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Supplementary Information for

Netrin1 Deficiency Activates MST1 via UNC5B Receptor, Promoting Dopaminergic Apoptosis in Parkinson's Disease

Eun Hee Ahn, Seong Su Kang, Qi Qi, Xia Liu, Patrick Mehlen and Keqiang Ye *

Email: kye@emory.edu

This PDF file includes:

Figures S1 to S7
Figure Legends for Figures S1 to S7

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Supplementary Information Text
Fig. S1.

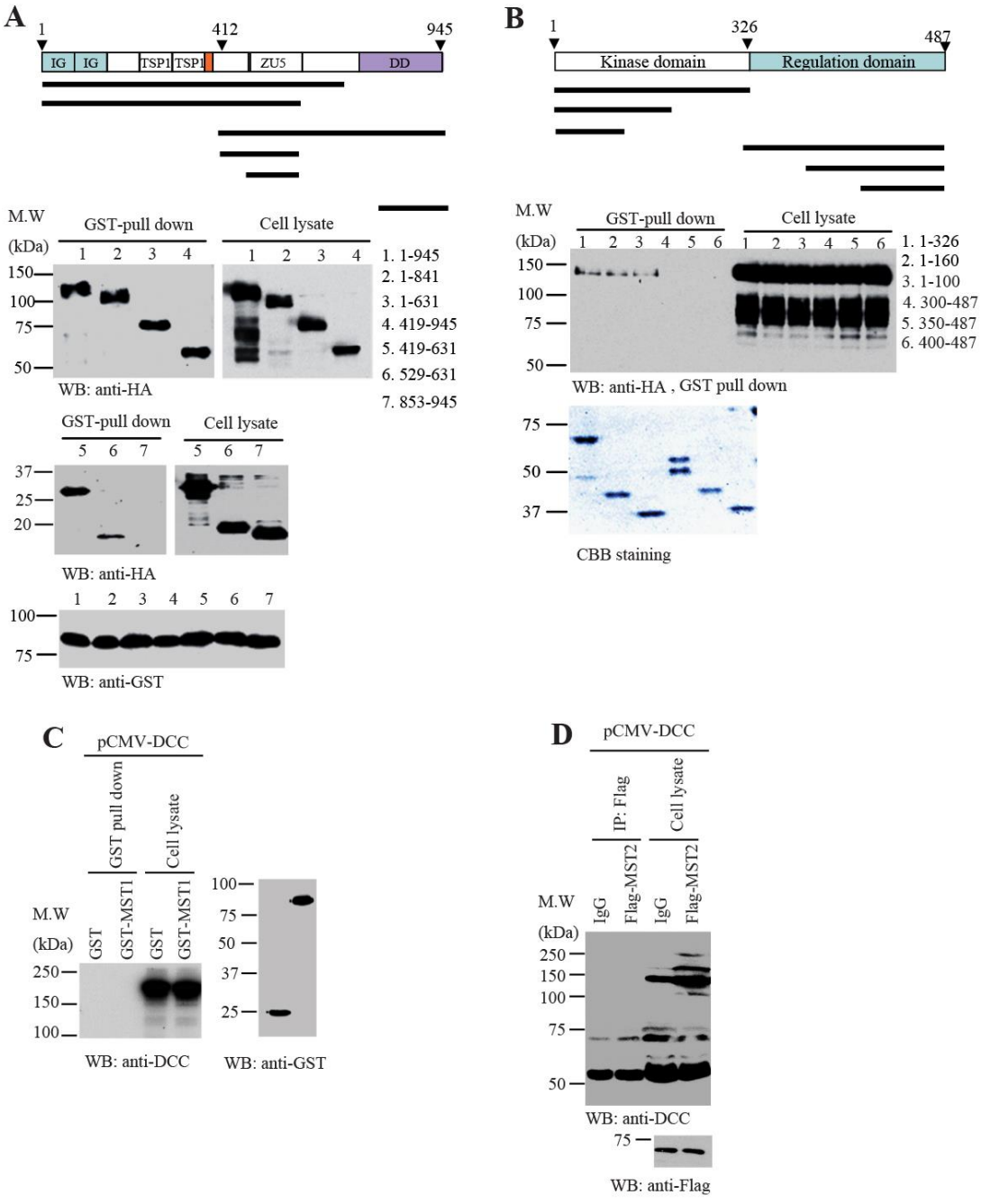

Fig. S1. UNC5B binds to MST1.

(A and B) The mapping of the domains on UNC5B receptor and MST1 for the specific interaction sites. Diagrams of UNC5B and MST1 were shown in the A&B top panels. GST pulldown assays showed that UNC5B 419-945 fragment interacted with MST1 of kinase domain (a.a.1-326) (the middle 2-3rd panels).

