protein is present at higher relative abundance will have substantially stronger signal. Ryan did not provide a quantitative cutoff for this, but drew a bar graph to illustrate the difference in magnitude.

?It may be better to use total summed signal to noise instead or normalized relative abundance in this case. Normalized relative abundance assumes the same amount of protein in all samples, when we would expect lacZ to have less protein because we didn't pull down Nrf1.

?Ryan agreed with my concerns about the samples and dataset.

Why didn't we detect Nrf1 in the dataset? Is it an enzyme digestion problem?

?He also noticed this. He wasn't sure.

Hierarchical clustering

Rachel performed this using the free Broad Institute GENE-E program. Ryan also uses Gene cluster 3.0 with Java Tree View. Perseus from Matthias Mann, and R, can also do hierarchical clustering. The samples would be expected to cluster by group, and the mix standard should have a consistent blue shade throughout. However, the samples don't cluster by group.

Comparison with Takahisa Nakamura's project (*Cell Reports* 2015)

?Taka's analysis was label-free, which presents its own challenges. Each sample was an independent run on the mass spectrometer. Sometimes a protein would be present in one replicate/sample and not in another.

How do I batch analyze the dataset for statistical significance? What sort of adjustment do I make for multiple comparisons?

?They don't provide advanced data analysis services, because Ryan doesn't have the expertise for it.

Outcome

He will do 2 more plexes for us free of charge.

Data analysis

Data analysis

I developed a custom data analysis pipeline to process the Excel file output from TCMP. The sections below correspond to the sections in my proteomics protocol, BWS proteomics protocol.docx.

Dataset management in Excel

Normalization

I normalized to the mix standard to compare values across the two runs by dividing the normalized relative abundance or total summed signal to noise value for each sample and protein by the corresponding value for the mix standard from that run.

Filtration

I filtered the hit list to include only proteins with ≥2 peptides in each run (252 included, 381 excluded).

I am not sure if the number provided by TCMP is unique or total peptides.

I did not receive information on % coverage by the detected peptides, so I cannot use coverage as an additional criterion.

Including only proteins with multiple corresponding peptides means the identification of the protein itself is confident, because multiple peptides corresponding to it have been identified, and that the identification of the protein is repeatable, because ≥2 peptides were detected in each of the 2 mass spectrometry runs. For example, Hist2h2be is the most increased in cholesterol animals on average, but

only one peptide was detected in each run. Similarly, Igtp was the second most increased in cholesterol animals. The increase was primarily in run 2, in which only one peptide was detected. Filtering can easily be accomplished in Excel by clicking the dropdown box in the bottom right of the column header (worksheets from TCMP are in pivot table format), and selecting filter by greater than 1. Filtering the dataset with this criterion left a set of 252 proteins.

Transformation and background subtraction

∆**Cholesterol**=log2((HA chol/mix)/(HA cont/mix))-log2((HA cont/mix)/(lacZ/mix))

Background subtraction was not effective in these samples because protein abundance was greater in lacZ than HA in many cases. Proteins with an HA fold change (HA cont/mix)/(lacZ/mix)<1 could be excluded (meaning that the proteins were higher abundance in lacZ samples, without the tag we were pulling down). I kept them in for this analysis.

Statistical analysis

A fold change of 1.5 (log2=0.58) is commonly used as a threshold for biological significance (Blagoev Nature Biotechnology 2004, Margolin PLoS One 2009).

33 proteins had ∆Cholesterol>0.58.

ANOVA performed in R for the 33 hits with ∆Cholesterol >0.58. I performed ANOVA in R on the untransformed total summed signal to noise/mix values for all the hits. See attached R code.

Hit list

See excel workbook for full data

		Cholesterol fold change			**HA fold change**	
Protein ID (Uniprot)	Gene Symbol	(HA chol/mix)/(HA cont/mix)	log2((HA chol/mix)/(HA cont/mix))	t test	(HA cont/mix)/(lacZ/mix)	log2((HA cont/mix)/(lacZ/mix)
sp|P70124|SPB5_MOUSE	Serpinb5	1.07	0.10	0.74	0.39	-1.3
sp|O70456|1433S_MOUSE	Sfn	1.33	0.41	0.40	0.49	-1.0
sp|P17182|ENOA_MOUSE	Eno1	1.56	0.64	0.23	0.63	-0.6

sp|Q02105|C1QC_MOUSE	**C1qc**	**2.39**	**1.26**	**0.01**	**1.06**	**0.**
sp|P98086|C1QA_MOUSE	**C1qa**	**2.25**	**1.17**	**0.01**	**1.02**	**0.**
sp|P10649|GSTM1_MOUSE	Gstm1	1.15	0.20	0.65	0.55	-0.
sp|P22892|AP1G1_MOUSE	Ap1g1	1.52	0.60	0.63	0.76	-0.
sp|P14106|C1QB_MOUSE	**C1qb**	**2.08**	**1.06**	**0.02**	**1.05**	**0.**
sp|Q8BTM8|FLNA_MOUSE	Flna	1.57	0.65	0.01	0.79	-0.
sp|P00186|CP1A2_MOUSE	Cyp1a2	1.38	0.46	0.17	0.74	-0.
sp|P11352|GPX1_MOUSE	Gpx1	1.30	0.38	0.20	0.71	-0.
sp|P52480|KPYM_MOUSE	Pkm	1.42	0.51	0.15	0.79	-0.
sp|P20852|CP2A5_MOUSE	Cyp2a5	1.58	0.66	0.01	0.93	-0.
sp|Q91X77|CY250_MOUSE	Cyp2c50	1.25	0.33	0.38	0.74	-0.
sp|P59999|ARPC4_MOUSE	Arpc4	1.12	0.17	0.72	0.67	-0.
sp|Q8VDD5|MYH9_MOUSE	Myh9	1.11	0.15	0.83	0.67	-0.
sp|P05064|ALDOA_MOUSE	Aldoa	1.08	0.11	0.65	0.65	-0.
sp|P17742|PPIA_MOUSE	Ppia	1.22	0.29	0.44	0.74	-0.
sp|Q8CIM7|CP2DQ_MOUSE	Cyp2d26	1.21	0.27	0.24	0.74	-0.
sp|P20029|GRP78_MOUSE	Hspa5	1.33	0.41	0.11	0.81	-0.
sp|P27773|PDIA3_MOUSE	Pdia3	1.46	0.54	0.13	0.89	-0.
tr|E9Q616|E9Q616_MOUSE	Ahnak	1.15	0.20	0.57	0.70	-0.
sp|Q8QZR3|EST2A_MOUSE	Ces2a	1.13	0.18	0.43	0.70	-0.
sp|P11589|MUP2_MOUSE	Mup2	0.96	-0.06	0.91	0.61	-0.
sp|P60710|ACTB_MOUSE	Actb	1.02	0.02	0.96	0.65	-0.

sp|Q60605|MYL6_MOUSE	Myl6	1.07	0.10	0.89	0.69	-0.5
sp|P97872|FMO5_MOUSE	Fmo5	1.40	0.49	0.00	0.90	-0.
sp|P56654|CP237_MOUSE	Cyp2c37	1.18	0.24	0.35	0.76	-0.
sp|P57780|ACTN4_MOUSE	Actn4	1.04	0.05	0.87	0.67	-0.5
sp|P68369|TBA1A_MOUSE	Tuba1a	1.05	0.08	0.78	0.69	-0.5
sp|P68368|TBA4A_MOUSE	Tuba4a	0.99	-0.02	0.96	0.65	-0.
sp|O08807|PRDX4_MOUSE	Prdx4	1.42	0.50	0.12	0.94	-0.
sp|Q4LDG0|S27A5_MOUSE	Slc27a5	1.27	0.34	0.51	0.85	-0.

