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Analysis of the interaction of FLAG-TIFA with TRAF6, TRAF2 and c-IAP1 by co-immunoprecipitation

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Protocol status: Working
This protocol is working well in our laboratory and has been successfully used to define the key regions on TIFA that are required for its interaction with TRAF6, TRAF2 and c-IAP1 upon ADP-heptose stimulation.

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Abstract

ALPK1 (alpha-kinase 1) is an atypical protein kinase that is activated during infection. In particular, ALPK1 is activated by binding of nucleoside-diphosphate-heptoses such as ADP-heptose to its N-terminal domain, enabling ALPK1 to phosphorylate TIFA (TRAF-interacting protein with FHA domain) at Thr9. This induces the interaction of phosphorylated Thr9 with the forkhead-associated domain of another TIFA molecule, leading to the “head-to-tail” polymerisation of TIFA into “TIFAsomes” that recruit TRAF6 (tumour necrosis factor receptor-associated factor 6) and TRAF2, and TRAF2 in turn recruits c-IAP1 (cellular inhibitor of apoptosis protein 1). TRAF6 and c-IAP1 are E3 ligases that generate the Lys63-linked ubiquitin chains required to recruit and activate TAK1 (transforming growth factor β-activated kinase 1), which in turn activates MAPK (mitogen-activated protein kinase) cascades that lead to the activation of JNK (c-Jun N-terminal kinases). One role of JNK is to activate the transcription factor AP-1 (activator protein 1). Another function of TAK1 is to activate the canonical IκB kinase (IKK) complex, which activates the transcription factor NF-κB (nuclear Factor kappa-light-chain-enhancer of activated B cells). In this protocol, FLAG-tagged wildtype and mutant or truncated forms of TIFA are overexpressed in TIFA knockout HEK-Blue cells and their interaction with TRAF6, TRAF2 and c-IAP1 assessed by co-immunoprecipitation.

Guidelines

This protocol uses reverse transfection in 6-well plate format and yields sufficient sample to run up to four SDS-PAGE gels for the immunoprecipitated samples (three gels are required to detect TIFA, TRAF6, TRAF2 and c-IAP1).

Materials

Laboratory Equipment

Pipetman 4-Pipette Kit (Gilson, #F167360)
Stripettor Ultra Pipet Controller (Corning, #4099)
Thermomixer comfort 5355 (Eppendorf)
CB150 Cell Culture Incubator (Binder)
BioMAT 2-SF Cell Culture Hood (Conditioned Air Solutions)
Micro STAR 17 Benchtop Microcentrifuge (Fisherbrand)
Accuspin Micro 17 Refrigerated Benchtop Microcentrifuge (Fisherbrand)
QBT2 dry block heater (Grant)
NuPAGE XCell SureLock Midi-Cell system (Invitrogen)
Trans-Blot Transfer Cell (BioRad)

Laboratory Supplies

15 cm Nunc cell culture dishes (ThermoFisher, #168381)
6-well Nunc cell culture plates (ThermoFisher, #140675)
50 ml conical centrifuge tubes (Greiner, #227261)
Serological pipettes (ThermoFisher, #10710810)
Safe-Lock 1.5 ml microcentrifuge tubes (Eppendorf, #30123611)
Safe-Lock 2.0 ml microcentrifuge tubes (Eppendorf, #30123620)
Spin-X 0.22 micron columns (Costar, #8161)
NuPAGE Bis-Tris 4-12% 20-well midi gels (ThermoFisher, #WG1402BOX)
80-well cooling chamber for 1.5 ml tubes (Diversified Biotech, #CHAM-8000)

Biological Material

TIFA knockout HEK-Blue cells (Invivogen, #hkb-kotifa) (store in liquid nitrogen)