(**C and D)** DCC does not bind to MST1 or MST2. Immunoprecipitation representative images showed that DCC did not interact with either MST1 or 2. Three independent studies were conducted in all of experiments.

Fig. S2.

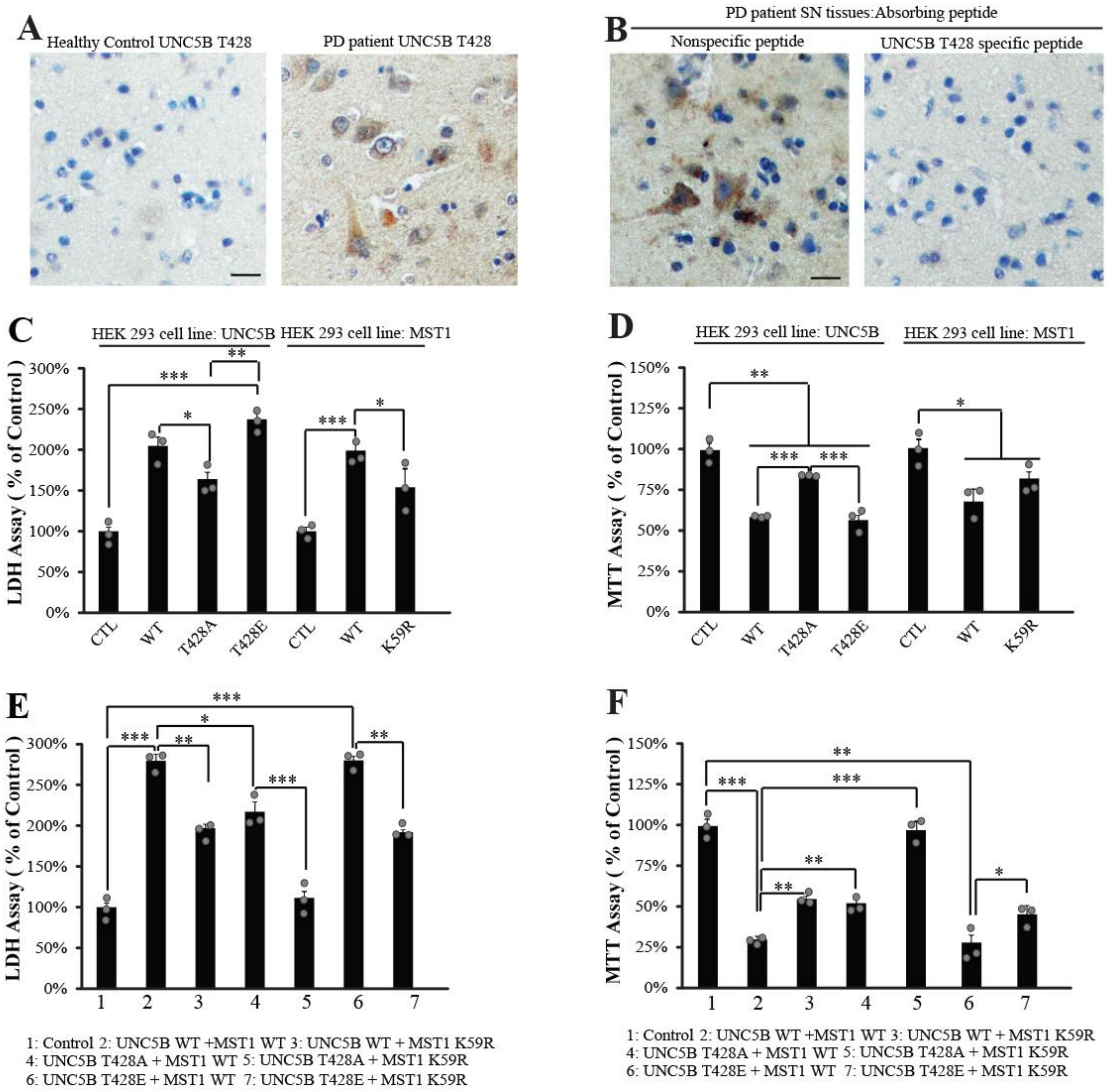
A
Healthy Control UNC5B T428
PD patient UNC5B T428
B
PD patient SN tissues:Absorbing peptide
Nonspecific peptide
UNC5B T428 specific peptide
C
HEK 293 cell line: UNC5B
HEK 293 cell line: MST1
LDH Assay (% of Control)
CTL
WT
T428A
T428E
K59R
D
MTT Assay (% of Control)
E
F
1: Control 2: UNC5B WT +MST1 WT 3: UNC5B WT + MST1 K59R
4: UNC5B T428A + MST1 WT 5: UNC5B T428A + MST1 K59R
6: UNC5B T428E + MST1 WT 7: UNC5B T428E + MST1 K59R

Fig. S2. MST1 phosphorylation of UNC5B mediates its pro-apoptotic activity.