Other proteins of interest

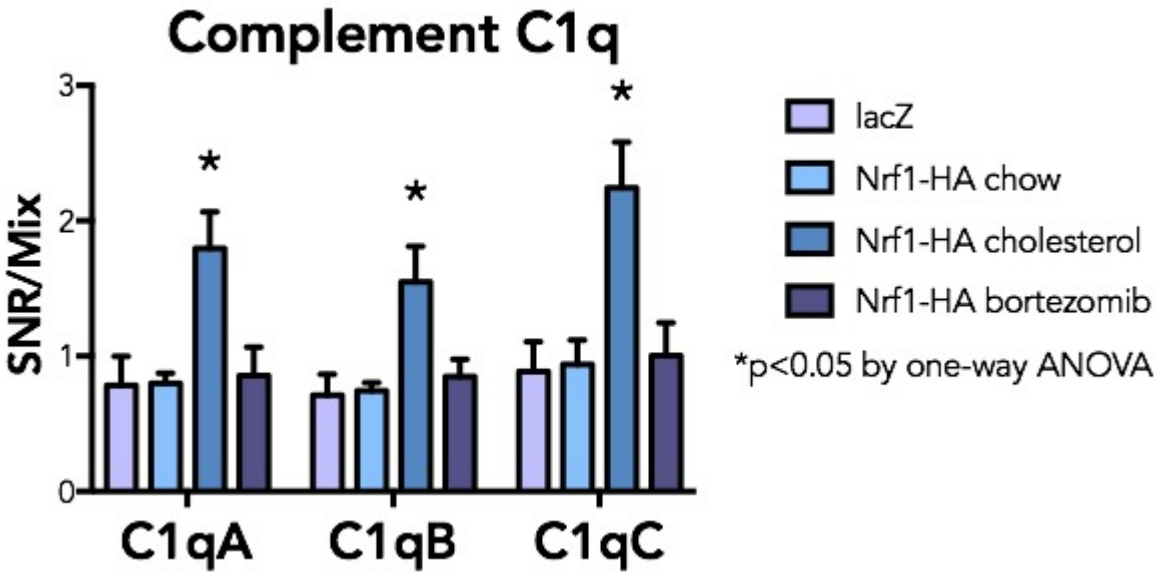
- The second most abundant protein across all the samples was Cltc (Clathrin heavy chain 1). Clint1 (clathrin interactor 1), Clta (Clathrin light chain A), COPA (Coatomer subunit alpha), Snx9 (Sorting nexin-9), Mvp (major vault protein), Sec16a were also present. Note that the endocytosis-related proteins like Clathrin were mostly present in the lacZ 3 sample.
- The most abundant proteins, like C1qC and Clathrin, were about 20% normalized relative abundance.
- We also see COP A (coatomer protein A) in the analysis from March 11 (20160311 57219 band 4 proteins.xlsx).
- VPS8 (Vacuolar protein sorting-associated protein 8 homolog) 20160311 57221 band 6 proteins.xlsx
- SEC24C, ESYT1: 3/16 band 1
- ER markers: Calreticulin, MTTP, VCP, ASGR, GRP78
- E3 ubiquitin ligases: Trim21
- Pro-Lamin A/C: This does not necessarily indicate nuclear contamination of the microsomal fraction, because it is pro-lamin, so probably being produced in the ER before transport to the nucleus.
- We did not see some expected interactions such as SCAP, Insig or Hrd1
- **We did not detect Nrf1. Alex Bartelt did not detect Nrf1 in his TCMP analysis either. It may be an enzyme digestion problem.**

C1q

The proteins highest in relative abundance in the Nrf1-HA cholesterol-diet samples vs. the mix standard were Complement C1q A, B, and C.

I performed one-way ANOVA analyses in R and Prism for Complement C1q A, B, and C. Nrf1-HA cholesterol significantly higher than all other groups, except C1qB p=0.06 vs HA bort.

C1q figure produced in Prism. PNG export image 2"x1" 300 dpi.

