Supplemental Figures to

Assembly dynamics and stoichiometry of the apoptosis signal-regulating kinase (ASK) signalosome in response to electrophile stress

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(A)ASK1 (B)ASK2 (C)ASK3 (D)ASK1 endogenous

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(A)ASK1 (B)ASK2 (C)ASK3 (D)ASK1 endogenous

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Figure S1. ICC images of the three ASK proteins. ICC results showing proper localization of the three ASK proteins with no evidence of aberrant aggregation.
**Figure S2.** Dose- and time-dependent activation of the ASK1 MAPK pathway. (A) ASK1-TAG cells were treated with increasing concentrations of HNE for 1hr. Activation of ASK1 was observed at all concentrations of HNE used (compare to EtOH) and maximal activation of the downstream kinase JNK and p38 was seen at 50µM. (B) ASK1-TAG cells were treated with 50µM HNE for the times indicated. Activation of ASK1 was detected at all time points but activation of the downstream kinases was strongest at 1-2hrs.
Figure S3. ASK1-ATAD3A interaction test. (A) The interaction of ATAD3A and ATAD3B with ASK1 seen in the over-expressing (ASK1-TAG) cells was confirmed by Co-IP Western. (B) However, in the context of endogenous expression in parental HEK-293 cells (top two panels) and RT-4 cells (which have a higher expression of ASK1), ASK1 and ATAD3A did not co-purify (compare lanes 3 and 5). Additionally, activation status of ASK1 did not seem to produce an association (compare lanes 8 and 10). In fact, there was a higher level of ATAD3A precipitation with the pre-immune IPs than with the ASK1 IPs (compare lanes 2 and 4 with 3 and lanes 7 and 9 with 8).
Figure S4. ASK1 knockdown IPs. An si-RNA knockdown of ASK1 was performed in 293 cells. (A) Western blot analysis of cells treated with non-targeting siRNA and ASK1-targeting siRNA shows that ASK1 expression was successfully knocked down. This can be seen at the whole cell lysate level (left panel) and the IP level (right panel). LRP normalized (B) and SID normalized (C) PRM analysis of non-targeting and ASK1-targeting siRNA treated cells show the decrease of ASK1 with siRNA treatment. The majority of the interacting proteins showed no difference with the sole exceptions of ASK2 and ASK3. Black bars denote the mean of each replicate and blue connecting lines show the si-RNA dependent drop in the effected peptides.
LRP-based results

Figure S4
Figure S4

LRP-based results
LRP-based results

Figure S4
LRP-based results

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**Figure S4**
LRP-based results

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LRP-based results

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LRP-based results

Figure S4
LRP-based results

Figure S4
SID-based results

**Figure S4**
SID-based results

Figure S4
USP9X - LAQQISDEASR

Normalized Peak Area

si-NEG  si-ASK1  IP-Control

SID-based results

Figure S4
Figure S5. SID calibration curve data. Linear regression curves of all 26 SID peptides with theoretical concentration on the x-axis and peak area ratio on the y-axis.
Figure S5.
Figure S5.
Figure S5.
Figure S5.
Figure S6. HNE EC_{50} determination. (A) EC_{50} values for HNE were found for each cell line using a WST assay. (B) ASK1 activation below (5\mu M) and above (20\mu M) the EC_{50} value.
Figure S7. (A) Western- and (B) PRM-based analysis of the amount of target protein purified in each cell line for the HNE concentration-response experiment.
Figure S8. HNE induced dynamic ASK1 complex changes. All peptides normalized by LRP for the (A) ASK1 IPs, (B) ASK2 IPs, (C) ASK3 IPs, (D) ASK1 Endogenous IPs were plotted as grouped scatter plots. The mean value for each condition is depicted as a bar. A red bar means that the peak areas for this condition were enriched over the negative control based on the Wilcoxon rank sum test with p<0.05 or detection in at least half of the replicates for a condition and no detection in the negative control samples. Concentration-response trends were tested in two ways: (1) the Jonckheere-Terpstra test was applied to detect an ordered trend in peak area – the results of this test are listed below the protein name and peptide sequence on each graph and (2) A Kruskal-Wallace test with post-hoc Dunn’s analysis was performed – all significant pairwise comparisons are listed on the graphs with bars connecting the significantly different conditions.
Figure S8.
Figure S8.