General Reagents

ADP-L-heptose triethylammonium salt (Invivogen, #tlrl-adph-l) (-80 °C)
Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, #11960-085) (4 °C)
200 mM L-Glutamine (Gibco, #25030024) (-20 °C)
Penicillin-Streptomycin 100X stock (Gibco, #15140122) (-20 °C)
Opti-MEM I Reduced Serum Medium (Gibco, #31985062) (4 °C)
Lipofectamine 2000 (ThermoFisher, #11668019) (4 °C)
Anti-FLAG M2 Affinity Resin (Millipore, #A2220) (-20 °C)
Phosphate-buffered saline (PBS) (Gibco, #10010023) (4 °C)
5 M NaCl (Sigma-Aldrich, #S6546) (room temp)
1 M DTT (ThermoFisher, #P2325) (-20 °C)
1 M Tris-HCl (pH 7.5) (ThermoFisher, #15567027) (room temp)
0.5 M EGTA (pH 8.0) (ThermoFisher, #J60767.AD) (room temp)
0.5 M EDTA (pH 8.0) (Sigma-Aldrich, #03690) (room temp)
10% (v/v) Triton X-100 (Sigma-Aldrich, #93443) (4 °C)

Foetal Bovine Serum (FBS) (Sigma-Aldrich, #F7524) (-20 °C)
Sucrose powder (VWR, #27480.360) (room temp)
MOPS SDS running buffer 20X stock (Formedium, #SDS5000) (room temp)
NuPAGE LDS sample buffer 4X stock (ThermoFisher, #NP0007) (room temp)
Precision Plus Protein Standards (Bio-Rad, #1610374) (-20 °C)
2-mercaptoethanol (Sigma-Aldrich, #M6250) (room temp)
Trypsin-EDTA solution (Gibco, #25200056) (4 °C)
Halt Protease and Phosphatase Inhibitor Cocktail, EDTA-free (ThermoFisher, #78441) (-20 °C)
Bovine serum albumin (BSA) (Sigma-Aldrich, #810533) (4 °C)
Ponceau S solution (Sigma-Aldrich, #P7170) (room temp)
Tris-Glycine Transfer Buffer 10X stock (Cell Signalling Technology, #12539) (room temp)
Tris-Buffered Saline with Tween 20 (TBS-T) 10X stock (Cell Signalling Technology, #9997) (room temp)
Methanol (VWR, #20847.318) (room temp)
SureLock Midi Pre-cut 0.45 µm PVDF Membranes and Filters (ThermoFisher, #STM2006) (room temp)
Super-Signal West Pico Chemiluminescent Substrate (ThermoFisher, #34577) (4 °C)

Antibodies
p-NFκB1 (p-Ser932) (Cell Signalling Technology, #4806) (-20 °C)
GAPDH (Cell Signalling Technology, #2118) (-20 °C)
TIFA (Proteintech, #11333-1-AP) (-20 °C)
FLAG (Abcam, #ab205606) (-20 °C)
TRAF6 (Abcam, #ab40675) (-20 °C)
TRAF2 (Abcam, #ab126758) (-20 °C)
c-IAP1 (Abcam, #ab108361) (-20 °C)
Anti-rabbit IgG (Cell Signalling Technology, #7074) (-20 °C)

Solutions (Recipes provided within the main text)
Antibiotic-Containing Culture Media (4 °C)
Antibiotic-Free Culture Media (4 °C)
Lysis Buffer (use immediately)
Wash Buffer (4 °C, use within 1 month)
Salt Wash Buffer (4 °C, use within 1 month)
Blocking Solution (4 °C, use within 1 week)
Transfer Buffer (4 °C)

Before start

Before starting this procedure, a confluent 15 cm dish of TIFA knockout HEK-Blue cells is required (cultured in 20 ml of Antibiotic-Containing Culture Media), which is sufficient for 18 transfections in 6-well plate format (i.e. 9 plasmids, each with and without ADP-heptose). The cells should be cultured by incubation at 37 °C with 5% CO2 and tested regularly for mycoplasma using a MycoAlert Mycoplasma Detection Kit (Lonza, #LT07-318). The cells should be passaged once confluent at a ratio of 1:10 (v/v), and not used beyond 30 passages.