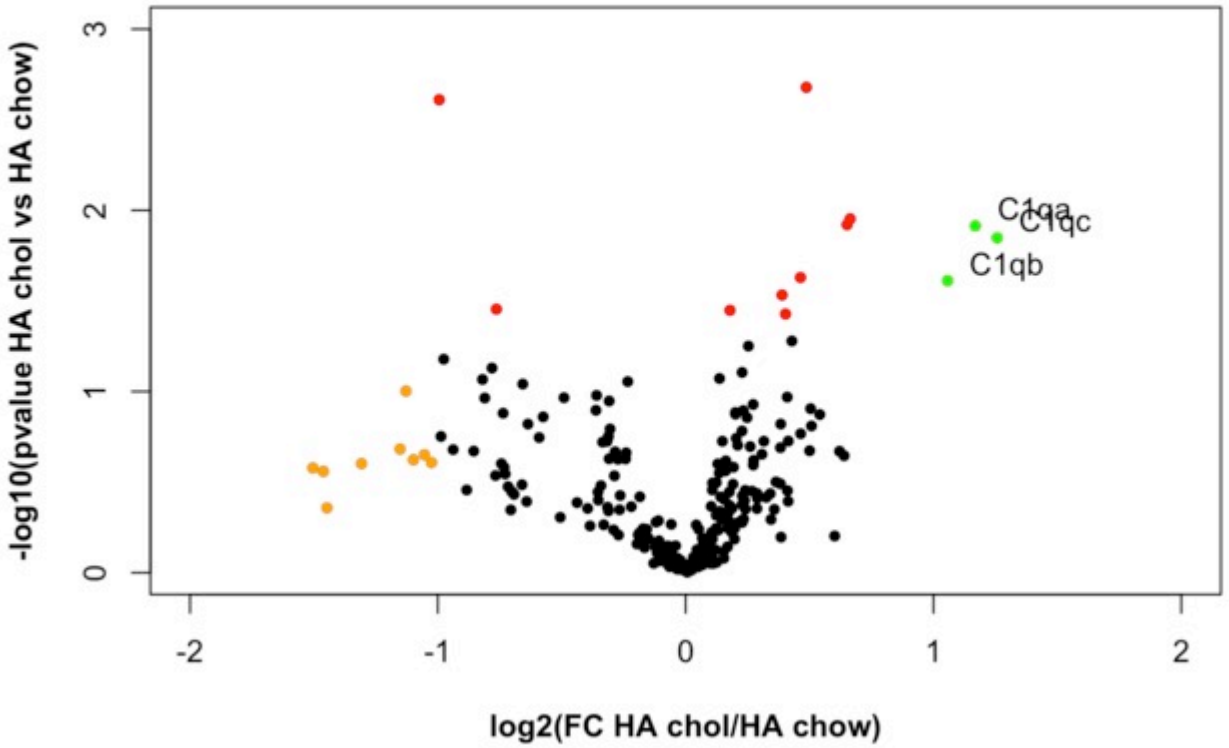


C1q may simply be increased in cholesterol-fed liver, and may not be specifically interacting with Nrf1. Should check C1q levels in inputs by Western blot.

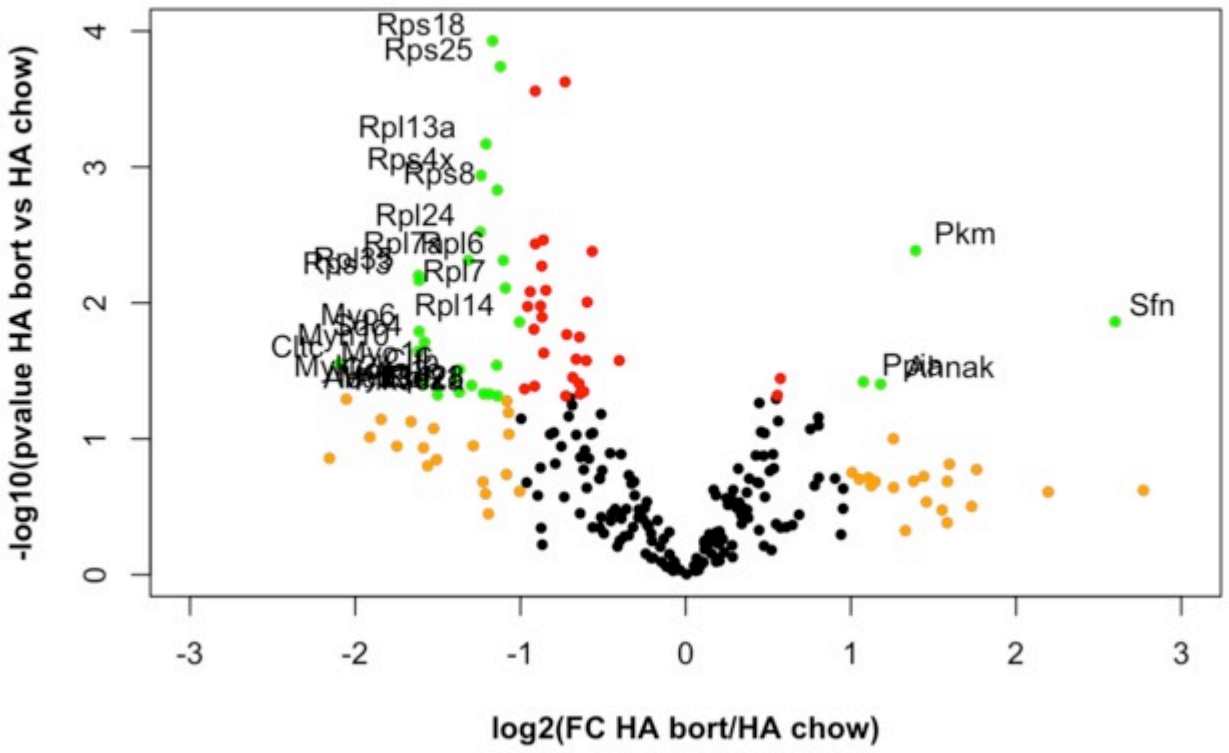
Plots

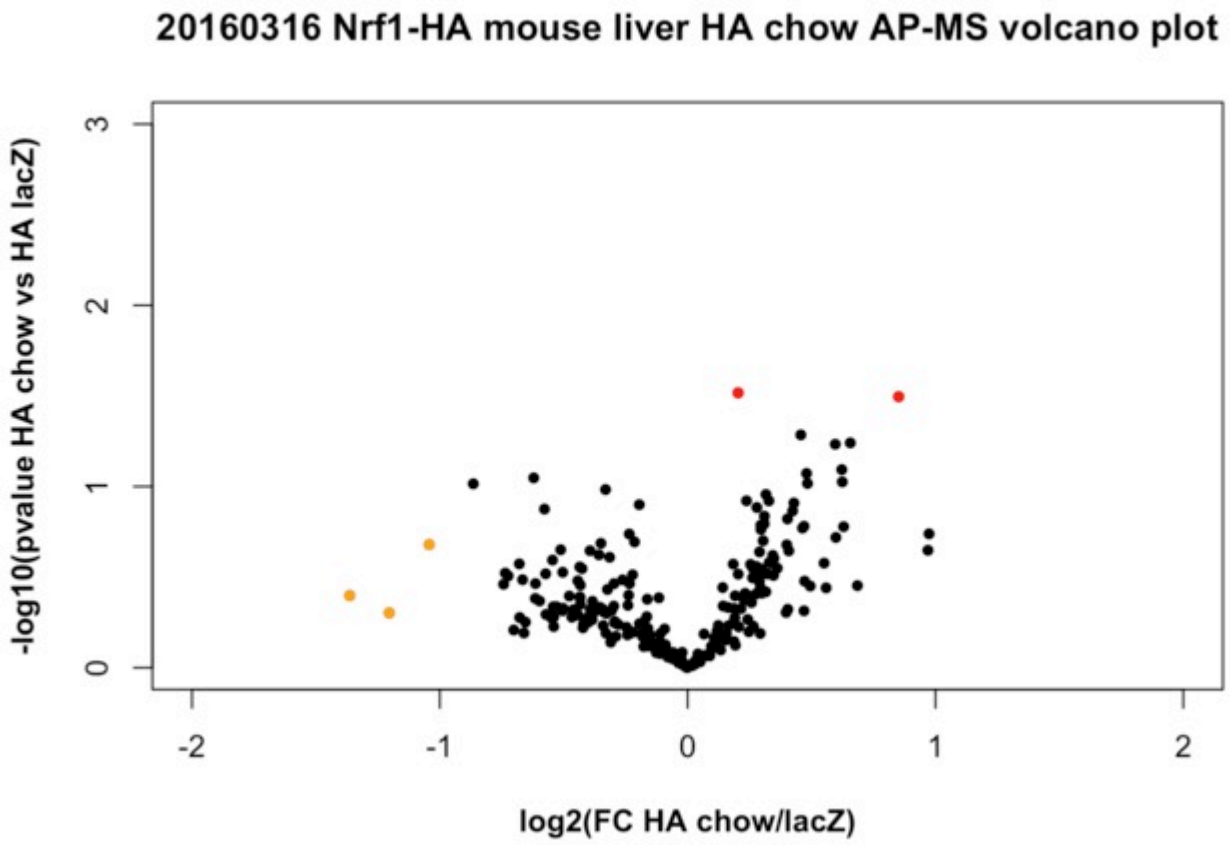
Produced in R (see attached code). Colored points on volcano plots: red if pvalue<0.05, orange if log2FC>1, green if both