ASK1 IPs

GSTM1 - RPWFAGNK

JT p-value: NA

Normalized Peak Area

GSTM1 - RPWFAGNK

μM HNE

Control

3.8e+05

3.8e+05

3.8e+05

3.8e+05

GTP1 - FQDGDLTYQSNTILR

JT p-value: 1.02e-01

Normalized Peak Area

GTP1 - FQDGDLTYQSNTILR

μM HNE

Control

6e+06

4e+06

2e+06

2e+06

HSP90AA1 - APFDLFENR

JT p-value: 3.5e-01

Normalized Peak Area

HSP90AA1 - APFDLFENR

μM HNE

Control

2.5e+07

2.0e+07

1.5e+07

1.0e+07

HSP90AA1 - LGIHEDSQNR

JT p-value: 2.39e-02

Normalized Peak Area

HSP90AA1 - LGIHEDSQNR

μM HNE

Control

1.0e+08

7.5e+07

5.0e+07

2.5e+07

Figure S8.
Figure S8.

ASK1 IPs

HSP90AA1 - DQVANSAFVER

JT p-value: 1.73e-01

Normalized Peak Area

μM HNE

0 10 50 Control

3e+07

6e+07

9e+07

HSP90AB1 - VVVITK

JT p-value: 2.39e-02

Normalized Peak Area

μM HNE

0 10 50 Control

5.0e+07

1.0e+08

1.5e+08

HSP90AB1 - NPDDITQEEYGEFYK

JT p-value: 4.44e-02

Normalized Peak Area

μM HNE

0 10 50 Control

5.0e+06

1.0e+07

1.5e+07

HSP90AB1 - LGIHEDSTNR

JT p-value: 8.8e-03

Normalized Peak Area

μM HNE

0 10 50 Control

2.5e+07

5.0e+07

7.5e+07

1.0e+08

*
Figure S8.
Figure S8.
Figure S8.
Figure S8.

A

PRDX1 - LVQAFQFTDK

\[ JT \ p-value: 5.58e-02 \]

Normalized Peak Area

\[ \mu M \ HNE \]

PRKAA2 - SIDDEVVEQR

\[ JT \ p-value: NA \]

Normalized Peak Area

\[ \mu M \ HNE \]

PSMC1 - GVILYGPPGTGK

\[ JT \ p-value: 2.39e-02 \]

Normalized Peak Area

\[ \mu M \ HNE \]

PSMC2 - ALDEGDIALLK

\[ JT \ p-value: 1e+00 \]

Normalized Peak Area

\[ \mu M \ HNE \]

ASK1 IPs
Figure S8.

**PSMC2** – GVLLFGPPGTGK

*JT p-value: 5.58e-02*

**PSMC3** – GVLMYGPPGTGK

*JT p-value: NA*

**PSMC5** – GVC[+57]TEAGMYALR

*JT p-value: NA*

**SMN1** – NTAASLQQWK

*JT p-value: 1.46e-02*
Figure S8.

- **USP9X - VVIQSNDDIASR**
  - JT p-value: 1.64e-01

- **YWHAE - NLLSVAYK**
  - JT p-value: 1.05e-03

- **YWHAE - HLIPAANTGESK**
  - JT p-value: 1.29e-02

- **YWHAE - DSTLIMQ LLR**
  - JT p-value: 1.19e-02

**ASK1 IPs**
Figure S8.
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Figure S8.
Figure S8.

**ASK2 IPs**
Figure S8.

**ASK2 IPs**

- HSP90AB1 - NPDDITQEEYGEFYK
  - JT p-value: 1.26e-01
- HSP90AB1 - LGIHEDSTNR
  - JT p-value: 1.69e-01

- HSPA1A - FGDPVVQSDMK
  - JT p-value: 2.81e-01
- HSPA1A - LLQDFFNGR
  - JT p-value: 1.69e-01
Figure S8.
Figure S8.