Antibiotic-Containing Culture Media (500 ml bottle)

Reagent	Final concentration	Amount to add
DMEM	Not applicable	500 ml
FBS	10% (v/v)	50 ml
20 mM L-Glutamine	2 mM	5.6 ml
Penicillin-Streptomycin 100X	1X	5.6 ml

Antibiotic-Free Culture Media (500 ml bottle)

Reagent	Final concentration	Amount to add
DMEM	Not applicable	500 ml
FBS	10% (v/v)	50 ml
20 mM L-Glutamine	2 mM	5.6 ml

(Recipes for preparing a 500 ml bottle of Antibiotic-Containing Culture Media or Antibiotic-Free Culture Media)

It is also necessary to have endotoxin-free plasmids ready for transfection. The plasmids listed in the table below encode FLAG-tagged TIFA under a UbC promoter and were purified using NucleoBond Xtra Midi Endotoxin-Free kits (Macherey-Nagel, #740420) and stored at -20 °C in water. Each plasmid was assigned a unique identifier (DU) and are available upon request (mrcppureagents.dundee.ac.uk).

Expressed	Plasmid	DU Number
FLAG-TIFA	pcDNA5D FRT TO (UbC Pr)-FLAG-TIFA	DU71017
FLAG-TIFA[T9A]	pcDNA5D FRT TO (UbC Pr)-FLAG-TIFA[T9A]	DU71055
FLAG-TIFA[1-157]	pcDNA5D FRT TO (UbC Pr)-FLAG-TIFA[1-157]	DU79169
FLAG-TIFA[1-165]	pcDNA5D FRT TO (UbC Pr)-FLAG-TIFA[1-T165]	DU79189
FLAG-TIFA[1-172]	pcDNA5D FRT TO (UbC Pr)-FLAG-TIFA[1-Q172]	DU79168
FLAG-TIFA[E178A]	pcDNA5D FRT TO (UbC Pr)-FLAG-TIFA[E178A]	DU71057

(Plasmids encoding wildtype, mutant or truncated FLAG-tagged TIFA under the control of a UbC promoter)

Prepare complexes of lipofectamine 2000 and plasmid in 6-well culture plates (Day 1)

27m

1 **Prepare a mastermix for each plasmid and add them to the relevant wells of 6-well culture plates:**

1.1 Calculate the number of wells to be transfected with each plasmid, plus one extra. Each plasmid will be transfected in duplicate (i.e., 2 wells per plasmid, and so prepare a mastermix for 3 wells). 1m

1.2 Each well to be transfected requires 2.5 µg of plasmid in 100 µl of OptiMEM. For a 3-well mastermix of plasmid encoding WT TIFA, add 7.5 µg of this plasmid to 300 µl of OptiMEM in a 1.5 ml microcentrifuge tube. Invert the tube five times to mix thoroughly. 5m

2 **Prepare diluted lipofectamine 2000 and add to each of the diluted plasmids:**

2.1 Calculate the number of wells to be transfected plus 10% extra (for 18 wells, prepare for 20 wells). 1m

2.2 Each well requires 6.25 µl of lipofectamine 2000 in 100 µl of OptiMEM. Prepare a mastermix for 20 wells by adding 125 µl of lipofectamine 2000 to 1875 µl of OptiMEM in a 2.0 ml microcentrifuge tube. Invert the tube five times to mix thoroughly. 5m

2.3 Add 300 µl of the diluted lipofectamine 2000 into each of the tubes containing diluted plasmids (because it is a 3-well mastermix). Invert 5 times and wait 5 min. 10m

2.4 Add 200 µl of each diluted plasmid-lipofectamine mixture to relevant wells of the 6-well culture plates, remembering to change tips for different plasmids. 5m

Add single-cell suspension to the 6-well plates containing lipid-DNA complexes (Day 1)

14m

3 **Prepare a single-cell suspension of the TIFA knockout HEK-Blue cells:**

3.1 Aspirate the culture media from a confluent 15 cm cell culture dish of TIFA knockout HEK-Blue cells and add 5 ml of PBS to rinse. 2m