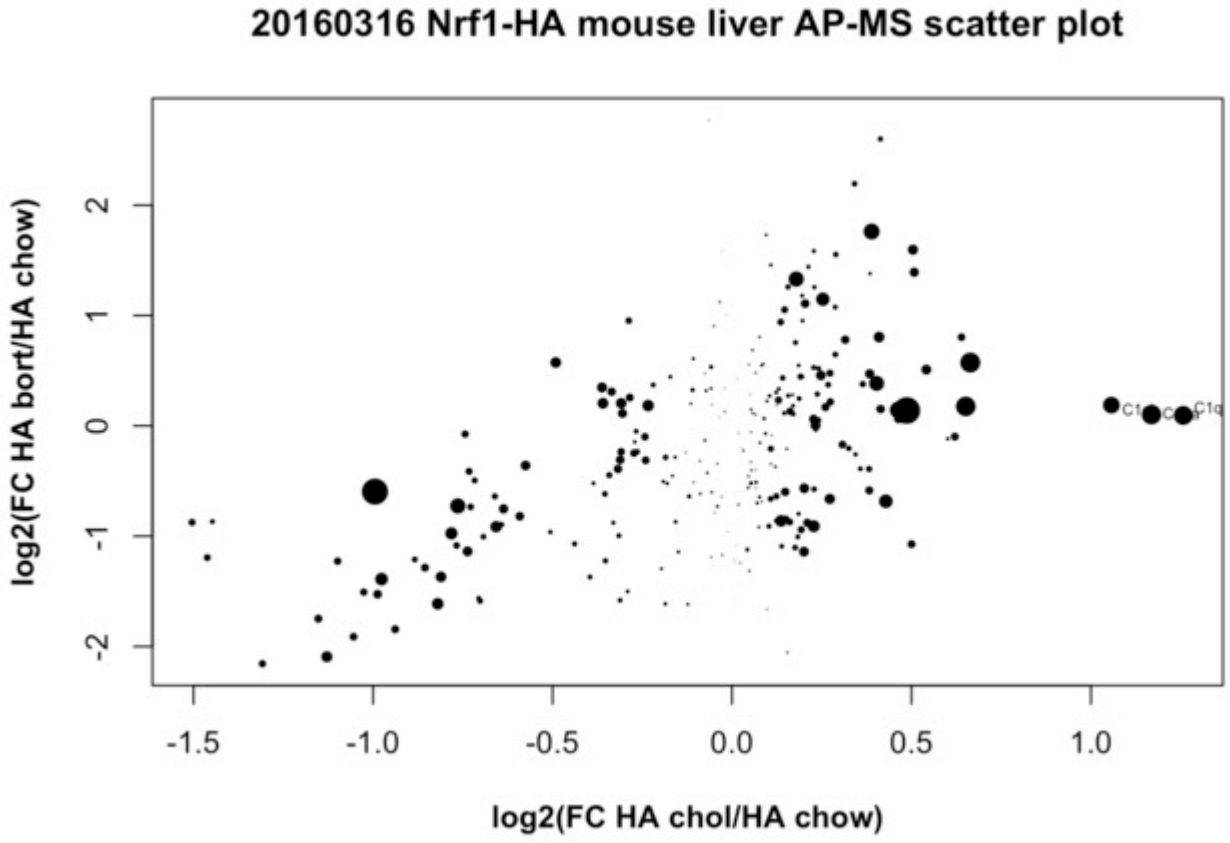
20160316 Nrf1-HA mouse liver cholesterol AP-MS volcano plot



20160316 Nrf1-HA mouse liver bortezomib AP-MS volcano plot







This scatter plot is similar to the plot Matt Lynes and Alex Bartelt generated for the Nature Nrf1 brown fat paper. Dot size depicts

statistical significance for ttest of cholesterol vs chow. I think the volcano plots are more useful.

Hierarchical clustering in Morpheus

See BWS proteomics protocol.docx. Morpheus and GENE-E are free heat map and cluster analysis tools from the Broad. GENE-E is no longer supported, so Morpheus should be used.

Data input: Paste log2 transformed data into a new Excel worksheet, using "paste values" to omit formulas and rows that have been filtered out (such as proteins with ≤2 peptides). Retain just the sample names as column headers, gene names in left column, and log2 data. **Dataset was filtered to include 252 proteins with ≥2 peptides as described above, and sorted by average cholesterol/ average control ratio.**

Heat map: View ->Preferences->color scheme->global to display abundance from dark blue (0) to white (highest)

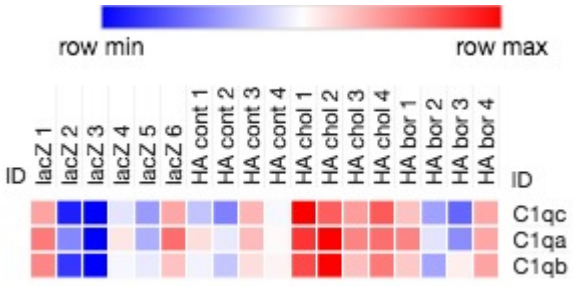
Hierarchical clustering: cluster columns, one minus Pearson correlation, do not group columns.

Relative color compares among samples within a given protein, global calculates a single color intensity scale based on all values in the dataset.