(A and B) Immunohistochemistry shows increase of p-UNC5B T428 expression levels in PD patient's brain paraffin slides. Confirmation of the specificity of the anti-pUNC5B T428 antibody. The anti-pUNC5B T428 antibody was pre-incubated with a peptide containing pUNC5B T428 or non-specific peptide before immunohistochemistry. The signal was blocked by pUNC5B T428 peptide but not nonspecific peptide. Scale bar: 20 μm. 3 independent blinder tests were conducted in all of experiments.

(**C and D)** The LDH assay (C) and MTT assay (D) showed the cytotoxicity and viability of different UNC5B WT, UNC5B T428A, UNC5B T428E, MST1 WT, or MST1 K59R constructs individually transfected human cells.

(**E and F)** MST1 phosphorylation of UNC5B mediates its pro-apoptotic effect. HEK293 cells co-transfected with various UNC5B and MST1 plasmids showed the cytotoxicity (E) and viability (F).

Error bars represent the mean ± SEM. Statistical significance was determined using a two-way ANOVA followed by post hoc Bonferroni test for multiple group comparison. *$p < 0.05$; **< 0.01; ***$P < 0.001$.

Fig. S3.

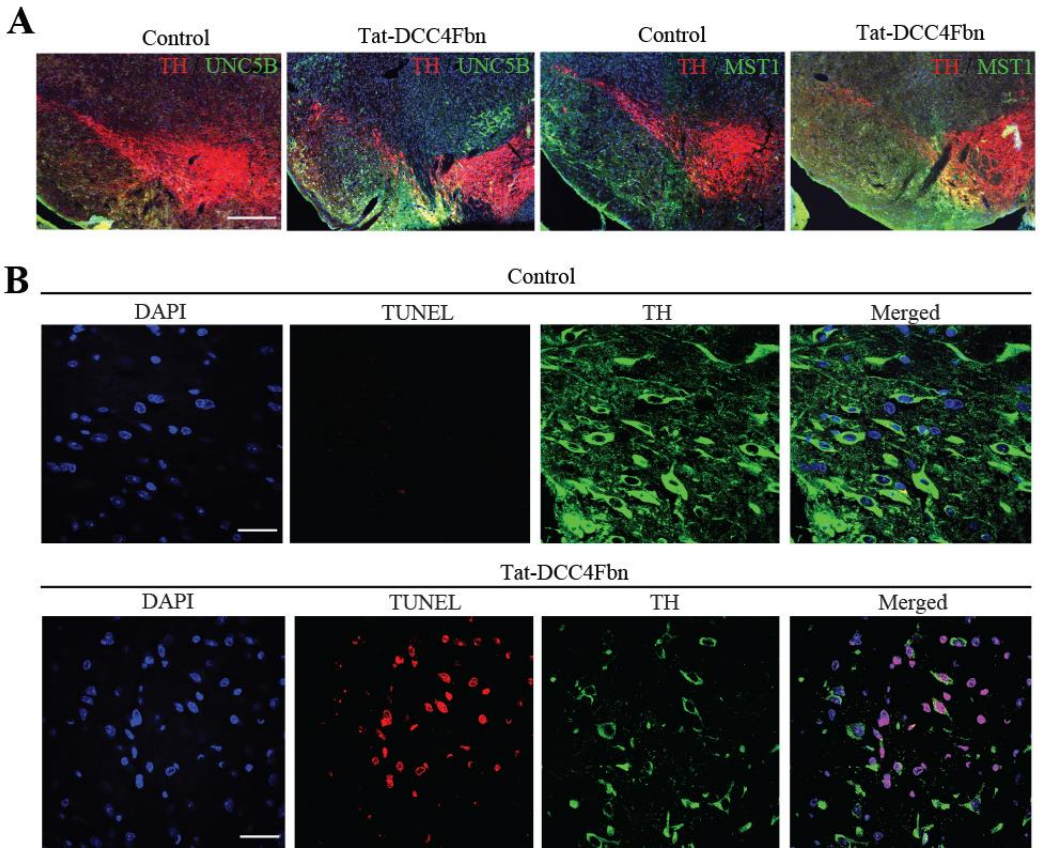

Fig. S3. Tat-DCC-4Fbn robustly induces UNC5B and MST1 protein expressions in SN regions, triggering dopaminergic cell death

(A) Immunofluorescent co-staining representative images showed that TH loss (Red) was correlated with the UNC5B (Green) or MST1 (Green) expression escalation in the SN region by DCC-4Fbn. Scale bar: 1000 μm.