HSPA4 - EDIYAVEIVGGATR

JT p-value: 5.78e-01

MAP3K4 - SIILQLLNAAGK

JT p-value: NA

MAP3K4 - VVPQVETVDTLR

JT p-value: NA

MAP3K4 - LGPIEAIQK

JT p-value: 7e-01

ASK2 IPs
Figure S8.

**MAP3K7 - LSLGASR**

JT p-value: 3.33e-01

**MAPK14 - DLLIDEWK**

JT p-value: 1e+00

**NACC1 - VEALPEQVAPESR**

JT p-value: 5.71e-01

**PARK7 - GAEEMETVIPVDVMR**

JT p-value: NA

ASK2 IPs
Figure S8.

ASK2 IPs

PPP5C – VTISFMK

JT p-value: NA

PRDX1 – LVQAFQFTDK

JT p-value: 9.26e-02

PRKAA2 – SIDDEVVEQR

JT p-value: NA

PSMC1 – GVILYGPPGTGK

JT p-value: 6.59e-02
ASK2 IPs

**Figure S8.**

**PSMC1 – TMLELLNQLDGDFSR**

JT p-value: NA

**PSMC2 – ALDEGDIALLK**

JT p-value: 7.33e-03

**PSMC2 – GVLLFGPPGTGK**

JT p-value: 3.06e-02

**PSMC3 – GVLMYGPPGTGK**

JT p-value: 5.78e-01
Figure S8.
Figure S8.

**ASK2 IPs**

**USP9X - VVIQSNDDIASR**

JT p-value: 3.51e-01

**YWHAE - NLLSVAYK**

JT p-value: 1.26e-01

**YWHAE - HLIPAAINTGESK**

JT p-value: 1.28e-01

**YWHAE - DSSLIMQLLR**

JT p-value: 2.81e-01
Figure S8.
Figure S8.

ASK2 IPs
Figure S8.

**Actin - GYSFTTTAER**

$JT \ p\text{-value: } 1.69e-01$

Normalized Peak Area

![Graph showing data points and trend lines for HNE concentration against normalized peak area.](image)

**ASK2 IPs**
Figure S8.
Figure S8.
Figure S8.
Figure S8.
Figure S8.
Figure S8.
Figure S8.
Figure S8.
Figure S8.
Figure S8.
Figure S8.

ASK3 IPs
Figure S8.

Actin - GYSFTTTAER

JT p-value: 4.31e-01
Figure S8.

ASK1 Endogenous IPs

- **PDCD6 – AGVNFEFTGVWK**
  - JT p-value: NA

- **PDCD6 – YITDWNQNVFR**
  - JT p-value: NA

- **ARRB2 – VYTITPLLSDNR**
  - JT p-value: NA

- **ASK1 – LDGFGETTVLDR**
  - JT p-value: 5.2e-01
Figure S8.

**ASK1 Endogenous IPs**

- **ASK1 - VAQASSSQQYFR**
  - JT p-value: 6.18e-01

- **ASK2 - EPQPGLEPR**
  - JT p-value: 5.2e-01

- **ASK2 - AALGVLGPEVEK**
  - JT p-value: 4.31e-01

- **ASK3 - SESSQGGAAGGPEAGAR**
  - JT p-value: 2.86e-01
Figure S8: ASK1 Endogenous IPs
Figure S8. ASK1 Endogenous IPs
Figure S8.