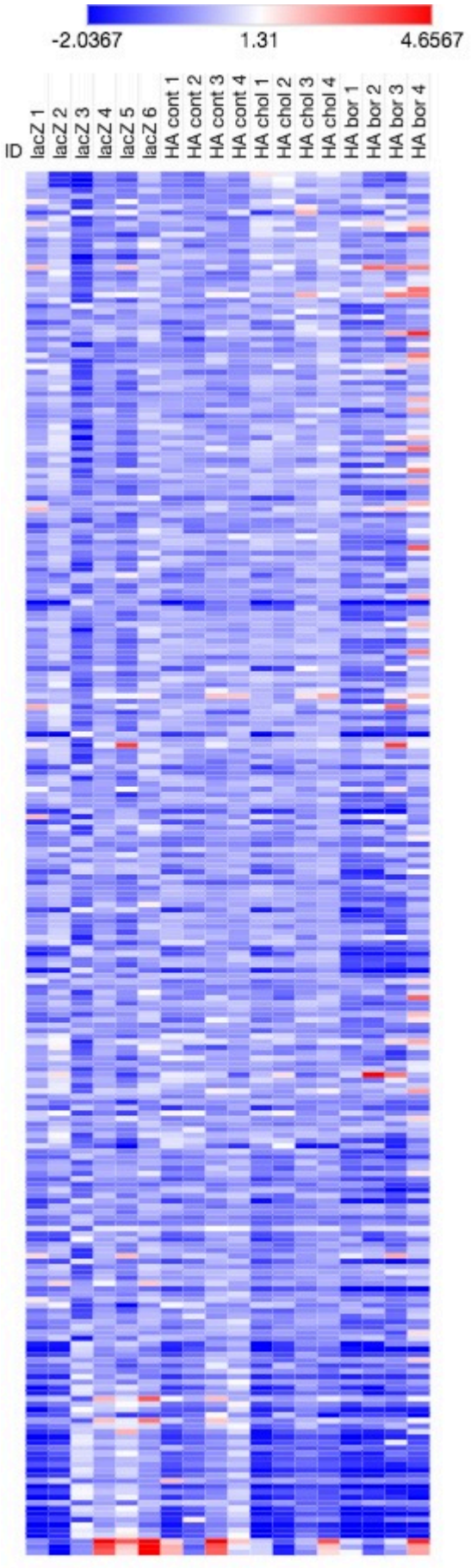
Note how lacZ3 separates from the other samples due to high levels of select proteins, predominantly associated with the clathrin endocytosis machinery.
Also note that the samples do not cluster appropriately by group.

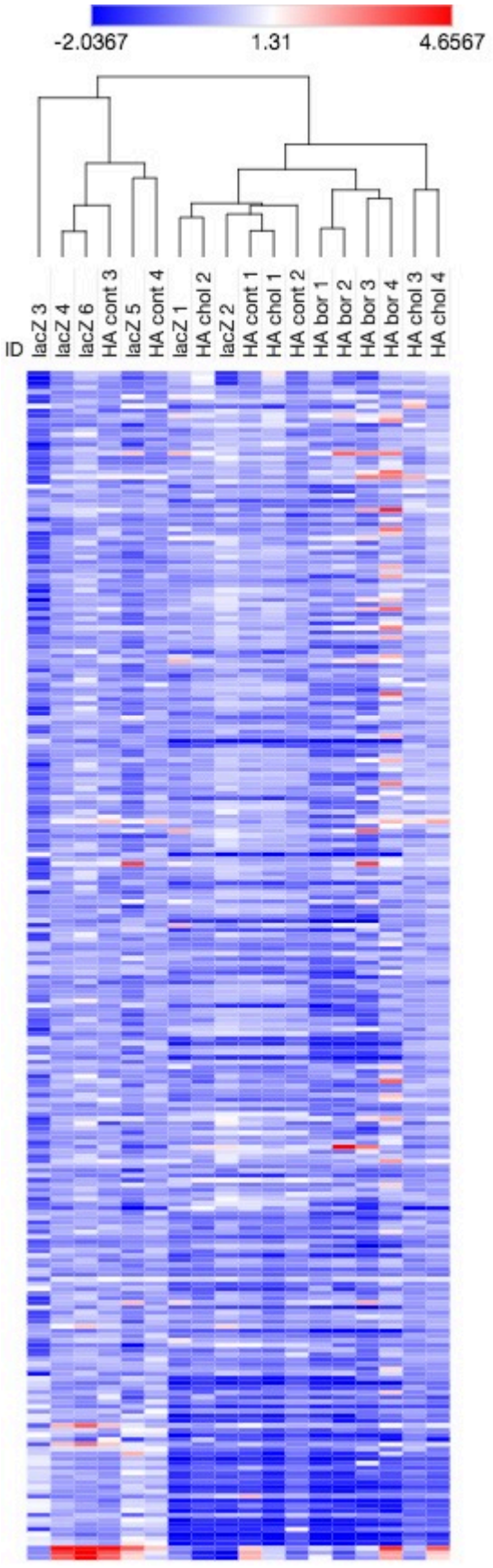
Filtered Total Summed Signal to Noise/Mix

C1q only



Full dataset


-2.0367
1.31
4.6567
ID
lacZ 1
lacZ 2
lacZ 3
lacZ 4
lacZ 5
lacZ 6
HA cont 1
HA cont 2
HA cont 3
HA cont 4
HA chol 1
HA chol 2
HA chol 3
HA chol 4
HA bor 1
HA bor 2
HA bor 3
HA bor 4


-2.0367
1.31
4.6567
ID
lacZ 3
lacZ 4
lacZ 6
HA cont 3
lacZ 5
HA cont 4
lacZ 1
HA chol 2
lacZ 2
HA cont 1
HA chol 1
HA cont 2
HA bor 1
HA bor 2
HA bor 3
HA bor 4
HA chol 3
HA chol 4

DAVID pathway analysis

david-d.ncifcrf.gov

Methods

See pathway analysis protocol for full details on methods.