3.2 Aspirate the PBS and add 3 ml of trypsin-EDTA solution. Return the dish to the incubator until the cells have visually detached, which typically takes 2 min. 3m

3.3 Add 16 ml of antibiotic-free culture media and pipette up and down with a serological pipette until a single-cell suspension has been produced (the total volume should be 19 ml). 2m

3.4 Transfer the cell suspension to a 50 ml conical centrifuge tube and add 18 ml of antibiotic-free culture media, bringing the total volume to 38 ml. 1m

4 **Add the single-cell suspension to the 6-well plates that contain the lipid-DNA complexes:**

4.1 Add 2 ml of cell suspension to each required well in the 6-well plates. After plating, ensure cells are evenly distributed by moving the plates in a figure-of-8 motion. Return the cells to the incubator for 24 h. 3m

4.2 To maintain the cell line, add 2 ml of cell suspension to a 15 cm cell culture dish and top up with 18 ml of antibiotic-containing culture media. Return the dish to the incubator, which should be confluent in 4 days. 2m

Stimulation of cells and preparation of whole cell extracts (Day 2) 1h 22m

5 **Prepare the required reagents:**

5.1 Prepare 1 mM ADP-heptose by adding 347 µl of PBS into a 250 µg vial. Vortex for 30 seconds. 2m

5.2 Prepare sufficient lysis buffer to account for a 20% surplus. Since each well requires 0.5 ml of this lysis buffer, for 20 wells make sufficient volume for 24 wells (i.e. a total of 12 ml). 10m

Lysis Buffer (50 ml)

Reagent	Final concentration	Amount to add
1M Tris-HCl (pH 7.5)	50 mM	2.5 ml
Sucrose powder	270 mM	4.6 g
10% (v/v) Triton X-100	1% (v/v)	5 ml
0.5 M EDTA (pH 8.0)	1 mM	100 µl
0.5 M EGTA (pH 8.0)	1 mM	100 µl
1 M DTT	2 mM	100 µl
Halt Cocktail 100X	1X	0.5 ml
Water	Not applicable	41.7 ml

(Recipe for preparing 50 ml of Lysis Buffer)

6 **Preparation of whole cell extracts:**

6.1 Stimulate the relevant cells for 20 min with 10 µM ADP-heptose by adding 22 µl of the 1 mM stock solution and returning the 6-well culture plates to the incubator (the volume of culture media should be 2.2 ml). 25m

6.2 After 20 min, remove the 6-well culture plates from the incubator and place them onto a tray of ice. Aspirate the culture media carefully, gently add 2 ml of ice-cold PBS to wash and aspirate, being sure to remove as much residual liquid as possible. Add 0.5 ml of ice-cold lysis buffer to each well. 10m

6.3 Carefully scrape the cells into the lysis buffer, making sure to use a different scraper for each well. 10m

6.4 Transfer each cell lysate to a pre-chilled 1.5 microcentrifuge tube on ice and centrifuge at 18,000 xg for 20 min at 4 °C and transfer each supernatant (cell extract) to a new 1.5 ml microcentrifuge tube on ice. 5m

Note: Bradford assays can be performed at this stage to allow the normalisation of cell extracts based on protein concentration, but it is not included within this protocol.

7 **Prepare an aliquot of each whole cell extract for SDS-PAGE analysis:**

7.1 Transfer 50 µl of each of the cell extracts (i.e. 10% by volume) to a new pre-chilled 1.5 ml microcentrifuge tube on ice and add 16.7 µl of 4X LDS sample buffer containing 10% (v/v) 2-mercapethanol. 10m

7.2 Heat to 75 °C for 5 min, centrifuge at 18,000 xg for 1 min at room temp and store at -20 °C until analysis. 10m

Immunoprecipitation, washing and elution of FLAG-tagged TIFA (Day 2) 7h 14m

8 **Immunoprecipitation of FLAG-TIFA from the whole cell extracts:**

Note: Ideally step 8 should be performed immediately after cell lysis, but if this is not possible then cell extracts can also be stored at 4 °C overnight and step 8 performed the following day.