ASK1 Endogenous IPs
Figure S8. ASK1 Endogenous IPs

HSP90AA1 - LGIHEDSQNR

$JT \ p-value: 7.22e-01$

HSP90AA1 - DQVANSAFVER

$JT \ p-value: 8.31e-01$

HSP90AB1 - VVVITK

$JT \ p-value: 9.43e-01$

HSP90AB1 - NPDDITQEEYGEFYK

$JT \ p-value: 8e-01$
**Figure S8.**

**ASK1 Endogenous IPs**

- **HSP90AB1 - LGIHEDSTNR**
  - JT p-value: 9.43e-01

- **HSPA1A - FGDPVVQSDMK**
  - JT p-value: 1.06e+00

- **HSPA1A - LLQDIFFNGR**
  - JT p-value: 3.51e-01

- **HSPA1A - SAVEDEGLK**
  - JT p-value: 8.31e-01
ASK1 Endogenous IPs

**Figure S8.**
Figure S8.

ASK1 Endogenous IPs

HSPA4 - EDIYAVEIVGGATR

JT p-value: NA

MAP3K7 - LSLGASR

JT p-value: NA

NEDD4 - ATVLEDSYR

JT p-value: 1.06e+00

NEDD4 - LWIEFEDGEK

JT p-value: 4.67e-01
Figure S8.

ASK1 Endogenous IPs

**NEDD4 - EGFFELIPQDLIK**

JT p-value: NA

**PARK7 - GAEEMETVIPVDVMR**

JT p-value: NA

**PPP5C - VTISFMK**

JT p-value: NA

**PRDX1 - LVQAFOQTSDK**

JT p-value: 5.2e-01
Figure S8.

ASK1 Endogenous IPs

**PSMC1 - GVILYGPPGTGK**

- **JT p-value:** $4.31e^{-01}$

**PSMC2 - ALDEGDIALLLK**

- **JT p-value:** $6.66e^{-01}$

**PSMC2 - GVLLFGPPGTGK**

- **JT p-value:** $9.43e^{-01}$

**PSMC3 - GVLMYGPPGTGK**

- **JT p-value:** $6.68e^{-01}$
ASk1 Endogenous IPs

**Figure S8.**

- **PSMC5 - GVC[+57]TEAGMYALR**: JT p-value: 8.31e-01
- **QARS - EAATQAQQTGLGSTIDK**: JT p-value: NA
- **SMN1 - NTAASLQQWK**: JT p-value: 2.2e-01
- **TRAF2 - GPNDALLR**: JT p-value: NA
Figure S8. ASK1 Endogenous IPs

TRAF3 – VTELESVDK
JT p-value: NA

USP9X – VVIQSNDDIASR
JT p-value: 9.43e-01

YWHAE – NLLSVAYK
JT p-value: 6.18e-01

YWHAE – HLIPAANTGESK
JT p-value: 7e-01
Figure S8.

ASK1 Endogenous IPs
Figure S8.

ASK1 Endogenous IPs
ASK1 Endogenous IPs

Figure S8.
Figure S9. HNE induced dynamic ASK1 complex changes. All peptides normalized by SID for the (A) ASK1 IPs, (B) ASK2 IPs, (C) ASK3 IPs, (D) ASK1 Endogenous IPs were plotted as grouped scatter plots. The mean value for each condition is depicted as a bar. A red bar means that the peak areas for this condition were enriched over the negative control based on the Wilcoxon rank sum test with p<0.05 or detection in at least half of the replicates for a condition and no detection in the negative control samples. Concentration-response trends were tested in two ways: (1) the Jonckheere-Terpstra test was applied to detect an ordered trend in peak area – the results of this test are listed below the protein name and peptide sequence on each graph and (2) A Kruskal-Wallis test with post-hoc Dunn’s analysis was performed – all significant pairwise comparisons are listed on the graphs with bars connecting the significantly different conditions.
Figure S9.

pan-p38\_ink - ILDFGLAR

*JT p-value: NA*

PARK7 - DGLILTSR

*JT p-value: 7.43e-03*

PRDX1 - IGHPAPNFK

*JT p-value: 2.82e-02*

TRX1 - VGEFSGANK

*JT p-value: 3.5e-01*
Figure S9.

USP9X - LAQQISDEASR

JT p-value: 9.12e-02

Normalized fmol/IP

µM HNE

ASK1 IPs
Figure S9.
Figure S9.
Figure S9.
Figure S9.