Functional annotation tool

Step 1: Paste in the names from the “Gene symbol” column of the TCMP spreadsheet

Step 2: select identifier: OFFICIAL_GENE_SYMBOL

Step 3: List Type: Gene List

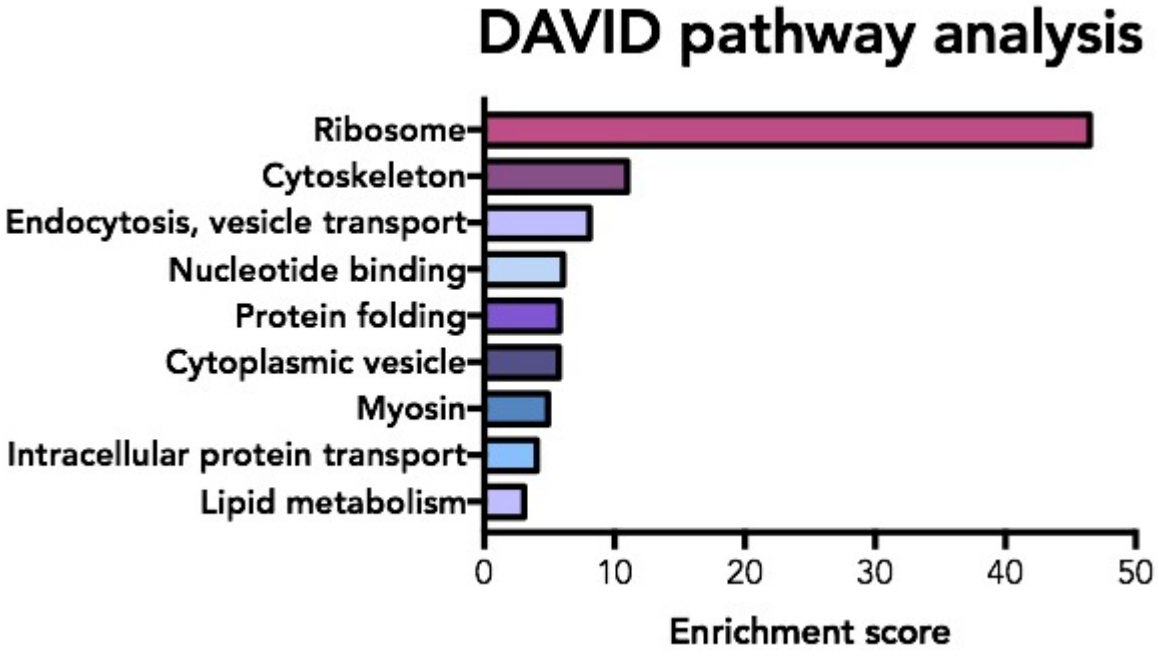
Make horizontal bar graph in Prism.

Results

Ribosome pathways are strongly enriched because ribosome proteins have a strong affinity for magnetic beads.

- See figure 1 in Kazak L, Chouchani ET, Jedrychowski MP, Erickson BK, Shinoda K, Cohen P, Vetrivelan R, Lu GZ, Laznik-Bogoslavski D, Hasenfuss SC, et al. A Creatine-Driven Substrate Cycle Enhances Energy Expenditure and Thermogenesis in Beige Fat. Cell 163:643–55 (2015). They did not enrich with magnetic beads in this analysis, but ribosome proteins may also be generally abundant in the cell.
- Investigation of "Bead-ome": Keilhauer EC, Hein MY, Mann M. Accurate protein complex retrieval by affinity enrichment mass spectrometry (AE-MS) rather than affinity purification mass spectrometry (AP-MS). Mol. Cell. Proteomics 14:120–35 (2015).

Vesicle transport and endocytosis pathways are enriched in the dataset. As seen in the heat maps above, this is probably due mostly to the lacZ 3 sample.



I am unable to compare pathways differentially enriched among the samples using DAVID. DAVID only accepts gene lists as input, and in the TCMP analysis, if a TMT signal is detected, the protein is quantified in all the samples. I could select only proteins that are significantly enriched in a given group as inputs for DAVID, but I'm not sure how to batch-analyze the dataset for statistical significance yet.

DAVID also assumes that the whole cell is represented. This is just the liver microsomal fraction, so significance calculations for these pathways may be inflated.

In the future, with an improved dataset, I will plan to do a deeper pathway analysis using MetaCore and Ingenuity.

Discussion

Wnt signaling basic info

Wnt secretion: Wnt must first be secreted. It may pass through the Golgi and then get loaded onto a lipoprotein for secretion.
See Clevers H. Wnt/beta-Catenin Signaling in Development and Disease. Cell 127:469–480 (2006).
"Figure 1. Wnt Secretion. To be secreted, Wnt proteins in the endoplasmic reticulum (ER) need to be palmitoylated by the action of Porcupine. Wnt proteins also re- quire Wntless (Wls/Evi) in order to be routed to the outside of the cell. Loading onto lipoprotein particles may occur in a dedicated endo/ex- ocytic compartment. The retromer complex may shuttle Wls between the Golgi and the endo/exocytic compartment."
It could also be ER to endosome cholesterol transport.

Annexin A1 Tethers Membrane Contact Sites that Mediate ER to Endosome Cholesterol Transport

Emily R. Eden, Elena Sanchez-Heras, Anna Tsapara, Andrzej Sobota, Tim P. Levine, Clare E. Futter

Canonical: Key result is nuclear translocation of beta catenin

Complement

See Janeway's Immunology

C1q

From InterPro: “C1q is a subunit of the C1 enzyme complex that activates the serum complement system.”

“Complement C1q Activates Canonical Wnt Signaling and Promotes Aging-Related Phenotypes,” 10.1016/j.cell.2012.03.047

C1q is a complex that includes C1qa.

Could Nrf1 be interacting with this protein in the ER, before it is targeted to plasma membrane? Nrf1 could be regulating synthesis of lipoproteins in the ER. This could also place Nrf1 in a metaflammation/lipid metabolism axis. See Bugno 5.5 for inflammation TNF alpha

NEJM complement articles, search mendeley and downloads for complement

Serum C1q comes from macrophages.

Lipoprotein metabolism

Is there a connection between Wnt signaling and lipoprotein metabolism? -> Yes.

- **LRP6 was previously identified as a gene that is a “functional regulator of cellular cholesterol homeostasis.”** Interestingly, this was in the setting of cholesterol depletion in HeLa cells. We did not pull down TMEM97, but we did get TMEM 205, 214, 33.
- **LRP5/6 is cleaved as part of Wnt signaling. TGF-beta family members like GDF11 and myostatin are proteolytically processed. Nrf1 is also proteolytically processed.**
- Bartz F, Kern L, Erz D, Zhu M, Gilbert D, Meinhof T, Wirkner U, Erfle H, Muckenthaler M, Pepperkok R, et al. Identification of Cholesterol-Regulating Genes by Targeted RNAi Screening. Cell Metab. 10:63–75 (2009).
- Sheng: cholesterol activates Wnt signaling. Sheng R, Kim H, Lee H, Xin Y, Chen Y, Tian W, Cui Y, Choi J-C, Doh J, Han J-K, et al. Cholesterol selectively activates canonical Wnt signalling over non-canonical Wnt signalling. Nat. Commun. 5:4393 (2014).
- Go G-W, Srivastava R, Hernandez-Ono A, Gang G, Smith SB, Booth CJ, Ginsberg HN, Mani A. The combined hyperlipidemia caused by impaired Wnt-LRP6 signaling is reversed by Wnt3a rescue. Cell Metab. 19:209–220 (2014).
- Check Cravatt papers for Wnt proteins
- Niphakis MJ, Lum KM, Cognetta AB, Correia BE, Ichu T-A, Olucha J, Brown SJ, Kundu S, Piscitelli F, Rosen H, et al. A Global Map of Lipid-Binding Proteins and Their Ligandability in Cells. Cell 161:1668–1680 (2015).
- Köberlin MS, Snijder B, Heinz LX, Baumann CL, Fauster A, Vladimer GI, Gavin A-C, Superti-Furga G. A Conserved Circular Network of Coregulated Lipids Modulates Innate Immune Responses. Cell 162:170–183 (2015).
- Ouchi N, Higuchi A, Ohashi K, Oshima Y, Gokce N, Shibata R, Akasaki Y, Shimono A, Walsh K. Sfrp5 is an anti-inflammatory adipokine that modulates metabolic dysfunction in obesity. Science 329:454–457 (2010).
- *Apoer2/Lrp8* and *Vldlr* are putative Nrf1 target genes (Bugno table 4, Tsujita MCB 2014).