(**B**) Tat-DCC-4Fbn i.p. injection increases the TUNEL positive dopaminergic neuronal cells in mice. TUNEL (Red) and TH (Green) were co-stained on the brain sections. Scale bar: 50 μm.

Fig. S4.

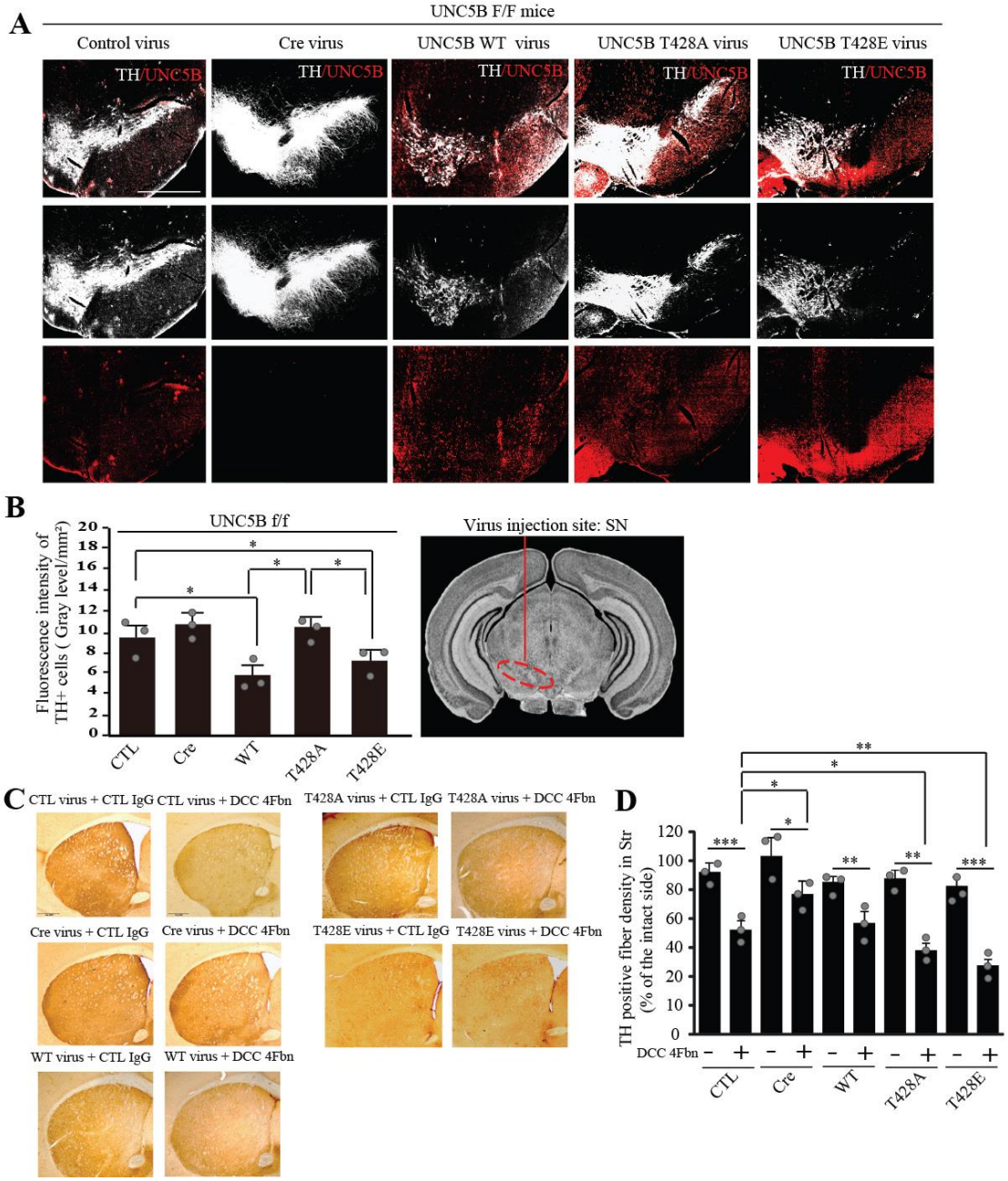
A
UNC5B F/F mice
Control virus
Cre virus
UNC5B WT virus
UNC5B T428A virus
UNC5B T428E virus
TH/UNC5B
B
UNC5B f/f
Fluorescence intensity of TH+ cells (Gray level/mm²)
CTL
Cre
WT
T428A
T428E
Virus injection site: SN
C
CTL virus + CTL IgG
CTL virus + DCC 4Fbn
T428A virus + CTL IgG
T428A virus + DCC 4Fbn
Cre virus + CTL IgG
Cre virus + DCC 4Fbn
T428E virus + CTL IgG
T428E virus + DCC 4Fbn
WT virus + CTL IgG
WT virus + DCC 4Fbn
D
TH positive fiber density in Str (% of the intact side)
DCC 4Fbn

Fig. S4. UNC5B phosphorylation by MST1 is required for initiating dopaminergic neuronal cell death in the SN and Striatum.