8.1 The remaining cell extract (0.5 mg of protein) will be immunoprecipitated using 15 µl of anti-FLAG affinity resin per sample as described below. Prepare 20% more resin than needed (for 20 samples prepare 360 µl of resin, which is sufficient for 24 samples).

8.2 Add 720 µl of anti-FLAG M2 affinity resin slurry (50% resin by volume, i.e. 360 µl of resin) to a 1.5 ml microcentrifuge tube on ice using a pipette tip where the narrow end has been trimmed using a scalpel. 2m

8.3 Centrifuge the slurry at 2000 xg for 1 min at 4 °C to pellet the resin. Aspirate the supernatant and add 1 ml of ice-cold lysis buffer. Repeat the centrifugation and wash steps twice. 10m

8.4 After the final wash, resuspend the resin in the volume of ice-cold lysis buffer required to make a 25% slurry (i.e. for 360 µl of resin add 1080 µl of buffer). 2m

8.5 Use a trimmed pipette tip to add 60 µl of this slurry to each of the cell extracts. 5m

8.6 Incubate at 4 °C for ideally 5 h on a rotating wheel to immunoprecipitate the FLAG-tagged TIFA from each cell extract. Alternatively, incubate for 16 h and continue the following day. 5h

9 **Washing of FLAG-TIFA immunoprecipitates:**

9.1 Prepare wash buffers and store them at 4 °C for up to 1 month (see recipes below). They contain 0.1% (v/v) Triton X-100 to prevent resin from sticking to microcentrifuge tubes and 1 mM DTT to maintain proteins in their reduced state. At this concentration of DTT the FLAG resin remains functional. 10m

Wash Buffer (1 L)

Reagent	Final concentration	Amount to add
1 M Tris-HCl (pH 7.5)	50 mM	50 ml
10% (v/v) Triton X-100	0.1% (v/v)	10 ml
1 M DTT	2 mM	2 ml
Water	Not applicable	938 ml

Salt Wash Buffer (1 L)

Reagent	Final concentration	Amount to add
1 M Tris-HCl (pH 7.5)	50 mM	50 ml
10% (v/v) Triton X-100	0.1% (v/v)	10 ml
5 M NaCl	0.25 M	50 ml
1 M DTT	2 mM	2 ml
Water	Not applicable	888 ml

(Recipe for preparing 1 L of Wash Buffer and Salt Wash Buffer)

9.2 Centrifuge the samples at 2000 xg for 1 min at 4 °C to pellet the resin, carefully aspirate the supernatant and resuspend the resin in 1 ml of ice-cold salt wash buffer. 15m

9.3 Repeat centrifugation and wash steps twice with 1 ml of ice-cold salt wash buffer, followed by twice with 1 ml of ice-cold wash buffer to remove NaCl prior to SDS-PAGE. 45m

9.4 After the final wash, carefully aspirate all residual wash buffer such that the resin is dry. All following steps in this section are performed at room temperature, unless stated otherwise. 15m

10 **Elution of FLAG-TIFA and interacting proteins:**

10.1 Resuspend the resin in 50 µl of 1X LDS sample buffer (without 2-mercaptethanol) and incubate for 5 min at 37 °C (to prepare 1X LDS sample buffer, add 250 µl of 4X LDS sample buffer to 750 µl of water). 10m

10.2 Centrifuge the samples for 1 min at 1000 xg and transfer supernatants to Spin-X columns. Subject the columns to centrifugation for 1 min at 13,000 xg, which will remove the resin from the samples. 5m

10.3 Discard the resin and add 2.5% (v/v) 2-mercaptoethanol (i.e. 1.3 µl) to the flow-through. 5m

10.4 Heat to 75 °C for 5 min, centrifuge at 18,000 xg for 1 min and store at -20 °C until analysis. 10m

SDS-PAGE analysis (Day 3) 2h 20m

11 **Analyse the immunoprecipitated samples by SDS-PAGE:**

11.1 Load protein ladder (5 µl) into the first lane of each of three 20-well 4-12% Bis-Tris gels (this ladder contains pre-stained proteins with molecular masses of 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa). 2m