- How dows VLDLR relate to LRP for VLDL uptake? Is one more relevant in mice? Is the LRP that is cleaved as part of Wnt signaling related to the LRP that is involved in VLDL uptake? LRP8 (?) is the VLDLR?
- Vldlr and Apoer2 are also neuronal.
- http://www.ncbi.nlm.nih.gov/m/pubmed/24755222/
- LRP6 and 8 mutations linked to human disease.
- http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4125478/#R12
- http://www.ncbi.nlm.nih.gov/pubmed/8626535
- http://www.ncbi.nlm.nih.gov/pubmed/17314095
- http://www.ncbi.nlm.nih.gov/pubmed/22170052
- **Increasing VLDL/IDL uptake would be highly therapeutically desirable. IF Nrf1 regulates VLDL endocytosis, this could be major new therapeutic target, because it could prevent conversion to LDL.**
- Geerling JJ, Boon MR, van der Zon GC, van den Berg SAA, van den Hoek AM, Lombès M, Princen HMG, Havekes LM, Rensen PCN, Guigas B. Metformin lowers plasma triglycerides by promoting VLDL-triglyceride clearance by brown adipose tissue in mice. Diabetes 63:880–891 (2014).
- How did Brown Goldstein determine that Srebp goes to golgi? ATF6 also goes to Golgi with COPII coat. See Hetz C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nat. Rev. Mol. Cell Biol. 13:89–102 (2012).
- Why didn't Nrf1 come up in the macrophage sterol responsive network? -> These are only secreted proteins. Becker L, Gharib SA, Irwin AD, Wijsman E, Vaisar T, Oram JF, Heinecke JW. A macrophage sterol-responsive network linked to atherogenesis. Cell Metab. 11:125–135 (2010).

Results

Conclusion

Conclusion

In this experiment, we infected mice with lacZ control or Nrf1-HA adenovirus, fed the mice chow or Paigen cholesterol diet, with an additional Bortezomib treatment group, isolated liver microsomal fractions, performed an affinity purification for the HA tag, and submitted the samples for quantitative mass spectrometry analysis. The only proteins significantly increased in relative abundance in cholesterol-fed mice were Complement C1q A, B, and C. Pyruvate Kinase M2 and 14-3-3 sigma were increased in Bortezomib-treated mice, and 63 other proteins were significantly downregulated.

Major issues with the dataset

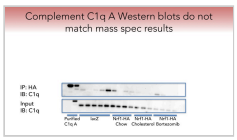
- **Presence of the same proteins in lacZ vs. Nrf1-HA mice, despite our HA IP, suggesting that the proteins identified are**

just background signal.

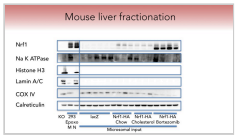
- **Animals do not cluster by treatment group**
- **Protein levels in the IP eluates are low and inconsistent. We need enough protein to see clear bands with Coomassie/ colloidal blue staining. This will require a more effective pulldown system.**
    - **Fractionation was not the limitation here. We did obtain adequate protein in our liver microsomal fractions. The fractions were not pure.**
    - **The HA IP is not effective.**
    - **We should perform on-bead digestion in the future so we don't have to elute.**
    - **Gel bands must be clearly visible with colloidal blue staining. Silver stained bands should show up within the recommended 2-3 minutes and should not take 10-15 minutes to visualize as they did here.**

Ryan will re-do the analysis for us free of charge, and I don't consider it wasted time, because I approached the dataset as an opportunity to learn proteomics data analysis. It is clear that we need to re-do the experiment with a better pulldown system.

Experiment Attached Images



2016-11-11_mouse_liver_IP_re-do_C1q_A.png



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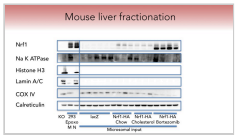

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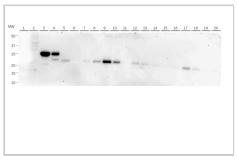

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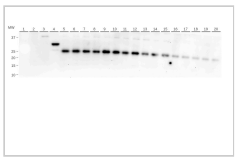

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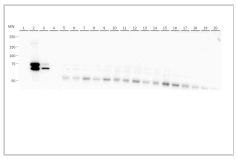

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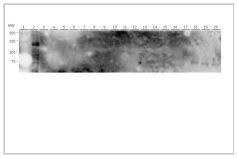

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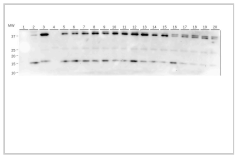

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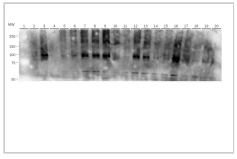

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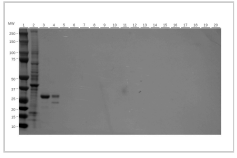

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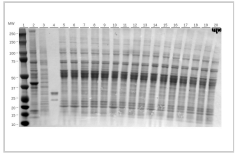

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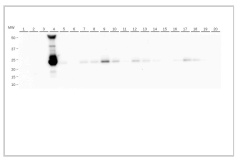

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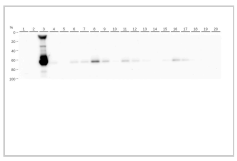

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2016-10-05_23-56-09_BWS_blot_1-2_calreticulin_8bit.png

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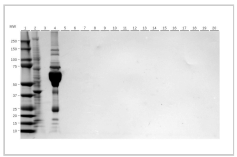

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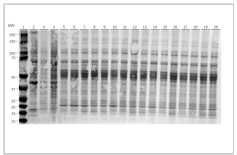

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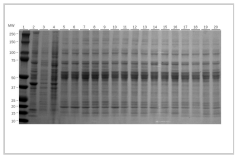

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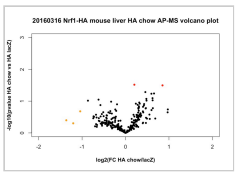
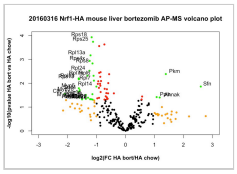


20160316_Nrf1-HA_mouse_liver_chow_AP-MS_volcano_plot.png



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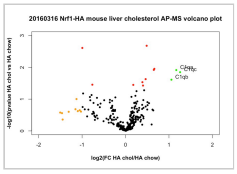