(A) TH and UNC5B immunofluorescent co-staining shows that TH cell survival in SN region is mediated by pUNC5B T428 (Cy5-white:TH and Alexa 594-red: UNC5B). Scale bar: 1000 µm. (**B)** The quantification bar graph of fluorescence intensity TH positive cells. N=3 independent experiments.

(**C)** TH immunohistochemistry images in the Striatum. Scale bar: 1000 µm.

(**D)** Quantification bar graph of the density of TH-positive fibres in the striatum. N=3 independent experiments.

Error bars represent the mean ± SEM. Statistical significance was determined using a two-way ANOVA followed by post hoc Bonferroni test for multiple group comparison. *p < 0.05; **< 0.01; ***P < 0.001.

Fig. S5.

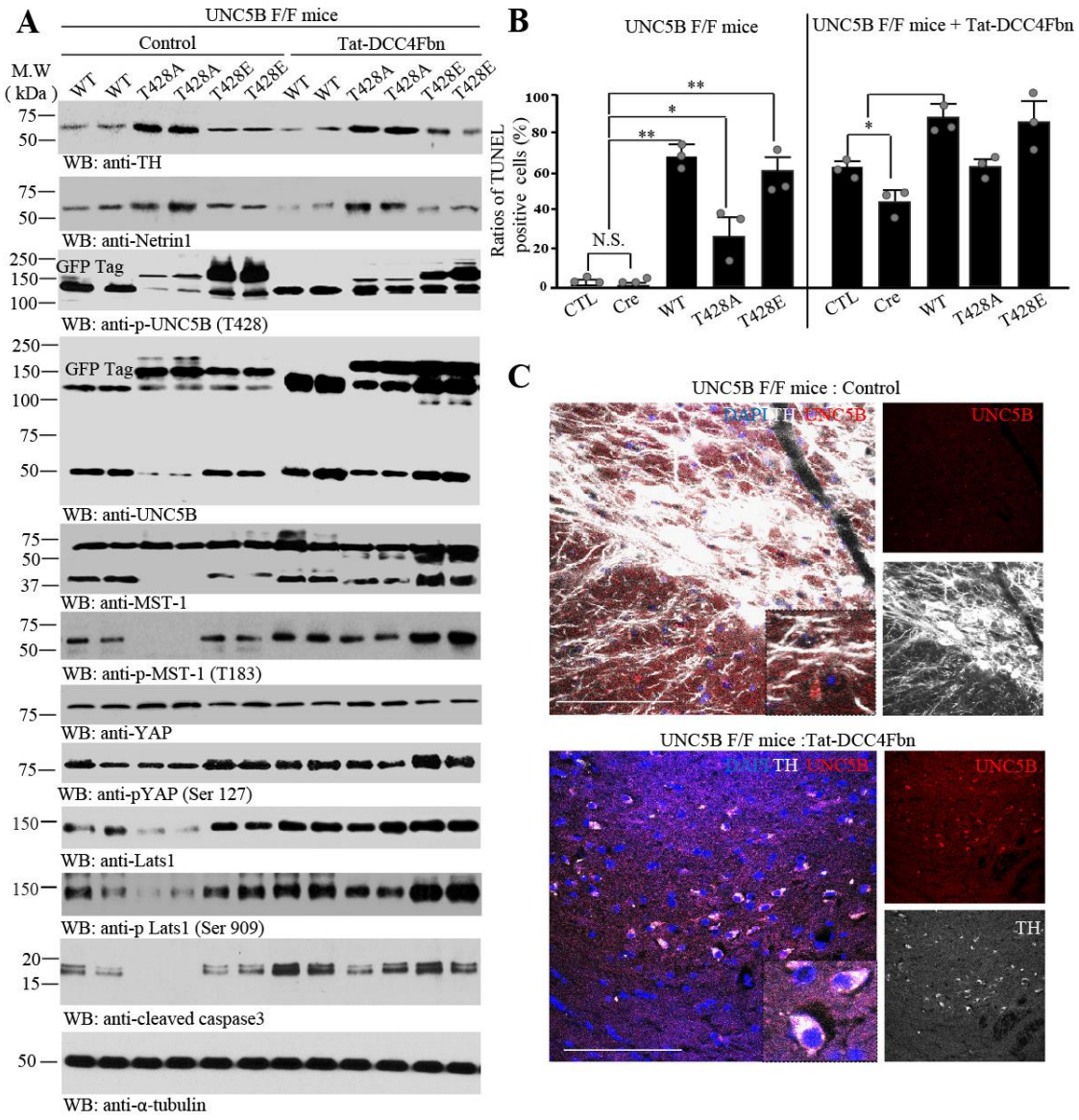
A
UNC5B F/F mice
Control
Tat-DCC4Fbn
M.W (kDa)
WT
T428A
T428E
WB: anti-TH
WB: anti-Netrin1
GFP Tag
WB: anti-p-UNC5B (T428)
WB: anti-UNC5B
WB: anti-MST-1
WB: anti-p-MST-1 (T183)
WB: anti-YAP
WB: anti-pYAP (Ser 127)
WB: anti-Lats1
WB: anti p Lats1 (Ser 909)
WB: anti-cleaved caspase3
WB: anti-α-tubulin
B
UNC5B F/F mice
UNC5B F/F mice + Tat-DCC4Fbn
Ratios of TUNEL positive cells (%)
N.S.
CTL
Cre
C
UNC5B F/F mice : Control
DAPI TH UNC5B
UNC5B
TH
UNC5B F/F mice :Tat-DCC4Fbn