**ASK3 IPs**

- **ASK1 - LSALSAGSNEYLR**
  - JT p-value: 3.71e-01

- **ASK3 - LWSAVSQYR**
  - JT p-value: 8.31e-01

- **pan_YWHA - VISSIEQK**
  - JT p-value: 5.2e-01

- **pan-p38_ink - ILDFGLAR**
  - JT p-value: 4.65e-01
Figure S9.
Figure S9.

**ASK1** - LSALSAGSNEYLKR

JT p-value: 3.51e-01

**ASK2** - GDNV.INTF.SGLLKR

JT p-value: 3.51e-01

**ASK3** - LWSAVSQYR

JT p-value: 7.22e-01

**pan_YWHA** - VISSIEQK

JT p-value: 7.22e-01

**ASK1 Endogenous IPs**
Figure S9.
Figure S9.

ASK1 Endogenous IPs
Figure S10. Enrichment of dynamic ASK1-interacting proteins in HEK-293 cells. The 14 proteins identified as dynamic interactors in the ASK1-TAG cell line were measured in IPs of endogenous ASK1 to determine if they were enriched in the ASK1 IPs compared to the negative control IPs. ND = not detected.
### Size Exclusion Chromatography methods

<table>
<thead>
<tr>
<th></th>
<th>This study</th>
<th>Noguchi et. al.</th>
</tr>
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</table>
| **SEC Buffer**       | 50mM HEPES pH 7.5  
10mM KCl  
150mM NaCl  
1mM EDTA  
1mM EGTA  
1.5mM MgCl$_2$  
10% glycerol  
0.1% CHAPS  
0.01% Brij35 | 50mM HEPES-KOH pH 7.5  
10mM KCl  
150mM NaCl  
1mM EDTA  
1mM EGTA  
1.5mM MgCl$_2$  
10% glycerol  
0.1% CHAPS  
0.01% Brij35 |
| **Lysis method**     | 5x10$^7$ cells/mL were lysed with a glass Dounce homogenizer in SEC buffer supplemented with 1mM DTT, and 1X Halt Protease & Phosphatase inhibitor. | 5x10$^7$ cells/mL were lysed with a glass Dounce homogenizer in SEC buffer supplemented with 1mM DTT, 1mM phenylmethylsulfonyl fluoride, and 5 ug/mL aprotinin. |
| **Spin processing**  | The lysate was centrifuged at 20,000g for 30 minutes for clarification prior to SEC. The resulting supernatant was frozen at -80°C. | The lysate was centrifuged at 10,000g for 10 minutes. The resulting supernatant was further centrifuged at 105,000g for 90 minutes. This supernatant was frozen at -135°C. |
| **SEC method**       | The stored lysate was loaded onto a Superose6 10/300 GL column pre-equilibrated with the SEC buffer. Proteins were eluted isocratically at 0.3mL/min and collected in 0.6mL fractions. | The stored lysate was loaded onto a Superose6 10/300 GL column pre-equilibrated with the SEC buffer. Proteins were eluted isocratically at 0.3mL/min and collected in 0.5mL fractions. |
| **Protein isolation**| ASK signalosome complexes were isolated via immunopurification with anti-HA beads. | Each fraction was precipitated using acetone/ethanol (1:4) |
| **Amount used for subsequent assays** | Samples from 2 column runs were used for Western analysis. Samples from 6 column runs were used for PRM analysis. | Samples from 1 or 4 column runs were pooled and analyzed by Western |

**Figure S11.** Size-exclusion fractionation of intact ASK1 complexes. (A) Methods comparison for the SEC assays performed in this study and Noguchi et. al. (B & C) Treatment of ASK1-TAG cells with ethanol (B) and 50µM HNE (C) yields similar distributions of ASK1 complexes with no shift to a higher molecular weight observed upon activation by HNE. (D) LRP-PRM analyses of ASK1, ASK2, and ASK3 in the SEC fractionated samples confirm the western results and show no change in complex localization upon activation by HNE.
Figure S11.
Figure S11.