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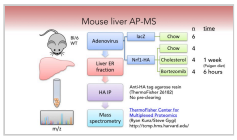

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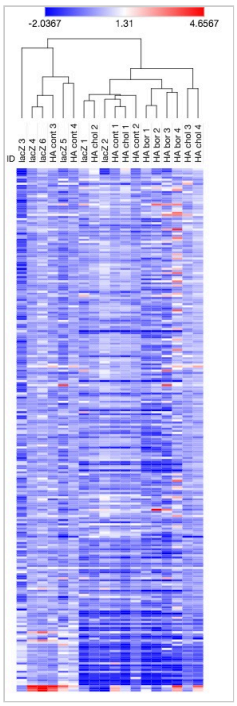

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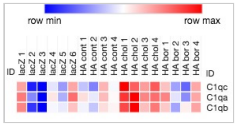

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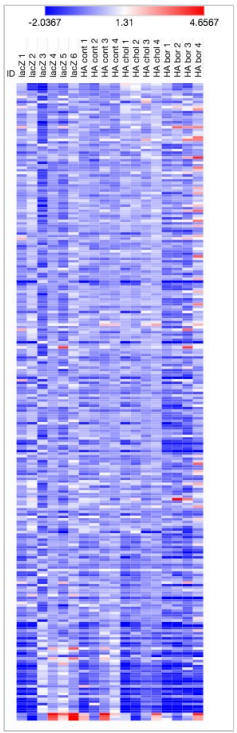

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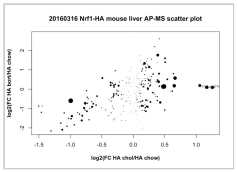

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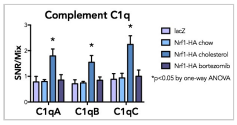
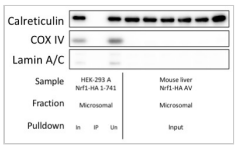


Complement_C1q_SNR.png



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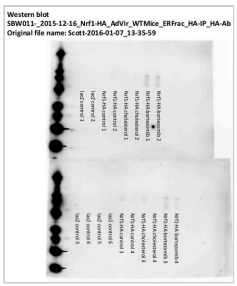

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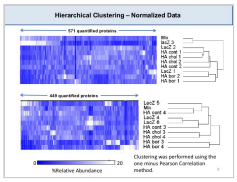

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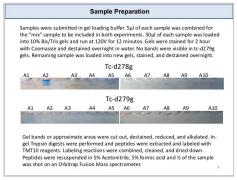

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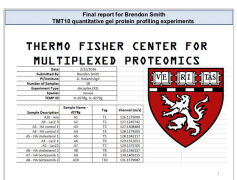

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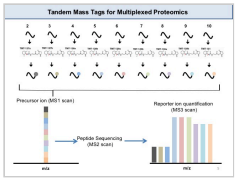

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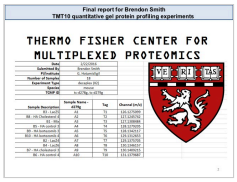

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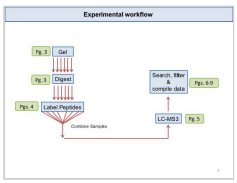

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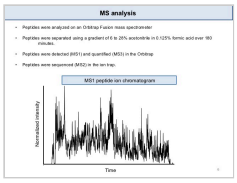

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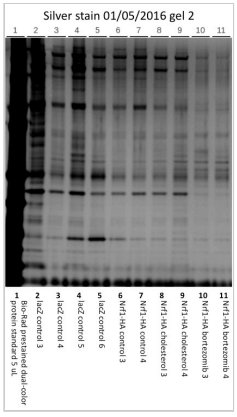
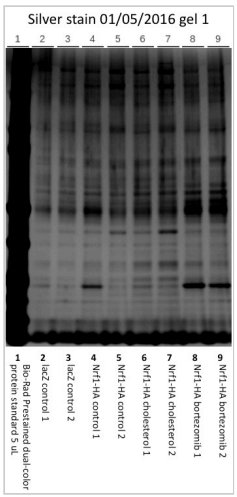


20160105_16-19-39_silver_stain_gel_2_labeled.png



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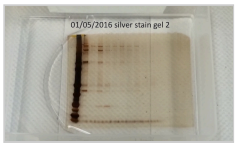

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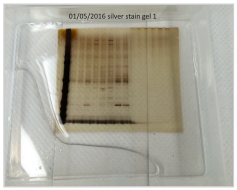

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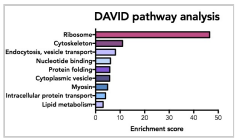

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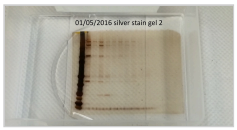

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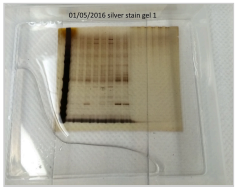

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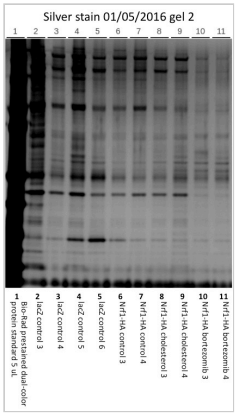

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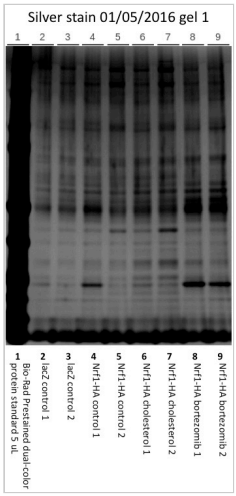

20160105_16-24-11_silver_stain_gel_1_labeled.png

Experiment Attached Files

1479501497.zip

signed_289661_645391_641.pdf

2016-03-03_blots_from_0227_fractionation.zip

2016-10-06_mouse_liver_IP_and_293_detergent_fractionation.zip

2016-11-11_mouse_liver_IP_re-do.zip

20160316_Nrf1-HA_mouse_liver_AP-MS_R.zip

20160105_16-19-39_silver_stain_gel_2.zip

20160105_16-24-11_silver_stain_gel_1.zip

Sample_Submission_Form___The_Thermo_Fisher_Center_for_Multiplexed_Proteomics_20160111.pdf

Linked Resources

20151216-18 Nrf1-HA mouse liver fractionation and affinity purification (Experiment)

Gel and Western blot protocol Brendon Smith (Protocol)

20160227 HEK-293 A Nrf1-HA 1-741 subcellular fractionation (Experiment)

20160928 HEK-293 A Nrf1-HA 1-741 detergent fractionation and Nrf1 IP (Experiment)

Proteomics Brendon Smith (Protocol)