Fig. S5. UNC5B phosphorylation by MST1 mediates dopaminergic cell apoptosis.

(A) Unphosphorylated UNC5B T428A mutant losses its pro-apoptotic activity. Immunoblotting analysis of the SN brain tissue lysates with the following antibodies: TH, Netrin1, p-UNC5B T428, UNC5B, MST1, p-MST1 T183, YAP, p-YAP S127, LATS1, p-LATS1 S909 and active caspase-3 levels.UNC5B f/f mice were injected with 3 different UNC5B virus (UNC5B WT; UNC5B T428A;UNC5B T428E), followed by Tat-DCC 4Fbn or vehicle administration.

(**B)** The TUNEL assay quantification bar graph N =3 per group.

(C). Induction of UNC5B by netrin-1 deprivation elicits TH loss in the SN of UNC5B f/f mice. TH(White) and UNC5B(Red) immunofluorescent co-staining representative images from the mice with or without Tat-DCC 4Fbn i.p. injection. Scale bar: 50 μm.

Error bars represent the mean ± SEM. Statistical significance was determined using a two-way ANOVA followed by post hoc Bonferroni test for multiple group comparison. *$p < 0.05$; **< 0.01; n.s., not significant.

Fig. S6.

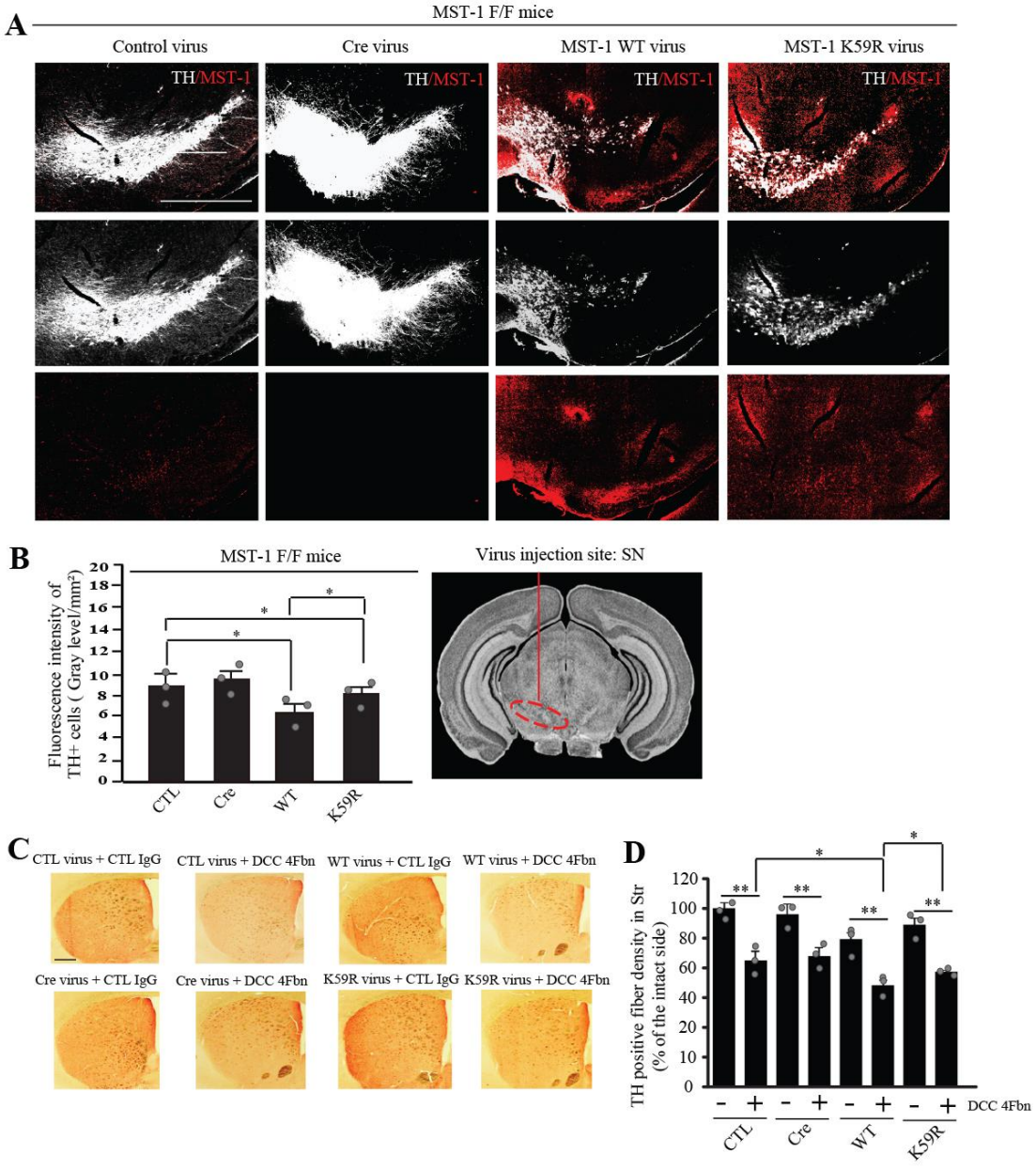
A
MST-1 F/F mice
Control virus
Cre virus
MST-1 WT virus
MST-1 K59R virus
TH/MST-1
B
MST-1 F/F mice
Virus injection site: SN
Fluorescence intensity of TH+ cells (Gray level/mm²)
CTL
Cre
WT
K59R
C
CTL virus + CTL IgG
CTL virus + DCC 4Fbn
WT virus + CTL IgG
WT virus + DCC 4Fbn
Cre virus + CTL IgG
Cre virus + DCC 4Fbn
K59R virus + CTL IgG
K59R virus + DCC 4Fbn
D
TH positive fiber density in Str (% of the intact side)
DCC 4Fbn
CTL
Cre
WT
K59R