11.2 Load 12.5 µl of each sample (i.e. 25%) into the remaining lanes of each of the gels, noting the order. 8m

11.3 Run the gels in MOPS buffer at 160 V until the dye front has reached the bottom (typically 1 h). 1h

12 **Analyse the whole cell extracts by SDS-PAGE:**

12.1 Analyse the whole cell extracts that were prepared prior to immunoprecipitation in the same way using two (not three) duplicate 20-well gels, also loading the protein ladder in the first lane. 1h 10m

Western transfer of resolved proteins and immunoblotting (Days 3-4) 22h 48m

13 **Wet transfer of resolved proteins onto PVDF membranes (Day 3):**

13.1 Activate the pre-cut PVDF membranes in 100% methanol and wash once with water followed by once with transfer buffer. The recipe for transfer buffer is given below. Transfer buffer can be reused up to 5 times. 1m

Transfer Buffer (1 L)

Reagent	Final concentration	Amount to add
Transfer Buffer 10X stock	1X solution	100 ml
Methanol	20% (v/v)	200 ml
Water	Not applicable	700 ml

(Recipe for preparing 1 L of Transfer Buffer)

13.2 Place a sponge pad at the cathode side of the transfer cassette, followed by filter paper, the SDS-PAGE gel, activated PVDF membrane, another filter paper and then another sponge pad. 1m

13.3 Close the transfer cassette and place it into the transfer cell and fill with transfer buffer. 1m

13.4 Transfer proteins from the SDS-PAGE gels to the activated PVDF membranes at 90 V for 90 min at 4 °C (it is recommended to place the transfer tank in a cold room for this step). 1h 30m

Note: It is possible to leave the transfer tank overnight at this stage, with the apparatus set to stop running after 90 min.

14 **Immunoblotting on PVDF membranes (Days 3-4):**

14.1 Remove the PVDF membranes from the transfer tank and re-activate the PVDF membranes in methanol, wash once with water and stain with Ponceau S solution to visualise proteins and hence evaluate the transfer quality. After staining, wash twice with water. 10m

14.2 Cut membranes with a scalpel such that proteins of different molecular weights can detected at the same time. For the immunoprecipitations: Gel 1: TIFA and TRAF6; Gel 2: TRAF2; Gel 3: c-IAP1. For the whole cell extract samples: Gel 1: TIFA and GAPDH; Gel 2: FLAG, GAPDH and p-NFκB1. 10m

14.3 Destain the cut membranes by incubation in TBS-T for 5 min. Bands might not be visible for the immunoprecipitation samples, except the protein ladder and immunoglobulin heavy and light chains. 5m

14.4 Block the membranes by incubation for 1 h in blocking solution, which serves to minimise the non-specific binding of antibodies to the membrane in later steps. Blocking solution can be stored at 4 °C for up to a week. 1h

Blocking Solution (50 ml)

Reagent	Final concentration	Amount to add
TBS-T	Not applicable	50 ml
BSA	5% (w/v)	2.5 g

(Recipe for preparing 50 ml of Blocking Solution)

14.5 Incubate the cut membranes with the relevant primary antibody solution on a shaker for 16 h at 4 °C. All primary antibodies are prepared as 25 ml solutions at 1:1000 (v/v) in blocking solution and stored at -20 °C when not in use. The antibodies can be re-used up to 10 times. 16h

14.6 Wash the membranes five times (10 min per wash) with TBS-T on a shaker. 1h

14.7 Incubate membranes at room temp for 1 h with 25 ml of anti-rabbit horseradish peroxidase-conjugated secondary antibody solution. The secondary antibody (anti-rabbit IgG) solution is prepared fresh each time by diluting 1:5000 (v/v) in blocking solution. 1h

14.8 Wash the membranes five times (10 min per wash) with TBS-T on a shaker. 1h

14.9 Develop the immunoblots using ECL reagent and detect the resulting chemiluminescence using a ChemiDoc MP, remembering to also take a merged colorimetric image. 1h

Protocol references

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