Fig. S6. Kinase death of MST1 protects dopaminergic neuronal loss from DCC-4Fbn.

(A) Kinase-dead MST1 K59R mutant fails to trigger dopaminergic neuronal loss. TH and MST1 immunofluorescent co-staining showed that TH neurons were protected in MST1 K59R virus injected group (Cy5-white:TH and Alexa 594-red: MST1). Scale bar: 1000 μm.

(**B)** The quantification bar graph of fluorescence intensity TH positive cells (Results shown as mean ± SEM; n=3 independent experiments, *p<0.05 by two-way ANOVA).

(**C)** TH immunohistochemistry images in the Striatum from mice infected with various indicated virus, followed by DCC-4Fbn treatment. Scale bar: 1000 μm.

(**D)** Quantification bar graph of the density of TH-positive fibres in the striatum. (Results shown as mean ± SEM; n=3 independent experiments, *p<0.05, **p<0.01 by two-way ANOVA)

Fig. S7.

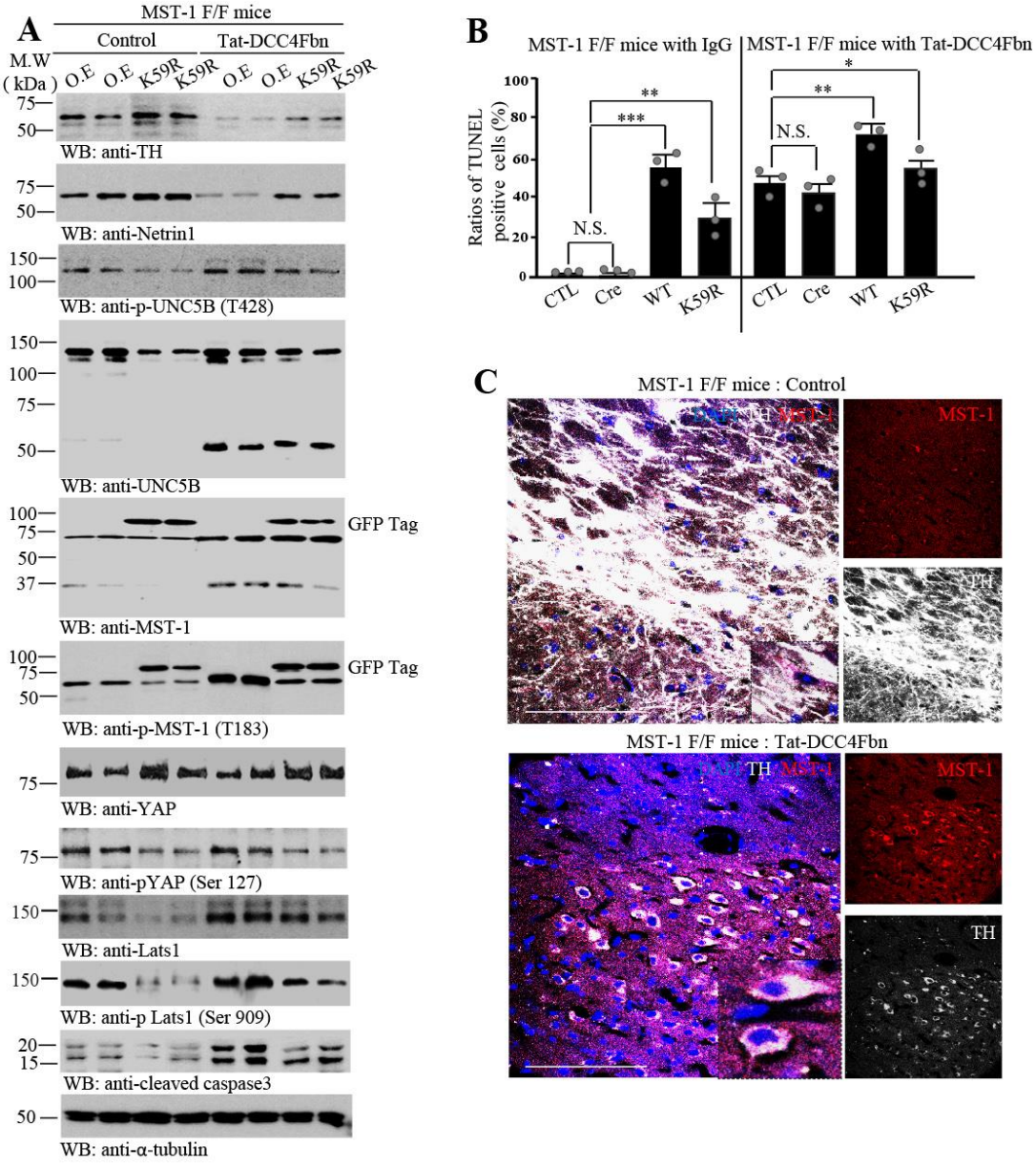
A
MST-1 F/F mice
Control
Tat-DCC4Fbn
M.W (kDa)
O.E
O.E
K59R
K59R
O.E
O.E
K59R
K59R
75
50
WB: anti-TH
75
50
WB: anti-Netrin1
150
100
WB: anti-p-UNC5B (T428)
150
100
75
50
WB: anti-UNC5B
100
75
50
37
GFP Tag
WB: anti-MST-1
100
75
50
GFP Tag
WB: anti-p-MST-1 (T183)
75
WB: anti-YAP
75
WB: anti-pYAP (Ser 127)
150
WB: anti-Lats1
150
WB: anti-p Lats1 (Ser 909)
20
15
WB: anti-cleaved caspase3
50
WB: anti-α-tubulin
B
MST-1 F/F mice with IgG
MST-1 F/F mice with Tat-DCC4Fbn
Ratios of TUNEL positive cells (%)
100
80
60
40
20
0
**
***
N.S.
*
**
N.S.
CTL
Cre
WT
K59R
CTL
Cre
WT
K59R
C
MST-1 F/F mice : Control
DAPI TH MST-1
MST-1
TH
MST-1 F/F mice : Tat-DCC4Fbn
DAPI TH MST-1
MST-1
TH

Fig. S7. MST1 kinase activity is required for Netrin1 deprivation-elicited dopaminergic neuronal loss.

(A) MST1 kinase-dead mutant (K59R) decreases dopaminergic neuronal loss from DCC-4Fbn treatment. Immunoblotting analysis of the SN brain tissues lysates with the following antibodies: TH, Netrin1, p-UNC5B T428, UNC5B, MST1, p-MST1 T183, YAP, p-YAP S127, LATS1, p-LATS1 S909 and active caspase-3 levels. MST1 f/f mice were injected with 2 different MST1 virus (MST1 WT and MST1 K59R) into the SN regions, treated with Tat-DCC 4Fbn or vehicle.

(**B)** The TUNEL assay quantification bar graph. We performed the 3 independent experiments.

(**C)** TH and MST1 immunofluorescent co-staining representative images from the SN region in MST1 f/f mice treated with or without Tat-DCC-4Fbn (i.p. injection). Scale bar: 50 μm.

Error bars represent the mean ± SEM. Statistical significance was determined using a two-way ANOVA followed by post hoc Bonferroni test for multiple group comparison. *$p < 0.05$; **< 0.01; ***$P < 0.001$; n.s., not significant.